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Quantification of short-chain fatty acids in cecal material by gas chromatography-mass spectrometry

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**Quantification of Short-Chain Fatty Acids in Cecal Material by Gas Chromatography-Mass
Spectrometry**

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Thesis

Submitted to the Department of Chemistry

Eastern Michigan University, Ypsilanti, MI

in partial fulfillment of the requirements

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MASTER OF SCIENCE

Chemistry

Thesis Committee

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ABSTRACT

Quantification of Short-Chain Fatty Acids in Cecal Material by Gas Chromatography-Mass Spectrometry

Probiotic bacteria in the human colon that produce C₂ – C₄ short chain fatty acids (SCFA) and *Lactobacillus* species that produce lactate in addition to SCFAs are known to have positive health benefits. These organic acids were extracted with ether from murine cecal material (from Dr. Gary Huffnagle, Univ. of Mich.) and derivatized for quantitative measurement by gas chromatography-mass spectrometry. Antibiotic-treated animals gavaged with *Candida albicans* had a lower level of cecal butyrate relative to untreated animals, but a higher level of butyrate when treated mice were also administered *Lactobacillus johnsonii* NF-1. More recent studies have demonstrated a butyrate-lowering effect of antibiotic-/*C. albicans* treatment relative to antibiotic or *C. albicans* treatment alone, but a corresponding increase in the level of lactate. It is proposed that the steady state levels of small organic acids provide a marker of human health and disease.

TABLE OF CONTENTS

	<u>Page</u>
Approval.....	ii
Acknowledgments.....	iv
Abstract.....	v
INTRODUCTION.....	1
1. Gastrointestinal tract (GI).....	1
2. Microbiota in Humans.....	2
3. Health benefits of short chained fatty acids.....	3
4. Sources of SCFA production.....	5
5. Analysis of SCFA of gas chromatography/mass spectrometry.....	7
6. Gas chromatography mass spectrometric detection of analytes.....	8
7. Use of mouse model system.....	9
EXPERIMENTAL PROCEDURES.....	10
1. Preparation of standards.....	10
2. Preparation of pentafluoro benzyl bromide esters of fatty acids.....	10
3. Hydroxyl fatty acid analysis.....	11
4. Conversion of hydroxyl fatty acids to their (bis)-TMS ester derivatives by use of BSTFA.....	11

5. Processing of biological samples.....	12
6. Extraction of cecal material for SCFA and lactate analysis.....	13
7. Conversion of short chain fatty acids in cecal material to PFB-ester derivative.....	14
8. Conversion of lactate and other SCFA in cecal material to (bis)-TMS derivative.....	15
9. Materials.....	15
10. GC conditions for butyrate and short chain fatty acid PFB derivatives.....	16
11. GC conditions for lactate (bis)-TMS derivatives.....	17
12. Extraction of analytes using C18 cartridges.....	17
RESULTS.....	19
1. PFBB derivative reaction of short chain fatty acid standards and calibration curve reproducibility.....	19
2. PFBB derivative reaction of butyrate in biological cecal sample and short chain fatty acid verification in samples.....	21
3. Biological sample spiking with butyrate standards.....	24
4. Butyrate analysis in biological samples (August 2008).....	25
5. Butyrate analysis of experimental samples as a function of time of residence in GI tract.....	27
6. Butyrate analysis in Abbott mice.....	28
7. Calibration curve of lactate-TMS derivative by GC/MS.....	32
8. Analysis of lactate present in cecal material of biological results.....	35
9. Additional short chain fatty acids.....	36

DISCUSSION.....40

REFERENCES.....47

LIST OF TABLES

<u>Tables</u>	<u>Pages</u>
1. Chemicals used for the research project, with corresponding identification and vendor source.....	16
2. Solvents used for research project, with corresponding identification and vendor source.....	16
3. Raw data for butyrate-PFB calibration curve.....	20
4. Experimental groups for the Abbott 5/6 experiments.....	28

LIST OF FIGURES

<u>Figures</u>	<u>Pages</u>
1. Diagram of Human Gastrointestinal System.....	1
2. Common short chain fatty acids produced in GI system.....	4
3. Biochemical pathway of butyrate production associated with various nutrient sources and organisms.....	6
4. Reaction of SCFA with PFBB yielding SCFA-PFB ester linked derivative.....	7
5. Reaction of hydroxyl-fatty acid with BSTFA forming (bis)-TMS derivative.....	8
6. Calibration curve for butyrate-PFB derivative.....	20
7. Repeat calibration curve for butyrate-PFB derivative from April of the following year.....	21
8. Overlay of GC select ion monitor of m/z 181 fragment from butyrate-PFB standard sample and butyrate-PFB peak found in biological sample.....	22
9. Mass spectra of butyrate-PFB peaks found at retention time 8.1 minute from both a calibration standard and a biological sample exposed to the same reaction assay.....	23
10. Reported mass spectrum of internal standard-PFB derivative chosen for analysis of SCFA.....	24
11. Experimental results from butyrate spiking experiment of pooled biological samples.....	24
12. Butyrate results from August 2008 analysis of biological samples.....	26

13. Butyrate results in cecal samples at days seven and fourteen of mice treated with <i>C. albicans</i> , the antimicrobial agent Cefoperazone, or both.....	27
14. Procedural details of Abbott 5 and Abbott 6 experiment treatment administration.....	29
15. Butyrate results for Abbot 5 from cecal material of female mice.....	30
16. Butyrate results for Abbot 6 from cecal material of male mice.....	31
17. Mass spectrum of lactate-TMS derivative selected from calibration standard. m/z 219 fragment represents the molecular ion for the derivative.....	32
18. Mass spectrum of 1,3-propanediol-TMS derivative selected from calibration standard.....	33
19. Lactate-TMS derivative calibration curve with m/z 219 selected for lactate-TMS, and m/z 147 selected for internal standard.....	33
20. Unsuccessful lactate-TMS calibration curve selecting same ion for lactate-TMS and internal standard analytes.....	34
21. Lactate analysis results of cecal samples from animals treated with antimicrobial agent followed by <i>C. albican</i> gavage at days seven and fourteen.....	35
22. Gas chromatograms of internal standard and propionate-PFB derivatives.....	37
23. Mass spectral results of dideuterated propionate used as	

internal standard versus propionate found in biological samples.....	38
24. Early propionate calibration curve with m/z 254 fragment ion selected propionate-PFB derivative, and m/z 256 fragment ion was selected for internal standard.....	38
25. Example of successful propionate calibration curve with m/z 254 fragment ion selected propionate-PFB derivative, and m/z 256 fragment ion selected for internal standard.....	39
26. Chromatogram of acetate-PFB identification.....	40
27. Mass spectra of acetate-PFB and the substance with the same retention time (7.02 min) in a cecal sample.....	54

LIST OF ABBREVIATIONS

GI	Gastrointestinal
SCFA	Short chain fatty acid
GC/MS	Gas chromatography/mass spectrometry
TMS	Trimethylsilyl
PFBB	Pentafluorobromobenzene
BSTFA	Bis(trimethylsilyl)fluoroacetamide
FID	Flame ionization detector
TCD	Thermal conductivity detector
MSD	Mass selective detector
GC	Gas chromatograph
m/z	Mass to charge ratio
EDIPA	N,N-Diisopropylethylamine
HCl	Hydrochloric acid
SPE	Solid phase extraction
IS	Internal standard
TMCS	Trimethylchlorosilane

INTRODUCTION

1.1 Gastrointestinal tract (GI)

The gastrointestinal tract in mammals contains a series of organs that are responsible for digestion of foods and absorption of nutrients into the body. The remaining substances that are not absorbed are then processed and removed via defecation. The GI system is commonly divided into the upper GI tract and lower GI tract. As shown in Figure 1, The upper portion of the GI tract begins at the mouth and extends past the stomach into the duodenum of the small intestines. The lower GI tract consists of most of the small and large intestines, and the anus. The large intestine itself is composed of the cecum, the colon, and the rectum.

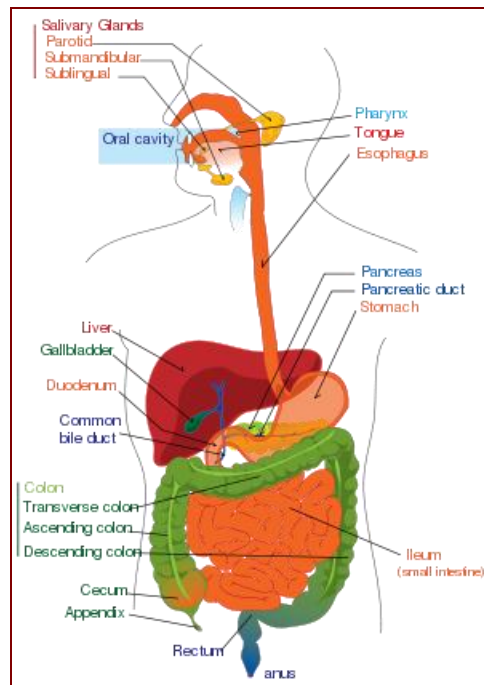


Figure 1. Diagram of Human Gastrointestinal System

http://www.newworldencyclopedia.org/entry/Gastrointestinal_tract (accessed November 2009)

Given the length of and diversity of tissue in the GI tract within the human body, it is no surprise that the physiological conditions of the various organs vary greatly with conditions such as pH and oxygen levels. The GI system is known to host a wide variety of microorganisms, commonly referred to as the microbiota. Along with the varying conditions of the GI tract, there is great variation in the concentration and identity of organisms residing in specific portions of the GI tract. It has been found that few organisms can survive the low pH in the stomach; therefore, a low concentration of bacterial cells is observed in this acidic environment. Further down the GI tract the concentration of microorganisms increases, with the highest concentration located in the colon. Anaerobic bacteria are found in the GI tract due to the very low levels of oxygen available in these deep organ tissues (1).

1.2 Microbiota in Humans

A highly diverse population of microorganisms resides specifically in the lower GI tract. A great level of diversity in microbiota exists as well from human to human, with adults exhibiting a high degree of complexity in the GI microbiota. One factor that affects the bacterial composition in the human GI tract is diet (2). The reason for this is that food intake provides nutrients for the host but also for the microorganisms that reside therein. Food nutrients that are not digested may serve as an energy source for the organisms living within the intestines and colon. A change in diet may consequently eliminate the preferred energy source of a particular organism, and that organism may no longer be able to survive within the GI tract. For example, undigested carbohydrates and proteins can enter the large

intestine or colon and be partially broken down by certain organisms. The leftover material may then serve as an energy source by a different set of organisms, which may then break down the by-products into short chain fatty acids (SCFA). These SCFA are known to have positive health benefits to the host (3).

1.3 Health benefits of short chained fatty acids

It is recognized that many GI-related diseases and health issues are caused by imbalances in the microbiota in the large intestines (4). The common known related diseases and illnesses include Crohn's disease, colitis, irritable bowel syndrome, and colon cancer. It has been reported that the large intestines can contain an excess of up to 200g of material, of which approximately half of this mass may be microbial biomass (5). In healthy individuals these organisms are non-pathogenic and provide positive health benefits (6). Not only do these organisms provide competition inhibiting pathogenic organisms from colonizing, their main function is to breakdown undigested food materials into SCFA. This is beneficial because the starches and fiber that they process would otherwise be removed by defecation with no benefit to the host system. Instead these organisms produce useful materials that the host can use. The presence of SCFA in the GI system has been found to provide several positive health benefits (7). The average daily SCFA production in the large intestines has been reported as 400mmol (8). Two of the most beneficial SCFA are believed to be butyrate and lactate (Figure 2).

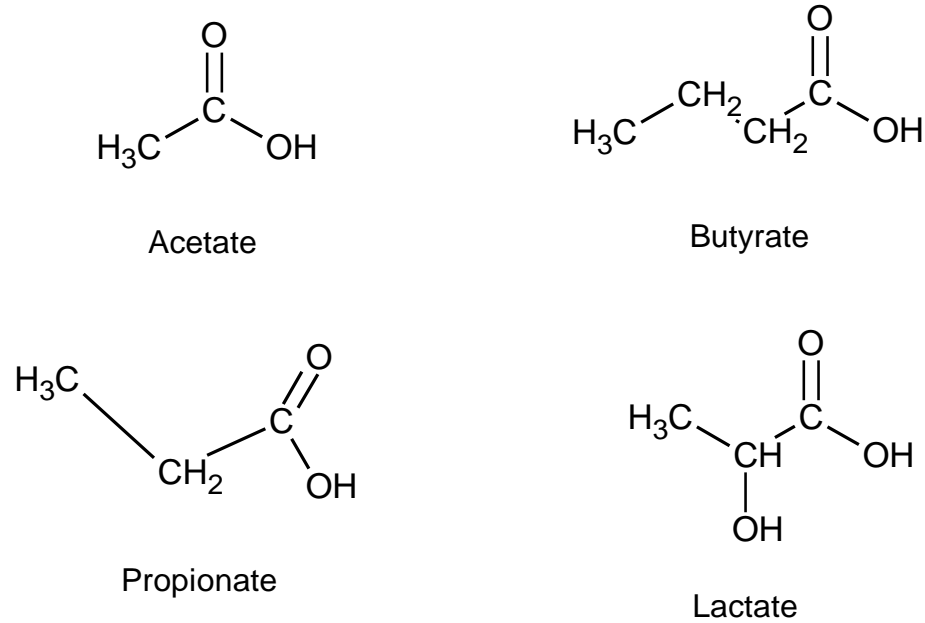


Figure 2. Common short chain fatty acids produced in GI system

In vivo experiments with GI epithelial cells and lactate-producing bacteria have demonstrated a modified T-cell immune response regulating the allergic immune response (9). Results of studies have provided evidence that lactate is commonly metabolized to butyrate (10). Therefore, lactate production may be necessary for the formation of butyrate. Butyrate has been found to lower the risk of colon cancer in humans (11). One possible explanation for this is that butyrate is the preferred energy source of epithelial cells in the colon (4). It has been reported that these cells derive 70% of their energy through the oxidation of butyrate (12). Butyrate and lactate are used by the host organism's cells for healthy cellular function. It has also been shown that butyrate may induce apoptosis in human colonic tumor cell lines (13). The lead theory for the etiology of ulcerative colitis is a failure of butyrate metabolism (14).

1.4 Sources of SCFA production

As stated earlier there are several dietary sources that lead to the formation of SCFA in the large intestines. Commonly these dietary sources enter the GI system as indigestible carbohydrates, polysaccharides, starches, fiber, and protein. Upon entering the large intestines they are used as an energy source for microorganisms residing in the GI tract. In certain instances they may directly be broken down to produce SCFA, or maybe converted into an intermediary component, which may then be converted by another organism to finally yield SCFA. Figure 3 displays how specific microorganisms are able to produce butyrate from any of a variety of nutrient precursors. Interestingly, despite the diversity of nutrient precursors used to form butyrate, all biochemical pathways contain crotonyl-CoA as a common intermediate. Acetyl-CoA, polyphenols, and the amino acids lysine and glutamate are able to directly form this compound. Carbohydrates and other proteins must first produce succinate, which passes through the Krebs's cycle to yield crotonyl-CoA. This pathway ultimately leads through the action of various enzymes to the production of butyrate and one equivalent of acetyl-CoA. Acetyl-CoA then reenters the pathway and leads to the formation of more butyric acid. Once the pathway has begun there is an alternative cycle that certain organisms possess. For these organisms, enzymes catalyze the reaction of acetyl-CoA with phosphate to form butyryl phosphate, which goes to yield butyrate.

Butyrate Production Pathway

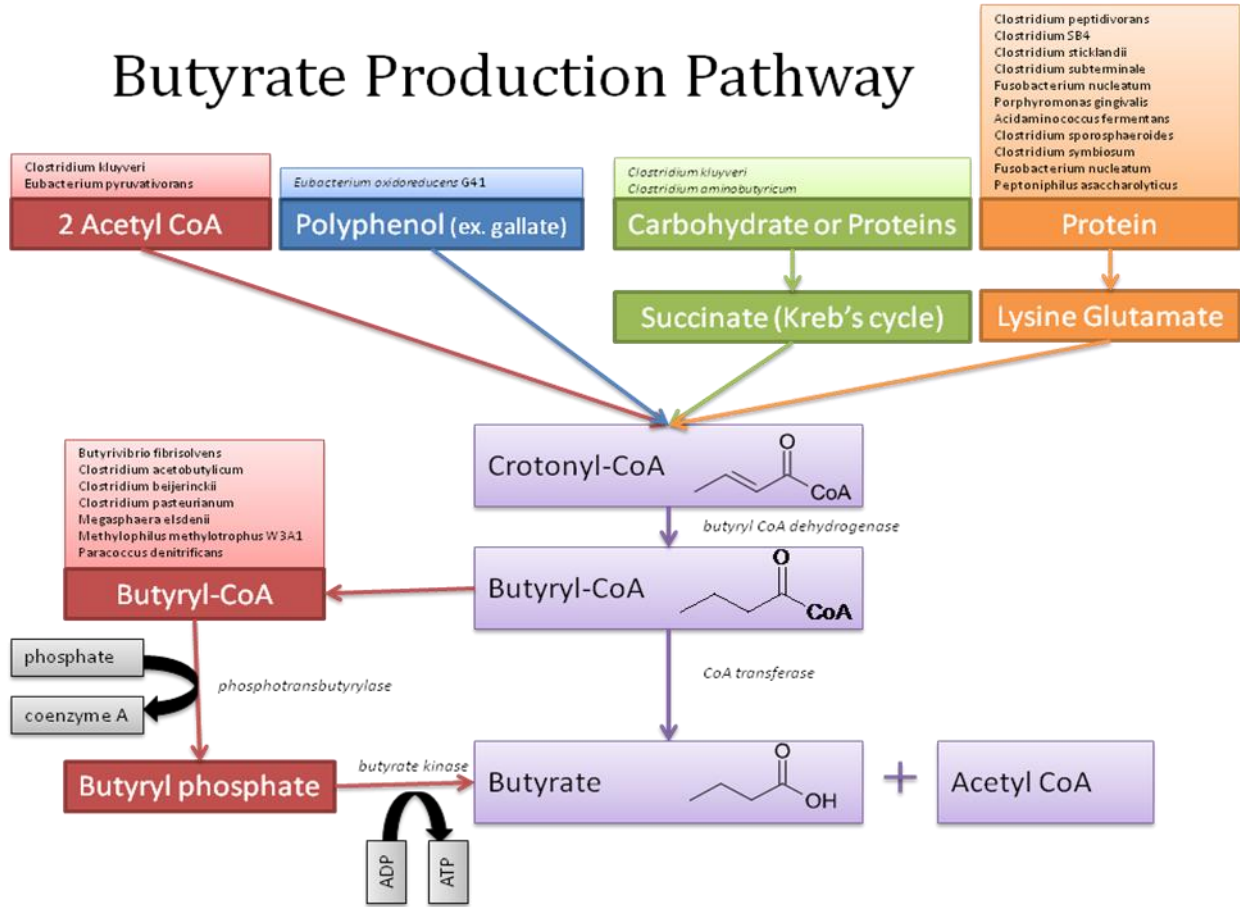


Figure 3. Biochemical pathway of butyrate production associated with various nutrient sources and organisms.

1.5 Analysis of SCFA of gas chromatography/mass spectrometry

Chemical derivitization and analysis by GC/MS is a technique that is commonly used for characterization of SCFA. Typically investigators prepare trimethylsilyl (TMS) derivatives of SCFAs (11). Alternatively, 2,3,4,5,6-pentafluorobenzyl bromide (PFBB) is used to prepare the pentafluoro-ester linkage with the SCFA as shown in Figure 4.

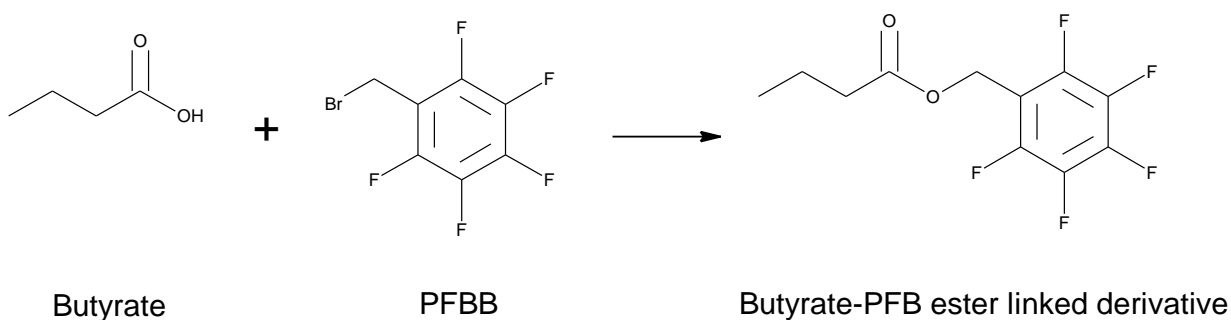


Figure 4. Reaction of SCFA with PFBB yielding SCFA-PFB ester linked derivative

The PFB group yields a derivative with a greater mass than that for the TMS derivative, thus improving GC/MS detection and peak quality. This reaction has been found to occur readily at room temperature and reaches completion in a relatively short amount of time. This derivative can then be prepared in solvent and injected onto the GC for resolution and characterization by a mass-selective detector. As shown in Figure 5, for short chain fatty acids like lactate, the TMS derivatization of the hydroxyl and carboxylate groups is carried out with N,O-bis(trimethyl-silyl) trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA) to form the bis-trimethylsilyl derivative (15).

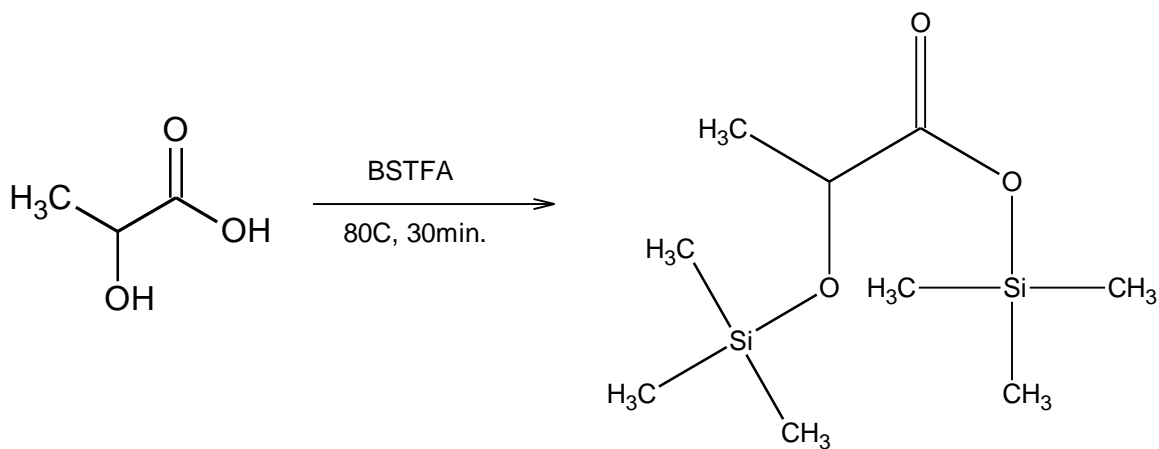


Figure 5. Reaction of hydroxyl-fatty acid with BSTFA forming (bis)-TMS derivative

An internal standard is also used in quantitative analysis. It is added at constant volume to all standards and samples. The internal standard normalizes for the loss of any analytes in the derivatization process and solvent loss prior to sample injection.

1.6 Gas chromatography mass spectrometric detection of analytes

A gas chromatograph is an instrument that is commonly used in analyses of organic materials. The column used in the current study involves ultra high purity helium as the mobile phase and a coated 30-meter capillary column for the stationary phase. A small aliquot of sample is injected into the port via glass syringe. This liquid sample is then vaporized into its gaseous form and swept onto the column by the mobile phase (helium gas). As the sample passes through the stationary phase column, its chemical components are separated based on their interaction with the column walls. The individual components of the sample traverse the column at different times (known as the retention time), based on the extent to which they interact with the stationary phase. Gas chromatographs are

compatible with several detectors such as flame ionization detector (FID), thermal conductivity detector (TCD), and the mass selective detector (MSD). A mass selective detector was used in this research project.

The resolved analytes exit the GC column and enter the mass spectrometer's ion trap through a heated transfer line. The materials are then bombarded by an electron beam, which fragments the compounds into charged species. The mass analyzer then calculates the mass-to-charge ratio of the charged particles and sorts the ions based on this ratio. Finally the sorted ions pass through a detector, which calculates the abundance of each of the ions. In the data analysis stage the operator can then identify the individual compound by the fragments.

1.7 Use of mouse model system

Model organisms are extensively used in biological research to investigate particular biological occurrences. They are commonly used to gain information related to human diseases and possible treatments for such disorders. Researchers have a wide variety of research organisms to choose from that are certified to meet specific qualifications for research and are readily available from commercial suppliers. In our studies, commercially available mice were housed in sterile environments that do not contain microorganisms that may interfere with the experimental procedures.

EXPERIMENTAL PROCEDURES

Preparation of standards

Stock solutions of all short chain fatty acids including acetate, propionate, butyrate, and lactate were prepared in acetonitrile. The concentrations of each of the materials varied from 200mg/L to 700mg/L. The stock solution of 200mg/L would be prepared to make standards for experiments when a lower concentration was needed to evaluate the lower limits of the calibration curve. The compound chosen for the internal standard for non-hydroxyl short chain fatty acid analyses was a dideuterated compound, propionic acid-2,2-d₂. Stock solutions of this material were prepared with acetonitrile as the solvent, at a concentration of 200mg/L. All stock solutions were stored at -20°C, and replaced on a monthly basis.

Preparation of pentafluoro benzyl bromide esters of fatty acids

A stock solution of 2,3,4,5,6-Pentafluorobenzyl bromide (PFBB) was prepared monthly by adding 0.355mL of PFBB to 11.55mL of acetonitrile yielding a concentration of 58mg/mL. This solution was also stored at -20°C. Standards of SCFA-PFB derivatives were prepared in glass test tubes on ice. For a five-point calibration curve, the typical volumes of SCFA stock solution selected were 1, 5, 10, 15, and 20µL, which encompassed a range of SCFA from 5.8µg to 116µg. After adding the stock solution, 10µL (2 µg) of the deuterated propionate stock solution were added to each test tube. A 25-µL Hamilton syringe was used to transfer aliquots of SCFA and internal standards. Subsequently, 100µL of the PFBB stock solution were added to each test tube, followed by 20µL of the catalyst N,N-diisopropylethylamine 100% (EDIPA). Each test tube

was vortexed at moderate speed for one minute and then returned to a test tube rack. The reaction was then allowed to proceed at ambient room temperature for twenty minutes.

After the twenty-minute reaction time, the solvent in each test tube was evaporated to dryness under a steady stream of nitrogen gas. The dried contents of each tube were re-suspended in 100 μ L of ethyl acetate and vortexed for an additional minute prior to injection onto the GC/MS system.

Hydroxyl fatty acid analysis

For fatty acids such as lactate containing an additional hydroxyl group, an alternative assay was developed to ensure that both the hydroxyl group and the carboxylic group were derivatized.

The derivatizing reagent was N,O-bis(trimethyl-silyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMS), and the subsequent derivative prepared was (bis)-TMS-lactate.

The lactate stock solution was prepared in acetonitrile following the same procedures used for other fatty acids. The internal standard compound that was chosen for this assay was 1,3-propandiol. The stock solution for this material was prepared following the same procedure as for the deuterated compound used for the butyrate analysis.

Conversion of hydroxyl fatty acids to their (bis)-TMS ester derivatives by use of BSTFA

For preparations of standards to be used as a calibration curve for lactate analysis, the following procedure was employed. A fixed quantity of the lactate stock solution was added via a 25- μ L glass Hamilton syringe to corresponding test tubes representing the five different

calibration levels. Typically the volumes of the stock solution used for the calibration levels were 1, 5, 10, 20, and 25 μL , encompassing a range from 5.8 μg to 145 μg . All calibration curves used for quantitative analysis were performed in duplicate. A 10- μL aliquot of the 1,3-propanediol internal standard stock solution was added to each of the test tubes via a 25- μL glass Hamilton syringe. 200 μL of neat BSTFA with 1% TMS would then be added to each test tube, followed by 25 μL anhydrous pyridine.

Each test tube was then covered securely with two squares of parafilm and submitted to vortexing for one minute. Optimum reaction conditions were achieved by placing the test tubes in an 80°C water bath for 30 minutes. Once the samples returned to room temperature, they were injected onto the GC column.

Processing of biological samples

All biological samples were stored at -80°C until processing. The first step involved acquiring the mass of the cecal material and the plastic vials in which they were stored using an analytical balance and measured out to 0.0001g. Samples were thawed on ice. Into each vial containing the biological sample, 500 μL of filtered DNAase-free water was added using a 1mL micropipetter. The contents of each vial was mixed by inversion until a homogenous solution was achieved (approximately two minutes). A vortex was not used to mix the sample for fear of cell lysis. After the homogenous solution was obtained, the biological samples were placed in a 10°C centrifuge for five minutes at a speed of 2500 Xg. If two distinct layers did not result, samples were submitted to centrifugation for five additional minutes.

When two distinct layers were present in all samples, the top cecal layer was transferred to a new plastic screw top vial. This was accomplished by use of an adjustable 100- μ L volumetric micropipetter. Initially 100 μ L aliquots were transferred, although smaller volumes could be used without compromising the quality of the results. When complete, each sample was capped and placed in the -80°C freezer until the extraction period. The final volume would then be recorded in the laboratory notebook along with the initial mass of material plus the vial. The remaining cells in plastic vials would then be discarded in a biological waste container, and the vials would then be cleaned, dried, and weighed empty. This mass was then recorded in the laboratory notebook and subtracted from the total mass of the vial plus biological material. The subtraction of those numbers yields the total mass of biological material processed.

Extraction of cecal material for SCFA and lactate analysis

Once the biological material had been processed it was then available for chemical extraction by ether. The processed cecal samples were thawed on ice. For each sample, two corresponding glass test tubes were labeled and placed in test tube racks. Into each test tube, 10 μ L of 0.6M HCl and 50 μ L of thawed cecal material were transferred using an adjustable micropipetter.

To each test tube containing acidified cecal material, 1mL of diethyl ether, dried overnight in sodium sulfate, was added. The samples were submitted to moderate vortexing for 30 seconds. The test tubes were then submitted to centrifugation for two minutes at a speed of 2000 Xg. The organic layer (upper) was transferred to the remaining clean glass test tubes, with

special care taken to not transfer any aqueous material (lower layer) into the second glass test tube. Since the extraction procedure is to be repeated three additional times, it is not essential to remove the entire organic layer after the first extraction.

This procedure was carried out three additional times, thus resulting in four ether extractions. The remaining tubes containing the aqueous layer were disposed of in a container containing a phenolic antimicrobial solution. Test tubes with the organic ether layer were placed aside to allow the ether to evaporate overnight. If no aqueous layer is present in the test tubes, evaporation typically required 24 hours. However, if moisture was present, an additional 24 hours was required for complete evaporation. Once all the ether is evaporated, samples were then ready for the derivatization procedure and analysis by GC/MS.

Conversion of short chain fatty acids in cecal material to PFB-ester derivative

When all ether was evaporated from the extraction test tube, the samples were then ready for conversion to PFB-ester derivative. These samples were prepared in the same manner as the standards, except no butyrate stock solution was added to the biological samples.

In the test tube used to collect the organic phase internal standard, PFBB stock solution, and the catalyst were added in the same volumes that were used for the calibration curve. If time allowed, the calibration standards were prepared at the same time as the biological samples to ensure that volumes and reaction time for both samples and standards were held constant.

Once the PFB-ester derivative had been prepared, the standards and cecal samples were then injected onto the GC for quantitative analysis of the fatty acid of choice.

For the extraction process determined for lactate, instead of choosing the PFBB assay for derivatization, the (bis)-TMS assay would be carried out and the standards and samples injected onto the GC column on the same day.

Conversion of lactate and other SCFA in cecal material to (bis)-TMS derivative

To the dried extracts of cecal material the internal standard, BSTFA, and pyridine were added in the exact same volumes as those used for standard calibration samples. All reaction conditions were carried out in the exact manner as those used for lactate calibration standards. If the number of biological samples is not too great, the calibration standards should be prepared at the same time to ensure the conditions are identical. After the derivatization reactions had been completed, the calibration standards and the biological samples were then injected onto the GC column for the quantitative analysis of lactate present in the biological material.

Materials:

Table 1 and Table 2 provide the materials and reagents used, respectively, for this research project, along with CAS and product numbers:

Table 1. Chemicals used for the research project, with corresponding identification and vendor source.

Material	CAS No.	Vendor	Product #
Butyric acid	107-92-6	Aldrich	B10350-0
L(+)-Lactic acid	79-33-4	Aldrich	L6402-1G
Acetic acid	64-19-7	Aldrich	A6283
Propionic acid	79-09-4	Fisher	A258-500
1,3-Propanediol	504-63-2	Aldrich	P50404-100G
Propionic acid-2,2-d ₂	14770-51-5	Aldrich	377929-1G
N,N-Diisopropylethylamine (EDIPA)	7087-68-5	Aldrich	550043-100ML
Pyridine (anhydrous)	110-86-1	Aldrich	270970-100ML
N,O-bis(trimethyl-silyl) trifluoroacetamide w/ 1% trimethylchlorosilane (BSTFA)	25561-30-2	Aldrich	T6381-25G
2,3,4,5,6-Pentafluorobenzyl bromide, 99+% (PFBB)	1765-40-8	Aldrich	101052-25G

Table 2. Solvents used for research project, with corresponding identification and vendor source.

Solvent	CAS No.	Vendor	Product #
Acetonitrile (anhydrous)	75-05-8	Aldrich	271004
Ethyl acetate	141-78-6	Aldrich	E7770
Diethyl ether	60-29-7	Aldrich	309966

GC conditions for butyrate and short chain fatty acid PFB derivatives

For the analysis of TMS-derivatives ultra high purity UHP helium was used as the carrier gas.

The injection mode was set to “Std Split/Splitless,” with the injector temperature at 250°C. The valve temperature was set at 275°C and the split ratio at 5:1. For the oven parameters, the

initial oven temperature was 50°C and was held for one minute, while the column flow was 1.4mL/minute. Two temperature ramps were used for the method: Ramp 1 proceeded at 10.0°C/minute until 100°C was attained; Ramp 2 proceeded at 40.0°C/minute until 230°C. The total time of the method was 9.25 minutes.

GC conditions for lactate (bis)-TMS derivatives

For the analysis of TMS-derivatives, ultra high purity UHP helium was used as the carrier gas. The injection mode was set to “Std Split/Splitless,” with the injector temperature at 250°C. The valve temperature was set at 275°C and the split ratio at 40:1. For the oven parameters, the initial oven temperature was 50°C and was held for 1 minute, while the column flow was 1.4mL/minute. Two temperature ramps were used for the method: Ramp 1 proceeded 10.0°C/minute and was held for two minutes at 100°C; Ramp 2: proceeded at 30.0°C/minute until reaching a temperature at 230°C. The total run time was 11.33 minutes.

Extraction of analytes using C18 cartridges

All biological media were stored in -80°C freezer until ready for extraction. When thawing biological medium, samples were placed on ice. When no ice was present in the sample, it was placed in a test tube rack. In a glass test tube, 500µL of the biological medium was transferred with a micropipetter. 50µL of 0.6M HCl was added to the test tube containing the medium, then moderately agitated for 30 seconds. Using a glass pipette the entire contents of the test

tube were transferred into a C18 solid phase extraction (SPE) column. Using a micropipetter, 500 μ L diethyl ether was transferred to the same C18 SPE column. The material was passed through the SPE column, and the eluent was collected in a new clean glass test tube. The material that had been collected contained a high composition of aqueous material that needed to be removed. This was accomplished by an organic solvent extraction, similar to that used for extracting cecal material.

A 1mL aliquot of diethyl ether was dried overnight in sodium sulfate to remove water, which was then added to each test tube containing the C18 eluent fraction. The samples were then moderately agitated by vortexing for 0.5 minutes, which allowed the layers to mix. After agitation the test tubes were placed in a refrigerated centrifuge for two minutes and spun at a speed of 2000 rpm. After centrifugation, two distinct layers were formed with the organic ether layer on top. Using glass pipettes, the organic layer was then transferred to a new glass test tube. Since the extraction procedure is to be repeated three additional times, it was not essential to remove the entire organic layer after the first extraction.

RESULTS

3.1 PFBB derivative reaction of short chain fatty acid standards and calibration curve reproducibility

Standards of the SCFA-PFB derivatives were prepared to verify that the reactions were feasible and that reproducibility could be achieved. The final procedure reported in the Experimental section was optimized over time as a result of varying reaction conditions and GC analysis parameters. Calibration curves were prepared with the standards of the various SCFA as shown in Table 3, to determine linearity and reproducibility of the assay. The calibration curves determined about one year apart were linear in the range from 0.2 to 5 μg (Figures 6 and 7). The ion chosen for peak area integration of butyrate was m/z 181 (see Figure 9 for mass spectrum), which represents the mass ion associated with the pentafluorobenzyl moiety, the prominent ion in the mass spectrum for the butyrate-PFB derivative. For the dideuterated internal standard the molecular ion, m/z 256, was selected for peak area integration (mass fragmentation pattern not shown).

Table 3. Raw data for butyrate-PFB calibration curve.

Cal Level	mass Butyrate derivatized (ug)	IS	butyrate	ratio	avg. ratio
0	0.00	none det.	none det.		
1	0.20	17419	5953	0.34	0.35
1	0.20	15932	5841	0.37	
2	1.00	5594	5639	1.01	1.02
2	1.00	5401	5561	1.03	
3	2.00	bad inj.	Bad inj		1.33
3	2.00	9947	13194	1.33	
4	3.00	18866	23532	1.25	1.69
4	3.00	20739	44246	2.13	
5	4.00	16731	33809	2.02	2.33
5	4.00	14985	39516	2.64	

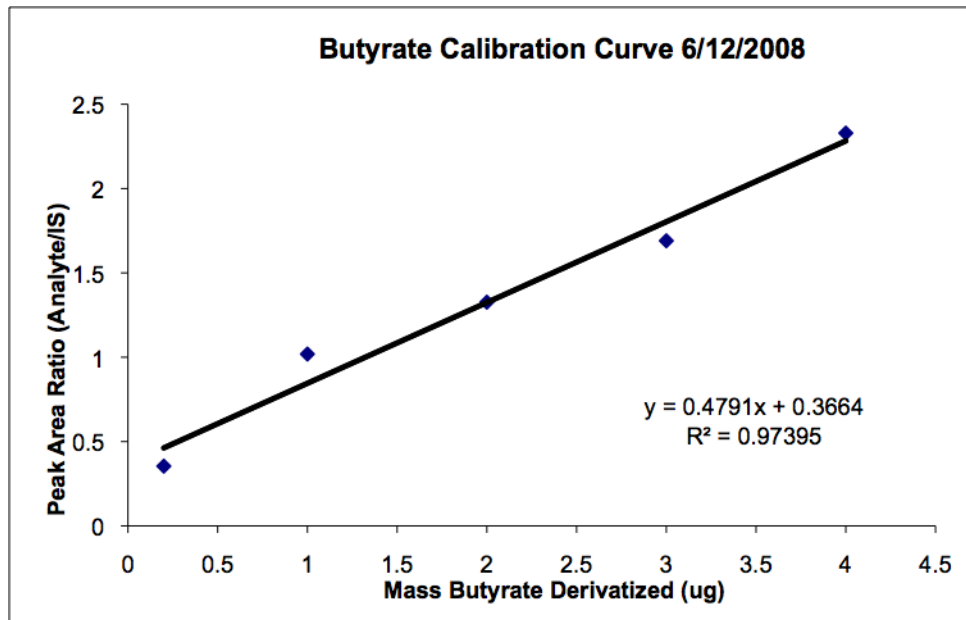


Figure 6. Calibration curve for butyrate-PFB derivative. Calibration points are based on the average peak area ratio of two standards.

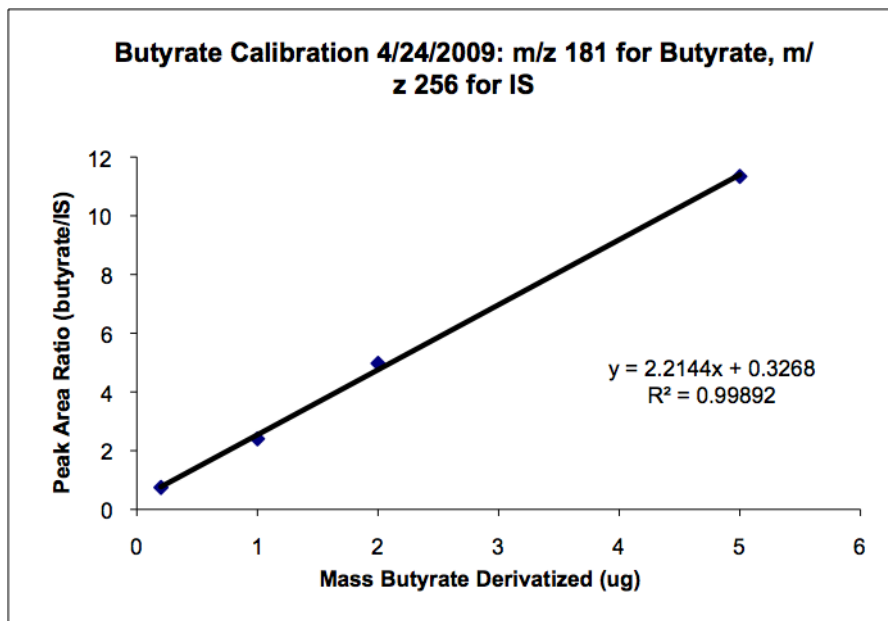


Figure 7. Repeat calibration curve for butyrate-PFB derivative from April of the following year. Calibration points are based on the average peak area ratio of two standards.

3.2 PFBB derivative reaction of butyrate in biological cecal sample and short chain fatty acid verification in samples

In order to verify that butyrate was being derivatized in biological samples, retention time verification experiments were performed with biological samples and standards. Identification was verified by preparing both standards and biological samples using the same assay on the same day. Verification could be confirmed by comparing both the total ion chromatograms for retention time verification and mass spectral data.

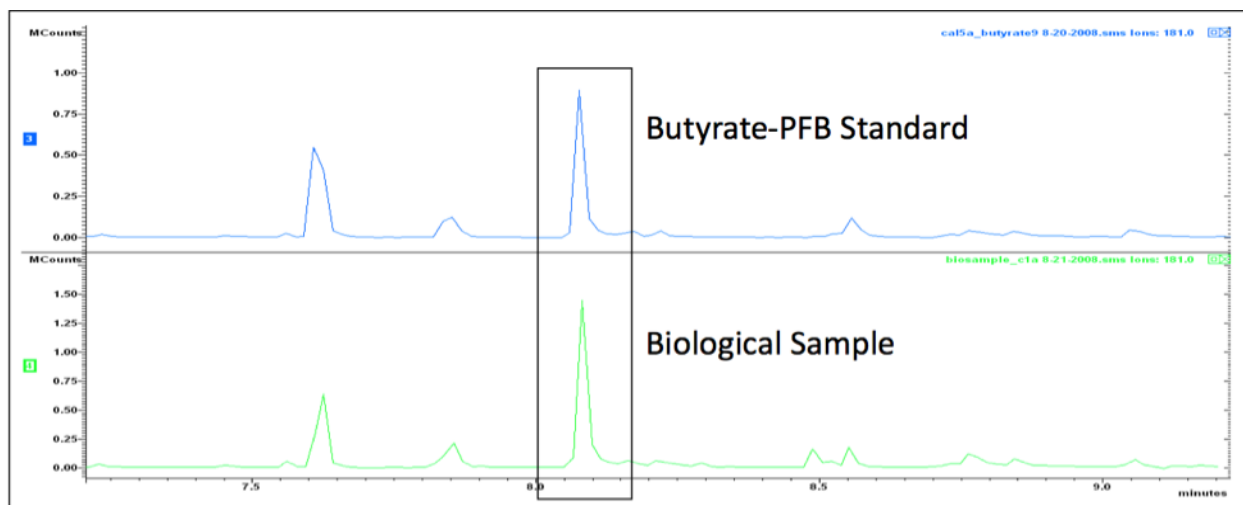


Figure 8. Overlay of GC select ion monitor of m/z 181 fragment from butyrate-PFB standard sample and butyrate-PFB peak found in biological sample.

Figure 8 indicates that the GC peak identified as butyrate-PFB in the standard samples is also present at the exact same retention time from the chromatogram of the biological samples that are reacted with the PFBB reagent. Therefore, we have retention time verification of the butyrate-PFB peak in the biological samples. Investigation of the mass fragmentation pattern of the underlying peaks would yield further confirmation of peak identity.

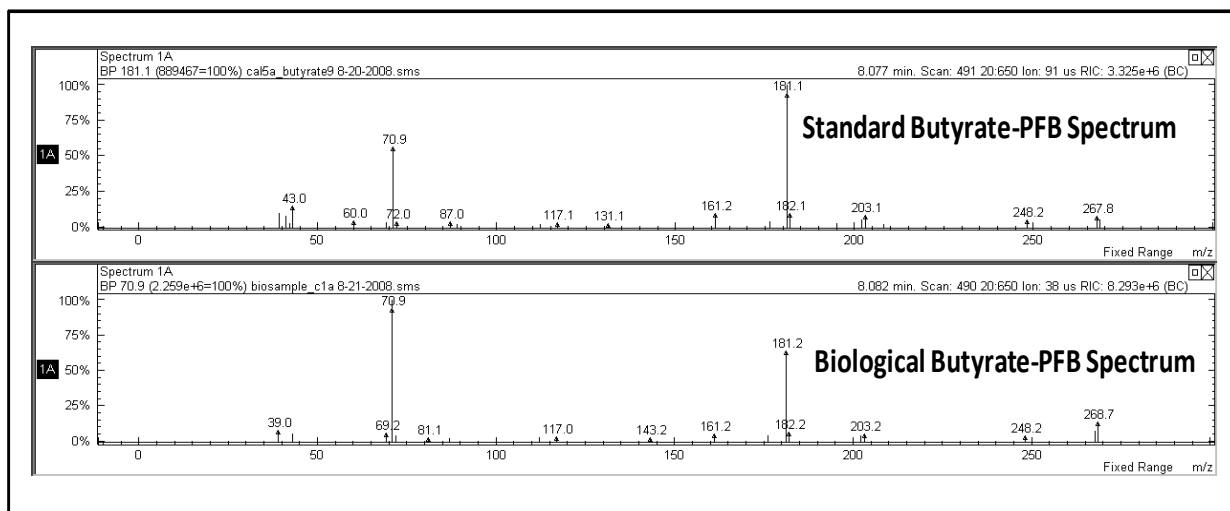


Figure 9. Mass spectra of butyrate-PFB peaks found at retention time 8.1 minute from both a calibration standard and a biological sample exposed to the same reaction assay.

Figure 9 provides mass spectral evidence that the butyrate present in the biological samples is able to react with PFBB yielding a derivative that is detected by GC/MS and provides robust peaks that are capable of being integrated. For both of the spectra we see the base peak of m/z 181. This fragment represents PFB ions that were cleaved from the molecular ion. The molecular ion is m/z 268 and is present in both samples and in similar fragment ratios.

Figure 10 displays the mass spectrum of the internal standard, which was used for the analysis of the short chain fatty acids whose only functional group is a carboxylic acid. Propionic acid-2,2-d₂ was subjected to a reaction with PFBB to form the internal standard-PFB complex.

The molecular ion for this derivative is m/z 256 rather than 254, due to the fact that two deuterium atoms are present in the internal standard compound. This is necessary due to the possible presence of propionate in the biological samples and for future quantification of the SCFA propionate.

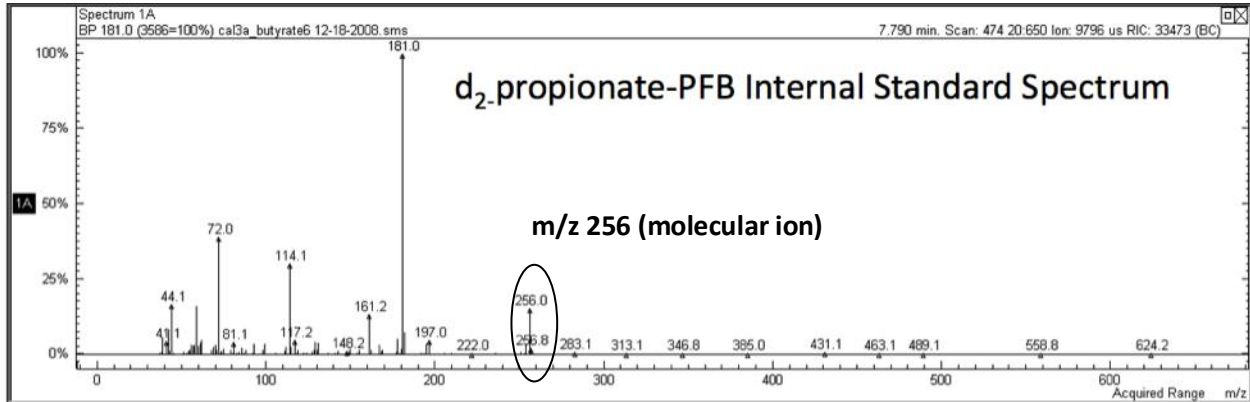


Figure 10. Mass spectrum of internal standard-PFB derivative chosen for analysis of SCFA.

3.3 Biological sample spiking with butyrate standards

To determine the extraction efficiency of the assay, a spiking experiment was performed with biological samples. For the experiment, a group of cecal samples were pooled together to give sufficient amount of material to complete the experiment. For each experimental set, 200 μ L of pooled sample was extracted following the reported procedure.

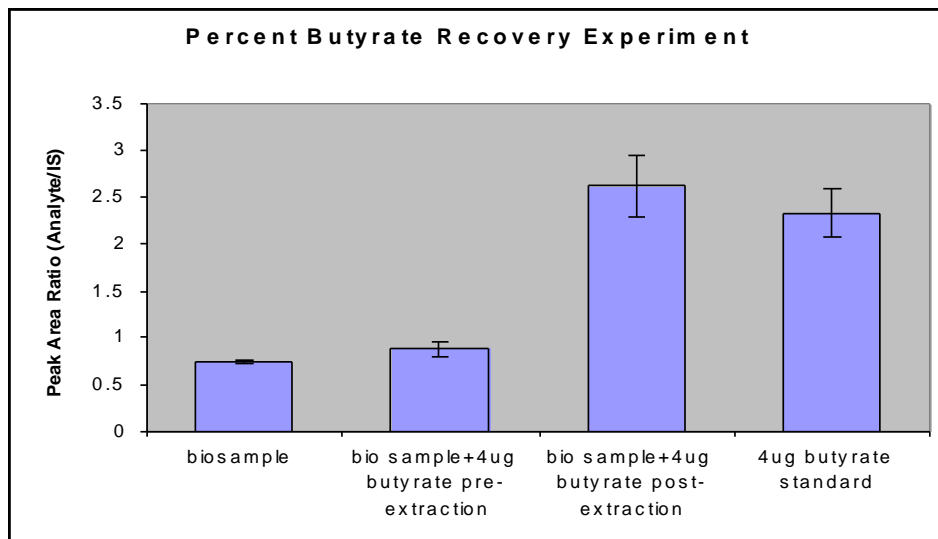


Figure 11. Experimental results from butyrate spiking experiment of pooled biological samples.

The peak area ratio data from this experiment indicated that an extraction efficiency of ten percent was achieved for butyrate extraction from biological sample. This was calculated by comparing the sample only cell to the spiked samples and the 4 μ g butyrate standard.

The sample represented in the first bar column had a peak area ratio of 0.7446, when substituted into the calibration curve for this experiment yielded 0.789 μ g butyrate derivatized. The amount of material used for extraction was 200 μ L, yielding 3.95ng of butyrate/ μ L of biological samples. Multiplying this value by the calculated extraction efficiency yields 39.5ng butyrate/ μ L cecal material. The early assay required that the original cecal material be re-suspended in a phosphate buffer solution by a dilution factor of 4.5. When this value is taken into account, the actual calculated concentration of butyrate was 177.8ng/ μ L or about 2.0mM.

This experiment represented an initial analysis with the biological cecal samples using an assay developed by past researchers. It was determined as a result of this experiment that acidification was necessary to improve extraction efficiency and that filtered DNA-free water should be used for dilution of the provided biological material.

3.4 Early (August 2008) butyrate analysis in biological samples

The result of the first quantitative measurement experiment performed with biological samples is shown in Figure 12. It should be noted that certain experimental samples displayed a wide

variation in the amounts of butyrate present upon repeated sampling, so it was deemed necessary to analyze each sample in triplicate. The samples that were run in duplicate showed a large standard deviation and standard error, as demonstrated by the error bars. However, when using 200 μ L of biological sample, as was done in this experiment, it is possible that not enough material exists to allow triplicate runs. Therefore, it was determined that future analyses be performed with 50 μ L of cecal material, as described in the Experimental Procedures.

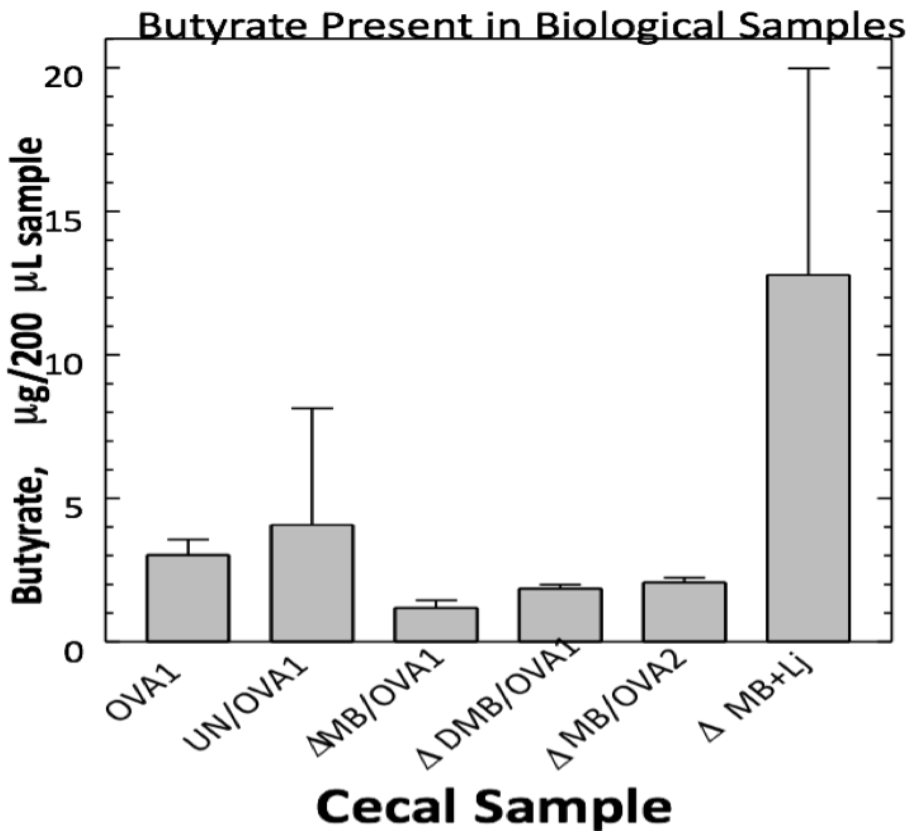


Figure 12. Butyrate results from August 2008 analysis of biological samples

3.5 Butyrate analysis of experimental samples as a function of time of residence in GI tract.

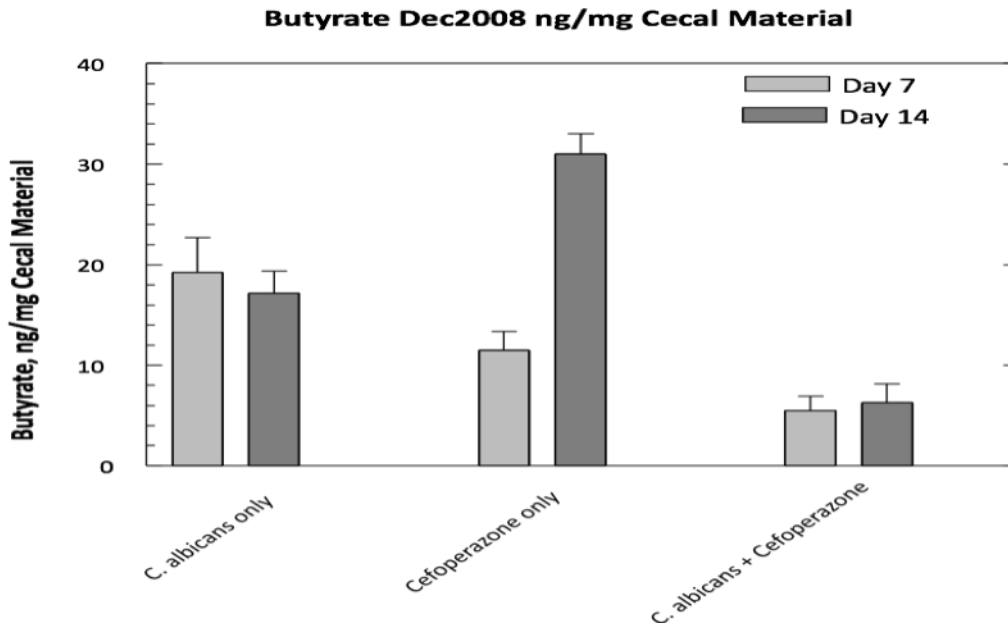


Figure 13. Butyrate results in cecal samples at days seven and fourteen of mice treated with *C. albicans*, the antimicrobial agent Cefoperazone, or both.

The mice chosen for this experiment were from Jackson Laboratories and were ordered at six weeks of age. The antimicrobial agent Cefoperazone was administered for seven days in the drinking water of the animals. Seven days after the first treatment of Cefoperazone, the antimicrobial treatment was terminated (Day 0), and selected animals were administered the yeast strain *C. albicans* by gastric gavage. The first animal harvest occurred seven days after the *C. albicans* gavage (Day 7), followed by a later harvest at day fourteen (Day 14). Each experimental value represents the combined results of three different organisms, which were extracted and analyzed in triplicate by GC/MS.

The results indicate that animals administered only the antimicrobial treatment displayed a large spike in butyrate from days seven to fourteen. This suggested to our collaborators at the

University of Michigan (Ann Arbor) that after the initial elimination of most of the butyrate-producing bacteria, there is a repopulation of these microorganisms and subsequent butyrate production in the cecal material of these organisms. It is also observed that the combination of the yeast and antimicrobial treatments yields a greater reduction of butyrate levels than either of the treatments alone. This reduced level of butyrate remains the same after day fourteen. This suggested to our collaborators that the pathogenic *C. albicans* in some way lowers the level of butyrate in the cecum.

3.6 Butyrate analysis in Abbott mice

The following results were analyzed in the spring and summer of 2009 and involved a different strain of mouse. These experiments were divided into two separate analyses designated Abbott 5 and Abbott 6 and were performed in consecutive months. The organisms chosen for these analyses were bred at the University of Michigan lab of Dr. Huffnagle and were not purchased directly from a laboratory animal supplier (unlike what was done in the prior experiment). For the experimental design, there were five different experimental groups as shown in Table 4.

Table 4. Experimental groups for the Abbott 5/6 experiments

Organism No.	Treatment
1,2,3	Untreated
4,5,6	ovalbumin (OVA)
7,8,9	Cefoperazone+ <i>C. albican</i> + OVA
10,11,12	Cef.+ <i>C. albican</i> + OVA+ <i>L. rhamnosus</i> (prebiotic food)
13,14,15	Cef.+ <i>C. albican</i> + OVA+ <i>L. rhamnosus</i> and <i>B.lactis</i>

Figure 14 provides detailed daily descriptions of how and when the various treatments were administered to the organisms in the two experiments.

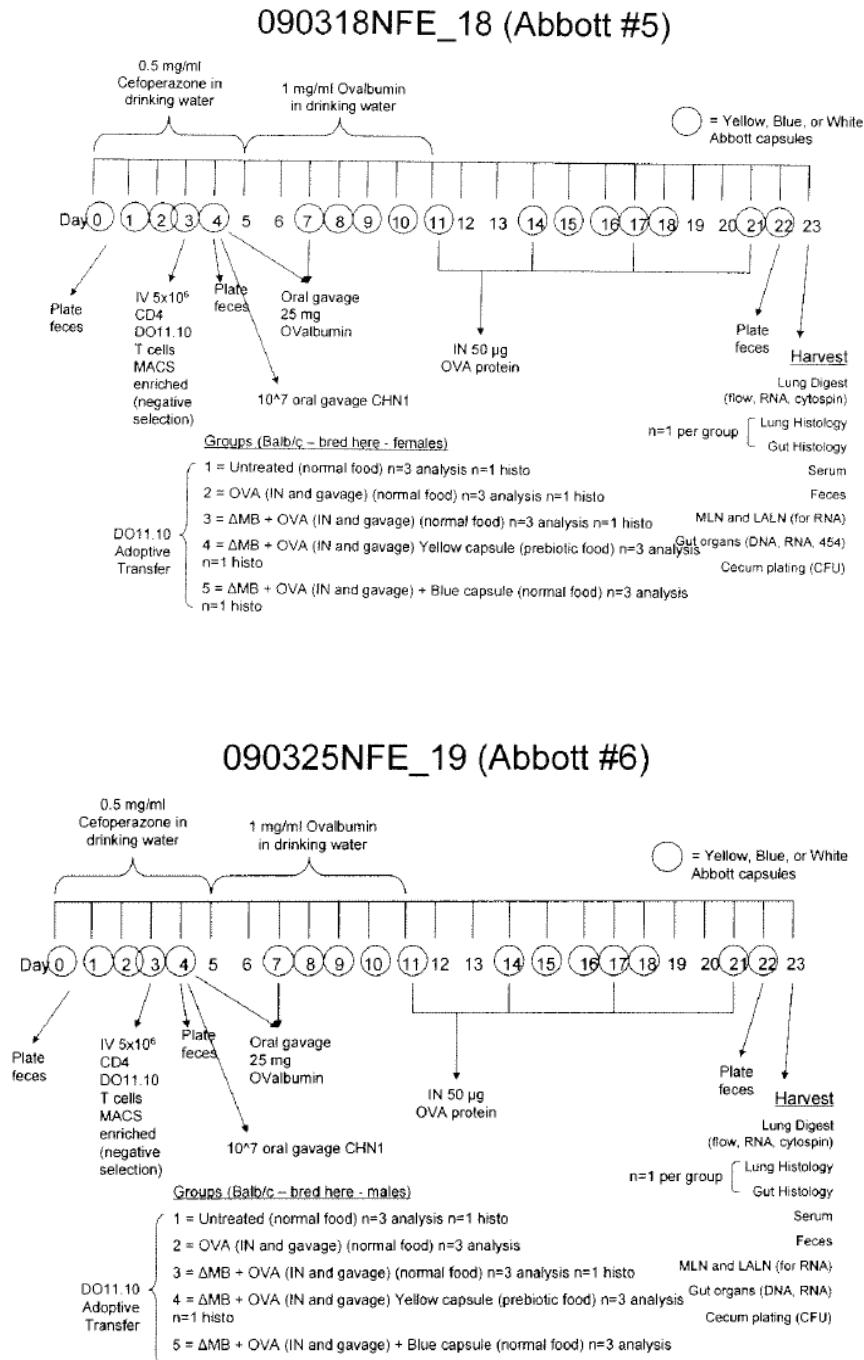


Figure 14. Procedural details of Abbott 5 and Abbott 6 experiment treatment administration.

(Provided by Nicole Robson, University of Michigan, Ann Arbor, MI)

The only substantial difference between the two series of experiments is that the Abbott 5 contained only female mice, and Abbott 6 contained only male mice. Figure 15 shows the results from the Abbott 5 analysis for butyrate present in the provided cecal material:

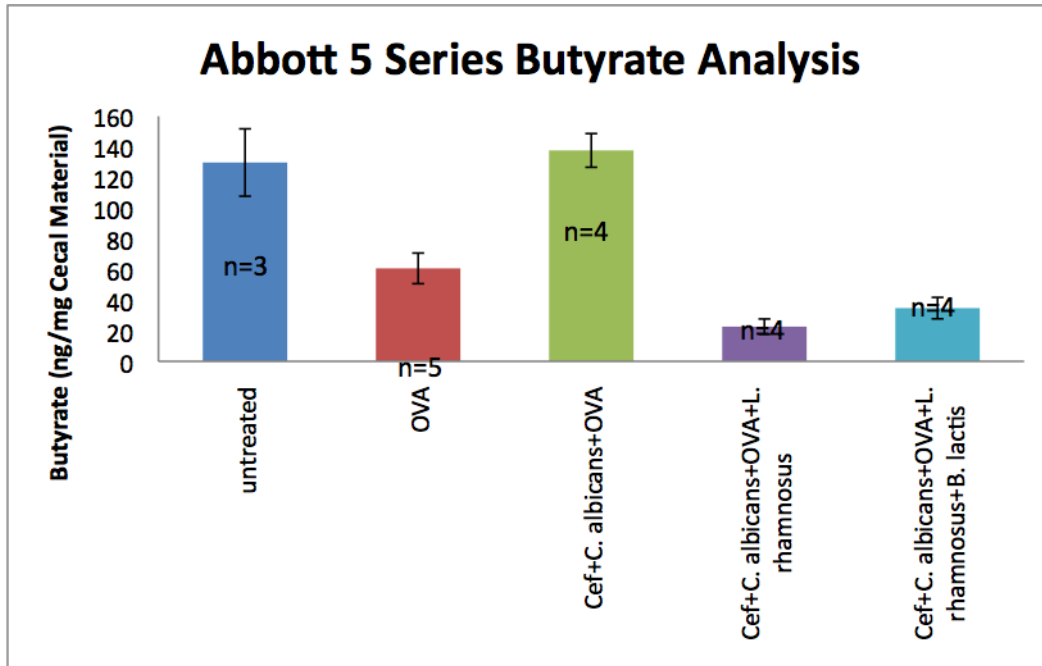


Figure 15. Butyrate results for Abbot 5 from cecal material of female mice.

Treatment with ovalbumin caused a marked decrease in the level of butyrate. Ovalbumin is given to stimulate immune function in hopes of amplifying the effect of subsequent treatment of mice with antibiotics, probiotic organisms and/or pathogenic organisms (*C. albicans*).

However, repopulation of the GI tract with organisms after a single cefoperazone treatment (to clear the GI tract of organisms) and coincident treatment with *C. albicans*, a pathogenic organism, caused an increase in the level of butyrate relative to ovalbumin controls. The fourth and fifth groups were administered an additional treatment of probiotic organisms;

Lactobacillus rhamnosus or *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, which greatly

diminished the level of butyrate produced in fluid extracted from the mouse cecum. These results are in opposition to earlier data, which indicate that probiotic organisms increase butyrate levels and that pathogenic organisms diminish the level of butyrate.

The results in the Abbott 6 experiment are shown in Figure 16 and are roughly equivalent to those of the Abbott 5 experiment except that the level of butyrate for all the male groups in the Abbott 6 experiment are less than those for the same treatment for female mice in the Abbott 6 experiment. The range of butyrate concentrations detected in all samples was decreased, as the lowest reported set was 10.42ng/mg and the highest was 18.48ng/mg cecal material. When compared to the Abbott 5 series, where the lowest reported butyrate concentration was 22.66ng/mg, these are greatly reduced. The other significant difference between the Abbott 5 and 6 experiments is that the negative effect of butyrate levels by ovalbumin was less pronounced in the Abbott 6 males as was the negative effect of probiotic organisms on butyrate levels.

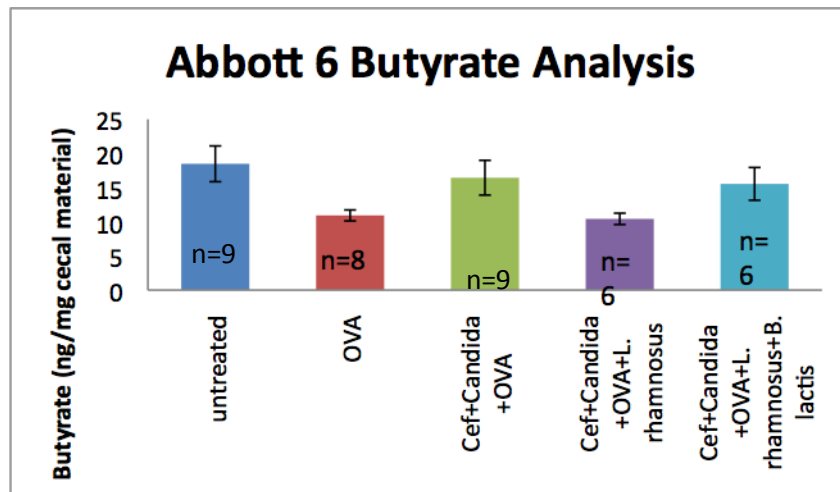


Figure 16. Butyrate results for Abbot 6 from cecal material of male mice.

3.7 Calibration curve of lactate-TMS derivative by GC/MS

Figure 17 shows a four-point lactate-TMS calibration curve and the corresponding mass spectra of the lactate and internal standard-TMS derivatives. For data analysis the molecular ion m/z 219 ion fragment was selected for the lactate-TMS analyte. For this analysis the molecular ion was a stable fragment that allowed for reliable selection for data analysis. The mass fragmentation pattern for the propanediol-TMS spectrum is shown in Figure 18, and the m/z 147 ion fragment was chosen to determine peak area for the internal standard.

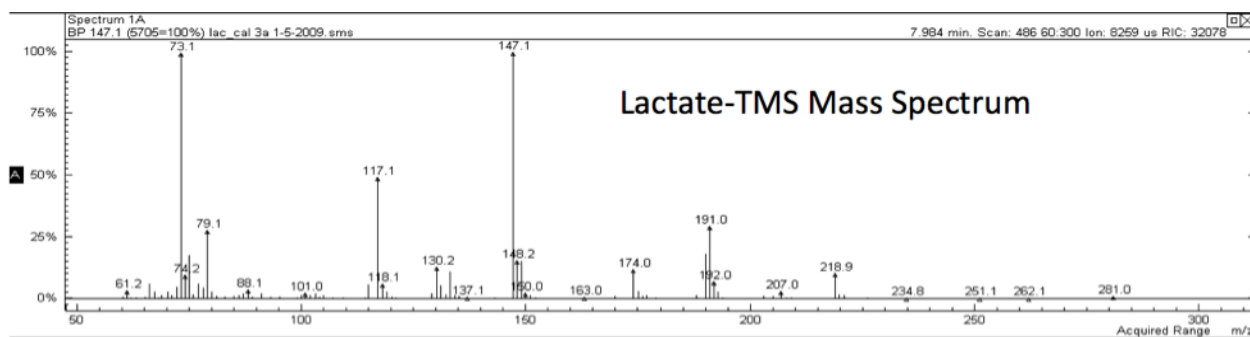


Figure 17. Mass spectrum of lactate-TMS derivative selected from calibration standard. m/z 219 fragment represents the molecular ion for the derivative.

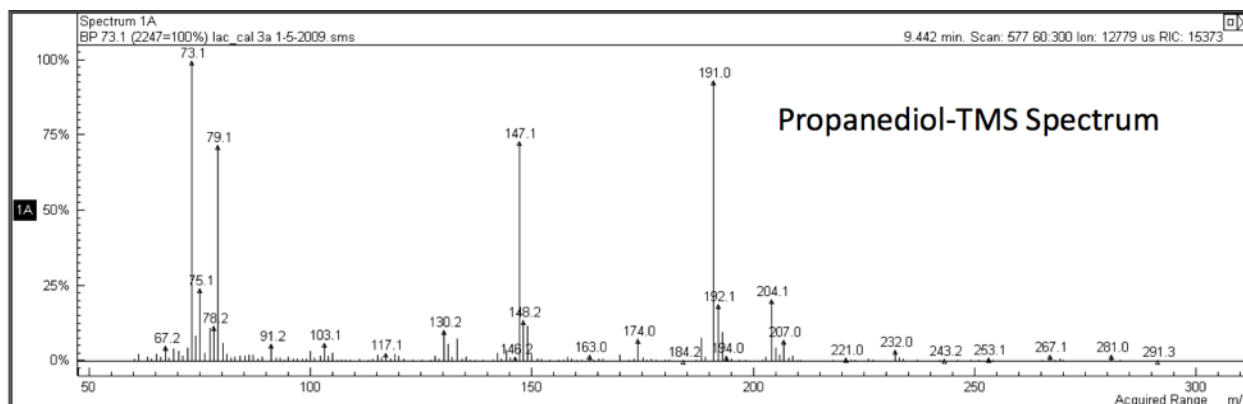


Figure 18. Mass spectrum of 1,3-propanediol-TMS derivative selected from calibration standard.

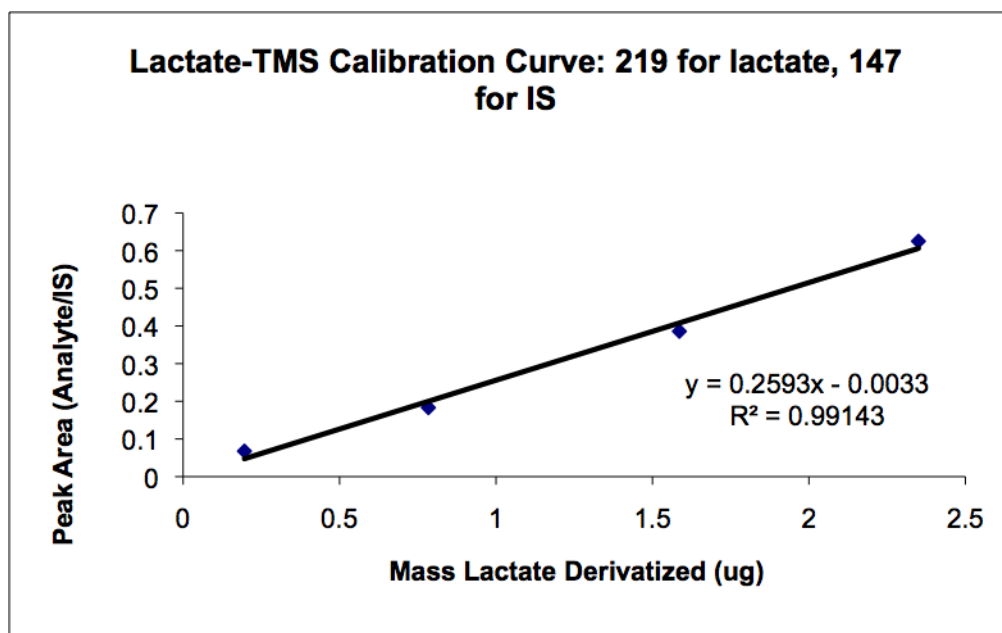


Figure 19. Lactate-TMS derivative calibration curve with m/z 219 selected for lactate-TMS, and m/z 147 selected for internal standard. Each calibration point represents the average peak area ratio of two standards.

The lactate-TMS assay was developed in a similar manner as for the butyrate-PFB method. Several standards and attempted calibration curves were prepared until consistent results could be achieved (Figure 19). The lactate-TMS method does lack the consistency observed

with the butyrate method. For example, it was observed that in a group of standards that were prepared at the same time under exact conditions, one or more standard/s did not react to form the derivative complex. Figure 20 is an example of a calibration curve that was not successful due to lack of analyte formation in one or more standards.

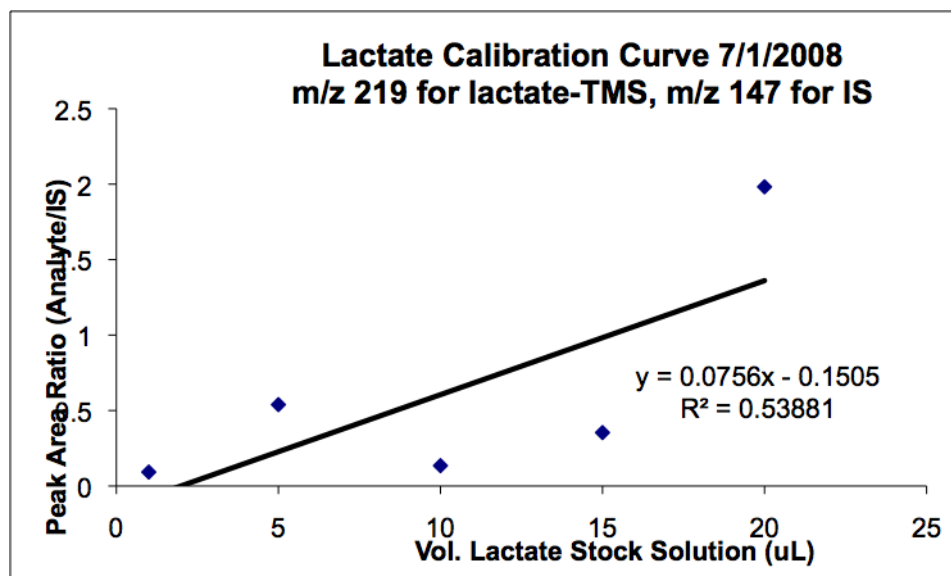


Figure 20. Unsuccessful lactate-TMS calibration curve selecting same ion fragments for lactate-TMS and internal standard analytes.

Due to the inconsistencies with the lactate-TMS formation, it was decided to place emphasis on completing biological samples by analyzing for butyrate. Also since the lactate assay would require separate extractions, there were limitations on how much cecal material was available from each organism. If an analysis were performed for lactate and butyrate, there would not be enough cecal material to rerun any errant biological samples if necessary.

3.8 Analysis of lactate present in cecal material of biological results

Figure 21 shows the lactate analysis of the same biological samples analyzed for butyrate at days seven and fourteen, after treatment with Cefoperazone and/or *C. albicans*.

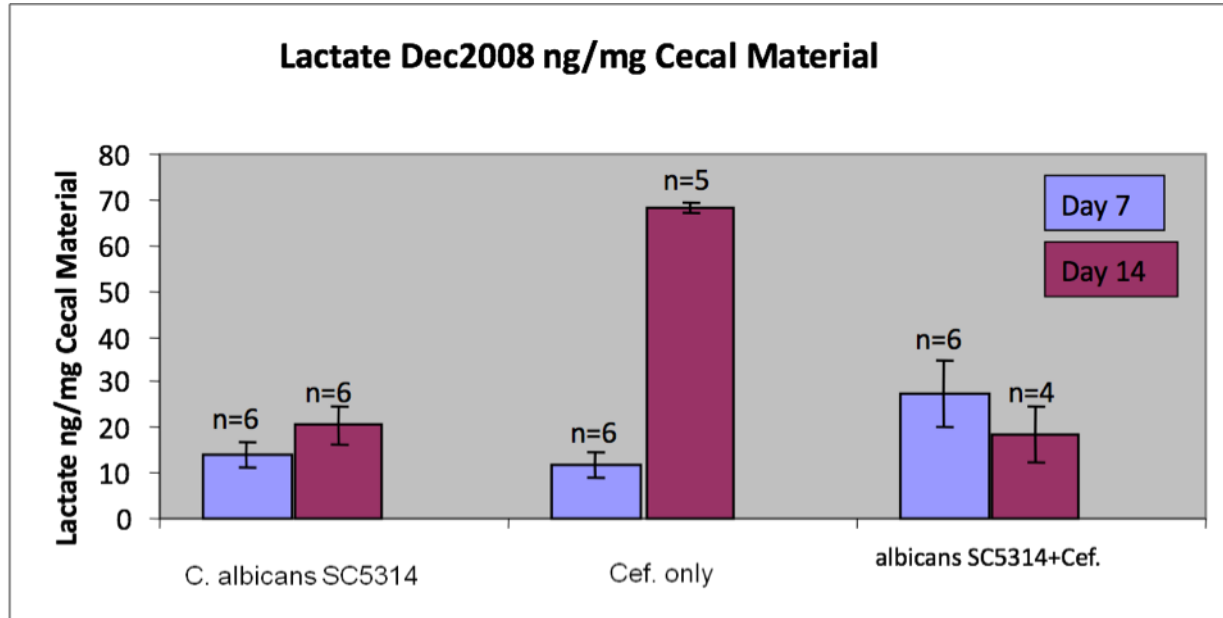


Figure 21. Lactate analysis results of cecal samples from animals treated with antimicrobial agent followed by *C. albican* gavage at days seven and fourteen.

These were the same biological samples analyzed for butyrate, except separate extractions were performed and the lactate-TMS assay was carried out. The mice chosen for this experiment were Jackson mice ordered at six weeks of age. The antimicrobial agent Cefoperazone was administered for seven days in the drinking water of the animals. Seven days after the first treatment of Cefoperazone, the antimicrobial treatment was terminated (Day 0), and selected animals were administered the yeast strain *C. albicans* by gastric gavage. The first animal harvest occurred seven days after the *C. albicans* gavage (Day 7), followed by a

later harvest at day fourteen (Day 14). Each experimental cell represents the combined results of three different organisms, which were extracted and analyzed in duplicate by GC/MS.

The relative magnitude of the lactate levels for all the groups were similar as for the butyrate levels that were described earlier. The lactate levels increase almost seven-fold from day seven to day fourteen, indicating that lactate-producing microorganisms repopulate the gastrointestinal system and continue to produce lactate in the cecum. The Day 14 results indicate that samples treated with the yeast strain showed lower levels of lactate than the animals treated only with Cefoperazone. It is also observed that the yeast strain inhibited lactate production when both administered alone or in combination with the antimicrobial agent.

3.9 Additional short chain fatty acids

Standards and calibration curves for propionate and acetate have been prepared and could be used for the quantification of these analytes in biological samples if desired. The internal standard used in the analysis of propionate is deuterated propionate, which has only two mass units more than the short chain fatty acid propionate. As expected when reacted with PFBB, both derivatives coelute as shown in Figure 22. Therefore, mass spectral data is necessary to distinguish between the two materials, these spectra are shown in Figure 23.

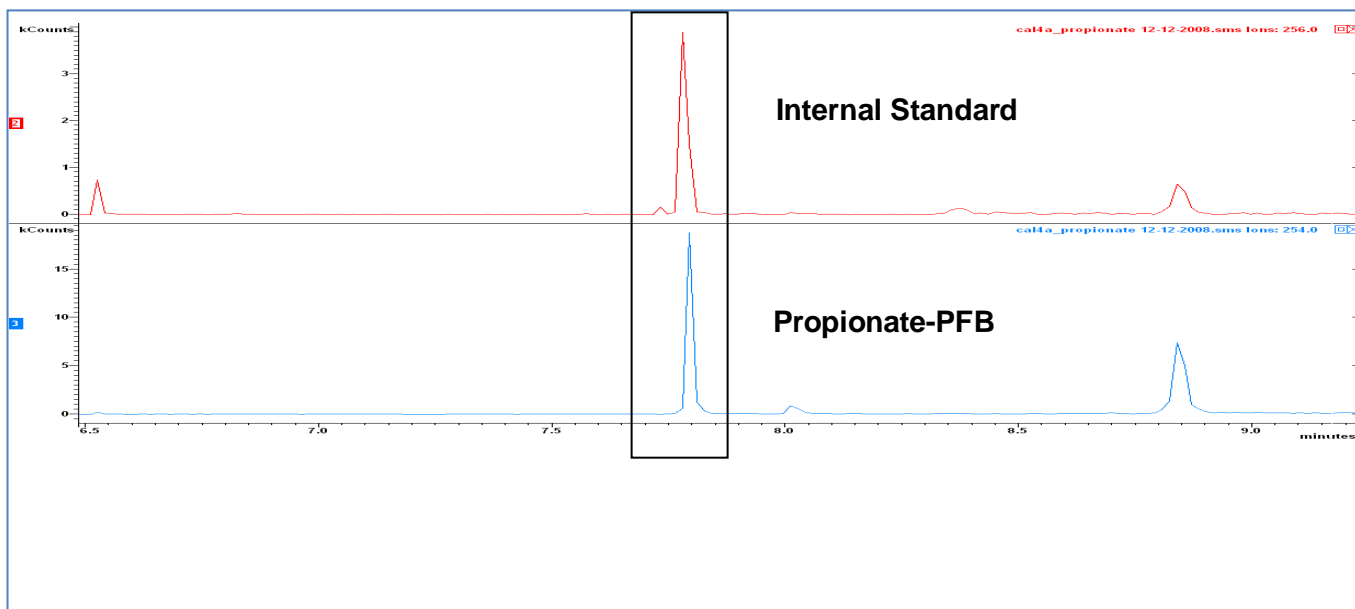


Figure 22. Gas chromatograms of internal standard and propionate-PFB derivatives.

Two examples of propionate-PFB calibration curves are given in Figures 24 and 25. The mass ion used to integrate the propionate-PFB was m/z 254, which is the molecular ion for the undeuterated propionate-PFB derivative. The mass ion used to integrate the deuterated propionate-PFB was m/z 256, which is its molecular ion. Figure 23 shows the mass spectra of propionate and deuterated propionate samples.

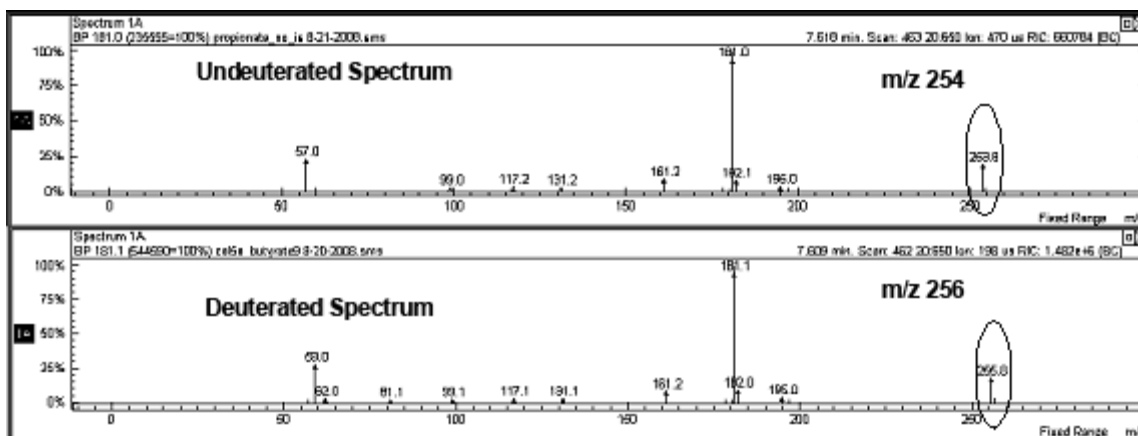


Figure 23. Mass spectral results of dideuterated propionate used as internal standard versus propionate found in biological samples.

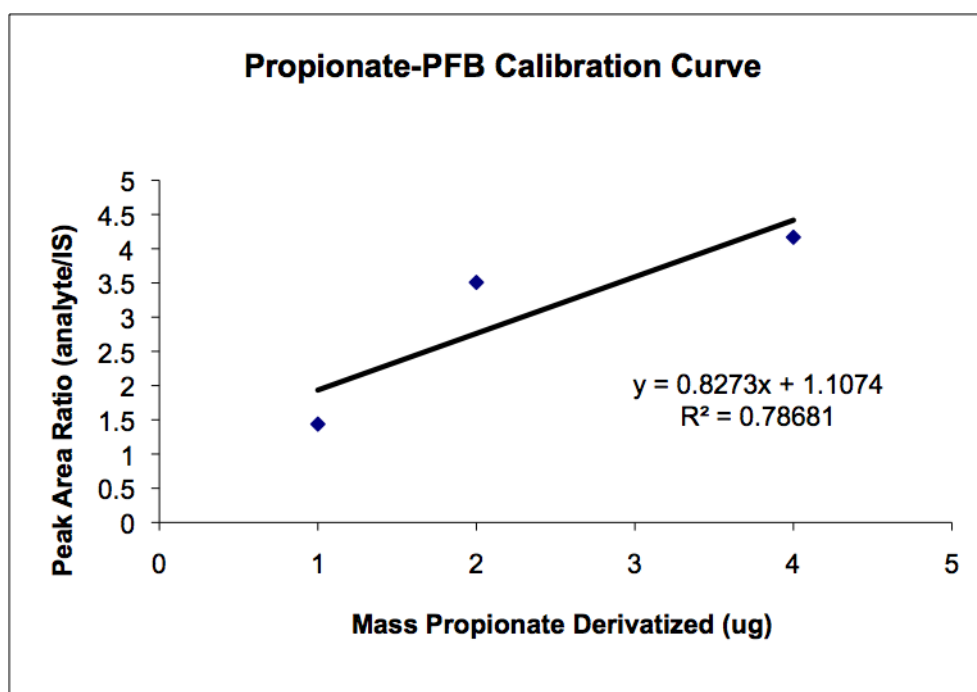


Figure 24. Early propionate calibration curve with m/z 254 fragment ion selected propionate-PFB derivative, and m/z 256 fragment ion was selected for internal standard.

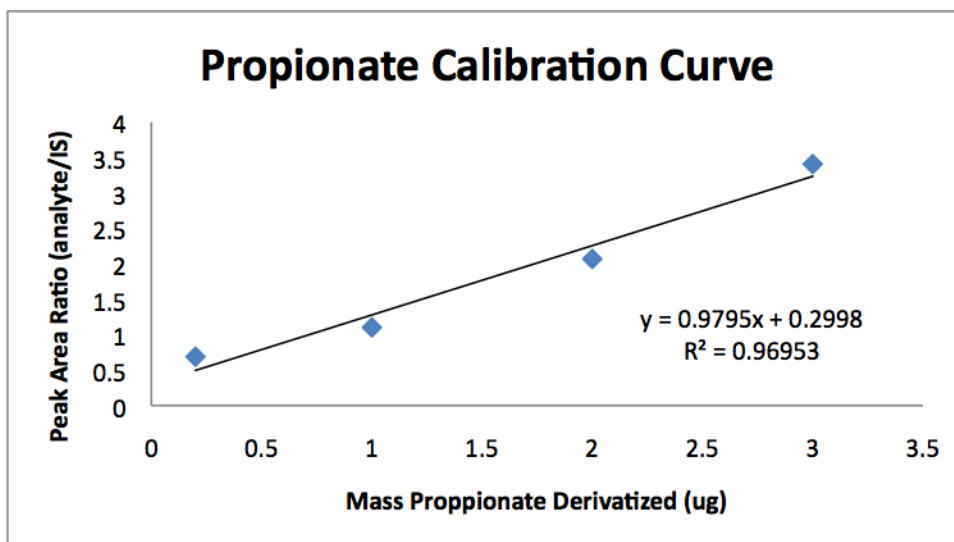


Figure 25. Example of successful propionate calibration curve with m/z 254 fragment ion selected propionate-PFB derivative, and m/z 256 fragment ion selected for internal standard.

Another short chain fatty acid of interest is acetate. Figure 26 shows the results of acetate standards that have been carried out following the PFBB derivatization assay. Ethyl acetate is used as the solvent to re-suspend the sample, and so it was important to show that no acetate (obtained by hydrolysis of ethyl acetate) was observed in the ethyl acetate blank at a retention time that was coincident with that of acetate standard. Interestingly, the biological sample contained a peak that had the same retention as that of acetate-PFB standard and so provides evidence that acetate can be quantified in cecal material. Figure 27 shows that the acetate-PFB mass fragmentation spectrum is qualitatively the same as that for the substance in the cecal sample that has the same retention time.

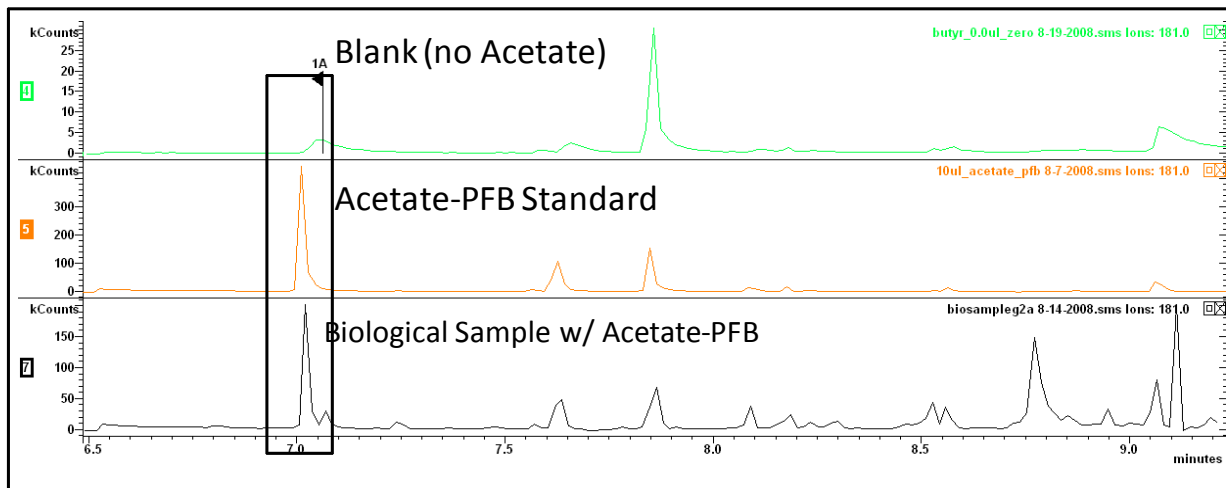


Figure 26. Chromatogram of acetate-PFB identification.

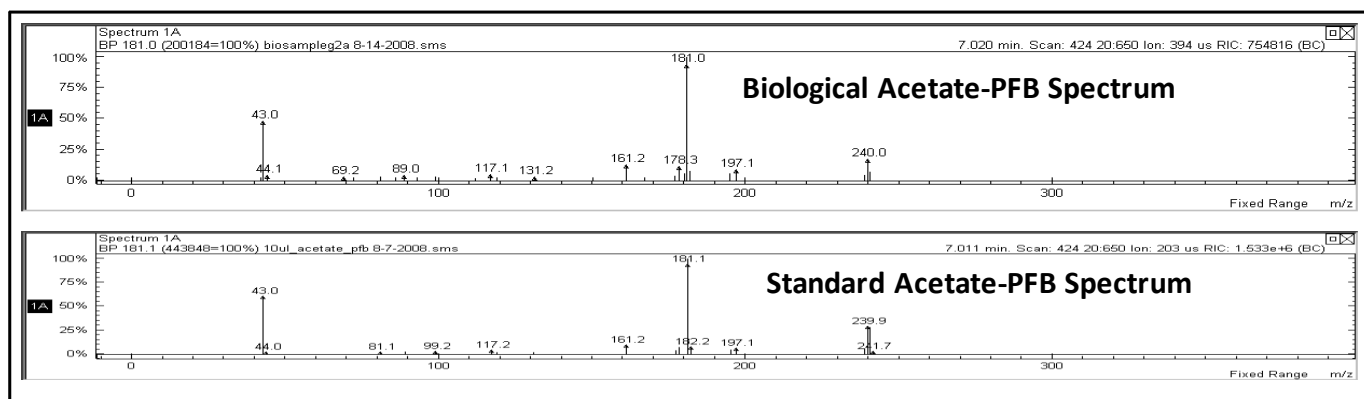


Figure 27. Mass spectra of acetate-PFB and the substance with the same retention time (7.02 min) in a cecal sample.

DISCUSSION

Pentafluorobenzyl derivatization has been a well-established method for analyzing short chain fatty acids such as propionate and butyrate by GC/MS (12). This approach has been used in the current study to evaluate standards and calibration curves of these two analytes. Literature articles commonly report TMS derivatives being prepared when analyzing these types of fatty acids involving preparation of the fatty acid in an ester linkage to a trimethylsilyl group (13). TMS derivatization requires a greater reaction time and an increase in temperature above ambient conditions. Through internal trials and replications of standard calibration curves, optimal ratios of derivatizing reagent, catalyst, and SCFA were determined and are reported in the Experimental section.

The use of deuterated propionate as an internal standard has been found to be effective in the quantification of SCFA such as butyrate. Since these two compounds have similar molecular weights and chemical properties, the dideuterated propionate has proven to be an acceptable internal standard for the quantification of various SCFA. By use of the internal standard assay presented, calibration curves of standards have been achieved with correlation coefficients near 1.0. The use of glass micro syringes was needed in order to achieve linear calibration curves; plastic micropipette instruments were not able to transfer small aliquots of materials accurately for calibration. After five-point calibration curves had been prepared repeatedly with consistent results and correlation coefficients near 1.0, it was determined that three-point calibration curves, prepared in duplicate, were sufficient for quantitative analyses of biological cecal material. The advantage of only three points is that, for certain experiments, up to thirty biological samples could be prepared for analysis, and all calibration curves are required to be

analyzed on the same day as the biological samples. Therefore, a reduction in total samples required for analysis could save time and allow for more biological samples to be prepared.

Whereas quantitative analyses of propionate and acetate were not performed with the biological cecal material in this project, they have been qualitatively identified in cecal materials provided to us. It has been verified in standards that both of these compounds readily react with the PFBB reagent to form the ester-linked derivative.

Preparation of the lactate-TMS derivative has been found to be less reliable than the SCFA-PFBB derivative. As presented, calibration curves could be achieved with success and acceptable correlation coefficients. However, not all standards appeared to react with the BSTFA reagent consistently. Initial assays involved adding stock solutions of both lactate and internal standard, followed by removing the solvent and re-suspending in BSTFA and pyridine. This procedure did not produce quantifiable results but could be used to obtain qualitative data. The assay as currently used no longer involves re-suspension of the lactate and internal standard in BSTFA, but rather requires that all reagents are together for reaction in the water bath. After reaction of the materials, no evaporation of solvent is allowed to take place, and the samples are ready for injection onto the GC. This change has improved reproducibility; however, as stated in section 3.6, formation of the derivatives in both standards and biological samples does not always occur. A possible explanation considered is the amount of moisture present in the laboratory in which the samples are being prepared. Due to inconsistent humidity levels in the lab space, moisture could enter into the reaction with BSTFA. It is known that even small amounts of moisture could disrupt this reaction due to the fact that water will

readily react with BSTFA. If moisture levels could be reduced in the reaction conditions, greater consistency may be achieved.

Another solution proposed by a former member of the laboratory is to replace the BSFTA containing 1% trimethylchlorosilane (TMCS), with material containing greater than 10% (TMCS). It has been suggested that the greater composition of TMCS may also improve reaction efficiency and conversion to the lactate-(bis)-TMS ester derivative.

Initial extractions of biological cecal material were performed without acidification of the cecal material. This resulted in poor extraction efficiencies. Literature articles support the concept of extracting biological materials prior to extraction of fatty acid species (14). Protonation of the carboxylates in the extracted material with 10 μ L of 0.1M HCl leads to increased amounts of butyrate that are now able to enter the organic layer for subsequent derivative conversion and analysis.

The quantification of butyrate was made the priority of most analyses. The assay performed more consistently and with greater reproducibility when compared to the lactate-TMS method. The cecum of a mouse is a small compartment of the gastrointestinal system; therefore, a limited amount of material was available to perform multiple analyses with enough repetitions. Hence, focus was placed on the butyrate-PFBB assay, with a minimum of three replications per organism. As seen in earlier results, a sample processed in duplicate did not provide the sampling number to achieve the tight standard deviation and standard error needed to determine a difference in butyrate levels of various samples. The butyrate-PFBB assay did provide results consistent with levels of butyrate reported in previous literature. The lower

limit of detection was determined to be 0.5µg of butyrate derivatized, which yields a concentration of 4.2µmol butyrate/kg cecal matter, based on the average cecal mass. This limit of detection is consistent with that previously reported (15) and below the expected levels of butyrate in the cecal material of both germ-free and wild-type mice.

Butyrate levels had been measured in organisms exposed to various experimental treatments. It was found in both the December 2008 and Abbott 5 experiments that butyrate-producing organisms are able to repopulate and continue to produce butyrate after cefoperazone treatment. As seen in the seven and fourteen day harvests from December 2008, the levels of butyrate increased three times in organisms that were harvested after Day 14 compared to organisms harvested only seven days after the end of the antimicrobial treatment. This experiment also indicated that the combination of Cefoperazone, and *C. albicans* inhibited the butyrate production more than either treatment alone, and that the butyrate levels remained low even after fourteen days. This may indicate that when the yeast is exposed to organisms that already have a decreased level of microorganisms in the GI tract, the yeast strain is able to populate the system and prevent repopulation of butyrate producing anaerobic bacteria. With the known benefits of butyrate for healthy functional cellular activity, these organisms may have digestive problems and be at greater risk to immune and other diseases.

In the Abbott 5 series it was reported that the butyrate concentrations of organisms treated with cefoperazone, *C. albicans*, and ovalbumin returned to the same approximate levels of those seen in untreated animals. The comparison of this experiment to the one performed in December 2008 may not be valid due to the fact that the Abbott 5 animals were harvested

twenty-four days after the beginning of all treatments. This extra time may be enough to allow for repopulation of butyrate producing microorganisms. These organisms were also exposed to ovalbumin, which was added to induce an allergic response in the animals tested. As previously noted, the mice chosen for the Abbott experiments were bred in house at the University of Michigan, and the Jackson mice used for the December 2008 experiment were purchased at six weeks of age. This may also make comparisons between the experiments difficult at best.

It is unclear why the Abbott 6 series did not produce comparable butyrate concentrations in any of the sample cells. After data analysis of this group was complete, multiple extractions were repeated to verify the initial results obtained. The second analysis confirmed the results and continued to yield low butyrate concentrations in all samples analyzed. As stated, the only difference between Abbott 5 and Abbott 6 is in the sex of the animals harvested. This may or may not be the reason for the conflicting results seen between these two analyses. However, it is interesting that the earlier indicated that probiotics induce a higher level of butyrate and that a pathogenic organism drives down the level of butyrate; however, the later Abbott studies demonstrate the complete opposite effect of probiotics (a decrease) and pathogenic bacteria (an increase) on butyrate levels. Obviously, the housing of the animals, the gender differences in the animals, and prior ovalbumin treatment may all be mitigating influences on the system that must be characterized. However, the current study defines the conditions that are necessary for a consistent and reproducible assay of butyrate in cecal samples.

Future directions of this project could lead to the incorporation of multiple SCFA analysis with one assay quantifying multiple chemical compounds. As reported in the literature, lactate is a

SCFA that has known health benefits when present in the GI system. Therefore, the assay could be further developed to remove inconsistencies in the reaction and improve reaction conversions. This may be accomplished by replacing BSFTA with 1% TMCS with 10% TMCS.

REFERENCES

1. Martin F.J., Wang Y., Sprenger N., Yap I.K., Lundstedt T., Lek P., Rezzi S., Ramadan, Z., Van Blander P., Fay L.B., Kochnar, S., Lindon J.C., Holmes E., Nicholson J.K. *Molecular Systems Biology*. 4(157), (2008).
2. Wollowski I., Rechkemmer G., Pool-Zobel B.L. *The American Journal of Clinical Nutrition*. **73** (2001). P. 451S-55S.
3. Pomare E.W., Branch W.J., Cummings J.H. *Journal of Clinical Investigation*. **75**(5), (1985). P. 1448-54. Noverr M.C., Huffnagle G.B. *Clinical and Experimental Allergy*. **35** (2005), p. 1511-1520
4. Cummings J.H. Macfarlane G.T. *Nutrition*. **13** (1997). p. 476-478
5. Tappenden, K.A., Deutsch, A.S. *Journal of the American College of Nutrition*. **26**(6), (2007) p. 679S-683S.
6. Topping, D.L., Clifton, P.M. *Physiological Reviews*. **81** (2001), p. 1031-64
7. Cummings, J.; Quantitative short chain fatty acid production in humans.
In: Cummings, J.; Binder, H.J.; Soergel, K, editors. Short-chain fatty acids. Boston: Kluwer Academic Publisher; **1994**
8. Ratajczak C., Duez C., Grangette, C., Pochard, P., Tonnel, A., Peste, J. *Journal of Biomedicine and Biotechnology*. **2007** (2007).
9. Blaut M., Clavel T. *The Journal of Nutrition*. **137** (2007). P. 751S-755S
10. Archer, S.Y.; Meng, S.; Shei, A.; Hodin, R.A.; *Proceedings of the National Academy of Sciences of the United States of America*. **95**(12) (1998), p. 6791-6796
11. Hague, A.; Butt, A.J.; Paraskeva, C.; *Proceedings of the Nutritional Society*. **55** (1996), p. 937-943
12. Roediger, W.E.; *Lancet*. **2** (8197), 1980, p. 712-715
13. Moreau, N.M.; Goupry, S.M.; Antignac, J.P.; Monteau, F.J.; Le Bizec, B.J.; Champ, M.M.; Martin, L.J.; Dumon, H.J.; *Journal of Chromatography B*. **784** (2003), p. 395-403.
14. Hanada, Y.; Imaizumi, I.; Kido, K.; Tanizaki, T.; Koga, M.; Shiraishi, H.; Soma, M.; *Analytical Science*. **18** (2002), p. 655

15. Adams, M.A.; Chen, Z.; Landman, P.; Colmer, T.D.; *Analytical Biochemistry*. **266** (1999), p. 77-84
16. Bettinardi, N; Colombo, C; Corbetta, C. *Clinical Chemistry*. **3** (1999), p. 406-407
17. Hoverstad, T; Midtvedt, T; *Journal of Nutrition*. **116** (1986), p. 1772-1776