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Short chain fatty acid production by probiotic organisms in the gastrointestinal tract

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Short chain fatty acid production by probiotic organisms in the gastrointestinal tract

Abstract

Interest in determining the mechanism by which pro biotic bacteria in the gastrointestinal tract produce short chain fatty acids (SCFA) has increased over the past few years. This study uses gas chromatography and mass spectrometry to characterize lactate production in aerobic test tube cultures, aerobic bioreactor cultures, and anaerobic bioreactor cultures. To collect anaerobic bioreactor samples, a novel *in vitro* anaerobic model was developed. The pH and colony forming units of each bacterial sample was also measured in order to understand the correlation between lactate production and bacterial growth. The purpose of this study was to analyze production of SCFA in co-cultures and to develop an anaerobic model to analyze SCFA production by anaerobic bacteria. 4

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SHORT CHAIN FATTY ACID PRODUCTION BY PROBIOTIC ORGANISMS IN
THE GASTROINTESTINAL TRACT

By

Elyssa Rautiola

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Eastern Michigan University

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ABSTRACT

Interest in determining the mechanism by which probiotic bacteria in the gastrointestinal tract produce short chain fatty acids (SCFA) has increased over the past few years. This study uses gas chromatography and mass spectrometry to characterize lactate production in aerobic test tube cultures, aerobic bioreactor cultures, and anaerobic bioreactor cultures. To collect anaerobic bioreactor samples, a novel *in vitro* anaerobic model was developed. The pH and colony forming units of each bacterial sample was also measured in order to understand the correlation between lactate production and bacterial growth. The purpose of this study was to analyze production of SCFA in co-cultures and to develop an anaerobic model to analyze SCFA production by anaerobic bacteria.

INTRODUCTION

The Gastrointestinal Tract

The human gastrointestinal (GI) tract is populated by an array of microbial communities. These communities function together with the host in a mutualistic fashion to support host metabolism and various other processes including development of the immune system, energy production, and vulnerability to disease (1). The bacteria are present in concentrations of up to 10^{11} – 10^{12} cells/g which equates to up to 100 trillion bacteria overall (2). The bacteria not only vary greatly in number, but also in the type of species. The most common types include *Bacteroidetes*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Clostridium*, *Lactobacillus*, and *Enterococcus* (8).

Microbes that have a positive influence on microbe-microbe and host-microbe interactions are considered probiotics. These microbes are non-pathogenic and have beneficial effects on host health (3). A study investigating the effects of *Lactobacillus rhamnosus* on microbiota composition demonstrated that daily consumption of probiotics can improve health and decrease allergic disorders (8). Of particular interest is the effect of probiotics in the GI tract as they have a positive influence on the production of SCFA (3).

There are both aerobic and anaerobic bacteria present in the GI tract. Those bacteria that can only grow in the presence of oxygen are considered obligate aerobes. The oxygen is necessary for them to undergo aerobic respiration to produce ATP. Bacteria that can undergo either aerobic respiration or fermentation are facultative anaerobes while those that grow only in the absence of oxygen are considered obligate anaerobes and undergo fermentation, producing SCFAs (11).

Short Chain Fatty Acids

Short chain fatty acids are fermentation products from the bacteria residing in the GI tract (see Figure 1). These bacteria use carbohydrates as their primary source of energy. SCFA are carboxylic acids that have other functional groups attached to their carbon atoms. The amount of SCFAs produced depends on different factors such as site of fermentation, diet, time spent in the GI tract, and the composition of the bacteria present (7). Although most of the SCFAs are absorbed in the colon, 10 to 20% are excreted in the feces (4). There are several types of SCFAs produced as a result of metabolic activity, including acetic, propionic, butyric, and lactic acids. Of these SCFAs, butyrate and lactate are of particular interest. Butyrate has been shown to have anti-inflammatory properties along with inhibiting growth and inducing apoptosis of human colonic carcinoma cells (5). It is also the preferred source of energy for host cells in the GI tract and is involved in many cellular processes such as repair of the gut mucosal lining, stimulation of the autonomic nervous system and production of hormones associated with the GI tract (12).

Lactic acid is another short chain fatty acid that is important to monitor. Bacteria in the GI tract that produce lactate include lactobacilli, bifidobacteria, enterococci, streptococci and *Eubacterium* (9). Although lactate is an important SCFA, it does not appear to be a major fermentation product as lactate is also used by other bacteria in the environment, especially the butyrate producing bacteria. This being the case, by putting both lactate-producing and butyrate-producing bacteria together, a significant amount of lactate will be converted to butyrate, maximizing the amount of butyrate produced (9).

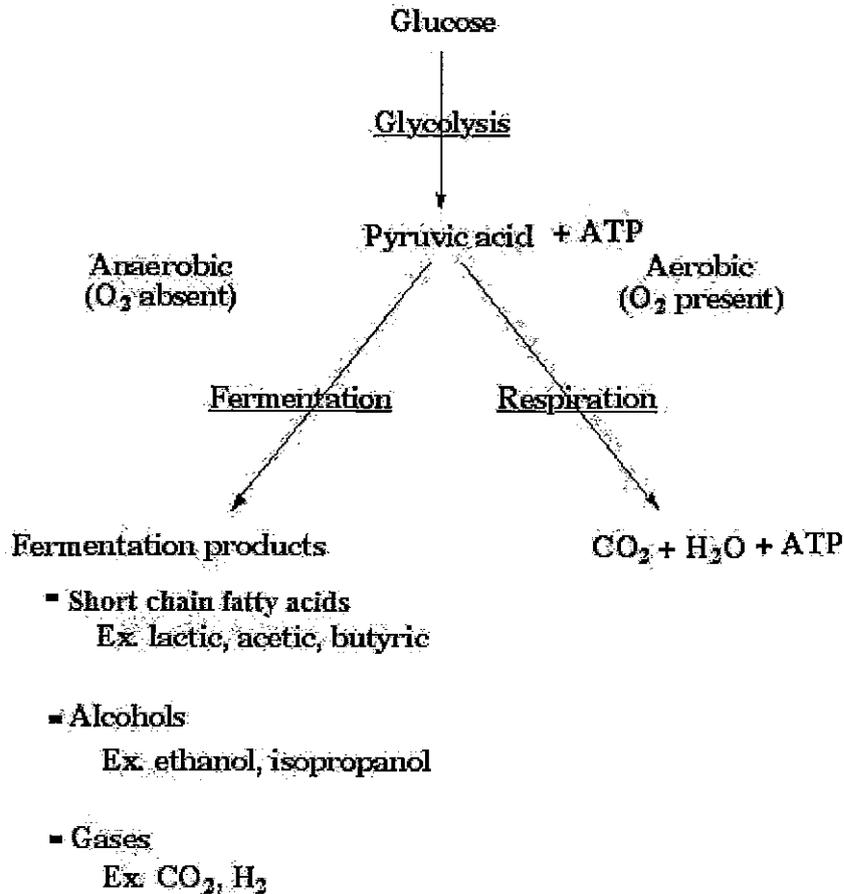


Figure 1. Diagram of the production of SCFA

Biofilms

The mucosal layer that exists along the entire GI tract is the site at which biofilms are found (15). A biofilm is an organized bacterial community embedded in an extracellular matrix (ECM) containing carbohydrates, proteins, phospholipids and nucleic acids (6). Not only does the mucosal layer act as a home for biofilms, it provides a lubricated surface for digestive contents to move along. Therefore, persistence of a bacterial species within the GI tract is dependent on its ability to remain attached to the mucosal layer via biofilm (15). The biofilm enables the bacteria to survive in unfavorable

conditions via expression of bacterial phenotypes not normally expressed in planktonic cells (10). Living in close quarters to one another, bacteria have the benefit of using one another's metabolic products. This is especially the case with the butyrate producers using the fermentation product of lactate producers to fuel their own metabolic reactions.

Research Objective

There are two main objectives of this study; the first objective is to analyze SCFA levels in bacterial samples to determine when bacteria produce the most SCFA and to determine how lactate levels differ in co-cultures of bacteria versus monocultures. The second objective is to develop a novel *in vitro* anaerobic model to closely mimic the conditions and characteristics of biofilms in the gut to study SCFA production by anaerobic bacteria.

MATERIALS AND METHODS

Part I: Characterization of short chain fatty acids in test tube cultures

Preparation of standards and biological samples

The lactate standard was prepared in acetonitrile to a final concentration of 200 mg/L. The internal standard used was 1,3-propanediol which was also prepared in acetonitrile to a final concentration of 200 mg/L.

Biological samples (see Table 1) were prepared as test tube cultures. These cultures were inoculated in single test tubes with 10 mL of pre-reduced reinforced clostridial media (RCM) and grown overnight. Then, 100 μ L of each overnight was put into tubes with 10 mL RCM for growth in an anaerobic jar at 37-39°C. These tubes were left to grow for either 24, 48, or 72 hours. The bacterial samples were then analyzed via Gas Chromatography/ Mass Spectrometry (GC-MS) to determine the amount of SCFA present.

Extraction of SCFA from biological samples

Extraction of the SCFA from the biological sample was necessary for analysis by GC-MS. In this process, the SCFA are removed from the media and isolated to be easily quantified. To do this, the sample was acidified with 0.6 M HCl and vortexed for one minute. Ether was then added and the solution was centrifuged for 5 minutes at 2000 g. The resulting top layer containing the ether and SCFA was extracted off and saved. This process was repeated for a total of five times to ensure all the SCFA were collected. The ether was left to evaporate, leaving only the SCFA behind. Once all the ether was gone, the internal standard (1,3-propanediol) and catalyst (anhydrous pyridine) were added. The

resulting solution was vortexed and the derivitizing agent (MTBSTFA in TBDMCS) was added. The samples were then covered and placed in a heating block at 70°C for 4 hours. Once removed, ethyl acetate was added, the samples were vortexed and then analyzed via GC-MS.

Gas Chromatography Mass Spectrometry

Measurement of the amount of short chain fatty acid produced by a bacterial strain was achieved using GC-MS. The injector type used was a standard split injector with an injector temperature of 270 °C. Helium was chosen as the carrier gas and had a flow rate of 0.6 mL/min. The column temperature was 70 °C for the first minute, and then increased at the rate of 20 °C per minute to 160 °C where it was held for 3.5 min. The temperature was then raised to 280 °C at a rate of 35 °C per minute. The total run time of a sample was 12.43 minutes.

There were 4 peaks of interest when analyzing the mass spectrum. The internal standard produces ions that have a mass to charge ratio of 219 and 247 and lactate produces ions that have a mass to charge ratio of 233 and 261. When examining mass spectrum from these molecules, there should be peaks at those specified values. The mass spectrum for the lactate and the internal standard are shown respectively in Figures 2 and 3.

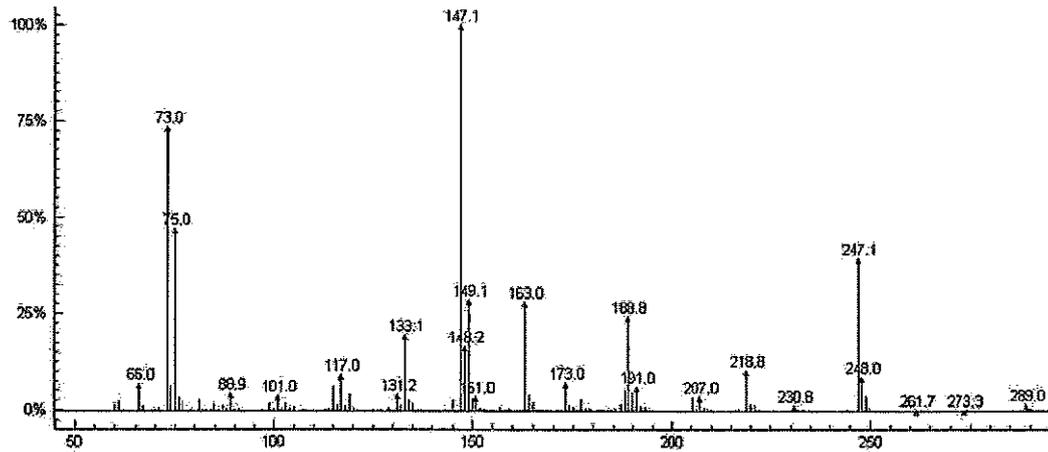


Figure 2. Mass spectrum of 1,3-propanediol

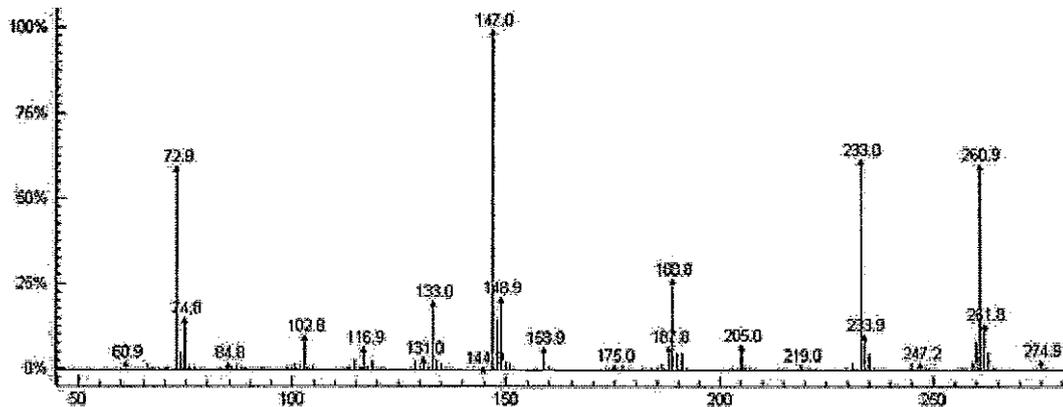


Figure 3. Mass spectrum of lactate

Part II: Development of the model

Anaerobic environment

A vinyl anaerobic chamber (Coy Laboratory Products Inc.) was used to create an anaerobic environment of 0 to 5 ppm oxygen. Nitrogen and hydrogen gas were pumped in to the chamber to purge the system of oxygen. To ensure that all the oxygen was removed from the environment, a palladium catalyst was used. The catalyst exists as part of a heated fan box that is used to maintain a temperature of 37° C within the anaerobic

chamber. As the hydrogen gas circulates throughout the chamber, the catalyst binds it with oxygen to form a molecule of water. The catalyst is prepared by baking in an oven for 2 hours at 165° C. The catalyst lasts for about 2 weeks before it needs to be replaced with a freshly baked catalyst.

Bacterial growth

Within the chamber, a CDC Biofilm Reactor was set up with a fresh flow of media being introduced through use of a peristaltic pump (Pharmacia Fine Chemicals) (Figure 4). To have a visual confirmation of the media's oxygen content, resazurin (an indicator) was added to a ratio of 1:1000. Reduced brucella broth with starch was used to grow the bacteria. The ingredients that went in to making 900 mL this media include 25.5 g brucella broth, 0.225 g L-cysteine, 0.45g sodium thioglycolate, 0.9 g starch, 0.09 g sodium sulfate, and 900 µL resazurin. These were mixed into 900 mL deionized water and then put in the autoclave.

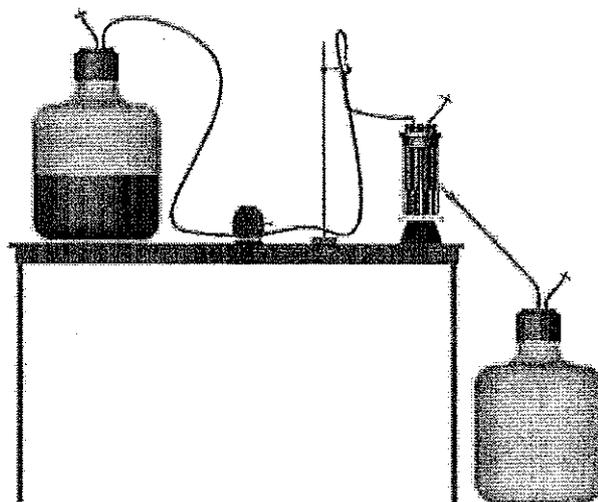


Figure 4. Schematic of CDC Biofilm Reactor set up

Bacterial strains

Table 1 displays the microbial strains used, their main fermentation products and their growth requirements.

Table 1. List of bacterial strains used (13)

Strain	Fermentation Product(s)	Growth Requirements
<i>Clostridium propionicum</i> ASF 500	Butyrate	Obligate anaerobe
<i>Enterococcus faecalis</i> OG1S	Lactic acid	Facultative anaerobe
<i>Eubacterium plexicaudatum</i> ASF 492	Butyrate and lactic acid	Obligate anaerobe
<i>Lactobacillus johnsonii</i> NF-1	Lactic acid	Facultative anaerobe
<i>Lactobacillus rhamnosus</i> ATCC 53103	Lactic acid	Facultative anaerobe

The bacteria in Table 1 were grown in reduced brucella broth under anaerobic conditions in a CDC biofilm reactor for a period of 72 hours. The media was inoculated at 0 hours and then time points were taken every 24 hours, measuring the pH, optical density at 600 nm, and colony forming units. The peristaltic pump was turned on 24 hours after inoculation. Pellets and supernatants were saved from each sample in order to perform quantitative PCR and to measure the presence of SCFAs via Direct Analysis in Real Time Mass Spectrometry (DART-MS), which is another form of mass spectrometry similar to GC-MS.

Quantitation of bacterial strains

To measure the colony forming units, the samples taken from the CDC Biofilm Reactor were diluted before being plated. This was done to ensure individual colonies could be isolated. For this experiment, 10^{-2} , 10^{-4} , and 10^{-6} dilutions were made via serial dilutions. To prepare the 10^{-2} dilution, 10 μ L of the undiluted sample were added to 990

μL of 1X PBS. For the 10^{-4} dilution, 10 μL of the 10^{-2} dilution were added to 990 μL of 1X PBS. Finally, for the 10^{-6} dilution, 10 μL of the 10^{-4} dilution were added to 990 μL of 1X PBS. Then, 100 μL of each dilution was plated on BHI plates and left to grow in the anaerobic chamber.

During the counting process, the plate with 30 to 300 colonies was used. This is the reason for the three dilutions; one of the three plates should have the number of colonies within that range. Once the appropriate plate was selected, the number of exact colonies was counted. From there, back calculation accounting for the dilutions could be done to determine the number of living bacterial organisms present in the CDC Biofilm Reactor at that given time period.

Collection of the supernatant and pellet for future quantitation

Supernatants and pellets were collected at each time point. To obtain the supernatant, 10 mL of the sample was spun down in the centrifuge for 5 minutes at 2000 rpm. The supernatant was then transferred to a falcon tube and placed in the freezer at -20°C for later analysis by DART-MS. To obtain the pellet, 1 mL of the sample was spun down for 3 minutes at 12×1000 ref. The resulting supernatant was discarded and the pellet frozen at -20°C until further use for quantitative PCR.

RESULTS

Part I: Characterization of short chain fatty acids in test tube cultures

In order to be able to quantitate the amount of recovered lactate for biological samples (see Table 1), a calibration curve was made using the GC-MS. This was done by measuring the peak area ratio (ratio of lactate to internal standard) for varying amounts of lactate standards, ranging from 1.5 μg to 6 μg of lactate.

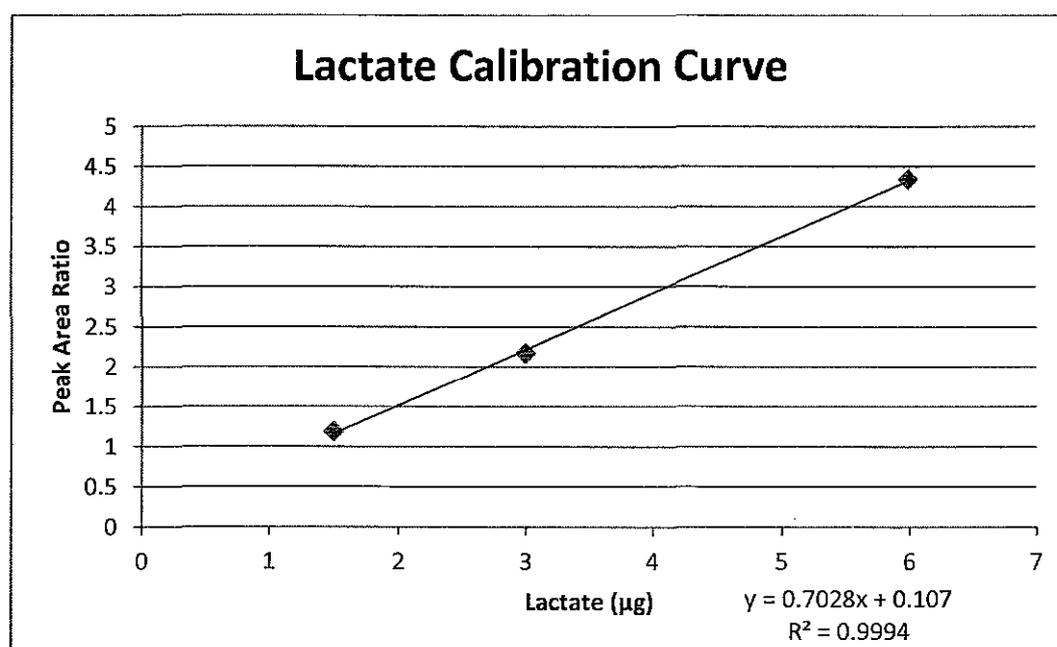


Figure 5. Lactate Calibration Curve

The lactate calibration curve had a best fit line with the equation $y=0.7028+0.107$ and a correlation coefficient of 0.9994. This graph can be used when determining the amount of recovered lactate from biological samples by substituting the measured peak area ratio in to the best fit line equation to obtain the unknown amount of lactate in micrograms.

Production of lactate as a function of bacterial growth

One aspect that was examined was the amount of lactate produced at varying points in bacterial growth. There was interest in seeing during what times in particular the bacteria produce the most lactate. To do this, *Lactobacillus johnsonii* was grown for 72 hours anaerobically in tube tubes. Supernatant samples were collected from the 24, 48 and 72 hour time points and measured for the presence of lactate using GC-MS.

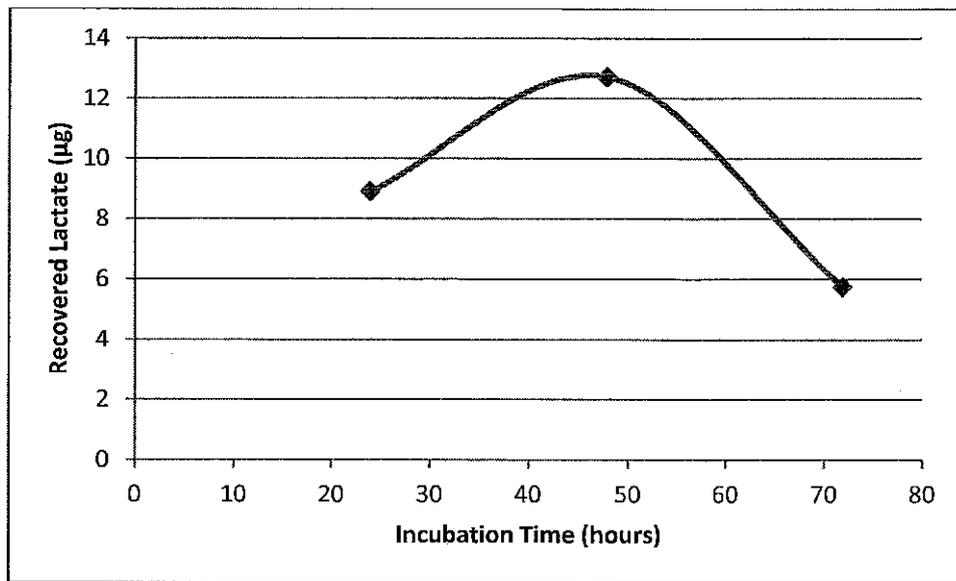


Figure 6. Graph of the production of lactate by *L. johnsonii* over 72 hours

Figure 6 shows the production of lactate by *Lactobacillus johnsonii* at three different time points. The greatest amount of lactate produced was seen 48 hours after inoculation with a value of 12.70 µg. The lowest amount of recovered lactate was 5.74 µg which was collected 72 hours after inoculation.

Comparison of SCFA production between microbial strains

A second area of interest was determining which microbial strains produce the most SCFA when grown under the same conditions. To study this, *Enterococcus faecalis*, *Eubacterium plexicaudatum* and *Lactobacillus johnsonii* were grown anaerobically for 24 hours in individual test tubes. The supernatants of these known lactate-producers were then collected and analyzed via GC-MS. The amount of lactate produced by each organism is shown in Figure 7.

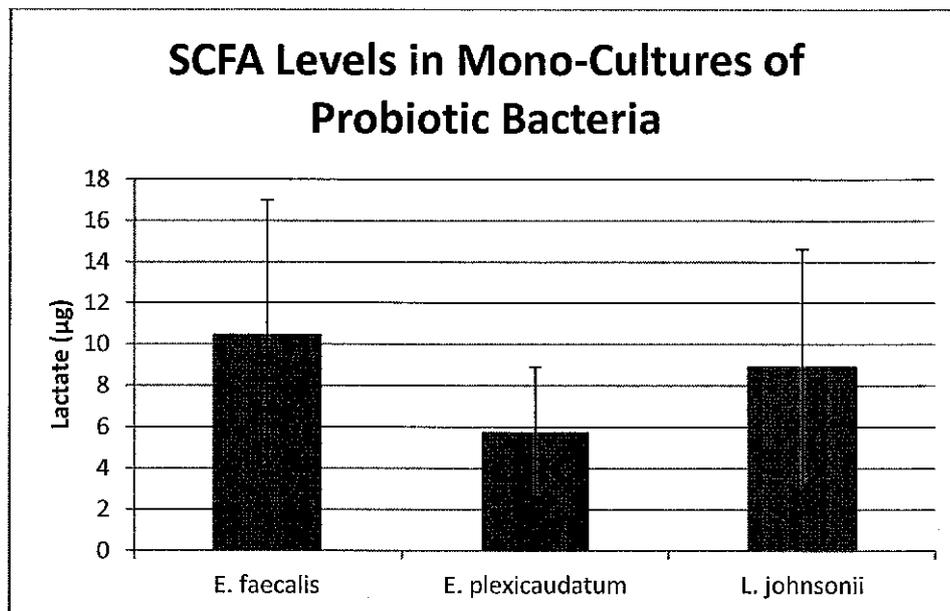


Figure 7. The amount of lactate present in samples collected from monocultures of known lactate-producers

As seen in Figure 7, *E. faecalis* produced an average of 10.44 µg of lactate whereas *L. johnsonii* produced an average of 8.90 µg and *E. plexicaudatum* produced an average of 5.74 µg.

Effects of co-culturing on lactate levels

A third aspect that was investigated during the experiment was the effect of culturing a known lactate producer with a known butyrate producer. According to previous studies, the lactate produced by the one organism should be used by the butyrate producer (9). Therefore to test this knowledge, *E. faecalis*, a known lactate producer was cultured with *C. propionicum*, a known butyrate producer for 48 hours. A graph depicting lactate levels measured by GC-MS in monocultures of *E. faecalis* and *C. propionicum* and their co-culture is shown in Figure 8.

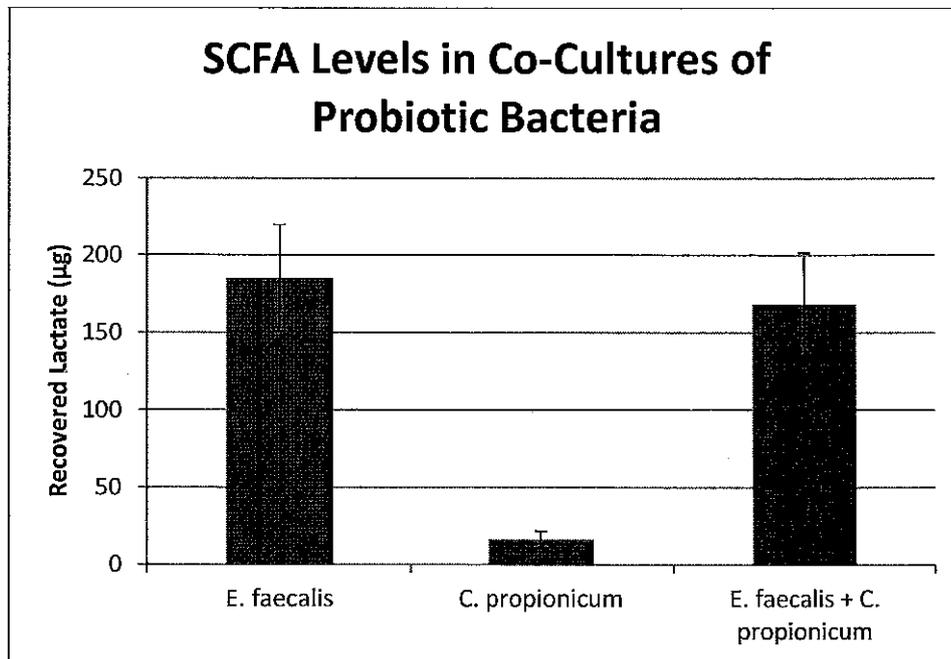


Figure 8. Lactate levels in monocultures of *E. faecalis* and *C. propionicum* and their co-culture

E. faecalis produced 185.2 µg of lactate when grown alone which is significantly higher than *C. propionicum* which produced only 16.36 µg. When grown together, 168.4

µg of lactate were recovered. The error bars represent the mean ± the standard deviation of triplicates. The co-culture of these bacteria did in fact have less recovered lactate than the amount of lactate recovered from the monoculture of *E. faecalis*. This suggests that *C. propionicum* used some of the lactate to produce butyrate. However, the difference between the lactate levels of the co-culture and the *E. faecalis* monoculture is not great enough to confirm the suggested mechanism that butyrate-producers use lactate.

Part II: Development of the model

Aerobic environment

For this section of the experiment, the CDC Biofilm Reactor was under aerobic conditions with fresh media flow. *Enterococcus faecalis* and *Lactobacillus rhamnosus* were grown together for a period of 78 hours. Time points were taken where the pH and colony forming units (cfu) were measured. The number of colony forming units allows for proper characterization of the role of each organism in the production of SCFA. The supernatants from the 24, 48 and 72 hour samples were saved and SCFA levels were measured using direct analysis in real-time mass spectrometry (DART-MS).

The pH was measured at each time point because previous research has shown that lowering the pH of the environment can trigger different protein expression in *Lactobacillus reuteri* (14). By monitoring the pH of different bacterial strains, it can be determined if this trend is common among all microbes.

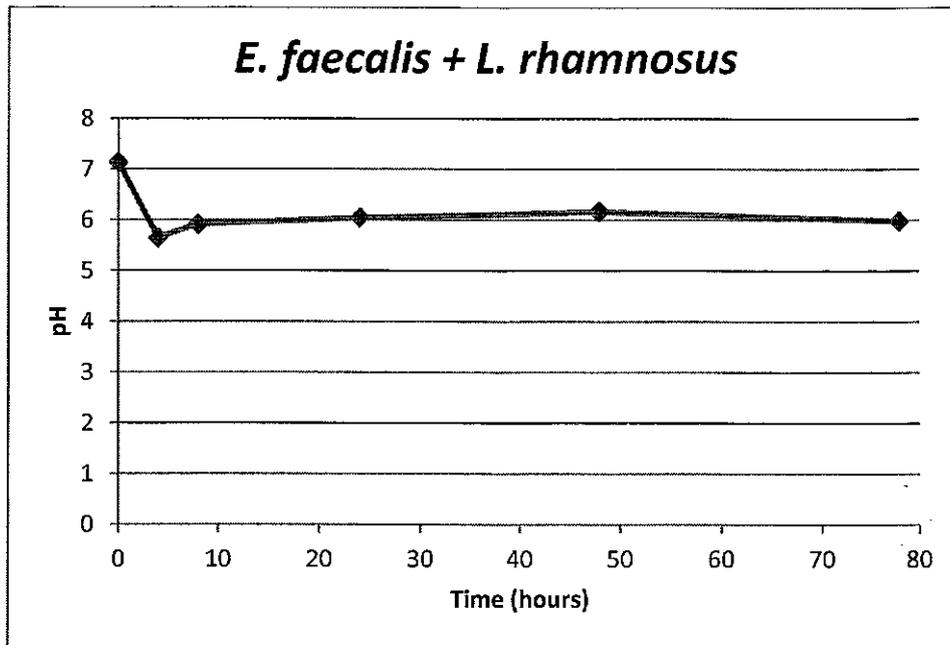


Figure 9. Graph of Time vs. pH of a co-culture sample of *E. faecalis* and *L. rhamnosus*.

As shown in Figure 9, the highest pH was seen at inoculation with a pH of 7.13. As the bacteria were left to grow, the pH dropped and then remained within a range of 5.6 to 6.2. After 72 hours, the media had a pH of 5.98.

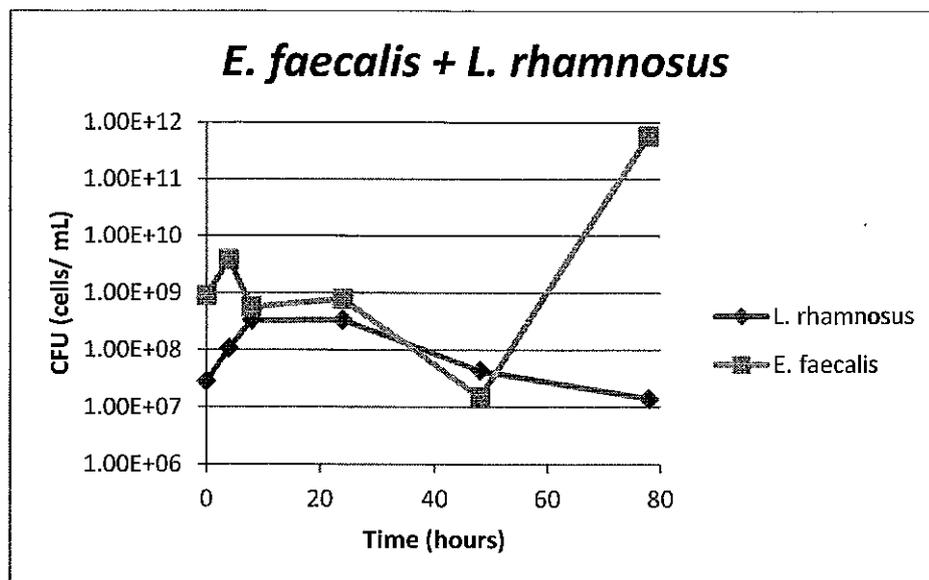


Figure 10. Graph of Time vs. CFU of *E. faecalis* and *L. rhamnosus*.

With the exception of the 48 hour reading, *E. faecalis* had the highest number of colony forming units when compared to *L. rhamnosus* (Figure 10). *E. faecalis* had the greatest growth between the 48 and 78 hour time points, with a final cfu count of 5.70×10^{11} . *L. rhamnosus* had the greatest number of cfu after 24 hours with a value of 7.81×10^8 . *L. rhamnosus* appears to have a steadier growth curve, while *E. faecalis* appears more sporadic.

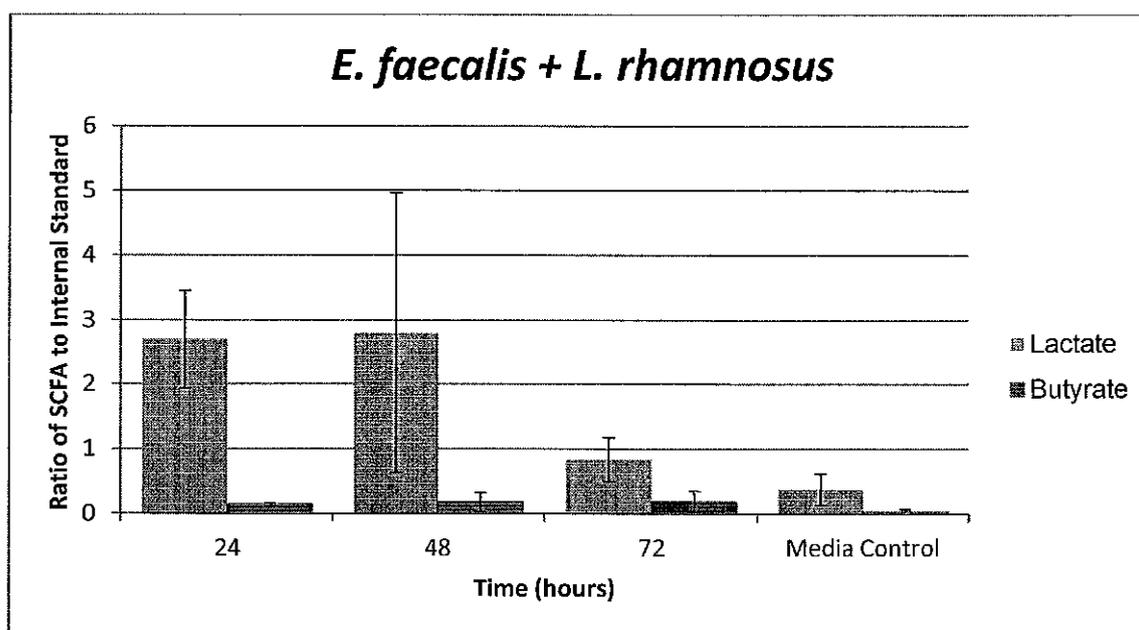


Figure 11. Graph of Time vs. Ratio of SCFA to Internal Standard of *E. faecalis* and *L. rhamnosus*

Figure 11 displays the amount of SCFA detected at each time period. The highest ratio of lactate to internal standard was exhibited after 48 hours, measuring 2.80 ± 2.16 . The highest ratio of butyrate to internal standard was seen after 72 hours, measuring 0.18 ± 0.16 . The error bars represent the mean \pm the standard deviation of the three samples.

The values were corrected for the blank which represents the level of lactate or butyrate in the media without the bacteria.

Anaerobic environment

For the anaerobic portion of the experiment, the CDC Biofilm Reactor was under anaerobic conditions with fresh media flow after 24 hours of bacterial growth. The two strains used were *Clostridium propionicum* and *Eubacterium plexicaudatum*. These two strains were grown for 72 hours. Every 24 hours, a sample was taken where the pH and colony forming units were measured. The supernatant was saved for analysis of SCFA production by DART-MS, and the pellet was saved for quantitative PCR. Quantitative PCR will be used to identify the bacterial strain and number of bacterial genomes within the sample.

Table 2. Time point measurements from *Clostridium propionicum* samples

Hours	Run 1		Run 2		Average	
	pH	cfu	pH	cfu	pH	cfu
0	6.90	0	6.82	0	6.86	0
24	5.72	2.10E+06	5.57	2.00E+07	5.645	1.11E+07
48	5.68	1.60E+09	5.51	5.30E+06	5.595	8.03E+08
72	5.66	2.50E+06	5.78	2.70E+06	5.72	2.60E+06

Table 2 shows the pH and cfu for the two time-course runs of *Clostridium propionicum*. Both runs had similar readings for pH, starting out at about a neutral reading and then decreasing to about 5.6 after 24 hours. The colony forming units differed slightly between the two runs, but on average, there was an increase in cfu up to 48 hours and then a decrease to the 72 hour measurement.

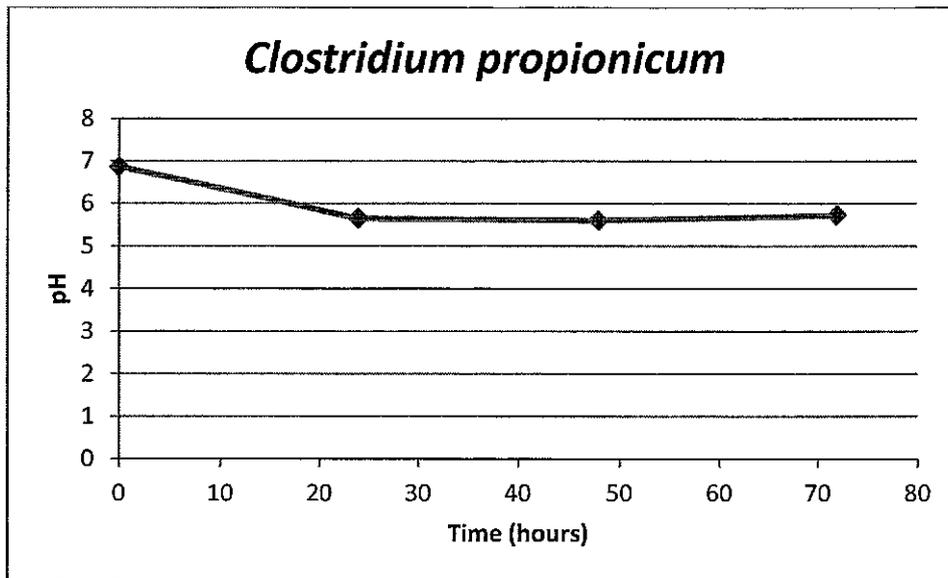


Figure 12. Graph of Time vs. Average pH for *Clostridium propionicum*

At the time of inoculation, the media had an average pH of 6.86 (Figure 12). As seen before, the pH dropped slightly but then remained between a pH of 5.5 and 5.7 for the rest of the readings.

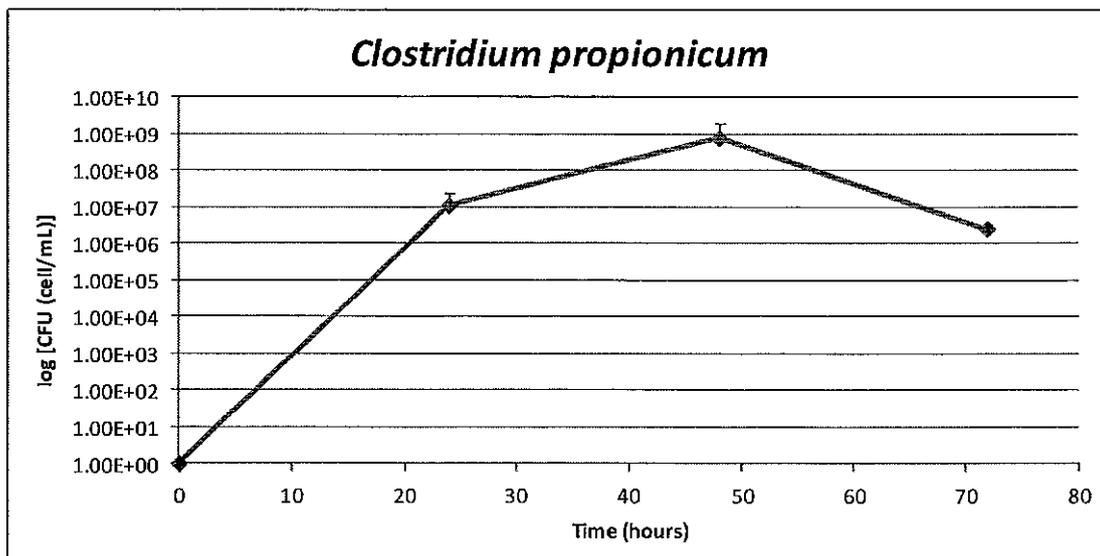


Figure 13. Graph of Time vs. CFU for *Clostridium propionicum*

The greatest growth occurred between 0 and 24 hours (Figure 13), while the greatest number of colony forming units was seen after 48 hours, measuring an average of 8.03×10^8 cfu. The number of cfu then dropped to 2.60×10^6 at 72 hours after inoculation.

Table 3. Time point measurements from *Eubacterium plexicaudatum* samples

Hours	Run 1		Run 2		Average	
	pH	cfu	pH	cfu	pH	cfu
0	7.01	0	6.92	0	6.97	0
24	7.11	0	5.45	9.50E+04	6.28	4.75E+04
48	5.59	3.50E+04	5.51	8.00E+04	5.55	5.75E+04
72	5.57	3.00E+05	5.73	3.30E+06	5.65	1.80E+06

Table 3 contains the data from the two time-course runs of *Eubacterium plexicaudatum* and their average measurements. For both runs, the pH followed similar trends, starting off about neutral and decreasing to a value around 5. The colony forming units had a slightly different trend. The first run didn't see much initial growth until 48 hours after inoculation, while the second run began seeing growth after 24 hours.

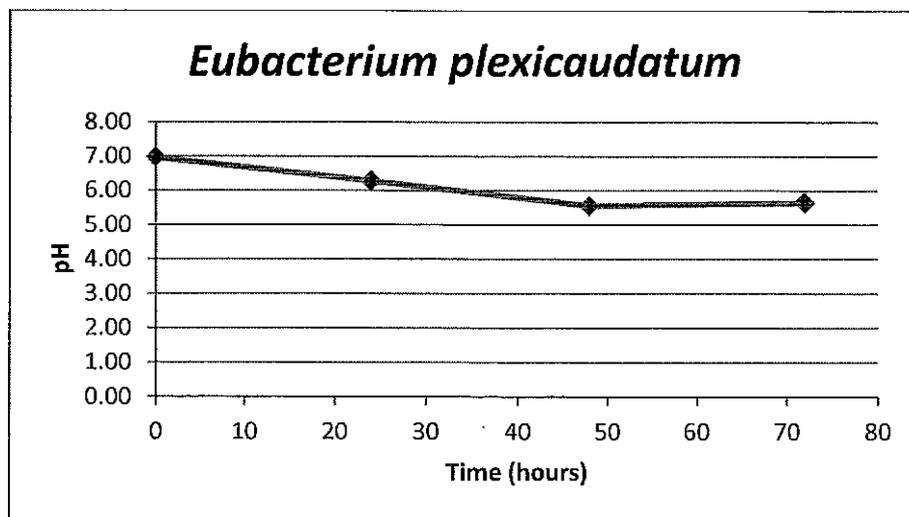


Figure 14. Graph of Time vs. Average pH for *Eubacterium plexicaudatum*

Eubacterium plexicaudatum seems to follow the pH trend that has been described before as shown in Figure 14. At inoculation, the media was neutral but then decreased slightly as the bacteria began to grow. The pH then averaged between 5.5 and 6.3 for the following readings.

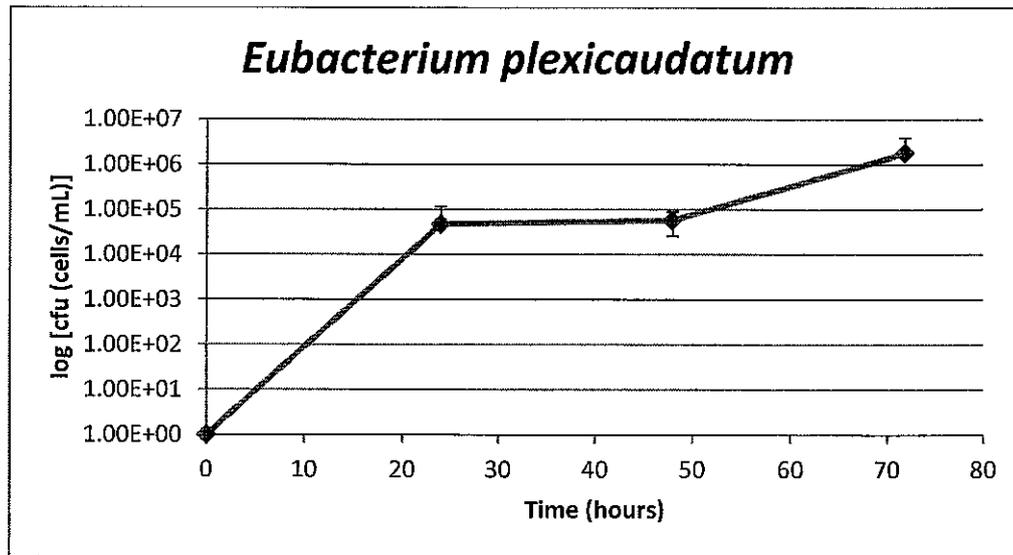


Figure 15. Graph of Time vs. Average CFU for *Eubacterium plexicaudatum*

Figure 15 shows the average growth curve of *Eubacterium plexicaudatum* over a period of 72 hours. The most growth was seen after 72 hours with an average cfu count of 1.80×10^6 .

DISCUSSION

Production of lactate at various points in growth

To look at lactate production at various points along the bacterial growth curve, *Lactobacillus johnsonii*, a known lactate producer, was grown for 72 hours. After analysis of three time points (24, 48, and 72 hours), the lactate production appeared to peak at 48 hours. A reason for the drop in lactate production after that time point could be that the media was sufficiently depleted of its nutrients. If there were not enough nutrients to sustain bacterial life, they would die and consequently not produce any lactate. This lack of continual feeding in test tube cultures was accounted for by introducing a continuous flow of media to the bacterial samples. The continuous flow of media also attempted to more closely mimic the environment representative of the biofilm conditions in the GI tract.

Lactate levels in co-cultures

When *E. faecalis*, a known lactate producer, was co-cultured with *C. propionicum*, a known butyrate producer, the amount of recovered lactate was less than the amount recovered when *E. faecalis* was cultured alone. Although the decrease in the amount of recovered lactate was not significant, this observation is consistent with literature (9). The decrease in recovered lactate suggests that *C. propionicum* used some of the lactate present, thus converting it to butyrate. This would decrease the overall amount of lactate present.

It would be insightful to run this again to check if a larger decrease in the production of lactate can be seen in the co-culture, and so that the cfu and pH can be measured. With this additional information, we would be able to know the number of

each organism present which can help determine which microbe is primarily responsible for the conditions observed.

Aerobic conditions

By measuring the colony forming units and lactate levels from a co-culture of two lactate producers, *E. faecalis* and *L. rhamnosus*, the relationship between the two measurements could be established. Interestingly, the 24 hour sample had few colony forming units, but had high levels of lactate present. Opposite to this was the 72 hour sample which had the most colony forming units, but had the lowest levels of lactate of each organism. This inverse relationship between the two measurements seems contrary to common sense—one would think that the more bacteria there are, the more SCFA should be produced. An explanation for what was observed could be the result of competition among the bacteria. The sample with the highest number of cfu would experience a greater level of competition for nutrients than the sample with the fewest cfu. This competition for nutrients could result in decreased metabolic activity among the bacteria, leading to lower levels of lactate being produced. The opposite would be true as well; if competition is low between bacteria, their metabolic processes will greatly increase leading to the high production levels of lactate.

Development of the anaerobic environment

In order to truly visualize how bacteria function in the GI tract, an anaerobic model needed to be developed. This type of model had never been produced by this group before, so its development started at ground zero. Once the basic concept of the model was developed, construction took place and several difficulties arose that we had to smooth out. The first main issue was that the system didn't want to remain anaerobic.

The protocol given to us from the manufacturer to fill the anaerobic vinyl chamber with hydrogen and nitrogen gases was apparently incorrect, causing a seal to leak. This leak caused the hydrogen and nitrogen gases to escape the chamber and allowed oxygen to enter. Once the seal was located and patched, a new protocol was developed to obtain the correct amount of hydrogen and nitrogen gases for our chamber.

A second issue was with creating reducing conditions within the media. When the media was first pulled out of the autoclave, the resazurin (indicator of oxygen in the media) indicated the media was anaerobic. However, by the time we got the media to the anaerobic chamber, the media turned aerobic. Resazurin turns the media pink in the presence of oxygen or it turns the media a yellow-orange in the absence of oxygen, allowing visual confirmation of the media's oxygen content. The media was then left in the chamber with the intention to have it become anaerobic but after a couple of days, it remained aerobic. To combat this issue, reducing agents such as L-cysteine, sodium thioglycolate and sodium sulfate were added. These agents can be oxidized, thus removing the free oxygen from the media so that it remained anaerobic at all times.

A third issue that we had to overcome was contamination. The nature of the anaerobic vinyl chamber makes the workspace difficult to clean. On top of that, to obtain a sample from the CDC Biofilm Reactor, we have to open the closed and sterile system to collect the culture. Normally when one has to open a closed and sterile system, a flame is used to create an air bubble free of bacteria. Since we are working in a hood with hydrogen and nitrogen gases, we are unable to use a flame. Therefore, our main defense against contamination was the use of sterile technique.

Anaerobic growth conditions

With the successful completion of an anaerobic model, monocultures of anaerobic bacteria were grown to visualize growth patterns under these conditions. *Eubacterium plexicaudatum* and *Clostridium propionicum* were the two strains chosen to analyze because they are both obligate anaerobes. When the pH was measured, a common trend was seen where the pH at inoculation was about neutral but then dropped slightly as the bacteria began to grow. A reason for this could be that as the bacteria began to grow, they started introducing short chain fatty acids to the media which would result in a lower pH. The pH would lower because acids have a low pH so if you were to add acids to a non-buffered solution, the pH would drop. There is interest in looking at the correlation between colony forming units and pH change for this reason. There is also interest in looking at pH because of previous literature suggesting that changes in pH lead to changes of transcription (14). When *C. propionicum* had the highest number of cfu, the pH was the lowest. *E. plexicaudatum* saw a similar trend; when the number of cfu was the highest, the pH was second lowest. This suggests that under anaerobic conditions, when there is the most bacterial growth, SCFA production is at its maximum which would account for the lowest pH measurements.

CONCLUSION

Analysis by GC-MS allowed for reproducible results for lactate in test tube cultures. Although detection of lactate in monocultures and co-cultures was pretty good, there were some inconsistencies between trials. This lack of precision makes it difficult to draw strong conclusions about the different levels of lactate measured in monocultures versus co-cultures. In these experiments, only lactate was measured. Other SCFA should be measured to have a more complete picture of SCFA metabolism in bacteria. Butyrate in particular will be important to quantitate as it is the preferred energy source for human cells in the GI tract.

The current *in vitro* anaerobic model that was developed to better resemble the conditions within the GI tract is rather effective. By successfully creating an anaerobic system, we have been able to grow facultative and obligate anaerobes in a biofilm reactor which better recreates the environment of the GI tract compared to test tube cultures. The constant flow of media through the bioreactor acts to represent the flow of food and nutrients through the GI tract. Implementation of the coupons within the bioreactor will allow for true biofilm growth. Collecting samples of the biofilm will allow for analysis and better characterization of the production of SCFA in the GI tract.

Growth between the aerobic and anaerobic biofilm reactor models was similar. With regard to pH, there was a highly reproducible trend seen with all microbial samples—the pH was about neutral at inoculation and then decreased to a pH between 5 and 6 once bacterial growth commenced. The number of colony forming units did however differ slightly between the two models. *E. faecalis* and *L. rhamnosus* measured

high values for cfu in the aerobic model while *C. propionicum* and *E. plexicaudatum* measured moderate values for cfu in the anaerobic model. The difference in growth may be a result of the different models, but it is very likely that the small difference is a result of differences in standard microbial growth, meaning certain bacteria simply don't produce as many cfu as others. It will be very interesting to see if there is any difference in lactate production between the two models once analysis on the DART-MS is completed.

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