Culturing *Clostridium difficile* and the human gut microbiome

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Table of Content

Samenvatting

In dit verslag wordt het onderzoek naar het aërobisch kweken van *Clostridium difficile* beschreven. Ook wordt er een methode beschreven om het humane intestinale microbioom in kaart te brengen met behulp van culturomics, het bestuderen van microbiële samenstellingen door op grote schaal bacteriën te kweken.

C. difficile is een spoorvormende, gram positieve staaf en kan onder normale omstandigheden alleen anaeroob gekweekt worden. *C. difficile* is namelijk zeer gevoelig voor lage zuurstofgehaltes. Door twee verschillende stoffen, ascorbinezuur en glutathion, die beide als antioxidanten werken toe te voegen aan medium werd *C. difficile* gekweekt onder aërobe incubatie. De groei werd weergegeven door een groeicurve op te stellen bij aërobische en anaërobische incubatie. Tevens werd er gekeken naar de verschillen in toxineproductie en spoorformatie onder aërobische en anaërobische condities en of deze gelijk zijn gebleven aan elkaar. Door *C. difficile* aeroob te kweken kan het kweekproces zeer vereenvoudigd worden.

Met behulp van Vero E6 cellen werd de toxineproductie aangetoond. Ook werd sporulatie aangeduid door middel van fluorescentiemicroscopie en met een sporulatietest waarbij *C. difficile* blootgesteld werd aan ethanol en vervolgens werd opgekweekt.
Naast dit onderzoek werd er onderzocht of er onbekende bacteriën, die aanwezig zijn in het intestinale microbioom, geïdentificeerd kunnen worden door een kweekmethode te optimaliseren om zodoende het microbioom te kunnen karakteriseren. Het intestinale microbioom bestaat uit verschillende bacteriën die van nature voorkomen in het lichaam en die ervoor zorgen dat pathogene bacteriën niet in staat zijn om te groeien.

Het optimaliseren van deze kweekmethode werd gerealiseerd door het combineren van culturomics, massaspectrometrie en sequencing. Dit werd gedaan door fecesmonsters met en zonder ethanol te behandelen en deze vervolgens op te kweken op verschillende voedingsbodems. Het behandelen van de monsters met ethanol zorgde ervoor dat bacteriën werden afgedood waardoor alleen de sporen opgekweekt werden. Identificatie van de gegroeide kolonies werd gedaan met behulp van MALDI-TOF. Kolonies waarvan er geen identificatie mogelijk was met MALDI-TOF zijn geïdentificeerd met behulp van 16s rRNA sequencing.
In dit onderzoek was het aërobisch kweken van *C. difficile* met behulp van stoffen met een antioxidante werking geslaagd. Bij het inoculeren van het medium met *C. difficile* kon na het incuberen bij 37°C gedurende de nacht, groei geobserveerd worden. Mogelijke zorgen waren dat het aëroob kweken invloed zou kunnen hebben op toxineproductie of sporulatie van *C. difficile*. Echter werd er geen verschil in toxineproductie aangetoond bij zowel, aerobe incubatie, als anaerobe incubatie. Ook werd er aangetoond dat *C. difficile* onder aerobe omstandigheden in staat is sporen te vormen.
Voor het kweken van de intestinale microbioom is er een optimale methode beschreven namelijk het kweken van fecesmonsters met en zonder een ethanol behandeling. Vervolgens werd het identificeren van kolonies met de MALDI-TOF methode vastgesteld en ten slotte werden kolonies die niet geïdentificeerd konden worden met de MALDI-TOF methode gesequenced met behulp van 16s RNA sequencing.
Vier verschillende bacteriële stammen konden niet geïdentificeerd worden met MALDI-TOF. Het uitvoeren van een 16s rRNA sequencing, resulteerde in een identificatie van de vier verschillende bacteriële stammen. Alhoewel er geen onbekende bacteriën werden gekweekt, is er wel een goede methode beschreven voor het kweken van bacteriën op grote schaal.

Introduction

Clostridium difficile is a gram positive, rod shaped bacterium that is an obligate anaerobe.[1] *C. difficile* resides in the intestinal tract and is the primary cause of hospital-acquired diarrhea.[1,2] It was first described by Hall and O'Tool in 1935 as being part of a healthy newborn infants flora.[1,3] It was then discovered that *C. difficile* could cause pseudomembranous colitis when patients are treated with antibiotics.[6]

A *Clostridium difficile* infection (CDI) normally presents itself after antibiotics use. Antibiotic use disrupts the normal intestinal flora, creating a niche for resistant *C. difficile* to grow. Symptoms resulting from a CDI may range from mild or moderate diarrhea, inflammatory lesions, fulminant pseudomembranous colitis, sepsis and even death.[5,6] A CDI can be life threatening, especially in elderly people with a underlying disease.[1]

The primary virulence factor of *C. difficile* is its ability to produce toxins. *C. difficile* can produce Toxin A, Toxin B and in some strains the binary toxin. These toxins can then cause the above-mentioned severe symptoms by triggering a cascade of host cell responses.[1,4]

C. difficile can produce spores. Vegetative cells are only able to survive in anaerobic conditions whereas spores can survive several harsh environments. *C. difficile* spores are also resistant to several disinfectants, heat, desiccation and antimicrobial agents.[1,6,7] This makes eliminating *C. difficile* spores more difficult. Spores can survive in hospital environments for several months to years.[8]

Transmission of *C. difficile* takes place through the fecal-oral route. Spores produced by *C. difficile* end up in the gastrointestinal tract, germinate and colonize the intestine continuing its life cycle.[9] Cultivation of *C. difficile* is of great importance to be able to diagnose a CDI. Since the normal habitat of *C. difficile* is anaerobic, *C. difficile* can only be grown anaerobically. *C. difficile* is in fact extremely sensitive to even low levels of oxygen in the environment. This requires laboratories to have anaerobic chambers or anaerobic jars to be able to cultivate *C. difficile*. But being classified as strict anaerobic also makes it harder to perform experiments where oxygen is needed. One example is experiments using fluorescence. Most fluorophores, like Green Fluorescent Protein (GFP) require oxygen to work. This can make using GFP or other fluorophores that require oxygen with *C. difficile* problematic.

C. difficile is not the only bacterium occupying the human gut. The intestinal microbiome consists of many different bacteria. Thus, there is a great variety of bacteria present in the intestinal flora. A balanced and diverse commensal flora is vital for our health and wellbeing and ensures that pathogenic bacteria are not able to grow.[10] Microbial cultures were first used to study the gut microbiota but later this switched to molecular techniques, particularly DNA sequencing.[12] For example, sequencing of the 16s ribosomal RNA (rRNA) gene is used to study the phylogeny and taxonomy of different bacteria. The 16s rRNA gene (1500 base pairs (bp)) is located in the bacterial genome. This method of sequencing is a common amplicon sequencing method to identify bacteria. Since the introduction of sequencing/metagenomics, which uses high-throughput sequencing to study metagenomes [13], culturing bacteria is considered old-fashioned by many researchers. As a result there has been little progress in microbial culturing in the clinical microbiology compared to molecular biology. Approximately 80% of the bacterial species discovered in the intestines by molecular diagnostics are uncultured or are considered unculturable. Yet a pure bacterial culture is essential when studying gut microbiota.[12]

The reason being that associations between the microbiota and human disease, which are reported in 16s rRNA studies, can only be functionally validated when the actual bacterial strain is isolated and tested in models. For example to study virulence, resistance and why a particular bacterium is associated with a disease. This explains why researchers still depend on culture-based diagnosis

despite the popularity of molecular methods. Thus 'Culturomics' came about, the study of microbial compositions by high-throughput culturing.

Culturomics allows for the isolation and identification of new bacterial species previously not detected by current molecular techniques by using selective or enriched culture media to cultivate bacteria. It gives a more comprehensive understanding of the microbial composition by high-throughput culturing while complimenting molecular techniques and giving another insight to the composition of microbial environments.[11] An application of culturomics is the culturing of the human intestinal microbiome.[12, 13]

With this study we aim to elucidate:

— Whether it is possible to grow the obligate anaerobic bacterium *C. difficile* under aerobic conditions.
— Whether there are unknown bacteria that are present in the intestinal microbiome that can be identified by optimizing a culturing method to characterize fecal microbiota.

The aerobic cultivation of *C. difficile* can have many positive effects. It can greatly facilitate the process of culturing *C. difficile*, which can permit all laboratories to cultivate *C. difficile* without utilizing anaerobic chamber and other expensive materials.

Optimizing a culture method will be achieved by combining 'culturomics', together with mass spectrometry, to rapidly identify bacteria living in the human intestine, and sequencing.

Hypothesis

Research has shown that it is possible to grow anaerobic bacteria in an aerobic environment.[14,16] In this study it is expected that *C. difficile* will be cultivated aerobically by creating a special medium that is supplemented with the antioxidants ascorbic acid and glutathione.

Research also suggests that high-throughput culturing can lead to extend the human gut microbiota repertoire.[11,12,13] In this study we expect that optimizing a method for high-throughput culturing and identification of bacteria will be achieved.

Experimental design

For *C. difficile* to grow aerobically, a special medium is created as described by D. Raoult *et al*.[14] As a base medium Brain Heart Infusion broth (BHI) will be used instead of Schaedler agar. Schaedler broth was not at our disposal at the time, therefore BHI was chosen as it is also a rich media which is frequently used to culture a broad range of gut bacteria. BHI is usually used for the cultivation of fastidious pathogenic microorganisms.[15] Antioxidants are then added to this liquid medium. By adding the antioxidants, it is expected that *C. difficile* will be able to grow aerobically.[14,16] Four different ribotypes (RT) of *C. difficile* will be used i.e. RT001, RT017, RT027 and RT078. These ribotypes were chosen to represent phylogenetic diversity among the different *C. difficile* types. Subsequently, if culturing under aerobic conditions is possible, the effects of oxygen on toxin production and spore formation will be investigated. Fluorescence microscopy will also be used to observe spore formation.

In addition to this research the human fecal microbiome will be cultured. This will be done by culturing stool samples. Samples will be treated with and without an ethanol shock. The ethanol shock ensures that vegetative cells are killed and that only spores will be cultured. Stool samples will be serially diluted and plated on different media plates to ensure that a diverse amount of bacteria will grow. TSS (trypcase Soy agar + 5% sheep blood), Brain heart infusion agar supplemented with yeast extract and CNA (columbia naladixic acid agar) plates will be used. Grown colonies will then be identified using MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time of Flight). MALDI-TOF is a technique in which an intact bacterium is shot by a laser.

Proteins are ionized and separated based on mass and charge. Small proteins reach the detector more quickly than large molecules. The mass / charge ratio is processed in a spectrum and compared to the spectra of known bacteria to obtain an identification. Subsequently the bacteria strain is identified or a 'not reliable identification' is given.[17,18] Bacteria that cannot be identified using MALDI-TOF will be re-streaked and saved in an isolate collection to sequence. Identifying colonies cultured from stool samples using culturomics will give a representation of the bacteria living in the gut and may lead to bacteria that have not yet been cultured as part of the gut microbiome.

Materials and Method

Aerobic cultivation of C. difficile

1.1 Aerobic growth of C. difficile in liquid medium

Aerobic cultivation of *C. difficile* in liquid medium supplemented with antioxidants.

Medium
C. difficile was grown aerobically by adding compounds with an antioxidant activity (ascorbic acid and glutathione) to Brain Heart Infusion (BHI) broth (Thermo-Fisher) supplemented with yeast extract (Sigma-Aldrich), prepared according to the supplier's recommendation. A 20x stock solution was made for ascorbic acid (Sigma-Aldrich) and glutathione (Sigma-Aldrich). The pH was set for both solutions at pH 7,5 using 37% HCl and 10M/1M NaOH. Both stock solutions were filter sterilized using a 0,2 μm filter. After autoclaving the BHI/Yeast extract broth, the antioxidants were added to the medium to obtain a final concentration of 10 mg/ml ascorbic acid and 1 mg/ml glutathione.

Strains
Four different types of *C. difficile* were tested i.e. RT001, RT017, RT027 and RT078. Each type was inoculated from a glycerol stock and plated on blood TSS (Trypcase Soy agar + 5% sheep blood) plates. These plates were incubated in an anaerobic chamber (Whitley Scientific minimacs anaerobic workstation) for 48 hours. All four types of *C. difficile* were inoculated by re-suspending a number of colonies, using an inoculation loop, in the medium supplemented with antioxidants. These test tubes were then incubated aerobically overnight without CO_2 at 37˚C. BHI/Yeast extract broth was also inoculated and incubated aerobically overnight without CO_2 at 37˚C to serve as a negative control. Furthermore a positive control was added by inoculating BHI/Yeast extract broth with antioxidants which was incubated in an anaerobic chamber. Non-inoculated medium was also incubated under aerobic and anaerobic conditions to serve as a sterility control.

Growth curve
To follow the growth kinetics/dynamics of *C. difficile* in an aerobic environment a growth curve was made. *C. difficile* RT078 was inoculated in BHI/yeast extract medium supplemented with antioxidants. The optical density (OD) was measured every hour for a total of nine hours while being incubated aerobically at 37˚C.

1.2 Toxin Expression aerobically grown C. difficile

C. difficile can produce toxins under normal conditions. To see if it is also the case under aerobic conditions toxin expression was observed by visually determining differences in the morphology of Vero E6 cells due to toxin production.[19]

To determine if *C. difficile* grown aerobically can still produce toxins Vero E6 cells were used. Vero E6 cells were seeded into a 96 wells plate at a density of 1x 10^5 cells per well and incubated overnight at 37˚C. *C. difficile* RT078 was grown aerobically overnight without CO_2 at 37˚C. 1 mL culture sample was collected and centrifuged for 5 minutes at 4000 rpm. The supernatant was collected and filter sterilized with a 0,2 μm filter. Supernatant samples were serially diluted 2, 10^1, 10^2, 10^3, 10^4, 10^5 fold in cell culture medium (BioWhittaker Dulbecco's Modified Eagle medium supplemented with 8% fetal calf serum and Penicillin/Streptomycin). 50 μl of each dilution was then added onto the Vero E6 cell monolayers and incubated for 1 hour at 37˚C. Anti-toxin (TechLab) was also added in 2 fold to supernatant samples and added onto the Vero E6 cell monolayers to serve as the negative control.

After 1 hour of incubation, the samples were removed and replaced with 200 µl of culture medium. After 24 hours and 48 hours of incubation at 37˚C, the end point titer was determined. The end point titer was defined as the dilution in which the Vero E6 cells morphology are identical to neutralized cells.

1.3 Sporulation Assay with Ethanol Shock

To assess if *C. difficile* still produces spores when cultivated aerobically an ethanol shock was applied to *C. difficile* samples to eliminate vegetative cells and allow spores to cultivate if present.

Culture samples were incubated for 24 hours and 48 hours at 37˚C. 1 mL culture sample was collected and an equal volume ethanol 95% was added to the sample. The sample was vortexed regularly over one hour. After applying the ethanol shock, samples were serially diluted 10^1 and 10^2 fold. Culture samples were then plated onto BHI/yeast extract plates supplemented with 0,1% taurocholate and incubated for 48 hours. Colony forming units were then observed.

1.4 Sample preparation for fluorescence microscopy

Fluorescence microscopy was used as well to determine if there was spore formation by *C. difficile* under aerobic incubation. 1 ml of overnight culture was transferred to an 1.5 ml eppendorf tube and centrifuged for 1.5 minutes at 4000 rpm. Supernatant was discarded and the pellet was re-suspended in 1 ml PBS. This was done twice. After centrifuging the sample once more, 900 µl of the supernatant was discarded and the pellet was re-suspended in the remaining 100 µl PBS. 1 µl FM4-64 (1 µg/µl- final conc. 10 µg/ml) or 1 µl MTG (0.1 µg/µl – final conc. 1 µg/ml) was added and mixed carefully. On a pre-reduced 1.5% agarose patch 2 µl sample was spotted and covered with a cover slip. An inverted fluorescence microscope (Leica AF6000) using the LAS AF software (Leica Microsystems) was used to view the slides and for imaging.

Culturomics gut microbiota

Culturing the human gut microbiota includes steps to process stools samples, cultivate different bacteria and identifying these bacteria using mass spectrometry and sequencing.

2.1 Processing of stool sample for anaerobic culturing

Two fecal samples were taken, weighing 250 mg each. One sample was treated with 2.5 ml 70% ethanol. The sample was frequently vortexed for over one hour. After the ethanol shock was applied, the sample was centrifuged at 4000 rpm for 10 minutes. Thereafter the supernatant was discarded and the pellet was re-suspended in PBS. This was repeated twice with final re-suspension at 100mg/ml. The sample was serially diluted 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 fold in PBS. 2.5 ml PBS was added to the other fecal sample. The sample was frequently vortexed for over one hour. This sample was also serially diluted 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 fold in PBS. 125 µl of the dilution samples were then plated onto TSS (trypcase Soy agar + 5% sheep blood), Brain heart infusion agar supplemented with yeast extract and CNA (columbia naladixic acid agar) plates. Plates were incubated 72-96 hours in an anaerobic chamber.

2.2 Identification of colonies using MALDI-TOF

Colonies were picked from the plates with a disposable inoculation loop and thinly spread within the spot on a target-plate (Bruker MSP 96 target polished steel BC). 1 µl matrix (Bruker Matrix HCCA, portioned) was then pipetted on each spot. Colonies were then identified using MALDI-TOF (*Bruker Daltonik MALDI Biotyper*). Colonies that could not be identified, giving a 'not reliable identification', were re-streaked to confirm purity and were run through the MALDI-TOF a second time.

2.3 16s rDNA and Sequencing

DNA was isolated from colonies that could not be identified with the QIAGEN QIAamp® DNA Purification Kit, used according to the manufacturer's instructions. Isolates were identified by PCR amplification of the full-length 16s RNA gene. The 16s7F (5'-AGAGTTTGATYMTGGCTCAG -3') forward primer and 16s1530R (5'-ACGGYTACCTTGTTACGACTT-3') reversed primer were used. For specific cycling conditions see appendix 1. PCR samples were loaded on a 0,8% gel. Positive PCR samples were then purified (Thermo Scientific GeneJet PCR purification Kit). 7.5 µl sample was added to 2,5 µl primer (100 µM) and samples were submitted for sequencing (Leiden Genome Technology Center).

Results

Aerobic cultivation of C. difficile

In this experiment aerobic cultivation of the strictly anaerobic bacterium *C. difficile* was performed. To cultivate *C. difficile* aerobically ascorbic acid and glutathione were added to BHI/yeast extract medium. These two different compounds with antioxidant activity were tested in varying concentrations. The same concentrations that were used in a paper by D. Raoult *et al.*[14] were initially applied. The concentrations used were 1,0 mg/ml ascorbic acid and 0,1 mg/ml glutathione. After having no success with these concentrations, a 10 times higher concentration was tested. Using a concentration of 10 mg/ml ascorbic acid and 1,0 mg/ml glutathione in BHI/yeast extract medium resulted in aerobic growth of *C. difficile*.

After incubating strains aerobically overnight at 37 ˚C growth was observed. Figure 1A shows the negative control, consisting of BHI/yeast extract medium, not supplemented with antioxidants. This resulted in *C. difficile* not growing. In figure 1B, growth can be observed in all four ribotypes of *C. difficile. C. difficile* RT001, RT017, RT027 and RT078 were used in this experiment.
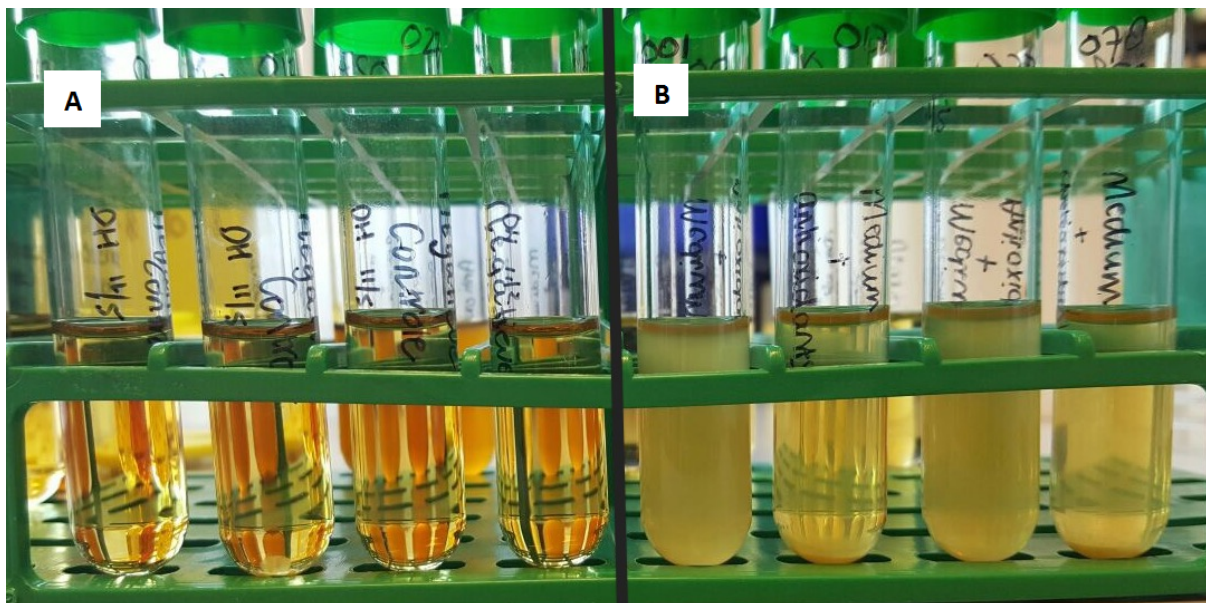

Figure 1: Aerobic overnight cultures of *C. difficile* at 37˚C.
A: Negative control; Aerobic incubation of *C. difficile* in BHI/yeast extract broth without antioxidants. From left to right: RT001, RT017, RT027 and RT078.
B: Aerobic incubation of *C. difficile* in BHI/yeast extract broth with antioxidants. From left to right: RT001, RT017, RT027 and RT078.

Growth of the aerobic culture was visually determined. This was then expressed in growth, more growth or abundant growth for each ribotype. This is illustrated in table 1 (figure 1 coincides with table 1, experiment 3). As can be seen in table 1, RT017 and RT027 show less growth in two of the tree experiments compared to RT001 and RT078. Both RT001 and RT078 grow very well in an aerobic environment, showing abundant growth in two of the tree experiments.
This experiment was performed a total of 3 times. Identification with MALDI-TOF confirmed that the cultivated bacteria were indeed *C. difficile* (see appendix 2).

Table 1: Results of aerobic growth of *C. difficile* determined visually and expressed in growth, more growth or abundant growth

	Experiment 1	Experiment 2	Experiment 3
RT001	+++	++	+++
RT017	+++	+	+
RT027	+	+	+++
RT078	+++	+++	++

+   Growth
++   More growth
+++   Abundant growth

Growth curve

In a separate experiment a growth curve was made to follow the growth of *C. difficile* in an aerobic and anaerobic environment. The Optical Density (OD) was measured every hour for 9 hours. *C. difficile* RT078 was used as the standard strain. Both growth curves are illustrated in figure 2. Aerobic growth is much faster then anaerobic growth and starts its exponential phase after approximately 3 hours of growth. Whereas the exponential phase for anaerobic growth starts after approximately 4 hours of growth. For both growth curves the death phase starts after 8 hours. It can also be observed that with aerobic growth a higher OD is achieved, around 1,1 in comparison to an OD of around 0,7 with anaerobic growth.
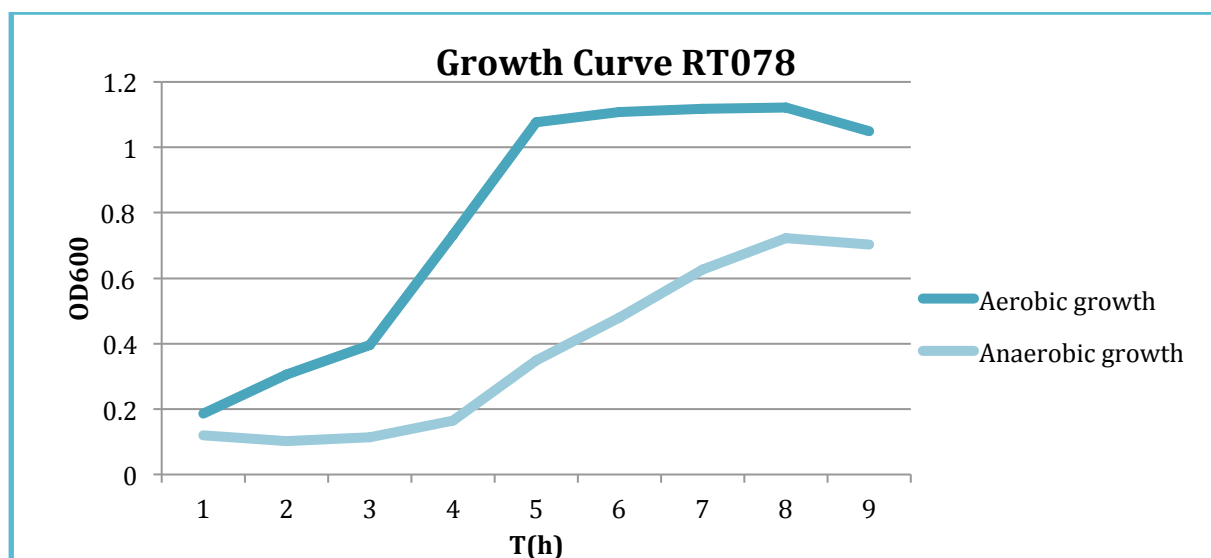

Figure 2: Growth curve *C. difficile* RT078 with aerobic and anaerobic incubation over a 9 hour time period.

Toxin production

With *C. difficile* growing successfully under aerobic conditions specific characteristics of *C. difficile* were evaluated under these aerobic conditions. Characteristics including toxin production and sporulation kinetics.

Toxin expression as determined by cytopathologic effects in Vero E6 cells was tested. *C. difficile* RT078 was used as the standard strain and was cultured aerobically and anaerobically overnight. The supernatant of these samples were serially diluted and then added to Vero E6 cells. Toxin expression as determined by cytopathologic effects in Vero E6 cells was observed after 24 hours and 48 hours of incubation. The cytopathologic effects in Vero E6 cells was visually determined by observing change in morphology of the Vero E6 cells i.e. cell rounding, under a microscope. In table 2 and 3 cell rounding is indicated with a (+) and no change in morphology of the Vero E6 cells are indicated with a (-). This is illustrated with the corresponding dilutions. After 48 hours of incubation toxin expression observed on Vero E6 cells is equal in both samples, aerobic and anaerobic cultured *C. difficile*. This experiment was performed three times with identical results in each replicate.

Table 2: Toxin expression as determined by cytopathologic effects in Vero E6 cells after 24 hours of incubation

24 hour incubation							
Supernatant dilution	1:1	10^1	10^2	10^3	10^4	10^5	10^6
Anaerobic samples	+	+	-	-	-	-	-
Aerobic samples	+	-	-	-	-	-	-

Table 3: Toxin expression as determined by cytopathologic effects in Vero E6 cells after 48 hours of incubation

48 hour incubation							
Supernatant dilution	1:1	10^1	10^2	10^3	10^4	10^5	10^6
Anaerobic samples	+	+	-	-	-	-	-
Aerobic samples	+	+	-	-	-	-	-

Sporulation

Another characteristic of *C. difficile* that was determined was spore kinetics. To ascertain if *C. difficile* could produce spores when incubated under aerobic conditions, samples were dyed with FM4-64 membrane dye and viewed with fluorescence microscopy after 24 hours and 48 hours of incubation. Figure 3a shows *C. difficile* RT078 after 24 hours of aerobic incubation and figure 3b shows the same image but with differential interference contrast fluorescence. Both images illustrate a formed spore still inside a cell. When the spore maturates, cel lysis occurs and the spore is released in its environment. This experiment was performed two times with identical results in each replicate. An anaerobic sample was also viewed as a control. The control had approximately the same amount of spores as the aerobic samples. The anaerobic sample did not differ much from the aerobic sample when looking at sporulation. Approximately ten images were taken of each sample throughout the slide to get a representative view.
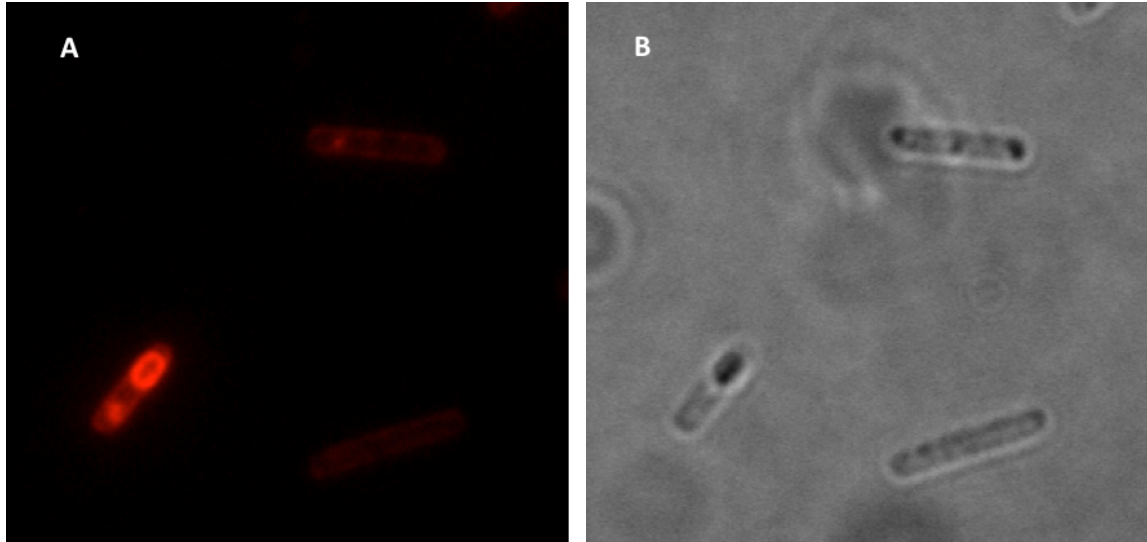
Figure 3a: Fluorescence of *C. difficile* RT078 spore formation under aerobic conditions after 24 hours of incubation using FM4-64 membrane dye.
Figure 3b: DIC fluorescence of *C. difficile* RT078 spore formation under aerobic conditions after 24 hours of incubation.

Figure 4 shows *C. diffcile* RT078 after 48 hours of aerobic incubation. In this figure spore formation is also observed.
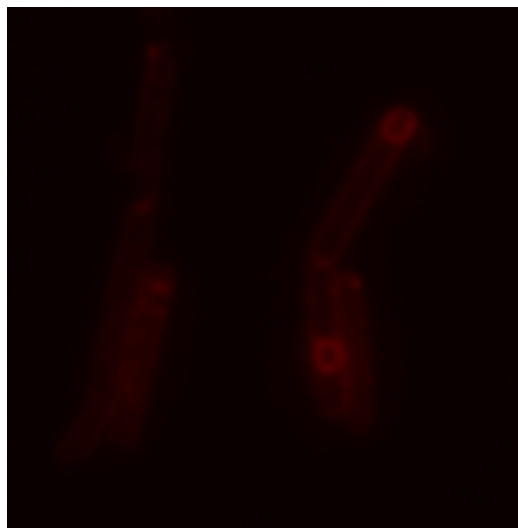

Figure 4: Fluorescence of *C. difficile* RT078 spore formation under aerobic conditions after 48 hours of incubation using FM4-64 membrane dye.

In addition to fluorescence microscopy *C. difficile* cultured aerobically was also treated with an ethanol shock to determine if spore formation still occurs. Treating samples with ethanol ensures that bacteria are killed only spores are cultivated. Growth is illustrated with a (+), no growth with a (-). As can be seen in table 4 both samples contain spores. It is also evident that there are more spores in the anaerobic sample. This experiment was performed three times with identical results in each replicate.

Table 4: Ethanol shocked *C. difficile* growth on BHI/yeast extract plates supplemented with 0,1% taurocholate.

	Undiluted	10^1	10^2
Anaerobic samples	+	+	+
Aerobic samples	+	+	-

Culturing the human fecal microbiota

Using selected and enriched media stool samples were cultured to identify bacteria living in the fecal microbiome. A method was developed to optimize high-throughput culturing to identify bacteria living in the human intestine. Stool samples were first treated with and without an ethanol shock. These samples were then serially diluted and incubated in an anaerobic chamber. The dilutions that showed the best growth after incubation were the 10^4, 10^5 and 10^6 fold dilutions for the untreated samples. The 10^1, 10^2 and 10^3 fold dilutions showed overgrowth and were not suitable for bacterial identification. Furthermore, the 10^1, 10^2 and 10^3 fold dilutions showed the best growth for the ethanol treated samples after incubation. In choosing colonies it is vital that there are enough colonies to pick from and that the colonies are not touching each other. This is to be sure that only one bacterium is being picked at a time for identification. This experiment was performed a total of six times.

In table 5 identified bacterial strains are illustrated. Colonies were picked from different plates and were then identified using MALDI-TOF. The most cultured bacteria were *Bifidobacterium adolescentis* and *Collinsella aerofaciens*. *Bifidobacterium adolescentis* was identified from TSS, BHI/yeast extract and CNA plates. *Collinsella aerofaciens* was identified from BHI/yeast extract and CNA plates.

Table 5: Identified colonies using MALDI-TOF

Identified colonies
Bacteroides ovatus
Bifidobacterium adolescentis
Bifidobacterium longum
Bacteroides uniformis
Butyricimonas virosa
Collinsella aerofaciens
Clostridium disporicum
Eubacterium tenue
Staphylococcus epidermidis
Streptococcus agalactiae
Streptococcus salivarius
Streptococcus gordonii
Streptococcus parasanguinis
Parabacteroides distasonis

When characterizing the human fecal microbiota four bacterial strains were unable to be identified after culturing. These four strains resulted in a 'not reliable identification'. These four samples were then submitted to 16s sequencing. A high fidelity 16s PCR was performed on these four strains and run on a gel with a 1KB+ ladder (Figure 5). Bands are illustrated around the 1600 bp mark confirming that the PCR was successfully performed.
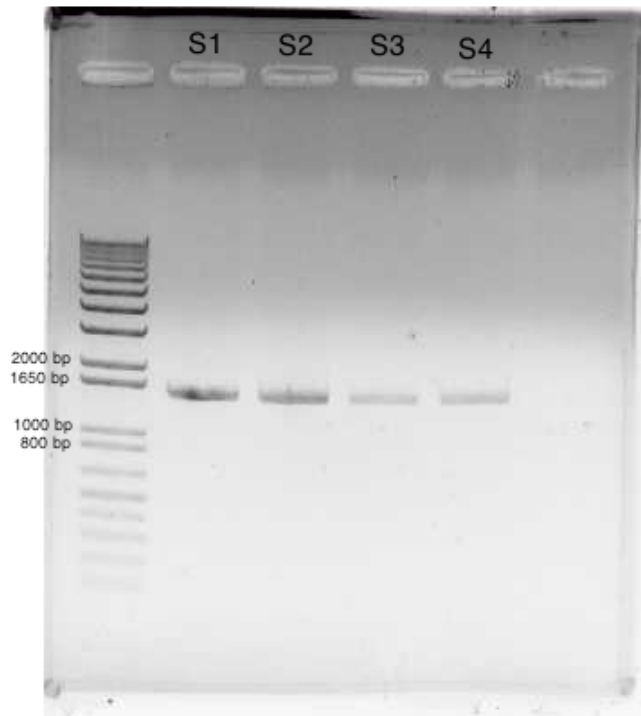

Figure 5: High fidelity PCR on four unidentified bacterial strains named; strain 1 (S1), strain 2 (S2), strain 3 (S3) and strain 4 (S4).

After a positive PCR result, PCR samples were purified and submitted to 16s rRNA sequencing. Each sequence was then identified using The Basic Local Alignment Search Tool (BLAST). This is illustrated in table 6. For full sequence see appendix 3.

Table 6: Results of 16s rRNA sequence and BLAST of strains 1,2,3 and 4.

	Identified as
Strain 1	*Staphylococcus warneri*
Strain 2	*Catenibacterium mitsuokai*
Strain 3	*Bifidobacterium adolescentis*
Strain 4	*Catenibacterium mitsuokai*

Discussion/Conclusion

The first aim of this study was to determine if *C. difficile* could be cultivated aerobically by supplementing BHI/yeast extract medium with antioxidants. It was first described by D. Raoult[14] that adding antioxidants to a medium allows for aerobic growth of bacterial species that are actually anaerobes. Although studies performed by D. Raoult did not specifically focus on *C. difficile*, it was still mentioned as being culturable among many other bacterial strains.

In this study, we succeeded in performing aerobic cultivation of *C. difficile* in liquid medium supplemented with antioxidants. Growth was visually determined and was observed after overnight incubation. 10 mg/ml ascorbic acid and 1 mg/ml glutathione were added to the BHI/yeast extract medium, making it possible for *C. diffcile* to grow. In a paper by D. Raoult *et al*.[14] lower concentrations of antioxidants were used namely 1,0 mg/ml ascorbic acid and 0,1 mg/ml glutathione. Furthermore in an earlier paper by D. Raoult *et al.*[15] lower concentrations of antioxidants were also used. Although cultivating *C. difficile* was achieved, a higher concentration of antioxidants than described in the above-mentioned papers, was needed.

Possible concerns were that the aerobic environment would have an effect on toxin production or sporulation despite observing growth under aerobic conditions. These concerns were tested out with a few experiments. Using cell culturing the effects of *C. difficile* toxin expression as determined by cytopathologic effects in Vero E6 cells were determined. This was also performed in another research using *C. difficile* mutants. The cytotoxic effect of toxins were also observed by cell rounding of Vero E6 cells.[19]
When culturing C. difficile aerobically, it could clearly be observed that *C. difficile* toxins had an effect on the morphology of the Vero E6 cells. Proving that *C. difficile* grown aerobically does show toxin expression as determined by cytopathologic effects in Vero E6 cells in the same matter as anaerobically grown *C. difficile*. However, it did take 48 hours of incubation to achieve the same results as anaerobically grown *C. difficile,* which showed results after only 24 hours of incubation. This could mean there are less toxins being produced when cultivating *C. difficile* in an aerobic environment.

C. difficile is known to be a spore forming bacterium and can survive outside of an anaerobic environment.[6] But what would the effect on spore formation be when cultivating *C. difficile* under aerobic conditions? To answer this question it was also tested whether it was possible for *C. difficile* to produce spores under aerobic conditions by performing a sporulation assay and by using fluorescence microscopy. Samples were ethanol shocked to kill vegetative cells so that only spores remain in the samples. If spore formation occurs, growth would be observed. Aerobic and anaerobic samples were compared. Looking at the sporulation assay results and the fluorescence microscopy of aerobically cultivated *C. difficile* it can be concluded that spore formation does indeed occur under aerobic conditions. On the other hand, spore formation did not occur in the 10^2 fold dilution as it did under anaerobic conditions. This could mean that there are less spores forming in aerobic culture than anaerobic culture. Another possibility is that spores germinate faster in aerobic conditions compared to anaerobic conditions.

The second aim of this study was to cultivate yet unknown bacterial strains from the human gut microbiome by optimizing a culturing method to characterize fecal microbiota. High-throughput culturing of stool samples was carried out and identifying grown colonies was done using MALDI-TOF. MALDI-TOF is a great method for trustworthy identification of bacterial strains within a small time frame, which is ideal when culturing in high numbers. A number of different bacteria were identified using MALDI-TOF. *Bifidobacterium adolescentis* and *Collinsella aerofaciens* were among the identified bacteria and were cultured the most out of all the samples. These two bacteria are known to inhabit the intestinal tract.[20,21]

However four bacterial strains could not be identified. Subsequently 16s rRNA high fidelity PCR was executed on these four bacterial strains. Thereafter these four strains were sequenced. All four bacterial strains were identified. Although no unknown bacterial strains were cultured, a good method was developed to ensure high-throughput culturing. Continuing with culturing of the gut microbiome is necessary to discover unknown bacterial strains and to get a better view of the different bacteria living in the human gut.

A definite benefit of aerobic culture of anaerobic bacterial strains are that one single medium could be used to grow both aerobic and anaerobic bacteria, all in an aerobic environment. Although in this study the aerobic culturing method is limited to liquid medium, it is still a very promising result. Next steps would be to look at the possibility of cultivating anaerobic bacterial strains on plates. This has been done before in a study by D. Raoult *et al*.[14] but these experiments need to be performed by others to see if this method can be replicated. Use of plates are much more common and would actually leave colonies to work with. A lot more experiments need to be repeated to determine if *C. difficile* does indeed grow the same way aerobically as it does anaerobically before by-passing the anaerobic chamber completely.

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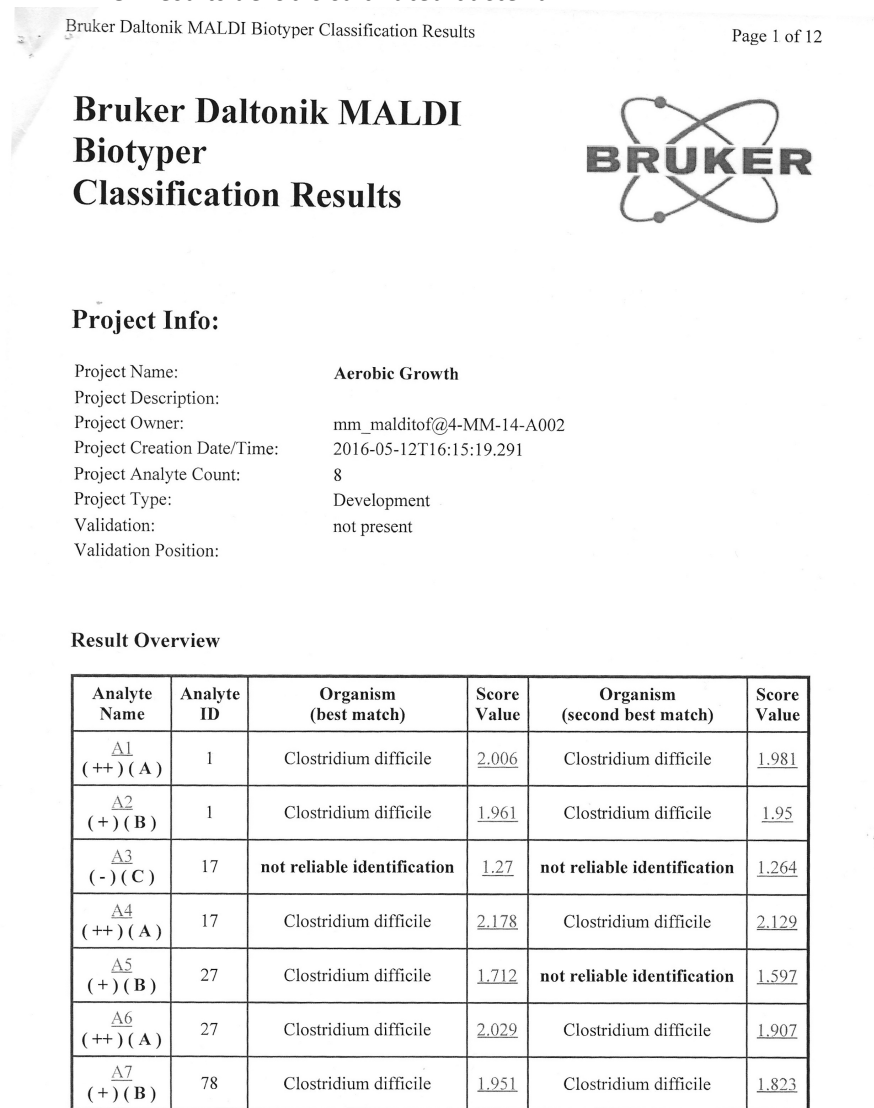
Appendix

1. *PCR cycle conditions*
   Program:
   1. 30'' 98 °C
   2. 10'' 98 °C
   3. 30'' 60 °C
   4. 2kb/min. + 15'' 72 °C
   5. 2'' 72 °C
   6. ~16 °C

2. MALDI-TOF *results aerobic cultivated bacteria*

Bruker Daltonik MALDI Biotyper Classification Results

BRUKER

Project Info:

Project Name:	**Aerobic Growth**
Project Description:	
Project Owner:	mm_malditof@4-MM-14-A002
Project Creation Date/Time:	2016-05-12T16:15:19.291
Project Analyte Count:	8
Project Type:	Development
Validation:	not present
Validation Position:	

Result Overview

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
A1 (++)(A)	1	Clostridium difficile	2.006	Clostridium difficile	1.981
A2 (+)(B)	1	Clostridium difficile	1.961	Clostridium difficile	1.95
A3 (-)(C)	17	**not reliable identification**	1.27	**not reliable identification**	1.264
A4 (++)(A)	17	Clostridium difficile	2.178	Clostridium difficile	2.129
A5 (+)(B)	27	Clostridium difficile	1.712	**not reliable identification**	1.597
A6 (++)(A)	27	Clostridium difficile	2.029	Clostridium difficile	1.907
A7 (+)(B)	78	Clostridium difficile	1.951	Clostridium difficile	1.823

file:///C:/Users/mm_malditof/AppData/Roaming/Bruker%20Daltonik/MALDIBiotyperA...   12-05-2016

A8 (+)(B)	78	Clostridium difficile	1.952	Clostridium difficile	1.891

3. *Strain 1*
TCGACGGCTAGCTCCATAAATGGTTACTCCACCGGCTTCGGGTGTTACAAACTCTCGTGGTGTGACGG
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GCGGTTTAGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATG
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20

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ATGTTATCCGGTATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTTATAGGTAGGTTATCCACGTGTTA
CTCACCCGTCCGCCGCTAACGTCAAAGGAGCAAGCTCCTTATCTGTTCGCTCGACTGCATGTATAG

Strain 2
GCTCCCTCCCTTGCGGGTTGGGCCGCCGGCTTCGGGTGTTGCCAACTCTCATGGTGTGACGGGCGGT
GTGTACAAGGCCCGAGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTC
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TTAGCAGGAGTTTCCTCCTGTTATCCCCCGCACAGGGGCAGGTTACCTATGTATTACTCACCCGTTCGC
CACTCATCTTCCGAAGAAGATTCGTTCGA

Strain 3
GGCTCCCCCAAAAGGTTGGGCCACCGGCTTCGGGTGCTACCCACTTTCATGACTTGACGGGCGGTGT
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CTCACCCAGGAGCAAGCTCCTGGGATCCCGTTCGACTGCA

Strain 4

TGCAGTCGAACGAATCTTCTTCGGAAGATGAGTGGCGAACGGGTGAGTAATACATAGGTAACCTGCC
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