Research letter

Impact of environmental conditions and gut microbiota on the *in vitro* germination and growth of *Clostridioides difficile*

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GRAPHICAL ABSTRACT

ABSTRACT

Clostridioides difficile is a spore-forming anaerobic Gram-positive bacterium responsible for a broad spectrum of intestinal symptoms and healthcare associated diarrhoea. The hypothesis of this work was that different *in vitro* conditions, notably pH and human faecal microbiota composition impact the germination or/and the growth of *C. difficile*. This study aimed to correlate growth kinetics of the bacterium with these two physiochemical parameters by using a static *in vitro* model. To better understand the initial gut colonization, several growth curve assays were carried out to monitor the behaviour of the spores and vegetative forms of a *C. difficile* strain 078 under different conditions mimicking the gut environment. When the faeces were added, no spore germination or growth was observed but *C. difficile* spores germinated *in vitro* when the pH was maintained between 6.6-6.9 regardless of four faeces donor. The evolution of microbiota studied by 16S rDNA profiling showed high proportions of *Enterobacteriaceae* and *E. coli*/*Shigella* when *C. difficile* grew regardless of the inoculated faeces. This model helped us to understand that the germination and the growth of *C. difficile* is strongly pH dependent, and further research is needed to evaluate the potential impact of the gut microbiota composition on *C. difficile*.

Keywords pH, *Clostridioides difficile*, static *in vitro* model, 16S rDNA profiling, gut microbiota, germination

INTRODUCTION

Clostridioides difficile, formally called *Clostridium difficile* (Lawson *et al.* 2016), is a spore-forming anaerobic gram-positive bacterium that is considered the leading cause of antibiotic-associated diarrhoea in hospitals (Centers for Disease Control and Prevention, 2019). The intestinal carriage of *C. difficile* can be asymptomatic, but in cases of infection it is associated with different clinical signs of disease that can vary from mild diarrhoea to pseudomembranous colitis (Crobach *et al.* 2018).

Transmission of *C. difficile* infection (CDI) occurs by the faecal-oral route. After ingestion, the spores can survive to stomach acid and germinate in the small intestine. Vegetative forms continue along the digestive tract until they reach the colon where they can multiply, colonize the epithelium and secrete their toxins (A, B and/or binary toxin CDT) (Awad *et al.* 2014; Monot *et al.* 2015; Samarkos, Mastrogianni and Kampouropoulou, 2018). Several factors are considered to be needed for spore germination in the gut, such as the presence of primary bile acids (PBAs) in the small intestine (Sorg and Sonenshein 2008, 2009; Giel *et al.* 2010; Kochan *et al.* 2018a), a pH ranging between 6.5 and 8.5 (Kochan *et al.* 2018b; Wetzel and McBride 2020), the presence of amino acids (glycine and/or L-alanine) (Sorg and Sonenshein 2008; Kochan *et al.* 2017, 2018a; Shrestha and Sorg 2018), the availability of calcium (Kochan *et al.* 2017, 2018a), a temperature of 37 °C (Kochan *et al.* 2018a; Shrestha and Sorg 2018), and an anaerobic atmosphere (Sorg and Sonenshein 2008; Crobach *et al.* 2018; Shrestha and Sorg 2018). In contrast, several other factors could prevent *C. difficile* germination, including the presence of secondary bile acids (SBAs) (Sorg and Sonenshein 2009; Crobach *et al.* 2018), a pH <5.5 or >10.5 (Kochan *et al.* 2018b), and resistance to colonization by competitive microbiota interactions (Britton and Young 2012; Pérez-Cobas *et al.* 2015; Crobach *et al.* 2018). The resistance of colonization is mediated by the presence of short-chain fatty acid (SCFA)-producing bacteria such as *Ruminococcaceae*

and *Lachnospiraceae* (Pérez-Cobas *et al.* 2015) and the presence of bacteriocin-producing bacteria (*Clostridium scindens*) (Buffie *et al.* 2015; Pérez-Cobas *et al.* 2015).

Recent studies have compared the gut flora between patients suffering from CDI and *C. difficile* negative subjects. Patients with CDI presented a decrease in bacterial diversity and richness (Milani *et al.* 2016; Han *et al.* 2019; Hernandez 2019; Jeon *et al.* 2019), which is associated with an increase in Proteobacteria, *Enterobacteriaceae* and *Escherichia* spp. and a reduction in *Lachnospiraceae*, *Ruminococcaceae*, *Alistipes*, *Bacteroides* and *Prevotella* spp. (Milani *et al.* 2016; Han *et al.* 2019; Hernandez 2019; Jeon *et al.* 2019). The decrease in *Lachnospiraceae* and *Ruminococcaceae* leads to a reduced concentration of SCFAs and SBAs (Pérez-Cobas *et al.* 2015).

The present study aimed to focus on the growth kinetics of *C. difficile* and evaluate the impact of physicochemical parameters. The first objective was to examine different *in vitro* conditions for the germination and growth of *C. difficile* through a static model. The second objective was to study the impact of addition of human faecal microbiota on *C. difficile* multiplication *in vitro*. This study describes a static *in vitro* model to study the intestinal pathology of *C. difficile* and provides answers as to the minimal conditions to be used to study the behaviour of the bacterium.

MATERIALS AND METHODS

Bacterial strain and spore production

The *C. difficile* strain used in this study belonged to PCR ribotype 078. It was initially isolated from piglet faeces (Rodriguez *et al.* 2012) and characterized for the presence of toxin genes, toxin activity in cell lines and PCR ribotyping (Rodriguez *et al.* 2012). It was internally labelled (S0756) and stored frozen (-80 °C) in a solution of 20 % glycerol 80 % BHI (VWR 24388.295; Brain Heart Infusion, Oxoid CM1032).

For spore production and purification, the strain stored at -80 °C was placed in regenerated antibiotic-free BHI broth and incubated at 37 °C for 7 days under strict anaerobic conditions. Then, the spores were harvested by centrifugation (Eppendorf, Centrifuge 5810R, with swing-bucket rotor) (20 min at 4,500 x g) and using an ethanol treatment (1 ml; 95 % ethanol at room temperature) on the pellet for 1 h. Next, two washing steps with sterile water were performed (5 min at 4,500 x g), and the final solution was diluted with 1 ml of sterile water and stored at 4 °C. To determine the final spore concentration (ISO 7218:2007), 10-fold dilutions were made, and 100 µl of each dilution was spread on blood agar (Thermo Fisher Scientific, PB5039A) and on homemade cycloserine cefoxitin fructose agar taurocholate (CCFAT) (Delmée *et al.* 1987) and were incubated anaerobically for 48 h at 37 °C.

Genome sequencing and characteristics

A ten-microliter aliquot of the stored bacteria was regenerated in BHI broth for genome sequencing. The solution was incubated anaerobically at 37 °C for 48 h and further subcultured on blood Agar. The extracted DNA from a colony was sequenced using Illumina MiSeq (GIGA, Belgium). The raw reads were trimmed by Geneious v.10.2.3, and a *de novo* assembly was performed using the software SPAdes implemented in Geneious v.10.2.3. Annotation was performed using the Pathosystems Resource Integration Center (PATRIC 3.6.12) (Brettin *et al.* 2015) and the genome assembly is available in Genbank.

Faeces acquisition and dilution

Faecal samples were obtained at random and anonymously from 4 healthy donors aged between 26 and 66 years and without a history of antibiotic or probiotic use in the two months prior to recruitment. The donors had no concomitant illness. The age and sex of the donors are described in supplementary_tableA1. Written informed consent was obtained from all the participants involved in the study.

The absence of *C. difficile* was determined by both direct culture and faecal enrichment as described previously (Rodriguez *et al.* 2012). A solution of 20 % fresh faeces and phosphate buffer were obtained with the described protocol (Bondue *et al.* 2020) and were kept at -80 °C until further testing.

C. difficile growth curves using two different types of culture media in an anaerobic chamber

An initial suspension of 30 ml of BHI broth containing 1.5 $\log_{10}$ colony-forming units (CFU)/ml of *C. difficile* spores was incubated in an anaerobic chamber (Concept Plus, Anaerobic workstation, LedTechno) at 37 °C for 144 h in triplicate. Bacterial counts on CCFAT were carried out at 0 h, 8 h, 24 h, 32 h, 48 h, 56 h, 72 h, 80 h, 96 h, 104 h, 120 h, and 144 h. These results were used to perform a reference BHI growth curve of *C. difficile* spores S0756.

A second *C. difficile* growth curve was generated using the same conditions as previously described but with SHIME nutritional medium instead of BHI. This medium contains 70 % feed (ProDigest, PDNM001B) and 30 % pancreatic juice (PJ) (ProDigest, Pancreatic enzymes, PDDE001, Bile salts, PDDE002, $NaHCO_3$, PDDE007) (supplementary data_tableA2). Briefly, the intial suspension consisted of 30 ml of SHIME medium containing 1.5 $\log_{10}$ CFU/ml *C. difficile* spores in duplicate and these suspensions were stabilized at a pH of 7.4 on the first day and placed in an anaerobic chamber for 96 h at 37 °C without further control of the pH. Enumerations on CCFAT were carried out at 0 h, 6 h, 24 h, 32 h, 48 h, 56 h, 72 h, 80 h, 96 h. The obtained results were used to generate a reference SHIME growth curve of *C. difficile* spores S0756.

A third assay was performed in the anaerobic chamber using the previously diluted human faeces. A total of 1 ml of these diluted stools was added to 30 ml of SHIME medium and incubated at 37 ºC for 24 h. Then, the samples were centrifuged (5 min at 4,500 x g)

(Eppendorf, 5810/5810R), the supernatants were removed, and an additional 40 ml of SHIME medium was added for 80 h of incubation. In three out of the seven samples, 1.77 $\log_{10}$ CFU/ml ± 0.5 *C. difficile* spores were inoculated. In the three other samples, 5 $\log_{10}$ CFU/ml ± 0.7 *C. difficile* vegetative forms were added. The seventh tube was used as a negative control. Enumerations were carried out after 0 h, 8 h, 24 h, 32 h, 48 h, 56 h, 72 h, and 80 h of growth time on CCFAT. The assay was repeated for the fecal samples of the four recruited donors. Detailed information about the specific conditions of each assay performed in the anaerobic chamber is given in Table 1.

A two-ways ANOVAs with mixed effect model for paired samples (Prism version 9), combined with a Benjaminy Yekutieli False Discovery Rate correction (Q=0.05), were used to identify significant growth differences between co-culture assays combining *C. difficile* and faeces suspension. We removed *C. difficile*/D1 assays from the analysis as microbial counts results were considered as outliers.

C. difficile growth curves in microbial fermenters under controlled conditions

Three experiments were conducted using fermenters with control of growth conditions (Molly *et al.* 1994). In order to take into account, the gut microbiota diversity, the faeces from the 4 different donors were not pooled and we assumed that one donor was one replicate in this part of experiment. The system was programmed to automatically control the temperature (37 °C), agitation (300 rpm) and the microbial fermenters were kept under anaerobic conditions using nitrogen flushing. Fermenters containing 40 ml of diluted faeces (from each of the 4 donors) and 560 ml of SHIME medium were connected to pH pumps (0.5 M HCl and 0.5 M NaOH) and pH probe (ProDigest, Ghent, Belgium). In the first experiment, eight fermenters (2/donors) were inoculated with *C. difficile* spores, and the pH was not controlled for 66 h. In the second experiment, four fermenters (1/donors) were inoculated with *C. difficile* spores, and the pH was controlled for 94 h. In the third experiment, four fermenters

were inoculated with *C. difficile* vegetative forms, and the pH was controlled for 94 h. Detailed information about the specific conditions of each assay performed in the microbial fermenters is given in Table 2. Bacterial counts on CCFAT were performed twice a day. A rapid test "C. DIFF Quik Check Complete" (Abbott, Belgium, Wavre) for toxins and glutamate dehydrogenase antigen detection (GDH) was performed at 30 h, 52 h and 76 h on one replicate during the three experiments.

DNA extraction and qPCR targeting of *C. difficile*

Total DNA extractions were performed using different commercial kits according to the type of sample the manufacturer's recommendations. The samples from the calibration curve and BHI growth curves were extracted with a DNeasy Blood & tissues kit (Qiagen, Belgium). The samples from the *C. difficile* growth curves in microbial fermenters under controlled conditions were extracted with a PSP stool DNA *Plus* kit (Isogen Lifesciences B.V, Netherlands). The rest of the samples were extracted with QiAamp Power Fecal Pro DNA (Qiagen, Belgium).

A specific qPCR targeting the 16S rDNA gene of *C. difficile* (157 bp, F64-R220) was performed in all samples using the 16S rDNA gene primers *16S*-Forward [5'TTGAGCGATTTACTTCGGTAAAGA3'], *16S*-Reverse [5' CCATCCTGTACTGGCTCACCT 3'] and *16S*-Probe [5'FAM-CGGCGGACGGGTGAGTAACG-TAMRA 3'] (Mutters *et al.* 2009; Stewart and Hegarty 2013). Standard DNA was obtained from the purified PCR products and quantified using PicoGreen (Thermo Fischer Scientific, Waltham). Tenfold dilutions of these standard DNA were performed to generate a standard curve. qPCR was run using CFX-96 (Biorad, Belgium, Temse) in a final reaction volume of 12.5 µl. Genome equivalent values were deduced from CT to 16S copy transformed values considering 13 16S rDNA copies by genome and converted to bacterial concentrations. These results were calculated as "bacteria/ml".

Once the CCFAT count (CFU/ml) and results of qPCR 16S (bacteria/ml) were obtained, the growth rate (μ) was calculated using two points from the exponential phase with the formula $\ln(X_1)-\ln(X_0)/ (t_1-t_0)$, where X_1 is the second point of the exponential phase and X_0 is the first point of the exponential phase (Raina M. Maier 2015). The growth potential (δ) was calculated at three specific points (24 h, 48 h and 72 h), according to the formula explained by Lebrun *et al.* (Lebrun *et al.* 2020).

16S rDNA amplicon sequencing

With total bacterial DNA extracted from 4 faeces donor and from the growth curves of the fermenters, 16S rDNA profiling targeting the V1-V3 hypervariable region was performed as described previously (Rodriguez *et al.* 2015; Ngo *et al.* 2018; Gérard *et al.* 2021). Sequencing libraries are available in the GenBank repository under the PRJNA716140 bioproject.

RESULTS

***C. difficile* genome characteristics**

Genome analysis of strain S0756 (PCR ribotype 078) confirmed the presence of PaLoc with all toxin genes (*tcdA, tcdB, tcdC, tcdR, tcdE*), and the presence of CdTLoc (*cdtR, cdtB, cdtA*) specific to *C. difficile*. The number of copies of the 16S rDNA gene was 13. The genome annotation is available in the GenBank repository under the PRJNA716140 bioproject and GCA_017592625.1 assembly. In supplementary_tableA3, the main characteristics of the genome and the analysis of average nucleotide identity between *C. difficile* strain 630, M120 and S0756 are showed.

***C. difficile* growth curves using two different types of culture media in an anaerobic chamber**

Several growth curve assays were carried out to study the behaviour of the spores and vegetative cells under different conditions (Figure 1). Between 24 h and 72 h, the growth potential (δ) in BHI broth was 5.1 ± 0.1 $\log_{10}$ CFU/ml. The growth rate (μ) based on CCFAT

counting for the BHI growth curve was 0.17 (± 0.01) h^{-1}. An exponential and stationary phase was observed in the growth curve generated from the SHIME medium when it was tested without faeces. During the first 24 h, δ was 2.1 ± 0.46 $\log_{10}$ CFU/ml. The μ based on CCFAT counting was 0.2 (± 0.06) h^{-1}. When *C. difficile* spores were put in contact with faeces, no growth was observed in any of the donor samples. However, when the *C. difficile* vegetative forms were inoculated with these four different donor faecal samples, there were two phases: the first phase was negative growth the first 24h (δ -2.72 ± 1.3 $\log_{10}$ CFU/ml), and the second phase was positive growth from 24 h to 72 h (δ 1.05 ± 0.9 $\log_{10}$ CFU/ml). Supplementary_tableA4 details the growth rates and all growth potentials that were calculated as a function of CCFAT enumeration and the qPCR results, respectively.

Concerning statistical analysis of growth assays (a two-ways ANOVAs with mixed effect model for paired samples), D2 and D4 were significantly different (P<0.05) at 48h in growth curve with spores. With vegetative forms, D2 were significantly different (P<0.05) with D3 and D4 at 24h, 48h and 72h. Supplementary_figureA1 details CCFAT enumeration and the qPCR results for each faecal donor.

***C. difficile* growth curves in microbial fermenters under controlled conditions**

In the first experiment, a drop in pH from 6.8 ± 0.11 to 5.6 ± 0.18 in the fermenters was observed between 0 h and 6 h (Supplementary_figureA2). The pH remained stable at 5.6 ± 0.04 over the next 60 h. No growth was observed with CCFAT counting or *C. difficile* quantification using qPCR. *C. difficile* rapid detection tests performed at 20 h, 42 h and 66 h were all negative for glutamate dehydrogenase antigen and toxins A and B. No germination or growth were observed with the tested parameters. In the second experiment, the pH was maintained in the range of 6.6 to 6.9. Figure 2 illustrates the second and third experiments performed with *C. difficile* spores and vegetative cells and represent the mean of the four replicates donors. The δ at 72 h was 2.0 ± 0.6 $\log_{10}$ (CFU/ml) in the second study. In the third

study, the δ at 72 h was 2.3 ± 1.3 $\log_{10}$ (CFU/ml). In the second and last experiments, the C. DIFF Check Quick Complete tests performed after 30 h, 52 h and 76 h were positive for the GDH and the toxins. In supplementary_tableA5, all growth potentials were calculated based on CCFAT counting and qPCR results, respectively. Supplementary_figureA3 details CCFAT enumeration and the qPCR results for each faecal donor.

16S rDNA amplicon sequencing

The 16S rDNA profiling of the four faecal samples collected is illustrated in Figure 3. Due to the limited number of donors, no statistical analysis was performed, and only a descriptive analysis was used to compare the microbiota evolution in the fermenters.

The relative abundances in the bacterial groups at the genus level were almost identical for each of the four donors, with three dominant taxa (*Prevotella*_9, *Faecalibacterium* spp. and *Lachnospiraceae*). Specifically, for donors 2 and 4, important abundances of *Faecalibacterium* spp. and *Lachnospiraceae* were observed, which constituted more than 50 % of the total relative abundance. *Prevotella*_9 appeared to be dominant in the other two donors, with an important proportion in donor 1. Supplementary_figureA4 and tableA6, shows the diversity, the richness, and the amount of total flora of theses 4 faeces donors.

Second, 16S rDNA profiling of the samples from the fermenters was performed to detect possible variations in the microbial communities. The microbiota composition was determined on days 0 and 2 for the experiment without pH control, and on days 0, 2 and 4 for the assay with pH control (Figure 4). All four profiles followed the same profile evolution. Some differences were detected on day 2 when the pH was 5.6, with an increase in *Escherichia coli* in donors 1, 3 and 4 (Figure 4A). Regarding the microbiota composition after *C. difficile* inoculation and pH control, no differences were detected between the vegetative form and spore inoculation on days 2 and 4. Only an important increase in *E. coli* and the *Enterobacteriaceae* family were observed in donors 1, 2 and 3 on the second day of the assay

(Figure 4B and 4C). Regardless of the amount (CFU) of inoculated *C. difficile*, control of the pH in our static *in vitro* test directly influenced the gut microbiota because the system was closed, and no nutritive medium was added after initiation of the assay. When the pH was controlled between 6.6-6.9, on day 2 and on day 4, the model also allowed the growth of the *Enterobacteriaceae* family (*Escherichia-Shigella spp*.).

DISCUSSION

C. difficile spores germinate in different proportion in different media (BHI and SHIME medium). The μ based on CCFAT counting for the BHI growth curve was similar to the results obtained in the work of Connor *et al.* (2018). BHI medium is a nonselective medium that is suitable for growing many bacteria. During the first 24 h, the δ for SHIME medium without faeces was 2.1 ± 0.46 $\log_{10}$ CFU/ml. SHIME medium mimics the nutrient matrix of a normal person and the pancreatic juice and bile acids mimics the enzymes necessary for the digestion of proteins and fat. PBA favours the germination of *C. difficile* spores (Sorg and Sonenshein 2008, 2009; Giel *et al.* 2010; Kochan *et al.* 2018a).

Concerning microbiological enumeration ($\log_{10}$ (CFU/ml)), the growth curve of *C. difficile* in co-culture with donor 2, 3, 4 were significantly different in several point. The growth curve of *C. difficile* in co-culture with donor 1 were excluded of this analysis but after vegetative and spore inoculation, no growth of *C. difficile* was visualized with this donor. This study didn't allow us to conclude if it was due to the abundance of *Prevotella* spp. known to protect the microbiota against *C. difficile* (Han *et al.* 2019; Jeon *et al.* 2019; Martinez *et al.* 2022). These data allowed us to limit conclusion that the microbiota influence the growth of *C. difficile* (Martinez *et al.* 2022).

Concerning the difference in the microbiological counting ($\log_{10}$ (CFU/ml)) and 16S rDNA gene copy number results, several hypotheses were formed: not all bacteria could be counted by the conventional plate count method (Kadiroğlu, Korel and Ceylan 2014); in one

CFU, the number of bacterial cells can be underestimated (Kadiroğlu, Korel and Ceylan 2014); and the genetic method counts both viable bacteria and dead bacteria (Kadiroğlu, Korel and Ceylan 2014). More importantly, the two methods showed the same growth kinetics rate.

Our findings confirmed the observations from previous studies (Paredes-Sabja *et al.* 2008; Wheeldon *et al.* 2008; Kochan *et al.* 2018b), which suggested that *C. difficile* is not able to germinate and grow below a pH of 5.6. Paredes Sabja *et al.* (2008) showed that *C. difficile* strain JIR8094 (derivative of strain 630) had an optimal pH for germination of 6.0. NCTC 11204 (001) and *C. difficile* R20291 (027) had an optimal pH for germination of 6.5-7.5 (Wheeldon *et al.* 2008). Kochan *et al.*(Kochan *et al.* 2018b) showed that when *C. difficile* spores were incubated at a pH between 6.5 and 8.5, they exhibited good germination efficiency, whereas the opposite effect was observed at a pH below 5.5 or above 10.5 (Kochan *et al.* 2018b). Wetzel and McBride (2020) (Wetzel and McBride 2020) also showed how an acidic pH (5.5) had a negative impact on *C. difficile* 630△*erm* strain sporulation efficiency, cell morphology, cell motility and toxin production. Our *in vitro* results also confirmed that pH is a key factor for *C. difficile* germination.

Several other studies have linked *C. difficile* infection and/or colonization with gastrointestinal pH and suggested some risk factors for the development of disease, such as the use of PPIs (proton pump inhibitors) or antibiotics (Bavishi and DuPont 2011; Shimizu *et al.* 2021). The use of PPI increases the pH in the stomach to 5, and the relative risk range for developing CDI is 1.2 to 5 times higher (Bavishi and DuPont 2011). Jackson *et al.* (2016) also showed that PPIs modified the composition of the gut microbiota, with a reduction in diversity and an increase in the contents of the *Enterococcaceae* and *Staphylococcaceae* families. After using antibiotics in mice for one week, the average pH values of the colon was

7.93 (Shimizu *et al.* 2021), which can lead to gut colonization by opportunistic bacterial pathogens such as *C. difficile*.

The result of the 16S rDNA faecal profiling have shown that there was a heterogeneity of the donors. The donor 1 and 3 showed a high *Prevotella* (67.7 % and 27.3 % respectively) relative abundance. In the literature, the abundance of *Prevotella* can reach 35 % (MetaHIT Consortium (additional members) *et al.* 2011). The controlled system in this study induced an increase of *Escherichia*/*Shigella* spp. and *Enterobacteriaceae*. In these conditions, *C. difficile* was able to grow. The state of dysbiosis is defined as a decrease in the obligate anaerobic bacteria and an increase in the relative abundance of facultative anaerobic bacteria, such as *Enterobacteriaceae* (Lupp *et al.* 2007; Winter, Lopez and Bäumler 2013), or as reduced diversity, a decrease in anti-inflammatory species such *Faecalibacterium prausnitzii*, and an increase in other populations such as *Enterobacteriaceae* (Mahnic *et al.* 2020). On healthy intestine, *Enterobacteriaceae* ranges from 0.01 % to 10.99 % (Winter, Lopez and Bäumler 2013; King *et al.* 2019). In the gut microbiota of CDI patients compared to healthy people, diversity is significantly lower (Zhang *et al.* 2015; Amrane *et al.* 2019; Han *et al.* 2019; Hernandez 2019; Jeon *et al.* 2019). An increase in *Proteobacteria* (Zhang *et al.* 2015; Amrane *et al.* 2019; Han *et al.* 2019; Jeon *et al.* 2019), *Enterobacteriaceae* (Song *et al.* 2013; Khanna *et al.* 2016; Sangster *et al.* 2016; Han *et al.* 2019; Hernandez 2019), and *Escherichia*/*Shigella* (Zhang *et al.* 2015; Jeon *et al.* 2019) has been described and is associated with a decrease in *Actinobacteria* (Amrane *et al.* 2019; Jeon *et al.* 2019).

Our results present some limitations. No statistical analysis was performed in fermenters data because we chose to test three conditions with four different donors instead of pooling the faeces in order to study the effects of the individual faeces. The data were analysed with growth rate and growth potential to show difference between our samples. In addition, these results can only be transposed to the *C. difficile* 078 studied strain. The

ribotype 078 and 014 are among most present in hospital and in Europe (Rodriguez *et al.* 2016). The ribotype 078 possesses the virulence genes (Rodriguez *et al.* 2016). No control of the evolution of microbiota without *C. difficile* was implemented in this experiment but the low proportion ($1/10^6$) between the *C. difficile* and total flora probably did not have any effect on microbiota. No repetition of theses experimentations could be carried out.

In conclusions, when the faeces were added, no spore germination or growth was observed in the fermenters or the anaerobic chamber. When the pH was controlled between 6.6-6.9, a growth was observed. *C. difficile* can germinate and grow with addition of faeces mimicking the descending colon when all favourable conditions are present *in vitro*. The system induced a modification of microbiota with an increase of *Escherichia*/*Shigella* spp. and *Enterobacteriaceae*. It was shown that regardless of the donor microbiota, when all the conditions are met, *C. difficile* can germinate, grow, and produce its toxins in a static *in vitro* model. In order to confirm the link between gut microbiota and its evolution and *C. difficile,* a complete *in vitro* dynamic digestive tract model will be used. More research is needed to establish in particular a link between *Enterobacteriaceae* abundance and the growth of *C. difficile,* and the predisposition factors for asymptomatic colonization and/or CDI is also required. This model helped us to understand the parameters governing the germination and the growth of *C. difficile* and to study the impact of the gut microbiota on *C. difficile* infection pathogenesis.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

FUNDING

This work was supported by "FSR 2019 – crédit sectoriel de recherche en sciences de la santé" granted by the University of Liege for the MICO-CDI project.

AUTHOR CONTRIBUTIONS

Elisa Martinez: Methodology, investigation, conceptualization, funding acquisition, data analysis, writing. **Cristina Rodriguez:** methodology, writing. **Sebastien Crèvecoeur:** investigation. **Sarah Lebrun:** methodology, investigation. **Véronique Delcenserie:** methodology, supervision, validation. **Bernard Taminiau:** methodology, funding acquisition, data analysis, supervision, validation. **Georges Daube:** methodology, funding acquisition, supervision, validation.

ETHICAL STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Hospital-Faculty Ethics Committee of the University of Liège, Belgium (2016/331-BE7072016306919) (date of approval, 2016).

ACKNOWLEDGEMENTS

We want to thank Fanny Bellot who worked with us on the *in vitro* static experiments.

REFERENCES

Amrane S, Hocquart M, Afouda P *et al.* Metagenomic and culturomic analysis of gut microbiota dysbiosis during *Clostridium difficile* infection. *Sci Rep* 2019;**9**:12807.

Awad MM, Johanesen PA, Carter GP *et al. Clostridium difficile* virulence factors: Insights into an anaerobic spore-forming pathogen. *Gut Microbes* 2014;**5**:579–93.

Bavishi C, DuPont HL. Systematic review: the use of proton pump inhibitors and increased susceptibility to enteric infection: Systematic review: proton pump inhibitors and bacterial diarrhoea. *Aliment Pharmacol Ther* 2011;**34**:1269–81.

Bondue P, Lebrun S, Taminiau B *et al.* A toddler SHIME® model to study microbiota of young children. *FEMS Microbiol Lett* 2020;**367**:fnaa135.

Brettin T, Davis JJ, Disz T *et al.* RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci Rep* 2015;**5**:8365.

Britton RA, Young VB. Interaction between the intestinal microbiota and host in *Clostridium difficile* colonization resistance. *Trends Microbiol* 2012;**20**:313–9.

Buffie CG, Bucci V, Stein RR *et al.* Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nature* 2015;**517**:205–8.

Centers for Disease Control and Prevention. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Service, CDC; 2019. DOI: 10.15620/cdc:82532.

Connor MC, McGrath JW, McMullan G *et al.* Development of an optimized broth enrichment culture medium for the isolation of *Clostridium difficile*. *Anaerobe* 2018;**54**:92–9.

Crobach MJT, Vernon JJ, Loo VG *et al.* Understanding *Clostridium difficile* Colonization. *Clin Microbiol Rev* 2018;**31**, DOI: 10.1128/CMR.00021-17.

Delmée M, Vandercam B, Avesani V *et al.* Epidemiology and prevention of *Clostridium difficile* infections in a leukemia unit. *Eur J Clin Microbiol* 1987;**6**:623–7.

Gérard A, El-Hajjaji S, Burteau S *et al.* Study of the microbial diversity of a panel of Belgian artisanal cheeses associated with challenge studies for *Listeria monocytogenes*. *Food Microbiol* 2021;**100**:103861.

Giel JL, Sorg JA, Sonenshein AL *et al.* Metabolism of Bile Salts in Mice Influences Spore Germination in *Clostridium difficile*. Ratner AJ (ed.). *PLoS ONE* 2010;**5**:e8740.

Han S-H, Yi J, Kim J-H *et al.* Composition of gut microbiota in patients with toxigenic *Clostridioides* (*Clostridium*) *difficile*: Comparison between subgroups according to clinical criteria and toxin gene load. Popoff MR (ed.). *PLoS ONE* 2019;**14**:e0212626.

Hernandez DR. Gut Check: *In Vitro* Diagnostics for Gut Microbiome Analysis. *Clin Microbiol Newsl* 2019;**41**:57–62.

Jackson MA, Goodrich JK, Maxan M-E *et al.* Proton pump inhibitors alter the composition of the gut microbiota. *Gut* 2016;**65**:749–56.

Jeon YD, Ann HW, Lee WJ *et al.* Characteristics of Faecal Microbiota in Korean Patients with *Clostridioides difficile*-associated Diarrhea. *Infect Chemother* 2019;**51**:365–75.

Kadiroğlu P, Korel F, Ceylan C. Quantification of *Staphylococcus aureus* in white cheese by the improved DNA extraction strategy combined with TaqMan and LNA probe-based qPCR. *J Microbiol Methods* 2014;**105**:92–7.

Khanna S, Montassier E, Schmidt B *et al.* Gut microbiome predictors of treatment response and recurrence in primary *Clostridium difficile* infection. *Aliment Pharmacol Ther* 2016;**44**:715–27.

King CH, Desai H, Sylvetsky AC *et al.* Baseline human gut microbiota profile in healthy people and standard reporting template. Goel A (ed.). *PLoS ONE* 2019;**14**:e0206484.

Kochan TJ, Foley MH, Shoshiev MS *et al.* Updates to *Clostridium difficile* Spore Germination. Margolin W (ed.). *J Bacteriol* 2018a;**200**, DOI: 10.1128/JB.00218-18.

Kochan TJ, Shoshiev MS, Hastie JL *et al.* Germinant Synergy Facilitates *Clostridium difficile* Spore Germination under Physiological Conditions. Ellermeier CD (ed.). *mSphere* 2018b;**3**:e00335-18, /msphere/3/5/mSphere335-18.atom.

Kochan TJ, Somers MJ, Kaiser AM *et al.* Intestinal calcium and bile salts facilitate germination of *Clostridium difficile* spores. Koehler TM (ed.). *PLOS Pathogens* 2017;**13**:e1006443.

Lawson PA, Citron DM, Tyrrell KL *et al.* Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O'Toole 1935) Prévot 1938. *Anaerobe* 2016;**40**:95–9.

Lebrun S, Van Nieuwenhuysen T, Crèvecoeur S *et al.* Influence of reduced levels or suppression of sodium nitrite on the outgrowth and toxinogenesis of psychrotrophic *Clostridium botulinum* Group II type B in cooked ham. *Int J Food Microbiol* 2020;**334**:108853.

Lupp C, Robertson ML, Wickham ME *et al.* Host-Mediated Inflammation Disrupts the Intestinal Microbiota and Promotes the Overgrowth of *Enterobacteriaceae*. *Cell Host Microbe* 2007;**2**:119–29.

Mahnic A, Breskvar M, Dzeroski S *et al.* Distinct Types of Gut Microbiota Dysbiosis in Hospitalized Gastroenterological Patients Are Disease Non-related and Characterized With the Predominance of Either *Enterobacteriaceae* or *Enterococcus*. *Front Microbiol* 2020;**11**:120.

Martinez E, Taminiau B, Rodriguez C *et al.* Gut Microbiota Composition Associated with *Clostridioides difficile* Colonization and Infection. *Pathogens* 2022;**11**(7), 781. DOI:10.3390/pathogens11070781

MetaHIT Consortium (additional members), Arumugam M, Raes J *et al.* Enterotypes of the human gut microbiome. *Nature* 2011;**473**:174–80.

Milani C, Ticinesi A, Gerritsen J *et al.* Gut microbiota composition and *Clostridium difficile* infection in hospitalized elderly individuals: a metagenomic study. *Sci Rep* 2016;**6**:25945.

Molly K, Woestyne MV, Smet ID *et al.* Validation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) Reactor Using Microorganism-associated Activities. *Microb Ecol Health Dis* 1994;**7**:191–200.

Monot M, Eckert C, Lemire A *et al. Clostridium difficile*: New Insights into the Evolution of the Pathogenicity Locus. *Sci Rep* 2015;**5**, DOI: 10.1038/srep15023.

Mutters R, Nonnenmacher C, Susin C *et al.* Quantitative detection of *Clostridium difficile* in hospital environmental samples by real-time polymerase chain reaction. *Journal of Hospital Infection* 2009;**71**:43–8.

Ngo J, Taminiau B, Fall PA *et al.* Ear canal microbiota - a comparison between healthy dogs and atopic dogs without clinical signs of otitis externa. *Vet Dermatol* 2018;**29**:425-e140.

Paredes-Sabja D, Bond C, Carman RJ *et al.* Germination of spores of *Clostridium difficile* strains, including isolates from a hospital outbreak of *Clostridium difficile*-associated disease (CDAD). *Microbiology* 2008;**154**:2241–50.

Pérez-Cobas A, Moya A, Gosalbes M *et al.* Colonization Resistance of the Gut Microbiota against *Clostridium difficile*. *Antibiotics* 2015;**4**:337–57.

Raina M. Maier ILP. Bacterial growth. *Environ Microbiol* third edition. Academic Press, 2015, 37–56.

Rodriguez C, Fernandez J, Van Broeck J *et al. Clostridium difficile* presence in Spanish and Belgian hospitals. *Microb Pathog* 2016;**100**:141–8.

Rodriguez C, Taminiau B, Brévers B *et al.* Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of *Clostridium difficile* at hospital admission. *BMC Microbiol* 2015;**15**:181.

Rodriguez C, Taminiau B, Van Broeck J *et al. Clostridium difficile* in young farm animals and slaughter animals in Belgium. *Anaerobe* 2012;**18**:621–5.

Samarkos M, Mastrogianni E, Kampouropoulou O. The role of gut microbiota in *Clostridium difficile* infection. *Eur J Intern Med* 2018;**50**:28–32.

Sangster W, Hegarty JP, Schieffer KM *et al.* Bacterial and Fungal Microbiota Changes Distinguish *C. difficile* Infection from Other Forms of Diarrhea: Results of a Prospective Inpatient Study. *Front Microbiol* 2016;**7**, DOI: 10.3389/fmicb.2016.00789.

Shimizu K, Seiki I, Goto Y *et al.* Measurement of the Intestinal pH in Mice under Various Conditions Reveals Alkalization Induced by Antibiotics. *Antibiotics* 2021;**10**:180.

Shrestha R, Sorg JA. Hierarchical recognition of amino acid co-germinants during *Clostridioides difficile* spore germination. *Anaerobe* 2018;**49**:41–7.

Song Y, Garg S, Girotra M *et al.* Microbiota Dynamics in Patients Treated with Fecal Microbiota Transplantation for Recurrent *Clostridium difficile* Infection. Berg G (ed.). *PLoS ONE* 2013;**8**:e81330.

Sorg JA, Sonenshein AL. Bile Salts and Glycine as Cogerminants for *Clostridium difficile* Spores. *J Bacteriol* 2008;**190**:2505–12.

Sorg JA, Sonenshein AL. Chenodeoxycholate Is an Inhibitor of *Clostridium difficile* Spore Germination. *JB* 2009;**191**:1115–7.

Stewart DB, Hegarty JP. Correlation between virulence gene expression and proton pump inhibitors and ambient pH in Clostridium difficile: results of an *in vitro* study. *J Med Microbiol* 2013;**62**:1517–23.

Wetzel D, McBride SM. The Impact of pH on *Clostridioides difficile* Sporulation and Physiology. *Appl Environ Microbiol* 2020;**86**:13.

Wheeldon LJ, Worthington T, Hilton AC *et al.* Physical and chemical factors influencing the germination of *Clostridium difficile* spores. *J Appl Microbiol* 2008;**105**:2223–30.

Winter SE, Lopez CA, Bäumler AJ. The dynamics of gut-associated microbial communities during inflammation. *EMBO Rep* 2013;**14**:319–27.

Zhang L, Dong D, Jiang C *et al.* Insight into alteration of gut microbiota in *Clostridium difficile* infection and asymptomatic *C. difficile* colonization. *Anaerobe* 2015;**34**:1–7.

FIGURE LEGENDS

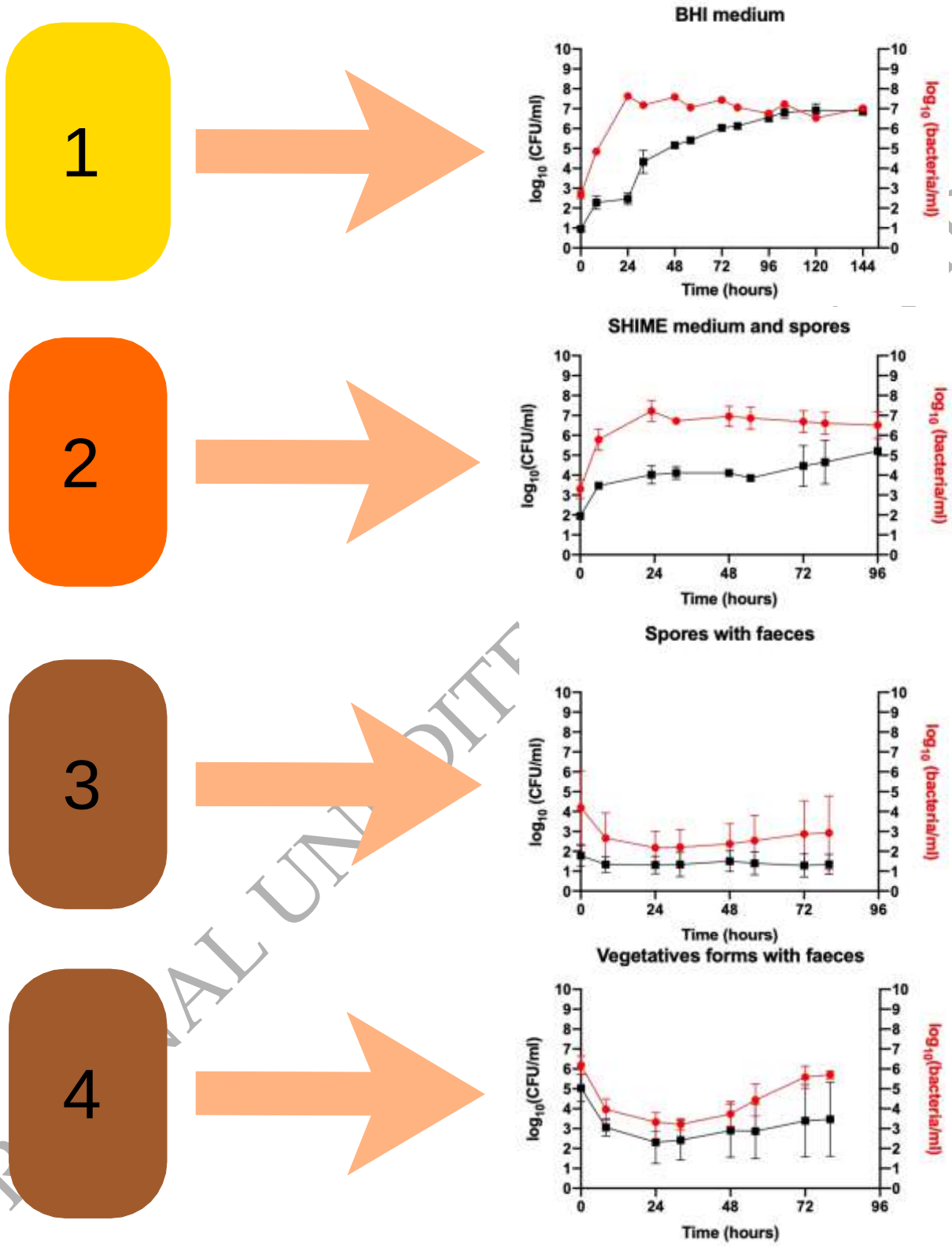

Figure 1 – Growth curve of *C. difficile* in different media. On the left axis in black, the results of classical microbiology are expressed as $\log_{10}$ (CFU/ml) and on the right axis in red, the results of the qPCR targeting 16S rRNA gene of *C. difficile* are expressed as $\log_{10}$ (bacteria/ml). (1) Growth curve of *C. difficile* spores in BHI (n=3); (2) Growth curve of *C. difficile* spores in SHIME medium (n=2); (3) Mean of the four growth curve of *C. difficile* spores in SHIME medium and faeces (n=3); (4) Mean of the four growth curve of *C. difficile* vegetative forms in SHIME medium and faeces (n=3). (●) Quantification of the 16S rDNA genes of *C. difficile* expressed as $\log_{10}$ (bacteria/ml). (■): Quantification of *C. difficile* on CCFAT expressed as $\log_{10}$ (CFU/ml).

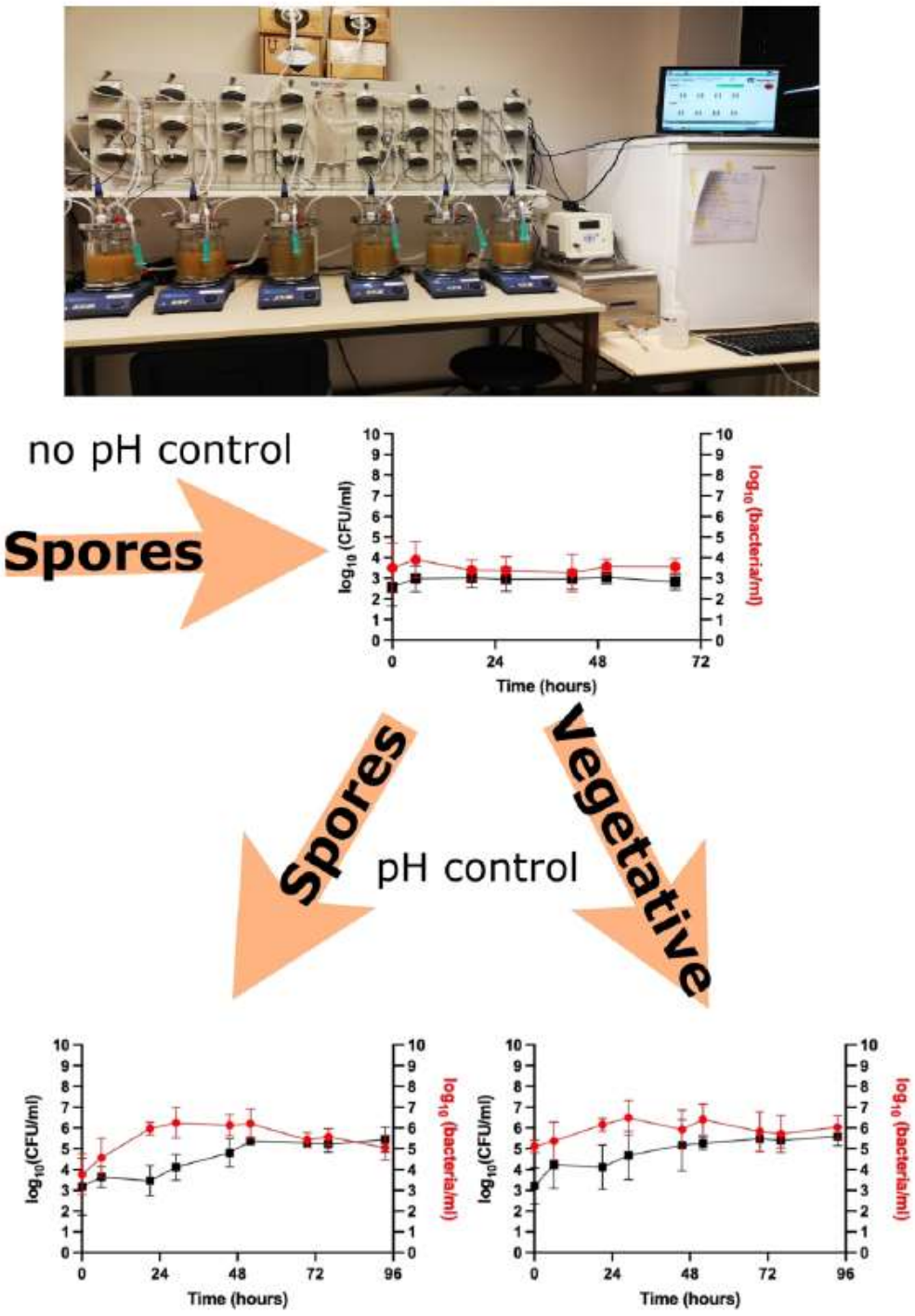

Figure 2 – Growth curve of *C. difficile* in SHIME medium with and without pH control and faeces. On the left axis, the results of classical microbiology are expressed as $\log_{10}$ (CFU/ml), and on the right axis, the results of qPCR targeting 16S rDNA specific to *C. difficile* are expressed as $\log_{10}$ (bacteria/ml). (S) Mean of the four growth curve of *C. difficile* spores in SHIME medium and faeces; (V) Mean of the four growth curve of *C. difficile* vegetative

forms in SHIME medium and faeces. (●) Quantification of the 16S rRNA gene-specific regions of *C. difficile* expressed as $\log_{10}$ (bacteria/ml). (■): Quantification of *C. difficile* in CCFAT expressed as $\log_{10}$ (CFU/ml).

All microbiological enumeration results are described as colony forming units/ml (CFU/ml), and all qPCR of 16S rDNA targeting specific *C. difficile* results are reported as bacteria/ml.

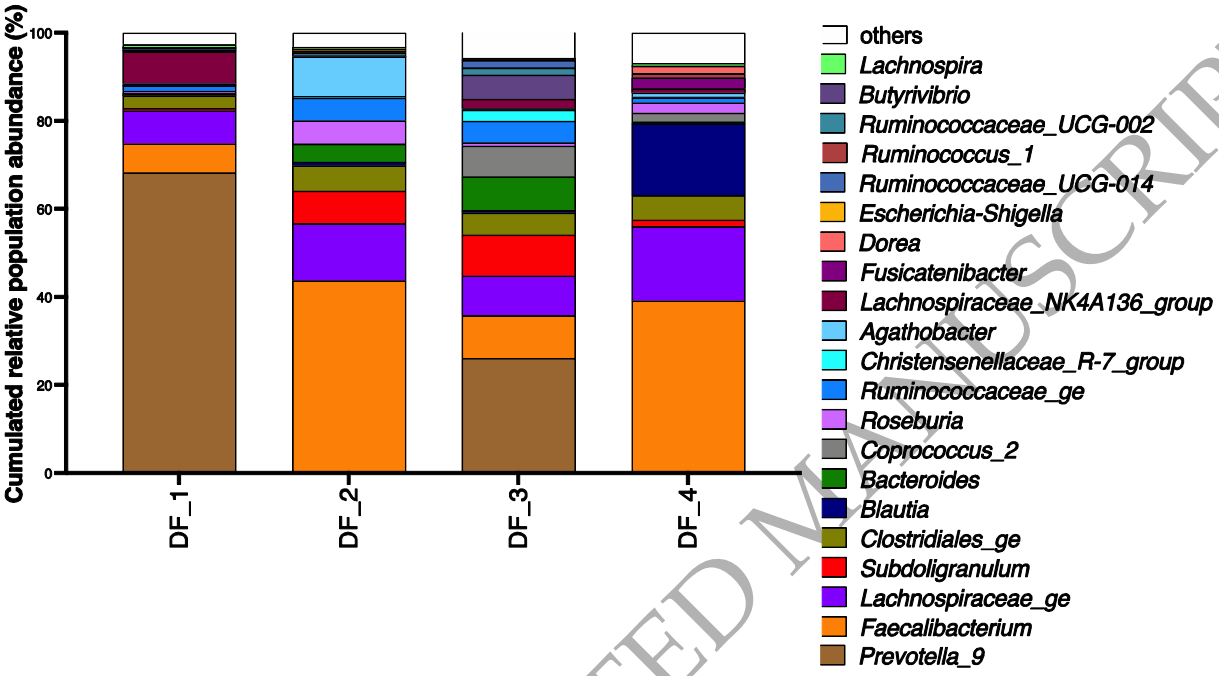

Figure 3 – Cumulative relative population abundance of bacterial genera in the four donor feces (DF).

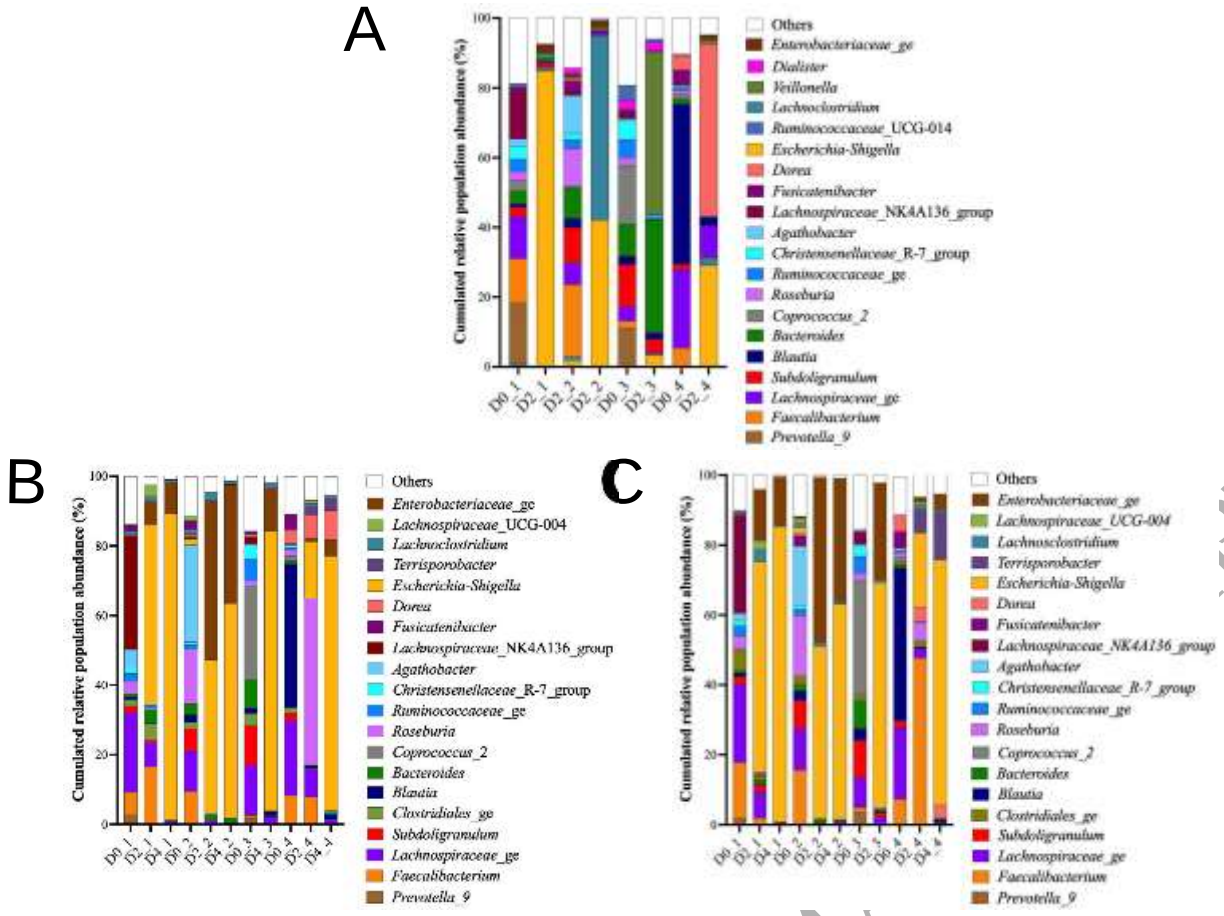

Figure 4 – Cumulative relative population abundance in the fermenters mimicking the descending colon (A) on day 0 and day 2 with *C. difficile* spores without pH control, (B) *C. difficile* spores with pH control between 6,6-6,9 on day 0, day 2 and day 4 and (C) vegetative cells. Two fecal samples from donor 3 are missing.

Tables:

Table 1 – Specific detailed information of the growth conditions in a static batch in an anaerobic chamber.

Study	pH initial	pH control	T (°C)	Spores inoculation (log_{10} CFU/ml)	Vegetative forms inoculation (log_{10} CFU/ml)	Replicates	Duration (h)
1	7	No	37 °C	1.5	NR	Triplicate	144 h
2	7.4	No	37 °C	1.5	NR	Duplicate	96 h
3	7.2	No	37 °C	1.77	NR	Triplicate	80 h
4	7.2	No	37°C	NR	5	of each 4 donors	80 h

(1) Growth curve of *C. difficile* spores in BHI; (2) Growth curve of *C. difficile* spores in SHIME medium; (3) Growth curve of *C. difficile* spores in SHIME medium and feces; (4) Growth curve of *C. difficile* vegetative forms in SHIME medium and feces.

Table 2 – Specific detailed information of the growth conditions in the microbial fermenters under controlled conditions.

Study	pH initial	pH control	T (°C)	Spores inoculation (log_{10} CFU/ml)	Vegetative forms inoculation (log_{10} CFU/ml)	Replicates	Duration (h)
1	6.9	No	37 °C	2.54	NR	4 donors	60 h
2	6.9	6.6-6.9	37 °C	3.18	NR	4 donors	76 h
3	6.9	6.6-6.9	37 °C	NR	3.22	4 donors	76 h

NR: not realized