INVESTIGATION OF MICROFLORA STABILITY WITHIN THE HUMAN INTESTINAL TRACT USING TERMINAL RESTRICTION FRAGMENT (TRF) LENGTH POLYMORPHISM ANALYSIS

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ABSTRACT

In a previous study investigating the effects of probiotics on healthy individuals undergoing antibiotic therapy, the study population was found to be divided into two normal distributions of microflora stability: those that were considered "stable" and those that were considered "unstable"[1]. To further investigate the validity of this control group distribution, the prokaryotic microflora community within the intestinal tract of three subjects was studied through the collection of fecal samples over a fourteen-day period. Following DNA extraction, the 16S ribosomal RNA gene from each of the samples was amplified by PCR, digested by Hae III, and then subjected to Terminal Restriction Fragment (TRF) Analysis. The TRF pattern data was analyzed using Bray-Curtis similarity, and the results supported the validity of the bimodal distribution in the original study population with the intestinal microflora for two of the three subjects being categorized as stable. Unfortunately, the subject exhibiting instability unknowingly took antibiotics within sixty days prior to sample collection, a violation of the protocol that may have contributed to her instability in intestinal microflora.

INTRODUCTION

The gastro-intestinal tract (GIT) serves a functional role in human physiology and health. It is home to many different bacterial species, with gram-negative *Bacteriodes* and *Fusobacterium*, and gram-positive *Bifidobacteria*, *Clostridia*, and *Lactobacillus* being the most dominant species. *Bifidobacterium* and *Lactobacillus* are able to play a vital role in preventing the proliferation of pathogenic bacteria by fermenting carbohydrates within the intestinal tract thereby lowering its pH.[2] The intestinal epithelium with an optimal intestinal flora represents a barrier to the invasion or uptake of pathogenic microorganisms, antigens and harmful compounds from the gut lumen. In healthy individuals this barrier is stable, ensuring host protection and providing normal intestinal function and immunological resistance.[3]

It is estimated that one kilogram of bacteria inhabit the adult intestinal tract[4]. Even with the large quantity of bacteria in the GIT, the distribution of each microbe varies among individuals. The differences between each individual are directly influenced by factors such as stress and diet. In a previous clinical study in the Environmental Biotechnology Institute (EBI), at California Polytechnic State University, San Luis Obispo, it was noted that the control group was divided into two normal distributions: 60% stable and 40% non-stable[1]. (See Figure1). This observation raised a question as to whether the existence of the two groups was valid or if the non-stable group was present due to experimental error.

This study's aim is to help provide further data to validate the findings on the population in Engelbrektson's study. Terminal Restriction Fragments (TRF) analysis was conducted on DNA extracted from human fecal samples collected over a fourteen-day period. The data was analyzed via Bray-Curtis similarity.[5]

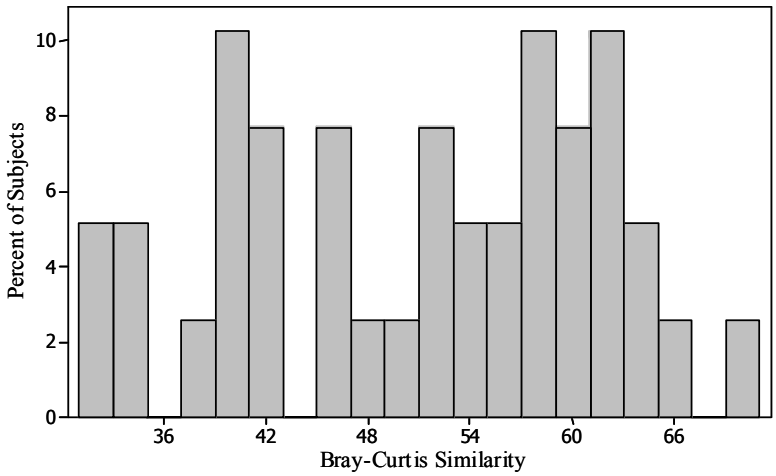


Figure 1. Average Bray-Curtis similarity of TRF patterns from each subject across the three initial samples (days 1, 7, 14). Note the two distributions. An estimated cut-off for Stable/Unstable categories was made at 50% similarity. Subjects with average similarity >50% during the first three sampling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

MATERIALS AND METHODS

Collection of Fecal Samples

Fecal samples were collected from three subjects, ages 20-23, once a day for fourteen days. Samples were colleted in plastic wrap, manually homogenized, and then transferred to a 1.5 mL centrifuge tube. These samples were stored at 0^{o} C until DNA extraction. During the time of collection, food intake, stress level, and qualitative characteristics of feces were recorded. Subjects were prohibited from undergoing antibiotic treatment during the period of sample collection and sixty days prior to the beginning of the study.

DNA Extraction

DNA samples were extracted from fecal matter in triplicate using MoBio PowerSoil™ DNA kit, and the success of each extraction was determined by 1.5% 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'), were fluorescently labeled with phosphamide dye, and used for each reaction. Reactions were carried out using 1μL DNA, 1μL 10X buffer, 3 μL 10 mM dNTP, 2 μL 20mg/mL BSA, 7 μL 25 mM $MgCl_2$, 1 μL K2R, 1 μL Ba2F, 29.7 μL PCR H_2O, and 0.3 μL 5U/mL TaqGold®. Reaction temperatures and times were 94 ^oC for 10 minutes; 30 cycles of 94 ^oC for one minute, 46.4 ^oC for one minute, 72 ^oC for two minutes; and 72^o C for 10 minutes. Success of PCR was determined by 1.5% agarose gel electrophoresis. If PCR was unsuccessful, 1μL of extracted DNA was diluted 1:10 and subjected to PCR again. All reactions were performed in triplicate and combined using MoBio UltraClean® PCR Cleanup Kit. Amplicon concentration was determined by A_{260} UV fluorometer following EBI protocol.

Enzyme Digests and TRF Pattern Generation

A 40- μL enzyme digest was performed on each PCR product using the New England Biolabs restriction endonuclease Hae III. The volume of enzyme and DNA varied between samples. Digests performed on some sample were successful using only 0.4 μL of Hae III, however, other samples required volumes up to 0.8 μL . 4 μL of buffer was used for each digest. The samples were digested for 4 hours at 37 ^oC and inactivated for 20 min at 80 ^oC. The digestion products were ethanol precipitated and resuspended in 20 μL of fomamide 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 program into Microsoft Excel. TRF peaks representing less than 0.5% 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[5]. Statistical analyses were performed using PrimerV and Excel.

RESULTS AND DISCUSSION

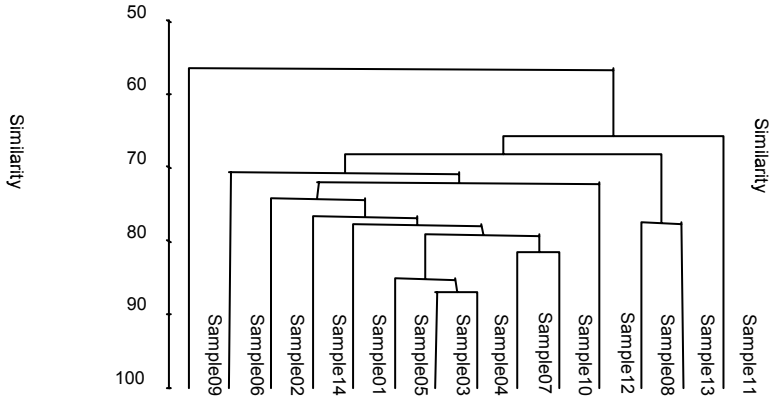
A Bray-Curtis similarity matrix, formed on Primer V, calculated the percent similarities of each TRF pattern, in relation to the other thirteen. This data was used to create a daily progression bar graph that compared the similarities between each consecutive day of each subject as well as how the similarities of one subject compared to the similarities of the other two. The least similar consecutive samples for subject 1 were from days 8 and 9, resulting in 47.6% similarity. There are no documented extrinsic factors that may have contributed to this decrease in similarity. The most dissimilar samples of subject 2 were between days 10 and 7 and were 42.0% similar. This decrease in similarity may have resulted from the inability to collect a sample between days 9 and 10, thereby causing a longer intestinal incubation period for sample 10. Subject 3 showed a 47.9% similarity between days 8 and 9, and like subject 1, there are no known explanations to account for the decrease in similarity between these two days.

Another comparison performed using the Bray-Curtis Similarity Matrix was the average percentage of the three most dissimilar samples (minimum three) from each subject. A percentage below 50% similarity indicated intestinal instability regardless of whether or not the overall average percent similarity was above 50% because any sign of instability is significant. Subject 1's minimum three average, calculated for samples 8, 9, and 11 was 52.1% whereas her overall similarity between all the samples was 69.9%. Both averages were above 50%, indicating that subject 1 did not have any significant fluctuations in intestinal microflora. Subject 2 showed a 64.5% overall similarity between samples, however, her minimum three of 48.2%, determined from samples 7, 10, and 14, categorized her as unstable. Subject 3 had an overall similarity of 69.8% and a minimum three of 54.5%, calculated from samples 7, 8, and 13, indicating that she, like subject 1, did not exhibit any major fluctuations in intestinal microflora during the period of sample collection.

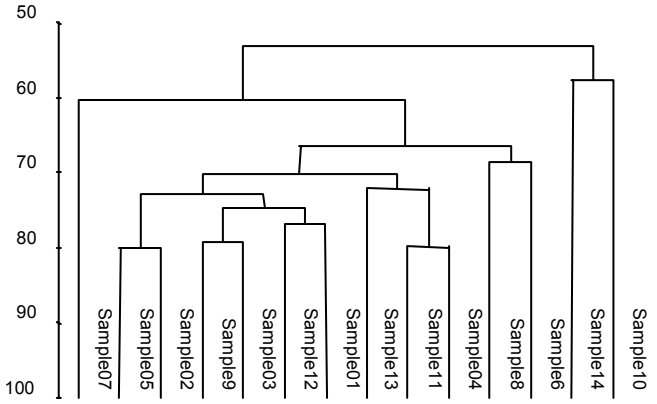
Using the Bray-Curtis Similarity data, dendrograms were constructed for all three subjects using Primer V. The dendrogram delineates the similarity and dissimilarity between samples by clustering like samples together on the same branch.

Figure 3: Dendrograms representing overall relationships between all 14 samples using Bray-Curtis similarities for Subjects 1,2, and 3.

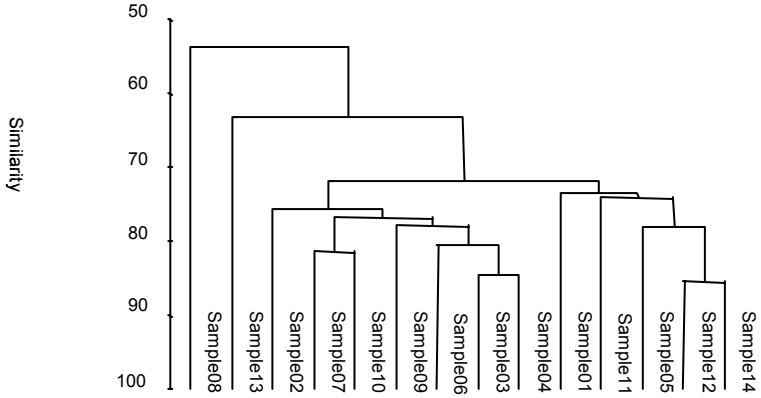
Subject 1



Subject 2



Subject 3



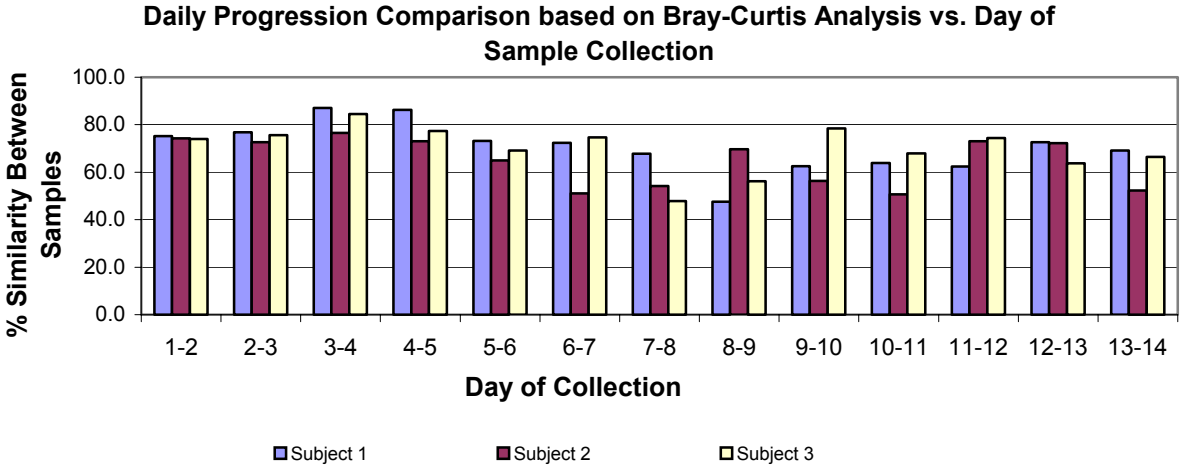


Figure 4: Daily progression bar graph indicating the percent similarity (based on Bray-Curtis analysis) between consecutive TRF pattern/samples.

The persistence of a TRF peak is defined as the number of samples, out of the fourteen, in which a specific peak is observed. For example, subject 1 had a total of 17 peaks with a persistence of 1 because each of those peaks only appeared once in all of her TRF patterns. For each persistence group, the average peak area was determined in order to study the relationship between peak frequency and the relative size of certain bacterial populations within the human intestine. It was observed that infrequently occurring populations had smaller average peak areas while more frequently occurring populations had larger average peak areas. This suggests that frequently occurring bacterial populations are more likely to be present in higher numbers than populations that occur less often.

Table 1: Calculated average peak area (%) from daily persistence (total number of peaks presented for each group).

	Subject 1		Subject 2		Subject 3	
Persistence	Total number of peaks	Average Peak area (%)	Total number of peaks	Average Peak area (%)	Total number of peaks	Average Peak area (%)
1	17	1.90	19	1.29	25	0.82
2	5	0.78	14	0.97	12	0.95
3	11	0.86	3	1.10	4	0.77
4	6	1.51	4	1.29	3	1.16
5	5	0.85	3	1.74	2	1.17
6	2	1.07	2	0.81	3	1.07
7	2	1.49	3	1.69	2	0.93
8	1	0.90	4	1.27	1	2.84
9	2	2.17	4	2.56	4	1.58
10	5	1.41	2	1.78	2	1.29
11	2	1.68	2	2.26	2	1.53
12	3	1.49	6	3.06	5	1.47
13	7	4.05	2	6.38	11	2.51
14	11	4.32	8	5.08	10	5.00

Figure 5: Persistence of peaks (days present in the collection period) vs. Number of TRF Peaks. NOTE: Bubble width indicates average peak area.

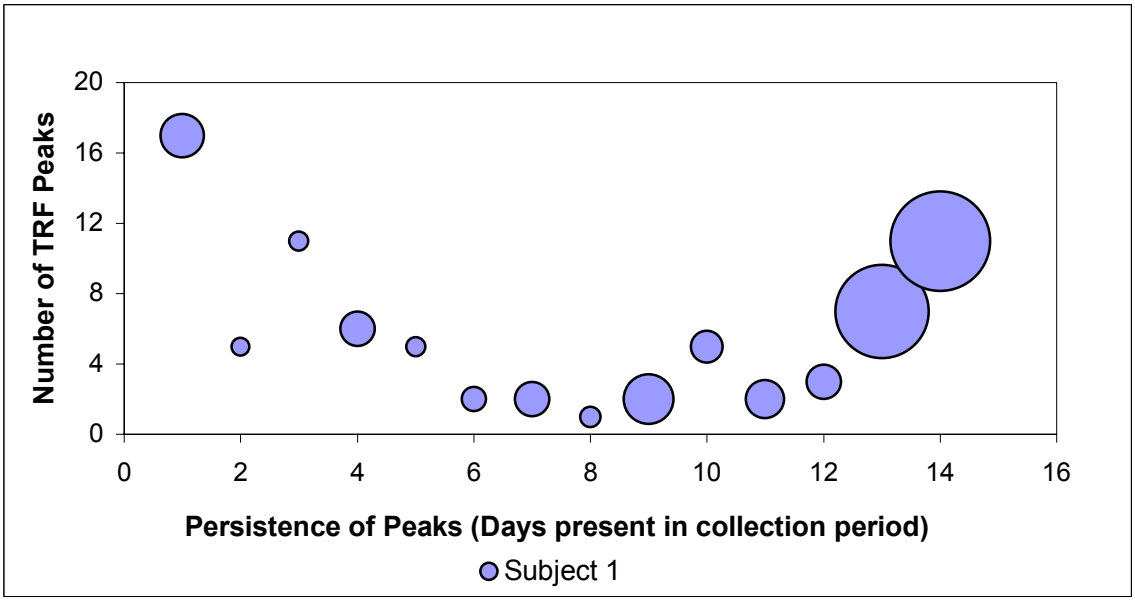
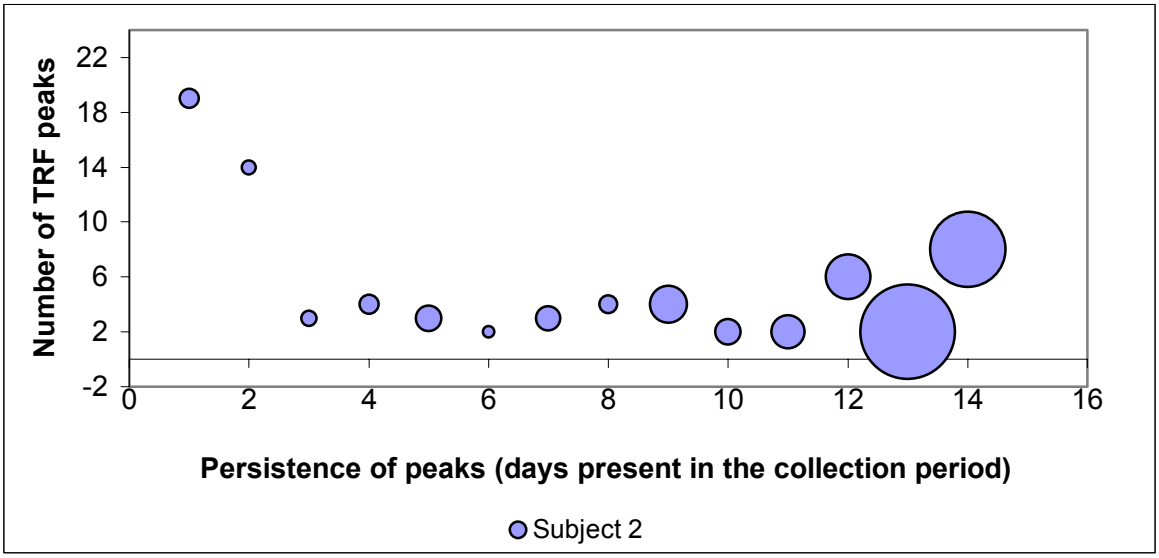
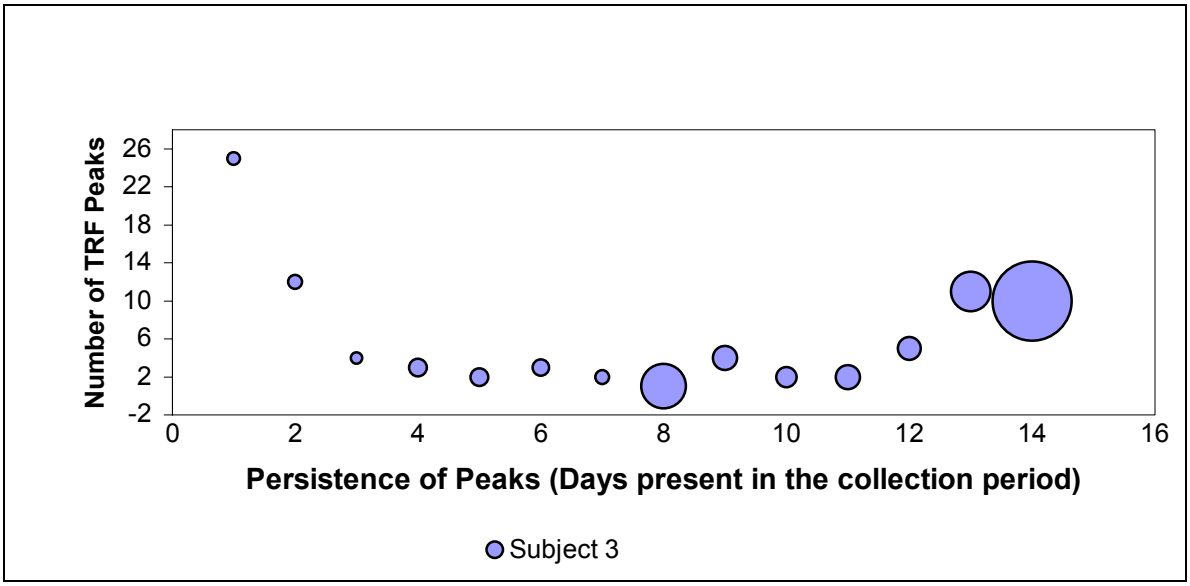


Figure 5 continued:





When the number of TRF peaks was plotted against persistence, two extremes, with a persistence of 1 and 14 respectively, were visible. Groups near the extremes yielded the highest number of TRF peaks whereas groups with a moderate persistence gave a lower yield. Peaks with a larger average area tended to have greater persistence compared to peaks with smaller average areas. Groups with a higher

persistence are believed to represent bacterial species more dominant in the GIT. The presence of these groups may be a large factor in stabilizing bacterial microflora in the GIT. Likewise, groups with a lower persistence represent bacterial species less dominant in the GIT. Thus, these groups may contribute to any bacterial instability occurring within the intestines.

CONCLUSIONS

The final analysis determined that the microbial communities within the GIT of subjects 1 and 3 were stable, according to the Bray-Curtis similarity requirements with average similarity above 50%. Subject 2 was only considered unstable in her minimum three value, however, her overall stability was above 50% indicating that the three most unstable days may have just been outliers. Subject 2 had been taking 200 mg Minocycline as an oral acne treatment prior to collection, thereby unintentionally violating the 60 day abstinence from antibiotics indicated in the protocol which could have been the cause of her instability. Possible side effects of this antibiotic are an upset stomach (taking it with food is recommended) and diarrhea[6]. In addition, there are many external factors in addition to diet and stress that make it difficult to pinpoint the precise cause of subject 2's instability or to even correlate a specific activity with instability or stability in a subject.

From this study, 2 of the 3 subjects adhered to strict protocol and both had a greater than 50% Bray-Curtis similarity, categorizing them as stable. Still, the greatest factors in predicting a subject's GIT microflora stability are based on the higher percentage of total peak area and higher persistence of TRF peaks. Even with this information, no definite conclusion can be made regarding the intestinal microflora stability in a whole population. In order for a statistically valid conclusion to be drawn, the study should be completed on more subjects.

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