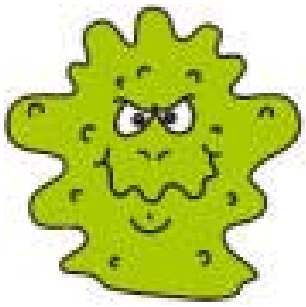


16S rDNA Terminal Restriction Fragment (TRF) Pattern Analysis of *Clostridia* Species in Feces of Volunteers Administered Antibiotics Alone and in Conjunction with Probiotic Bacteria

By: Litz Aguilar and Leisah Burrow

This paper is submitted in partial fulfillment of the graduation requirements of Cal Poly, San Luis Obispo

Abstract

Ingesting probiotic bacteria has become common practice in the United States. Most commonly used probiotic strains are from the genera *Bifidobacterium* and *Lactobacillus*. These genera have been used extensively in the food industry and have an established history of being safe for consumption. Physiologic effects of probiotic consumption may be inferred by observing changes in the fecal flora. In particular, the levels of *Bacteroides* and *Clostridium* species in the feces are commonly altered by probiotic ingestion. The goal of this study was to evaluate the effect of probiotics on *Clostridium* species in volunteers administered antibiotics. Fecal samples were collected from volunteers at the start of the study and after all subjects had finished a 7-day course of Augmentin. Half of the subjects also consumed a mixture of probiotic bacterial strains during the 7-day antibiotic course; the other subjects were given a placebo. Fecal samples were analyzed by DNA extraction and PCR with *Clostridium coccoides* sub-group specific primers. PCR products were digested with a restriction endonuclease, and species distribution was analyzed by Terminal Restriction Fragment Length Polymorphism (TRF) using capillary gel electrophoresis. After antibiotic treatment, the relative proportion of species represented by TRF peaks had not changed significantly, although one of the less abundant TRFs decreased in relative abundance. Consumption of probiotics during antibiotic treatment had a mixed effect on the stability of species distribution after antibiotic treatment.

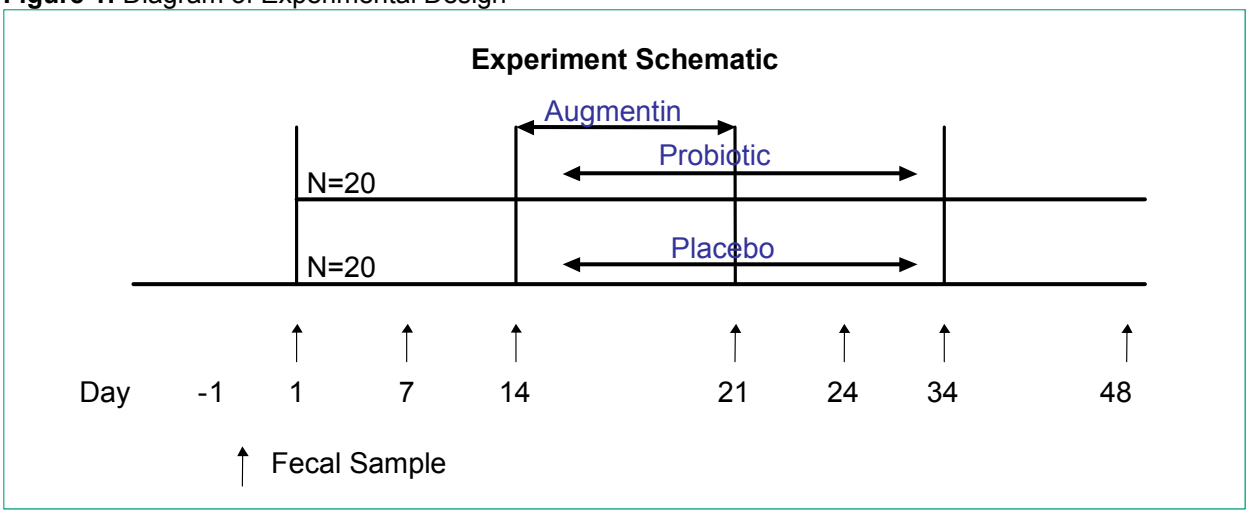
Introduction

Probiotics are found in a variety of foods and supplements, especially in yogurt, which usually contains live cultures of *Lactobacillus*, a common probiotic genus (1). The physiological effects of probiotics are not well established, and clinical uses for probiotics are still under investigation. Probiotics may provide various benefits for intestinal health. For example, it is anticipated that probiotics may stabilize the normal communities within the gut.

A major effect of antibiotic treatment is the disruption of native gut microflora. These microflora offer a natural protection against intestinal pathogens. This study was designed to evaluate the effect of probiotics on the fecal flora of people taking an antibiotic. One of the objectives was to determine the impact of probiotic therapy during and after antibiotic therapy on fecal bacterial communities. The antibiotic administered in this study was Augmentin (amoxicillin and clavulanic acid) and the test product was a capsule containing a dried bacterial preparation of probiotic bacteria in the genera, *Lactobacillus* and *Bifidobacterium.*Members of the genera Clostridiae are gram-positive, spore-forming, anaerobic bacilli. The *Clostridium coccoides* group, which forms one of the largest groups within the *Clostridium* sub-phylum, is part of the indigenous microflora of the intestine. This group's rRNA cluster contains a vast consortium of organisms with a variety of phenotypes, including almost 20 genera (1). According to estimates, the *C. coccoides* group constitutes up to one-half of the fecal bacterial population (2). Thus changes in the population structure of these organisms could have important health effects. TRF analysis is a rapid way of estimating populations structure and PCR primers directed to the *C. cocciodes* group are in the literature (2).

The clinical portion of this study was conducted over 48 days. There were a total of 40 healthy patients, 20 of which took the antibiotic and probiotic, and 20 of which took only antibiotic with a placebo. During this time period, fecal samples were taken starting at day 1. Three baseline (no treatment) fecal samples were obtained at days 1, 7, and 14. All subjects then took a 7-day course of Augmentin. Fecal samples were also collected at day 21, 25, 34, and 48. On day 14, one group starting taking a probiotic capsule, the other group a placebo. Probiotic and placebo treatment continued until day 34. See Figure 1. In our analysis, we only obtained data from a subset of the 40 patients, four people on probiotics, and three people on placebo.

Figure 1: Diagram of Experimental Design



Materials and Methods

Extraction of bacterial DNA

Fecal samples were collected from 40 healthy adult subjects divided into two groups of probiotic and placebo (20 each). Samples were collected and delivered to the hospital within 8 hours of collection, and aliquots of 3 grams were stored at -80° C. Samples were extracted using the MoBio Ultraclean® soil DNA kit following manufacture's protocol. Success of each extraction was determined by measuring DNA concentration in the extraction product with a Spectramax spectrophotometer.

PCR Amplification

PCR was performed using 16S rDNA primers homologous to highly conserved regions on the 16s rRNA gene. The reverse primer CcocR (5'-AAG CGT TCT TAC TTT GAG TTT C-3'), and the forward primer CcocF (5'-AAA TGA CGG TAC CTG ACT AA-3'), which was fluorescently labeled with a phosphamide dye, was used for each reaction. Reactions were carried out using 1 µL of 10x Buffer, 3 µL of 10 mM dNTP, 2 µL 20 µg/mL BSA, 7µL 25 mM $MgCl_2$, 1.25 µL CoccR, 1.25 µL CoccF, 29.7 µL PCR water, and 0.3 µL 5 U/µL TaqGold®. Reaction temperatures and times were 92°C for 10 min; 30 cycles of 92° C for 30 sec, 50°C for 20 sec, 72°C for 20 sec; and 72°C for 10 min. All reactions were performed in triplicate and then combined using a MoBio Ultraclean® PCR Cleanup Kit following manufacture's protocol. Amounts of DNA in each sample were again determined using Spectramax spectrophotometer.

Enzyme Digest and TRF Pattern Generation

An enzyme digest was performed on each PCR cleanup product using the New England Biolabs restriction endonuclease HaeIII. Each 40 µL digestion used 75 ng of

DNA, 1 U of enzyme, and 4 µL of buffer. The samples were digested for 4 hours at 37° C and inactivated for 20 min at 65° C. The digestion products were ethanol precipitated and resuspended in 20 µL of formamide and 0.25 µL of CEQ 600 base pair standard. Terminal restriction fragment profiles were obtained using Beckman Coulter 8000x DNA Analysis system. TRF peaks were identified by matching to a sequence database.

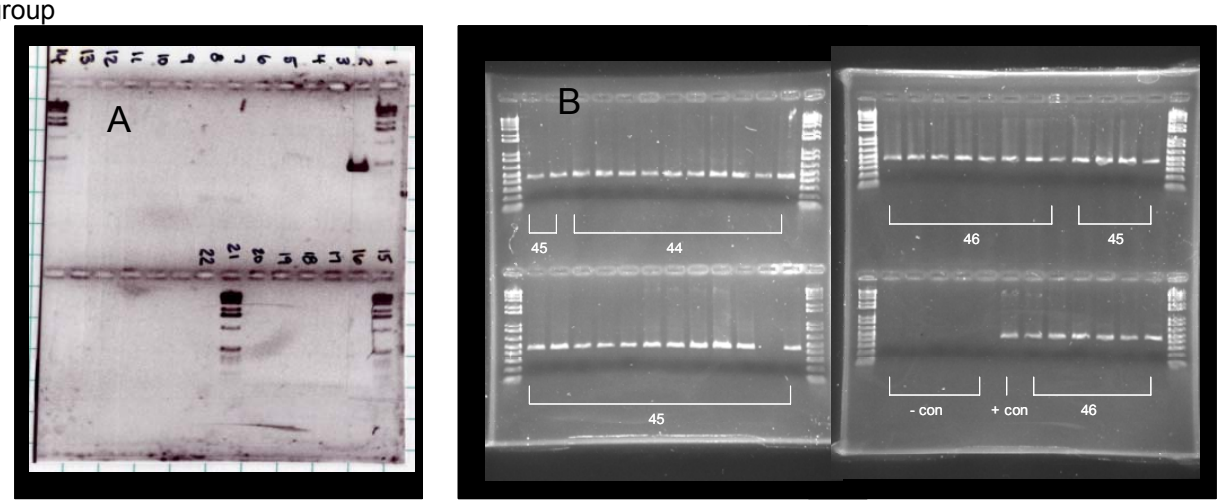
Data Analysis

Terminal restriction fragment (TRF) patterns are a practical way to categorize bacterial communities. The method can be used to identify both species dominance and species richness within the samples (Clement, 1998). Generation of a TRF pattern involves extraction of DNA from a sample, PCR using a labeled primer, digestion with a restriction endonuclease, ethanol precipitation to clean up the digestion product, and use of a capillary gel electrophoresis system to generate the TRF pattern. This pattern is then analyzed using a variety of statistical techniques. From the pattern, the different phylotypes of bacteria present in each sample can be identified.

Results and Analysis

The first step of our research began with the optimization of the group primers. To accomplish this we attempted several PCR conditions, varying [$MgCl_2$], BSA, and dNTP concentrations. We also applied a temperature gradient in order to determine the correct annealing temperature for the primers. Once all the conditions were working satisfactorily, we began to analyze the primers on positive and negative controls(See figure 2A). Once the positive and negative controls were confirmed by PCR and gel electrophoresis, we began to use the primers on our patient subset. (See figure 2B)

Figure 2. A) PCR with *C. cocciodes* primers on control organism DNA
B) Example of labeled PCR of fecal samples from the placebo group



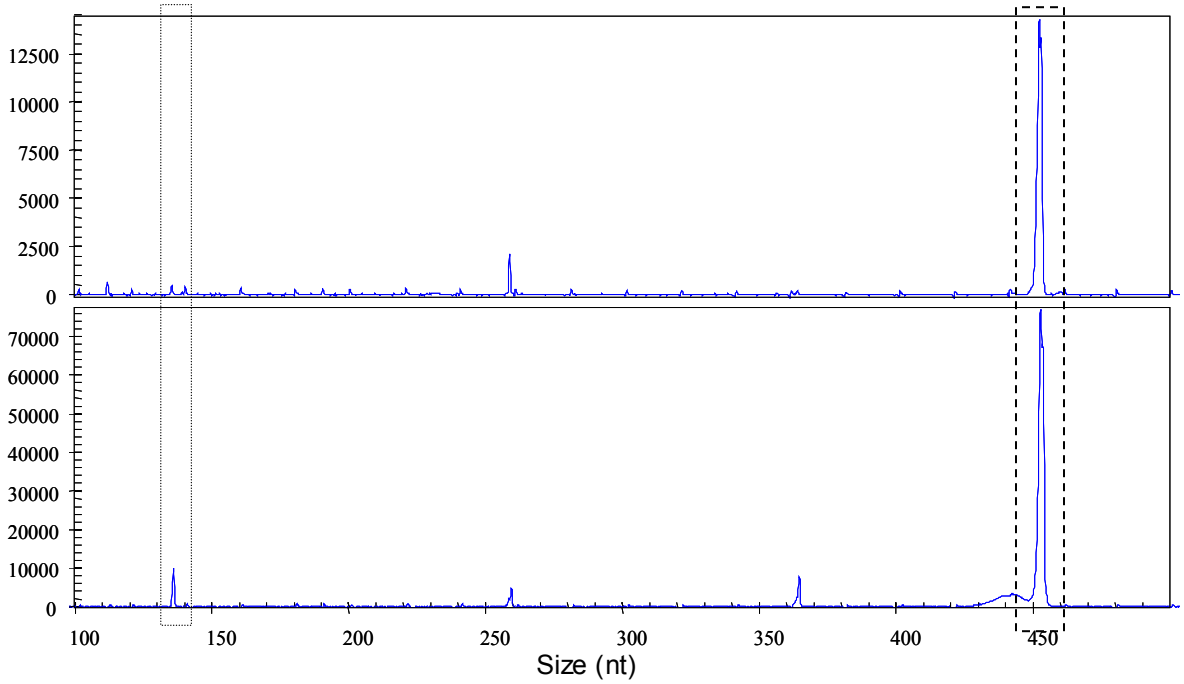
+/- *clostridium* controls

Placebo group persons 44,45,46
(Shown in duplicates and triplicates)

The method we used to analyze our results was Terminal Restriction Fragment Length Polymorphism (TRF). This is a PCR-based tool that allowed us to study the phylotypes that were represented within our patient samples. Figure 3 shows two different TRF patterns from the patient subset. Statistical analysis of the TRF patterns follow in figures 4 and 5. The fragments we chose to analyze were fragment sizes 111-

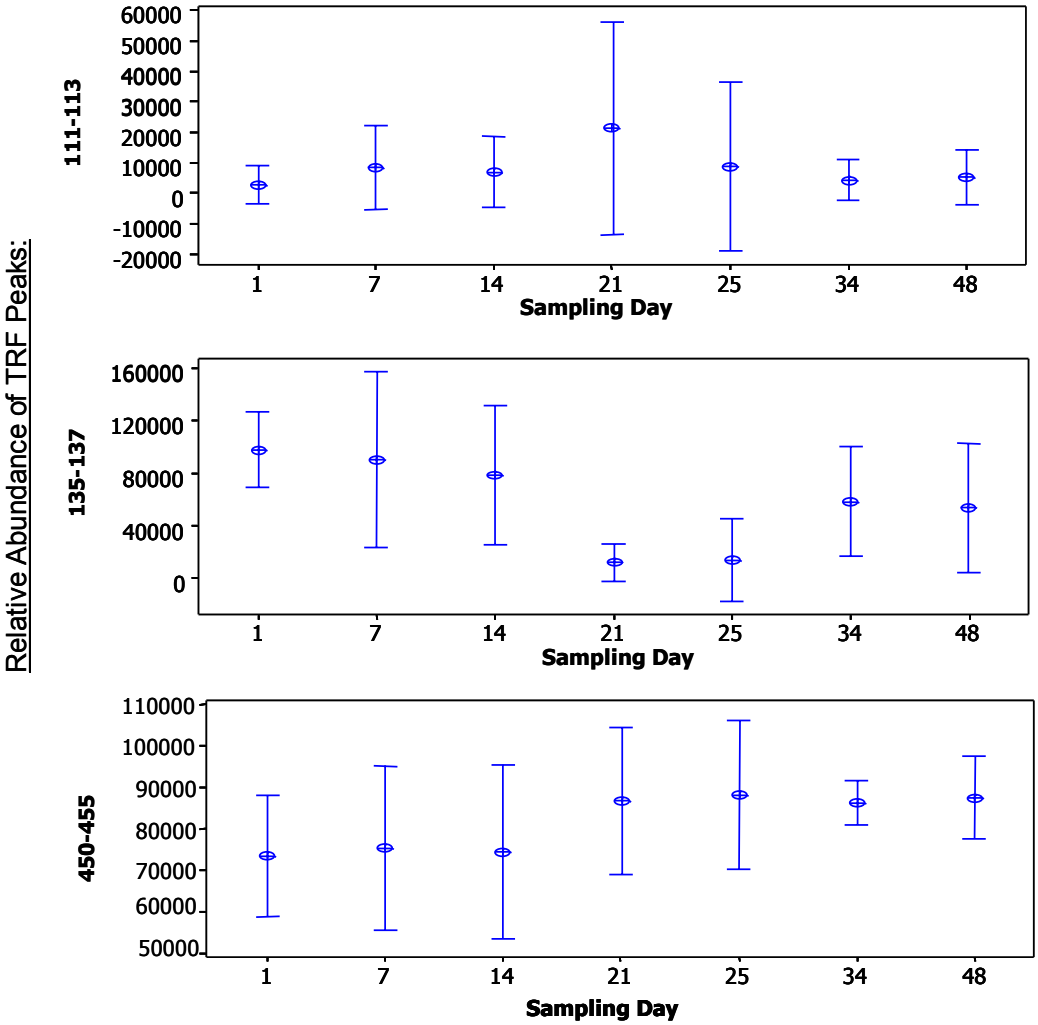
113 nt., 135-137 nt., and 450-455 nt. We chose to analyze these particular fragments because they were the most recurring peaks in most of the patterns.

Figure 3. Example TRF Patterns indicating TRF groups used for further analysis.



One of the types of statistical analysis we applied was relative abundance. TRF peak 135-7 exhibits a normal clostridia population distribution up to day 14. Once the antibiotic was administered, a noticeable drop in this distribution occurred at day 21. There was a return to normal distribution levels beginning on day 34. We also notice that the variation decreased on day 21. For Peaks 111-3 there was a slight increase in population distribution beginning at day 21, and then a rapid return to normal level. However there was a large increase in variation of the population at day 21 and 25. Peaks 450-55 showed a wide range of population distribution due to the undigested DNA fragments (Figure 4).

Figure 4. Relative abundance of TRF groups over time in the study. Error bars represent 95% confidence intervals.

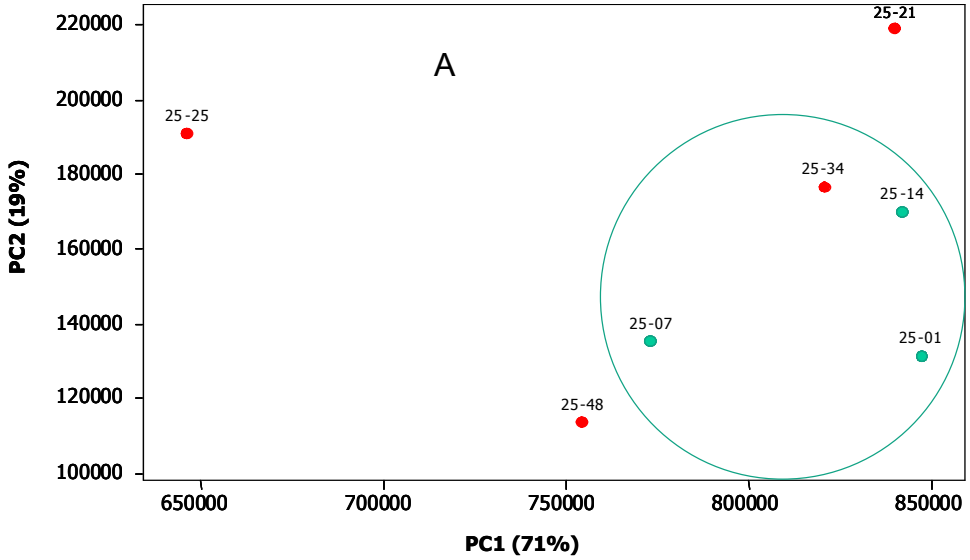


In figure 5, principle component analysis (PCA), we begin to see the effects of using a probiotic in conjunction with an antibiotic. The circle on the figures represent the normal range distribution of intestinal flora, this is an estimation of where the normal range of intestinal flora is expected to return after taking the probiotic treatment. We specifically chose patients 25 (probiotic group) and 46 (placebo group) because they showed a good representation of the effect of probiotic/ placebo treatment. As showed in fig 5A, subject 25 at day 34 returned within the expected normal range distribution. Where as in fig. 5B, a placebo was administered instead of a probiotic. The subject

never returned to normalcy, meaning that in the days following the first three base-line days, the subject never re-entered into the normal range distribution.

PC1 and PC2 represent first two largest measures of variation in the population being analyzed. Fig. 5a and 5b, both exhibit that most of the variation in these populations are occurring in PC1. In “A” PC1 is 71%, where as PC2 is 19%. In “B” PC1 is at 86% and PC2 is at 8%. Thus the variation is mostly all within the PC1 component.

Figure 5. Principal Components Analysis of TRF groups in individual subjects during the study.
A) Probiotic subject #25 showed a return to pre-antibiotic population structure by day 34.
B) Placebo subject #46 showed an incomplete return to normal population structure by day 34



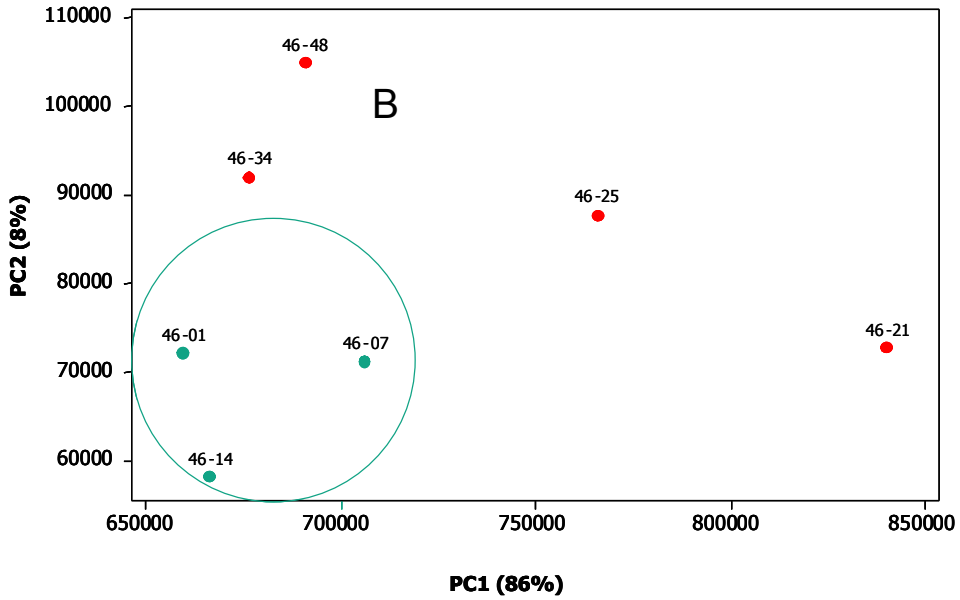


Table 1 shows an overall summary of the patient subset, as determined by PCA. The data at this point is inconclusive due to the small size of the subset, and incomplete data for some patients.

Table 1. Summary of return to normal population structure after antibiotic treatment, as shown by PCA. Days with no data, "nd"; days with different population structure, "–"; days with a somewhat similar population structure "+"; days with a very similar population structure, "++".

Probiotics	Day 21	Day 25	Day 34	Day 48
5	–	nd	–	nd
6	+	–	nd	nd
24	nd	nd	–	++
25	+	–	++	++
Placebo				
44	+	–	–	nd
45	–	nd	–	–
46	–	–	+	+

Discussion

All TRF patterns showed dominance of TRFs at 450-55 nucleotides. This represents PCR products that did not contain the *Hae*III cut site (GGCC). There are two explanations for this: either many different types of clostridia are represented in these few peaks, or human gut clostridia are very similar. The latter choice is not supported by the literature based on culturing clostridia from human feces (1). Thus, TRF patterns using this enzyme do not present an accurate representation of the diversity of clostridia in the human gut. In spite of this drawback, some of the less abundant types of clostridia (TRF group 135-7) showed a decrease in relative abundance after antibiotic

treatment. Yet another low abundance TRF group (111-13) showed an increased variation in relative abundance after antibiotic treatment.

More of the subjects in the probiotic group showed a return to normal clostridium population structure after 48 days of the study than subjects in the placebo group. Because of the low number of subjects analyzed thus far, this result is inconclusive at this point.

Conclusion

There are many ways in which to continue the direction of this research. First is to choose a new enzyme to digest the 16S rRNA PCR product to better represent the expected diversity of clostridia in the human gut. We might also look into a different gene, other than 16S rRNA, as a target for PCR that will give a better representation for the diversity of clostridia. Also to look for another statistical method for determining return to a normal population structure other than PCA. Of course analyzing the rest of the samples from this study (there were a total of 40 subjects) is imperative for a conclusive result.

There was also one small thing we failed to catch during the duration of our Sr. Project. A factor that may have been the cause of many months of excruciating frustration of optimizing primers, is that the reverse primers were ordered backwards. Therefore the target sequence was off, and the primers never should have been able to be optimized. However the primers were optimized, beautifully, if you noticed the gels at the beginning of the results section, ahem. So, whether or not all this data should be thrown in the trash, will be left to individual discretion. Editors note: when the corrected primers were used the results were the same – with fragments 10 bp smaller – very strange…

Reference

1. Collins M.D., Lawson P.A., Willems A., Cordoba J.J., Fernandez-Garayzabal J., Garcia P., Cai J., Hippe H., Farrow J.A. 1994. The Phylogeny of the Genus Clostridium: Proposal of Five New Genera and Eleven New Species Combinations. International Journal of Systematic Bacteriology. 44(4): 812-26

2. Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., and Tanaka, R. 2002. Development of 16S rRNA-Gene-Targeted Group-Specific Primers for the Detection and Identification of Predominant Bacteria in Human Feces. Applied and Environmental Microbiology 68: 5445-5451.

Acknowledgements

There are many people that helped us through this project whom we would like to thank:

Anna Engelbrekston, she may be loud and intense at times, but she was also the driving force of this project. Without her, we wouldn't have even had a project or the opportunity to work with an incredible lab. She spent countless hours with us in training and explaining, and she never once tried to strangle us for screwing up (which we rarely did anyways). It was a pleasure to work with her, and we gained valuable knowledge and experience due to her expertise and patience.

Dr. Kitts, although he gives horrible exams in microbial physiology, he redeemed himself by giving us free pizza. He is one of the most incredible professors we have had the honor to learn from. He can be a little aloof at times, but he used to be a physics major, which explains it. He spent many hours helping us analyze our data, re-explaining the data, and editing our abstracts, grants, and poster. He is extremely dedicated to his students and allowed us a lot of freedom in the project, to just think and flow from one part to the next. His commitment and devotion was a guidance throughout the year.

The labbies of EBI, without you, the overall experience would not have been complete. There was a lot of laughter and support, all of which helped us to get though our last year of college. The memories will be with us always.

And lastly, the PCR Gods. Once you learned how these Gods like to be worshiped (by standing on your left leg, and hopping around in small circles while repeating the mantra "please work"). If you do this and everything else correctly, the PCR Gods will *usually* be in your favor.