EFFECT OF *P*-CRESOL ENRICHMENT IN ANAEROBIC GUT CULTURES

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ABSTRACT OF THE THESIS

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p-Cresol is an aromatic organic compound that is widely used in the manufacture of chemical products such as disinfectants and preservatives. It is a toxic compound and considered as pollutant in the environment. Studies have discovered anaerobic microbial degradation of *p*-cresol under nitrate-reducing, sulfate-reducing, iron-reducing, and methanogenic environments (Bossert *et al*., 1986; Londry *et al*., 1999; Muller *et al*., 2001; Lovley *et al*., 1993; Haggblom *et al*., 1990). *p*-Cresol is found naturally in the human gut system as a product of tyrosine and phenylalanine metabolism.

Clostridium difficile infection (CDI) is a disease affecting the gut system with symptoms ranging from diarrhea to inflammation of colon. The infection usually occurs following a disruption of healthy microbial community such as after antibiotic treatment. The organism colonizes by producing toxins that cause death on the epithelial cells. *C. difficile* produces *p*-cresol and is able to tolerate higher concentration of *p*-cresol that would be too toxic for other microorganisms. It is one of the strategies for *C. difficile* to suppress the growth of other microorganisms and to maintain its dominance in the gut system during infection. Antibiotic treatment is effective for CDI but has 20-40% chance of recurrence. Fecal microbiota transplantation (FMT) has become an alternative

treatment that is able to resolve recurrence of infection. It restores the microbial community in the gut back to its normal healthy state, which is hard to achieve with the antibiotic treatment. *p*-Cresol degradation has been found in various anaerobic environments and this study aims to investigate if there are any microorganisms in the human gut that are able to degrade *p*-cresol that can potentially be used to develop a more targeted FMT treatment for CDI.

Anaerobic gut cultures were set up from stool samples of two donors under sulfate-reducing and methanogenic conditions with *p*-cresol amendment as carbon source. Four batches were set up throughout the experiment with different adjustments on the method to get a better representation of the gut culture. *p*-Cresol degradation was not observed in any of the gut cultures that were set up. On the other hand, *p*-cresol was produced in most of the gut cultures, both in the active cultures and the background controls. As a comparison to the gut cultures, two different environmental cultures were set up under methanogenic condition from freshwater sediments and anaerobic digestate from a waste water treatment plant and were able to degrade 0.5 mM *p*-cresol within 12 days of incubation. It confirmed *p*-cresol degradation that has been found in various environmental cultures in previous studies.

High-throughput Illumina sequencing of 16S rRNA gene showed the change of microbial community in the gut cultures after incubation. It confirmed the presence of five phyla that has been reported to be found in the human gut, which are Firmicutes, Bacterioidetes, Verrucomicrobia, Proteobacteria, and Actinobacteria. The gut cultures showed a pattern of a decrease in Firmicutes and an increase in Bacteroidetes except for one batch from a post-antibiotic treatment stool sample. This batch had a different

microbial composition on day 0 compared to other batches from healthy stool samples suggesting that antibiotic treatment can affect microbial community in the human gut as other studies have reported. The fourth batch were set up with different stool sample handling method and composition of media and showed a more balanced ratio of Firmicutes and Bacteroidetes before and after incubation, suggesting that the methods used in the gut culture set up could affect the microbial community composition in the culture.

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CHAPTER I

LITERATURE REVIEW

1.1 *para*-Cresol

para-Cresol (*p*-cresol) is an aromatic organic compound with a structure of a phenol with one methyl group and is also known as 4-methylphenol in IUPAC nomenclature (Figure 1.1). It is a colorless solid with a tar-like odor (HMDB, 2017). *p*-Cresol is an important synthetic precursor in the manufacturing process of many chemical products such as synthetic resins, disinfectants, antioxidants, preservatives, fumigants, explosives, and other products (Du *et al.*, 2016). It is derived from industrial processes such as coal gasification and coal tar fractionation. Besides industrial activities, animal farming is also a major source of *p*-cresol since it is present in animal waste. *p*-Cresol is one of the primary odor-causing volatile organic compounds in animal farms (Borhan *et al.*, 2012).

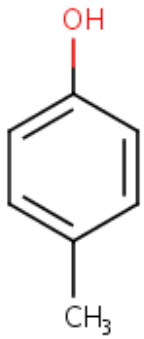

Figure 1.1 *para*-Cresol

(Source: https://toxnet.nlm.nih.gov/)

p-Cresol is considered a toxic compound. Acute human exposure to *p*-cresol can cause irritation of eyes, skin, or mucous membranes, breathing difficulty, irregular and rapid respiration, and weak pulse. Effects on the central nervous system can cause confusion and respiratory failure. It also targets and can damage the liver, kidney, pancreas, and cardiovascular system (NIOSH, 2017). *p*-Cresol is a possible human carcinogen and considered as a uremic toxin (HSDB, 2017). Higher serum levels of *p*-cresol have been found in patients with chronic kidney disease and it has been associated with increased cardiovascular mortality (HSDB, 2017).

1.2 Anaerobic microbial degradation of *p*-cresol in the environment

The presence of *p*-cresol in the environment is regarded as a pollutant due to its toxicity. Anaerobic microorganisms in the environment have been known to have the ability to degrade a variety of aromatic organic compounds. Therefore, studies have been conducted on *p*-cresol anaerobic degradation and found its activity in nitrate-reducing, sulfate-reducing, iron-reducing, and methanogenic environments.

Bossert *et al.* (1986) isolated two strains from a polluted river sediment, working as a co-culture, that can degrade *p*-cresol in a nitrate-reducing environment. The co-culture of two strains, PC-07 and PB-04, was able to degrade 1.8 mM of *p*-cresol in one week. The strain PC-07 metabolized *p*-cresol into *p*-hydroxybenzoate and PB-04 utilized *p*-hydroxybenzoate as substrate. Degradation of *p*-cresol was present only if the two strains were grown together as the *p*-cresol degradation to *p*-hydroxybenzoate yielded no carbon for PC-07 and it could only get the carbon as *p*-hydroxybenzoate was metabolized by PB-04. Further investigation in this study looked into the degradation pathway of *p*-

cresol in the nitrate-reducing environment. Bossert *et al.* (1989) characterized a *p*-cresol methylhydroxylase that mediated the oxidation of *p*-cresol to *p*-hydroxybenzaldehyde, that would later be converted to *p*-hydroxybenzoate through a dehydrogenase enzyme.

Three different bacteria have been found to degrade *p*-cresol in sulfate-reducing environments. Bak and Widdel (1986) investigated *p*-cresol degradation by *Desulfobacterium phenolicum* sp. nov., which was isolated from a marine sediment. It was able to degrade 2 mM of *p*-cresol within six days. Londry *et al.* (1999) found *p*-cresol metabolism by *Desulfotomaculum* sp. strain Groll. In this organism, the metabolites produced and enzyme activity found suggested a methyl oxidation pathway with an end product of *p*-hydroxybenzoate, similar to what was reported by Bossert *et al.* (1989). A study by Muller *et al.* (2001), however, found evidence for a different pathway of *p*-cresol degradation in *Desulfobacterium cetonicum*. In this strain, the methyl group of *p*-cresol was activated by the addition of fumarate, forming 4-hydroxybenzylsuccinate. This intermediate can then be metabolized into 4-hydroxybenzoyl-CoA.

Degradation of *p*-cresol under methanogenic conditions was reported in the study by Haggblom *et al.* (1990) using cultures originating from freshwater pond sediment. The culture was able to degrade 1 mM of *p*-cresol, initially within 21 to 30 days, and after two additional amendments, within 6 to 10 days. *p*-Hydroxybenzaldehyde and *p*-hydroxybenzoate were found as intermediates suggesting the pathway of methyl oxidation for the degradation of *p*-cresol. The same cultures in this study were also able to degrade *p*-cresol under nitrate-reducing and sulfate-reducing conditions. Degradation of *p*-cresol under methanogenic conditions was also reported by Kennes *et al.* (1997) in an up-flow anaerobic sludge blanket (UASB) reactor using a mixed culture of sludge

from wastewater treatment. *p*-Cresol degradation has also been found under iron-reducing conditions as observed in the study by Lovley *et al.* (1993). *Geobacter metallireducens* was able to use 0.5 mM *p*-cresol as an electron donor in the presence of Fe (III) oxide. The pathway of *p*-cresol degradation by the bacteria was later investigated by Peters *et al.* (2007) and Chaurasia *et al.* (2015), and it was found that the degradation was also initiated by methyl hydroxylation.

All of these studies showed that different anaerobic microorganisms were found in the environment that has the ability to degrade *p*-cresol. The number of microorganisms in the culture may have affected the amount of *p*-cresol that could be degraded and the time it took to degrade in these different studies, but it showed that anaerobic microbial degradation of *p*-cresol is present in the environment. The two main pathways of anaerobic *p*-cresol degradation that have been observed are initiated with hydroxylation or the addition of fumarate, both of which occur at the methyl group (Figure 1.2).

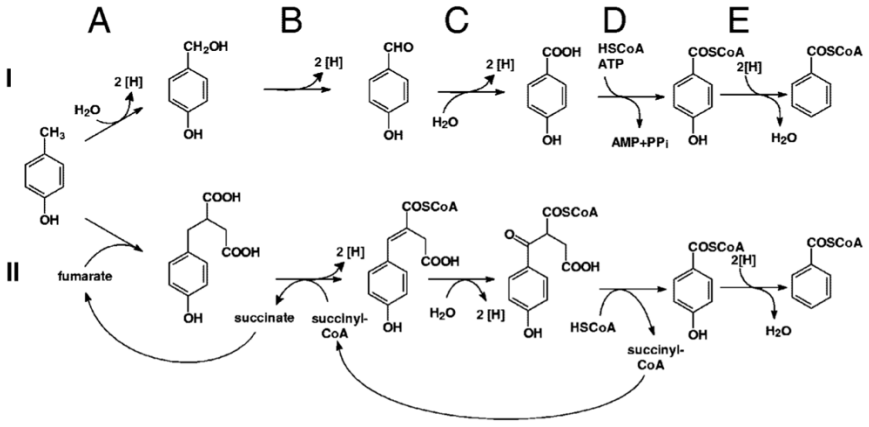

Figure 1.2. Two pathways of *p*-cresol metabolism in anaerobic bacteria. I. Methyl group hydroxylation. II. Oxidation via addition of fumarate. (Source: Peters *et al.*, 2007)

1.3 *p*-Cresol in the human gut system

p-Cresol is produced naturally in the human gut system as one of the end products of amino acid metabolism by gut microbiota. It is the main product of tyrosine metabolism by anaerobic bacteria in the intestine (Mathus *et al.*, 1995; Smith & Macfarlane, 1996; Vanholder *et al.*, 1999; Gryp *et al.*, 2017), predominantly in the colon. *p*-Cresol can also be produced indirectly from phenylalanine through the decarboxylation of 4-hydroxyphenylacetic acid that is produced in the metabolism of phenylalanine (Vanholder *et al.*, 1999). Anaerobic bacteria from different genera (*Clostridium*, *Faecalibacterium*, *Eubacterium*, *Anaerostipes*, *Ruminococcus*, *Bacteroides*, *Bifidobacterium*) have been reported to produce *p*-cresol in the human gut (Gryp *et al.*, 2017). The reaction of tyrosine degradation to *p*-cresol is (Mathus *et al*., 1995):

L-tyrosine + α-ketoglutarate + $3H_2O \rightarrow$ *p*-cresol + glutamate + $2HCO_3^-$ + H_2 + $2H^+$

Detoxification of phenols, including *p*-cresol, occurs in the mucosa of the colon or in the liver. *p*-Cresol is mainly sulfated into *p*-cresol sulfate while a small fraction of it is glucuronated into *p*-cresyl glucuronide (Gryp *et al.*, 2017). However, *p*-cresol has also been found in urine (around 30 μg/ml) and stool samples (around 50 μg/g) of healthy adults (King *et al.*, 2009). Increased levels of *p*-cresol in urine or feces has been considered as an indicator of health problems as it is associated with several conditions such as large bowel health (King *et al.*, 2009), uremia (Vanholder *et al.*, 1999), or *Clostridium difficile* infection (Dawson *et al.*, 2011). Changes in the intestinal microbiota can result in the overgrowth of bacteria that are *p*-cresol producers (Vanholder *et al.*, 1999; Dawson *et al.*, 2011).

1.4 Human gut microbiota

The perspective on human-associated microorganisms has shifted in the past decade, from the pathogenic and disease-causing point of view into an understanding that microorganisms have always been a part of the human system and that most of the relationships between the microbiota and the host are either commensal or mutualistic (Dethlefsen *et al.*, 2007). This shift has been enabled by the advancement of gene sequencing technologies that allow culture-independent methods to explore the microbial diversity in the human body. Human microbiome research has been focused mainly on the gut system, with about 17,000 studies published on the gut/colon/intestinal microbiome, with about 10,000 of those published between 2011 and 2016 (Lloyd-Price *et al.*, 2016).

The human gut microbiota provides functions such as protection from enteropathogens, extraction of nutrients and energy from food, and support of normal immune function (Lozupone *et al.*, 2012). There is no consensus yet to define the composition of a healthy human gut microbiome. It is a complex system, and even among healthy individuals, there are variations of microbial composition affected by different factors such as age, genetics, diet, and environment. However, efforts are still being conducted to have a better understanding of the gut microbiota. One approach is to compare the microbiota of healthy individuals with patients with a specific disease and finding features that broadly distinguish the two populations (Stojanovic, 2013; Lloyd-Price *et al.*, 2016).

Culture-independent sequencing studies of the human gut microbiota have shown that each individual contains a high diversity of more than 1,000 species-level

phylotypes. At the higher taxonomic level, however, these species belong to only a few phyla. The dominant bacterial phyla that are found in the human gut of most individuals are Firmicutes and Bacteroidetes which comprise 90% of the human gut microbiota, followed by Actinobacteria, Proteobacteria, and Verrucomicrobia (Tremaroli & Backhed, 2012; Lozupone *et al.*, 2012).

Microorganisms in the intestinal lumen are present in site-specific communities, and each community consists of certain families and genera that are found in the same habitat in most individuals (Dethlefsen *et al.*, 2007). The microorganisms that reside in the small intestine differ from what is found in the large intestine according to Kamada *et al.* (2013). This is driven by the different environment of the two locations. The small intestine is richer in nutrients compared to the large intestine that is located at the end of the gut system. The small intestine is dominated of Proteobacteria spp. (mainly Enterobacteria), Lactobacillales, and Erysipelotrichales (especially *Turicibacter* spp.). The nutrient-poor large intestine lacks these microbes and consists mainly of Bacteroides and Clostridiales that are able to utilize undigested fibers.

Human gut microbiota can have a significant impact on human health as studies observed dysbiosis, which is a significantly different microbial composition and is related to a disease (Stojanovic, 2013). Dysbiosis has been associated with several diseases such as irritable bowel syndrome, inflammatory bowel disease, obesity, colorectal cancer, and *Clostridium difficile* infection (Stojanovic, 2013; Guinane & Cotter, 2013).

1.5 *p*-Cresol in relation to *Clostridium difficile* infection

Clostridium difficile infection (CDI) has become a health concern in the United States with nearly 500,000 diagnoses and approximately 30,000 deaths reported annually (Hudson *et al.*, 2017). *C. difficile* is a spore-forming Gram-positive obligate anaerobe that produces a toxin. CDI can occur asymptomatically in individuals that have a neutralizing immune response against the toxin. The symptoms of CDI range from diarrhea, fever, nausea and belly pain, to the more severe pseudomembranous colitis that can lead to death. The risk of CDI primarily increases after a broad-spectrum antibiotic treatment that significantly reduced the diversity of the gut microbiota (Dawson *et al.,* 2008; Lee *et al.*, 2016, Hudson *et al.*, 2017).

A common treatment for CDI is the administration of antibiotics such as metronidazole and vancomycin; however, antibiotic treatment for CDI is frequently followed by the recurrence of infection. Recurrences are reported in 20 – 40% of CDI cases after one episode, after which it increases to 60% (Lee *et al.*, 2016; Hudson *et al.*, 2017). An alternative treatment for CDI is fecal microbiota transplantation (FMT) from a healthy donor. The main idea of FMT is to reintroduce healthy gut microbiota and recover the ability to resist colonization by *C. difficile*. Different methods of FMT administration have been used in CDI treatment, from oral capsule, nasogastric tubes, nasoduodenal tubes, colonoscopy, or enema. One clinical study on FMT for CDI treatment was conducted by Lee *et al.* (2016) on 219 patients, and the treatment was able to resolve CDI in 70% of the patients following the first treatment and 91% of the patients with subsequent treatments. More studies have followed to look into FMT as

CDI treatment with similarly successful results in overcoming the infection, reducing infection recurrence, and restoring the diversity of gut microbiota (Hudson *et al.*, 2017).

Although regarded as a safe and effective treatment for CDI, FMT still has safety concerns; as the method introduces the whole microbiota of a healthy donor, there are risks of transferring pathogens or infectious diseases such as norovirus. Understanding the mechanism and function through which FMT is able to resist *C. difficile* colonization can lead to the development of a more targeted approach. Hudson *et al.* (2017) identified several mechanisms of *C. difficile* colonization resistance: nutrient availability and competition for resources, bile salt metabolism, production of anti-*C. difficile* compounds, and maintenance of the intestinal epithelial cell barrier. The study also looked into known probiotics that can improve those mechanisms and therefore may have the potential to be used in CDI.

CDI is also related to the presence of *p*-cresol in the human gut. *C. difficile* is able to produce *p*-cresol from tyrosine and can tolerate higher concentrations of *p*-cresol that would inhibit the growth of other anaerobic bacteria (Dawson *et al.*, 2008). The study by Dawson *et al.* (2008) discovered that hypervirulent strains of *C. difficile* were able to tolerate higher concentrations of *p*-cresol compared to the historical *C. difficile* strains. The study suggested that the production and tolerance of *p*-cresol could be one of the strategies of *C. difficile* when colonizing the host. In relation to the development of a more targeted approach to treat CDI based on the efficiency of FMT, it is possible that if there are bacteria in the human gut microbiota that are able to degrade *p*-cresol, they can be used as one of the probiotics in CDI treatment.

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CHAPTER II

p-CRESOL ENRICHMENT IN ANAEROBIC GUT CULTURES AND ENVIRONMENTAL CULTURES

Abstract

p-Cresol is present in the human gut as a product of amino acid metabolism (Smith and Macfarlane, 1996). The compound is related to *Clostridium difficile* infection (CDI) as the organism can produce and tolerate high concentration of *p*-cresol. Fecal microbiota transplantation has been able to effectively treat CDI and prevent recurrence of infection. *p*-Cresol microbial degradation has been found in various anaerobic environments, and therefore it is possible that there are microorganisms in the human gut that can degrade *p*-cresol.

Gut cultures were set up from stool samples of two different donors under methanogenic and sulfate-reducing conditions. *p*-Cresol was added to the gut cultures as substrate. Batch 1 was set up with stool sample from a post-antibiotic treatment female donor, and batch 2 was from a healthy male donor, both amended with 1 mM or 0.25 mM *p*-cresol. Batch 3 was set up from a healthy female donor and amended with 0.25 mM, 0.1 mM *p*-cresol, and an additional treatment of 0.25 mM amino acid mixture (tyrosine and phenylalanine). Batch 4 was set up from a healthy female donor with an addition of yeast extract, tryptone, and sodium chloride in the methanogenic media and 0.25 mM *p*-cresol amendment. The stool sample was also handled differently for batch 4, using potassium phosphate buffer to make a slurry prior to inoculation. The result showed no *p*-cresol degradation activity in any of the gut cultures. *p*-Cresol was produced in most of

the gut cultures, both in active cultures and background controls, especially in batch 3 and batch 4. Environmental methanogenic cultures were also set up as a comparison, using freshwater pond sediments and anaerobic digestate from a wastewater treatment plant as inocula. The environmental cultures were able to degrade 0.5 mM of *p*-cresol within 12 days of incubation.

2.1 Introduction

p-Cresol is present naturally in the human gut system as a degradation product of the amino acids tyrosine and phenylalanine (Smith and Macfarlane, 1996). It is considered a toxic compound and most microorganisms cannot tolerate high levels of *p*-cresol. This compound can be conjugated in the liver, mainly transformed into *p*-cresol sulfate. When *Clostridium difficile* infection occurs, *p*-cresol plays a role as the organism is able to produce the compound and can tolerate higher levels of it (Dawson *et al.*, 2008). Thus, *p*-cresol is produced during the infection, and it would suppress the growth of other microorganisms in the system.

C. difficile infection is usually treated with antibiotics such as vancomycin and metronidazole; however, there are cases where antibiotic treatment is followed by a reoccurrence of infection. An alternative treatment that is becoming popular for *C. difficile* infection is fecal transplantation from a healthy donor. This method has been effective in treating the infection and reducing the chance of reoccurrence (Bakken *et al.*, 2011). An antibiotic treatment would wipe out a large number of microorganisms in the gut system while a fecal transplant would introduce a healthy microbial community into the gut system. The efficacy of a fecal transplant indicates how the diverse microbial

community from a healthy donor can restore the balance in the gut system (Fuentes *et al.*, 2014). Therefore, it is possible that there are bacteria in the human gut system that are able to degrade *p*-cresol.

p-Cresol degradation has been reported in various anaerobic environments such as sulfate-reducing marine sediments (Bak and Widdel, 1986), nitrate-reducing river sediments (Bossert *et al.*, 1986), iron-reducing river sediments (Lovley *et al.*, 1993), and methanogenic freshwater pond sediments (Haggblom *et al.*, 1990). In this chapter, anaerobic gut cultures were set up under sulfate-reducing and methanogenic conditions and amended with *p*-cresol to see if there is any degradation activity occurring in the culture. Environmental anaerobic cultures were also set up and amended with *p*-cresol as a comparison to the gut culture. *p*-Cresol degradation is expected to be found in the environmental cultures. The degradation is hypothesized to be found in the gut cultures as well because the compound is present in the human gut, can be a potential carbon source, and microbial degradation of *p*-cresol has been reported in various anaerobic environments.

2.2 Material and Methods

Anaerobic gut enrichment culture set up

Stool sample for this experiment was obtained from two donors: female and male. A post-antibiotic treatment stool sample from the female donor was used in the first batch of the gut enrichment culture, while the other batches were set up with stool samples from the two donors in healthy conditions. Fresh stool was collected and kept in a closed mason jar at room temperature before being used as inoculum for batch 1, 2, and 3. The

stool sample collection procedure was modified slightly for the fourth batch to provide a better anaerobic condition for the sample. It was submerged in a N_2-degassed potassium phosphate buffer in a mason jar upon collection and kept in that condition before being used as inoculum.

Two different types of media were used in the gut enrichment culture, a sulfate-reducing and methanogenic medium. The sulfate-reducing medium was prepared according to Widdel and Pfennig (1981) with modification of no sodium chloride addition. The methanogenic medium was prepared according to Healy and Young (1979). For batch 4, YE/MH/NaCl media was prepared by adding 0.75 g/L yeast extract, 5.25 g/L Mueller Hinton broth, and 5 g/L sodium chloride into methanogenic media and YE/tryptone/NaCl media was prepared by adding 1% yeast extract, 1% tryptone, and 5 g/L sodium chloride into methanogenic media. All media were prepared anaerobically, first by boiling to push out oxygen and purging the liquid with mixed gas (30% CO_2 / 70% N_2) as the medium was prepared. Twenty percent inoculum was added into the media and stirred for thirty minutes to make a homogenous slurry of gut culture. Fifty ml of culture was dispensed into serum bottles as it was purged with mixed gas and then sealed with butyl rubber stoppers and crimped.

A total of four batches were set up sequentially for the gut cultures and modified as summarized in Table 2.1. Each treatment consisted of three actives and two sterile controls. Two background controls were set up for each type of medium. Active cultures were amended with *p*-cresol as substrate (or amino acid in batch 3). Sterile controls were also amended with the substrate and were autoclaved three times on consecutive days to kill all the microorganisms. Background controls were not amended with any substrate.

Batch 1 and 2 were set up with the same method and treatments to compare between healthy and post-antibiotic cultures. Batch 3 was set up with a lower concentration of *p*-cresol treatment to avoid having the microorganisms inhibited by a high level of *p*-cresol. An additional treatment of amino acids was also used in batch 3 without *p*-cresol amendment. Batch 4 was set up with only one concentration of *p*-cresol amendment but with four different types of media. All cultures were incubated at 37°C. Samples were taken anaerobically using 70% N_2:30% CO_2 flushed syringes and centrifuged at 15,000 x g for 5 minutes. Then 650 μl of the supernatant was filtered using Costar Spin-X centrifuge tube filters at 13,000 x g for 3 minutes. The filtrates were transferred to HPLC vials for *p*-cresol analysis.

Tabel 2.1. Summary of different treatment in four batches of gut enrichment culture

Batch No.	**Inoculum**	**Types of media**	**Treatments for each type of media**
1	Post-antibiotic female, enclosed in a mason jar	-Sulfate-reducing -Methanogenic	-1 mM *p*-cresol -0.25 mM p-cresol
2	Healthy male, enclosed in a mason jar	-Sulfate-reducing -Methanogenic	-1 mM p-cresol -0.25 mM p-cresol
3	Healthy female, enclosed in a mason jar	-Sulfate-reducing -Methanogenic	-0.25 mM p-cresol -0.1 mM p-cresol -0.25 mM amino acid (tyrosine & phenylalanine)
4	Healthy female, submerged in potassium phosphate buffer	- YE/MH/NaCl media without sulfate - YE/MH/NaCl media with sulfate - YE/tryptone/NaCl media without sulfate - YE/tryptone/NaCl media with sulfate	-0.25 mM p-cresol

Anaerobic environmental enrichment culture set up

A methanogenic anaerobic culture was set up using two different sources of environmental samples as inocula; sediments from Passion Puddle pond on Cook Campus of Rutgers University and anaerobic digestate from a wastewater treatment facility in New Jersey. The anaerobic culture was set up using the same method as the gut culture, with 0.5 mM of *p*-cresol added as a substrate in the active cultures and sterile controls and no addition in the background control. The Passion Puddle culture was incubated at room temperature (25°C), while the anaerobic digestate culture was incubated at 37°C. Sampling and preparation were the same as for the gut cultures.

p-Cresol concentration measurement

The concentration of *p*-cresol was measured using reversed-phase high-performance liquid chromatography (RP-HPLC). The stationary phase used for the HPLC was a Kinetex 5μm C18 100A (250 x 4.6 mm) (Phenomenex) with a core-shell silica solid support and a trimetylchlorosilane (TMS) end-capping. The mobile phase used for measuring *p*-cresol was methanol-water-acetic acid (60:38:2). The flow rate used for RP-HPLC was 1 ml/minute with UV-detection at 280 nm. The retention time for *p*-cresol was 4.9 minutes.

Total gas production measurement

Total gas production volume in each culture bottle was measured using a glass syringe that had been previously flushed with N_2 to maintain anaerobic conditions. After the culture bottles had been set up, any excess gas in the headspace from flushing the

bottles with mixed gas was zeroed out by removing the excess gas through the glass syringe. The next measurement of excess gas from the headspace is considered as the total gas produced by the culture and measured in volume (ml) with the glass syringe.

Methane production measurement in methanogenic cultures

Methane concentration in the headspace of each culture bottles was measured using gas chromatography (GC) analysis. As described above, excess headspace gas from each culture bottle was measured using a glass syringe before GC analysis. Then 0.5 ml of the excess headspace gas was pushed back into the bottle while the remaining was removed. A gas-tight syringe was used to take 0.5 ml sample from the culture bottle and inject it into the GC. The methane concentration would be shown as a percentage and was then used to calculate the amount of methane that was present in the headspace of the culture bottle.

2.3 Results and Discussion

p-Cresol concentration in the gut culture

Batch 1 and Batch 2

The batch 1 *p*-cresol concentrations are presented in Figure 2.1. Both sulfate-reducing and methanogenic conditions showed an increase of *p*-cresol concentrations in the active cultures, in both concentrations of *p*-cresol amendments. An average of a 0.2-0.3 mM increase was observed in the 1 mM *p*-cresol amendment, while there was an average of a 0.1 mM increase in the 0.25 mM *p*-cresol amendment. The background controls of both types of media had no detectable *p*-cresol on day 0 and remained

unchanged after incubation. However, the sterile controls showed similar increases as the actives. If the production was only observed in the active cultures, it could be considered to be the result of microbial activity. Having the same results in sterile controls could indicate that the results were caused by abiotic factors. In this case, it is possible that the result is caused by the analysis method. The month-10 samples of batch 1 were initially run on a different HPLC instrument than day 0 samples due to instrument availability. Month-10 samples were kept frozen and were run again on the same instrument as day 0 in order to compare the data. The data presented in Figure 2.1 are from the second run that used the same HPLC instrument as day 0 samples. This could have affected the analysis results.

In batch 2, the *p*-cresol concentration in the active cultures of both treatments under both methanogenic and sulfate-reducing conditions did not change substantially. However, *p*-cresol was detected on day-0 in the background controls of both sulfate-reducing and methanogenic conditions of batch 2 (Figure 2.2.). The *p*-cresol concentration in the background controls under methanogenic conditions increased 67% from 0.195 to 0.326 mM, while the background controls under sulfate-reducing conditions changed much less (17%). A small decrease was observed in the average sterile controls of 1 mM *p*-cresol under sulfate-reducing conditions. This could be caused by an error in the analysis in one of the sterile controls that resulted in a 0.2 mM higher concentration compared to the other on day 0, while the sample after incubation showed a more similar result among the two bottles.

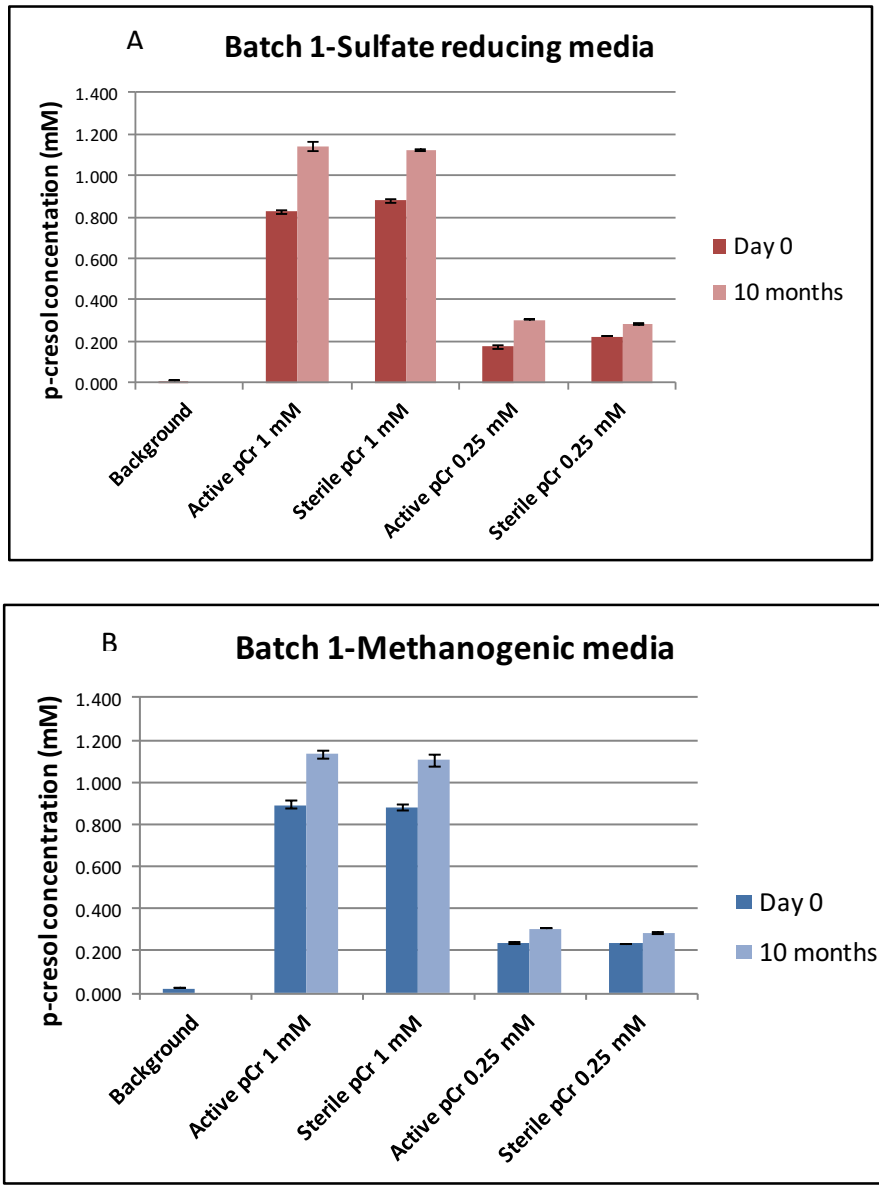
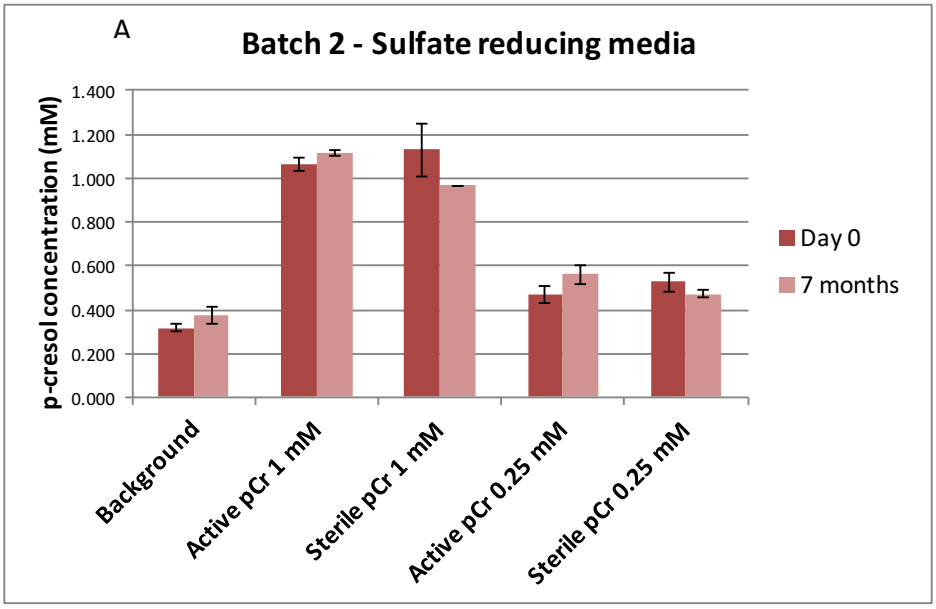


Figure 2.1. *p*-Cresol concentrations in batch 1 sulfate-reducing (A) and methanogenic (B) gut cultures. The error bar indicates standard deviation of the replicates.



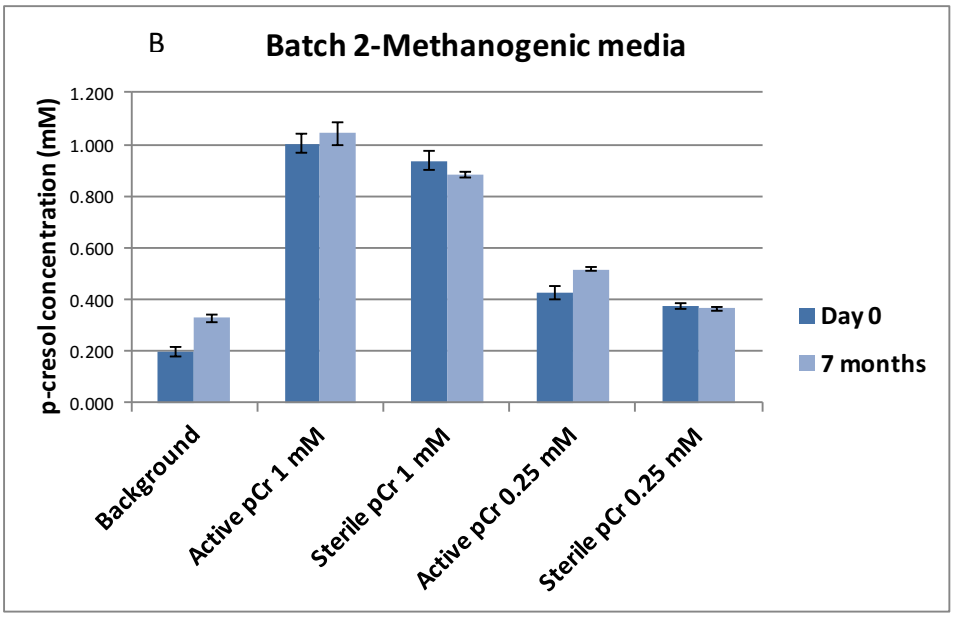


Figure 2.2. *p*-Cresol concentrations in batch 2 sulfate-reducing (A) and methanogenic (B) gut cultures. The error bar indicates standard deviation of the replicates.

Batch 1 and 2 were set up with the same methods and given the same treatments to have a comparison between a healthy and post-antibiotic treatment gut culture. Batch 1 not degrading *p*-cresol might be expected as antibiotic treatment would decrease microbial diversity in the system. Studies have reported the loss of diversity in the gut microbial community in stool samples within 3-4 days after antibiotic administration (Dethlefsen and Relman, 2011) and by one week after the end of the antibiotic course, it began to regain diversity but some changes in composition compared to the original state were still observed. A similar pattern of reduced microbial diversity after antibiotic treatment was also reported in other studies on the human gut through fecal samples (Bartosch *et al.*, 2004; Jakobsson *et al.*, 2010; Perez-Cobas *et al.*, 2012). However, batch 2, which represents a healthy gut culture, also did not degrade the *p*-cresol. During the set up method for batch 1 and 2, as the stool sample inoculum was added into the media and stirred into a slurry, all parts of the slurry was distributed into the serum bottles as culture. It also included some undigested solids from the inoculum. This might result in a high level of organic material in the culture, and the microorganisms might prefer to consume those organic materials that are easier to degrade instead of the *p*-cresol.

The concentration of *p*-cresol that was added into the culture was also a consideration. Between 0.5 – 1.8 mM of *p*-cresol was added as treatment in previous studies of *p*-cresol anaerobic microbial degradation in the environment (Bossert *et al.*, 1986; Haggblom *et al.*, 1990; Lovley *et al.*, 1993). But these concentrations were used in anaerobic culture with environmental samples as inocula. According to Hafiz and Oakley (1976) and Dawson *et al.* (2008), *C. difficile* was able to grow with 0.47 mM *p*-cresol, but other anaerobic gut bacteria could not survive 0.9 mM *p*-cresol. The concentration

could be too high for the gut culture in this experiment so that it became too toxic for most of the gut microorganisms to grow and selected for *p*-cresol-tolerant microorganisms instead that are not degrading it.

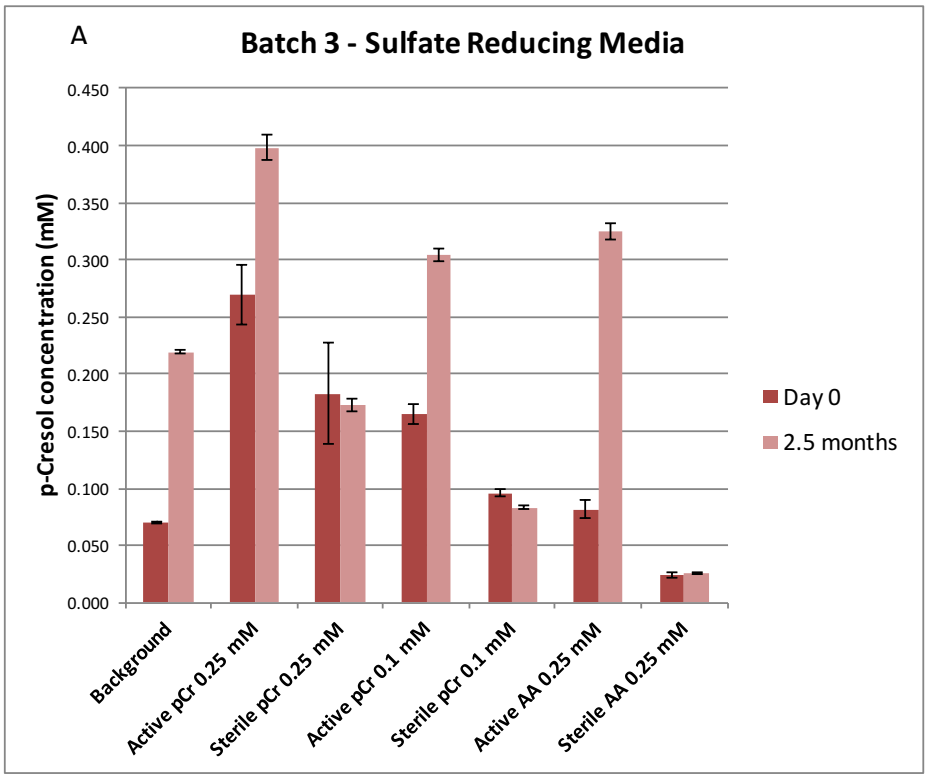
Batch 3

Batch 3 was set up with a modified method from batch 1 and 2, first to reduce the amount of solids of the inoculum in the culture. This was done by making a larger volume of the medium-inoculum slurry and letting most of the solids settle to the bottom before dispensing the culture into serum bottles, taking only the top liquid part of the slurry. Lower concentrations of *p*-cresol (0.25 and 0.1 mM) were used as substrate for batch 3. In addition to *p*-cresol, one set in batch 3 was amended with an amino acid mixture (0.25 mM) of tyrosine and phenylalanine. Degradation of tyrosine produces *p*-cresol as an end product. Phenylalanine can be converted to 4-hydroxyphenylacetic acid by intestinal bacteria and decarboxylated into *p*-cresol (Vanholder *et al.*, 1999). The amino acid treatment was intended to have *p*-cresol added into the culture through the natural process of amino acid degradation. The concentration of *p*-cresol was hoped to be similar to what is occurring in the gut system and that it would be degraded by some microorganisms in the system.

The results for batch 3 are shown in Figure 2.3. The background controls under sulfate-reducing conditions of batch 3 showed an average increase in *p*-cresol concentration after 4 months of incubation from 0.07 to 0.219 mM. There was also an increase in the background controls under methanogenic conditions after 2.5 months of incubation but much smaller, from 0.275 to 0.362 mM. The active cultures of sulfate-

reducing batch 3 that were treated with 0.25 mM and 0.1 mM *p*-cresol also showed an increase in *p*-cresol concentration, with the amount of increase similar to the background controls. This was also observed in the *p*-cresol-treated active cultures of methanogenic batch 3. The amino acid treatment resulted in a substantial increase in the *p*-cresol concentration in the active cultures under both sulfate-reducing and methanogenic conditions. Under sulfate-reducing conditions, it increased from 0.081 mM to 0.325 mM, while under methanogenic conditions there was already 0.285 mM and it increased to 0.597 mM.

The results of batch 3 still showed no degradation of *p*-cresol and it was produced instead in the background controls and all *p*-cresol-treated actives. The amino acid treatment also produced *p*-cresol and in a much higher amount. It suggests that *p*-cresol is produced through amino acid degradation. The amino acid treatment was expected to produce a moderate amount of *p*-cresol through the natural process of amino acid degradation and it was also expected that the *p*-cresol would be degraded by other microorganisms in the community. However, the result of the amino acid treatment in batch 3 showed a substantial accumulation of *p*-cresol, exceeding the highest concentration of *p*-cresol treatment given in the batch. If *p*-cresol degradation was occurring in the active cultures at a slower rate than the production of *p*-cresol, it could not be concluded from this experiment.



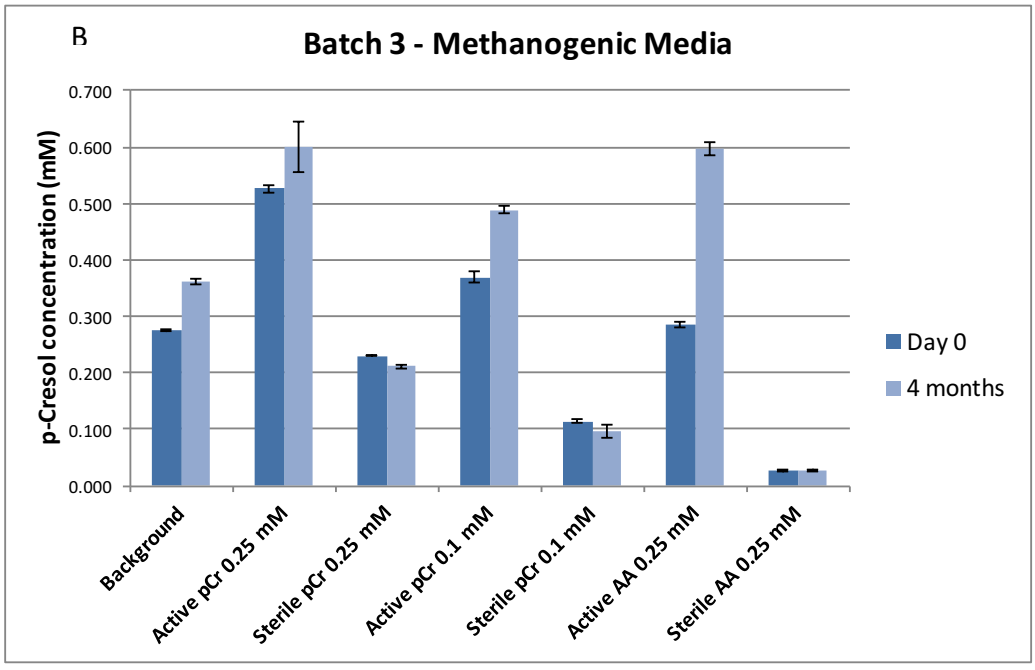


Figure 2.3. *p*-Cresol concentrations in batch 3 sulfate-reducing (A) and methanogenic (B) gut cultures. The error bar indicates standard deviation of the replicates. (AA: amino acids)

Batch 4

Batch 4 was set up with the consideration of nutrient availability in the medium of previous batches. Defined minimal medium was used in all previous batches of gut culture to make sure that *p*-cresol would be the only source of carbon that is available in the medium in order to drive the community to degrade it. All previous batches did not show *p*-cresol degradation activity. Another possibility is that the defined minimal medium that was used in those batches did not provide enough nutrients for the gut microorganisms in the inoculum and that may have repressed their growth and may have selected smaller groups of microorganisms to dominate the culture. With this consideration, batch 4 was set up with a richer medium containing beef extract, yeast extract, and sodium chloride.

Batch 4 under sulfate-reducing conditions in both YE/MH/NaCl and YE/tryptone/NaCl media showed an increase of *p*-cresol concentration after three weeks of incubation in both background controls and active cultures, as shown in Figure 2.4. In the YE/MH/NaCl medium, the background controls had an increase from 0.099 to 0.284 mM while the active cultures increased from 0.321 to 0.506 mM. In the YE/tryptone/NaCl medium, the increase in *p*-cresol was not far from what was observed in the YE/MH/NaCl medium, from 0.081 to 0.340 mM for background controls and from 0.301 to 0.620 mM for active cultures. Batch 4 under methanogenic conditions also produced *p*-cresol, and it was still observed after 11 months of incubation. The background controls increased from 0.110 to 0.559 mM and a similar amount of *p*-cresol formation (around 0.4 mM) was also observed in the active culture, from 0.336 to 0.757 mM.

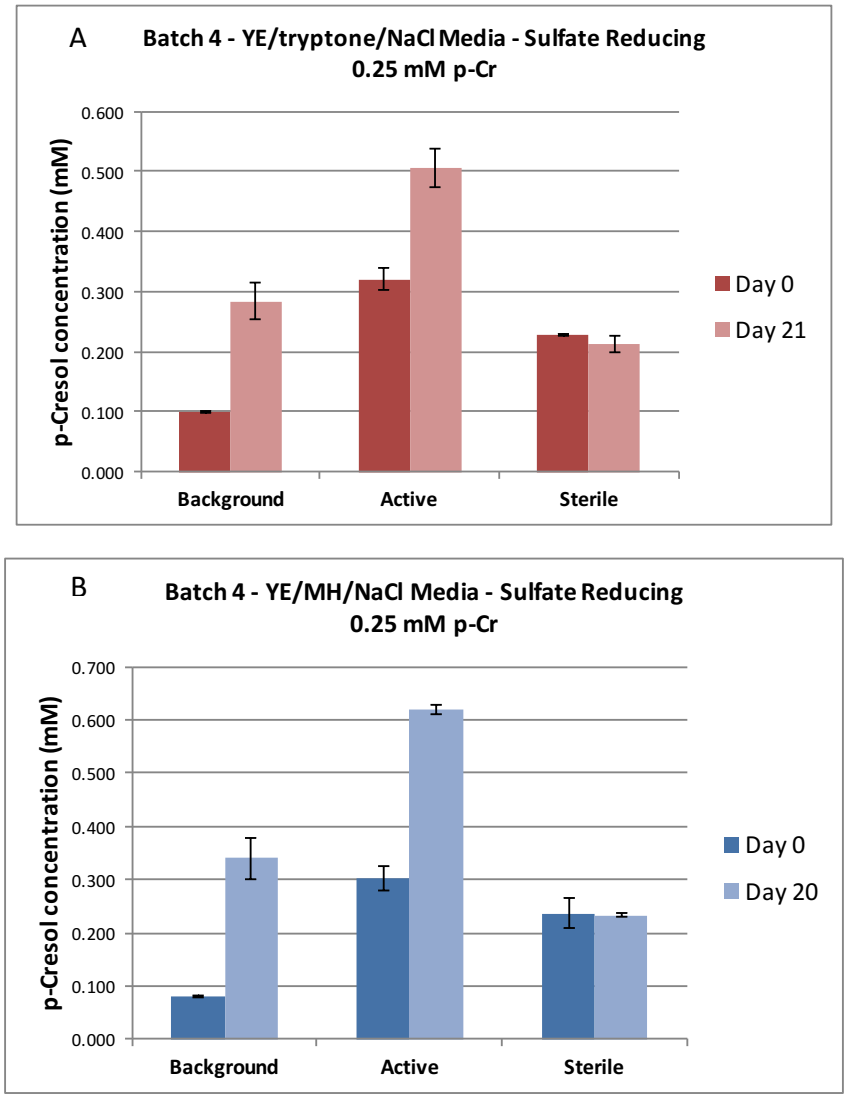


Figure 2.4. *p*-Cresol concentrations in batch 4 sulfate-reducing YE/tryptone/NaCl (A) and YE/MH/NaCl (B) medium gut cultures. The error bar indicates standard deviation of the replicates.

Another modification in batch 4 set up was how the stool sample was handled prior to inoculation. As mentioned in the method, for batch 4 the stool sample was submerged in a degassed buffer in a closed jar right after collection. The purpose of this modification was to maintain a more anaerobic environment that would help the microorganisms in the sample to stay active before transferring to the culture medium.

The result of batch 4 showed no degradation of *p*-cresol and instead it was formed in the culture, both in the actives and the background controls, and under both sulfate-reducing and methanogenic conditions.

p-Cresol degradation in environmental cultures under methanogenic conditions

The active cultures of both Passion Puddle and anaerobic digestate culture showed a loss of *p*-cresol concentration after almost two months of incubation. In order to confirm and maintain *p*-cresol degradation activity, both cultures were re-fed with *p*-cresol (0.5 mM) two times. The first re-feed resulted in almost complete degradation of *p*-cresol after 14 days of incubation as shown in Figure 2.5. Total headspace gas of the culture was also measured after the first re-feed and 3.5 ml and 3.6 ml of cumulative headspace gas volume were observed in the active cultures of Passion Puddle (Figure 2.6.) and anaerobic digestate, respectively, while there was no substantial increase of total headspace gas in the background and sterile controls. There were 1.8 ml of total headspace gas in the background controls of anaerobic digestate cultures, but most of it was produced earlier in the incubation period at day-2 instead of gradually accumulated during 14 days of incubation.

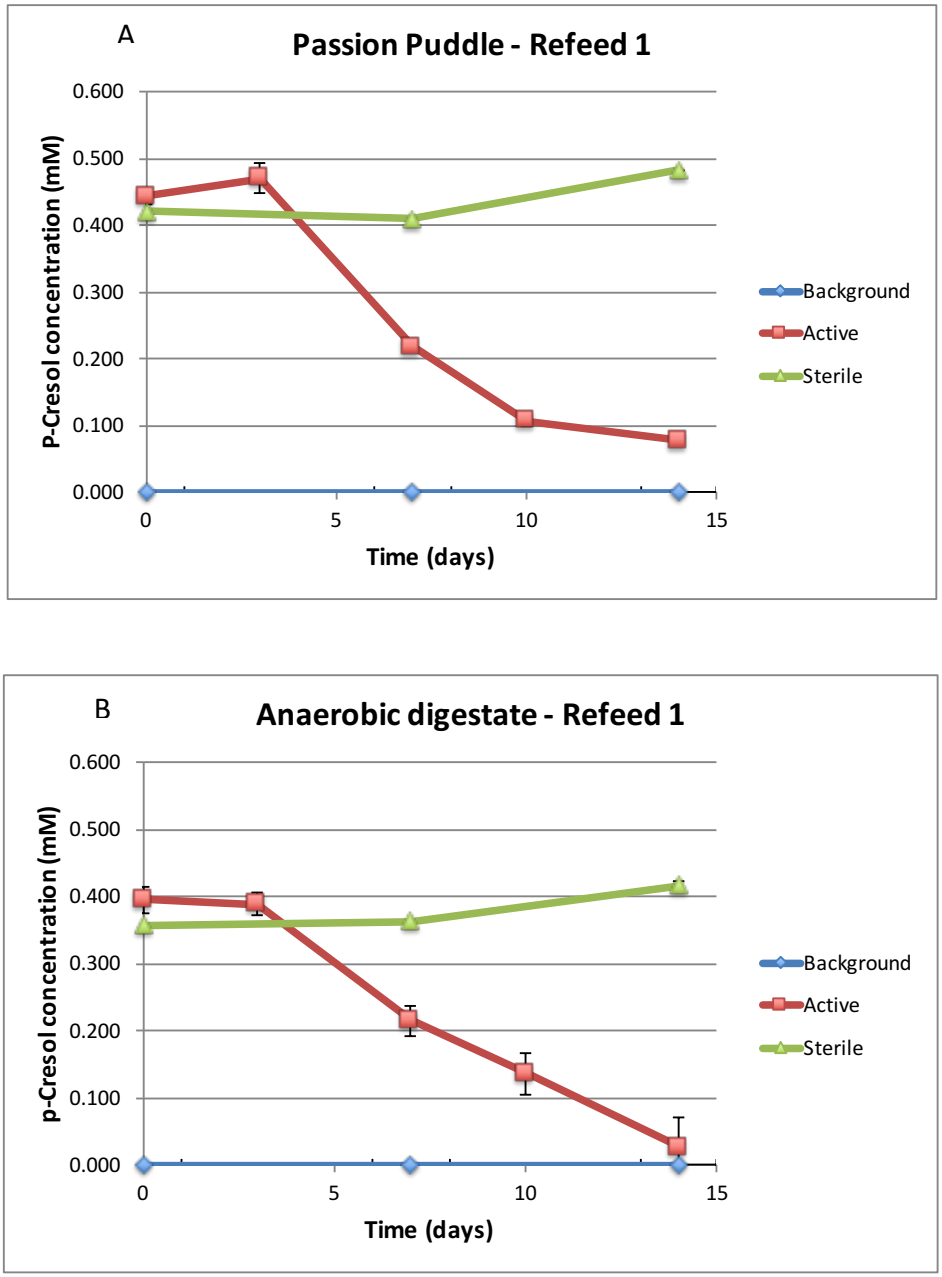


Figure 2.5. *p*-Cresol concentrations in first re-feed of Passion Puddle (A) and anaerobic digestate (B) cultures. The error bar indicates standard deviation of the replicates.

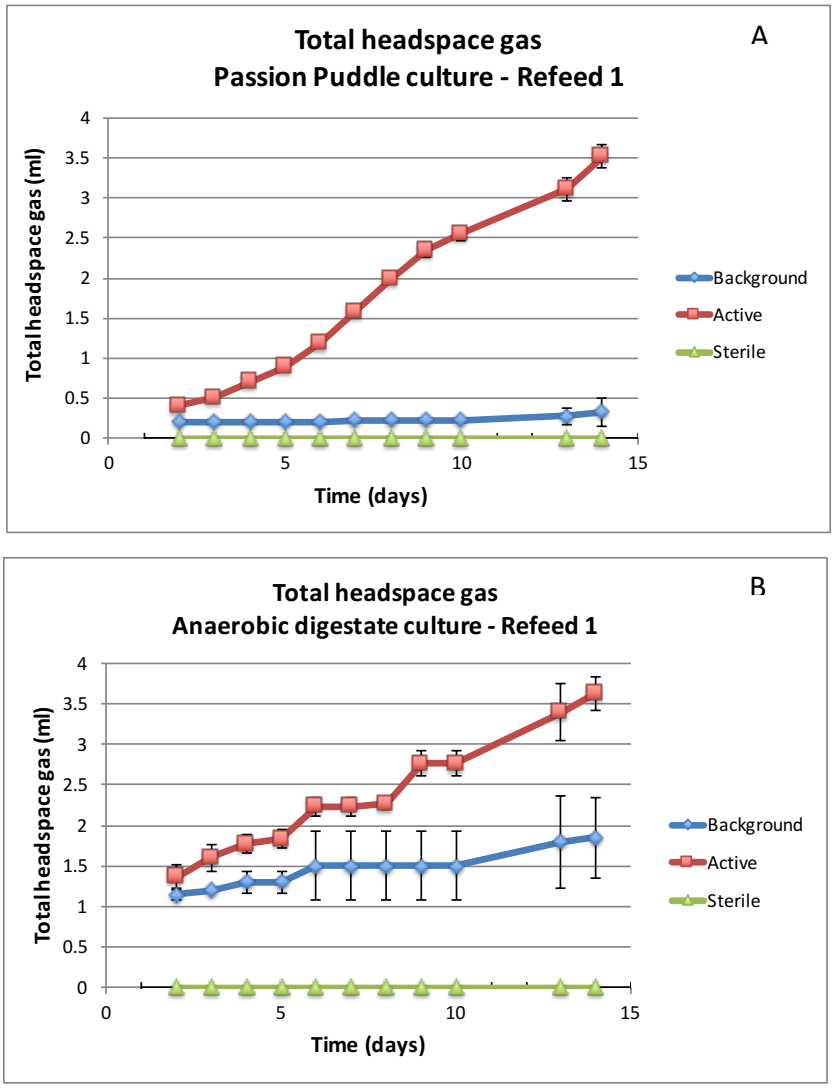


Figure 2.6. Total headspace gas production of Passion Puddle (A) and anaerobic digestate (B) cultures. The error bar indicates standard deviation of the replicates.

A second re-feed of 0.5 mM *p*-cresol resulted in complete degradation of *p*-cresol after 12 days of incubation as shown in Figure 2.7. GC analysis measured the amount of methane produced in the headspace of the culture during the second re-feed. After 12 days, a total of 4.1 ml of methane was produced in the active Passion Puddle cultures (Figure 2.8.) while the background controls produced 1 ml of methane. The anaerobic

digestate cultures also produced more methane in the active cultures but with smaller difference in comparison to the background controls. It was 3.3 ml in the actives while the background controls produced 1.9 ml. The sterile controls did not produce gas and were not sampled for GC analysis.

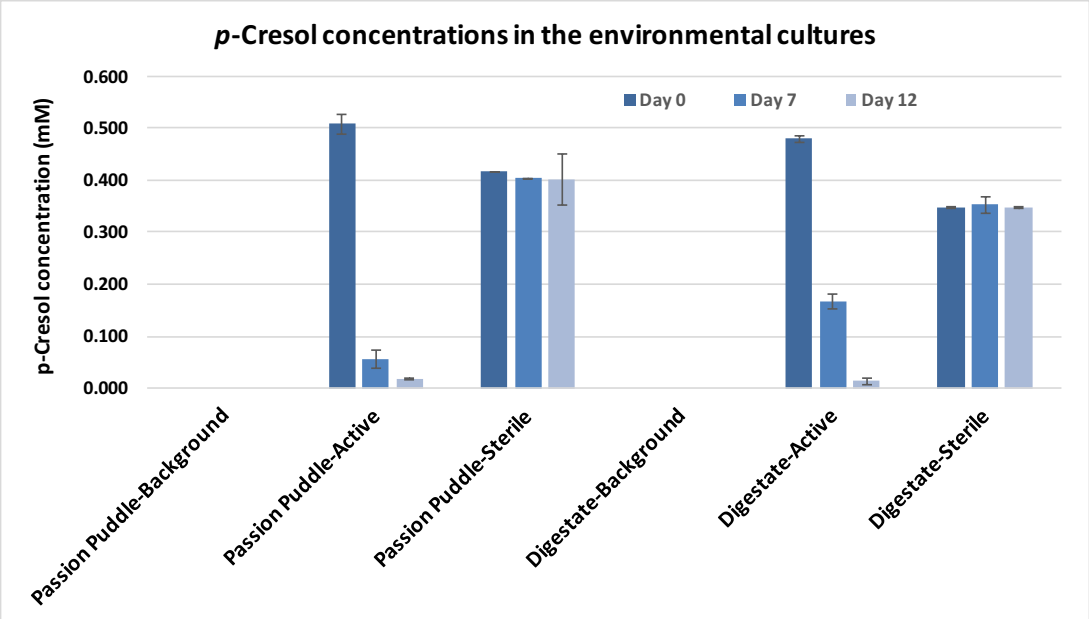


Figure 2.7. *p*-Cresol concentration in the second re-feed of Passion Puddle and anaerobic digestate cultures. The error bar indicates standard deviation of the replicates.

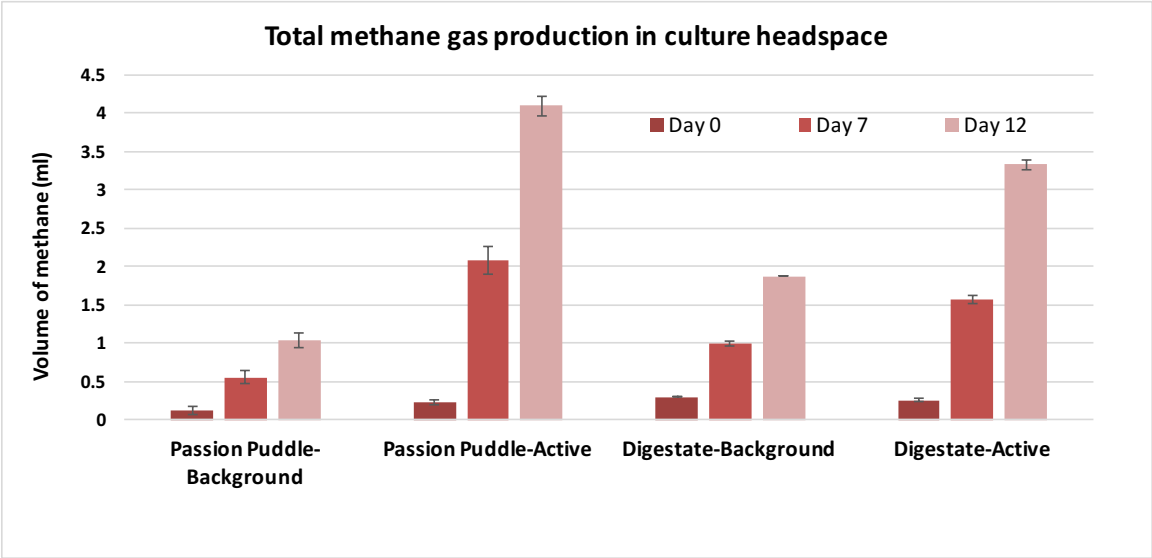


Figure 2.8. Methane gas production of Passion Puddle and anaerobic digestate cultures. The error bar indicates standard deviation of the replicates.

The Passion Puddle and anaerobic digestate cultures were able to degrade *p*-cresol. No *p*-cresol was detected in the background controls on day-0, and no increase of *p*-cresol was produced during incubation. This indicates that although *p*-cresol did not accumulate in the environment that was sampled, there are bacteria in the microbial community in both Passion Puddle and anaerobic digestate that can utilize *p*-cresol. This confirms previous studies that found *p*-cresol degradation in anaerobic environmental samples. The lack of *p*-cresol production in Passion Puddle cultures could be caused by the unavailability of amino acid in the cultures. In the gut cultures, *p*-cresol could be produced from amino acids that were carried over by the stool samples.

The Passion Puddle and anaerobic digestate cultures originated from ecosystems with much longer retention time compared to the human gut system. The anaerobic digestate solid retention time is usually between 20-40 days (Bolzonella *et al.*, 2004), while the sediment retention time in freshwater ponds or lakes are much longer and can range from weeks to years (Percent *et al.*, 2008). On the other hand, food retention time in the human gut is only around 48-66 hours (Tottey *et al.*, 2017). This allows the microbial community from the environmental samples to develop capabilities in utilizing different types of chemical compounds as resources, and this may include the ability to degrade aromatic compounds. Environmental cultures might also adapt better with the *in vitro* condition of the anaerobic set up in this experiment, while there is a bigger difference between the more continuous conditions in the gut system and the *in vitro* condition of the batch culture set up.

2.4 Conclusions

p-Cresol degradation did not occur in any of the gut cultures that were set up in this experiment. Oppositely, a formation of *p*-cresol was observed in the background control and *p*-cresol-treated actives of most of the gut cultures in this experiment. This could indicate that the *in vitro* conditions in the experiment might have selected for microorganisms that tolerate the presence of *p*-cresol and are able to produce *p*-cresol by utilizing resources that were carried over from the inoculum. Degradation of *p*-cresol occurred in the environmental culture set up of both Passion Puddle and anaerobic digestate with complete degradation after twelve days of incubation.

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CHAPTER III

CHANGES IN THE MICROBIAL COMMUNITY OF METHANOGENIC GUT CULTURES

Abstract

p-Cresol degrading Passion Puddle cultures were mixed with batch 1 and batch 3 of the gut cultures to see if the active Passion Puddle cultures can degrade *p*-cresol that was present in the gut culture. The results showed no degradation of *p*-cresol. Production of *p*-cresol was observed in the active cultures and background controls of the batch 1 mixed culture, indicating metabolism of organic material that was present in the gut cultures. In another set up, active Passion Puddle cultures were mixed with the filtrate of the batch 4 gut culture. The purpose of the filtration was to remove organic material and microorganisms from the gut culture so that *p*-cresol degradation can occur. However, there was also no *p*-cresol degradation in these second mixed cultures.

Batch 1 and batch 3 of the gut cultures were inoculated into methanogenic media with an amino acid amendment to reproduce natural *p*-cresol production. A second treatment was also set up with an amino acid and antibiotic amendment to see if the antibiotic would suppress the production of *p*-cresol. The results showed no production of *p*-cresol in the amino acid amended cultures. This inactivity of bacteria in the gut culture inoculum presumably was because the culture conditions were not able to trigger their growth and activity.

High-throughput Illumina 16S rRNA gene sequencing of the gut cultures confirmed the presence of five phyla: Firmicutes, Bacteroidetes, Verrucomicrobia,

Proteobacteria, and Actinobacteria. Firmicutes and Bacteroidetes were the dominant phyla in cultures from a healthy individual while the culture from a post-antibiotic treatment individual was dominated by Proteobacteria and Verrucomicrobia on day 0. The healthy cultures (batch 2 and 3) showed a decrease of Firmicutes and an increase of Bacteroidetes after incubation while the post-antibiotic cultures had the opposite result of a large increase in Firmicutes. This suggested that antibiotic treatment can alter the gut microbiota. Different stool sample handling methods in batch 4 resulted in a different composition of the bacterial community on day 0 with a more equal proportion of Firmicutes and Bacteroidetes compared to the other batches. The Passion Puddle cultures had a different composition compared to the gut cultures and were dominated by Proteobacteria.

3.1 Introduction

Results from experiments set up in Chapter II did not show degradation of *p*-cresol in gut cultures and there was production of *p*-cresol instead observed in some of the gut cultures. *p*-Cresol degradation was observed in the environmental cultures, degrading up to 0.5 mM of *p*-cresol in twelve days. The gut and environmental cultures gave opposite results but were set up with the same method. The next question that arose from the results was whether the active environmental cultures would be able to degrade *p*-cresol that was present in the gut cultures. Experiments were set up to see if there is *p*-cresol degradation activity when the active environmental cultures are inoculated into the gut culture. The potential of this experiment is to show if the active *p*-cresol-degrading

microorganisms in the environmental cultures can perform within the human gut microbial community.

The production of *p*-cresol in the gut cultures was not unexpected. Some anaerobic bacteria in the human gut can produce *p*-cresol according to Gryp *et al.* (2017) and they come from different genera such as *Clostridium*, *Faecalibacterium*, *Eubacterium*, *Anaerostipes*, *Ruminococcus*, *Bacteroides*, and *Bifidobacterium*. Among those groups, the genus *Clostridium* had the most species that were reported to produce *p*-cresol. *Clostridium* have the advantage as spore-forming bacteria to survive in changing environments. It is possible that bacteria from this genus were the ones that survived and thrived in the cultures set up in this experiment. In order to confirm this, methanogenic cultures were set up using the gut cultures as inoculum, and aromatic amino acids were amended as a substrate for *p*-cresol production. Another set of cultures was amended with aromatic amino acids and an antibiotic. The antibiotic was used to suppress the growth of some bacteria to see if that would affect the production of *p*-cresol. Two different antibiotics were selected that would target different groups of bacteria, a broad-spectrum antibiotic and another that is effective for *Clostridium*.

High-throughput sequencing of the 16S rRNA gene was performed to compare the bacterial community in the gut cultures and environmental cultures before and after incubation. The sequencing results would give information on the bacterial groups that were present when the cultures were set up and after incubation. Different bacterial communities are expected to be found between post-antibiotic and healthy gut cultures, and also between gut and environmental cultures. This chapter presents and discusses the results of these experiments.

3.2 Material and Methods

Mixing of gut and environmental culture set up

Batch 1 and batch 3 of the gut cultures treated with 0.25 mM *p*-cresol were selected to be mixed with the active *p*-cresol degrading Passion Puddle cultures. Ten ml of batch 1 and batch 3 cultures (actives, background controls, and sterile controls) were anaerobically transferred to sterile, degassed serum bottles using 70% N_2:30% CO_2 flushed syringes. Background controls of batch 3 were diluted with fresh methanogenic media (1:1) to minimize the concentration of *p*-cresol that had accumulated. Then 5 ml of active Passion Puddle cultures were inoculated into each transferred gut culture. Sterile controls were autoclaved after inoculation to kill all microorganisms. The mixed cultures were incubated at room temperature (25°C).

Mixing of gut culture filtrate and environmental culture set up

Batch 4 of the gut cultures with methanogenic media added with 0.75 g/L yeast extract, 5.25 g/L Mueller Hinton broth, and 5 g/L sodium chloride was selected to be mixed with the active Passion Puddle cultures. Three ml of the actives and sterile controls of batch 4 were filtered and transferred to sterile, degassed serum bottles anaerobically using 70% N_2:30% CO_2 flushed syringes. Then 6 ml of active Passion Puddle cultures were inoculated into each filtrate. Sterile controls were autoclaved after inoculation to kill all microorganisms. The mixed cultures were incubated at room temperature (25°C)

Amino acid amendment gut culture set up

Batch 1 and batch 3 of the gut cultures with 0.25 mM *p*-cresol treatment were used in this set up. Five ml of the active cultures and sterile controls of batch 1 and batch 3 were transferred anaerobically using 70% N_2:30% CO_2 flushed syringes to serum bottles with 10 ml methanogenic media. Three different treatments were set up for each batch: 0.25 mM amino acid amendment (tyrosine and phenylalanine), 0.25 mM amino acid + 5 μg/ml ampicillin, and 0.25 mM amino acid + 10 μg/ml metronidazole, plus one background culture was set up with no treatment. Each treatment and background control consisted of three active cultures and two sterile controls. Sterile controls were autoclaved after inoculation to kill all microorganisms.

p-Cresol concentration measurement

The concentration of *p*-cresol was measured using reversed-phase high-performance liquid chromatography (RP-HPLC). The stationary phase used for the HPLC was a Kinetex 5μm C18 100A (250 x 4.6 mm) (Phenomenex) with a core-shell silica solid support and a trimetylchlorosilane (TMS) end-capping. The mobile phase used for measuring *p*-cresol is methanol-water-acetic acid (60:38:2). The method used for RP-HPLC was 1 ml/minute flow rate with UV-detection at 280 nm. The retention time for *p*-cresol was at 4.9 minutes.

DNA extraction of gut cultures and environmental cultures

Samples from all cultures were taken on day 0 when the cultures were set up. Final sampling was taken from the following cultures on Table 3.1 that were selected for DNA extraction. DNA was extracted using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) following the manufacturer's protocol.

Table 3.1. Cultures selected for DNA extraction

Culture	**Type of medium**	**Incubation length on final sampling**
Batch 1 (female, post-antibiotic)	Methanogenic	20.5 months
Batch 2 (male, healthy)	Methanogenic	17.5 months
Batch 3 (female, healthy)	Methanogenic	15.5 months
Batch 4 (female, healthy, stool sample handling in potassium phosphate buffer)	Methanogenic	10.2 months
Passion Puddle	Methanogenic	8.7 months

16S rDNA Illumina sequencing analysis

The V4 variable region of 16S rRNA gene was amplified with PCR using 515 / 806 primers. The forward primer was barcoded for sequencing purpose. HotStarTaq® Plus Master Mix Kit (Qiagen, USA) was used for the reactions and the PCR condition for this primer set was 3 minutes of initial denaturation (94°C); 28 cycles of 30 seconds of denaturation (94°C), 40 seconds of annealing (53°C), and 1 minute of elongation (72°C); and 5 minutes of final elongation (72°C). The amplification products were run through

agarose gel (2%) electrophoresis to confirm if amplification was successful and to check for band intensity. Multiple samples were combined based on molecular weight and DNA concentrations with equal proportions. Purification of samples was performed using calibrated AMPure XP® beads (Beckman Coulter, Inc., USA). The purified combined samples were then used to prepare Illumina DNA library.

High-throughput sequencing was performed using MiSeq® System (Illumina, USA) at Mr. DNA Lab (Shallowater, TX, USA) following the manufacturer's guidelines. The data resulted were processed with the sequence analysis pipeline at Mr. DNA Lab (Shallowater, TX, USA). The pipeline includes joining sequences, depleting barcodes, removing sequences less than 150 bp, and removing sequences with ambiguous base calls. The sequence data were also denoised, and operational taxonomic units (OTUs) were generated from the sequences. OTUs were determined by clustering at 3% divergence (97% similarity). Taxonomic classification of final OTUs was assigned using BLASTn against database derived from Ribosomal Database Project-II and National Center for Biotechnology Information (http://rdp.cme.msu.edu, http://www.ncbi.nlm.nih.gov).

3.3 Results and Discussion

Mixing of environmental *p*-cresol-degrading cultures with gut cultures

Passion Puddle active cultures were mixed with gut cultures to see if the environmental cultures were able to degrade and utilize *p*-cresol that was available in the gut cultures. Batch 1 (post-antibiotic female) and batch 3 (healthy female) were chosen as a comparison of healthy and post-antibiotic gut cultures from the same individual. Both

environmental and gut cultures had been set up with the same methanogenic media and the same method. However, the cultures came from very different sources and would have different microbial compositions of their communities. The results of this mixing experiment are presented in Figure 3.1.

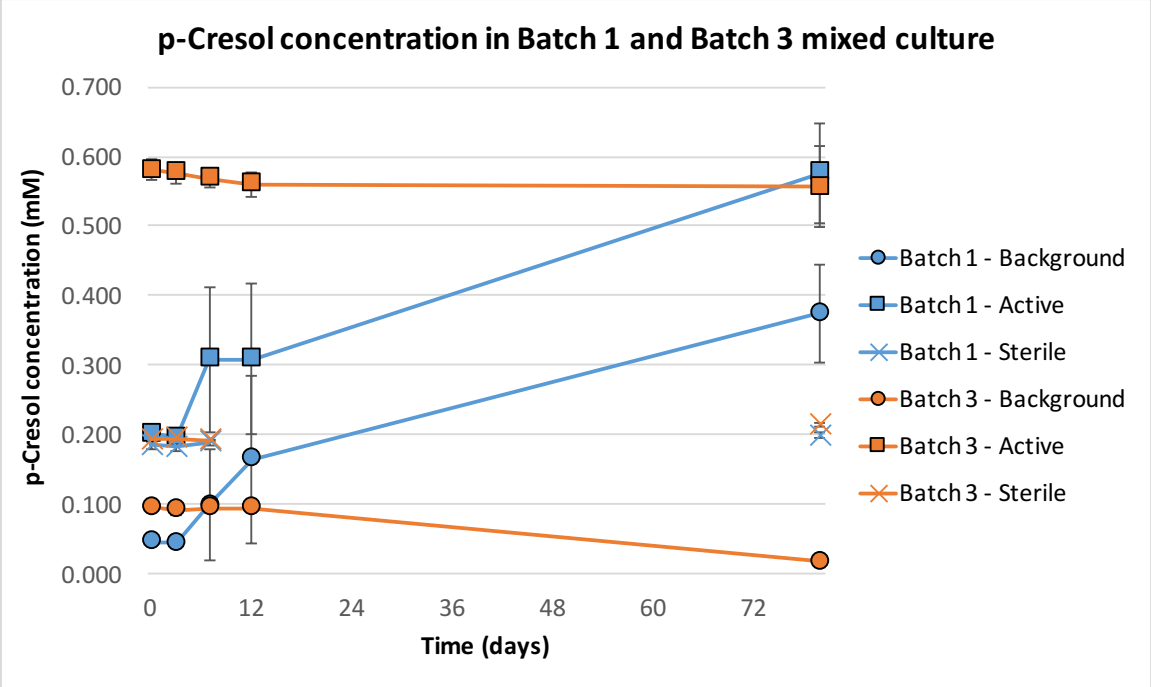


Figure 3.1. *p*-Cresol concentration in the mixed cultures of active Passion Puddle and batch 1 and batch 3 of gut cultures. The error bar indicates standard deviation of the replicates.

In the active batch 1 (post-antibiotic female) mixed cultures, an increase in *p*-cresol concentration was observed after 7 days of incubation (from 0.2 mM to 0.3 mM). The concentration remained the same on day 12. A final sampling after 80 days of incubation showed the concentration of *p*-cresol had increased to 0.58 mM, which is almost three times that of the concentration on day 0. The background controls of batch 1 mixed cultures had a similar pattern. There was only 0.05 mM of *p*-cresol in the

beginning, and on day 7 it increased to 0.1 mM and 0.165 on day 12. The final sampling on day 80 measured 0.38 mM of *p*-cresol in the background controls. The sterile controls of batch 1 mixed cultures remained constant with 0.18 of *p*-cresol from day 0 to day 80.

The actives and background controls of batch 3 (healthy female) mixed culture showed a different result. There was no change in *p*-cresol concentration. The *p*-cresol concentration in active cultures was 0.58 mM on day 0 and was 0.56 on day 80. There was 0.1 mM of *p*-cresol in the background controls on day 0, and it slightly decreased to 0.02 on day 80. The sterile controls remained constant with 0.2 mM of *p*-cresol.

The increase of *p*-cresol could be caused by utilization of amino acids from the remaining organic material in the batch 1 gut culture. Batch 1 was set up with more solids in the inoculum. When the Passion Puddle cultures were mixed with batch 1, some of the bacteria might become active due to the presence of more organic material compared to its previous culture conditions, as the sediment samples had less organic material compared to the gut culture. There was no *p*-cresol production in the batch 3 mixed cultures and no degradation activity as well. The organic materials that are present in the gut culture could make the *p*-cresol less favorable to utilize for the bacteria. There was gas production in all of the active mixed cultures and background controls. This suggests the activity of bacteria utilizing other organic material in the culture, but not *p*-cresol. Another possibility is that there were only a few of the *p*-cresol degraders and they could either get out-competed by the microorganisms in the gut culture or were not able to acclimate to the new environment, therefore becoming inactive.

A second mixing experiment was set up. By using only the filtrate of the gut culture, the Passion Puddle active cultures would not have to compete with the

microorganisms in the gut culture, and additional particulate organic material would be eliminated while the *p*-cresol would remain in the filtrate. Batch 4 was used in this set up as it had a high *p*-cresol concentration (0.76 mM in the actives and 0.28 mM in the sterile controls on final measurement before the set up). The results of the second mixing experiment are presented in Figure 3.2.

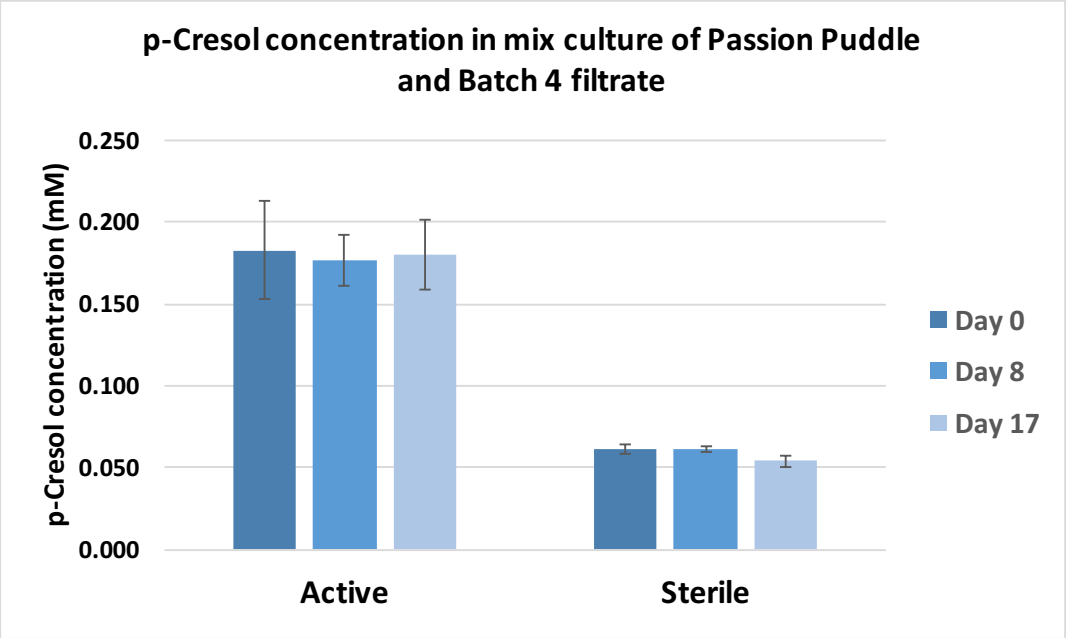


Figure 3.2. *p*-Cresol concentration in the mixed culture of active Passion Puddle and filtrates of batch 4. The error bar indicates standard deviation of the replicates.

The day 0 *p*-cresol concentration showed that the mixing had diluted the concentration as expected (1:3 dilution). There was 0.18 mM of *p*-cresol in the actives and 0.06 mM in the sterile controls. Samples were taken on day 0, 8, and 17 with the result showing no change in *p*-cresol concentration, both in the actives and sterile controls. It is possible that the Passion Puddle cultures did not acclimate well with the transfer. Another possibility is that the microorganisms were not active and the

conditions of the culture in this set up might not be conducive for the *p*-cresol degrading bacteria to be active. The pH of all of the cultures were not monitored throughout the incubation. Although sodium bicarbonate was included in the medium as a buffer, it is possible that changes in pH could occur in any of the cultures during the incubation period and when the cultures were mixed the pH may not be suitable for the activity of the *p*-cresol degrading bacteria.

Effects of amino acid and antibiotic amendment to gut cultures

The gut cultures were amended with amino acids to re-confirm *p*-cresol production. Batch 1 and batch 3 were selected to compare healthy and post-antibiotic gut cultures from the same individual. Two additional treatments included antibiotics alongside the amino acid amendment. *p*-Cresol is produced from amino acid metabolism by anaerobic bacteria. It is an end product of tyrosine metabolism and produced indirectly from phenylalanine metabolism (Vanholder *et al.*, 1999; Gryp *et al.*, 2017). Amino acids were added in this experiment as a substrate for *p*-cresol production. Clostridia could be the group that produced *p*-cresol in the gut culture as many species in this group were reported as *p*-cresol producers (Gryp *et al.*, 2017). Therefore, metronidazole was selected as one of the antibiotics to be added as it is one of the common antibiotics used for CDI treatment (Hudson *et al.*, 2017). If the amino acid amended cultures produced *p*-cresol and the metronidazole amended did not, this would suggest that the Clostridia are the ones that produce *p*-cresol. A broad spectrum antibiotic, ampicillin, was added in the other set ups as a comparison to the metronidazole treatment (Wexler *et al.*, 1985). The concentrations of metronidazole and ampicillin used in this experiment were determined

based on the minimum inhibitory concentration range in the study by Wexler *et al*. (1985) which tested the antibiotics on various isolates of anaerobic bacteria. A background set up was not amended with amino acid or antibiotics.

The amino acid-amended cultures were expected to produce *p*-cresol. Therefore, as all of the treatments were sampled on the same days, the amino acid cultures were analyzed first for *p*-cresol concentration. The result in Figure 3.3 showed no production of *p*-cresol in both batch 1 and batch 3 amino acid-amended cultures. The concentration of *p*-cresol in the actives and sterile controls of batch 1 remained stable (0.1 mM) from the beginning until 27 days of incubation. The same result was obtained for batch 3, with the active cultures showing 0.29 mM on day 0 and 0.32 mM on day 27. The slight increase is much less than the *p*-cresol that was produced on amino acid-amended cultures of batch 3 in Chapter II. The sterile controls remained constant also with 0.1 mM of *p*-cresol.

According to Payne *et al.* (2012), microbial growth in batch fermentation models of gut culture is dependent on the inoculation density and substrate depletion rate. The amino acids were provided as a substrate in this experimental set up. Therefore, it is possible that the cultures did not respond to the substrate due to the microorganisms that were inoculated. It was expected that there would be a sufficient number of microorganisms in the inoculated gut cultures, especially since batch 3 was from a healthy sample. However, it is possible that the bacteria were not active anymore and required specific nutrients to be reactivated that were not supplied in the methanogenic medium that was used in the experimental set up.

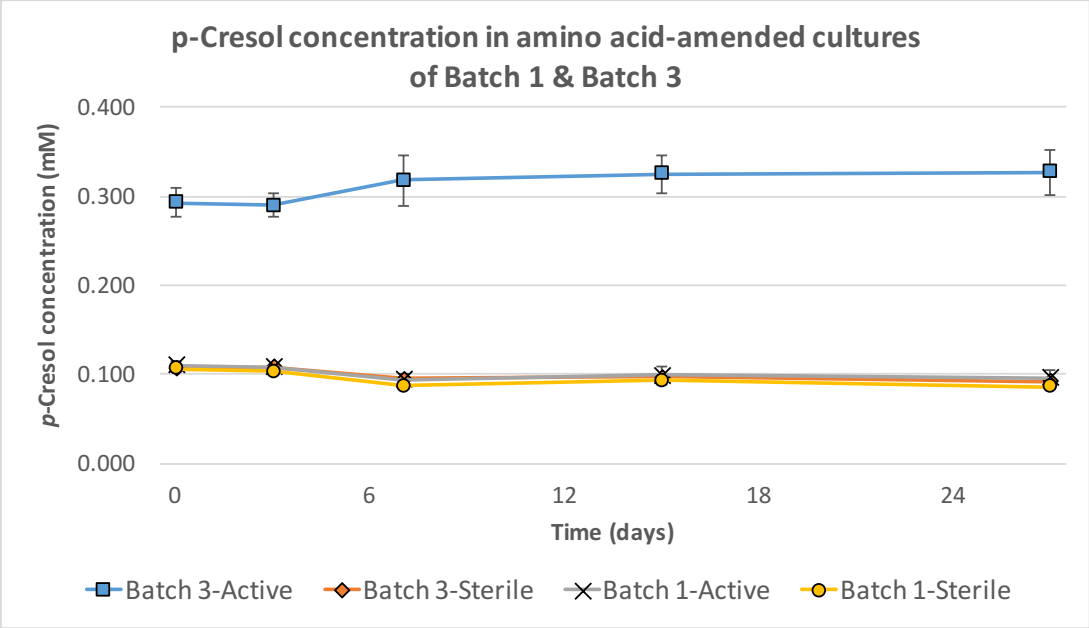


Figure 3.3. *p*-Cresol concentration in the amino acid-amended cultures. The error bar indicates standard deviation of the replicates.

Microbial community changes based on 16S rRNA Illumina high-throughput sequencing

The experimental results in Chapter II showed no degradation of *p*-cresol in any of the gut cultures, and when *p*-cresol was produced, it occurred in both active cultures and background controls. The production of *p*-cresol and its increasing concentration in the cultures could affect the microbial community. In cultures where the *p*-cresol concentration did not change substantially, it is also interesting to see if there is any change in the microbial community before and after incubation. Day 0 and the final sample of background controls (with an additional final sample of an amino acid treatment from batch 3) from all batches of methanogenic gut cultures were selected for

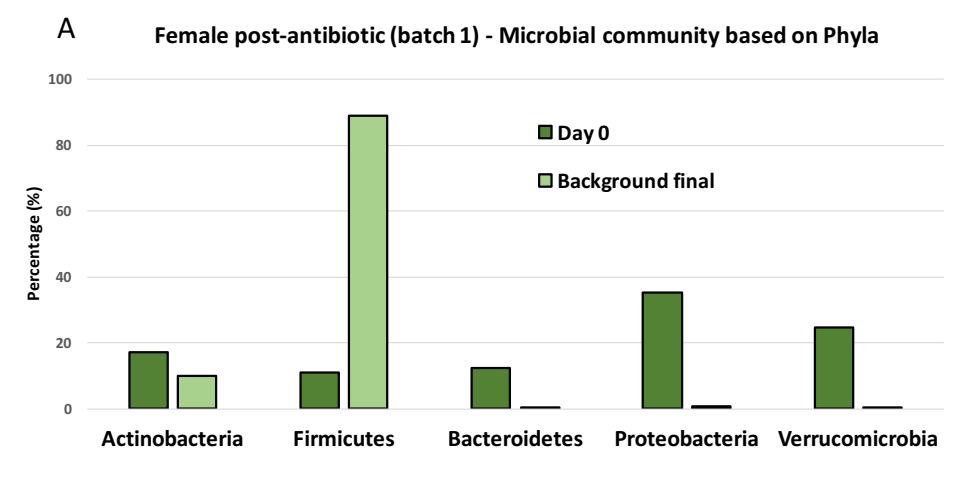
DNA sequencing as the *p*-cresol production was also observed in the background controls. Day 0 and final sample of active Passion Puddle cultures were also sequenced as a comparison with the gut culture community. High-throughput Illumina sequencing of the 16S rRNA gene would reveal the bacterial groups that are present in the community. It can give an estimation of what is present, but since it is based on DNA information, it cannot conclude whether those bacteria are active or not.

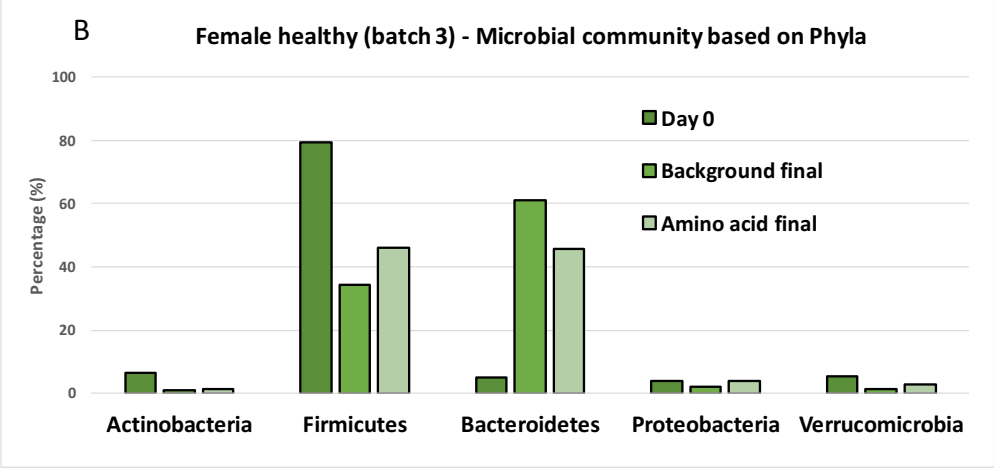
The sequencing result confirmed that human gut bacterial microbiota consists mainly of five phyla (Tremaroli & Backhed, 2012; Lozupone *et al.*, 2012), with Firmicutes and Bacteroidetes as the two dominant phyla, followed by Actinobacteria, Verrucomicrobia, and Proteobacteria as shown in Figure 3.4. The percentage refers to the relative abundance of a specific phylum in the sample. The female post-antibiotic culture (batch 1) had an increase of Firmicutes from 10.8% to 89% (Figure 3.4.A). Bacteroidia, Proteobacteria, and Verrucomicrobia decreased each to less than 1%. The female healthy culture (batch 3) had the opposite result of batch 1, with the Firmicutes decreasing from 79.3% to 34.5% in the background control and 46% in the amino acid treatment (Figure 3.4.B). Bacteroidetes in batch 3 increased from 4.8% to 61% in the background control and 45.6% in the amino acid treatment. In the female healthy culture with potassium phosphate buffer treatment on stool sample (batch 4), Firmicutes and Bacteroidetes were the two dominant phyla and the percentage did not change much after incubation (Figure 3.4.C).

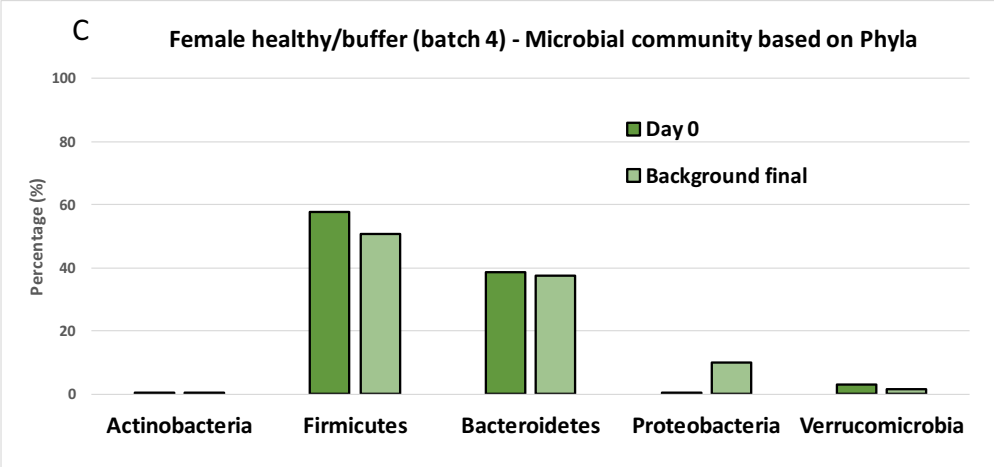
Batch 2 can be a comparison of gut cultures from a different individual (male, healthy) and it was set up the same way as batch 1 and batch 3. The result is similar to

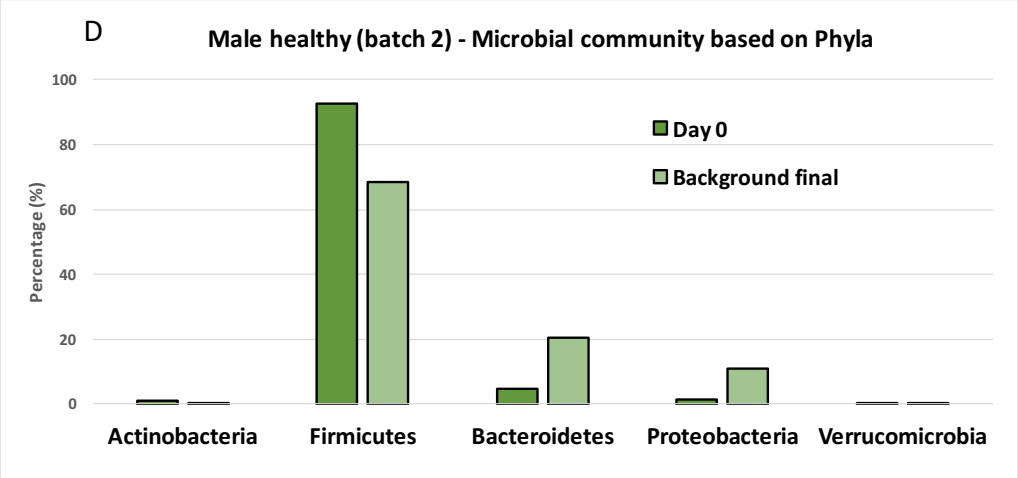
batch 3, which was also from a healthy individual. Firmicutes decreased from 92.5% to 68.3% and Bacteroidetes increased from 4.6% to 20.4% (Figure 3.4.D).

The Passion Puddle culture showed Proteobacteria as the dominant phylum, followed by Bacteroidetes, Firmicutes, and Chloroflexi which was not found in the gut culture. There was no substantial change after incubation except a decrease in Bacteroidetes from 28% to 13% (Figure 3.4.E).









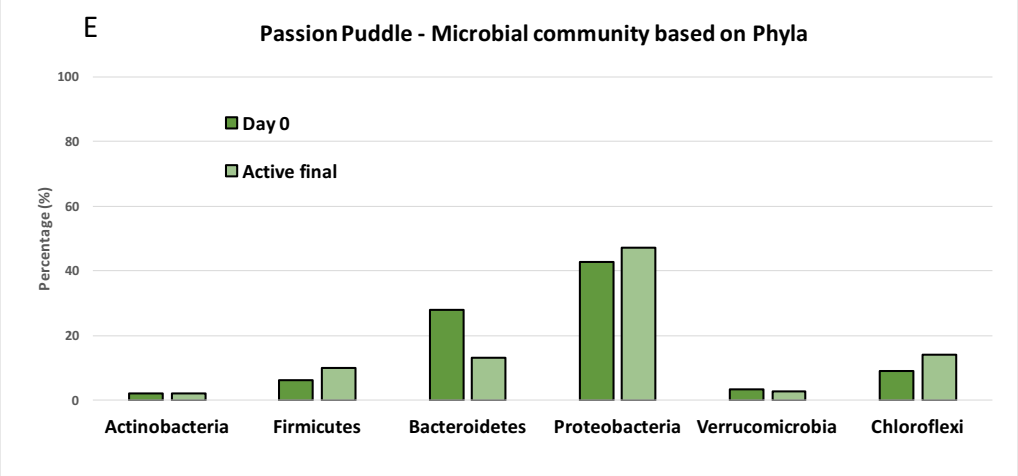


Figure 3.4. Percentages of different phyla for female post-antibiotic culture (batch 1) (A), female healthy culture (batch 3) (B), female healthy culture with potassium phosphate buffer treatment (batch 4) (C), and male healthy culture (batch 2) (D) and Passion Puddle active cultures (E), based on day 0 and final sampling of each batch.

Figure 3.5 shows the relative abundance of different classes in the bacterial community for each sample. The blue and yellow bars dominate the gut cultures and represent Clostridia (blue) and Bacteroidia (yellow). Clostridia made up most of the Firmicutes phylum, followed by smaller percentages of Bacilli, Erysipelotrichia, and Negativicutes. Day 0 of batch 1 (female, antibiotic) had the most distinct pattern compared to the other gut samples as it was dominated by Gammaproteobacteria and Verrucomicrobia. After incubation, batch 1 became dominated by Clostridia. Batch 3 (female, healthy) had the opposite results, with Clostridia dominating on day 0 and the percentage decreased after incubation followed along with an increase in the percentage of Bacteroidia. Batch 4 (female, healthy, potassium phosphate buffer treatment of stool sample) had a more equal composition of Clostridia and Bacteroidia on day 0 and both groups still dominated after incubation with a decrease in Clostridia and increase in Negativicutes and Betaproteobacteria.

Batch 2 (male, healthy) showed a similar pattern to batch 3 with Clostridia dominating on day 0 and decreasing after incubation. Bacilli were observed on day 0 and decreased as Negativicutes increased. Bacteroidia also increased in percentage but were still less than Clostridia. The Passion Puddle sample consisted of more diverse classes compared to the gut culture. Figure 3.5 presents the Passion Puddle results in reference to the classes that were found in the gut cultures only. Passion Puddle was dominated by Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria. Clostridia and Bacteroidia were also present on day 0 (5.7% and 8.9%) and after incubation (9.4% and 4.8%). Classes not included in the graph are Sphingobacteria (14.3% on day 0) and

Anaerolineae (10% on final sampling). The results showed that the environmental sample had a different composition of its bacterial community compared to the human gut.

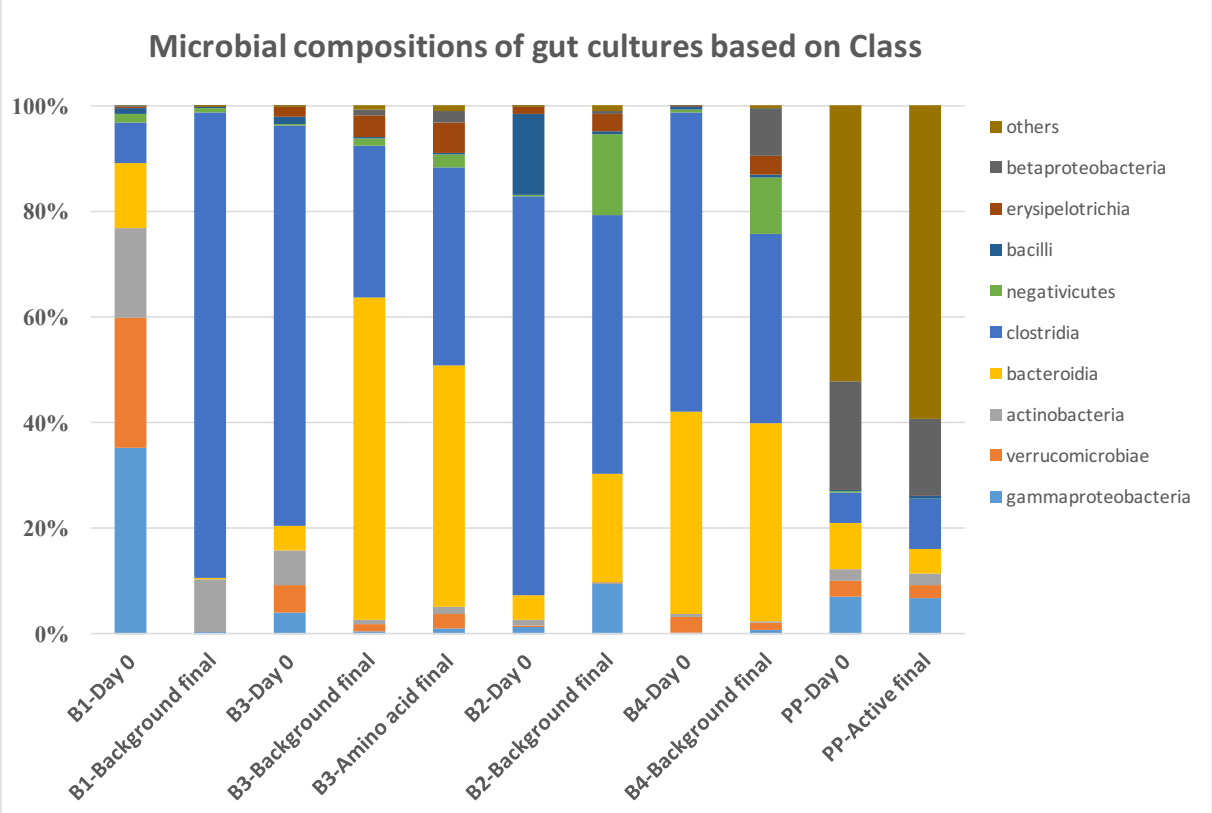


Figure 3.5. Microbial composition of different classes in batch 1, 2, 3, and 4 of the gut cultures and Passion Puddle active cultures, based on day 0 and final sampling of each batch.

The microbial community in the gut cultures changed from day 0 when the cultures were set up to the final sampling. Comparison between cultures from post-antibiotic (batch 1) and healthy (batch 3 and 4) stool samples from the same individual showed changes in the bacterial community. The healthy cultures started with a high percentage of Firmicutes dominated by Clostridia that decreased after incubation. The post-antibiotic cultures showed the opposite results. It started with a community

dominated by Proteobacteria and Verrucomicrobia, and it changed with a high increase of Firmicutes (also mainly Clostridia) that became highly dominant at the end of incubation. Jakobsson *et al.* (2010) studied changes in the microbial community based on 16S rRNA in the human gut by using stool samples. The study found in one of the three individuals of the post-antibiotic treatment samples, that there was an increase in Proteobacteria a week after the antibiotic treatment, while all of the healthy samples were dominated by Firmicutes. Stool samples for all of the gut culture batches were taken from one sampling time point, and therefore it cannot be concluded that this result was caused by antibiotic treatment. However, the similarity with the result of other study comparing post-antibiotic treatment effect on the gut microbiota suggested that it is a possible explanation.

The day 0 result of batch 4 compared to the other gut culture batches can be related to the handling of stool samples after collection. Unlike the other three batches, the stool samples of batch 4 were submerged in degassed potassium phosphate buffer right after collection and turned into slurry with the buffer right before inoculation to the media. Batch 4 also had a different media composition that is more complex compared to the other gut culture batches. Looking at the data in Figure 3.5, the bacterial community of batch 4 started with Firmicutes and Bacteroidetes with a more balanced percentage (57.9% and 37.5%), unlike batch 3 and batch 2 where Firmicutes were much higher (79.3% and 92.5%). After incubation, there was less change in the bacterial community compared to the other three batches. The result possibly indicated that the method for stool sample handling could affect the types of microorganisms that get into the culture. It should be noted that the stool samples for batch 4 and the other batches were taken at

different time points and thus were not a direct method comparison. The solids in the slurry were allowed to settle before transferring to the media during the set up of batch 4. Only the top liquid part was used as inoculum. This would reduce the amount of organic material that went into the culture compared to previous batches, and it could affect the bacterial community composition. Macfarlane and Macfarlane (2004) stated that methods of sampling, transport, and storage in cultivation techniques in human gut microbiota vary in different studies and it can lead to differences in results, while freezing of stool samples is avoided as it has been shown to reduce bacterial viability.

Differences in gut microbiota are also influenced by diet pattern. A long-term healthy, diverse diet promotes a more diverse gut microbiota (Claesson *et al.*, 2012). Short-term changes of diet pattern can also affect the microbiota composition; as reported by David *et al*. (2014), five days of dietary change to an animal-based diet increased the abundance of Bacteroidetes and decreased Firmicutes. This suggested that the bacterial community composition difference found in this experiment can also be influenced by diet pattern of the individual.

Clostridia were expected to be present in these gut cultures as the results in Chapter II showed production of *p*-cresol in many of these cultures. Many of the bacterial species that are mentioned in the literature as *p*-cresol producers are from this group (Vanholder *et al*., 1999; Dawson *et al*., 2008; Van der Meulen *et al*., 2008), although Gryp *et al.* (2017) also mentioned three species from Bacteroidetes that are reported to produce *p*-cresol as well. Clostridia also consists of spore-forming bacteria and this gives an advantage for the group to survive the change of environment from human colon, going to the mason jar, and eventually inoculated into fresh medium. However, the

results also showed Clostridia to decrease in percentage compared to day 0 in all of the healthy gut cultures (batch 2, 3, and 4) and at the same time, the Bacteroidia increased. This could suggest that the culture conditions enabled the Bacteroidia to grow and balance the presence of Clostridia. The bacterial community profile that was obtained in this experiment is based on DNA and therefore cannot confirm if those bacterial groups are active or not in the samples (Payne *et al.*, 2012). However, the noticeable percentages of Clostridia in the community could indicate that the group might contribute to the production of *p*-cresol in the cultures.

3.4 Conclusions

The mixing of active *p*-cresol degrading Passion Puddle cultures with batch 1 and batch 3 of the gut cultures did not result in *p*-cresol degradation. *p*-Cresol was formed in actives and background controls of batch 1 mix cultures, but did not form in any of the batch 3 mix cultures. Mixing with the filtrate of batch 4 also did not resulted in *p*-cresol degradation, that might be caused by inactive bacteria in the inoculum or inability of the bacteria to acclimate to the new culture conditions.

Amino acid amendment to batch 1 and batch 3 gut cultures did not result in a production of *p*-cresol in any of the cultures. This could be caused by inactive bacteria in batch 1 and batch 3 cultures that did not get sufficient nutrient requirements to become active and utilize the substrate. With this result, the experimental set up with amino acid and antibiotic amendments was not able to provide insight on bacterial groups that could be involved in *p*-cresol production in the previous gut cultures set up.

Illumina sequencing with 16S rRNA confirms Firmicutes and Bacteroidetes as dominant phyla in gut cultures, followed by Verrucomicrobia, Proteobacteria, and Actinobacteria. The Passion Puddle cultures were dominated by Proteobacteria, followed by Bacteriodetes, Firmicutes, and Chloroflexi, which were not found in the gut culture. Post-antibiotic gut cultures resulted in a different bacterial composition compared to healthy gut cultures, with Proteobacteria and Verrucomicrobia as dominant phyla on day 0. It also showed a large increase in Firmicutes percentage after incubation, whereas the healthy gut culture had the Firmicutes decreased and Bacteroidetes increased. Batch 4 that was set up with different stool sample handling method and different medium showed a more balanced ratio of Firmicutes and Bacteroidetes with less change in the composition after incubation, suggesting that the method used in culture set up could affect the microbial composition in the culture.

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CHAPTER IV

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

Degradation of *p*-cresol was not observed in any of the gut cultures that were set up. Production of *p*-cresol occurred in most of the gut cultures, both in active cultures and background controls. The culture conditions in this experiment might have selected for microorganisms that tolerate the presence of *p*-cresol and are able to produce it from organic materials that were carried over from the inoculum.

p-Cresol degradation was observed in the environmental cultures of both Passion Puddle and anaerobic digestate under methanogenic conditions, with complete degradation of 0.5 mM *p*-cresol within twelve days of incubation. Mixing of *p*-cresol degrading cultures from Passion Puddle with the gut cultures did not result in *p*-cresol degradation. Mixing Passion Puddle cultures with the filtrate of the gut cultures also did not result in *p*-cresol degradation. This might be caused by inactivity of bacteria in the inoculum or the inability of bacteria to acclimate to the culture conditions.

The second experiment set up in Chapter III with amino acid amendment to gut cultures did not produce *p*-cresol in either batch 1 and batch 3. Therefore, the experiment set up with amino acid and antibiotic amendments was not able to give information on bacterial groups that might be involved in *p*-cresol production in the previous experiments in Chapter II. This might be caused by inactivity of the inocula. The medium, substrate, and conditions provided in the cultures might not be sufficient to trigger bacterial growth and activity.

High-throughput Illumina sequencing of the 16S rRNA gene confirmed the presence of five phyla in the human gut microbiota, specifically Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria, and Actinobacteria. It also indicated changes in the bacterial community before and after incubation, with a decrease in Firmicutes and an increase in Bacteroidetes in all gut cultures except batch 1. Gut cultures from post-antibiotic stool samples showed a different composition on day 0 compared to cultures from healthy individuals. This confirmed that antibiotic treatment affects the microbial community in the human gut. Batch 4, set up with a different stool sample handling method and different medium, showed a different composition of bacterial community on day 0 with a more balanced ratio of Firmicutes and Bacteroidetes. The composition also did not change as much as the other batches after incubation suggesting different method in setting up the gut culture could affect the bacterial community composition. The bacterial community in environmental cultures had a different composition than the gut cultures. Proteobacteria was the dominant phyla and Chloroflexi was present only in the environmental cultures.

4.2 Future work

The gut cultures in this experiment did not show *p*-cresol degradation. There are methodological adjustments that can be used in the future for research in *p*-cresol degradation in the human gut. Different compositions of microbial community between batch 4 and the other batches suggested that the sample handling method prior to culture set up could be optimized. The stool sample microbiota came from an environment with high nutrient availability. Therefore, another aspect to consider in gut culture set up is to

provide enough nutrients to maintain the gut microbiota diversity and avoid selecting only certain groups of microorganisms. Another possibility is to test for *p*-cresol degradation activity using isolates of known strains that are present in the human gut. Nowak and Libudzisz (2007) reported degradation of *p*-cresol using isolates of intestinal lactic acid bacteria *Lactobacillus* sp. This approach can be applied to a wider range of known human gut bacterial isolates to explore *p*-cresol degradation activity in the human gut.

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