AN INVESTIGATION OF MICROBIAL FLORA STABILITY OF THE HUMAN GASTROINTESTINAL TRACT USING TERMINAL RESTRICTION FRAGMENT POLYMORPHISM ANALYSIS OF THE 16S RIBOSOMAL RNA GENE

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ABSTRACT

This study is a follow-up study designed to answer the question of whether or not the average individual exhibits microbial stability. This question arose from a previous study conducted to determine whether or not pro-biotics, when taken simultaneously with antibiotics, could lessen the side effects of antibiotics by facilitating re-growth of beneficial intestinal flora. The results of the pro-biotic study were inconclusive due to a bimodal distribution of stable and non- stable subjects. The question of microbial stability was addressed using 14 fecal samples collected from one healthy subject with no dietary restrictions or medical treatments. DNA was extracted from the 14 fecal samples, and amplified by PCR. An enzyme digest was used to restrict the 16S rRNA gene. Terminal Restriction Fragment (TRF) patterns were used to monitor the changes in the microbial composition of the Gastro Intestinal Tract (GIT) throughout the 14 day period. The subject's GIT micro-flora was deemed stable after Bray-Curtis similarity analysis of TRF pattern data showed that day to day similarity was above 50%.

INTRODUCTION

The Gastro Intestinal Tract (GIT) is made up of the small intestine, the large intestine and the colon. The GIT is calculated to have a surface area of 150-200 m^2. This much area is necessary for all of the interaction and absorption that takes place in the GIT during the digestive process. The main function of the GIT is the absorption of water and nutrients. The GIT is colonized by an estimate of 10^{14} bacteria, which create the necessary environment for these digestive processes to occur smoothly [1]. The GIT micro-flora is an essential component of a healthy body. GIT micro-flora is responsible for degrading certain food components, producing B-vitamins, preventing the colonization of pathogens and producing essential digestive and protective enzymes[1,2]. Intestinal microbes are also thought to improve lactose tolerance, carry anti-tumor properties, neutralize certain toxins, and reduce blood lipid levels[2]. The main types of bacteria that colonize the GIT are lactobacilli, enterobacteria, streptococci, *Bifidiobacterium*, *Bacteriodes*, and clostridia [3]. *Bifidiobacterium* are known to be the major component of the microbial barrier to infection [3].

A previous study at the Environmental Biotechnology Institute (EBI), at California Polytechnic University, San Luis Obispo, was conducted to determine whether or not the consumption of probiotics along with antibiotics would aid in reducing some of the side effects of antibiotics by facilitating a quicker restoration of beneficial GIT bacteria. The results of the study were inconclusive since the control group was split into two normal distributions: 60% stable, 40% unstable [4](**Figure 1**). This study brought about the question of whether the human population differed in their degree of GIT microbial stability, or if the unstable group was a result of experimental error. Our study

set out to determine the answer to this question by Terminal Restriction Fragment analysis of the 16S rRNA gene from fecal samples collected over a fourteen day period [7]. The data was further analyzed and interpreted by Bray- Curtis similarity analysis [5].

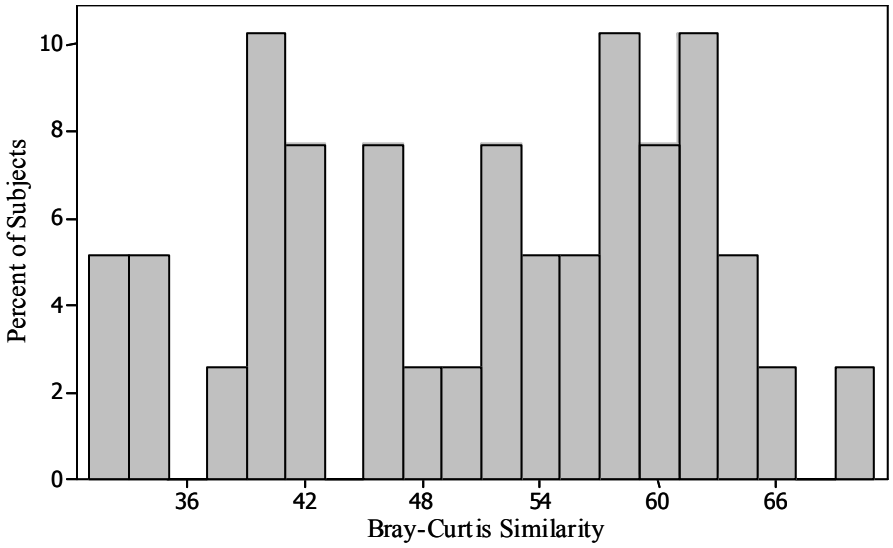

Figure 1. Average Bray-Curtis similarity of TRF patterns from each subject across the three initial samples (days 1, 7, 14). Note the two distributions. An estimated cut-off for Stable/Unstable categories was made at 50% similarity. Subjects with average similarity >50% during the first three sampling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

METHODS

Collection of Fecal Samples [6]

Fourteen samples were collected from a healthy, 20 year old subject over an eighteen day period. Antibiotics had not been used 60 days prior to the start of collection, nor during collection. There were several days skipped during the collection period due to non-consecutive bowel movements. Samples were collected at the subject's home and then stored in the freezer at 0ºC (for no more than five days), until DNA extraction was performed. Samples were collected using saran wrap over the toilet bowl as a catching device. The catching device was implemented to avoid toilet water and urine

contamination. The saran wrapped samples were then placed into a plastic zip lock bag and thoroughly homogenized. After homogenization 1.5 ml of sample was placed into a centrifuge tube and frozen until DNA extraction was performed.

Extraction of Bacterial DNA

DNA from the samples was extracted in triplicate, to ensure an abundant supply of sample. The MoBio Powersoil® DNA kit was used for the extractions and manufacturer's protocol was followed. The success of each extraction was determined by the visualization of DNA by agarose gel electrophoresis.

PCR Amplification

The DNA obtained from the fourteen samples was amplified by PCR in triplicate. 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 with phosphamide dye at the Cy5 position, were used for each reaction. Reactions were carried out using 1µL of a 10:1 diluted extraction product, 5µL of 10x Buffer , 3µL of 10 mM dNTP, 2µL 20 µg/mL BSA, 7 µL 25 mM $MgCl_2$, 1 µL Ba2FD4, 1 µL K2R, 29.7 µL of water, and 0.3 µL 5 U/mL TaqGold®. Reaction temperature and times were: 94ºC for 10 mi, 30 cycles of 94 ºC for 1 min, 46.5 ºC for 1 min, 72ºC for 2 min, and 72ºC for 10 min. Success of PCR was determined by visualization of products by 1.5% agarose gel electrophoresis. Since all reactions were performed in triplicate, the triplicates were combined into fourteen samples using MoBio Ultraclean® PCR clean up Kit following the manufacturer's protocol. PCR product quantification was performed by measuring the fluorescence of the Cy5

incorporated fluorescent label from the forward primer, using the A_{260} UV fluorometer following EBI protocol.

Enzyme Digest and TRF Pattern Generations

An enzyme digest was performed on each PCR product using the New England Biolabs restriction endonuclease *Hae*III. Each 40 µL digestion used variable amounts of DNA ranging from 3.26 to 12.12 µL, 0.4 µL of enzyme, and 4 µL of buffer. The samples were digested for 4 hours at 37ºC and inactivated for 20 minutes at 80ºC. The digestion products were ethanol precipitated to remove excess salts. After precipitation the digestion products were re-suspended 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 present in 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. Statistical analyses were performed using PrimerV and Excel.

RESULTS AND DISCUSSION

Bray -Curtis:

A Bray- Curtis similarity matrix was created in Primer V in order to track the similarity between the samples throughout the daily progression of the study period. Each sample TRF pattern was compared to the other 13 patterns. This data was then used to construct a daily

progression bar graph comparing the TRF pattern similarity between consecutive days of collection (**Figure 2**). Day 1 was compared to day 2, day 2 to day 3 and so on. The average similarity between all fourteen TRF patterns was 65. 69%. The most dissimilar comparison between two adjacent samples was 54.19% for days 3 and 4. Since the most dissimilar comparison had a value of 54.29% similarity, the subject was deemed stable as this value was above the stable criteria of 50%.

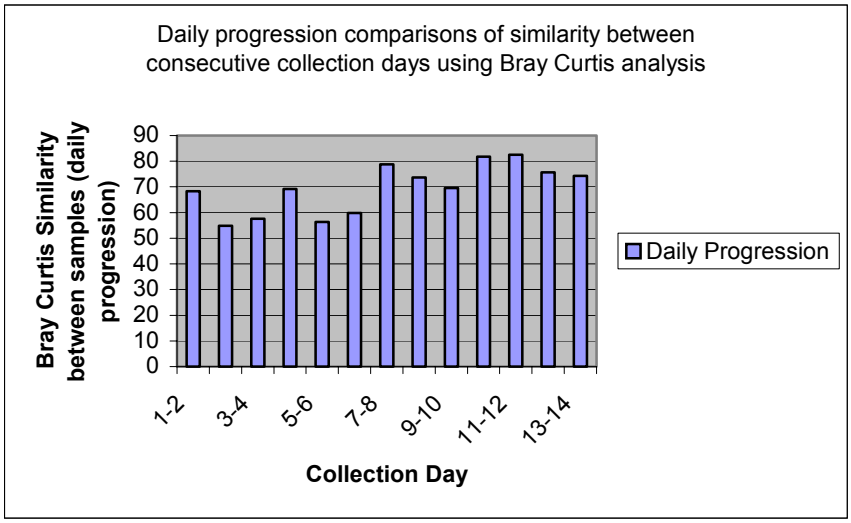

Figure 2: Daily progression bar chart displaying percent similarity (based on Bray-curtis analysis of TRF patterns) between fourteen consecutive days of sample collection.

To further support the 50% criterion another analysis was performed based on Bray-Curtis similarity data. Since it is possible for the natural fluctuations in GIT micro flora to result in a random set of 3 samples being on average less than 50% similar deeming the subject unstable, the lowest possible average of any three Bray-Curtis similarity scores was calculated to determine whether or not the data set carried such data that would support unstable flora. The lowest possible average similarity between three samples was 56.15%. This data demonstrates that no possible combination of percent similarity can be considered unstable because no possible combination of three patterns averaged less than 50% similar.

Bray- Curtis similarity data was used to construct a dendrogram in Primer V to allow better visualization of day-to-day similarity (**Figure 3**). The dendrogram clusters the most similar samples and separates the most dissimilar samples. Those days that are clustered lower on the dendrogram are most similar. Those days that branch off near the beginning of the dendrogram are least similar. These results indicate an overall similarity of about 56%. It is clear that consecutive samples deviated from each other resulting in random groupings of samples. The dendrogram supports the fact that changes in GIT micro flora did not happen in a gradual manner, but rather the changes between consecutive samples fluctuated at random around an equilibrium.

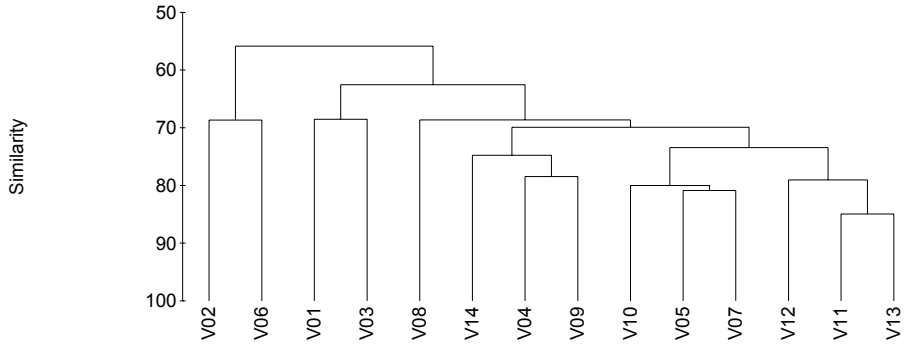

Figure 3: The dendrogram shows most similar samples clustered together. Since clustering of samples does not follow any kind of consecutive order, the dendrogram supports that changes GIT micro flora fluctuated at random around an equilibrium.

Individual TRF Peak Persistence and Abundance:

The persistence of a TRF peak was based on how many times it appeared throughout the fourteen day study. Those peaks that persisted the same number of days (in the same number of TRF patterns) were grouped together into persistence groups. For example, all peaks that persisted in all 14 TRF patterns were grouped together. All peaks that only appeared in one of any of the fourteen TRF patterns were clustered together into another persistence group. Once all peaks were clustered into their proper persistence groups, their areas were totaled and an average peak area of each persistence group was calculated **(Table1)**. A bubble graph of peak persistence (days present in the collection period) versus the number of TRF peaks was plotted in order to help visualize the relationship between peak frequency and relative size of certain bacterial populations within the human intestine (**Figure 4**).

Sample count	#TRFs	Average Peak Area
1	27	11817
2	18	11777
3	5	16092
4	3	8041
5	4	24602
6	4	14673
7	5	18303
8	0	0
9	0	0
10	2	59699
11	0	0
12	4	30528
13	1	59146
14	12	47655

Table 1: TRF peaks were grouped into their persistence groups by how many times they appeared in all fourteen TRF patterns. The average peak area of each persistence group was calculated.

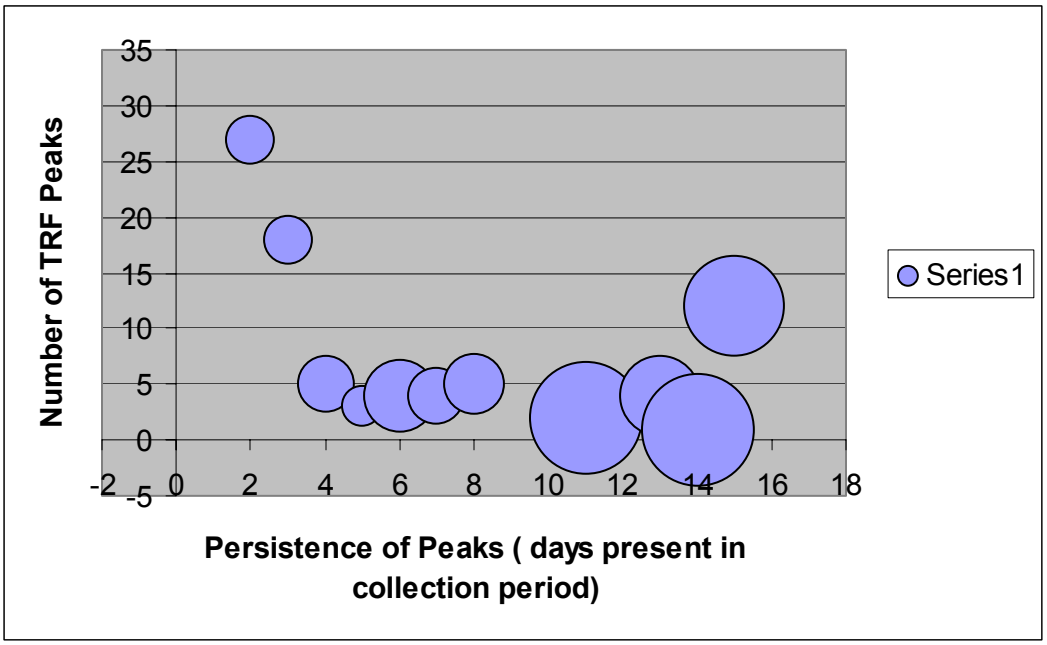

Figure 4: This bubble plot represents the persistence of peaks throughout the fourteen TRF patterns, and the number of TRF peaks that occurred in each persistency group. Most persistent peaks tend to have a greater abundance or average peak area as indicated by the diameter of the bubble.

Two extremes were visible in the bubble plot of peak persistence and abundance, peaks that were present in nearly all or absent in nearly all of the TRF patterns. Groups near the absent extreme had the highest number of TRF peaks, and the smallest areas. Groups near the greater persistence extreme had much larger areas, and were therefore both abundant and persistent. The peaks of greatest persistence are those of stable and dominant GIT micro flora as is visible by their larger average peak area. These highly persistent peaks have the greatest impact on the GIT. The peaks of least persistence and lower abundance are those bacteria with an insignificant impact on the human GIT.

CONCLUSIONS

The final analysis of the subject's GIT micro flora showed that the subject could be considered stable. Stability is a result of random fluctuations around a state of equilibrium and there is no definite pattern of consecutive day to day similarity. Organisms that are high in abundance, as well as persistence, dominate the human GIT and are responsible for upholding the stability of human GIT microbial communities.

In reference to the previous study, which asked the question of whether or not the average human GIT exhibits microbial stability, this subject falls into the stable category as her Bray-Curtis similarity analysis shows that her greatest dissimilarity was above 50%. However, because this study was performed on only one subject it does not answer the question of whether or not GIT microbial stability exists but only adds to the pool of similar studies that support this claim. In order to assess microbial stability of the human GIT more subjects must be studied, until at least 40 data sets can be considered.

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