EFFECTS OF SHORT-CHAIN FATTY ACIDS PROPIONATE, ACETATE, AND BUTYRATE ON THE GROWTH OF CLOSTRIDIUM DIFFICILE IN CO-CULTURE WITH AN ESCHERICHIA COLI ATOE MUTANT

by

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LIST OF ABBREVIATIONS

Abbreviation	Description
AAD	Antibiotic-associated Diarrhea
BHIyp	Brain-Heart Infusion with Peptone and Yeast
CD196	Patient Strain of *Clostridium difficile*
CDA	*Clostridium difficile* Agar
CDI	*Clostridium difficile* Infection
F/B	Firmicutes/Bacteroidetes Ratio
FMT	Fecal Matter Transplantation
GIT	Gastrointestinal Tract
GPCR43	G-Protein Coupled Receptor 43
HDACi	Histone Deacetylase Inhibitor
SCFA	Short-chain Fatty Acid

ABSTRACT

There are three major short-chain fatty acids (SCFAs) that play a role in gastrointestinal health: propionate, butyrate, and acetate. SCFAs have probiotic effects that lead to a healthy gastrointestinal environment such as providing energy, maintaining the epithelial lining, and roles in gene regulation and immunity. There is evidence that SCFAs function as important cell signals that affect the metabolism and overall physiology of the gastrointestinal tract. While SCFAs have been shown to reduce the amount of inflammation in association with GIT diseases, they also have been known to affect metabolic signaling processes and immunity. Determining which species of SCFAs are present could help us better understand their roles in the gut microbiome, metabolism, and cellular signaling propagation. High fiber diets and dietary supplementation of commensal strains such as *Lactobacillus* and *Bifidobacterium* have been shown to increase the amounts of SCFAs in the gastrointestinal tract. *Clostridium difficile (C. diff)* is a gram-positive, spore-forming, bacillus-shaped anaerobe. *C. diff,* normally a minor component of the gastrointestinal flora, is resistant to most broad-spectrum antibiotics. Dystopia of the normal flora, caused by antibiotic treatment for unrelated infections, causes an overgrowth of *C. diff*. In recent years there has been an increased number cases of *C. diff* infection (CDI) and antibiotic-associated diarrhea (AAD). The antibiotic-induced disruption in the natural gastrointestinal flora diminishes our own microbiome-mediated colonization resistance, allowing *C. diff* to proliferate, sporulate and produce toxins. This study aimed to examine if SCFAs play a role in the initial onset and growth of *C. diff* in a broth culture. Upon examining each of the three major SCFAs singly and in combination (1uM/SCFA concentration), no major effect on the growth of

C. diff was observed. Using spent media from BW25113 (WT) and BW25113 (*atoE*, SCFA mutant), *C. diff* showed a trend toward less growth and delayed onset of exponential growth as the concentration of spent media went up. Using mM concentrations in a 3:1:1 ratio of acetate, propionate, and butyrate respectively will mimic the human gastrointestinal tract more efficiently, and may have more of an effect on the growth of *C. diff*. It is suggested to try 30mM for acetate, and 10mM for propionate and butyrate.

I. INTRODUCTION

Roles of Short-Chain Fatty Acids in Gastrointestinal Health

Three major short-chain fatty acid (SCFA) products of carbohydrate metabolism by anaerobic bacteria are acetate, propionate and butyrate [1]. SCFAs have many beneficial effects on gastrointestinal tract (GIT) health such as lowering the pH, enhancing blood flow to the colon tissues, replenishing the mucous lining and decreasing inflammation. SCFAs are produced in the large bowel of the gastrointestinal system when commensal obligate anaerobes metabolize complex polysaccharides, (starches like fiber) that were not broken down in the upper gastrointestinal tract [2]. Obligate anaerobes of a healthy adult are mainly *Firmicutes* and *Bacteroidetes* as discussed before, and they exist in highest concentrations in the lower GIT [3]. Once dietary fiber and starches have been broken down in the upper GIT, these products are then hydrolyzed and fermented to end products that produce the SCFAs [4]. Different SCFAs are produced depending on the microbial community present. For example, *Bifidobacteria* are well known butyrate producers, while *Clostridia* are well characterized for producing both acetate and butyrate [5]. Each SCFA is known to affect cellular processes in a different manner, which is elaborated upon further in the next sections. Diets higher in fiber have also been shown to increase the number of *Firmicutes* and *Bacteroidetes*, in turn leading to increased concentrations of the three main SCFAs [6, 7]. Western diets are typically higher in fat concentration and lower in fiber, and have been shown to have reduced levels of SCFAs in comparison to diets of vegetarians and Eastern cultures. While this high fat diet initially led to lower numbers of all three SCFAs, the level of propionate and acetate were able to recover [8]. Diets with higher fiber content also led

to reduced risk of disease by lowering weight, liver fat amounts, cholesterol amounts, and triglycerides [8].

Butyrate is an important player in homeostasis and GIT immunity. The weak acid composition allows for ion exchange, and promotes the absorption of sodium and water [9] This can alleviate the symptoms associated with *Clostridium difficile* infection (CDI) and other antibiotic-associated diseases (AAD) diseases. Butyrate also fuels colonocytes by serving as the favored carbon source in the citric acid cycle [10]. Another powerful characteristic of butyrate is its ability to "hyperacetylize" histones leading to gene regulation effects in the nucleus [10]. This is commonly referred to as HDACi, or histone deacetylase inhibitor/inhibition. Carcinogenic conditions, chronic inflammation, colitis, and CDI are all sensitive to HDACi gene regulation [9, 11]. Lastly, butyrate promotes innate immune function by binding the G-protein coupled receptor 43 (GPCR43) and stimulating molecules such as T regulatory cells and macrophage development [1, 12-14]. GPCRs use SCFAs as signaling molecules to cause down regulation in pro-inflammatory molecules such as interleukins and cytokines [13]. Butyrate's role in controlling inflammation makes it of great importance when looking at diseases that are centered around inflammations such colitis, Crohn's disease, inflammatory bowel disease, and especially CDI.

Propionate is produced by *Bacteroidetes* via the succinate pathway and helps to promote the growth of probiotic commensals of the *Bifidobacteria* [15]. One of the main functions of propionate is to stimulate colonic blood flow and muscular contractions [10]. Both butyrate and propionate, when found intracellularly, act as a histone deacetylase inhibitors (HDACi). Propionate has similar roles in regulating inflammation to butyrate

by acting as a signaling molecule to GCPRs, but its anti-inflammatory potential is 8-10 fold less than that of butyrate [16]. Studies examining the roles of these two SCFAs in response to *Salmonella typhimurium,* showed that when used in combination, they were able to inhibit infection [17]. Due to propionate's ability to help butyrate in ameliorating inflammatory responses and anti-carcinogenic effects, propionate is being looked at as a potential additive in many prebiotics [16].

Acetate has the highest concentration of the three SCFAs in the colon, and is usually found in a ratio of 3:1:1 to propionate and butyrate, respectively [16, 17]. Acetate is not as effective as butyrate at stimulating an immune response by recruiting macrophages and other molecules to the primary site of infection, but it is still an important molecule for starting the initial response [1]. Acetate's HDACi ability has been questioned, but just like all three SCFAs, it stimulates GCPR43 activity and innate immune responses in the GIT [11, 13, 18]. Interestingly, higher levels of butyrate have been linked to lower levels of acetate in the distal colon [19]. This may be due to a phenomenon where butyrate-producing bacteria consume acetate, while the majority of bacteria in the gastrointestinal tract produce more amounts of acetate [5]. Uniquely, acetate is absorbed into the portal vein and becomes metabolized by tissues as the majority bypasses the liver, while propionate is absorbed by the liver, and butyrate serves as an energy source to epithelial cells [4, 10]. The acetate that is absorbed by the liver is used as a substrate for cholesterol synthesis, and also contributes to overall liver health [20]. A summary of all SCFAs is outlined in **Table 1**.

Table 1. Summary of SCFA Benefits in the Gastrointestinal Tract

SCFA	Known Effects	Benefits to GI Microbiome
Propionate	Increase contractile responses Histone Deacetylase (HDAC) inhibition	Improves blood flow Regulates Leukocytes & Macrophages
Acetate	Increase Cholesterol synthesis Used for cross-feeding	Maintains Gut Homeostasis Promotion of Butyrate-producing populations
Butyrate	Histone Deacetylase (HDAC) inhibition Stimulates Na+ & H_2O Absorption Energy source for colonocytes	Regulates Leukocytes & Macrophages Reduces diarrheal symptoms Epithelial and Mucosal Lining Maintenance

Determining which species of SCFAs are present could help us better understand their roles in metabolism, cellular signaling, gene regulation, and gastrointestinal immune responses. We can also look at their effects on bacterial hosts, such as our commensal GIT microbiome.

Commensal Bacteria of the Gastrointestinal Microbiome

Commensal bacteria play a crucial role in our GIT health and flora by maintaining homeostasis. Many of the commensal members of the GIT flora provide colonization resistance by blocking potential pathogens from adhering to the epithelial surface, and other members inhibit colonization by secreting antimicrobials call bacterocins [3]. The

commensal flora also provides several vitamins, minerals, and other metabolites including SCFAs to help maintain a healthy homeostasis in the gut [5, 21]. Studies have found through the use of metagenomics that two main members of the gastrointestinal microbiome include *Bacteroidetes* and *Firmicutes.* *Firmicutes* degrade polysaccharides and ferment amino acids while *Bacteroidetes* also play a major role in breaking down macromolecules for use by the gastrointestinal environment. [22]. The *Firmicutes* and *Bacteroides* ratio (F/B) has been studied in recent years, and although no conclusive data exist, there are a few studies that point toward a trend. Typically, with a reduced F/B ratio (higher levels of *Bacteroidetes)* there are increased levels of SCFAs and a trend to lower adiposity [7, 23]. Patients with obesity often show a higher F/B ratio, increased gastrointestinal inflammation symptoms, and lower levels of SCFA concentrations [7, 8]. A study by Mariat et al 2014, indicated that infant and elderly F/B ratios are very low in comparison to an adult ratio, but no specifications were made in regard to gender, weight, and medical history. These lower F/B ratios in the study also marked an increase in the levels of commensal pathogens and inflammation of the gastrointestinal tract [24]. While this is interesting, a higher F/B ratio for the adults in comparison to the other groups may only indicate that we are heavier in weight on average, and that our microbial communities are more diverse during this phase of life. In conclusion, most studies support that an equal balance of both *Firmicutes* and *Bacteroides* indicate a GIT in homeostasis.

Escherichia coli (*E. coli*), a commensal organism of the gastrointestinal flora, is well studied and characterized for its role in the gastrointestinal microbiome. In addition to being a commensal organism, *E. coli* can also behave as a pathogen, often associated

with opportunistic infections in the urinary tract [25]. *E. coli* competes for resources within the microbiome and has even been shown to completely suppress other pathogenic enteric anaerobes such as *Shigella* spp [26]. *E. coli* has been studied for many years and as a model organism due to its well characterized genome. The "Keio collection," constructed in Japan, is comprised of over 3000 non-essential gene knock-out mutant lines from the K-12 strain [27]. This gives researchers an invaluable tool to study specific genes and mechanistic associations. The *ato* gene family is not widely studied, but it contains many members involved in the synthesis, degradation, regulation and transport of SCFAs [28, 29]. The mutant *atoE* lacks an essential trans-membrane protein that is needed to transport SCFAs [30]. The entire family is commonly referred to as the ato-DABE operon, controlled by the regulator *atoC* [30].

Commensal *Clostridia spp.* are acidogenic bacteria that produce butyrate and acetate in the gastrointestinal microbiome. Commensal *Clostridia spp.* and are known to inhibit pathogenic strains of *Clostridia,* such as *C. difficile (C. diff),* via colonization resistance, competition for resources, and metabolite production [22, 31]. One way commensal *Clostridia spp.* maintain homeostasis is via bile salt modifications. Bile salts are synthesized in the liver, where they are conjugated with glycine or taurine. Several different *Clostridia spp.* have bile salt hydrolases on their cell surfaces which then remove the conjugated glycine or taurine, or modify the primary bile salt into a secondary bile salt. Secondary bile salts are inhibitory to the outgrowth of *C. diff* spores and can also affect the toxin production, while taurocholate in tandem with glycine has the opposite effect [32]. Sodium taurocholate has been shown to enhance the germination of

C. diff spores in culture by a factor of 10^5 [31, 33] as little as 1 minute exposure with glycine acting as a necessary co-germinant.

Lastly, *Clostridia spp.* partake in cross-feeding, or consumption of end products (like SCFAs) produced by other members of the GIT [6]. These cross-feedings strengthen the natural protective effect of the normal gastrointestinal flora, and may even provide food sources to niche populations that are otherwise unable to produce the metabolite themselves. Disruptions in microbial populations, whether caused by antibiotics, diet or another means, may weaken this protective cross-feeding effect. A summary of the strains for this experiment (BW25113 (WT), BW25113 (*atoE*), and CD196) are included in **Table 2.**

Table 2. Comparison of Strains

Strain Name	Characteristics	Role in Gastrointestinal Microbiome
Clostridium difficile (CD196)	Gram-positive bacillus Spore-forming Strict anaerobe	Commensal anaerobe Carbohydrate fermentation
BW25113 (WT)	Gram-negative bacillus Facultative anaerobe	Vitamin production Colonization resistance to pathogens
BW25113 *atoE*	Lacks transmembrane SCFA transporter	Unknown

Clostridium difficile Infection and Current Treatments

Clostridium difficile (C. diff) is a gram-positive, spore-forming, bacillus-shaped anaerobe that exists as a commensal in the GIT. *C. diff* has become a major emerging threat in the United States and other countries due to its difficulty in treatment. CDI is becoming well known in hospital environments as a nasty nosocomial pathogen with

resistance to many medications. Estimated costs for treatment of *C. diff* in 2015 were just short of $5 billion dollars with 500,000 new cases and 29,000 deaths [22]. Individuals who are very young, very old, or suffering from autoimmune disease are also more at risk for a recurrent infection. CDI can range from diarrhea to pseudo-membrane colitis, and even lead to death in severe cases [34, 35]. Traditional antibiotic use creates a dystopia of the normal gastrointestinal microbiome, affecting colonization resistance and important bacterial members who are needed to synthesize vitamins and other metabolites in order to maintain homeostasis, leading to an increased chance of CDI infection [36] Further treatment with antibiotics will perpetuate the dystopia of the gastrointestinal microbiome, and about 25% of patients will suffer from a second bout or recurrence of the disease [37]. The disruption in the natural gastrointestinal flora from antibiotic use allows the usually commensal *C. diff* to grow rapidly and produce toxins that are hallmarks of the disease. TcdA and TcdB are the two toxins that cause CDI colitis, and they are coded for on a pathogenicity locus [38]. The two toxins act in concert against the epithelium (causing inflammation), actin cytoskeletons, and can lead to apoptosis [22]. Another hallmark of CDI is sporulation, or the production of spores. *C. diff* lacks the common bile salt hydrolases found on many *Clostridia spp*., but the protease CspC receptor is important for bile acid-mediated germination of spores [33, 39]. For *C. diff* spores to germinate and grow into toxin producing cells, they require a germinant and/or co-germinant [22]. The secondary bile salt taurocholate in tandem with glycine, leads to the germination of *C. diff* spores.

Common antibiotics used in the treatment and prevention of *C. diff* include Clindamycin, Metronidazole, and Vancomycin. Clindamycin has since been abandoned

as a treatment for CDI due to its broad-spectrum nature, and its propensity to cause antibiotic resistant strains to colonize the gastrointestinal tract [34]. Metronidazole and Vancomycin both disrupt the gastrointestinal microbiome and causes changes in microbiome-mediated routes of colonization resistance. Metronidazole causes a smaller disruption than Vancomycin, and patients recovered colonization resistance faster when treated solely with Metronidazole [40]. Patients treated with Vancomycin took almost twice as long to recover their normal gastrointestinal microbial composition, and were vulnerable to Vancomycin-resistant nosocomial pathogens [40].

Thanks to the published studies of Nobel Laureate Metchnikoff noting the benefits of *Lactobacillus,* probiotic bacterial strains have been known and used for over 100 years to alleviate disease [41]. *Lactobacillus* and *Bifidobacteria* are two commonly used probiotic strains commonly found in the human gastrointestinal microbiome. *Lactobacilli* are widely available in many yogurts and milks available over the counter and have seen an increase in consumption over the last few years. *Lactobacilli* are known to provide protection from bacteria trying to cross the intestinal epithelium, and it has also been implicated as a signal carrier during immune processes [42]. Additionally, *Lactobacilli* have a positive effect on the growth of *Bifidobacteria [23]. Bifidobacteria* play a crucial role in acetate synthesis, vitamin B and other antioxidant synthesis, as well as helping to degrade non-digestible carbon sources (like fiber) [5, 23, 31]. *Lactobacillus* and *Bifidobacterium* strains also have the potential to decrease the populations of Bacteroides and Firmicutes, which an overgrowth of either can be associated with an unhealthy gastrointestinal flora. In one such study, *Lactobacillus* and *Bifidobacterium* were mixed as a "syn-biotic" and the F/B decreased, while reporting a 3-8 fold increase

in the amounts of SCFAs acetate and butyrate [23, 43]. When using *Lactobacilli, Bifidobacterium* or both in the treatment of CDI, these strains have been shown to stunt toxin production, while increasing the amounts of short chain fatty acid (SCFA) production [44, 45].

Currently, the most effective treatment for CDI comes from studies involving fecal matter transplantation (FMT). FMT comprises of a healthy donor sample, which will restore the microbial populations prior to dysbiosis. FMT is cheap, safe, and upwards of 90% effective [46]. Not only does FMT restore beneficial microbial populations, it also alters the fecal bile salt composition, which is known to affect the germination of *C. diff* spores (leading to recurrent bouts of infection) [47]. While FMT is still relatively new, it has promising results for the treatment of CDI and some other forms of colitis. FMT has also been studied in the treatment of diseases such as obesity, eczema, and behavioral disorders [5, 22]. Again, since FMT is relatively new and no laws on the regulation of donation exist, FMT is considered experimental and unforeseen side-effects may occur.

Statement of Purpose

The aim of this study is to examine the roles of SCFAs on the growth of *C. diff*. SCFAs can have antimicrobial effects, which may lead to a delay in the growth of *C. diff* and other harmful bacteria.

II. MATERIALS AND METHODS

Media Selections

All liquid cultures of BW25113 (WT), BW25113 (*atoE*), and CD196 were grown in BHI media supplemented with 5% yeast and 1% peptone (BHIyp) at 37°C in anaerobic conditions. The yeast and peptone provide additional nitrogen and amino acid sources. Plates were streaked from frozen stock cultures and grown overnight in order to ensure lack of contamination. 20mL scintillation vials filled with 12mL BHIyp, and were determined to provide an appropriate buffer from any accidental oxygen contamination. An example of the scintillation set up is provided in Figure 1A. All media preparations were allowed to reduce for 24 hours prior to inoculation. Once a colony had been selected from the frozen stock plate (BW25113 (WT), BW25113 (*atoE*), and CD196), it was inoculated into the scintillation vial and grown overnight at 37°C in anaerobic conditions. Agar plates for BW25113 (WT) and BW25113 (*atoE*) were BHIyp. Both BW25113 (WT)and BW25113 (*atoE*) showed similar phenotypes, expressing round, glossy and raised beige colonies. CD196 showed limited growth on BHIyp agar, so "Clostridium Difficile Agar" (CDA) was used to select for and differentiate CD196. CDA contains small amounts of cycloserine and cefoxitin, two antibiotics. Cycloserine inhibits the growth of gram-negative bacilli like *E. coli* while cefoxitin is broad spectrum and can act on both gram-negative and gram-positive bacteria (with the exception of *C. diff*) [48]. CDA also contains 7% horse blood to supply extra nutrients and encourage sporulation. Additional phosphates were added to buffer the pH, and fructose and peptone provided the carbon and nitrogen sources. Colonies were a gray-white color with irregular, undulated edges, as demonstrated in Figure 1B.

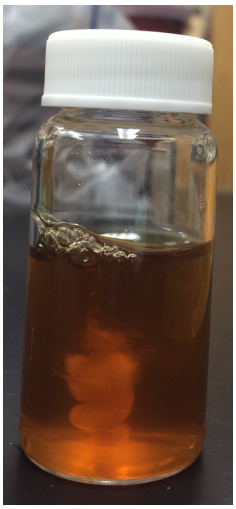
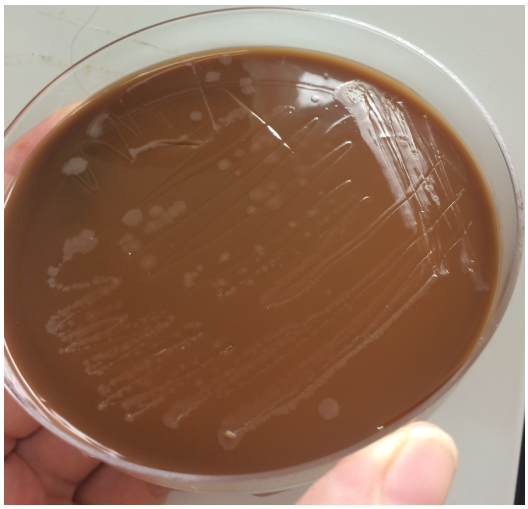
A  B 

Figure 1A. Scintillation Vial Set-Up. Scintillation vials of 20mL were used to ensure an anaerobic environment when working on the bench with the strict anaerobe CD196. Pictured is a biofilm attached to the bottom of the vial. **Figure 1B. CD196 Growth on CDA**. CDA with CD196 growth after 48 hours in anaerobic conditions.

Strain Selections

E. coli and *C. diff* are both present as a commensal organism in the lower GIT. *C. diff* is not as prevalent, and may experience colonization resistance from organisms such as *E. coli*. *E. coli* is well-studied organism, and the genome is widely available to researchers online. Using the Keio mutant collection, we have selected the *atoE* mutant which lacks a SCFA transporter. *C. diff* is also naturally occurring in a small percent of the population, and its ability to withstand the probiotic effects of SCFAs has not been studied previously to our knowledge. CD196 is a patient of PCR ribotype 027, which is notorious for many different *C. diff* outbreaks in the last century [32, 49]. This strain was provided by Manish Kumar, at Texas State University.

Anaerobic Growth in the PlasLabs Anaerobic Chamber

Studies used an anaerobic chamber Model 855-AC by PlasLabs for growth of initial cultures. The gas mixture inside the chamber included 85% Nitrogen, 10% hydrogen, and 5% carbon dioxide. A palladium catalyst system was used to sequester

trace amounts of oxygen. A "purge" of the entire chamber with the above-mentioned gas mix was performed 3x in accordance to the manufacturers manual prior to the insertion or any materials or cultures. Later studies indicated that oxygen was present in the chamber, so further work was continued in an anaerobic box, as discussed below. Even small amounts of oxygen showed the potential to inhibit the growth of CD196. The addition of sodium taurocholate and glycine to the BHIyp media was not effective in allowing for growth from spores. The highly anaerobic environment is difficult to reproduce in the laboratory environment, which has led to a slower pace when researching colonic models of CDI and the gastrointestinal microbiome.

Anaerobic Growth in the GasPackEZ Box

All studies involving spent media and SCFAs were performed using a BD GasPak EZ Gas Generating Chamber and BD EZ GasPaks. Samples were incubated at 37°C for the duration of the experiment, and gas packs were replaced upon the opening of the chamber. In the SCFA experiment, BW25113 (WT), BW25113 (*atoE*), and CD196 were only sampled once per hour to limit the amount of oxygen exposure while on the bench.

Spent media studies grew cultures from freezer stocks in an anaerobic environment for 24 hours as described above. After this period, samples were centrifuged for 5 minutes at 2000rpm and the pellet was used for plating/contamination check. The supernatants were then filter sterilized with a .22uM filter, before being mixed with fresh BHIyp to create the preferred concentrations (10, 25, 50, 75, and 100 percent). CD196 was sampled hourly to minimize contamination. Results were compared to BW25113 (WT) and BW25113 (*atoE*) grown in aerobic conditions, which

were sampled every 20 minutes using the BioTek PowerWave XS/XS2 microplate spectrophotometer at OD600.

SCFA experiments in the GasPackEZ box were conducted in a similar manner to the spent media experiments, with all cultures grown from a freezer stock and tested for contamination upon completion. SCFA vials (12mL) were degassed with the appropriate combination or single acid (1uM per acid) for 24 hours prior to inoculation. All SCFA studies were taken to 24 hours to ensure that no additional growth or contamination took place after the last sampling time point.

48 Hour Growth Curves using a Microplate Spectrophotometer

48-hour growth curves were generated using a BioTek PowerWave XS/XS2 microplate spectrophotometer at OD600. Only BW25113 (WT) and BW25113 (*atoE)* were able to be grown on the BioTek PowerWave XS/XS2, as even sealing with parafilm allowed sufficient amounts of oxygen to inhibit the growth of CD196. Sampling for these strains took place every 20 minutes over a 48-hour period. The software used for analysis was Gen5 version 1.05.

SCFA studies were also examined using the BioTek PowerWave XS/XS2 and Gen5 software. All vials were sampled from hours 2-12 hours via a 200uL deposit into a 96 well plate (OD630).

III. STANDARD GROWTH CURVES FOR CD196, BW25113 (WT), AND BW25113 (*ATOE)*

Growth for BW25113 (WT) BHIyp over a 48-hour period is shown in Figure 2. BW25113 (WT) consistently shows a trend toward diauxic growth in BHIyp media. Several mutants from the Keio collection implicated in the SCFA pathways were tested for baseline growth. BW25113 (*atoE)* showed a typical growth pattern with lag, log, and stationary phase, but the stationary phase slowly trends upward for several hours before making its final plateau, as seen in Figure 3. CD196 was grown in BHIyp for 8 hours and sampled every hour. OD600 values are plotted and show a very slow growth, with exponential growth taking place around 5 hours. This is consistent with CD196, as it tends to reach exponential growth around 6 hours, while reaching stationary phase around 8 or 9 hours (Figure 4). Sampling on the half-hour introduced too much oxygen to samples, and caused an extreme delay in CD196 growth.

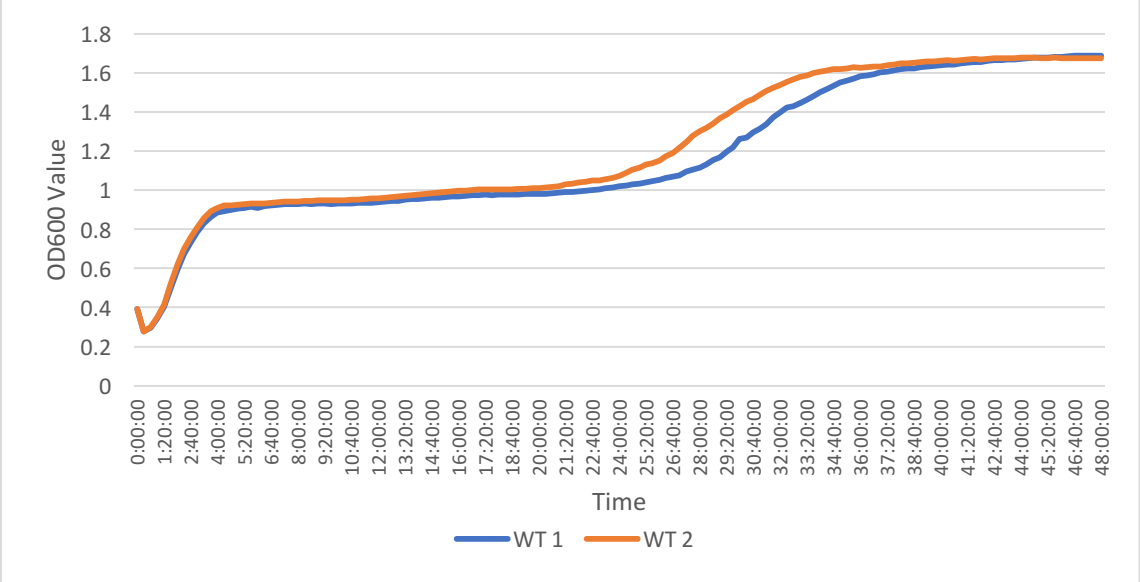


Figure 2. BW25113 (WT) in BHIyp. This data was taken on the microplate spectrophotometer at OD600. A diauxic trend is noted, possibly due to the addition of 5% yeast in BHI, which is already a very nutrient rich media. BW25113 (WT) reaches stationary phase at approximately 4 hours. The slight dip in OD values for the first hour is a common phenomenon due to condensation.

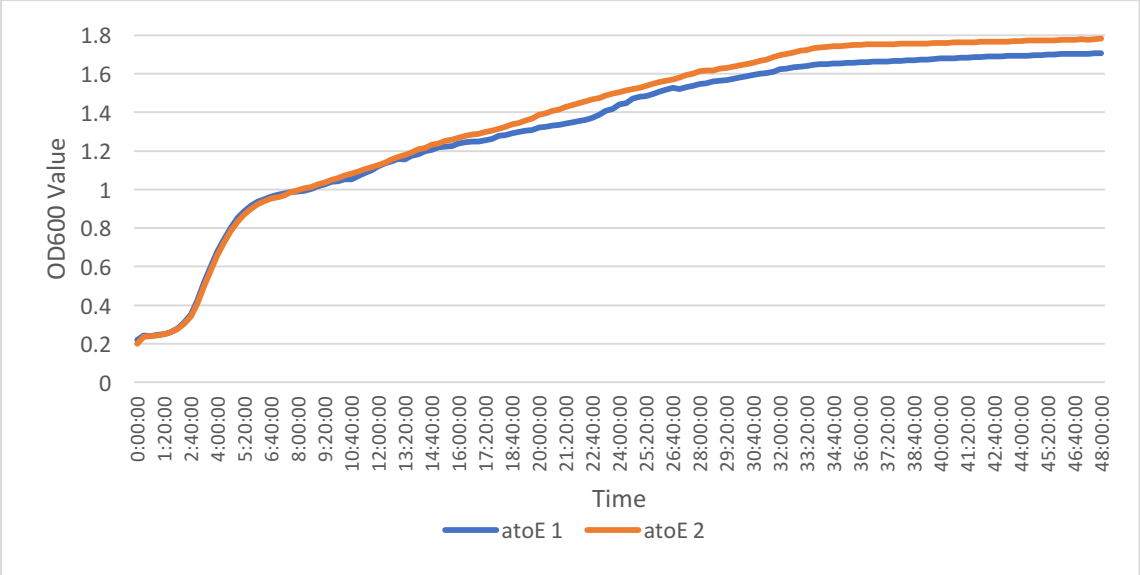


Figure 3. BW25113 (*atoE)* in BHIyp. This data was taken on the microplate spectrophotometer at OD600. BW25113 (*atoE)* reaches stationary phase around 6 hours, with a slight increase in OD600 over the next 24-hour period.

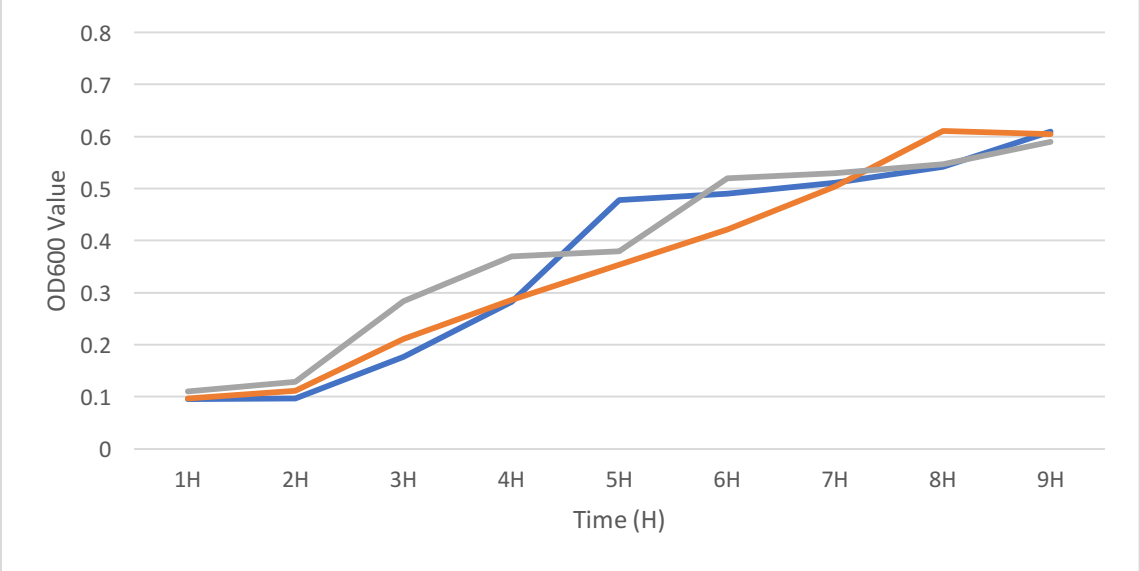


Figure 4. CD196 Growth in BHIyp. CD196 shows exponential growth around 5 hours with an average of .6 OD in the stationary phase.

IV. INCREASED CONCENTRATIONS OF SPENT MEDIA DELAY THE GROWTH OF BW25113 (*ATOE), (WT)* AND CD196

Spent media studies were conducted to examine if the metabolites produced by BW25113 (WT) and BW25113 (*atoE)* have any effect on the growth of CD196 (Figure 5 and Figure 6). Since BW25113 (*atoE)* cannot transport SCFAs across its cell membrane, we are using this line as a control in comparison to the wild type, which has no mutation affecting its ability to produce SCFAs. Metabolites of CD196 in spent media were also used to examine if there was any effect on the growth of BW25113 (WT) and BW25113 (*atoE)* (Figure 7 and Figure 8).

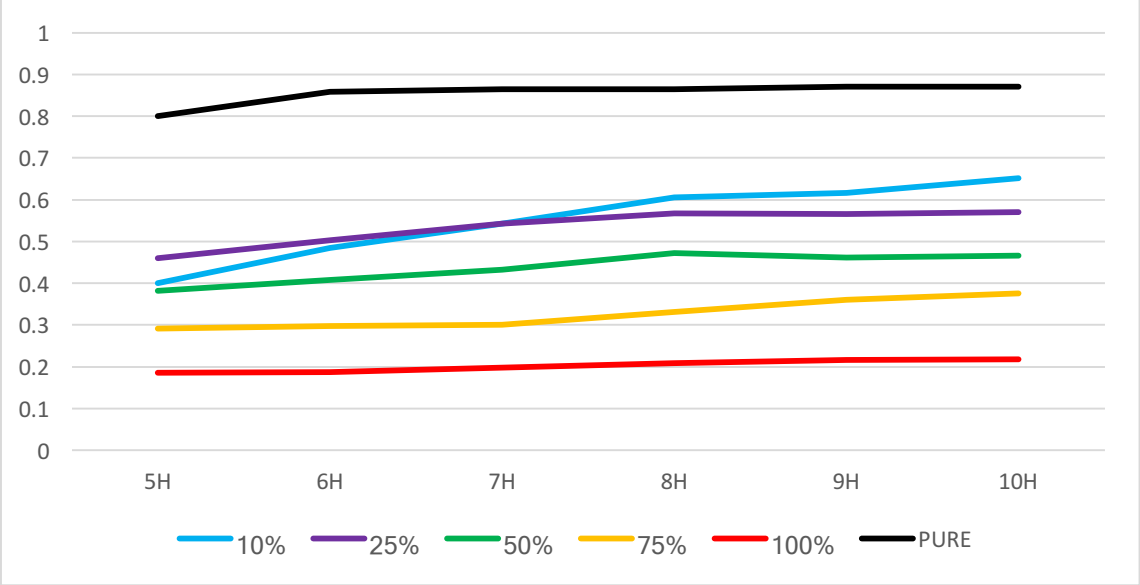


Figure 5. CD196 Growth in BW25113 (WT) Spent Media Concentrations. CD196 becomes completely inhibited and does not experience any growth in 100% SM.

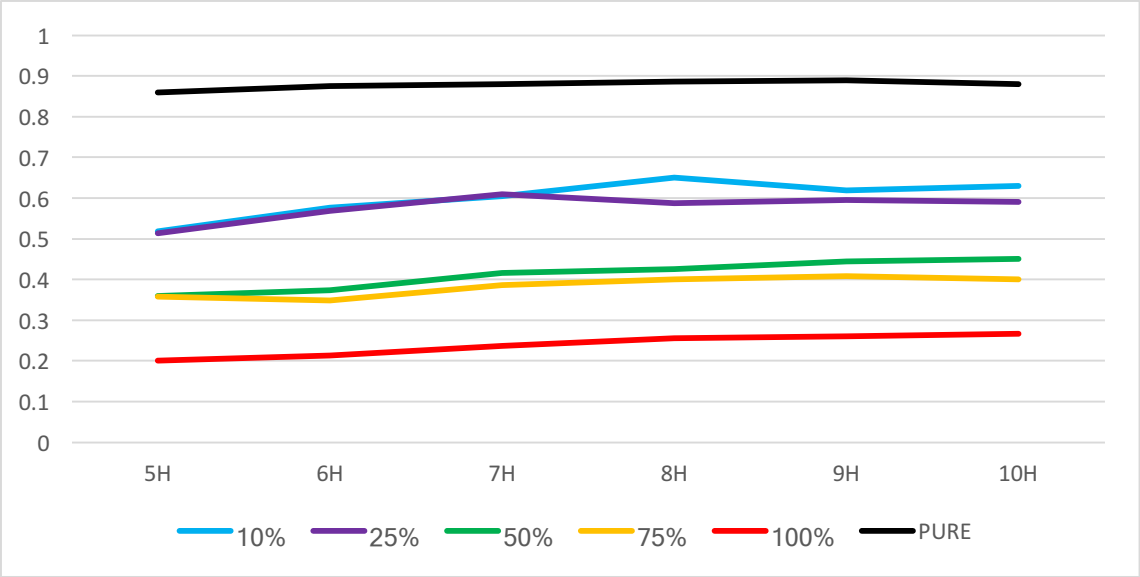


Figure 6. CD196 Growth in BW25113 (*atoE)* Spent Media Concentrations. CD196 did not show exponential growth in this study. As seen in the wild type, when the concentration of spent media increases, CD196 becomes completely inhibited and does not experience any growth in 100% SM.

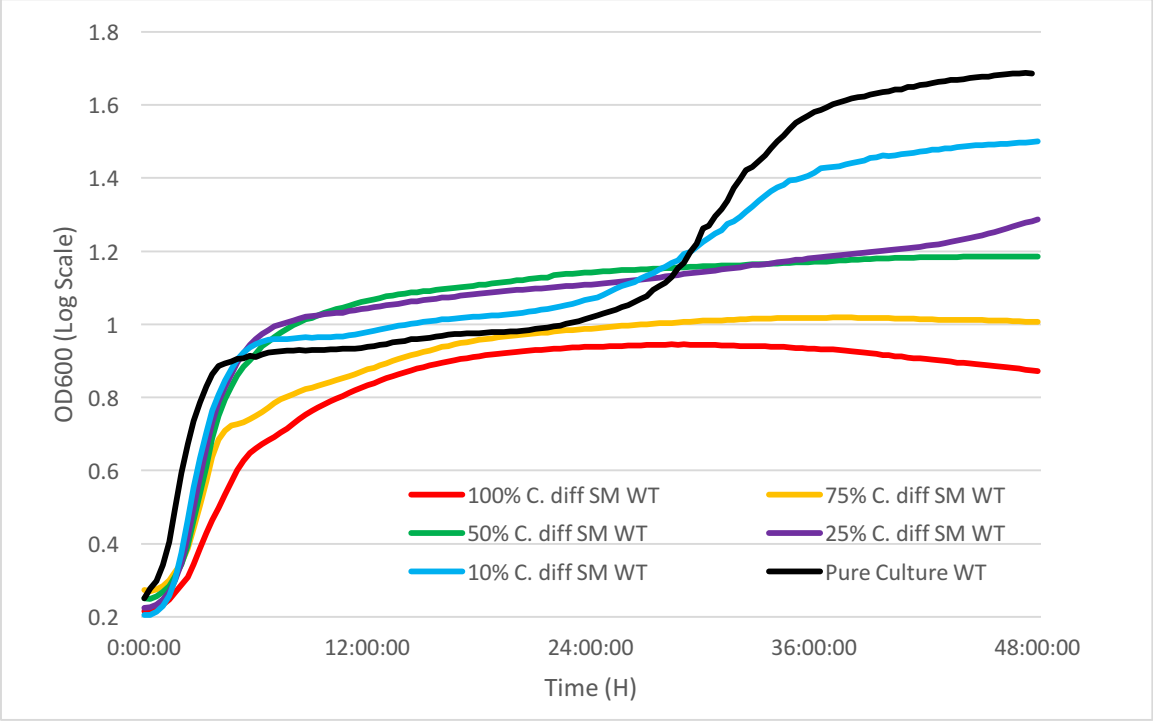


Figure 7. BW25113 (WT) in Various Concentrations of CD196 Spent Media (SM). As the concentration of SM for CD196 increases, there is a decrease in the OD values of BW25113 (WT).

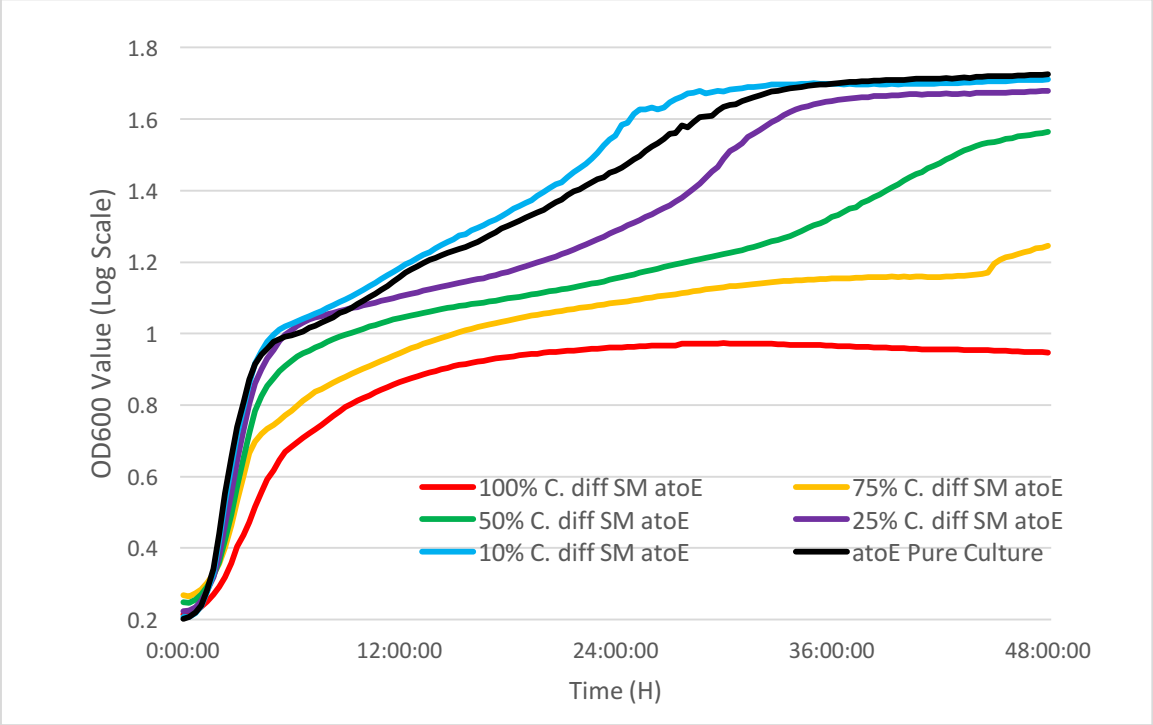


Figure 8. BW25113 (*atoE)* in Various Concentrations of CD196 Spent Media (SM). As spent media concentrations increase, the rate of growth and OD value for BW25113 (*atoE)* decreases.

V. SCFAs AND THEIR COMBINATIONS DO NOT AFFECT THE GRWOTH OF BW25113 (WT) OR BW25113 (*ATOE)*

SCFAs were examined singly and in combination to determine if acetate, butyrate or propionate have an effect on the growth of our chosen strains. SCFAs were added to BHIyp in the amount of 1uM prior to autoclaving media and inoculation. No observable differences were seen in the SCFA combinations or single SCFAs for BW25113 (WT) or BW25113 (*atoE)* (Figure 9 and Figure 10).

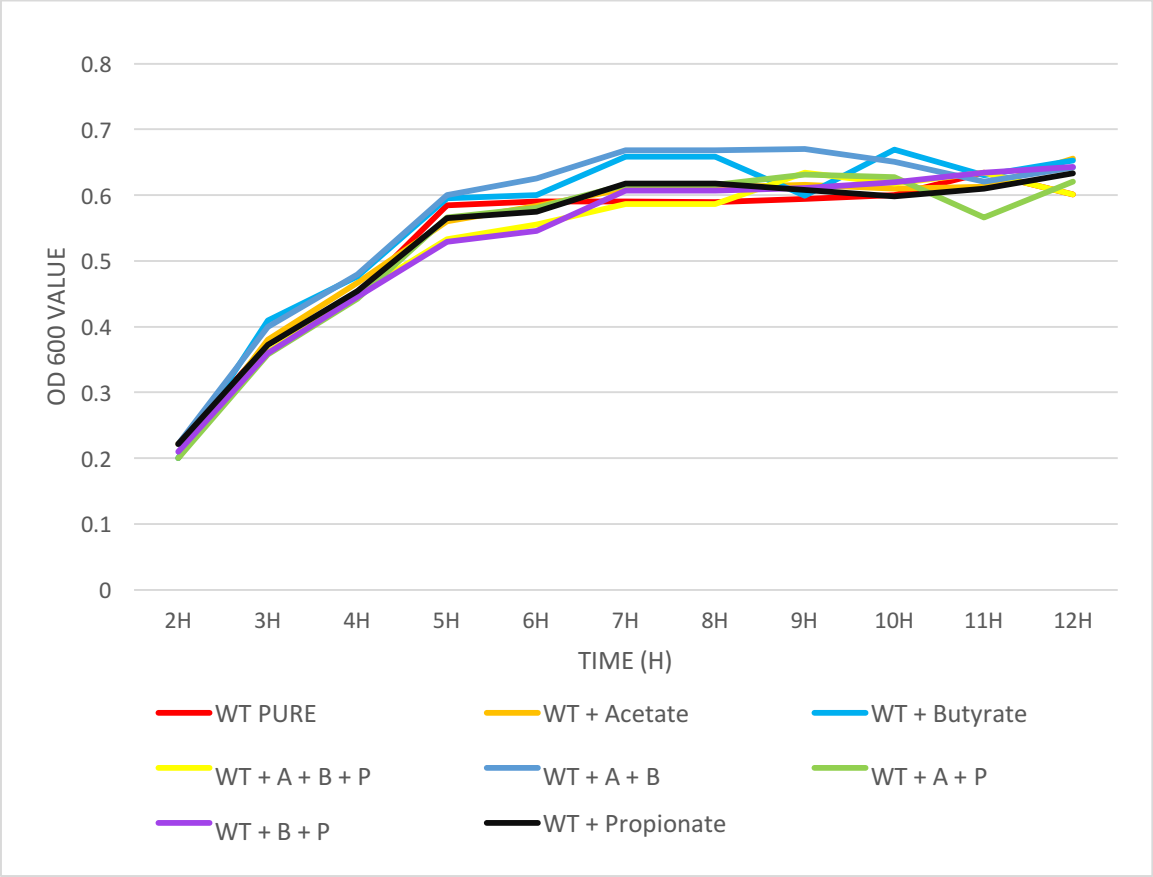


Figure 9. SCFAs and SCFA Combinations Effects on the Growth of BW25113 (WT).

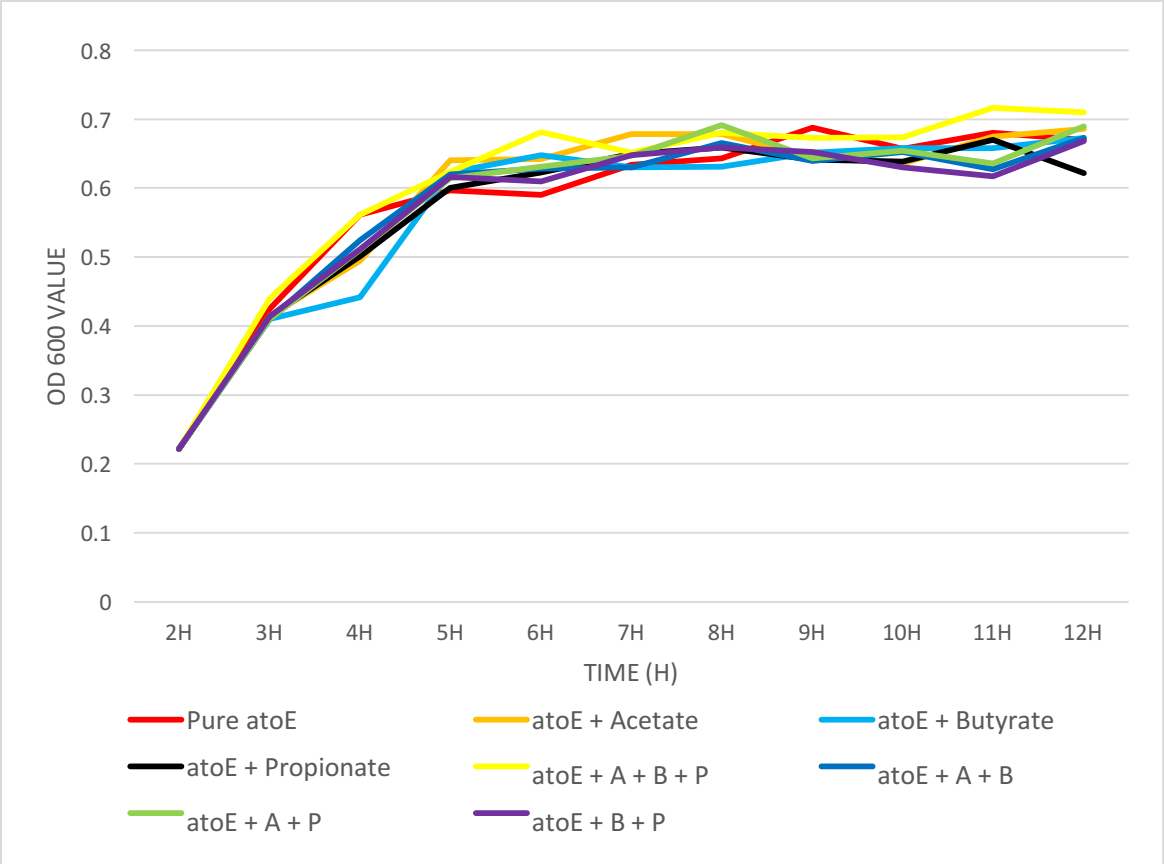


Figure 10. SCFAs and SCFA Combinations Effects on the Growth of BW25113 (*atoE)*.

All CD196 strains showed exponential growth around 6 hours, as seen in Figure 11. Acetate lowered the OD value for CD196, as well as the acetate combinations. This was not observed with BW25113 (*atoE)* or the wild type. Completion of the experiment was at 24 hours, and confirmed that all conditions were in stationary growth phase at hour 10.

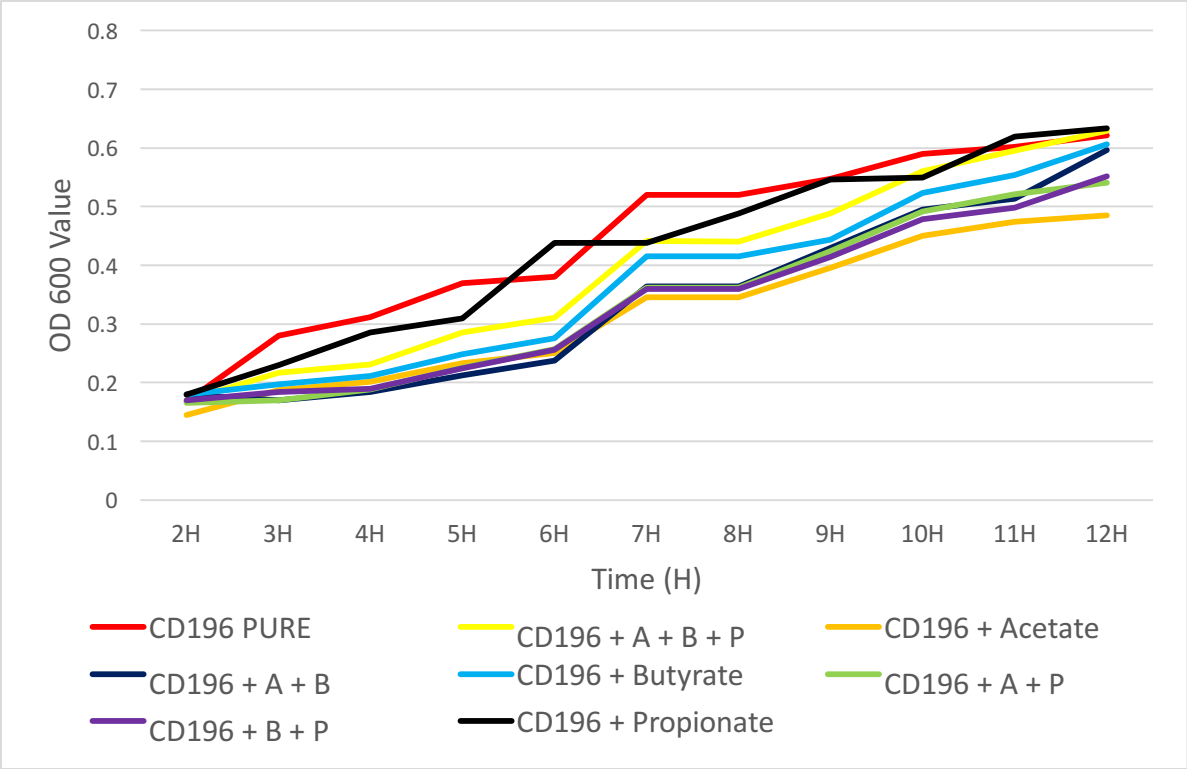


Figure 11. SCFAs and SCFA Combinations Effects on the Growth of CD196.

VI. RESULTS/DISCUSSION

Growth Curves for BW25113 (*WT*), BW25113 (atoE), and CD196

Studies into the baseline growth of all organisms are in Figures 2-4. BW25113 (*WT*) is showing a diauxic trend, where on the second day (~26 hours) a second phase of growth plateaus after a period of 8 hours (at hour ~34). This trend was not observed in LB broth (data not shown). BW25113 (*atoE)* enters exponential growth around 2 hours, and reaches stationary phase around 5 hours. BW25113 (*atoE)* also shows a trend toward diauxic growth, but the secondary growth phase is very extended and does not show true exponential growth. A firm stationary phase is reached after 32 hours. Both BW25113 *(WT)* and BW25113 (*atoE)* reach optical densities over 1.5. The similarities in growth for BW25113 (*atoE)* and the wild type are their diauxic growth, with this effect being stunted in BW25113 (*atoE)*. Perhaps the atoE receptor on the membrane is playing a role, as this is the only difference between BW25113 (*atoE)* and the wild type. BHIyp media is complex yet not firmly defined. When an organism utilizes additional carbon sources, a secondary growth phase is observed. The addition of yeast and peptone add additional nitrogen and amino acid sources, which should not cause this trend in BW25113 (*WT*) or BW25113 (*atoE)*. The sheep and beef components of BHI may contain sugars or other carbohydrates that allow for this trend in BW25113 (*WT*) and BW25113 (*atoE)*. CD196 was not able to be grown over 48 hours due to oxygen exposure and cost restraints. Several growth experiments indicate that the exponential growth phase is for CD196 takes place between 5-6 hours, while exponential growth if very short and stationary phase is reached within 1-2 hours. These results are shown on

Figure 4. CD196 does not reach optical densities over 1, and tends to reach saturation around .6-.7OD.

It should be noted that BW25113 cultures were grown over 48 hours in a mildly aerobic environment. To normalize all graphs and comparisons, BW25113 should be grown anaerobically with CD196, and sampled on the same time points.

Initial growth studies involved serial dilutions into PBS and plating, where all plates and tubes had been degassed in the PlasLabs anaerobic chamber for 24 hours. Oxygen contamination into the chamber affected the reproducibility of CD196 cultures, so further studies did not involve serial dilution and plating. The BD EZGasPak box holds ~20 plates and 10 scintillation vials. Plating to ensure lack of contamination was always performed for each growth curve. Future studies could use a larger BD Box, or place a gas pack in a sleeve of agar plates that have been inoculated. BD GasPak costs limited the scale of these studies.

Spent Media Studies

Both BW25113 and CD196 were examined in the presence of spent media. BW25113 (WT) and BW25113 (*atoE)* were first grown in varying concentrations of spent media while on the BioTek plate reader (over 48 hours). The trends of growth can be seen on Figure 7 and Figure 8. As the concentration of spent media increases, there is a decrease in the growth of BW25113 (WT) and BW25113 (*atoE)*. Most inhibition of growth is taking place at concentrations of 50% or higher, with 100% showing a significant inhibition of growth for both BW25113 (WT) and BW25113 (*atoE)*. BW25113 (WT) and BW25113 (*atoE)* lose their trend toward diauxic growth in spent media after 50% is reached. As the CD196 culture is grown overnight in BHIyp, there is

a depletion of nutrients taking place. In order to identify that inhibition of growth is not related to a lack of nutrients, concentrations should be monitored or doubled.

CD196 was also grown in spent media of BW25113 (WT) and BW25113 (*atoE)*. These studies were only sampled once per hour to limit the exposure to oxygen, and studies started to take readings at 5 hours, while ending at 10 hours post inoculation. Pure cultures appear to have already reached stationary growth by hour 5, as well as 10% and 25% spent media (SM). All lines showed a very slow growth from hour 5 until hour 8, but no concentration showed exponential growth. All CD196 lines were grown until 24 hours to confirm that no extra growth or contamination took place. CD196 samples all showed the same OD value at 24 hours as they did when the study halted at 10 hours. This study should be replicated, starting at hour 3 to catch all exponential growth phases for the lower concentrations, and any exponential growth in the higher concentrations. Also, nutrient concentrations need to be normalized before inoculation with CD196 to show this is not a depletion of nutrients causing an effect.

SCFA Effects on the Growth of BW25113 (WT), BW25113 (atoE), and CD196

The growth of BW25113 (*WT*) and BW25113 (*atoE)* and CD196 was examined in the presence of acetate, propionate, butyrate, and all combinations of these three acids. Vials were sampled from 2-12 hours via a 200uL deposit into a 96 well plate. No significant differences were seen in any of the SCFA combinations for BW25113 (*WT*) or BW25113 (*atoE).* CD196 pure culture had the most growth according to OD630 values. Combinations of acetate + propionate, propionate + butyrate, and acetate alone showed the lowest OD630 values for this 10-hour period, not reaching over 0.5 OD. Acetate was unable to reach 0.4 OD, and had the slowest growth of all conditions. All other

conditions were similar to pure culture experiments, with CD196 reaching stationary growth after 0.6 OD and 10 hours. Propionate showed the least effect when acting alone, and has similar growth to the pure culture.

These SCFA in this study were in uM concentrations. Further studies using mM concentrations following a similar model might yield stronger effects of the combinations on CD196 or to BW25113) and BW25113 (*atoE),* while also proving more physiologically relevant. Acetate concentrations should also be tripled, as acetate is available in the largest amounts in the lower GIT, and is often found in a 3:1:1 (30mM:10mM:10mM) ratio with propionate and butyrate. Since these preliminary findings indicate that acetate might be limiting the growth of CD196 the strongest, it would be interesting to see how more physiologically relevant concentrations affect the growth in comparison to the pure culture. Also, in order to quantify the amounts of SCFAs in each combination, the use of GC-MS would help to ensure proper concentrations.

VI. FUTURE DIRECTIONS

Media Modifications

In general, when working with BHIyp, the pH is around 7.0. The GIT exhibits an acidic environment, so it is proposed that all cultures be grown in acidified media and plates. pH may play a role in activating or allowing certain metabolic processes of *C. diff* to move forward. Using a pH meter, the pH levels could be monitored to ensure that the addition of SCFAs does not affect the media's overall pH. Secondly, an observed inhibition of growth was noticed in the spent media experiments. In order to prove this is an inhibitory effect caused by a metabolite, and not simply a depletion of nutrients, nutrient concentrations should be doubled to account for the extra time spent in culture.

The ability of *C. diff* to produce spores leads to difficulties in treatment, while also making recurrent infections very common. Spores were not looked at in this current experiment, but conjugated bile salts such as taurocholate and the co-germinant glycine can be added to the media to promote the growth of *C. diff* from spores. Secondary bile salts have been shown to have inhibitory effects on the outgrowth of *C. diff* spores and viable cells [33] . Looking at SCFAs and the outgrowth of *C. diff* from spores in the presence of bile salts may lead to new findings regarding recurrent infections of CDI.

Biofilm Formation in the Presence of SCFAs

E. coli and *C. diff* are both biofilm forming microorganisms, with E. coli forming biofilm in less than 24 hours, while C. diff starts to form a biofilm around 36 hours. Biofilms are formed when bacteria communicate about their surrounding environment, specifically when surroundings are favorable. Planktonic bacterial cells will attach to a surface, and once this attachment becomes irreversible, a solid biofilm has been formed

[50]. Biofilms have many advantages to their bacterial members, such as protection from the environment. It is believed that *C. diff* utilizes the buffering effects of the biofilm in order to establish recurrent infections with CDI. For example, *C. diff* will not grow in the presence of any oxygen, but may be able to grow in trace amounts of oxygen when present in a biofilm. *C. diff* may also be able to withstand antibiotic treatment when in a biofilm [25, 51]. Tests of biofilm formation in the presence of SCFAs may show inhibition or extra outgrowth of the biofilm.

Interactions with Probiotics and Other Commensals

Several probiotic strains are well characterized in the GIT, such as *Bifidobacteria* and *Lactobacillus*. These organisms are also anaerobic in nature, and would be easy to work with in tandem while examining CD196. Spent media studies may indicate if *Bifidobacteria* or *Lactobacillus* produce metabolites that discourage the initial outgrowth of CD196. Also, this study used BW25113 from the Keio collection due to its well-characterized genome and availability. Future studies that wish to look at competition for resources and utilization of SCFAs should use members of the *Bacteroides* or *Firmicutes*, since they represent the largest number of microbe families in the GIT.

Physiological SCFA Concentrations

This study looked at minimal SCFA concentration levels and what effect, if any, they may have of the ability of CD196 to establish growth. In order to model the GIT more efficiently, mM concentrations, not uM, should be utilized. As acetate, propionate, and butyrate are found in a 3:1:1 ration in the lower GIT, mM concentrations in this ratio are suggested (30mL acetate, 10mM propionate, and 10mM butyrate) for future studies.

This supports literature following the absorption and production of SCFAs in the GIT [4, 52]. These concentrations can be checked using gas chromatography/mass spectrometry.

Gene Families and SCFA Production

The *ato* gene family was focused upon due to the *atoE* mutant's inability to transport SCFAs across the cell surface. Other members of the *ato* family are involved in the synthesis and regulation of SCFA production, and may be better models for SCFA studies. The acetyl-CoA pathway is important for the synthesis of butyrate and acetate, while the succinate pathway leads to propionate production [53]. Several gene families regulate these two pathways, and may serve as a starting point for the interplay between SCFAs and CDI.

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