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

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ABSTRACT

Questions of microbial community stability were addressed using 14 consecutive fecal samples collected from two subjects with no dietary restrictions and under no medical treatments. DNA was extracted from the feces and bacterial 16S rRNA genes were amplified by PCR. Changes in the microbial ecology of the Gastrointestinal Tract (GIT) were observed through 16S rRNA gene Terminal Restriction Fragment (TRF) patterns. Both subjects' GIT microflora was considered stable after analysis of TRF pattern data, using Bray-Curtis similarity. Day-to-day similarity was above 50% and the most abundant organisms (as represented by the largest TRF peaks) were present in every sample.

INTRODUCTION

The human gastrointestinal tract (GIT) harbors a diverse community of microbes. The vast array of microflora that inhabit the human large intestine comprise a biomass of 10^{14} cells, of which there are an estimated 400 different species[1,2,3]. Non-spore forming, strictly anaerobic bacteria dominate the human gut. *Bacteriodes* and *Fusobacterium* are the predominant gram-negative microbes found in the gut. The most commonly found gram-positive bacteria are *Bfidobacteria* followed by *Clostridia*, *Lactobacillus* and a few gram-positive cocci in lesser amounts[3]. Although there may be large individual variation in the proportions of the major species from person to person, depending on diet or overall health of the human host, it is believed that the population sizes of different species from the same individual are relatively stable[3,4,5,6].

The dense microbial population results in the flora being of considerable physiological importance to the human host. These inhabitants play an important role in balancing host health and disease by modulating immune function, metabolizing carcinogenic agents, and providing a barrier to the invasion of the gut by pathogenic organisms like *Salmonella*. The decreased susceptibility to pathogenic infection is also known as "Colonization Resistance"[1], meaning that adequate colonization of the intestine with select organisms provides the host with better immunity to pathogenic organisms. Furthermore, the primary function of the human gut is uptake of water and nutrients, which is also aided by microbial activity. Specifically, microbes aid in digestion by fermenting a wide variety of sugars found in the host's diet; particularly, sugars that cannot be digested by the host alone, such as dietary fiber. These fermentation processes produce lactic acid and short-chain fatty acids that provide energy to the cells lining the colon wall, helping to improve absorption of minerals and positively influencing lipid and glucose metabolism in the liver[1].

There are many ways of studying the complex ecologies of the GIT microbes, most of which involve fecal specimens. The most tedious and error prone methods of evaluating the GIT microbial ecology is by direct plating. Many genetic methods are currently in use, all of which require PCR of the 16S rRNA gene to study prokaryotic communities since the gene's overall structure is highly conserved and several thousand bacterial sequences are available in genetic databases, even from intestinal bacterial species that cannot be cultured[1]. The amplified PCR products can either be sequenced

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individually or a pattern reflecting the bacterial community structure can be visualized through such methods as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis, Micro Arrays, and Terminal Restriction Fragment analysis (TRF), otherwise known as Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis[2,7].

Our investigation evolved from a previous clinical study in the Environmental Biotechnology Institute, at California Polytechnic State University, San Luis Obispo (EBI), sponsored by Danisco Corp. This study used TRF to analyze the effects of dietary probiotic bacterial supplements on human GIT microflora after antibiotic treatment. The study was complicated by a limited amount of control data to analyze the results (personal communication Anna Engelbrektson[8]). In the study 40% of the subjects appeared to have unstable GIT microflora according to data gathered from three control samples 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. The current study was designed to establish more complete sets of control data by sampling for fourteen days rather than at the three select days of 1, 7, and 14, as in the previous study. Because the preceding experiment was carried out using TRF, the most logical means of collecting our data was to follow the same protocol. Our study is not concerned with microbe identification, but rather the structure of the microbial communities present in the human GIT and subsequently human feces; that is, the regularity in which they are, or are not present and the relative abundance in which they are found.

Figure 1. Average Bray-Curtis similarity of TRF patterns from each subject across the three initial samples (days 1, 7, 14). Note the two distributions. An estimated cut-off for Stable/Unstable categories was made at 50% similarity. Subjects with average similarity >50% during the first three sampling days were deemed stable. (Figure taken from Engelbrektson[8])
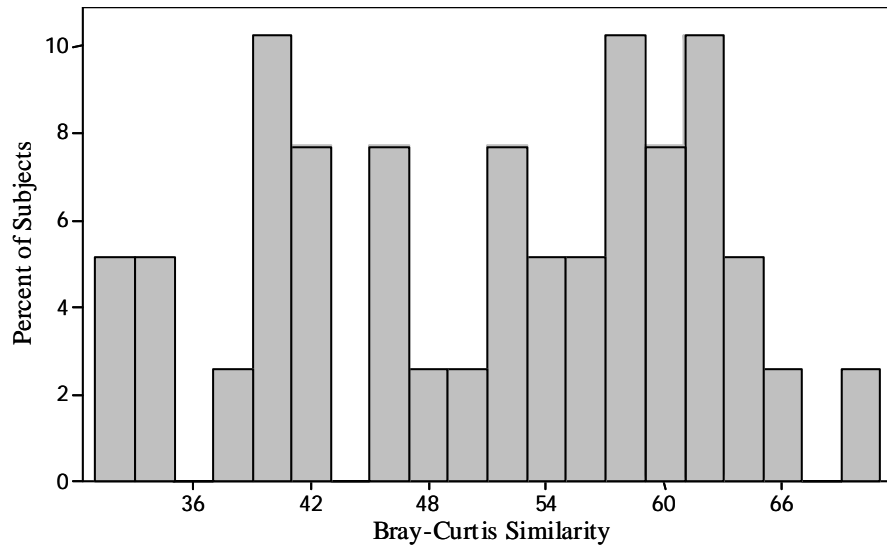
MATERIALS AND METHODS

Collection of Fecal Samples

Fecal samples were collected from two healthy adult subjects over a fourteen-day period.

Samples were collected in the subject's home and then stored at 0 °C for no more than five days

in the subject's home freezer 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 $MgCl_2$, 1 µL 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 A_{260} 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 excluded from analysis. TRF peak area was converted to relative abundance (percent of total area) and analyzed with a Bray-Curtis similarity measure[9]. Statistical analyses were performed using PrimerV and Excel.

RESULTS AND DISCUSSION

Fecal samples were collected from the two subjects until 14 samples were obtained. Fecal color and hardness was evaluated using a created pallet of pantone colors and a hardness scale[10]. Although this observational information was recorded, it was not found to be useful for any relevant extrapolations from the TRF patterns (data not shown). TRF patterns from each sample were then produced (**Figure 2a and 2b**) and evaluated for similarity. Figure 2 is a pictorial representation of the patterns found after the *Hae*III restriction digests were performed. The figure is a plot that displays the intensity of a dye signal that signifies the number of labeled PCR primers crossing the CEQ detector, verses the length, in nucleotides, of the crossing DNA fragments. Peaks found in the 50-300 nucleotide (nt) range correspond to digested 16S rRNA gene fragments and those peaks found between 450-500nt range correspond to undigested 16S rRNA genes. These TRF patterns visually aid in identifying the changes that had occurred between samples. These visual representations also allow for the overall stability of the GIT microflora to be observed and evaluated. It is apparent from TRF patterns of subject 1 that most amplicons were contained an *Hae*III restriction site as indicated by the smaller size and number of peaks found in the 450-500nt range. As for subject 2, many of the amplified 16S rRNA genes did not contain the *Hae*III restriction site and therefore a greater number of peaks were in the 450-500nt range. According to a database of 16S rRNA gene sequences the species most likely to lack *Hae*III cut sites are *Clostridium spp.*
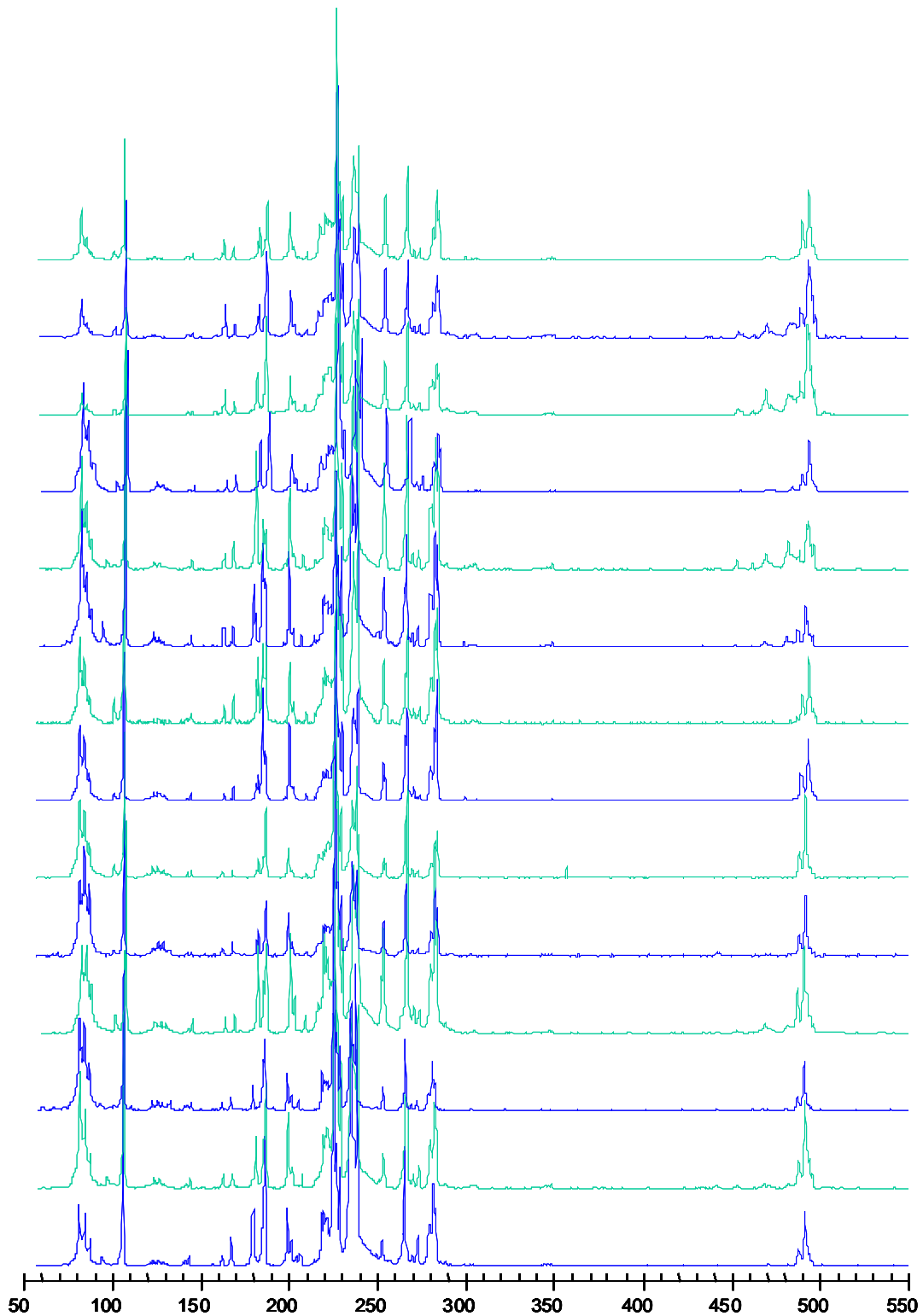
Figure 2a: Subject 1 TRF patterns with sample one at the bottom and sequentially going to sample fourteen at the top. Peak height represents the intensity of labeled PCR primer being detected and the x-axis is the length of the HaeIII digested 16S rRNA gene fragments in nucleotides.
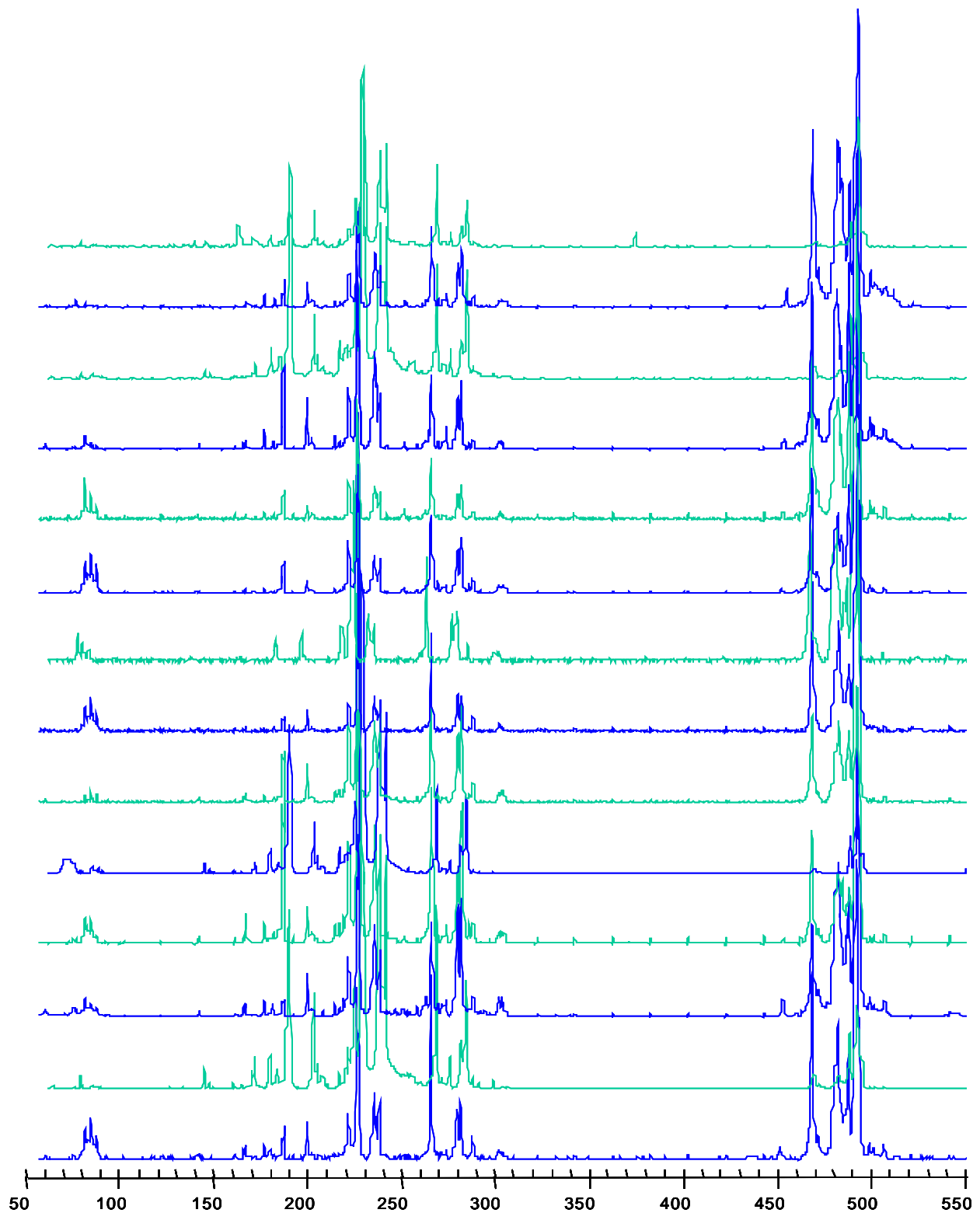
Figure 2b: Subject 2 TRF patterns with sample one at the bottom and sequentially going to sample fourteen at the top. Peak height represents the intensity of labeled PCR primer being detected and the x-axis is the length of the HaeIII digested 16S rRNA gene fragments in nucleotides.

TRF Pattern Anomalies in Subject 2 Data:

Two problems were encountered with the data acquired from subject two. First, some of the peaks that represented more than 1% of the total peak area went undetected by the CEQ software. Secondly, the internal *E.coli* standard was found to have contaminated sample 14 as a result of pipetting error.

To account for both of these mishaps, inspection of individual TRF patterns was carried out. The estimated peak area for each undetected peak was combined with the peak area of adjacent peaks. As a result, the peak representing the fragment of 226 nucleotides was combined with the fragment of 227 in all samples, as was also done in the case of fragments 235 and 236 being combined with 237. By performing these combinations, five peaks were replaced by two new peaks labeled 226-7 and 235-7. The solution to the second problem of *E. coli* digest standard contamination in sample 14 was to eliminate the *E. coli* 168 peak from the sample. This did not effect the ultimate question of GIT stability because of the lack of an *E. coli* 168 peak across all other days. After all of the anomalies were eliminated, analysis of the modified data from subject 2 went forward along with the analysis of data from subject 1.

Bray-Curtis:

A Bray-Curtis similarity matrix was created in PrimerV using the TRF pattern peak areas to give a percent similarity of each TRF pattern compared to each of the other 13 TRF patterns obtained. The resulting data was then used to create a daily progression bar graph indicating the percent similarity of each consecutive sample/TRF pattern to the next. The most dissimilar comparison between two adjacent samples for subject 1 was a 64.8% similarity between samples on days 11 and 12. The most dissimilar comparison between two adjacent samples for subject 2 was a 57.5% similarity between samples on days 6 and 7 (**Figure 3**). In the case of subject 2 days 4 through 7were found to have the least similarity. This major decrease in similarity between samples could be largely due to the fact that subject 2 did not have a bowel movement on days 3 and 6. Although 14 total samples were attained from subject 2, the longer "incubation" of samples 4 and 7 most likely attributed to the shift in similarity patterns before and after these samples.

Daily progression comparisons based on Bray-Curtis analysis verses the time in days of the samples collection
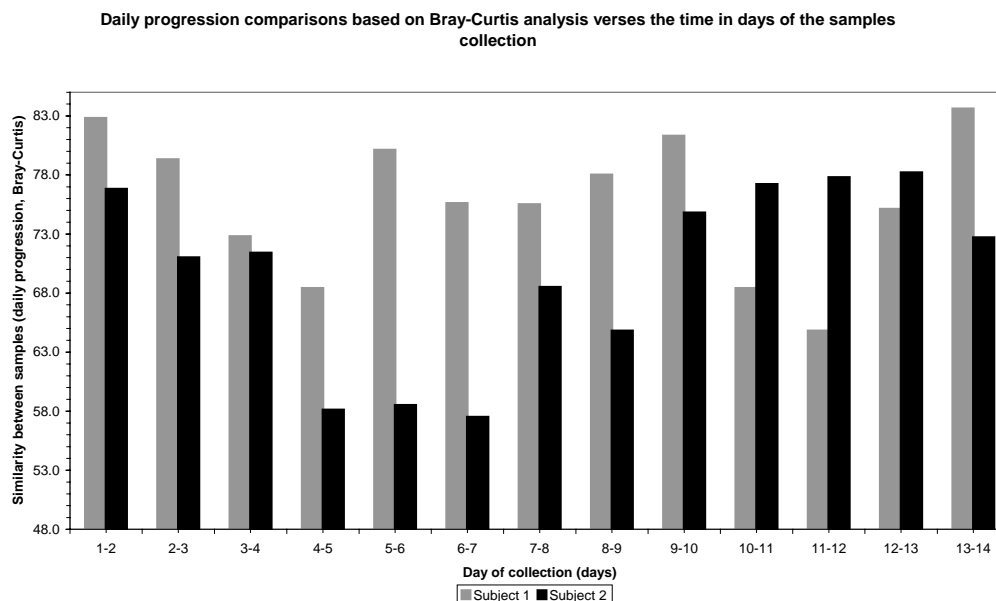

Figure 3: Daily progression bar chart indicating the percent similarity (based on Bray-Curtis analysis) of each consecutive TRF pattern/sample compared to the next for all days of sample collection. This view of the day-to-day similarity allows for the observation of how two consecutive samples relate to each other in sequential order based on their pattern of similarity.

The average percent similarity for all TRF patterns was 75.8% for subject 1 and 69.8% for subject 2. These percentages, being greater than 50%, indicate that both subjects' GIT microflora can be considered more stable than unstable (Figure 1). It is possible that the natural fluctuations in GIT microflora could result in a random set of 3 samples being on average less than 50% similar leading to a belief that a subject's flora is unstable based on Bray-Curtis similarity. Therefore, the lowest possible average of any three Bray-Curtis similarity scores was identified to determine if the any three samples could result in the subject being grouped under the 50% stability threshold, as was the case in Engelbrektson's study. The lowest possible average similarity between three samples for subject 1 was 61.3% and for subject 2 was 58.4%. Subject 1 and Subject 2 demonstrate no possible combination of percent similarity that can be considered unstable because no possible combination of three patterns averaged less than 50% similar.

Bray-Curtis Similarity Dendrogram:

To better represent similarity of overall variation in all TRF patterns rather than day-to-day similarity, a dendrogram was created in PrimerV using the Bray-Curtis similarity data (**Figure 4**). The

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dendrogram clusters those samples that are most similar and makes separations between groups of clusters that are dissimilar. Members of groups are more similar the lower they are clustered on the dendrogram. From these results, subject 1 had 69% overall similarity between all samples, while subject 2 had 65%. With both subjects, at higher similarity percentages, consecutive samples deviated from each other, resulting in random grouping of samples. That is to say, changes in GIT microflora did not happen in a gradual manner, but rather the changes between consecutive samples fluctuated at random around an equilibrium.
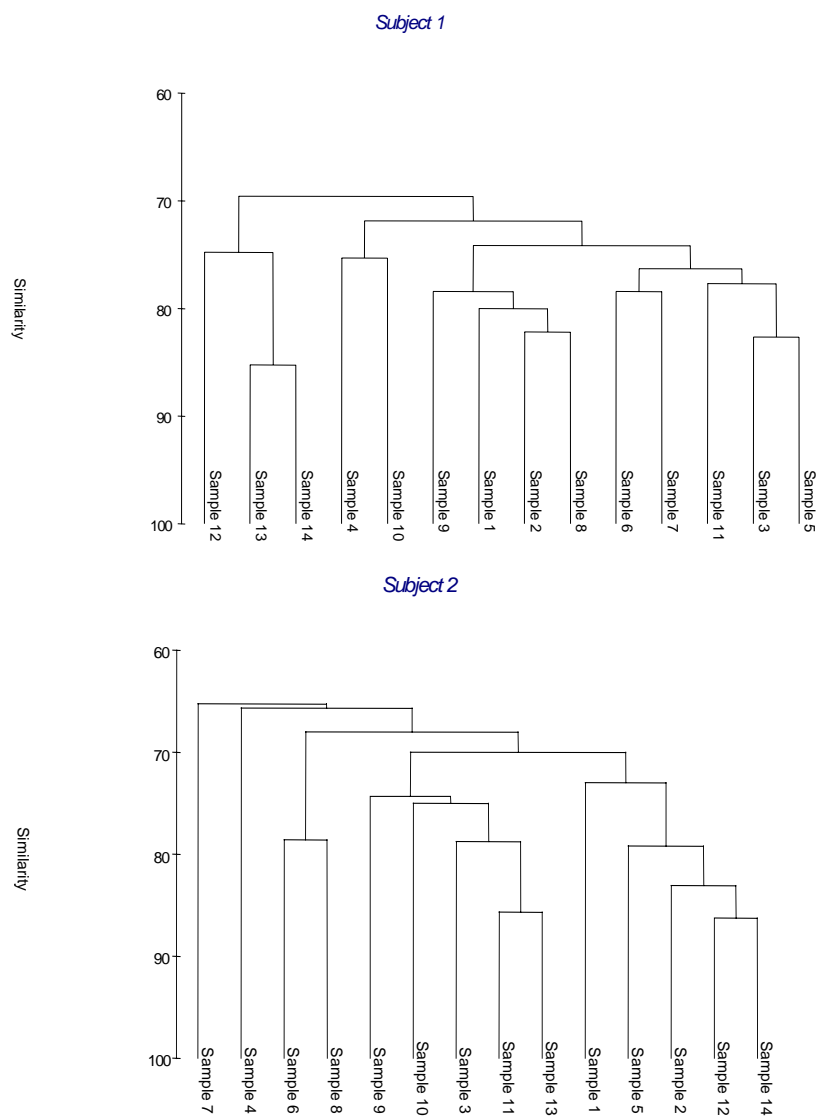

Figure4: The dendrogram depicts the affiliation of each sample across all 14 samples based on Bray-Curtis similarities. Subject 1 above and subject 2 below. There is no obvious relationship between consecutive samples based on their final clustering position, meaning that no more than 2 consecutive samples clustered together with more than 75% similarity.

Individual TRF Peak Persistence and Abundance:

The persistence of a TRF peak was defined as the number of TRF patterns (days) in which that particular peak was present. Peaks were then grouped based on their persistence. For example, all peaks that appeared in only one pattern were placed in one group and all peaks that appeared in every TRF pattern for all 14 days were considered another group. The number of peaks in each group was then totaled and an average peak area was calculated (**Table 1**). Correlating the total number of peaks to the number of days in the collection period that each group of peaks occurred provides a simple visual aid detailing the microbial population dynamics in the participating subject's GIT microflora (**Figure 5**).

Number of samples a peak was present in	Subject 1		Subject 2	
	Total number of peaks	Average Peak Area (%)	Total number of peaks	Average Peak Area (%)
1	9	1.46	5	1.31
2	4	1.59	7	2.08
3	5	1.78	5	1.86
4	0	0.00	8	2.42
5	2	1.94	5	1.77
6	2	1.80	2	1.84
7	1	1.80	3	2.12
8	2	2.27	1	1.51
9	0	0.00	1	2.01
10	3	3.10	2	1.58
11	5	2.06	1	4.34
12	4	3.22	0	0.00
13	4	2.81	2	7.32
14	10	5.36	9	6.72

Table 1: TRF peaks grouped by number of samples they appeared in and the average peak area is presented for each group.
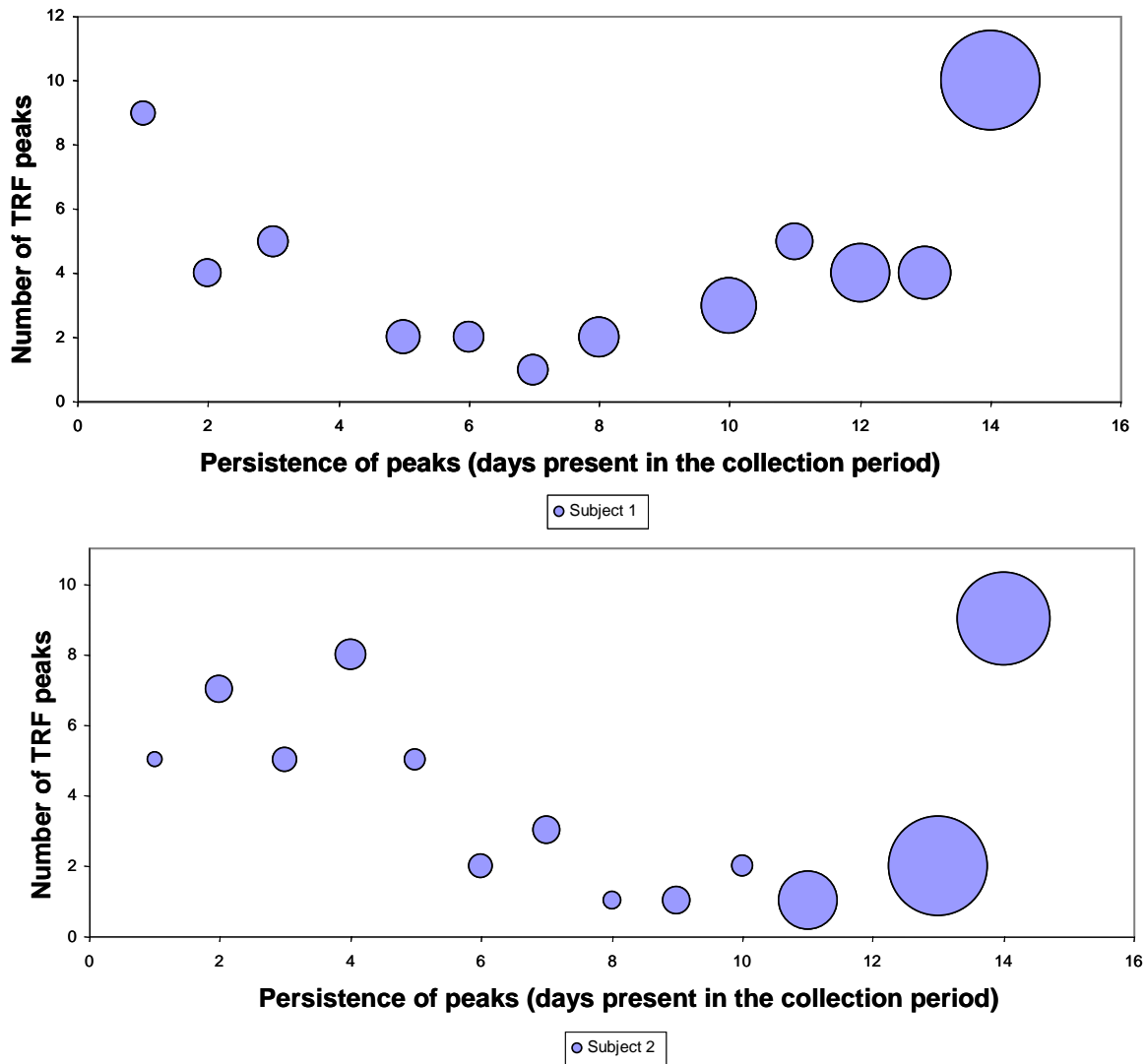
Figure 5: TRF Peaks were grouped by the number of days they were present on the x-axis (persistence) resulting in groups that contained peaks that were present in all/nearly all TRF patterns to be found farthest to the right, and groups that contained peaks that were absent from nearly all TRF patterns to be found farthest to the left. The number of peaks in each group is presented on the y-axis. The diameter of each symbol represents the average peak area for the group. Upper panel = subject 1; Lower panel = subject 2

Two extremes were visible when the number of TRF peaks was plotted against persistence; peaks that were present in nearly all or absent in nearly all TRF patterns. In general, groups near the extremes had the highest number of TRF peaks. This is consistent with the stable nature of the GIT microflora in these subjects. The large number of peaks found at the extremes is a direct result of two distinct microbial populations. The first population is comprised of those bacteria that are found in high abundance and persist, and the second being those bacteria that are found in low abundance and that

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are inconsistent. The first population is the dominant and stable microflora as indicated by the large average peak area of its members. The second population is the bacteria that are found at lower abundance and therefore appear inconsistent (**Figure 5**). At lower abundance (around 1%) the detection of these bacteria is sporadic; meaning the large number of TRFs at low persistence is an artifact of the detection limit. Bacteria at all levels of persistence fluctuate in relative abundance (**Figure 2a and 2b**), but because the bacteria found in low abundance are fluctuating above and below the 1% threshold they are only detected sporadically. The more abundant bacteria are fluctuating at greater distance from the threshold and are therefore being detected in spite of their fluctuations in abundance. Similarity measures and thus stability is less affected by TRF peaks in the low persistence group because they account for such a small percentage of the total peak area as represented by their symbol's small diameter (**Figure 5**).

CONCLUSIONS

Regarding the two subjects' samples involved in this study, the final analysis found that the microbial communities of their GIT could be considered stable. Most notably, stability is a result of random fluctuations around a state of equilibrium and organisms that are found in high abundance persist. Organism that are found at high abundance (large average TRF peak area) and with high persistence represent a greater percentage of the total peak area and therefore have a greater affect on determining the stability of a subject's GIT microflora.

With regard to the previous study, our results indicate the two subjects fall into the group that had greater than 50% Bray-Curtis similarity. Because there were only two subjects considered in this study, a solid conclusion regarding stability cannot be positively confirmed. It is possible that, by chance, both subjects happened to be stable. Differences in the sampling protocol may have contributed to these two subjects being deemed stable. Samples in this study were immediately homogenized and stored frozen for a maximum of 5 days, while the previous study samples were stored for up to 24 hours at 10°C and then frozen for up to 45 days. Overall, the data obtained from fourteen consecutive samples with more defined guidelines on their collection and storage was more dependable than the protocols defined by the previous study. The more stringent protocol could ultimately have promoted a more stable result.

To avoid future errors, changes that should be considered with subsequent subjects studied should include a more controlled collection protocol to decrease storage time of the samples followed by immediate DNA extraction. Ott et al. (2004) showed a clear effect of storage on the GIT microflora for 24 hrs at 4°C[2]. In addition, each TRF pattern needs to be carefully examined to ensure that the CEQ software recognized every single relevant peak as was illustrated by the problems encountered with data from subject 2, which required further manipulation to correct for errors in the CEQ software's original integrations of TRF peaks.

ACKNOWLEDGEMENTS

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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. Therefore, subjects of this experiment may be used as controls that will demonstrate the normal daily fluctuations in flora of the human large intestine. 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:

Guidelines:

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)

NOTE: NO ANTIBIOTICS can be taken sixty days prior to the initiation of fecal collection.

Finally, keep a diary and record the date of each bowel movement, its color and consistency/hardness. See attached pages for scales and sample diary page.

MATERIALS:
1. heavy duty plastic wrap
2. heavy duty resealable 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.
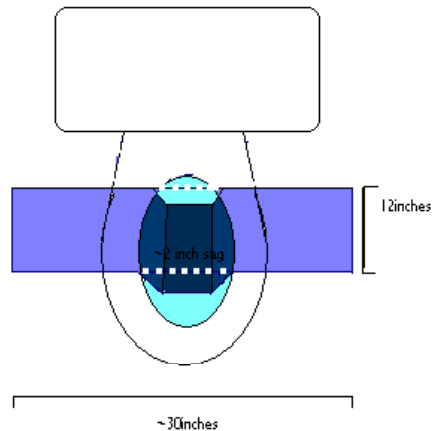
3.   Close the seat lid of the toilet onto the plastic wrap.

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, color and consistency of the sample using the provided scales in Table A1.

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 gel-freeze 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.

Table A1: <u>OBSERVATION DIARY PAGE</u>*

Sample Number	Date and Time of Sample	Color Evaluation	Hardness Evaluation	Daily Stress Log
1				
2				
3				
4				

*This is a sample of what the observation diary should be set up to look like. The scales for evaluating the fecal specimens are provided below.
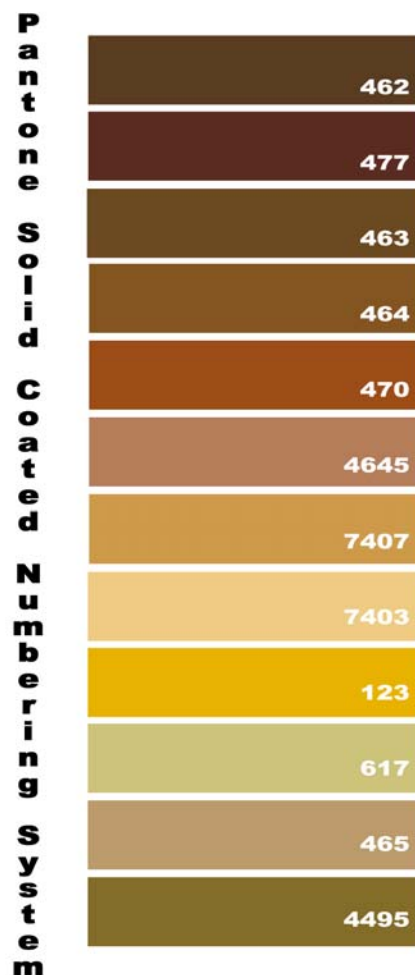
SCALES

1. <u>CONSISTENCY / HARDNESS OF FECAL SAMPLE:</u>
   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. <u>COLOR PALLETE USED FOR FECAL EVALUATION:</u>

3. <u>SCALE FOR THE EVALUATION OF DAILY STRESS LEVEL:</u>
   1. Completely relaxed
   2. Slightly stressed, mostly relaxed
   3. Semi-stressed
   4. Very stressed, hard to relax
   5. Extremely stressed