Analysis of Microflora in Human Gastrointestinal Tract by Using a PCR-based method called Terminal Restriction Fragment (TRF) Length Polymorphism

By

Amy M. Dinh

Biological Sciences Department

College of Science and Mathematics

California Polytechnic State University

San Luis Obispo

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Abstract

This experiment is an extension of previous laboratory experimentations to further the investigation and analysis of the microflora dynamics in the human GI tract. Fecal samples were collected from a healthy subject over a two-week period, in which the subject had routine dietary intake and did not take any antibiotics. DNA was extracted from fecal samples, 16S ribosomal RNA (rRNA) genes were amplified by PCR, and Cy5-labeled PCR products were analyzed by CEQ8000X for Terminal Restriction Fragment (TRF) Length Polymorphism. According to previous study (Kitts et al, 2005), subjects with a 50% or greater percentage of similarity as determined by Bray-Curtis Similarity, were deemed stable. Based on the 50% similarity cutoff, the microflora in the GI tract of this subject was stable.

Introduction

The human GI tract has a diverse community of microorganisms (Zoetendal et al, 1998). Plate count analyses of fecal samples estimate that there is an approximate range of 10^{10} to 10^{11}CFU per g (Zoetendal et al, 1998). However, conventional microbiological methods, such as plate count, have many limitations. These limitations include: only easily cultivated organisms are counted, and microorganisms that require more stringent growing conditions are not selected for, thus, leading to a biased conclusion based on incomplete and misleading data and/or results. Recent laboratory studies have applied

biotechnology approaches and advanced techniques, such as molecular methods based on 16S rRNA and PCR to quantify specific groups of bacteria in human feces (Zoetendal et al, 1998). Current studies suggest that the microflora in the human GI tract exhibit stable dynamics (Vanhoutte et al, 2004 and Zoetendal et al, 1998). The objective of this present study was to further extend previous studies by Kitts and colleagues, and to challenge the current findings. Specifically, the similarity of fecal microflora from seventeen (17) out of forty (40) subjects (Kitts et al, 2005) were less than 50% (Figure 1):

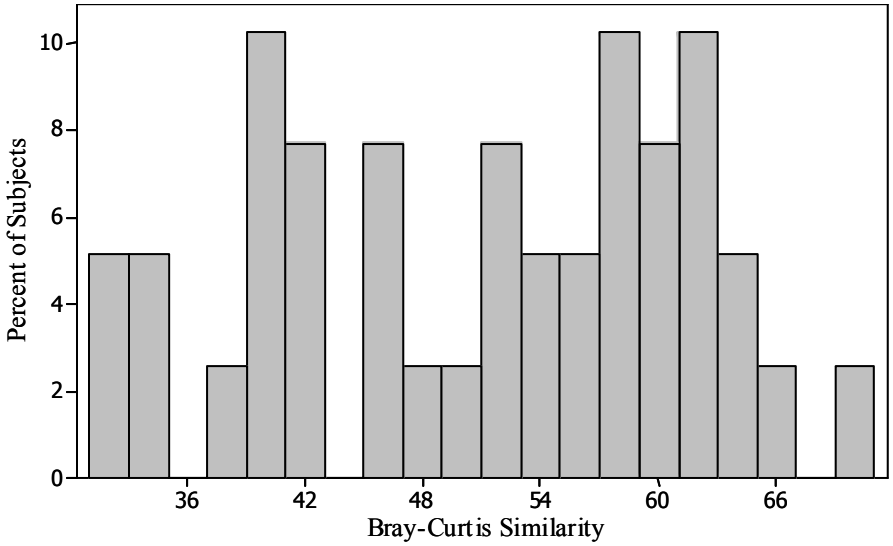

Figure 1. Average Bray-Curtis similarity of TRF patterns from each subject across the three initial samples (days 1, 7, 14). Note the two distributions. An estimated cut-off for Stable/Unstable categories was made at 50% similarity. Subjects with average similarity >50% during the first three sampling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

It was hypothesized that the microflora in some human GI tracts might not be stable due to varying degrees of dietary intake and stress levels. In this experiment, 16S ribosomal RNA (rRNA) Cy5-labeled PCR products were used in CEQ8000X to obtain Terminal Restriction Fragment (TRF) Length Polymorphism patterns (Kaplan et al, 2001).

Materials and Methods

Collection and processing of fecal samples

Fresh fecal samples were obtained from healthy human subject of 23 years of age for 14 days (Bunyea-Baron and Campanale, 2004). Subject was asked to not take any antibiotics for sixty (60) days from the initial fecal collection, until the end of the study period. Subject was also asked to keep a food diary, noting all types of food that were taken, including beverages, and to note stress level for that specific day. Upon collection of the fecal samples, they were manually homogenized and placed into sterile plastic containers (full filled), noting color. Samples were stored at -20°C and were processed within five (5) days.

Total DNA Extraction

Samples were extracted in triplicate using the MoBio Ultraclean® soil DNA kit following manufacture's protocol. Samples were stored at 4°C for further analysis.

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 1.0µL of a 1:10 diluted extraction product, 5 µL of 10X buffer, 3 µL 10m<u>M</u> DNTP, 2 µL 20µg/mL BSA, 7 µL 25m<u>M</u> $MgCl_2$, 1 µL forward primer, 1 µL reverse primer, 29.7 µL NanoPure H_2O, 0.3 µL 5U/µL *TaqGold*®. The reaction temperatures and times were: 94°C for 10 minutes; 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. All reactions were performed

in triplicate and then combined using MoBio Ultraclean® PCR Cleanup Kit following manufacture's protocol. DNA presence in each sample was determined by gel electrophoresis, and amounts of DNA in each sample were determined using a fluorometer.

Enzyme Digest and TRF Pattern Generation

An enzyme digest was performed on each PCR cleanup product using New England Biolabs restriction endonuclease *Hae*III. Each 40.0mL digestion used 175 ng/μL of DNA, 1 U of enzyme, and 4 mL of buffer. The samples were digested at 37°C for 4 hours, and inactivated at 80°C for 20 min. The digestion products were ethanol precipitated and re-suspended in 20 μL of formamide and 0.25μL of CEQ 600 base pair standard. Terminal restriction fragment profiles were obtained using 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 Excel. TRF peaks representing less than 0.5% of the total DNA presenting the samples were excluded from analysis. TRF peak area was converted to relative abundance (parts per million) and transformed by square root to deemphasize the importance of larger peaks for Principle Components, Euclidean, and Bray-Curtis analyses.

Results

Gel electrophoresis images indicated DNA from all samples were present and were available for application in further analyses. DNA concentration ([DNA]) was

determined by fluorometer and varied from approximately 30-70 µg/µL, as indicated in the following table:

Table 1. Fluorometry to Quantify Labeled with Cy5 PCR Products.

Sample, Day	[DNA], µg/µL
1	48.61
2	58.92
3	66.51
4	53.81
5	63.57
6	66.86
7	72.24
8	55.65
9	60.77
10	75.29
11	60.01
12	65.08
13	60.29
14	31.52

All samples produced good TRF data on the CEQ8000X. Bray-Curtis Similarity was used with TRF data to evaluate changes in a subject's fecal flora. Bray-Curtis Similarity takes the relative abundance of each TRF peak into account when comparing two patterns (Kitts et al, 2005). TRF data often contains zero values (a peak present in one pattern that is not seen in another) and covers 2.5 orders of magnitude variation in TRF peak area. Bray-Curtis Similarity was specifically designed for use with species abundance data sets and is less susceptible to bias introduced by large numbers of zero abundance data. Therefore, Bray-Curtis similarity is a preferable similarity measure when comparing TRF patterns (Kitts et al, 2005).

Bray-Curtis Similarity

$$100\left(1-\frac{\sum_{TRF=60}^{600}\left|Area_{TRF,d1}-Area_{TRF,d7}\right|}{\sum_{TRF=60}^{600}\left|Area_{TRF,d1}+Area_{TRF,d7}\right|}\right)$$

Figure 2. Formula for the similarity between samples used for TRF analysis. This case is set up to compare TRF peak area for TRFs from 60 to 600 nucleotides in one subject from day 1 (d1) to day 7 (d7).

It was determined that the minimum value of similarity for any two (2) samples from this subject was 57.0% and the maximum was 87.0 %. The average similarity was 68.1%, with standard deviation of ±3.2. The lowest combined three-day (3-day) average of similarity across all samples was 57.4%. A daily progression of the microflora dynamics in the GI tract was plotted (Figure 3), well above the 50% cutoff from the previous study. No two (2) consecutive days were less than 60% similar.

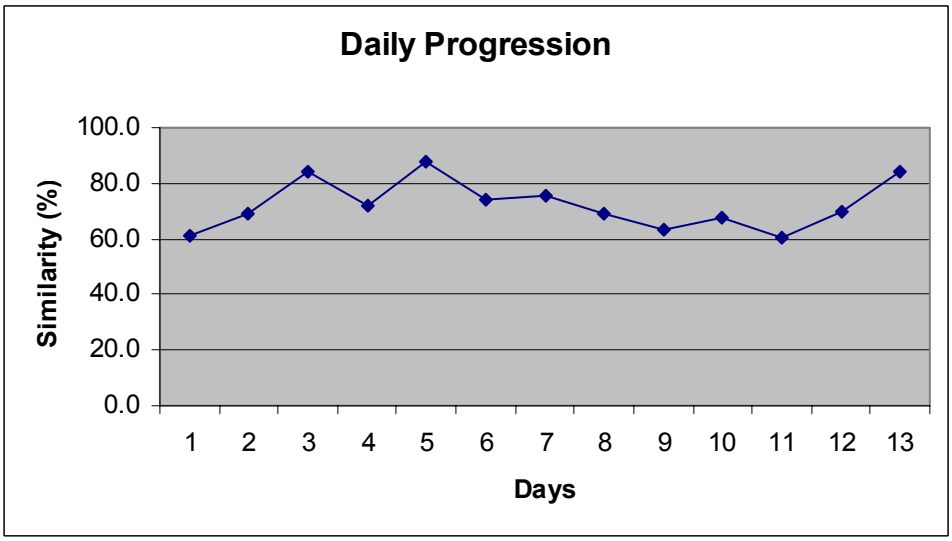

Figure 3. Daily Progression of Samples (i.e. Day 1 verses 2, 2-3, 3-4, etc.). The plotted daily progression indicates a relatively stable microflora. There was no evidence of instability.

A dendogram of Bray-Curtis Similarity was obtained to better identify which samples (one per day) were more similar to each other (Figure 4). No more than two (2) consecutive days were clustered together, indicating random fluctuations around an equilibrium.

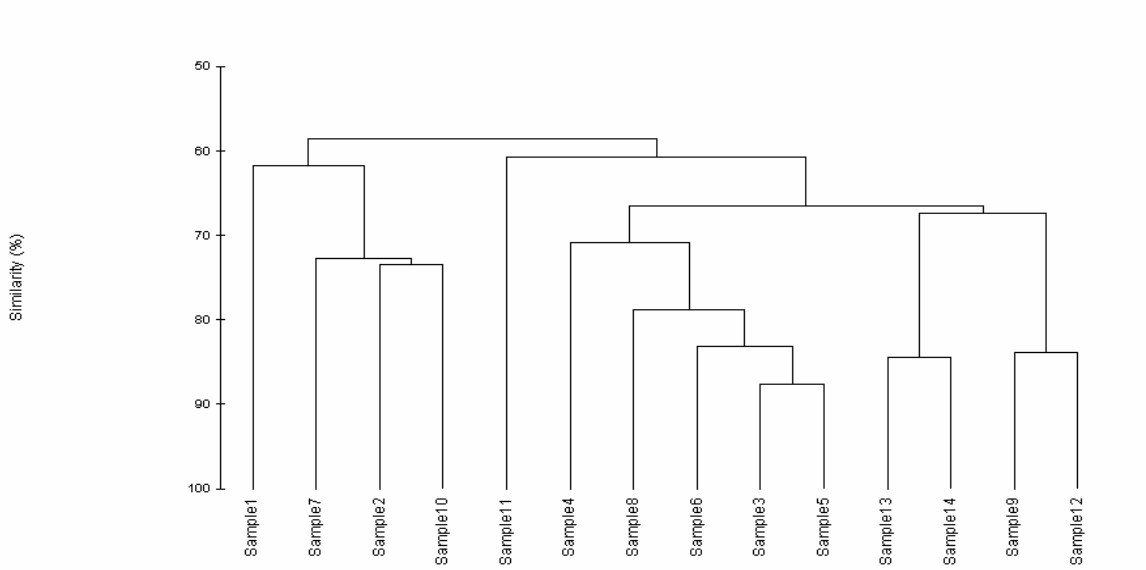

Figure 4. Dendogram of Samples based on Bray-Curtis Similarity. The cluster indicates which samples were more similar to each other.

TRF peak persistence and abundance were also plotted to empirically determine the factor(s) that contributed to the stability of the microflora in the GI tract (Table 2, Figure 5). Four (4) very large peaks were present in all samples. Many small TRF peaks showed up in only a few samples.

Table 2. Total TRF peaks Present in Samples (Days) and Their Corresponding Average Peak Area (%).

Days Present	Total TRF Peaks in Groups	Average Peak Area (%)
1	12	1.6
2	7	1.77
3	8	2
4	0	0
5	4	1.73
6	0	0
7	1	3
8	5	2.1
9	5	2.5
10	2	3.1
11	2	2.7
12	4	3.2
13	0	0
14	4	13.4

A bubble graph was plotted (Figure 5).

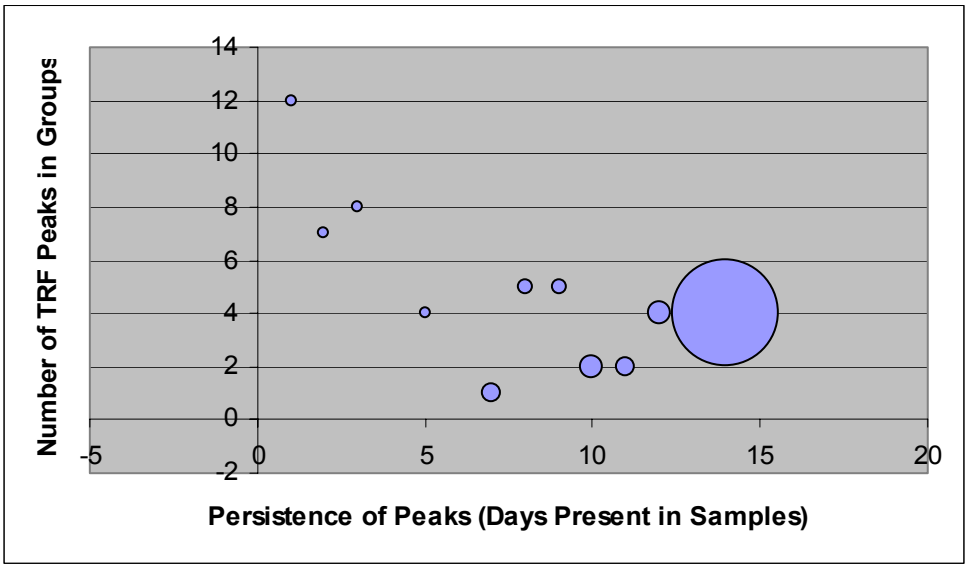

Figure 5. Persistence of Peaks vs. Number of TRF Peaks. Note: There are many small "bubbles" (width of bubbles were graphed), and one relatively very large "bubble."

Discussion

According to the data depicted in Figure 3, Daily Progression of Samples from day-to-day, a rather stable microflora was present in the GI tract of the subject over the course of the two (2) week study period. There were relatively little amounts of fluctuation between the samples. Based on Table 2 and Figure 5, the majority of TRF peaks in the samples had relatively small average peak area (%), rendering their impact on stability insignificant. However, there were a few TRF peaks, about four (4), which had an average area peak of 13.4% and were present in all samples. Thus, the relatively small amount of TRF peaks with large average peak area percentage had more impact, causing the microflora to be stable. Because no groups of consecutive sample grouped together in similarity (Fig. 4), it appears that the microflora varied randomly around an equilibrium. In conclusion, in this particular experiment, it was determined that the

microflora in the GI tract of this subject was stable. The hypothesis that some humans might have an unstable microflora was neither supported nor debunked by this experiment or the previous study by Kitts and colleagues (2005). A proof or disproof of this hypothesis awaits further studies like this one. A total of at least 55 subjects should be investigated.

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