16S Ribosomal RNA Gene Terminal Restriction Fragment Pattern Analysis Used to Asses the Stability of Bacterial Communities found in the Human Gastrointestinal Tract

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ABSTRACT

It has long been known that antibiotics can rapidly alter the population of the human gastrointestinal tract. Evaluation of the effects of probiotics has solicited base line data regarding the stability of microflora in healthy individuals. This study sought to examine the consistency subject's gastrointestinal flora using 16S rRNA gene Terminal Restriction Fragment (TRF) patterns on prokaryotic DNA extracted from feces. The data was intended to contribute to prior investigations utilizing the same methodology. The subject's gastrointestinal flora was considered stable over a two week period after analysis of TRF pattern data using Bray-Curtis similarity.

INTRODUCTION

Averaging as much surface area as a tennis court, it is no surprise that the human gastrointestinal tract harbors 10^{14} organisms representing greater than 400 species [1,2] Members of different genera and species are present in the gastrointestinal tract in amounts ranging from transient and minute to prevalent. The most common bacteria found in the large intestine include *Bacteroides, Bifidobacterium*, *Eubacterium*, *Enterococcus*, and the Enterobacteriacea which includes the infamous *Escherichia coli*. Although *E.coli* tends to be the hallmark coliform, it is dwarfed by the anaerobic *Bacteroides* spp. in both incidence and predominance in the large intestine. Following in a close second, anaerobic lactic acid bacteria from the *Bifidobacterium* genus are also well represented.[3] Many factors can affect the composition of flora in the intestine including host age, immune status, and diet but it is believed that the populations of different species within an individual are fairly stable.[2]

Favorable relationships between microorganisms and human hosts were first described in literature in 1892 in reference to lactic acid bacteria and the vagina. Shortly after, fecal ecology studies emerged.[4] Bacterial flora of the intestine have since been credited with conferring many benefits to their host including: synthesis and excretion of vitamins that are absorbed as nutrients by the host, aiding in digestion, maintenance of healthy immune stimulation, supposed anti-tumor properties and perhaps most notably preventing colonization by pathogens.[2,3]

The benefit of resistance to colonization by pathogens can occur either by synthesis of a compound that is antagonistic to non-resident microbes (i.e. acidity), or simply mechanical blockage and competition for nutrients. Resistance to colonization is illustrated by cases of

pseudomembranous colitis, or antibiotic-associated gastrointestinal disease. When a host is given antibiotics that inadvertently decimate the existing micro flora, existing pathogenic bacteria can flourish, or an arriving pathogenic bacterium is given the opportunity to colonize. In both cases disease can result from the pathogens' colonization. Such is the case for *Clostridium difficile*, which is present in the intestine of some healthy people and not at all in others. After taking antibiotic therapy -which lowers the quantity and diversity of the flora- small numbers of existing antibiotic resistant *C.difficile*, or newly acquired bacteria, are permitted to thrive. As the *C.difficile* population increases the cells begin to release greater amounts of enterotoxin and cytotoxin which then cause the symptoms of the disease. [5]

It is incidences such as that with *C.difficile* that are a focus of probiotic therapy and perhaps the motivation of studies concerning intestinal flora stability. Probiotic therapy is defined by the World Health Organization as, "Live microorganisms which when administered in adequate amounts confer a health benefit on the host." [6] Probiotics seeks to replace the previous microflora destroyed by antibiotics with 'friendly' bacteria that will provide the same benefits. This particular investigation has evolved from a clinical study in the Environmental Biotechnology Institute, at California Polytechnic State University, San Luis Obispo (EBI) concerning probiotics. The previous study analyzed the effects of dietary probiotic bacterial supplements on human gastrointestinal microflora after antibiotic treatment. Unfortunately, the study was complicated by a limited amount of normal flora data to analyze the results. In the study 40% of the subjects appeared to have unstable microflora according to data gathered from three control samples even before treatment began (**Figure1**). There were two distinct distributions of Bray-Curtis similarity for subjects in the study; a set above fifty percent similarity and another set below fifty percent similarity. The 40 % of the subjects deemed unstable were found in the distribution set below fifty percent similarity and those individuals above fifty percent similarity were considered stable. It was clear that more data concerning the stability of healthy individuals was needed to asses the effect of antibiotics and probiotics.

With a community of microbes perhaps more diverse than your local zoo and trillion times the magnitude, examining changes in the population diversity on any given day can prove to be an insurmountable task. Fortunately, feces have about 10^{11} organisms per gram making it quantitatively similar to that of the large intestine and much easier to access. [3, 5] Traditional methods of studying populations of organisms were based on phenotypic data such as gram

reaction and biochemical testing, and plate counts. While these methods have their strengths, when faced with such a huge and variant population are quite cumbersome and unreliable. Thankfully, molecular genetics has revolutionized this process with the arrival of the 16S ribosomal RNA sequence analysis, which was utilized for this study. [2]

The objective of this analysis was to determine if this subject could be deemed stable within the guidelines set by the previous analysis. Stability was defined as having 50% or greater similarity between specimens from two weeks of fecal sampling.

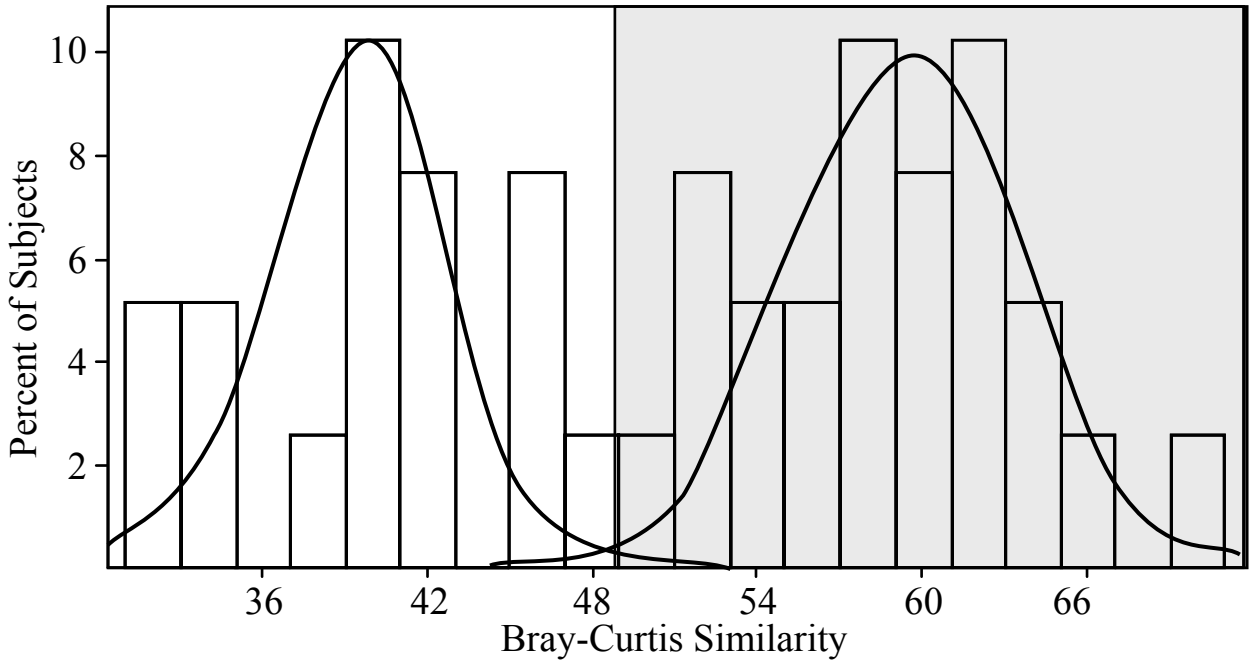

Figure 1. Average Bray-Curtis similarity of TRF patterns from each subject across the three initial samples (days 1, 7, and 14). Note the two distributions. An estimated cut-off for Stability was made at >50% similarity. Subjects with average similarity during their first three sampling days were deemed stable.

MATERIALS AND METHODS

Collection of Fecal Samples

Fecal samples were collected from one healthy adult female over a fourteen-day period. It should be noted that the subject had taken Ciprofloxacin, an antibiotic effective against Gram negative and positive organisms, 35 days prior to collection.

Samples were collected in the subject's home and then stored at 0 °C for no more than five days until DNA was extracted in the laboratory. Samples were collected using a catching device specially designed to avoid urine and toilet water contamination (Appendix 1).

Extraction of Bacterial DNA

Samples were extracted in triplicate using the MoBio Powersoil® DNA kit following manufacture's protocol. Success of each extraction was determined by agarose gel electrophoresis.

PCR Amplification

PCR was performed using 16S rDNA primers homologous to Eubacterial conserved regions on the 16S rRNA gene. The reverse primer 536-K2R (5'- GTA TTA CCG CGG CTG CTG G-3'), and the forward primer 46-Ba2F (5' GCY TAA CAC ATG CAA GTC GA-3'), which was fluorescently labeled a with phosphamide dye, were used for each reaction. Reactions were carried out using 1 µL of a 10-1 diluted extraction product, 5 µL of 10x Buffer, 3 µL of 10 mM DNTP, 2 µL 20 µg/mL BSA, 7 µL 25 mM MgCl2, 1µL K2R, 1 µL Ba2FD4, 29.7 µL water, and 0.3 µL 5 U/mL TaqGold®. Reaction temperatures and times were 94 °C for 10 min; 30 cycles of 94 °C for 1 min, 46.5 °C for 1 min, 72 °C for 2 min; and 72 °C for 10 min. Success of obtaining PCR product was ensured through agarose gel electrophoresis. All reactions were performed in triplicate and then combined using a MoBio Ultraclean® PCR Cleanup Kit following manufacturer's protocol. Amplicon concentration of each sample was determined by A260 UV fluorometer following EBI protocol.

Enzyme Digest and TRF Pattern Generation

An enzyme digest was performed on each PCR cleanup product using the New England Biolabs restriction endonuclease *Hae*III. Each 40-µL digestion used 150 ng of DNA, 1 U of enzyme, and 4 µL of buffer. The samples were digested for 4 h at 37 °C and inactivated for 20 min at 80 °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 (TRF) profiles were obtained using a Beckman Coulter CEQ 8000X DNA analysis system.

Data Preparation and Statistical Analysis

Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQ8000 into Microsoft Excel. TRF peaks representing less than 1.0% of the total DNA presenting the sample were considered not significant excluded from analysis. TRF peak area was converted to relative abundance (percent of total area) and analyzed with a Bray-Curtis similarity measure[8]. Statistical analyses were performed using PrimerV and Excel. [9]

RESULTS AND DISCUSSION

Fecal samples were collected daily, for period of fourteen days. Fecal consistency was evaluated and recorded but it was not found to be useful for any relevant extrapolations from the TRF patterns. TRF patterns are groupings of fragments of DNA based on length (in nucleotides) and quantity after the *Hae*III restriction digests were performed. The TRF patterns for each sample were produced and evaluated for similarity. The TRF patterns, although not yet quantitative data, allude to the overall similarity of each day's DNA extraction

Bray-Curtis Similarity Analysis

To compare each sample's TRF pattern to its surrounding samples' TRF pattern, a Bray-Curtis similarity matrix was created by converting TRF peaks into numerical data based on the individual area. The percentages are obtained by analyzing the area of each peak relative to the sum of all the other peaks in the analysis in a pair-wise fashion. For continuity, sample 14 was then compared to sample 1 to asses the progression through all 14 samples as well as the progression of the entire 14 day period. A graphical representation of this data displays the sequence of sample similarity in day-to-day order using Bray-Curtis Similarity (Figure 2).

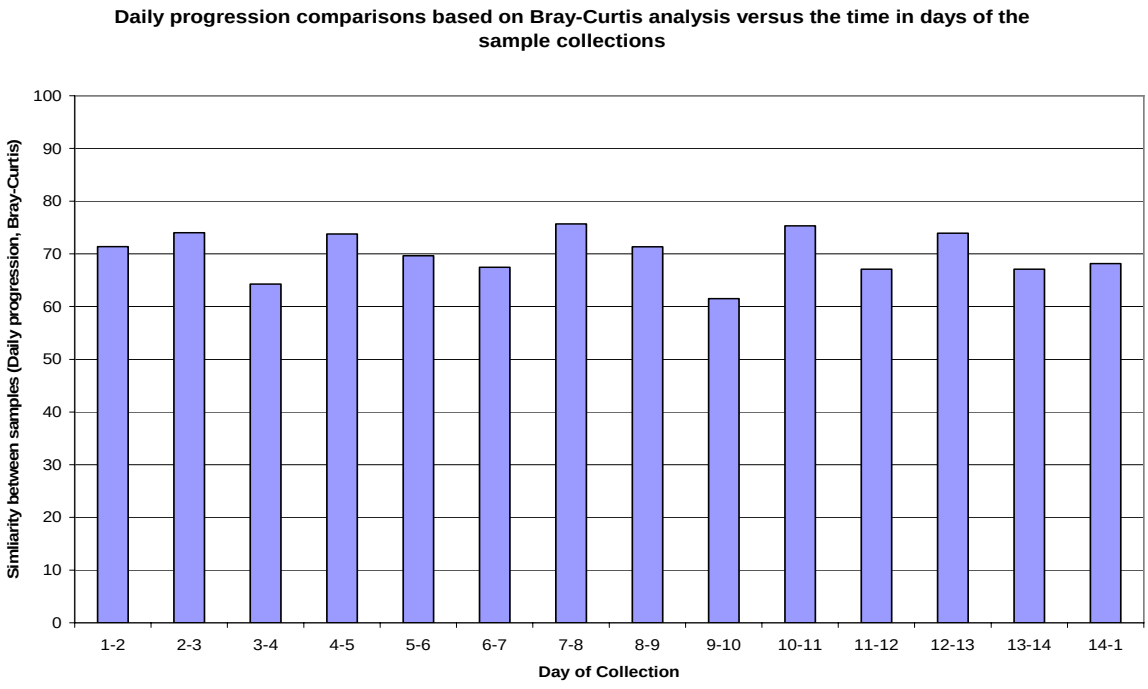

Figure 2. Daily progression chart indicating the percent similarity (produced using Bray-Curtis Analysis) of each consecutive sample's TRF pattern.

The average sample to sample similarity, excluding sample 14 to sample 1 is 70.20%. The average similarity between all 14 samples is 63.1%. Samples 9 and 10 were the least similar consecutive samples at 61.50% and samples 7 and 8 were the most similar consecutive samples at 75.69%. The three most divergent samples (4, 9, and 13) had an average similarity of 48.0% which fell below the stability cut off of 50%. These findings, although still qualifying as stable, are on the lower end of the data collected from four other subjects using the same methodology. The previous subjects' average similarity between all 14 samples were 65.7%, 75.8%, 69.8% and 63.4%.[7, 10, 11] Using this data the average similarity among all five subjects is 67.6% with a 95% confidence interval 62.9% to 72.2%

Bray-Curtis Similarity Dendrogram:

A dendrogram was created in PrimerV using the Bray-Curtis similarity data (**Figure 3**). The dendrogram organizes samples into clusters of similarity while separating samples of dissimilarity. Rather than simply showing day to day comparison, the dendrogram places all of the samples in relationship to one another. Those more alike are combined together closer to the bottom at a higher percent similarity, while those that are less alike appear individually. In this representation the samples appear to be clustered in three groups with samples 9 and 4 being very dissimilar from not only their consecutive samples but from each other. Of the three clusters, the samples within them are not clustered consecutively; meaning sample 8 is more similar to sample 10 that it is to sample 7. There is no obvious progression of dissimilarity among the samples, but rather an oscillation around a three separate 'normals.' The dissimilarity of samples 4 and 9 from the group can potentially be correlated to the food intake and stress journals that were kept during the collection period. While effect of stress on fecal flora is questionable; sample 4 was collected the day after the subject's Quantitative Analysis Final Exam. In this instance it might not necessarily be the stress that contributed to the variance of in micro flora but perhaps because the nervous subject did not consume much food on the day of the exam or the day prior. Peculiarly enough, sample 9 was collected the day after the subject-who normally eats at home- had dinner at a local restaurant. This could have contributed to the variance not only by the ingestion of different organisms than present in home-cooked food, but the different type of food that might have contributed to observance of a longer retention of the fecal sample. This is not to say that there were not other issues that could have

contributed to the dissimilarity of these and other samples (such as the consumption of left-over chicken soup of questionable integrity) but these two instances are mentioned because samples 4 and 9 were quite dissimilar.

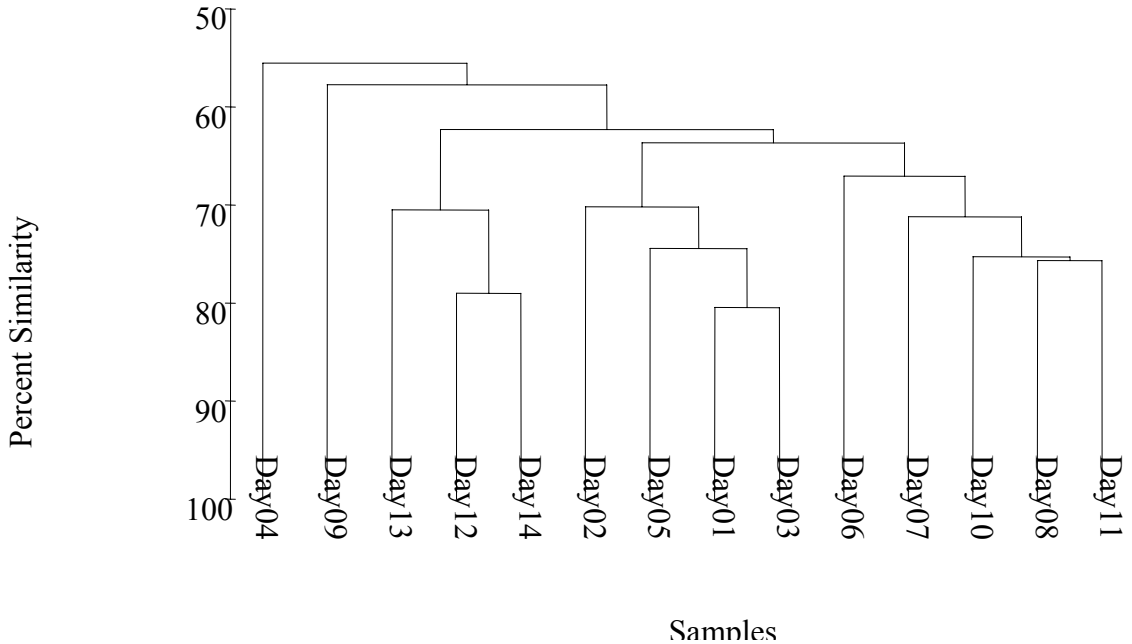

Figure 3. Dendrogram showing the clustering of samples based on similarity. Samples that were dissimilar are linked to the cluster higher up the y-axis (lower percent of similarity) and separation based on dissimilarity.

Individual TRF Peak Persistence and Abundance:

The persistence of a TRF peak was determined by the number of TRF patterns (samples) which that particular peak was present. For example, peaks that appeared in every sample were assigned a persistence of 14, while peaks that appeared in only one sample were assigned a persistence of 1. The number of peaks in each persistence group was totaled and an average peak area determined (Table 1). With this data, a bubble graph was created (Figure 4) to add perspective to the calculations. The graph shows on the y-axis the number of peaks in each group which the x-axis is the persistence of the peaks. The farthest to the left on the x-axis, are the peaks that were present in nearly every sample while the closer to the origin are the peaks that were only present in a few samples. The diameter of each bubble represents the Average Peak Area. As with the previous studies, the trend seems to be that a fewer peaks are present in every sample (few total number of peaks) but high in abundance (large average peak area) and

peaks present only transiently tend to be numerous and of low abundance. [7,10,11] However, in this study there were two a rather large peaks that appeared in 8 of the 14 samples (in samples 6 through 13, data not shown). Since the extracted DNA was processed in batches, contamination could explain this discrepancy. Although proper technique was utilized, a small amount of contaminating DNA once amplified through PCR has the potential to dramatically alter TRF patterns. It was noted that in the three prior studies, one had a similar situation of bacteria (or DNA) in high abundance that was only present in a few samples. [11]

Persistence	**Total Number of Peaks**	**Average Peak Area (% of total)**
1	27	0.93
2	14	0.95
3	12	1.39
4	3	1.92
5	3	0.82
6	5	1.61
7	2	1.49
8	2	5.02
9	3	1.30
10	3	1.20
11	3	1.69
12	4	1.43
13	5	5.48
14	7	5.82

Table 1. TRF peaks were grouped according to their presence in each of the samples.

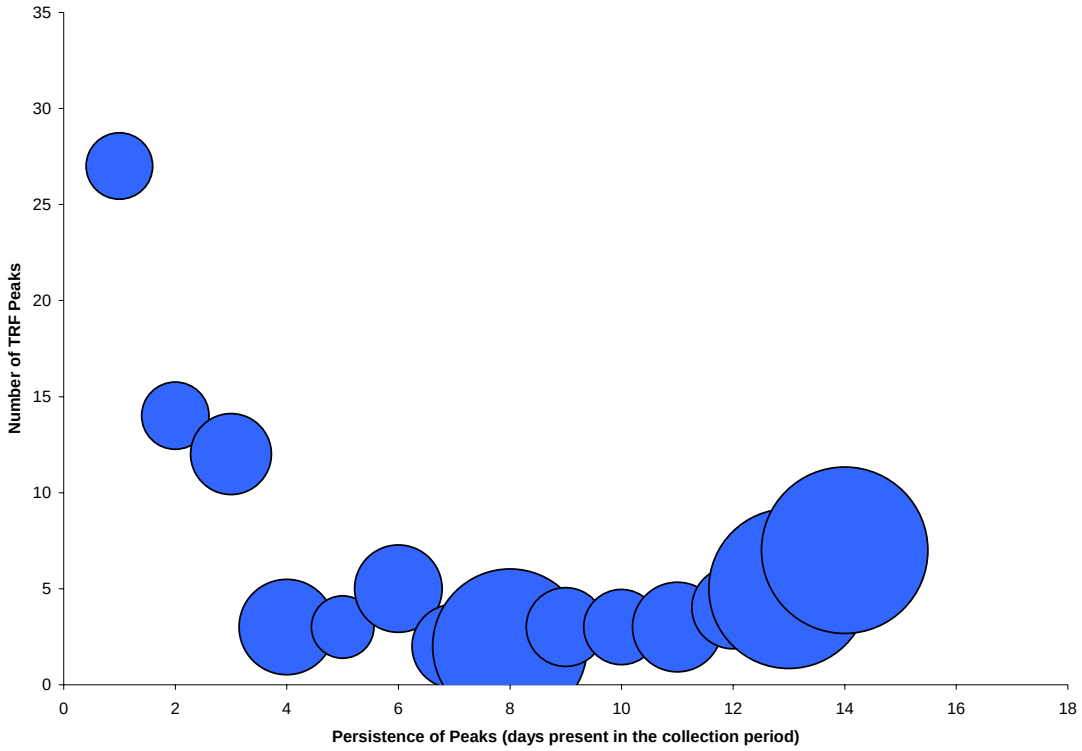

Figure 4: TRF Peaks were grouped by persistence on the x-axis and by quantity present on the y-axis. The diameter of each bubble is equivalent to the average peak area for the group, or the abundance. The data to the far left illustrates that peaks present in every sample tend to be abundant as opposed to peaks that appear in fewer samples tend to be less abundant

CONCLUSIONS

Using techniques and guidelines previously established, it can be concluded that the subject of this study has a stable community of micro flora in the large intestine. Having similar data to the other studies can not necessarily assume that any of the subjects have stable flora, but are at least stable relative to each other by exceeding the 50% similarity mark. Presumably, the Ciprofloxacin taken 35 days prior to beginning collection appears to have no dramatic effect on the stability of the subject's gastrointestinal flora in comparison to the four other subjects who had not taken antibiotics for at least 60 days prior to collection. [7, 10, 11] In addition, the variances in diet and gender did not create obvious discrepancies.

As with the other aforementioned studies it appears that the few peaks produced from the digestion of DNA that appeared in great abundance in every sample is what qualifies any of the subjects as having stable flora. If a subject where to be perpetually colonized with new arrivals, every sample would be extremely dissimilar, and display a great abundance of varying organisms with a relatively short persistence (essentially reversing the bubble plot). Although random organisms may briefly colonize the gastrointestinal tract existing in a scarce, short persistence, the hearty communities of organisms that persist are responsible for the subject maintaining a similarity of above 50% for the duration of the sampling period.

REFERENCES

1 Bengmark, S. Ecological control of the gastrointestinal tract, the role of probiotic flora. Gut. 1998. 42:2-7.

2 Collins, David M., and Glenn R. Gibson. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. The American Journal of Clinical Nutrition. 1999. Vol. 69, No. 5: 1052-10527.

3 "The Bacterial Flora of Humans." Kenneth Todar Univerisity of Wisconsin-Madison. 2002.

4 Holzapfel, Wilhelm H., Petra Haberer, Rolf Geisen, et al. Taxonomy and important features of probiotic microorganisms in food and nutrition. The American Journal of Clinical Nutrition. 2001. 73: 365-373.

5 Murray, Patrick R., Ken S. Rosenthal, and Michael A. Pfaller. Medical Microbiology. Elsevier Mosby. 2005.

6 Reid, Gregor, Jana Jass, M. Tom Sebulsky, and John K. McCormick. Potential Uses of Probiotics in Clinical Practice. Clinical Microbiology Reviews. 2003. Vol. 16, No. 4: 658-672.

7 Bunyea-Baron, B. and Campanale, J. "16S Ribosomal RNA Gene Terminal Restriction Fragment Pattern Analysis Used to Assess the Stability of Bacterial Communities found in the Human Gastrointestinal Tract." 2004.

8 Beals, EW. Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. Advances in Ecological Research. 1984. Vol. 14.

9 Kitts, Christopher L. 2001. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. 2:17-25.

10 Sanchez, Ellen. "Examination of the Stability of Bacterial Communities Within the Human Gastrointestinal Tract Through Terminal Restriction Fragment Length Polymorphism (TRFLP)." 2006.

11Cherepakho, Vera. "An Investigation of Microbial Flora Stability of the Human Gastrointestinal Tract Using Terminal Restriction Fragment Polymorphism Analysis of the 16S Ribosomal RNA Gene." 2005.

APPENDIX I

Collection Protocol

PURPOSE:

Through detailed observation and DNA analysis, the eubacterial species that occupy the human gut, specifically the large intestine, will be monitored for a period of two weeks. Detailed data will be obtained through terminal restriction fragment (TRF) analysis of DNA extracted from fecal material. By such experimentation, the fluctuations in baseline flora that occur in the human large intestine may be tracked. Results obtained specific to this study will be used to assist an ongoing clinical study seeking the
effects, if any, on patients taking probiotic supplements along with antibiotics compared to those patients taking only antibiotics.

FECAL COLLECTION:

Schedule a two week time period in which it would be most convenient to collect samples and record bowel movements. It is suggested that the two week time period be planned around vacations and other times that are known to not be of convenience to perform such a procedure. It is also suggested that females schedule their two weeks when they plan to not be menstruating which would complicate the procedure. An example schedule for the two week collection period is listed below. This schedule avoids having the samples frozen longer than five days and also avoids having to perform extraction procedures on weekends.

Start on a Friday:

Samples Collected on: Extract On:

Fri, Sat, Sun, Mon, Tue Wed (5 samples)

Wed, Thr, Fri, Sat, Sun Mon (5 samples)

Mon , Tue, Wed, Thr Fri (4 samples)

MATERIALS:

1. heavy duty plastic wrap
2. heavy duty re-sealable plastic bags
3. metal spatula
4. wooden dowel or any other rollable object such as a rolling pin
5. sterile 1.5mL snap-cap microcentrifuge tubes
6. gel freeze enzyme-safe microcentrifuge tube box

7. freezer (preferably not auto-defrosting)

PROTOCOL:

For collection of every bowel movement over the selected two week period.

1. Bladder must be completely emptied before starting a bowel movement because urine will alter the sample flora. Urination before bowel movement is essential.

2. A catching device must used to prevent the fecal sample from being contaminated by the organisms in the toilet water. Under the toilet seat, place a piece of plastic wrap (approximately 12 by 30 inches) across the toilet with edges folded over and adhering firmly to the outside of the toilet bowl. Cover the toilet bowl opening from the very back of the seat to approximately six inches from the front (see Figure 1). Make sure the wrap is able to catch the sample by ensuring that the wrap is not completely taut by allowing approximately 2 to 3 inches of sag in the plastic but be sure that the plastic wrap does not contact the toilet water. More than one layer of plastic wrap may be used if preferred.

3. Close the seat lid of the toilet onto the plastic wrap.

4. Sit on toilet and proceed with defection being careful to not urinate on the plastic wrap.

5. Place used toilet paper in the toilet bowl.

6. Lift the seat lid being careful that the weight of the fecal matter does not sink the plastic wrap into the toilet.

7. Lift the edges of the plastic from the sides of the toilet and place the entire catching apparatus and the fecal sample into a re-sealable plastic bag.

8. Record the date and consistency of the sample.

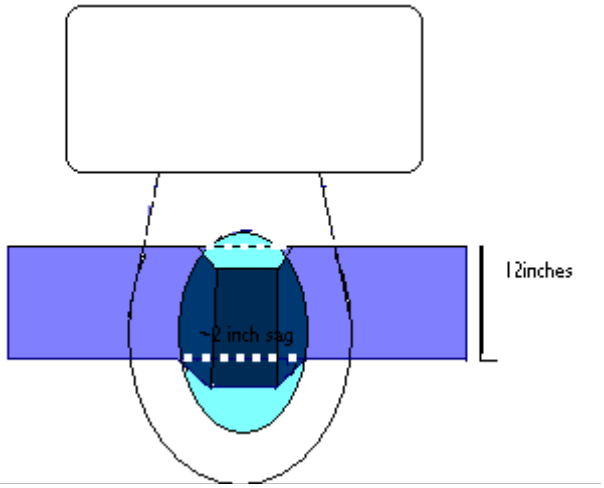

Figure 1. Collection apparatus.

9. Outside of the bag, manually mix the contents of the bag by whatever means necessary to thoroughly homogenize the sample. For samples that are abnormally difficult to homogenize, a wooden dowel or other rollable device may be used to assist in this process. Thorough homogenization takes approximately one to two minutes of constant manipulation. Due to varying bacterial abundance across the length of the fecal material, complete homogenization is required to ensure that the feces become uniform throughout.
10. When the sample is thoroughly homogenized, carefully reopen the bag to expose the sample and use a metal spatula to extract about 0.5mL of sample (approximately half of the tube) and place it into a 1.5mL snap-cap microcentrifuge tube and cap it.
11. Make sure the bag is completely sealed and throw it away outside.
12. Immediately label microcentrifuge tube (date) and place it in the gelfreeze tray in a freezer. Please note: auto-defrost freezers have drastic temperature changes that invalidate the results of the experiment. A freezer that does not auto-defrost is best, but if an auto-defrost freezer is all that is available, be sure to use the gel-freeze tray that minimizes the temperature fluctuations of the fecal samples. Samples may be kept frozen for one to five days but

DO NOT FREEZE SAMPLES MORE THAN FIVE DAYS!

13. When transporting samples to the lab they must be kept frozen. A small ice chest should be used to transport samples in order to ensure that they remain frozen.

SCALES

1. CONSISTENCY F FECAL SAMPLE:

1=Hard, dry mass
2=Hard, formed, dry stool: remains firm and soft
3=Soft, formed, moist: softer stool that retains shape
4=Soft, unformed: stool assumes shape of container, pudding-like
5=Watery: liquid that can be poured.

-Clapper et al. p.1525

2. SCALE FOR THE EVALUATION OF DAILY STRESS LEVEL:

1. Completely relaxed
2. Slightly stressed, mostly relaxed
3. Semi-stressed
4. Very stressed, hard to relax
5. Extremely stressed