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Characterization of short chain fatty acids in microbial cultures by DART-MS and GC-MS

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CHARACTERIZATION OF SHORT CHAIN FATTY ACIDS IN MICROBIAL CULTURES BY DART-MS AND GC-MS

by

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Abstract

Colonic bacterial degradation of starch and other carbon sources produces short-chain fatty acids (SCFA) such as butyrate and lactate, which are important in human health and disease. Analysis of the results of mono- and co-cultures in *in vitro* systems has led to the conclusion that diverse microbial organisms may each contribute in part to the complete metabolism to produce SCFA. GC- MS and DART- MS were investigated as analytical tools in the present study to evaluate the production of butyrate and lactate in a test tube and bioreactor mono- and co-cultures. The overall research goal is to investigate biological model systems and analytical methods to allow determination of lactate and butyrate levels under conditions that effectively emulate those that prevail in the gastrointestinal tract. These approaches will provide some understanding of the extent to which organisms work together to generate important biomolecules, in particular, the short-chain fatty acids.

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INTRODUCTION

GIT of humans and the process of digestion

As shown in Figure 1 (1), the human gastrointestinal tract (GIT) is a long, twisted tube that extends from the mouth to the anus and is composed of a number of organs that facilitate digestion.

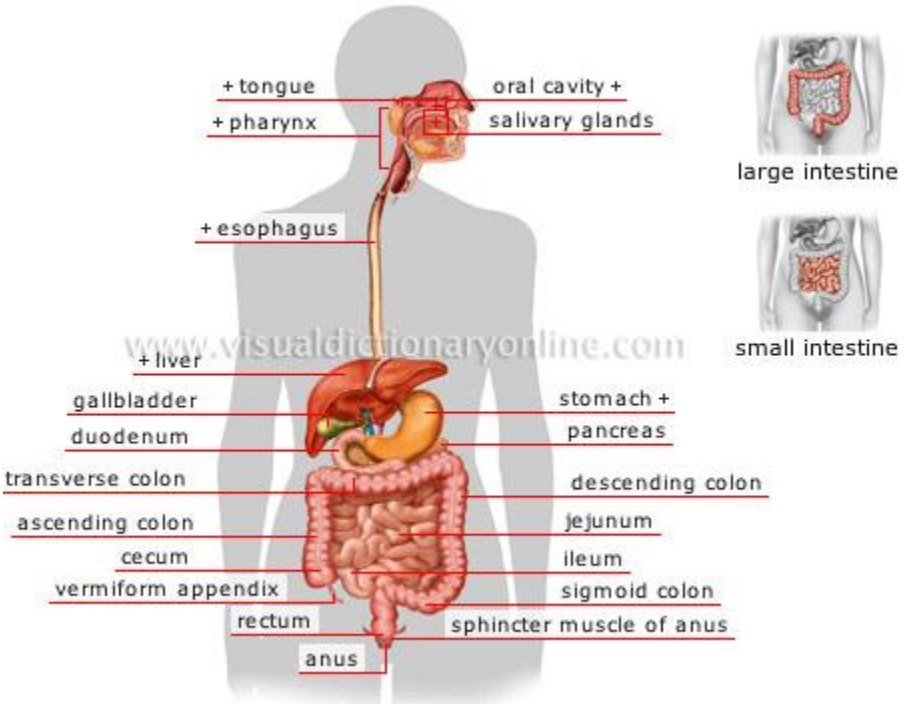

Figure 1. Characterization of the parts of the human digestive tract (1).

The process of digestion starts in the mouth where the food is digested partly due to the enzymes secreted by salivary glands that lyze the starch to simpler molecules (2). Chewing also helps in the partial digestion of food. Food reaches the stomach by the peristaltic movements of muscles in the esophagus. The food in the stomach then gets exposed to gastric acid (secreted by the stomach) and is further partially digested to produce chyme. The chyme then reaches the duodenum, jejunum, and the ileum, which compose the small intestine. The chyme is further

broken down in the small intestine by the bile, pancreatic enzymes, and other enzymes that aid in the digestion (2). Food then enters into the large intestine where it is dehydrated through the reabsorption of water. The microbes such as *Bacteriodes, E. coli, L. acidophilus,* and *Klebsiella* that reside in the large intestine also aid in the digestion of food (2). The rest of the solid waste enters into the rectum and is later expelled through the anus.

Gut microflora and its significance

The GIT is generally divided into three segments: the foregut, or stomach; the midgut, or small intestine; and the hindgut, which comprises the caecum and large intestine and contains a wealth of diverse aerobic and anaerobic microbes (3). The chief organisms among the anaerobes are *Bacteroides, Bifidobacteria, Eubacteria, Streptococci, and Lactobacilli*. Other microbes such as *Enterobacteria* are lesser in number (3), and they (*Enterobacteria*) play a major role in carbohydrate metabolism, digestion, immune system, and absorption of nutrients from undigested food (4). They also have effects on inflammation signaling, epithelial cell function, growth, and survival (4). The distribution of microflora in the human GIT is shown in Figure 2 (5).

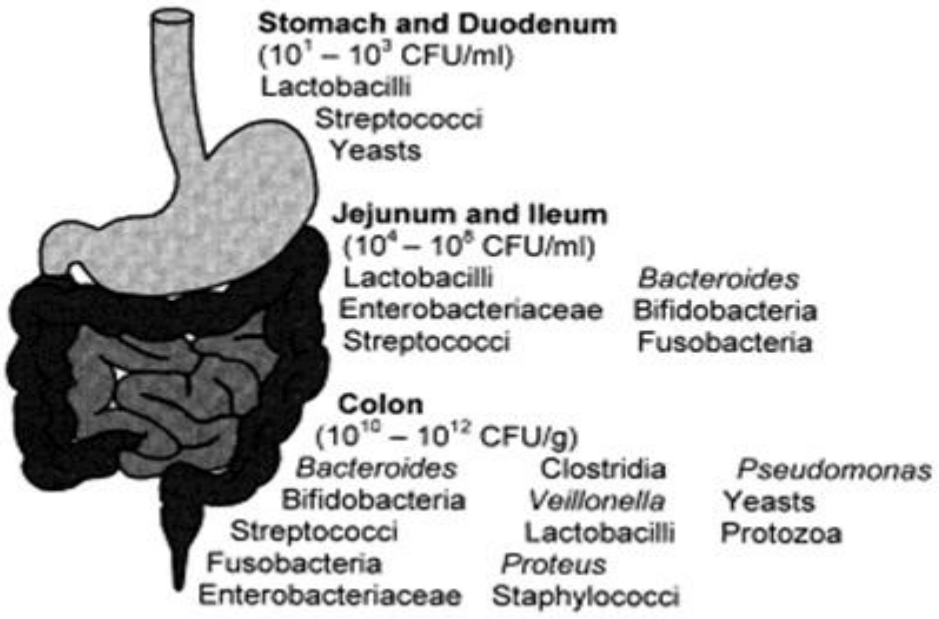

Figure 2. Microbial colonization of the human gastro-intestinal tract. CFU is the colony-forming unit, which determines the number of viable bacterial cells. From Reference 5.

These microbes exhibit a symbiotic relationship, which means that partners require one another for mutually beneficial purposes (6). An upset in the symbiotic balance leads to allergic or atopic disorders. In some cases, increased rates of autoimmune disease or conditions of immune dysregulation, including asthma, multiple sclerosis, and inflammatory bowel disease, (IBD) are observed. A dysbiotic microbiota leads to human ulcerative colitis (6).

SCFAs and their significance

Starch and other polysaccharides that are not acted by enzymes in the oral cavity become degraded in the colon by bacteroides and produce short chain fatty acids (SCFAs) (7). The most important short chain fatty acids are acetate, propionate, butyrate, and lactate, which is not a SCFA but is often grouped with them. They promote sodium ion transport and fluid absorption

in the colon and therefore prevent diarrhea (7). Acetate augments blood flow in the colon and thereby increases ileal movement (7). Previous studies indicated that butyrate serves as an energy substrate for the colonocyte and plays an important role in arresting colitis (7). Also, butyrate serves as energy fuel for colonic epithelial cells (8). Lactate undergoes fermentation in the presence of bacteria, lowers the osmotic load, and produces other SCFAs such as butyrate, which are later reabsorbed (7). The effects of SCFAs on colonic morphology and function are shown in Figure 3 (7).

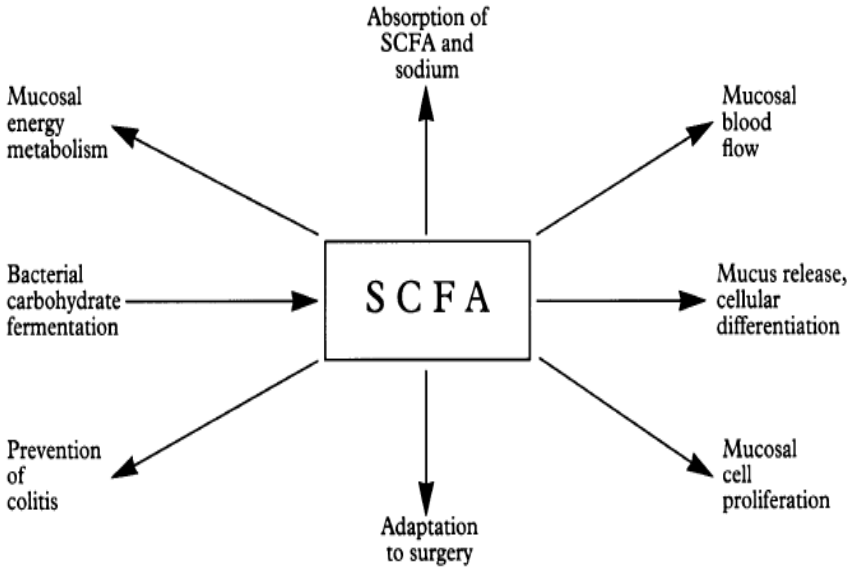

Figure 3. Production of SCFAs by bacterial carbohydrate fermentation and their various physiological roles (7).

SCFAs producers and pathophysiological significance of SCFAs producers

Previous studies have shown that obese mice and humans contain numerous members of the group Firmicutes such as Clostridium species and Bifidobacterium species (4) that produce a great amount of SCFAs. These SCFAs producers can be used as probiotics, which are defined as live microorganisms that when consumed in the diet in adequate amounts confer a health benefit

on the host (4). The organisms that comprise the symbiotic relationship can be used as prebiotics as they enhance T-cell responses, which are protective (4), and prevent innate immune signaling or lowering of inflammatory responses (4). These prebiotics are beneficial in treating intestinal functional and inflammatory disorders, systemic immune or allergic dysfunction, and metabolic disorders. Also, it is known that they harmonize intestinal nociception, psychological stress reactions, and endurance (4). Clinical studies revealed that they are useful in the treatment of acute viral gastroenteritis, post antibiotic-associated diarrhea, certain pediatric allergic disorders, enterocolitis, which leads to necrosis, and inflammatory bowel diseases such as Crohn's and postsurgical pouchitis (4). Prebiotic substrates when co-administered with live bacteria are known as "synbiotics."

Metabolism of SCFAs

Most polysaccharides and proteins undergo a fermentation process under anaerobic conditions in bacteroides and produce SCFAs and other organic acids (9). Hexose derived from polysaccharides undergoes metabolism in the glycolytic pathway to yield pyruvate, which is later transformed to several end products including SCFAs such as acetate, propionate, and butyrate, and other products like CO_2, H_2, CH_4, and H_2O. Oxidative decarboxylation of pyruvate yields acetate, and acetate produces acetoacetate, which is further reduced to yield butyrate. Lactate is produced from pyruvate by anaerobic bacteria. Its production is more favored when fermentable carbohydrates are available. The stoichiometry of anaerobic hexose metabolism to SCFAs and other products in the gut was determined by Miller and Wolin (9):

$$34.5\ C_6H_{12}O_6 \longrightarrow 64\ \text{SCFA} + 23.75\ CH_4 + 34.23\ CO_2 + 10.5\ H_2O$$

Figure 4 (10) provides a summary of the multiple products that are known to result from bacterial fermentation of digested polysaccharides and protein.

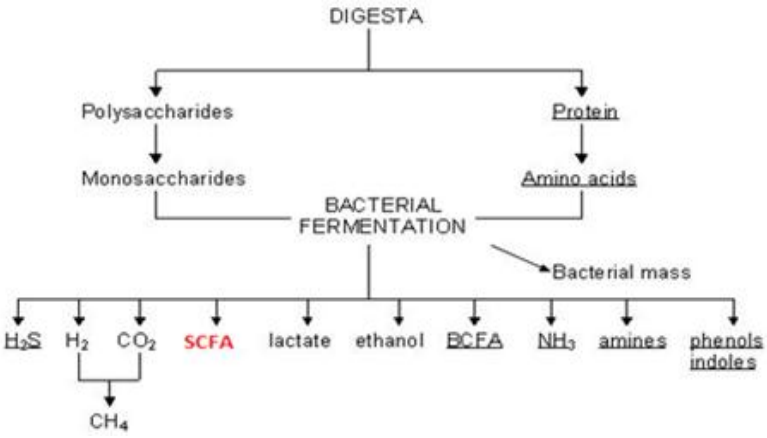

Figure 4. The products of polysaccharides and protein metabolism. Bacterial fermentation is a common metabolic route from diverse carbon sources to give a number of small molecules. From Reference 10.

Hexose also undergoes aerobic metabolism in bacteria by the pentose phosphate pathway after conversion to 6- phosphogluconate to produce intermediates useful for nucleic acids, co-factors, and amino acids.

Published procedures for characterization of SCFAs

Pouteau et al. (11) used GC-MS with positive chemical ionization to analyze SCFAs in plasma. In this technique, various proportions of labeled to unlabeled ^{13}C samples were prepared for isotopic enrichment for analysis by GC-MS. Solvent-extracted products were treated with 1-(tertiary-butyl-dimethyl-silyl) imidazole to prepare silylated derivatives of SCFAs for analysis by GC-MS. Good reproducibility was obtained, and the LODs were in the picomole range for SCFAs in plasma.

D. Elhottova et al. (12), in the detection of poly-β-hydroxy butyrate in environmental samples by GC-MS/MS, derivatized the prepared samples with N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) as shown in Figure 5 (12).

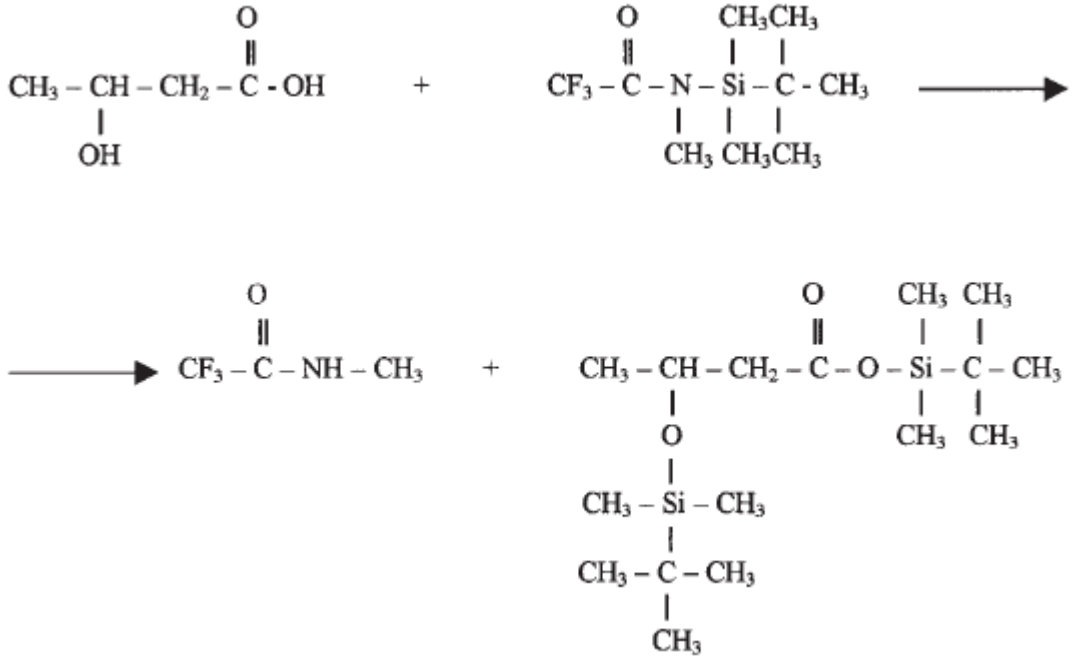

Figure 5. Derivatized products of poly-β-hydroxy butyrate when it is derivatized with MTBSTFA (12).

^{13}C-labeled lactate in blood was determined by Kou-Yi Tserng et al. (13) using GC- MS. They used n-propyl-amide heptafluorobutyrate as the derivatizing agent in their study. Based on silylated methods and with minor alterations, we studied in vitro cultures and bioreactor cultures using GC- MS. Direct Analysis in Real Time Mass Spectrometry (DART-MS) has been used to analyze γ- hydroxy butyrate (GHB) in urine samples (14). In various alcoholic and non-alcoholic drinks, GHB was analyzed using a DART- MS screening technique in negative ion mode. The LOD was determined to 0.05mg/ml. DART- MS showed 100% accuracy in results (13). Zhao et al. developed a method to quantify small molecules in plasma (15). The limit of detection for the compounds tested using DART- MS was from 0.5 to 2000 ng/mL. Hence, we

anticipated obtaining good reproducibility and sensitivity of our results using both GC-MS and DART-MS methods.

Instrumental analysis of product

Chromatography is a separation technique, where mixtures or compounds are separated by the way the molecules in the mixture interact with a stationary and a mobile phase (16). In gas chromatography, the stationary phase is a coating on the inside surface of the capillary column. The polarity of the coating determines how the separation occurs. On a non-polar stationary phase, more non-polar (or less polar) molecules are retained longer than are more polar molecules that are less like the stationary phase and thus spend more time in the mobile phase (17). In gas chromatography, the helium or nitrogen carrier gas (17) is used as the mobile phase. Generally, GC is used to separate volatile mixtures. For compounds like amino acids, steroids, fatty acids, and short chain fatty acids that are polar, especially those that can hydrogen bond with each other, derivatization is necessary to form volatile compounds. The selection of the column and thereby the stationary phase depends upon the nature of the analyte (17).

As the molecules are eluted from the GC column, they must be detected in some way. Mass spectrometry has the advantage as a detector of providing information regarding the identity of molecules. Electron impact ionization (18) is the most common ionization method in GC- MS. Molecules are bombarded with a high energy beam of electrons, resulting in the loss of an electron from the parent molecule. Because of the high energies involved, the molecules fragment extensively. The resulting pattern of fragmentation contains information about the nature and identity of the parent molecule. This pattern, called a mass spectrum, is displayed as a plot of abundance on the y-axis and mass-to-charge ratio on the x-axis.

Other ionization methods also exist for other types of mass spectrometry. These include but are not limited to electrospray and MALDI for intact proteins as well as many others (19). In clinical laboratory applications, mass spectrometry has great promise as an analytical method. Strathman and Hoofnagle provide an extensive review of such applications (20). Ambient ionization methods that require little or no sample preparation have particular appeal in the analysis of clinical samples. Desorption-electron spray ionization (DESI) has been reported for use in such applications (21) but is somewhat limited in terms of quantitation of small molecules. Direct analysis in real time (DART) is another ambient ionization method, first reported by Cody et al. in 2005 (22). DART is also ideal for molecules of molecular weight less than 1000 Daltons and requires no sample preparation. The sample of interest is introduced into the gap between the DART ionization source and the mass spectrometer inlet. The DART ionization mechanism produces primarily protonated species (MH^+) in positive ion mode and deprotonated species in ($M\text{-}H^-$) in negative ion mode (23). The DART ion source (white) and mass spectrometer orifice (silver) are shown in Figure 6.

Figure 6. The DART ion source, showing the gap where samples are introduced.

DART has been used in the identification of anti-malarial drugs (24). Bio-medically, it is used in the identification of alkaloids such as vomilenin and reserpine (25). Clinically, DART has shown promise in the diagnostic lab in the analysis of dried blood spots (DBS) for pharmacokinetic studies of several drugs (21). Ambient techniques like DART and DESI are limited by the positioning and shape of the samples (26). To resolve this problem, transmission mode DART (TM DART) can be used. The one-dimensional transmission DART (1D-TM DART) module is shown in Figure 7. The left panel is enlarged to show the screen onto which the samples are placed for reproducible introduction into the DART source.

Figure 7. Transmission DART screens.

In TM DART, the DART screen holding the samples sits upright in between the DART ion source and the mass spectrometer (MS) inlet. As a result, the strongly heated gas that emits from the DART ion source hits the sample which is placed as a spot at the center of the DART screen. The sample is desorbed from the mesh, ionized, and enters the MS inlet (26). Nine to ten samples can be analyzed at a time using 1D-TM DART and less sample is consumed (10 to 20µl), as sample is placed as a drop at the center of the screen. In TM DART, the sensitivity of the analyte can be improved as the sample is introduced very close to the MS inlet (26).TM DART has applications in quantitation of Central Nervous Stimulant (caffeine) in energy drinks

and pesticides in foods. It also has application in cellular metabolic studies, especially in microsomes (26). There is another method of approach in TM DART, x-z TM DART (26). It has the advantage of analyzing 96 samples in 20 minutes in a single run.

Previous research work in quantification of short chain fatty acids in lab

Earlier research group members worked on fecal samples of mice and established a method for quantification of SCFAs (27). Fecal samples were treated with 0.6 M HCl for acidification and the trimethylsilyl derivatives were created. Acidification leads to protonation of the samples. These protonated samples, which are thought to contain SCFAs, go into organic phase after performing extractions. Trimethylsilyl derivatives were created as derivatization makes the compounds volatile for separation GC separation. Silylation is the most common derivatization technique used in the analysis of SCFAs (28). Unfortunately, the results were not reproducible, and it was thought that the hydrophilic nature of the reagent yielded an unstable product that was easily hydrolysable under conditions where water was present. Alternatively, a tert-butyl dimethylsilyl derivative was prepared, and this gave considerably more consistent results. The pentabromobenzyl derivative was prepared for characterization of butyrate, which is thought to lead to hydrophobic product such that it is stable. It is thought that this hydrophobic product gives reproducible results.

Research goal

Preliminary work was accomplished to confirm the established method for analysis of SCFAs from *in vitro* microbial cultures using GC-MS. The main goal of this study is to compare GC-MS and DART-MS methods for analysis of SCFAs in microbial cultures. Two types of culture environments were investigated in the current study. In one method, bacteria were cultured alone or with another bacterial species in a test tube under anaerobic conditions. In the other method, a bioreactor was used to grow mono or co-cultures under aerobic conditions. One major difference between the two methods beyond a difference in oxygen content is that test tube cultures are static, whereas bioreactor cultures have a continuous flow of media through the system, which more effectively models the situation that occurs in the GIT. The ultimate goal of the research is to determine whether co-culturing of bacteria affects the production of SCFAs relative to individual cultures.

EXPERIMENTAL PROCEDURES

Microorganisms and conditions for growth

The microbial species shown in Table 1 were chosen for their ability to produce lactate (*B. longum, E. faecalis, L. johnsonii, L. rhamnosus, L. acidophilus,* and *E. plexicaudatum*) and /or butyrate (*Clostridial species, Eubacterium plexicaudatum*) as their fermentation products. The media used for the growth of the microbes in the current study was Brucella broth (Acumedia) with (1 g/L) added starch (BBS). This broth contains an enzymatic digest of casein (10 g/L), an enzymatic digest of animal tissue (10 g/L), yeast extract (2 g/L), sodium chloride (5 g/L), dextrose (1 g/L), and sodium bisulfate (0.1 g/L). The enzymatic digest of casein and animal tissue provide nitrogen and carbon sources. Dextrose serves as energy source, whereas yeast extract provides vitamins. Sodium chloride maintains the osmotic balance, and sodium bisulfate enhances the growth. Test tube cultures were grown under anaerobic conditions to facilitate fermentation that will yield lactate and butyrate, whereas bioreactor cultures were grown under aerobic conditions.

Table 1. The Microorganisms and growth conditions

Organism used	**Chamber used for growth**	**Incubation period (in hours)**	**Source of organism**
Bifidobacterium longum	Test tube	24	ATCC 15707
Clostridium species	Test tube	24	ASF 500
Enterococcus faecalis	Test tube Bioreactor	24 24, 48, 72	OGS-1
Eubacterium plexicaudatum	Test tube	24	ASF 492
Lactobacillus acidophilus	Test tube	24	NCFM
Lactobacillus johnsonii	Test tube Bioreactor	24 24, 48, 72	NF- 1
Lactobacillus rhamnosus	Bioreactor	24, 48, 72	

Test tube cultures were inoculated in single test tubes with 10 mL of Brucella broth and grown overnight. Then, 100 µL of each overnight culture was put into tubes with 10 mL Brucella for growth in an anaerobic jar at 37 to 39°C. These tubes were left to grow for 24, 48, or 72 hours. A vinyl anaerobic chamber (Coy Laboratory Products Inc.) was used under aerobic conditions. 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).

The chemical reagents and solvents used in the study are provided in Tables 2 and 3, respectively.

Table 2. Reagents and their sources used in this study

Reagent used	**Reagent grade**	**CAS #**	**Seller**
L-(+)-Lactic acid	Approx. 98%	79-33-4	Aldrich
Butyric acid	≥99%	107-92-6	Aldrich
Propionic-2,2-d2 acid-d	99 atom% D	14770-51-5	Aldrich
Pyridine anhydrous	Approx. 99.8%	110-86-1	Aldrich
N,N-diisopropylethylamine	>99%	7087-68-5	Aldrich
Pentafluorobenzyl bromide	99%	1765-40-8	Aldrich
N-tert-butyldimethylsilyl-N-methyltrifluoro acetamide with 1% tert-butyldimethyl chlorosilane	≥95%	77377-52-7	Aldrich
1,3-Propanediol	98%	504-63-2	Aldrich

Table 3. Solvents used in this study

Solvent	**Solvent grade**	**CAS #**	**Seller**
Acetonitrile	≥99.9%	75-05-8	Aldrich
Ethyl acetate anhydrous	99.8%	141-78-6	Aldrich
Diethyl ether	≥99.9%	60-29-7	Aldrich

Preparation and GC- MS analysis of the silylated derivatives of lactic acid and 1, 3–propanediol

The internal standard, 1, 3-propanediol was prepared as a stock in acetonitrile at 200 mg/L. Lactic acid was prepared as a 200 mg/L stock in acetonitrile. From the 200 mg/L of lactic acid in acetonitrile solution, 7.5 µL (1.5 µg), 15 µL (3.0 µg), 22.5 µL (4.5 µg), or 30 µL (6.0 µg) of the lactate stock was added to test tubes. To each of these solutions was added 10 µL (2 µg) of internal standard, 10 µL (9780 µg) of anhydrous pyridine, and 20 µL (207.2 µg) of MTBSTFA in tert-butyl dimethyl chlorosilane (TBDMCS). Test tubes were placed in a heat block at 70^{o}C for 3 hours. The silylated derivatives of lactate (left) and internal standard (right) putatively generated by this procedure are shown in Figure 8.

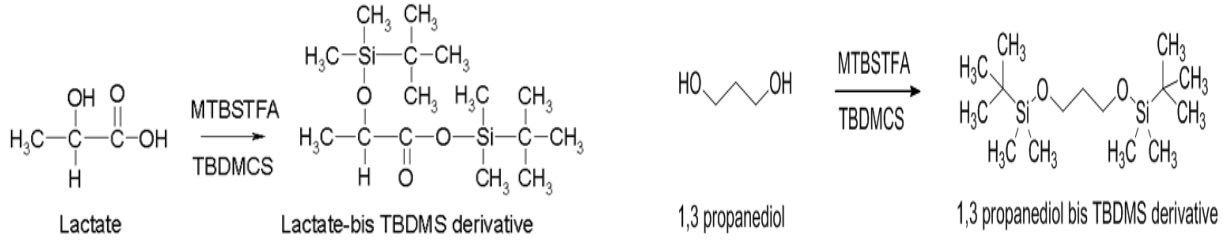

Figure 8. The formation of the bis tert-butyl dimethyl silyl derivatives of lactate and 1, 3-propanediol.

After drying of the product under nitrogen, 100 µL (9000 µg) of ethyl acetate was added for resuspension of the contents, and the test tube was vortexed for 1 minute prior to analysis by GC- MS.

A Varian 6800 gas chromatograph equipped with a Saturn 2200 mass spectrometer was used with a VF-5ms column (30 mx0.25 mm, film thickness), and the carrier gas was helium (Ultrapure, 99.99%). A 10 µL aliquot of sample was injected at 270 ^{o}C with a split ratio of

100:1. The oven temperature was held at 70 ^{o}C for 1 minute and increased at a rate of 20 ^{o}C/min to 160 ^{o}C, where it was held for 3.5 min. The temperature was finally increased to 250 ^{0}C at a rate of 35 ^{o}C/min. The column flow rate was 0.6 mL/min. The total run time was 12.43 min. Figure 9 shows the chromatogram for both 1, 3- propanediol and lactate as TBDMS derivatives.

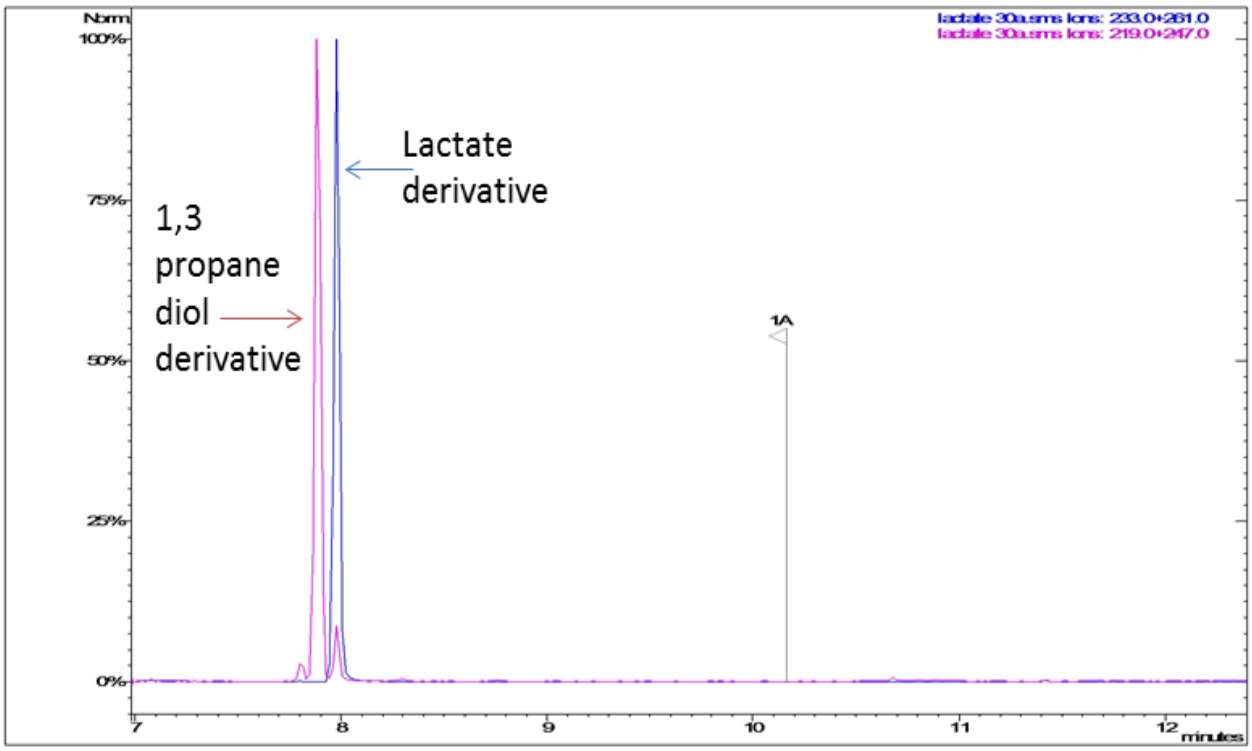

Figure 9. The selective ion gas chromatogram for the bis-tertbutyl-dimethylsilyl derivative of 1, 3-propanediol (left) and lactate (right).

The retention time for derivatized 1, 3-propanediol was mostly 7.85 min, whereas that for the bis-silylated lactate derivative was almost 7.93 min. The mass fragments produced from electron impact of the putative lactate 1, 3-propanediol derivatives and corresponding to the major peaks in their respective chromatograms are shown in Figures 10 and 11.

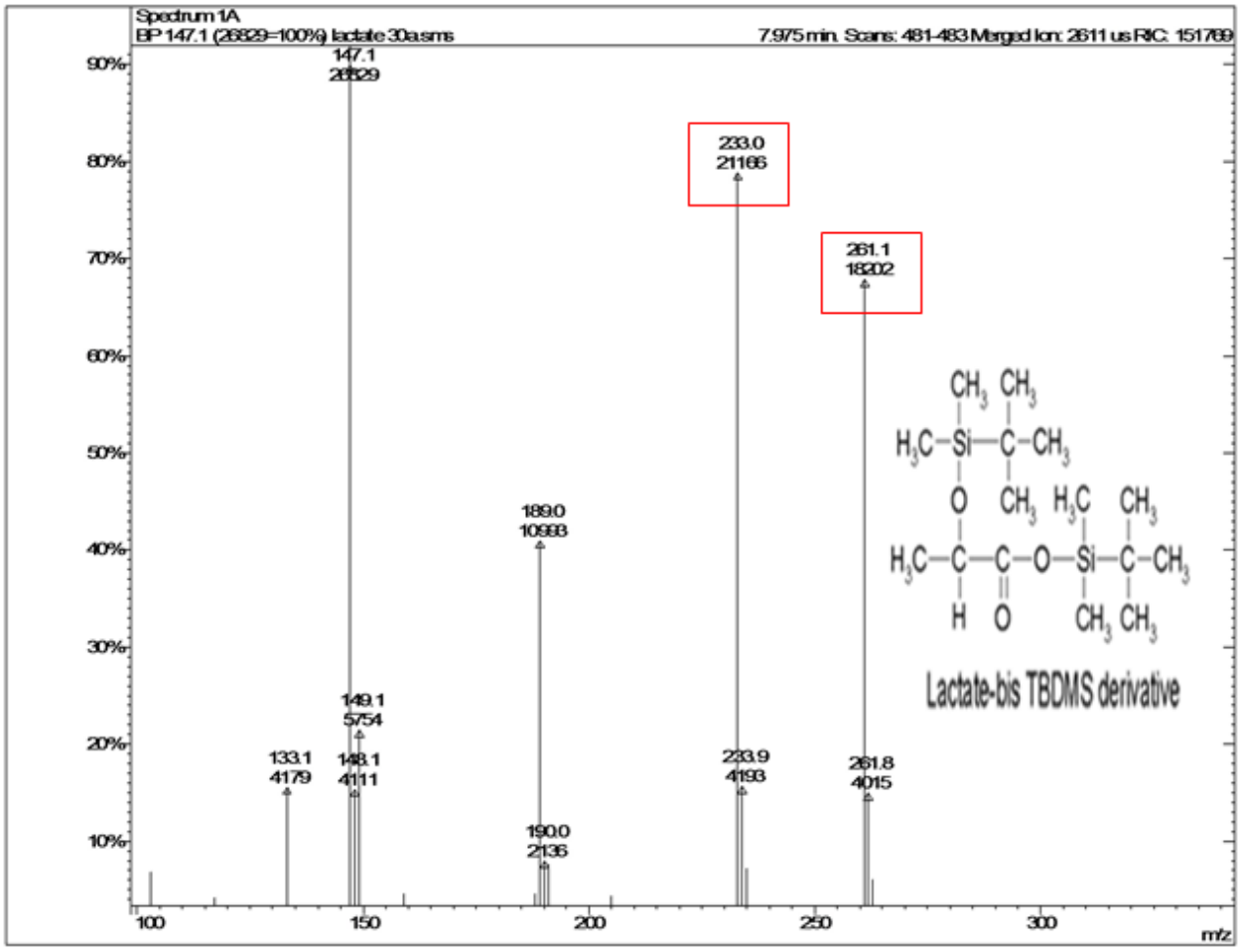

Figure 10. Mass spectrum for the derivatized lactate standard for the peak at 7.975 min peak in the Figure 7 chromatogram. The two boxed ions with *m/z* 233 and 261 were used to determine the peak area to quantify the lactate derivative. The peak area can be determined by integrating the eluted peak from the gas chromatogram.

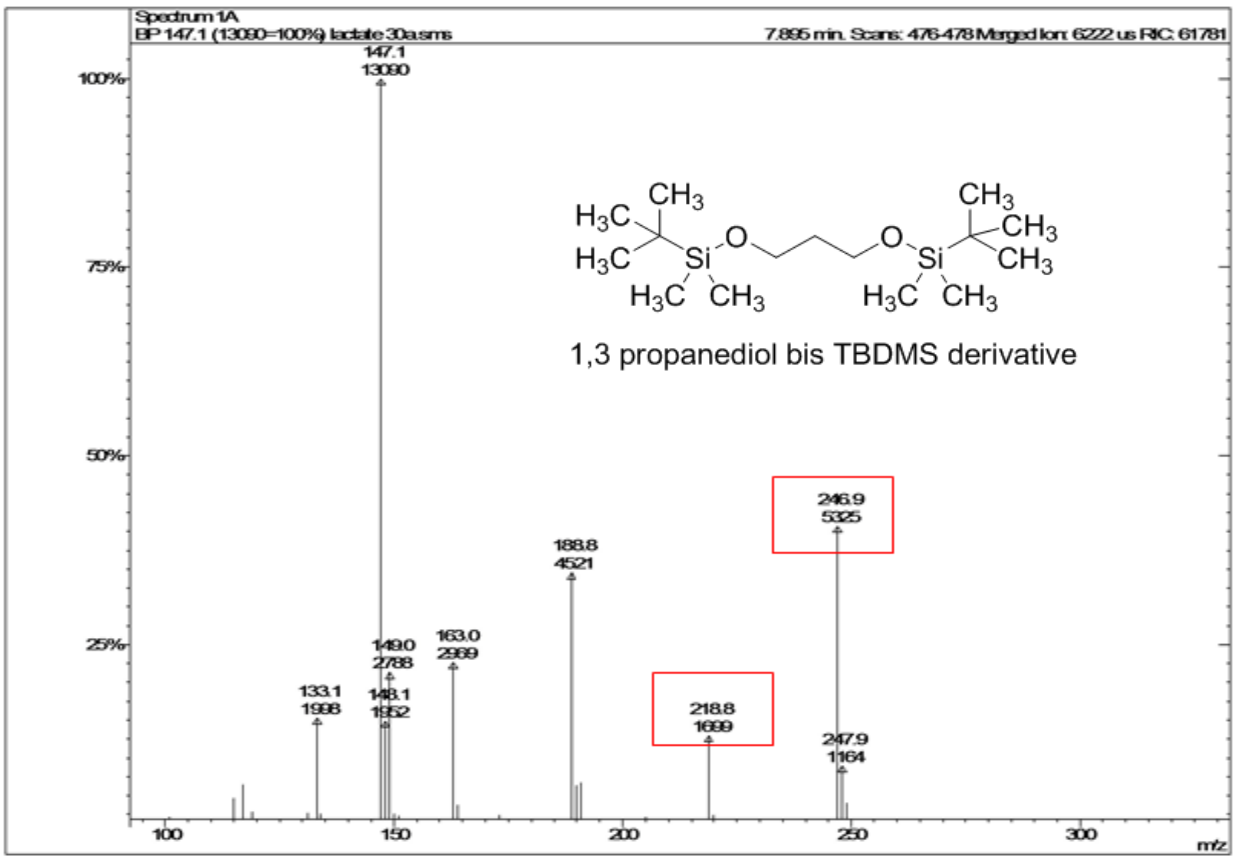

Figure 11. Mass fragmentation spectrum for the derivatized lactate standard for the peak at 7.895 min peak in the lactate chromatogram.

The ions with *m/z* 219 and 247 were used to quantify the internal standard, and the ions with *m/z* 233 and 261were similarly used to quantify the silylated lactate derivative. The ions with *m/z* 247 and 261 are believed to result from fragmentation of the tertiary butyl group in the derivatized internal standard and lactate derivative, respectively (12, 27, and 28).

The calibration curve for the lactate derivative is shown in Figure 12. The peak area ratio was calculated by dividing the peak area for the lactate derivative by that for the internal standard. The values shown in Figure 12 are the average and standard deviation for three

samples. There was no peak at 7.98 min in the chromatogram for the preparation containing internal standard but no lactate, which served as a blank.

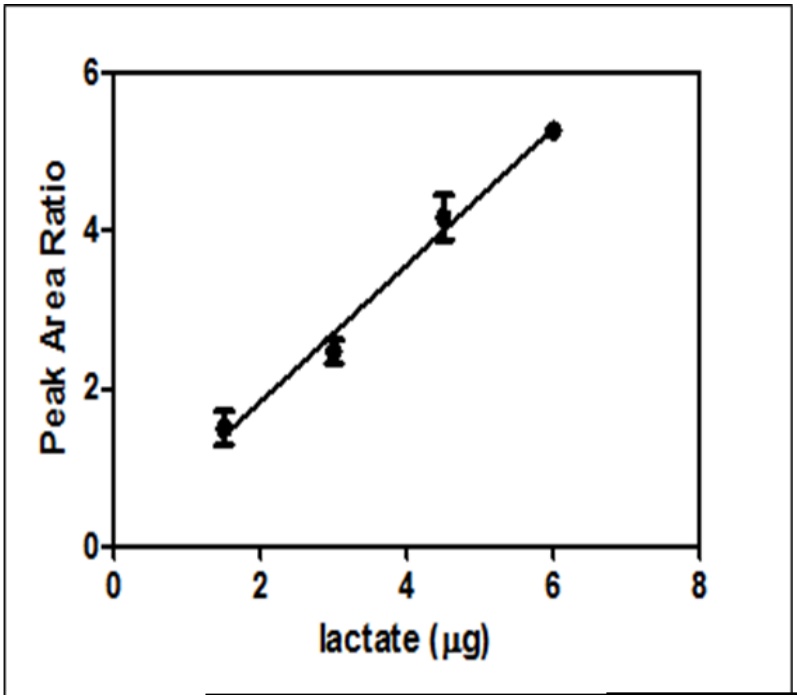

Figure 12. Calibration curve for lactate. $y = 0.870x + 0.0906$, $R^2 = 0.970$. The peak area ratio was determined from the peak area of the intensities of ions 233 and 261 for lactate divided by the peak area of the ions 219 and 247 for the internal standard (propanediol). Each value represents the mean and standard deviation for duplicate determinations.

Preparation of PFBB derivatives of butyric acid and dideuterated propionic acid and their analysis by GC- MS

The reaction of butyric acid with pentafluorobenzylbromide (PFBB) to yield a stable derivative is shown in Figure 13. Two hundred milligram per liter solutions of dideuterated (one of the deuterium atoms are contained in the carboxylic acid moiety) propionic acid (internal standard) and butyric acid were prepared in acetonitrile. To prepare a standard curve, 2 µL (4 µg), 5 µL (10 µg), 10 µL (20 µg), and 15 µL (30 µg) of butyrate standards were transferred to a test tube. To each of these tubes was added 10 µL (20 µg) of internal standard, 10µL (7820 µg) of N, N-diisopropylethylamine, and 100 µL (172800 µg) of pentylfluorobenzylbromide. All of the test tubes were set on the bench-top for 30 min to allow the reaction to take place, after which the solvent was removed under N_2 gas. The contents were resuspended by vortexing for 1 min in 100 µL (9000 µg) of ethyl acetate. This solution was transferred to vials for delivery to autosampler, which transfers this solution to GC-MS for analysis.

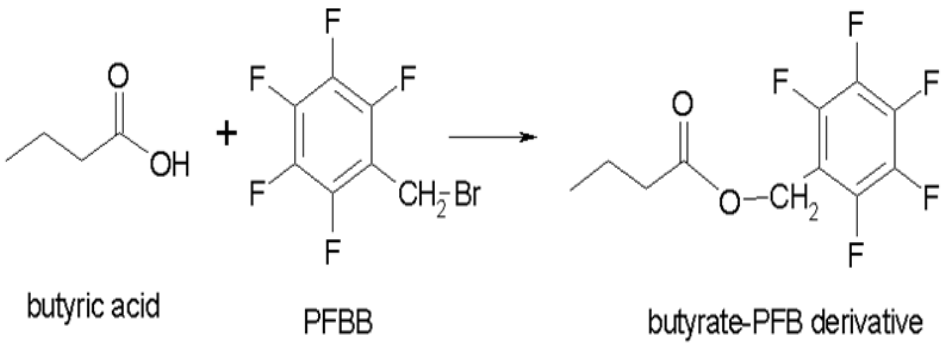

Figure 13. The preparation of the pentafluorobenzyl derivative of butyrate

For the analysis of butyrate, we used the same equipment and column that we used for lactate analysis. Ten microliters of sample was injected onto the column with a split ratio of 100:1 at 270 ^{o}C. The oven temperature was held at 50 ^{o}C for 1 minute and increased at a rate of 10 ^{o}C/min to 100 ^{o}C. The total time of analysis was 9.25 min. The column flow rate was 0.6 ml/min. The retention times for the butyrate and dideuterated under these conditions were 7.96 min and 7.3 min, respectively. The mass fragment with *m/z* 181 arises from the O-benzyl part of PFBB, and the ion with *m/z* 268 corresponds to the molecular ion (28). The ionic pair of *m/z* 181 and 254 arises from the corresponding O-benzyl and molecular ion that characterize the derivatized propionic standard. The ionic pair of *m/z* 181 and 256 arises from the corresponding O-benzyl and molecular ion that characterize the derivatized dideuterated propionic standard (28). A calibration curve was plotted (27, 28) for the range of standards for butyrate. Over time, the mass fragmentation spectrum for the derivative prepared from the butyrate standard gave variable results for its mass fragmentation spectrum, especially for the characteristic ion *m/z* 181. Calibration curves could not be constructed, and the butyrate assay was deemed unreliable. The PFB-butyrate derivative was abandoned as the basis for an assay to quantify butyrate in biological samples.

Preparation of biological samples for analysis of butyrate and lactate

All of the test tubes containing cultured samples were stored at -80 ^{o}C. Samples required for analyses were thawed, and 0.5 mL of each sample was added to clean test tubes. The biological samples were not used at a time and were preserved at -80 ^{o}C for future reanalysis. These samples were prepared in duplicate. Acidification of the sample was achieved by addition of 50 µL of 0.6 M HCl. A 0.5 mL aliquot of diethyl ether was added to the sample, and the contents were mixed by vortex for 30 to 60 s. The samples were submitted to centrifugation for 5 min at 2000 *g*. A Pasteur pipette was used to remove the ether layer, which was then

transferred to another clean test tube. A 1 mL aliquot of ether was added to the original test tube and again was submitted to centrifugation, after which the ether layer was extracted. This extraction procedure was repeated five times. All of the ether layers of each sample were combined into separate test tubes, and the collected ether samples were placed on the bench to allow for evaporation of the solvent at room temperature. Though the acids are volatile, the diethyl ether is evaporated more quickly due to its lower boiling point of diethyl ether (34.6 ^{o}C relative to butyric acid (163.5 ^{o}C) or lactic acid (119-125 ^{o}C). The derivatives of lactate and butyrate in the dried samples were then prepared for GC-MS as described above.

Preparation of samples for bioassay using DART- MS

An aliquot of 0.05 mL of thawed biological samples was transferred to 5 mL disposable tubes. To each of these tubes, 20 µL of trideuterated propionic acid in acetonitrile (2.06 g/L) was added as internal standard. This solution was prepared in acetonitrile by diluting 20 µL of trideuterated propionic acid stock in 10 mL of acetonitrile. The tubes were mixed in a vortex for 30 to 60 s and stored at -20 ^{o}C. The stored samples were thawed and mixed by vortexing again for 30 to 60 s before subjecting them to DART- MS analysis.

Preparation of samples for bioassay using transmission DART- MS

Ten microliters of the above mixture was placed as a drop on the stainless steel mesh of the TD apparatus. The stored samples were thawed and mixed by vortexing again for 30 to 60 s before placing the analyte on the DART screen.

Analyses were carried out on an AccuTOF JMS T100LC (JEOL USA, Peabody, MA) time of flight mass spectrometer with a direct analysis in real time (DART) ion source (Ionsense, Saugus, MA). Spectra were collected in negative ion mode, resulting in formation of singly-

charged M-H- ions from the SCFAs. The internal standard was observed as an M-2H- ion. Settings for the mass spectrometer include Orifice 1, 30 V (to minimize fragmentation); Orifice 2 and ring lens, 5 V; analyzer peaks voltage 600 V (to provide maximum sensitivity for low MW species); multichannel plate detector, 2500 V. The mass resolution was approximately 6000 (measured for reserpine at MH+ 609.281). Settings for the ion source include DART source grid voltage -530 V; ionization gas was He (UHP); gas heater temperature, 350 ºC. Transmission DART was run using the Ionsense transmission module with stainless steel mesh sampling strips to which sample was applied as described above. The module was introduced into the source at 0.5 mm/s. Mass calibration was performed within each transmission analysis with PEG-600 in methanol in the relevant mass range. All data were processed using TSSPro 3.0 (Shrader Laboratories, Detroit, MI).

RESULTS

GC- MS analysis of lactate and butyrate in test tube mono- and co-cultures of bacteria

Test tube cultures provided by Dr. Clemans' laboratory group containing one or two organisms were evaluated first to determine the extent to which the lactate assay could be used successfully to characterize the difference between cultures. In Figure 14 is shown the gas chromatogram for the co-culture containing *Clostridium propionicum* and *Lactobacillus johnsonii* after solvent extraction and treatment with reagents to prepare the lactate derivative. Figure 15 shows the mass spectrum for the derivatized lactate, and Figure 16 shows the mass spectrum for the derivatized internal standard for the same co-culture. The compound representing the peak at 7.978 min in the chromatogram possessed a mass fragmentation pattern that was essentially identical to that of the standard shown in Figure 10.

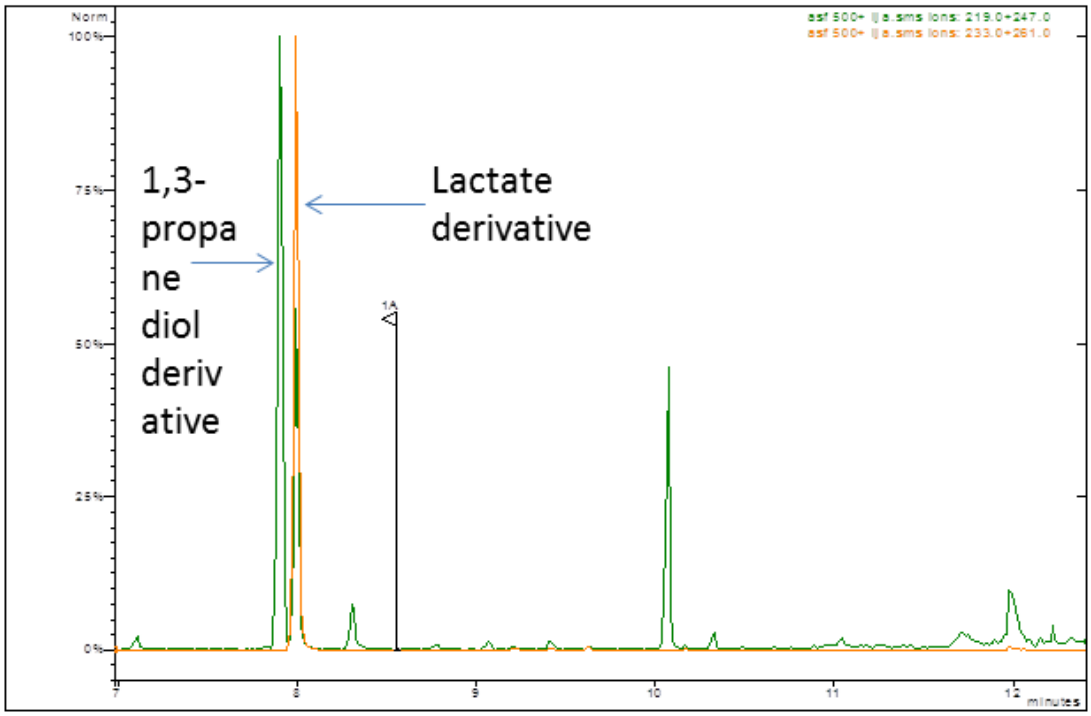

Figure 14. Selected ion gas chromatogram for the derivatized lactate and 1, 3-propanediol in media from the co-culture of *Clostridium propionicum* and *Lactobacillus johnsonii.*

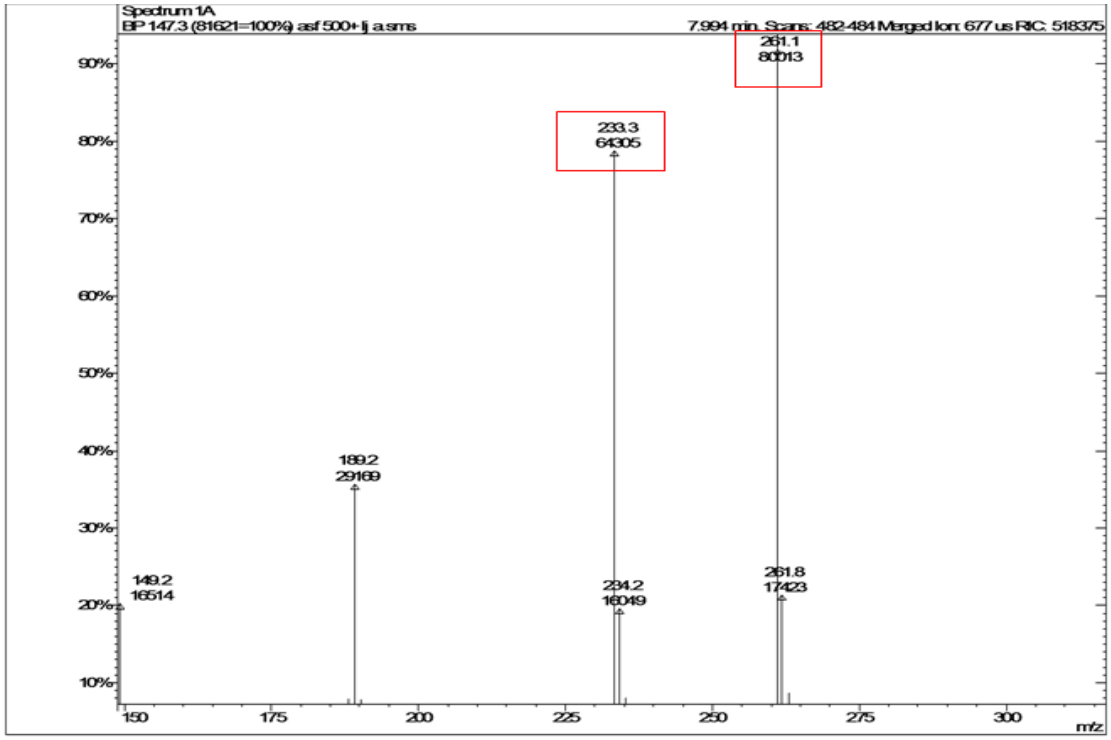

Figure 15. Mass spectrum for the lactate derivative at 7.994 min in Figure 14.

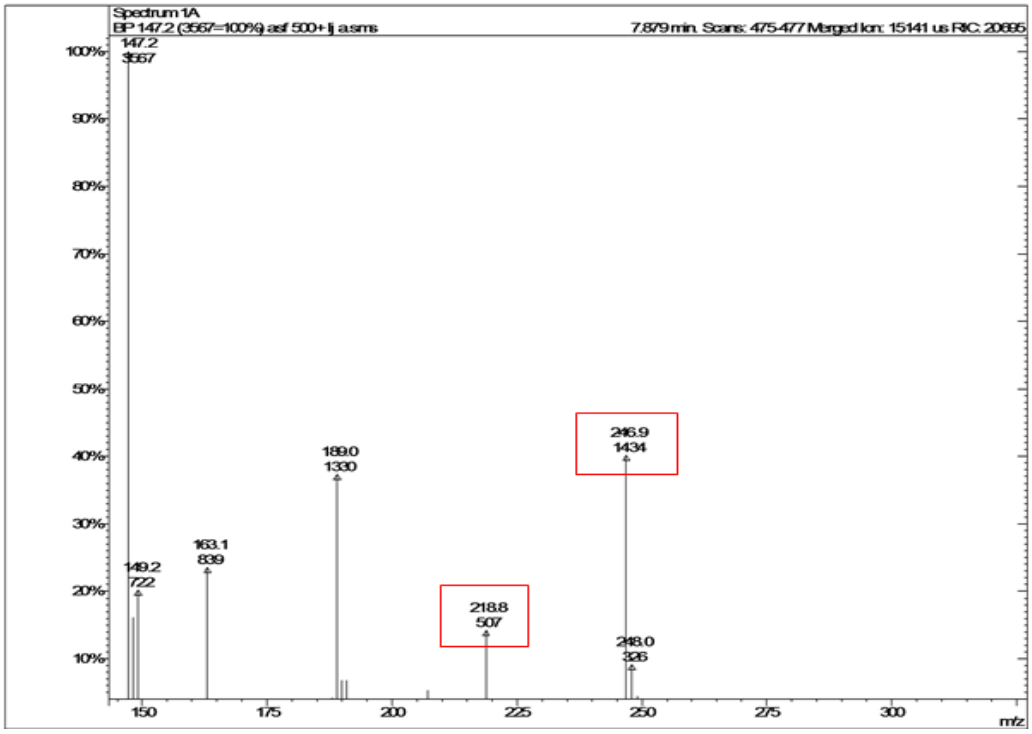

Figure 16. Mass spectrum for the internal standard derivative at 7.879 min in Figure 14.

As shown in Figure 17, the level of lactate was determined in duplicate for the mono- and co-cultures of various bacteria. The brucella broth (BBS) contains relatively negligible levels of lactate, whereas all of the cultures containing mono- or co-cultures of known lactate producers (27, 28) produce variable levels of lactate. The error in the measurement of the lactate was relatively small except for the co-culture *Clostridium propionicum* and *Lactobacillus acidophilus*, so these results provide the confidence needed to evaluate the differences that are expected to be seen between various organisms grown in culture. The limit of detection (LOD) was determined to be 0.431μg/500 μL, and the limit of quantification (LOQ) was determined to be 1.054 μg/500 μL. The results also indicate that there is a fair amount of lactate present in the cultures that contain a *Lactobacillus* species (*L. johnsonii* and *L. acidophilus*), or in the media cultured with *E. faecalis* as expected, since all of these organisms are known to produce lactate. Interestingly, the media of the co-culture containing *C. propionicum* and *E. plexicaudatum*, two species that are putative butyrate producers, also contain a good amount of lactate (average amount of lactate produced was 6.3 μg/500 μL).

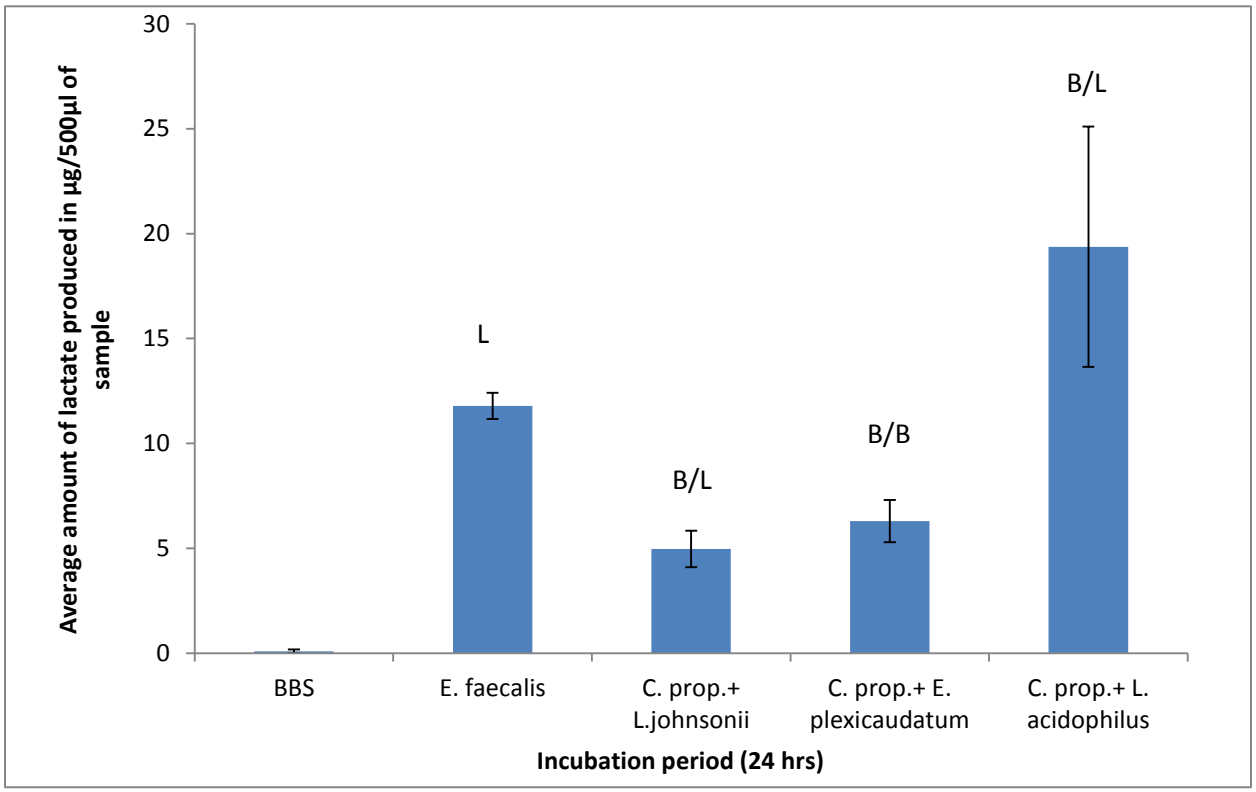

Figure 17. Lactate levels in mono- and co-cultures of bacteria grown in test tubes for 24 hours. BBS represents the Brucella broth with added starch containing no bacteria, *C.prop.* represents the *Clostridium* species. At the top of each bar, it was labeled as L, B/L, B/B which represents lactate producer, butyrate producer and lactate producer, and both are butyrate producers. The error bars indicate the standard deviation of the two measurements. All the values in the figure are the average of duplicate determinations.

The results of the experiments shown in Figure 16 are grouped together with those of another student in the lab for comparison and are shown in Figure 18. The LOD and LOQ were same for these set of results as the same blank was used by both students. Although the reproducibility of the assay varies between students, it is clear that all cultures containing lactate

producers (*L. acidophilus*, *E. faecalis*, *L.johnsonii*, and *B. longum*) possess greater amounts of lactate when compared to media (BBS: Brucella broth with starch) containing no bacteria but not in *L. johnsonii* culture as it looks pretty low that is below LOD. No definitive conclusions can be made about lactate production in *C. propionicum* cultures due to inconsistent results.

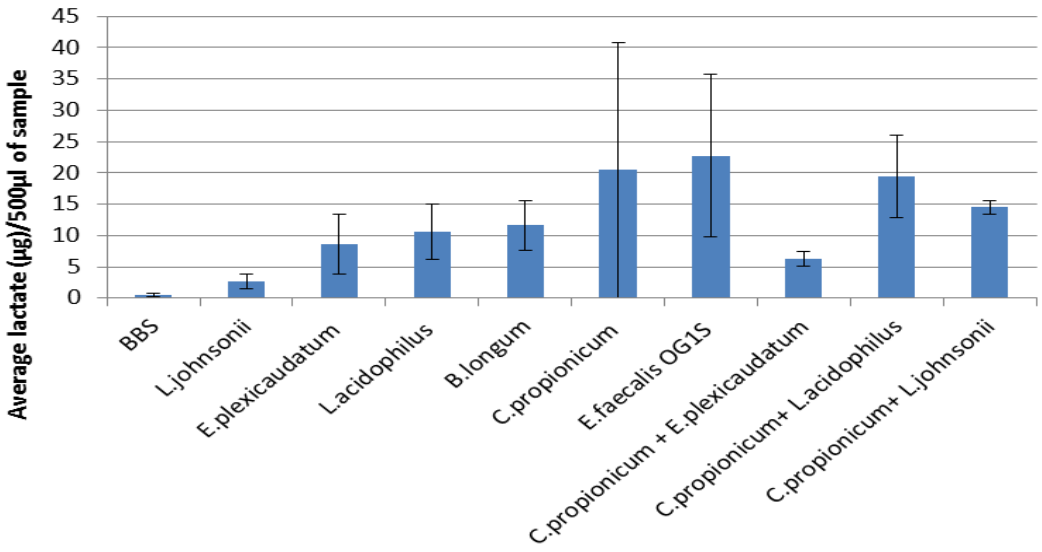

Figure 18. Lactate levels in mono-cultures or co-cultures of two bacterial species. The data shown originally shown in Figure 16 for BBS, *L. johnsonii*, *C. propionicum* and *E. plexicaudatum*, and *C. propionicum* and *L. johnsonii* cultures were included in the same figure as those results were obtained by another student in the lab.

Attempts were made to develop an assay for butyrate by preparing the pentafluorobenzylfluoride derivative as was formerly done by other students in the lab (27, 28). The butyrate derivative has a retention time of 7.8 min and is identified by the presence in the mass spectrum of the analyte of a prominent 181 ion, which characterizes the pentafluorobenzyl moiety (28). The 181 and 256 ions were used to identify the internal standard prepared as the deuterated propionic acid-PFB derivative, which has a retention time of 7.3 min. (28).

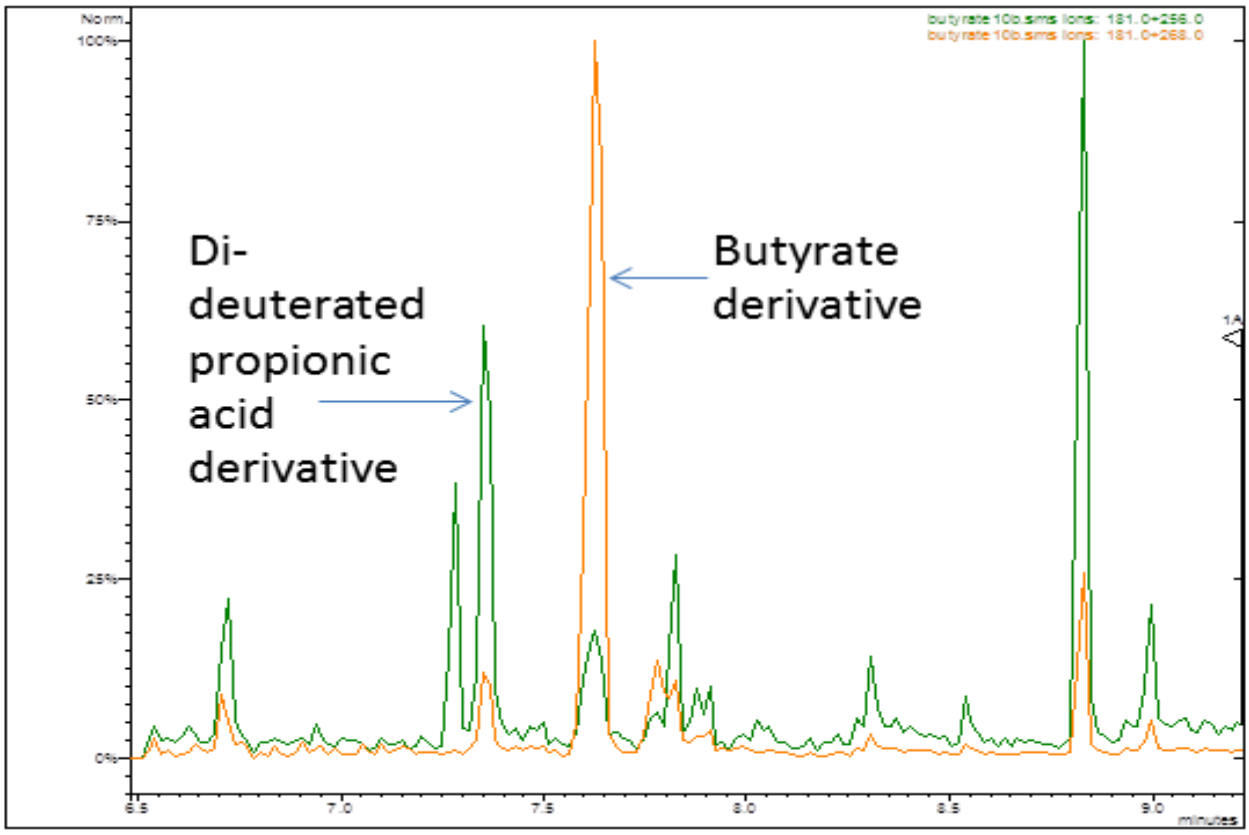

Figure 19. The selected ion chromatogram emphasizing the butyrate-PFB derivative (181 and 268 ions) and di-deuterated propionate-PFB derivative (181 and 256 ions).

The results in Figure 19 obtained more recently show the selected ion chromatograms for the butyrate standard (181 and 268 ions) with a retention time of 7.6 min, and the internal standard (181 and 256 ions) with a retention time of 7.3 min. As shown in Figure 19, there were no peaks in the region from 7.6 to 7.8 min that contained both the 181 and 268 ions that were previously characterized for identifying ions for butyrate-PFB derivative. The putative butyrate-derivative was identified only because the 268 ion was present in the mass fragmentation pattern for the analyte with a peak at 7.6 min as shown in Figure 20. In other work with the butyrate samples prepared with the PFBB reagent, the relative intensity of the 181 and 268 ions peak for the analyte at 7.6 min varied markedly upon repeated analysis. The internal standard was

identified on the basis of its retention time (Figure 21), but its mass fragmentation pattern contained relatively low levels of 181 ion and no characteristic 256 ion (Figure 22).

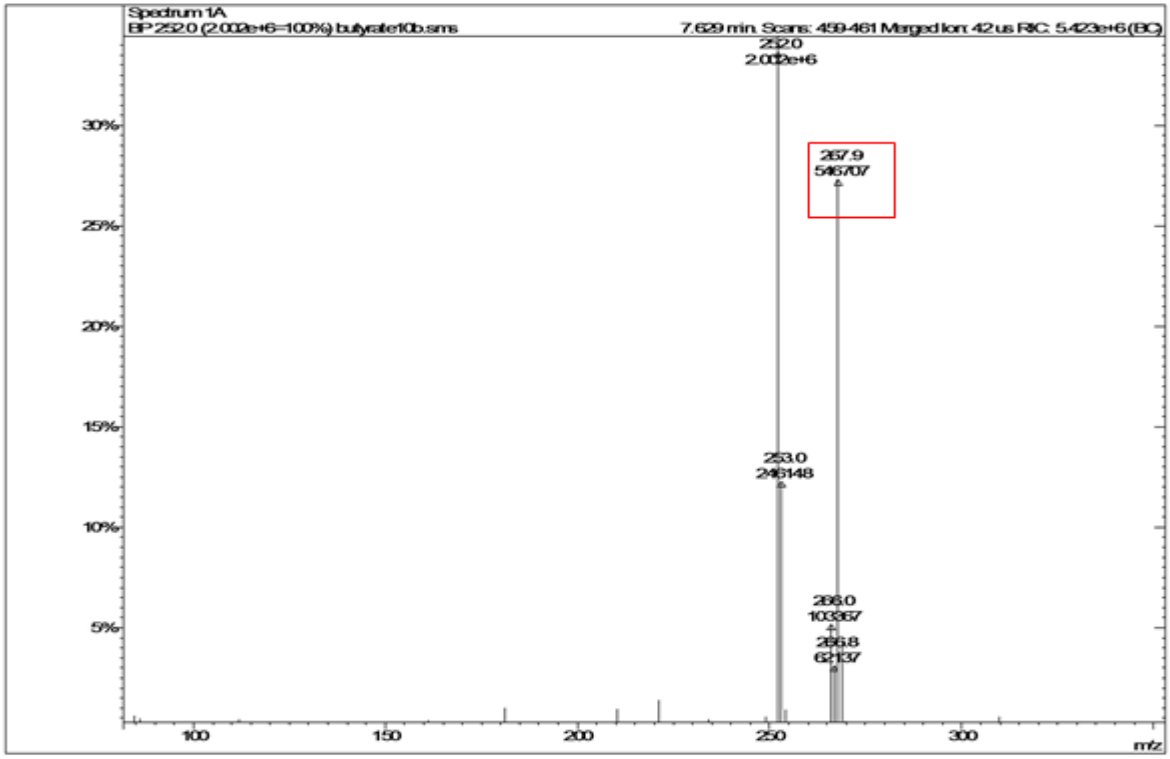

Figure 20. Mass spectrum for the PFB- butyrate standard for the peak 181at 7.629 min.

At other times, the prominent peak had a retention time of 7.8 min, but the peaks contained neither 181 nor 268 peaks. Moreover, in efforts to develop a butyrate calibration curve, the intensity of the 7.6-min peak containing the 268 ion did not demonstrate concentration dependence.

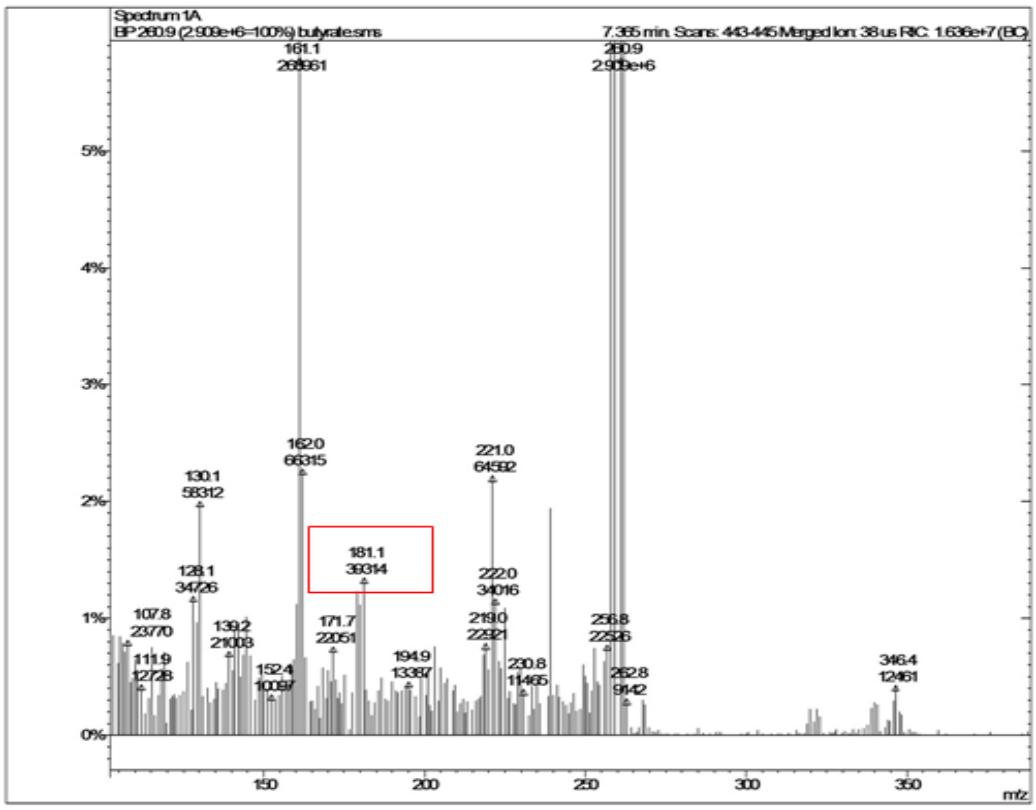

Figure 21. Mass spectrum for the PFB- dideuterated propionic standard showing the peak 181at 7.365 min.

In light of these inconsistent data for the butyrate and dideuterated propionic standard assays, it was obvious that a reliable assay for butyrate could not be developed. The acquisition of new stocks of the derivatizing agent and preparation of new stocks of standard did not improve the results. Moreover, when media from test tube cultures that had been previously assayed using this procedure were found to contain butyrate, again in subsequent analysis, there were no peaks observed in the appropriate portion of the chromatogram nor were there characteristic ions in the mass spectrum to indicate the presence of butyrate. Unreliable results for this butyrate assay were also obtained by another student in the laboratory (27). As butyrate assay involving synthesis of a PFB derivative was not reliable in our study, and GC- MS is

laborious and time consuming as it involves many steps. Rather than continuing with GC-MS methods, it was proposed that we try DART-MS and TM DART.

DART-MS analysis of bioreactor mono- and co-cultures under aerobic conditions

Given the lack of reliability for the butyrate assay in particular and the labor- and time-intensive nature of the preparation of chemical derivatives for analysis of SCFAs by GC-MS in general, it was recommended that an alternative methodology involving DART-MS be evaluated for SCFAs production in bacterial cultures. At this time, Dr. Lynne Shetron (EMU Clinical Laboratory Sciences) was in the early stages of developing a bioreactor chamber as a model system to evaluate the growth of mono- and co-cultures of bacteria. In these early experiments, the chamber was run under aerobic conditions, and the flow-through of media from the chamber was collected at two or three time intervals for later analysis. This circumstance provided a good opportunity to plan experiments to evaluate lactate and butyrate in appropriately paired cultures so that more meaningful comparisons could be made between the mono-cultures and co-cultures, which was not the case with the test tube cultures.

For these experiments, internal standard was added to an aliquot of the media, and one of two methods was used for application of the sample to the ion source in the DART-MS. In the first method, a capillary tube was dipped in the media and introduced into the ion source. This method of application was repeated two additional times so that the results could be evaluated as a triplicate determination for calculation of an average and standard deviation. The LOD and LOQ of this method were determined to be 0.128 and 0.246 respectively. The results for two *E. faecalis* cultures grown in the bioreactor under aerobic conditions are shown in Figure 22.

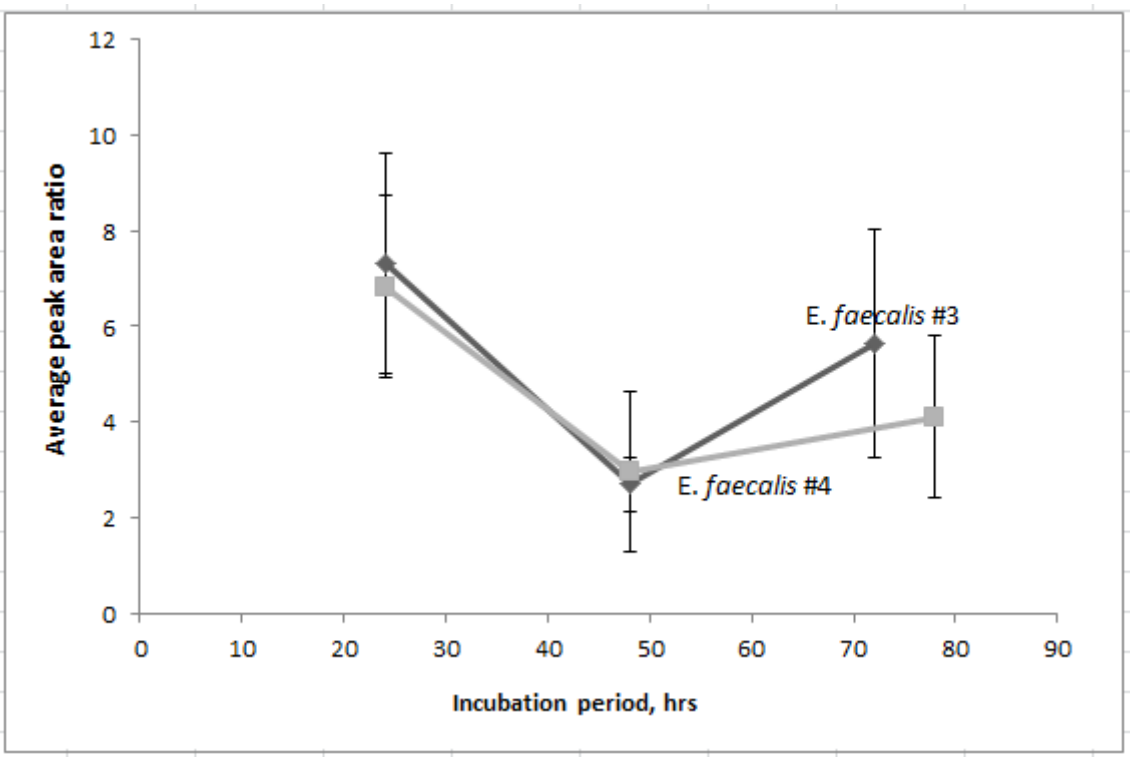

Figure 22. Lactate levels evaluated by "tube dip" DART-MS in two cultures of *E. faecalis* aerobically grown in a bioreactor. The values are provided as an average and standard deviation for three applications of media to which was added the internal standard (20 µL trideuterated propionic acid). The peak ratio was determined by dividing the intensity of the lactate detected by the intensity of the internal standard detected.

Table 4. The average peak area ratio and average standard deviation for the cultures *E. faecalis* #3 and *E. faecalis* #4 using tube dip method DART- MS.

Organism	Incubation period(hrs)	L/IS (Avg peak area ratio)	Avg std dev
E.faecalis #3	24	7.31	2.28
E.faecalis #3	48	2.7	0.56
E.faecalis #3	72	5.63	2.38
E.faecalis #4	24	6.83	1.89
E.faecalis #4	48	2.98	1.67
E.faecalis #4	78	4.11	1.69

Both cultures of *E. faecalis* at different hours of incubation by tube dip method were quantifiable and detectable as determined by the LOD and LOQ based on data shown in Table 4. It is rather difficult to make conclusions about the time course of lactate production in the mono-cultures and to make conclusions about the relative amount of lactate produced in mono-cultures due to the considerable variation in the amount of lactate measured at each time point.

Subsequent experiments were conducted to look at combinations of mono- and co-cultures to determine whether there were significant differences in the level of lactate in various cultures. The first of these experiments involving mono- and co-cultures of *L. johnsonii* and *E. faecalis*, two lactate producers, is shown in Figure 23.

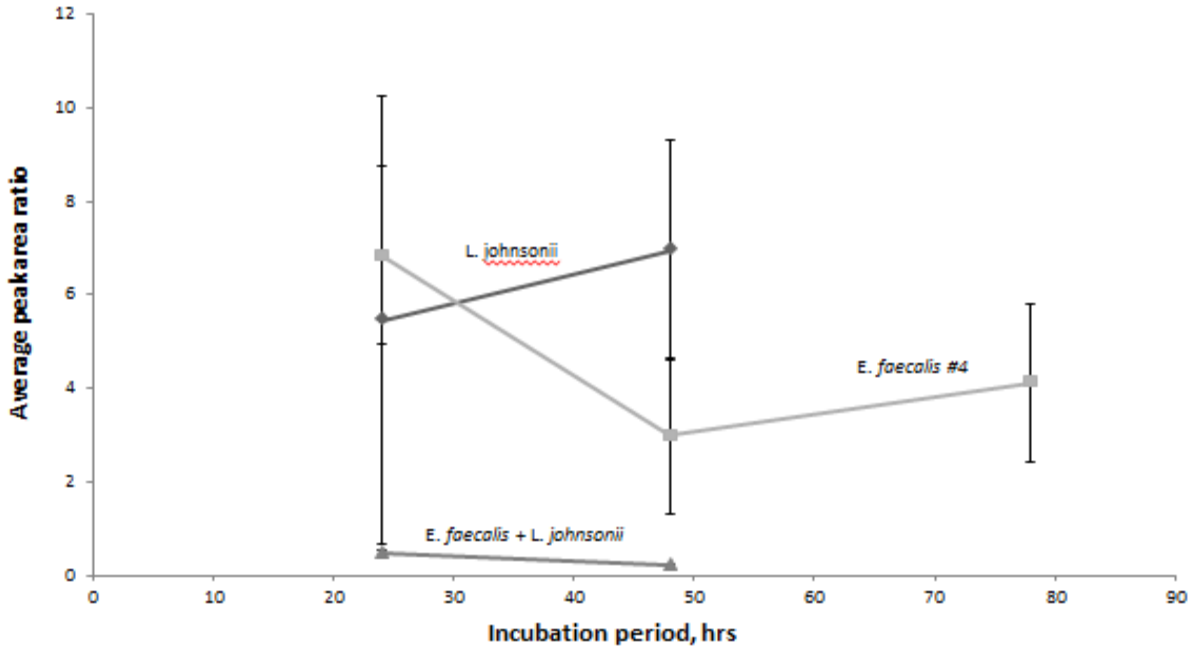

Figure 23. Lactate levels evaluated by "tube dip" DART-MS in mono- and co-cultures of *E. faecalis* and *L. johnsonii*. The values are provided as an average and standard deviation and the peak ratio was determined as for experiments shown in Figure 22.

Table 5. The average peak area ratio and average standard deviation for the cultures *E. faecalis* #4, *L. johnsonii* and co- culture *E. faecalis +L. johnsonii* using tube dip method DART- MS.

Organism	Incubation period (hrs)	L/IS (Avg peak area ratio)	Avg. Std. Dev
L.johnsonii	24	5.45	4.79
L.johnsonii	48	6.95	2.35
E. faecalis #4	24	6.83	1.89
E. faecalis #4	48	2.98	1.67
E. faecalis #4	78	4.11	1.69
E. faecalis+L. johnsonii	24	0.46	0.09
E. faecalis+L. johnsonii	48	0.21	0.04

For *L. johnsonii*, the lactate was detectable and quantifiable at 24 and 48 hours of incubation by tube dip method as judged by the comparison of values for the average peak area ratio to the LOD and LOQ respectively. The E. faecalis #4 was already determined to be quantifiable at each time point. Using the average peak area ratio values from the Table 5 and values for LOD and LOQ, the level of lactate in the co-culture at 24 and 48 hours of incubation is below the limit of quantification.

It is again rather difficult to make conclusions about the time course of lactate production in the mono-cultures and to make conclusions about the relative amount of lactate produced in mono-cultures due to the considerable variation in the amount of lactate measured at each time point. However, the considerably lower amount of lactate present in the co-cultured media, relative to the monocultures, particularly at the 48 hr time point is worth noting. These results also suggest that in co-cultures of *L. johnsonii* and *E. faecalis*, lactate might be consumed at higher level or produced at lower level relative to monocultures.

The same analysis was conducted with a different combination of lactate producers, *E. faecalis* and *L. rhamnosus,* as shown in Figure 24. The lactate was detectable and quantifiable in the *L. rhamnosus* culture at 24 and 48 hours of incubation, and the lactate levels are quantifiable at each time point in the *E. faecalis* culture as previously determined. In the *E. faecalis* + *L. rhamnosus* culture, the lactate was detectable and quantifiable at 24, 48 and 72 hours of incubation, when the values for the average peak area ratio as showed in Table 6 were compared to the LOD and LOQ. It is difficult to discern differences in the time courses for the mono-cultures given the considerable variation in the values at each time point. A relatively low level of lactate was consistently detected with both the mono-cultured and co-cultured media. There was a lower level of lactate in the co-cultures relative to the mono-cultures, but only at the 24 hr time point. This could be due to a higher level of lactate consumption or a lower level of lactate production in co-cultures.

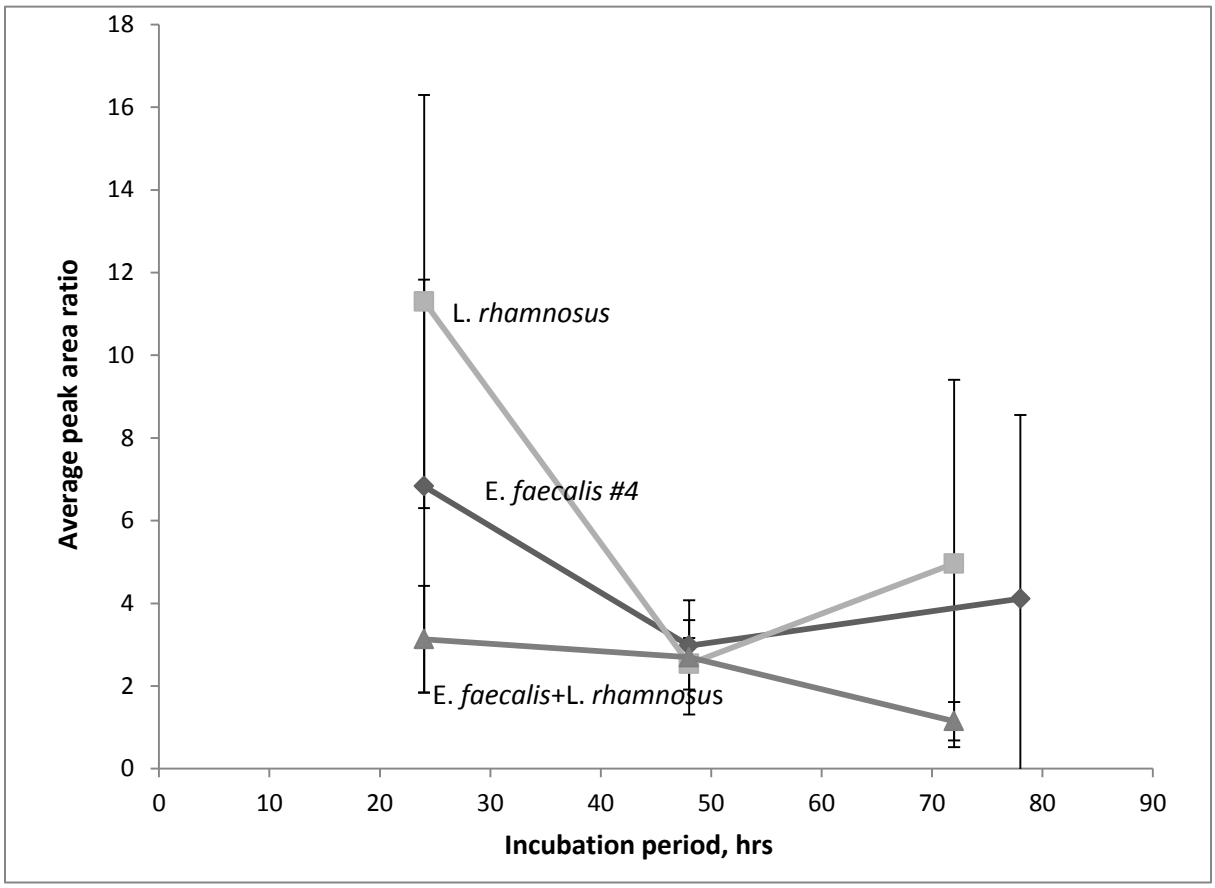

Figure 24. Lactate levels evaluated by "tube dip" DART-MS in mono- and co-cultures of *E. faecalis* and *L. rhamnosus*. The data for *E. faecalis* plotted here was taken from Figure 22 for *E. faecalis* #4. The values are provided as an average and standard deviation and the peak ratio was determined as for experiments shown in Figure 22.

Table 6. The average peak area ratio and average standard deviation for the cultures *E. faecalis* #4, *L. rhamnosus* and co- culture E. *faecalis* + *L. rhamnosus* using tube dip method DART- MS.

Organism	Incubation period(hours)	L/IS (Avg. peak area ratio)	Avg. Std. Dev.
E. faecalis #4	24	6.83	1.89
E. faecalis #4	48	2.98	1.67
E. faecalis #4	78	4.11	1.69
L. rhamnosus	24	11.29	4.99
L. rhamnosus	48	2.54	0.62
L. rhamnosus	72	4.96	4.44
E.faecalis + L. rhamnosus	24	3.13	1.29
E.faecalis + L. rhamnosus	48	2.69	1.39
E.faecalis + L. rhamnosus	72	1.15	0.47

In Figure 25, the lactate levels at 24 hrs for *E. faecalis* and *L. rhamnosus* were grouped together by whether they were determined in media from mono-cultures or by co-cultures. The results suggest that lactate is present at lower levels in co-cultures than in mono-cultures. In all cases, as for the test tube cultures, the level of lactate in brucella broth containing starch (BBS) with no bacteria is relatively low, which is in agreement with the earlier test tube data obtained by GC-MS (see Figure 16 and Figure 17).

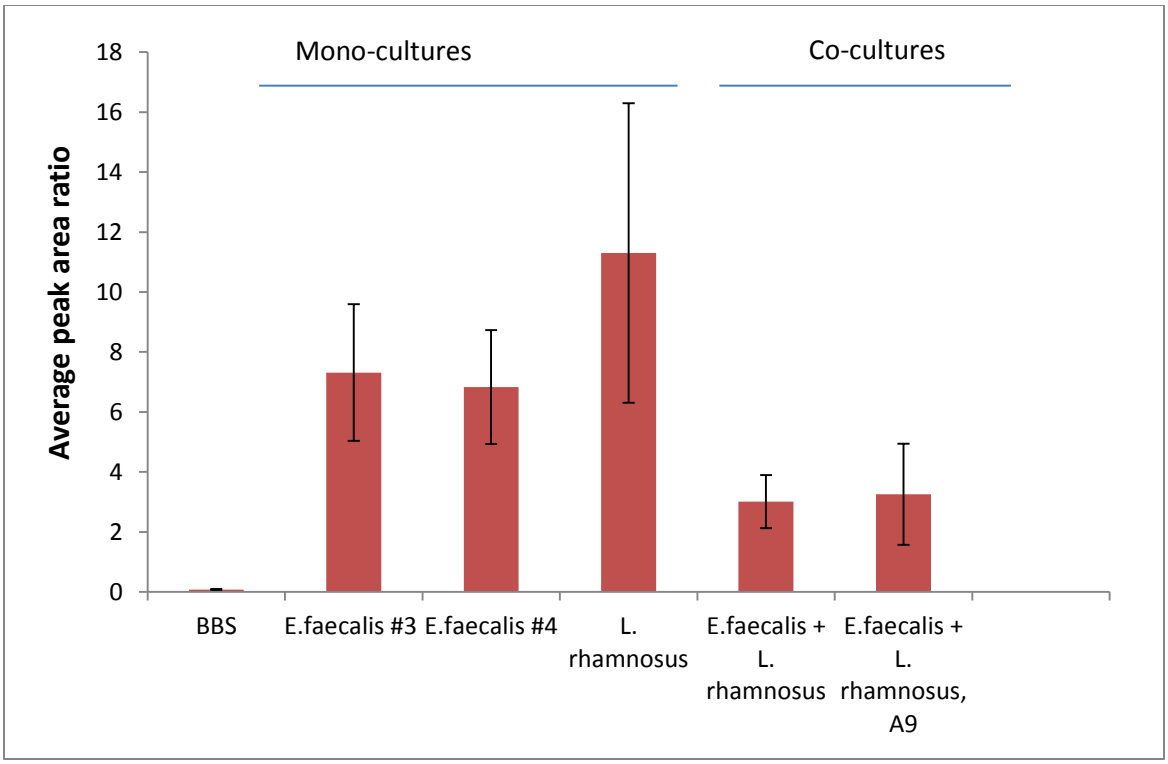

Figure 25. Lactate levels in 24 hr. aerobically grown bioreactor mono- and co-cultures by "tube dip" DART-MS method.

In light of the published finding (29) that co-culturing of lactate and butyrate producers gave much greater butyrate levels and correspondingly lower lactate levels than in mono-cultures, it was of interest to determine whether a similar trend is observed in the aerobically grown bio-reactor cultures in the present study. The results of butyrate analysis for bioreactor cultures evaluated by this "tube dip" method of DART- MS are shown in Figure 26. The LOD and LOQ cannot be determined for butyrate as it cannot be detected from the blank. Interestingly though, the level of butyrate in co-cultures is greater than what was detected in the mono-cultures, although there is also a considerable variation in the measurements in the co-cultures. The greater level of butyrate detected in *E. faecalis* and *L. rhamnosus* co-culture than in mono cultures was observed in two independent experiments. Taken together with the results shown in

Figure 26, these preliminary results indicate that there is a lower level of lactate in co-cultures at 24hours of incubation for *E. faecalis* and *L.rhamnosus*, and a greater level of butyrate in co-cultures relative to that in mono-cultures. It is also noteworthy that the levels of butyrate are considerably lower than those for lactate.

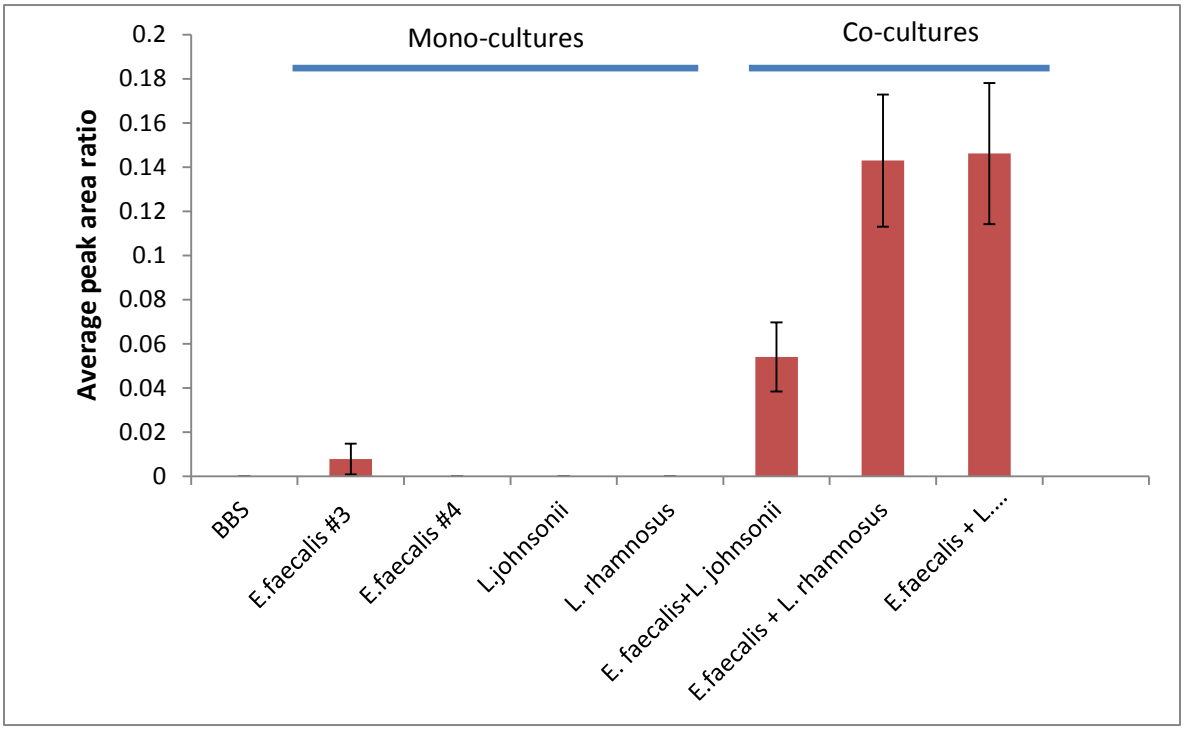

Figure 26. Butyrate levels in 24 hr. aerobically- grown bioreactor mono- and co-cultures by "tube dip" DART-MS method.

Given the considerable variation in the results obtained by the "tube dip" method, it was recommended that a different approach to introducing the sample to the DART-MS be attempted. In these experiments, the sample was uniformly applied to a screen, which is inserted at a constant distance from the ion source in hopes of increasing the reproducibility of the results. All of the samples that were formerly conducted with the "tube dip" method were subsequently

reanalyzed by this transmission DART-MS method. The LOD and LOQ of this method were determined to be 0.655 and 1.559 respectively.

The results obtained for the transmission DART showing the lactate levels over time for the two cultures of *E. faecalis*, the mono- and co-cultures of *E. faecalis* and *L. johnsonii*, and for the mono- and co-cultures of *E. faecalis* and *L. rhamnosus* are shown in Figures 27, 28 and 29 respectively.

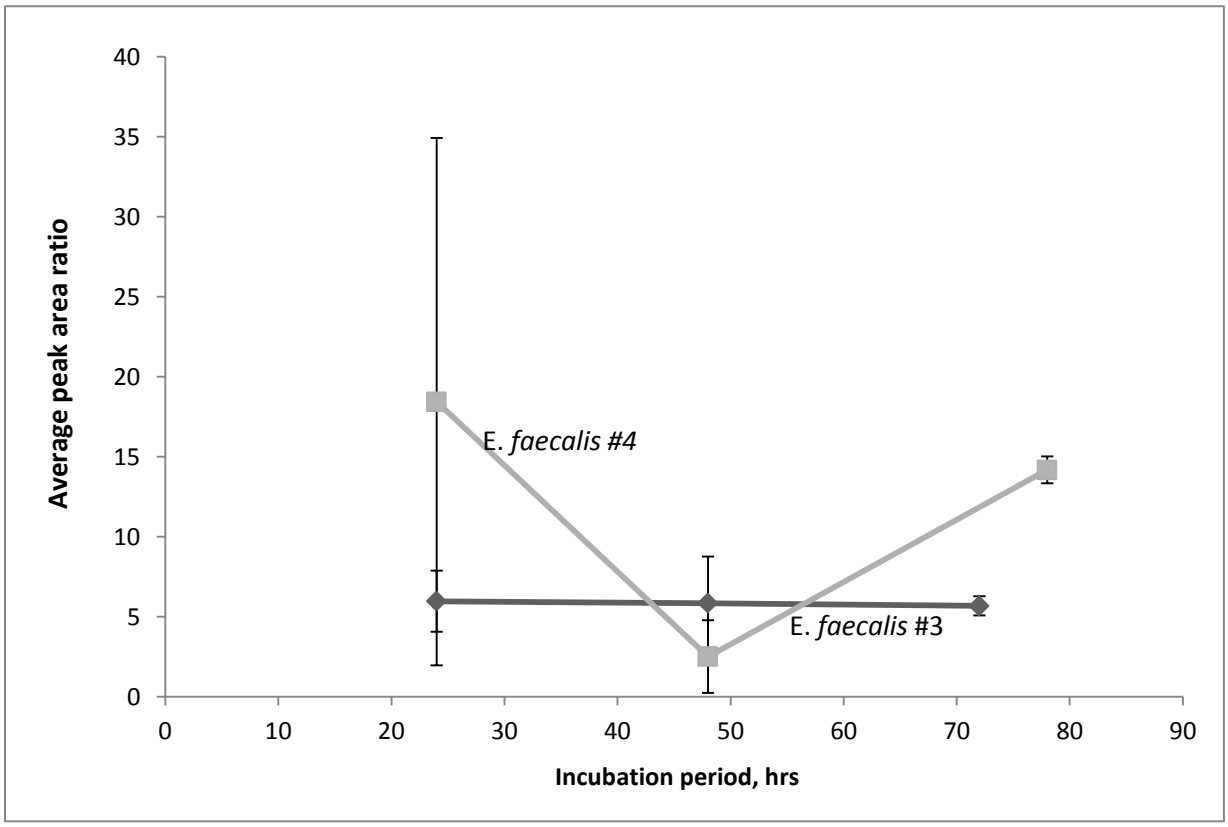

Figure 27. Lactate levels evaluated by transmission DART-MS in two cultures of *E. faecalis* aerobically grown in a bioreactor. The data points are the mean and the error bars are the standard deviation for triplicate determinations.

Table 7. The average peak area ratio and average standard deviation for the cultures *E. faecalis* #3 and E. *faecalis* #4 using transmission DART.

Organism	Incubation Time (hrs)	L/IS(Avg peak area ratio)	Avg. Std. Dev.
E.faecalis #3	24	5.97	1.91
E.faecalis #3	48	5.85	2.9
E.faecalis #3	72	5.68	0.59
E.faecalis #4	24	18.44	16.48
E.faecalis #4	48	2.51	2.27
E.faecalis #4	78	14.18	0.84

For the results shown in Figure 27, lactate can be quantified for *E. faecalis* #3 at all incubation times as judged by the comparison of the average peak area ratio values in Table 7 to the LOD and LOQ. The lactate in the culture of *E. faecalis* #4 at 24 and 78 hours, but not at 48 hours, is quantifiable. The average lactate level in culture media is fairly constant over time for one culture of *E. faecalis* (#3). However, there is a relatively low level of precision in the measurement of the 24 hr and 48 hr incubation. There is wide variation in the two measurements of the lactate level at 24 hr, so it is not possible to conclude anything about the level of lactate for *E. faecalis* #4 relative to that of *E. faecalis* #3. The measurement of lactate was conducted at 72 hr for #3 and 78 hr for #4, so it is difficult to compare the two samples in the amount of lactate produced even though the measurements were very precise.

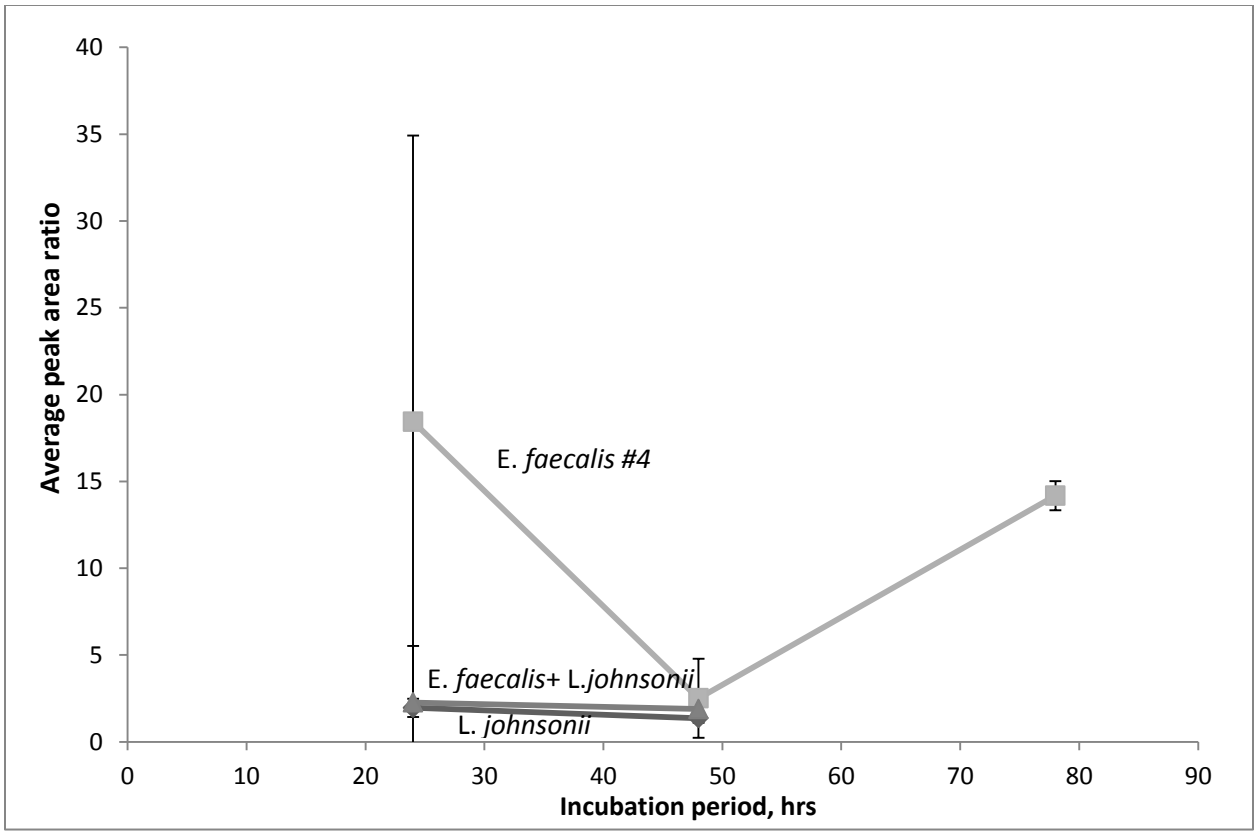

Figure 28. Lactate levels evaluated by transmission DART-MS in mono- and co-cultures of *E. faecalis* and *L. johnsonii*. The data points are the mean and the error bars are the standard deviation for triplicate determinations. The data for *E. faecalis* plotted here was taken from Figure 27 for *E. faecalis* #4.

Table 8. The average peak area ratio and average standard deviation for the cultures E. *faecalis* #4, *L. johnsonii* and co- culture E. *faecalis* + *L. johnsonii* using transmission DART.

Organism	Incubation Time(hrs)	Avg. L/IS	Avg. Std. Dev.
E.faecalis #4	24	18.44	16.48
E.faecalis #4	48	2.51	2.27
E.faecalis #4	78	14.18	0.84
L. johnsonii	24	1.96	0.53
L. johnsonii	48	1.36	0.27
E.faecalis + L. johnsonii	24	2.27	3.26
E.faecalis + L. johnsonii	48	1.89	0.39

Lactate levels were evaluated for mono-cultures and co-cultures of *E. faecalis* and *L. johnsonii* as shown in Figure 28. As shown in Table 8, the data pertaining to the co-culture of *E. faecalis* and *L. johnsonii* for lactate is not reliable as the standard deviation is greater than the signal at 24 hours. The co-culture at 48 hours of incubation is barely detectable and quantifiable. Lactate in the monoculture of *L. johnsonii* is barely detectable at 24 and 48 hours of incubation and cannot be quantified at 48 hours of incubation, as the average peak area value corresponding to it is below the LOQ value, and is barely quantifiable at 24 hours of incubation.

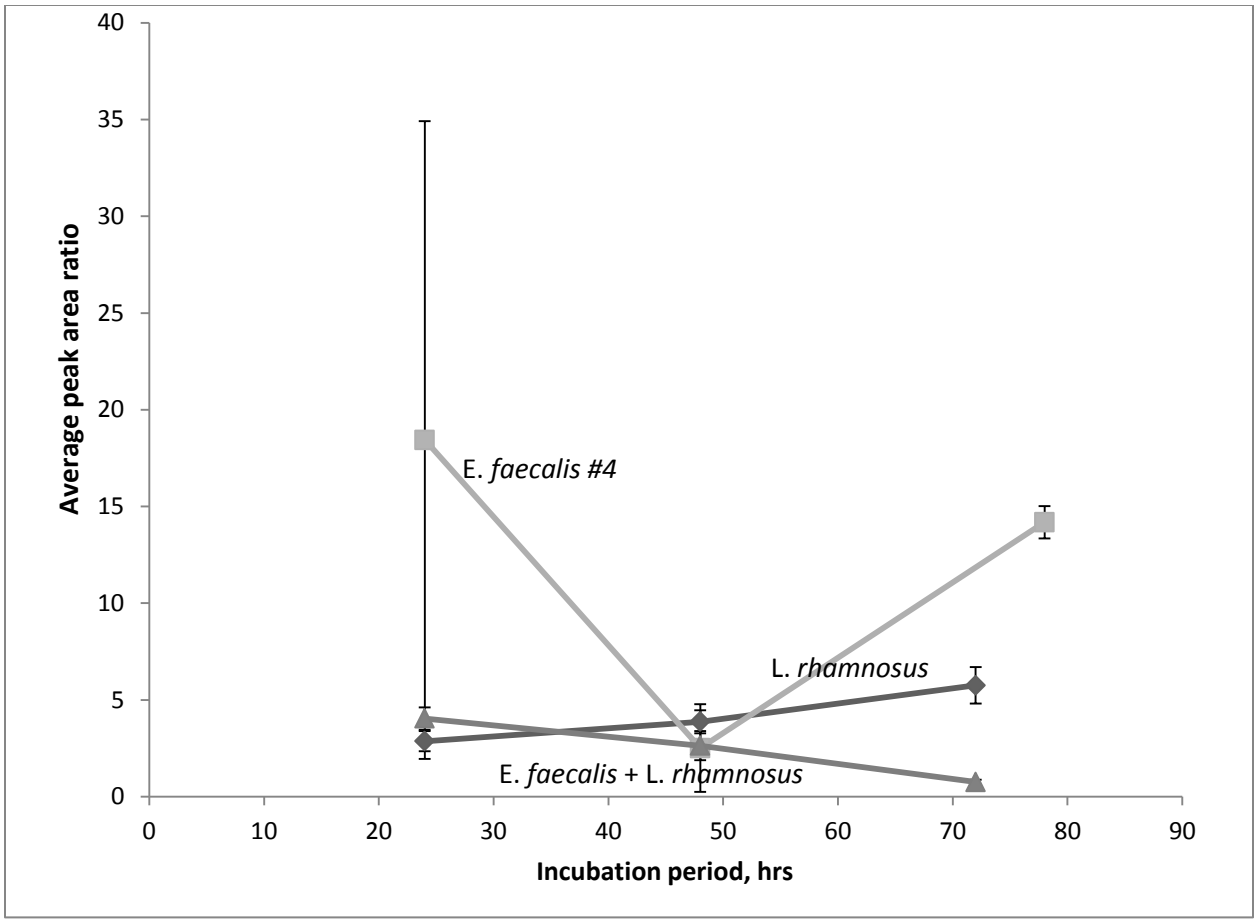

Figure 29. Lactate levels evaluated by transmission DART-MS in mono- and co-cultures of *E. faecalis* and *L. rhamnosus*. The data for *E. faecalis* plotted here was taken from the average of data for *E. faecalis* #4 in Figure27. The data points are the mean and the error bars are the standard deviation for triplicate determinations.

Table 9. The average peak area ratio and average standard deviation for the cultures *E. faecalis* #4, *L.rhamnosus* and co- culture *E. faecalis* + *L. rhamnosus* using transmission DART.

Organism	Incubation Time (hours)	L/IS (Avg.peak area ratio)	Avg. Std. dev
E.faecalis #4	24	18.44	16.48
E.faecalis #4	48	2.51	2.27
E. faecalis #4	78	14.18	0.84
L. rhamnosus	24	2.87	0.52
L. rhamnosus	48	3.87	0.59
L. rhamnosus	72	5.75	0.94
E.faecalis + L. rhamnosus	24	4.04	0.58
E.faecalis + L. rhamnosus	48	2.63	0.75
E.faecalis + L. rhamnosus	72	0.76	0.12

Lactate levels were evaluated in mono-cultures and co-cultures of *E. faecalis* #4, which was previously analyzed, and *L. rhamnosus* as shown in Figure 29. Using the data in Table 9 and the values for LOD and LOQ, it is possible to conclude that there are detectable levels of lactate in co-cultures *E. faecalis* and *L. rhamnosus* at 24 and 48 hrs of incubation, but the lactate level was barely detectable and not quantifiable at 72 hrs of incubation. The data shows that the monoculture *L. rhamnosus* can be detected and quantified at all time periods. The level of lactate in the co-culture at 72 hours of incubation was lower than that for either mono-culture.

Butyrate could not be detected in any of the mono- or co-cultures using transmission DART-MS.

CONCLUSIONS

The GC-MS assay for lactate provides reliable results and allows for the characterization of samples from different treatment groups that can easily distinguish two-fold differences between two groups, which is much less than what will be considered as significant in the model systems that will be evaluated. Unfortunately, the butyrate assay by GC-MS was found to be inconsistent. Further experiments will have to be done to determine whether the reagents used to derivatize lactate can also be used to derivatize butyrate. However, the more labor- and time-intensive lactate assay is undesirable for regular long-term measurement of SCFAs in biological samples. On the other hand, the use of sample media in DART- MS without substantial sample preparation makes this approach a much more convenient one in measuring SCFAs.

The "tube dip" method for analysis by DART-MS gave results that were somewhat better than by the transmission method, which employed application of the sample to screens. However, the transmission method led to more extensive drying of the sample and resulted in crystal formation on the screen most likely due to the salts in the media. Acidification and extraction of the sample would yield a preparation with less salt and hence possibly less variable results. Regardless of which method for DART-MS yields more reproducible results, DART-MS is more desirable in that it is a good deal less labor and time-intensive than GC-MS methods.

From the results of the earlier research group members (28) and from the DART- MS results, lactate production occurs in all cultures of organisms tested. Unfortunately, the butyrate production when analyzed by DART- MS at any other time period but 24 hours is significantly low to the point of not being measurable.

In the present study, it was found that co-culturing *E. faecalis* and *L. rhamnosus* for 24 hours gave significantly less lactate and significantly more butyrate than with either organism alone as observed by the "tube dip" method by DART-MS. The results are similar to those that were previously obtained by Duncan et al (29), who showed that co-culturing a lactate and butyrate producer gave a substantially greater amount of butyrate and no lactate when compared to the levels measured in mono-cultures of the lactate producer and butyrate producer (29). The big difference between the studies is that two lactate producers were used in the present study. The other important observation is that low amounts of butyrate relative to lactate were measured in the present study as judged by the relatively low intensity of the ions detected for butyrate. In the former study (29), the level of butyrate produced in the co-cultures was similar to the level of lactate produced in mono-cultures, suggesting a stoichiometric conversion of lactate to butyrate in co-culture. It is important to note that those experiments were conducted under anaerobic conditions. The experiments in the current study were conducted in an aerobic environment of the bioreactor. It is envisioned that a greater amount of butyrate may be produced when one of the lactate producers in the current study is replaced with a butyrate producer.

The discovery that two organisms gave greater amounts of butyrate than either one alone suggested that the two organisms worked together by a mechanism by which the lactate producer provided the butyrate producer lactate, the precursor to butyrate (29). However, Duncan et al. did not look at other parameters, like cell density and pH, which might have indirectly caused this change in butyrate production. These parameters will need to be monitored in our future studies. Moreover, any model system that is used should incorporate features that are prevalent in the gastrointestinal tract, including an anaerobic environment, continuous flow of media through the system, and the ability to produce bio-films, which are carbohydrate-filled layers that

are created by bacteria. The most effective system that can provide for these features is currently the bio-reactor.

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