STABILITY OF INTESTINAL TRACT MICROFLORA AS DETERMINED BY TRF PATTERN ANALYSIS OF 16S RIBOSOMAL RNA GENE

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ABSTRACT

To determine the stability of prokaryotic microflora in the intestinal tract, fecal samples were collected daily for 14 days. No antibiotics were taken during and in the sixty days prior to collection. Bacterial DNA was extracted, the 16S ribosomal RNA gene was amplified by PCR, and digested with the enzyme *Hae*III. Terminal Restriction Fragment patterns were obtained using the Beckman Coulter CEQ8000X, then analyzed by Bray-Curtis similarity. The average day to day similarity was over 74%, indicating that the subject's microflora was very stable.

INTRODUCTION

The human gastrointestinal tract contains numerous anaerobic prokaryotic bacteria that are needed to help maintain optimal health. Within the intestines, these organisms make up 1-1.5 kg of bacterial mass, with about 10^{11} individual bacteria per gram. The composition of this intestinal microflora tends to be fairly stable in most individuals, however diet, stress, medications, and disease can change the composition. (Toivanen 2001) *Bacterioides* is thought to make up at least 30% of the total flora, with the remainder a mix of bifidobacteria, clostridia, eubacteria, lactobacilli, gram-positive cocci, coliforms, methanogens and sulfate-reducing bacteria. In total there are an estimated 400-500 different species inhabiting the human gut. (Gibson 1999)

The bacteria aid in digestion of food, vitamin production, and prevention of pathogen colonization. (Matsuki et al, 1999) Traditionally, these organisms were studied by isolation and plate culture, and identified based on phenotypic characteristics. However, this approach is time consuming and not always accurate, because the physiological status and growth requirements of the microflora are not known. (Jernberg 2005)

The method used more commonly today involves examining Terminal Restriction Fragment (TRF) patterns. DNA is extracted from fecal samples, then amplified using fluorescently-labeled primers that target the 16S rRNA gene. After amplification, the fragments are digested with a restriction enzyme. Capillary gel electrophoresis with fluorescence detection is used to visualize the TRF patterns, which vary by taxonomic group. DNA standards of known sizes are used for comparison to determine the size of the TRF fragments. Statistical analysis of the patterns can be used to determine how similar they are to one another, and thus conclude whether or not there is overall stability. (Kitts, 2001)

One study conducted by the Environmental Biotechnology Institute at California Polytechnic State University used TRF analysis to determine the effects of probiotic dietary supplements on the stability of microflora in the intestines. Over the two-week study period, fecal samples were collected three times, on days 1, 7, and 14. DNA was isolated and TRF analysis performed. After analyzing the TRF patterns using Bray-Curtis similarity, it was determined that the subjects of the study fell into two groups: those with generally unstable intestinal flora (similarity below 50%); and those with generally stable intestinal flora (similarity above 50%) (see Figure 1)

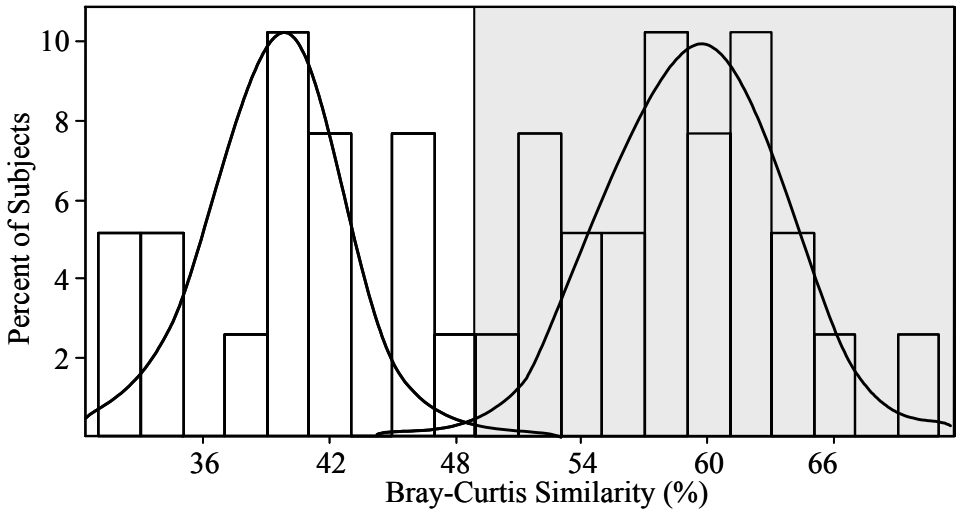

Figure 1. Average Bray-Curtis similarity for samples collected on days 1, 7, and 14. Note the two distributions. Those with a greater similarity that 50% are considered stable, while those below 50% are considered unstable.

Differences in microflora stability could be due to diet or stress level, but because samples were not collected every day, it was possible that the appearance of two groups is misleading. This study is part of a follow-up to obtain more complete control data sets, using the same method of TRF analysis, but with fecal sample collection every day of the 14-day period, rather than on just three of the days.

MATERIALS AND METHODS

Fecal Sample Collection

Fecal Samples were collected from a healthy 22 year old female for 14 consecutive days, following 60 days without taking antibiotics. A catching device was used that prevented the sample from coming in contact with the toilet, and care was taken to avoid urine contamination. After collection, samples were manually homogenized and frozen at -4°C for no more than five days before DNA extraction.

DNA Extraction

DNA was extracted from each sample using the MoBio PowerSoil® kit according to manufacturer's instructions. Presence of DNA was verified by agarose gel electrophoresis.

PCR Amplification

PCR was performed using 16S rDNA primers homologous to conserved regions on the Eubacterial 16S rRNA gene. Each reaction used 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. Reactions were carried out using 1 µL of extraction product, 5 µL of 10x Buffer, 3 µL of 10 mM dNTPs, 2 µL (20 µg/mL) BSA, 7 µL (25 mM) $MgCl_2$, 1 µL K2R, 1 µL Ba2F, 29.7 µL water, and 0.3 µL (5 U/mL) TaqGold®. The reaction mixture was heated to 94°C for 10 minutes, then underwent 30 cycles of 94 °C for 1 minute, 46.5 °C for 1 minute, 72 °C for 2 minutes. This was followed by 72 °C for 10 minutes, then the reaction mixture was held at -4 °C. Agarose gel electrophoresis was used to confirm the success of PCR. Reactions were performed in triplicate, then combined using a MoBio Ultraclean® PCR Cleanup Kit according to manufacturer's protocol. The concentration of amplicons in each sample was determined using a fluorometer.

Enzyme Digest and TRF Pattern Generation

The product of PCR cleanup was digested using New England Biolabs restriction endonuclease *Hae*III. Each 20 µL digestion used 75 ng of DNA, .5 µL of enzyme (*Hae*III), and 2 µL of buffer. The samples were digested for 4 hours at 37 °C, followed by 20 minutes at 80 °C to inactivate the enzyme. Following digestion, ethanol precipitation removed the excess salts, then the product was suspended in 20 µL of formamide and 0.25 µL of CEQ 600 base pair standard. The Beckman Coulter CEQ 8000X DNA analysis system was used to produce terminal restriction fragment (TRF) patterns.

Data Preparation and Statistical Analysis

Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQ8000X into Microsoft Excel. TRF peaks representing less than 0.5% of the total DNA were excluded. TRF peak area was converted to percent of total area, and analyzed by Bray-Curtis similarity.

RESULTS AND DISCUSSION

The subject collected fecal samples daily for 14 consecutive days, while keeping a food log. TRF patterns were produced and analyzed based on their similarity from sample to sample. Figure 2 shows the TRF patterns obtained using the CEQ8000X. Differences in these patterns indicate that changes in the microflora of the intestine may have occurred between the times the samples were collected. Because many of the same peaks are present in each sample, the subject appears to have stable gut microflora.

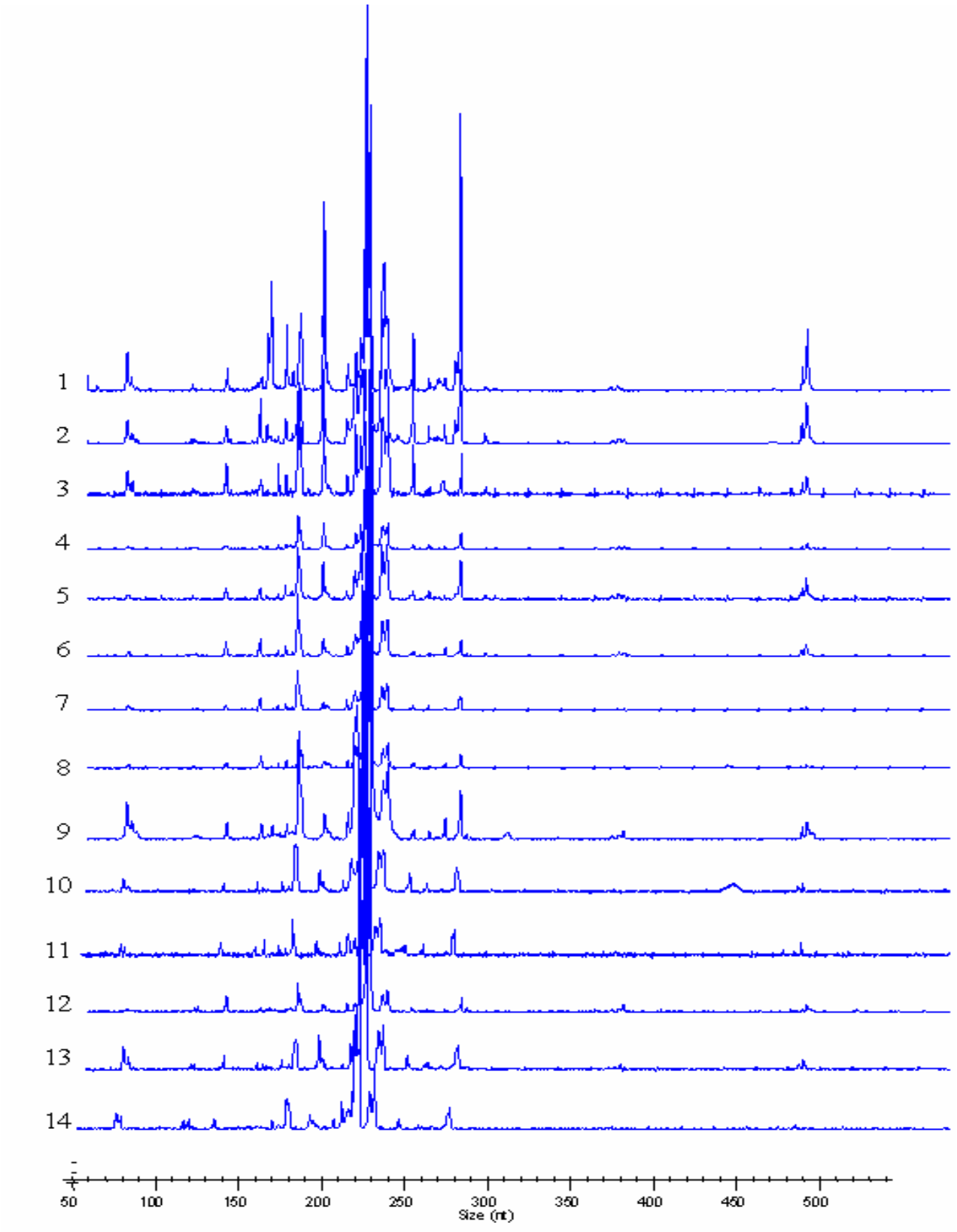

Figure 2. TRF patterns for samples 1 through 14. Peak height represents the intensity of labeled primer being detected

Bray-Curtis similarities were determined for each pair of TRF patterns using PrimerV to create a similarity matrix. A daily progression bar graph shows that similarity changed very little over the course of the study (Figure 3). The most similar consecutive samples were those collected on Days 7 and 8, with a Bray-Curtis similarity of 91.3%. The least similar consecutive samples were those collected on days 11 and 12, with a Bray-Curtis similarity of 66.0%.

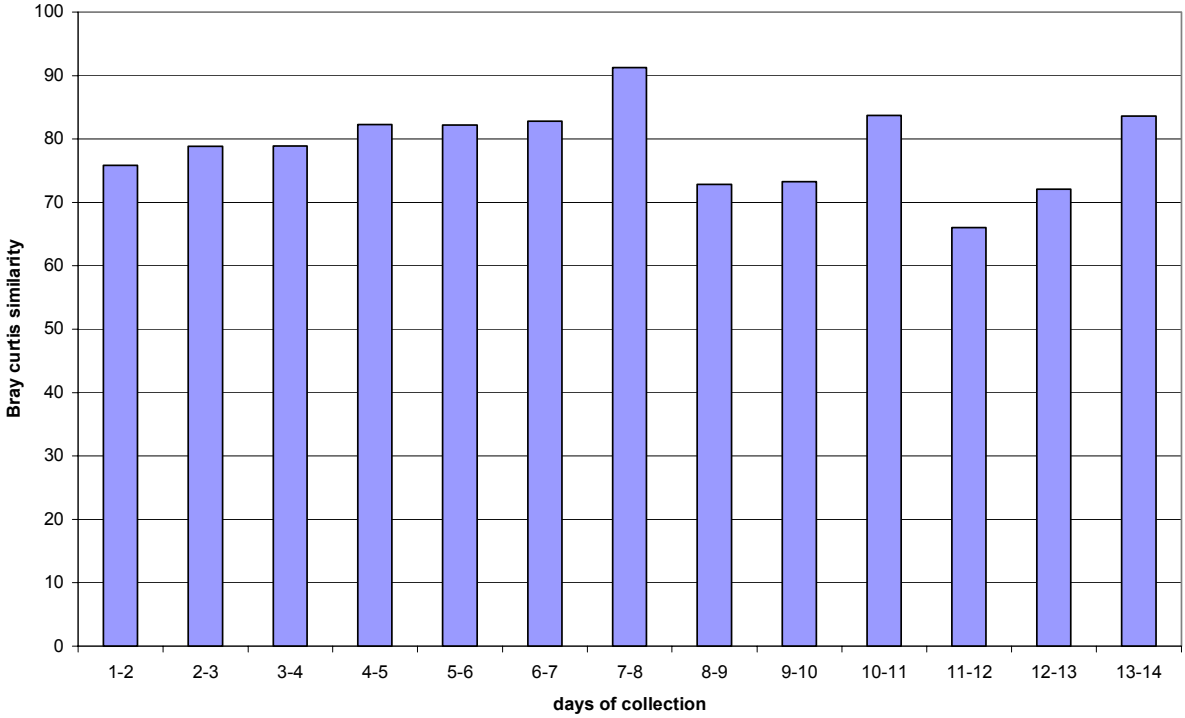

Figure 3. Similarity progression showing the TRF pattern Bray-Curtis similarity between each sample and the sample from the following day.

The overall average similarity was 74.3%, which is considered stable since it is greater than 50%. The three least similar samples, collected on Day 1, Day 2, and Day 12, had an average similarity of 65.9%, indicating that no possible combination of three samples could indicate an unstable microflora. To visualize similarity between all samples a dendogram was created in PrimerV using the information contained in the Bray-Curtis similarity matrix (Figure 4). The most similar samples are grouped together, and the more samples are separated, the less similar they are. The grouping of samples is random after the first two days, showing that

changes in microflora did not change gradually over the course of the study. However, the subject was slightly ill on the first two days of the study, which could have played a roll in the lower similarity of the samples collected those days.

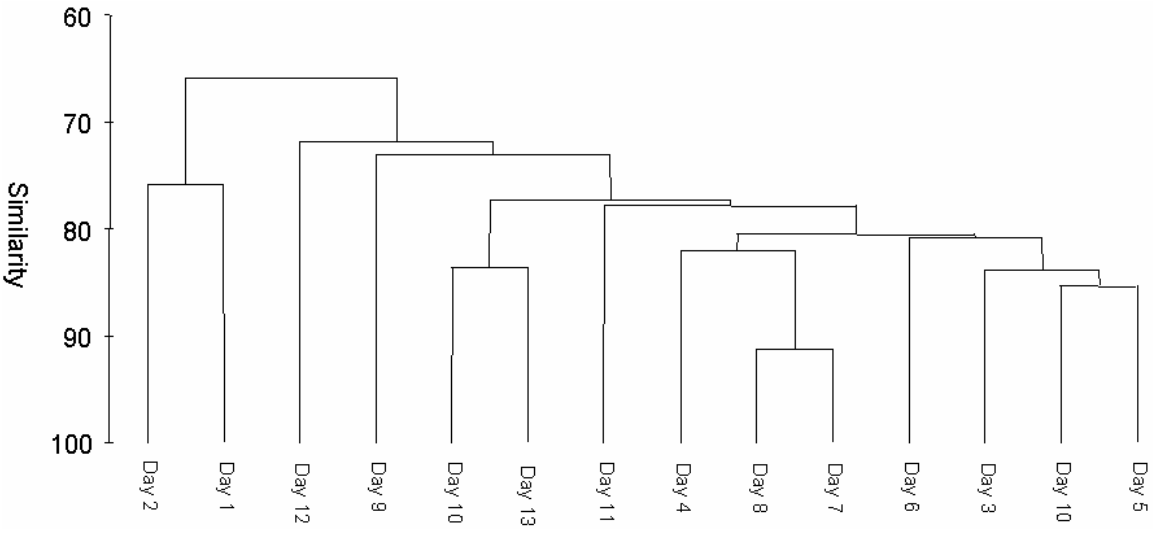

Figure 4. Bray-Curtis similarity dendogram. Note samples dispersed randomly rather than in sequential order.

Another method of analysis was to look at the presence of TRF peaks across all the samples. Table 1 lists the number of TRF peaks and the average peak area for TRF peaks present in a specific number of samples. This data supports the conclusion that the microflora is stable because the peaks with the greatest area were present on all 14 days of the study. While there are more individual peaks that could only be seen on one day, they do not contribute a significant area, so they do not indicate great changes in stability. In fact, the species with an abundance of less than 1% may be inconclusive, as they may be at the detection limit for the CEQ8000X. This data is visually represented in Figure 5.

The general trend shown in Figure 5 indicates the existance of a large number of different types of bacteria in low abundance that fluctuate from day to day. In addition, there is a group with much greater abundance that is present consistently.

Number of samples in which peaks are present	Number of Peaks	Area of peaks (%)
1	13	1.27
2	8	1.84
3	5	0.99
4	4	1.08
5	0	0.00
6	1	1.05
7	1	0.63
8	3	1.75
9	2	1.01
10	1	2.83
11	2	1.22
12	1	1.00
13	3	1.80
14	11	7.23

Table 1. Persistence of TRF peaks. The peaks are grouped by how many samples in which they appeared, and their area.

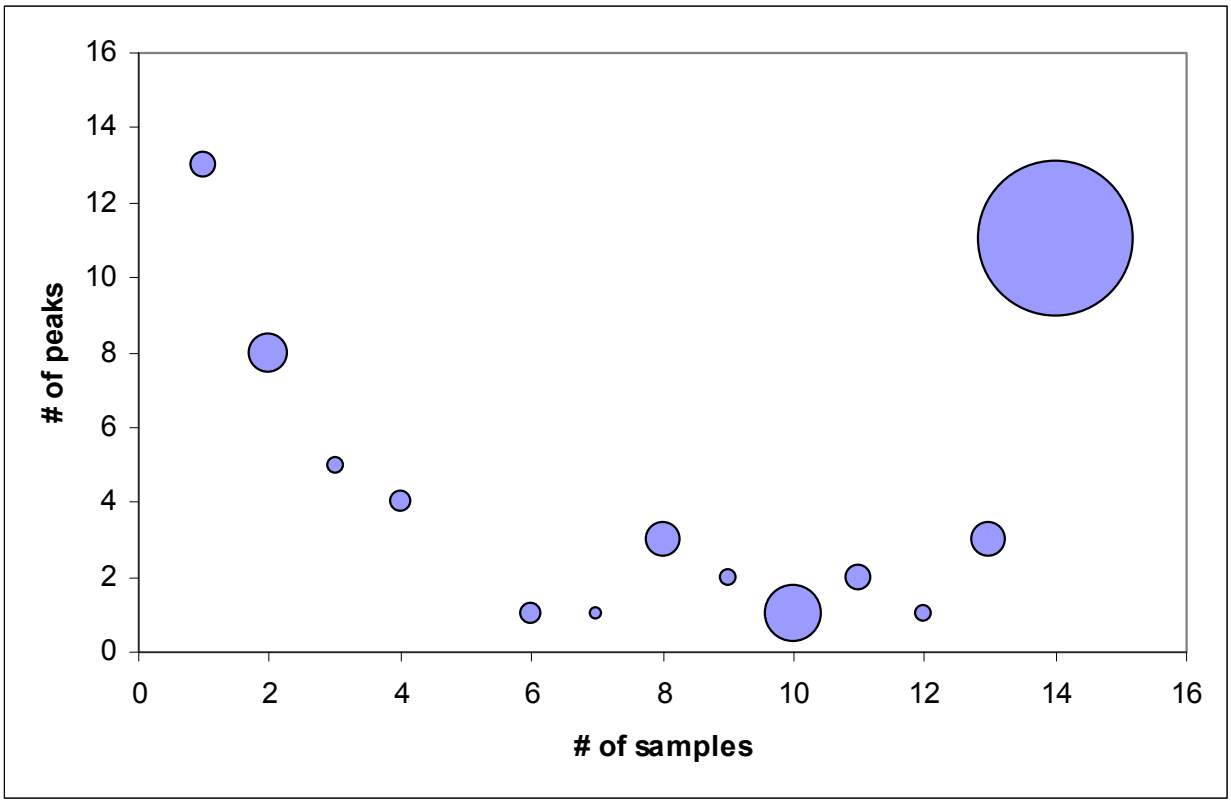

Figure 5. The number of peaks plotted agains the number of samples in which they appear. The size of the circle indicates the area made up by those peaks.

CONCLUSION

The individual in this study was found to have stable microflora, with an average overall similarity of greater than 50%. This stability was largely due to a group of very abundant microflora that did not fluctuate from day to day. While some organisms in the GIT do fluctuate in abundance, they make up a small enough portion of the total microflora that they do not affect the overall community structure.

Future considerations for additional follow-up to the study presented in the introduction include obtaining results from a greater number of people, representing a variety of lifestyles, ages, and genders. It would also be ideal to complete DNA extraction immediately upon collection of the sample, although this may not be logistically feasible.

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