

**ANALYSIS OF TREATMENT EFFECTS ON THE MICROBIAL ECOLOGY OF
THE GASTROINTESTINAL TRACT**

A thesis presented to the
Faculty of the Biological Sciences Department
California Polytechnic State University, San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Biological Sciences

By

Anna Lynn Engelbrekton

August 2005

OptiBac Probiotics
www.optibacprobiotics.co.uk

© 2005

Anna Lynn Engelbrekton

ALL RIGHTS RESERVED

APPROVAL PAGE

TITLE: Analysis of Treatment Effects on the Microbial Ecology of the
Gastrointestinal Tract

AUTHOR: Anna Lynn Engelbrekton

DATE SUBMITTED: August 2005

Christopher Kitts

Advisor

Signature

Raúl Cano

Committee Member

Signature

Rafael Jiménez-Flores

Committee Member

Signature

Mary Ellen Sanders

Committee Member

Signature

ABSTRACT

ANALYSIS OF TREATMENT EFFECTS ON THE MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT

ANNA L. ENGELBREKTSON

This work is a compilation of a manuscript, a final report submitted to Danisco Corporation, and a final report submitted to the California Central Coast Research Partnership (C3RP). All work was based on a Danisco funded study about the effect of probiotic treatment on the fecal bacterial communities of individuals undergoing antibiotic therapy. Both the molecular method of Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis and standard culturing were used to assess gastrointestinal microflora before, during, and after treatment.

The report to Danisco is the final report addressing both the culture and the 16S rDNA Bacterial TRFLP work. The C3RP report is a companion study specifically investigating *Lactobacillus* and *Bifidobacterium* communities using TRFLP with 16S rDNA genus specific primers. The manuscript is a discussion of analysis methods developed to analyze the complex data from studies of fecal flora.

The purpose of the Danisco study was to investigate the role of probiotics in the normalization of gastrointestinal flora after disturbance by antibiotic therapy. Fecal samples were collected from 40 individuals at seven time points over a 48-day period. Individuals were given a course of antibiotics, with half of the subjects concurrently consuming probiotics and the other half receiving a placebo. Bacterial DNA was extracted from each fecal sample and PCR was performed using 16S rDNA Bacterial primers. A restriction endonuclease was used to digest the PCR product and terminal

restriction fragment (TRF) pattern analysis was performed using capillary gel electrophoresis. Bacterial culturing was performed using five different selective media for *Bifidobacteria*, *Lactobacillus*, members of the *Bacteroides fragilis* subgroup, *Clostridium* species, and *Enterobacteriaceae*. This study was the basis of all the following work.

The goal of the C3RP study was to evaluate the effect of *Bifidobacterium* and *Lactobacillus* communities. DNA from the Danisco study fecal samples was amplified with *Bifidobacterium* and *Lactobacillus* specific PCR primers. As in all of the reports, species distribution for each genus was analyzed by Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of the PCR products.

In the manuscript, two different human trials are presented where fecal TRFLP data sets were analyzed to search for treatment effects. The relative merits of Principle Components Analysis and Cluster analysis based on grouped data were discussed in a comparison with analysis of data by subject using distance coefficients. Comparison to baseline within an individual before grouping by treatment provided the clearest indication of treatment effects as opposed to an evaluation of data grouped before analysis.

This Master's thesis resulted in research that has been presented at three national and two international meetings and a manuscript is currently in preparation for publication.

ACKNOWLEDGEMENTS

I would like to thank everyone in the EBI for their support and encouragement. Without help, none of this work would have been possible. I would especially like to thank all the senior project students who worked on the project; Leisah Burrow for her work with *Clostridium*, *Prevotella*, and *Bacteroides* primers, Litz Aguilar and Jessica Cooper for their work with *Clostridium* primers, Dominic Lung for his work with *Bifidobacterium* primers, and Hailinh Nguyen for his work with the difficult *Lactobacillus* primers. Without their assistance, I would have never finished such an enormous project. I would especially like to thank my fellow grad student, Kelly Wrighton. Her statistical expertise and moral support were vital to my success.

I would especially like to thank my committee. Mary Ellen Sanders conscientiously read and edited every report and poster I created and flew out here especially for my defense. Also, all the background material she provided was extremely helpful. Special thanks to Dr. Kitts, I would have gotten lost in all the statistics without his help. Seeing his love of data and analysis is an inspiration. He is truly devoted to every single one of his students. This department is a wonderful environment and I learned from everyone.

This project would not have been possible without funding from Danisco USA. The genus specific primer work was funded by the California State University Program for Education and Research in Biotechnology (CSUPERB) and by the California Central Coast Research Partnership (C3RP). This funding not only supported the work but also allowed me to grow as a scientist through both the research and by allowing me to attend numerous national and international conferences.

TABLE OF CONTENTS

LIST OF FIGURES	IX
LIST OF TABLES	XII
CHAPTER 1: ANALYSIS OF TREATMENT EFFECTS ON THE MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT	1
<i>Abstract</i>	2
<i>Introduction</i>	3
<i>Methods</i>	6
Probiotic-Prebiotic study design	6
Antibiotic-Probiotic Study Design	7
Bacterial culturing (Probiotic/Antibiotic Study)	9
Creation and normalization of TRF pattern data	10
Analysis of TRF pattern data	11
<i>Results</i>	11
Analysis of the Probiotic-Prebiotic Study TRF Data	11
Analysis of the Antibiotic-Probiotic Study TRF Data	20
Analysis of the Probiotic-Prebiotic Study Culture Data	24
<i>Discussion</i>	29
<i>Acknowledgements</i>	32
<i>References</i>	33
CHAPTER 2: THE EFFECT OF PROBIOTICS ON HEALTHY SUBJECTS UNDERGOING ANTIBIOTIC THERAPY	41
<i>Executive Summary</i>	42
<i>Background</i>	45
<i>Study Design</i>	46
<i>Methods</i>	47
Subject selection and sampling protocol	47
Bacterial culturing	48
TRF patterns	49
<i>Results</i>	53
<i>First Level of Analysis for TRF data – Principal Components Analysis</i>	54
Distance Measures	58
Identifying Changes in TRF Peaks	59
Stability of Fecal Flora	60
Bacterial Culture Analysis	63
<i>Effects of Antibiotics and Probiotics</i>	64

Effects seen with TRF data	64
TRF Peaks Changing with Antibiotic Treatment	65
Multi enzyme analysis	71
Effects Seen with Culture Data	75
Tracking Probiotic Strains with TRF data	78
<i>Conclusions</i>	82
<i>References</i>	85
CHAPTER 3: THE EFFECT OF PROBIOTICS ON THE SPECIES DISTRIBUTION OF <i>BIFIDOBACTERIUM</i> AND <i>LACTOBACILLUS</i> IN HEALTHY SUBJECTS UNDERGOING ANTIBIOTIC THERAPY	87
<i>Executive Summary</i>	88
<i>Introduction</i>	89
<i>Materials and Methods</i>	90
Study Design	90
Isolation of bacterial DNA	91
PCR Amplification	92
Enzyme Digest and TRF Pattern Generation	92
Data Preparation and Statistical Analysis	93
<i>Results</i>	93
<i>Bifidobacterium</i>	93
<i>Lactobacillus</i>	96
<i>Discussion</i>	97
<i>References</i>	99

LIST OF FIGURES

CHAPTER 1

1. Schematic of the Antibiotic-Probiotic experimental design. 8

Probiotic-Prebiotic Study TRF Data

2. PCA score plots of all subjects in each treatment group. 13
3. PCA score plots and cluster analysis dendrograms of two subjects from different treatment groups. 15
4. Formulas for the two distance measures used for TRF and culture analysis. 18
5. Interval plots of Bray-Curtis similarities of weeks 3 to 9, 3 to 12, and 9 to 12 for each treatment group. 19

Antibiotic-Probiotic Study TRF Data

6. PCA score plots of all subjects' days 1-21. 21
7. PCA score plots and cluster analysis dendrograms of two subjects from different treatment groups. 22
8. Average Bray-Curtis similarity from base line (days 1-14) for each day after treatment. 24

Probiotic-Prebiotic Study Culture Data

9. Interval plots of log colony-forming units (CFU) for all subjects' days 1-21 for each type of media. 25
10. Average Euclidian distance from base line (days 1-14) for each day after treatment. 27
11. Interval plots of difference from base line (days 1-14) for each day after treatment for each different medium. 28

CHAPTER 2

1. Schematic of the experimental design. 47
2. Electropherogram of TRFs from a single sample showing TRF peak size in nucleotides and area under the peak. 52

Principal Components Analysis

3. Principle Components Analysis score plot of all subjects days 1-21. 56
4. Principle Components Analysis score and loading plots for subject 50. 57

5. Principle Components Analysis score and loading plots for subject 42.	58
<i>Distance Measures</i>	
6. Formulas for the three distance measures used for TRF analysis.	59
<i>Stability of Fecal Flora</i>	
7. Average Bray-Curtis similarity of TRF patterns from each subject across the three initial samples (days 1, 7, 14).	61
<i>Bacterial Culture Analysis</i>	
8. Average Euclidian distance analysis of plate count pattern from each subject across the three initial samples (days 1, 7, 14).	63
<i>Effects Seen with TRF Data</i>	
9. Average Bray-Curtis similarity from base line (days 1-14) for each day after treatment.	65
10. Difference in TRF peak area from base line (days 1-14) for each day after treatment. TRF peaks that returned to baseline faster in the probiotic treatment group are presented.	69
11. Difference in TRF peak area from base line (days 1-14) for each day after treatment. TRF peaks that returned to baseline faster in the placebo treatment group are presented.	70
<i>Multi Enzyme Analysis</i>	
12. Comparison of multiple enzyme data for peaks representing enteric organisms.	72
13. Comparison of multiple enzyme data for peaks representing <i>Bifidobacteria</i> .	73
14. Comparison of the multiple enzymes for peaks corresponding with <i>Bacteroides</i> or <i>Prevotella</i> .	74
<i>Effects Seen with Culture Data</i>	
15. Average Euclidian distance from base line (days 1-14) for each day after treatment.	76
16. Interval plots of difference from base line (days 1-14) for each day after treatment for each different medium.	77
<i>Tracking Probiotic Strains with TRF Data</i>	
17. Interval plots of TRF peak area (including zeros) for each peak that corresponds to a probiotic strain.	79
18. Interval plots of TRF peak area (excluding zeros) for each peak that corresponds to a probiotic strain.	81

CHAPTER 3

1. Schematic of the Antibiotic-Probiotic experimental design. 91

Bifidobacterium

2. Average Bray-Curtis similarity from baseline (days 1-14) for each day after treatment. 94
3. Difference in TRF peak area from baseline (days 1-14) for each day after treatment. 95

Lactobacillus

4. Average Bray-Curtis similarity from baseline (days 1-14) for each day after treatment. 97

OptiBac Probiotics
www.optibacprobiotics.co.uk

LIST OF TABLES

CHAPTER 1

CHAPTER 2

1. TRF peaks discriminating between “Stable and Unstable” categories with TRF data. 62
2. TRF peaks associated with day 21 in patients that showed an antibiotic effect. 67
3. TRF peaks associated with enteric organisms in multiple subjects indicating the variation in bacterial strains from subject to subject. 71

CHAPTER 3

OptiBac Probiotics
www.optibacprobiotics.co.uk

**CHAPTER 1: ANALYSIS OF TREATMENT EFFECTS ON THE MICROBIAL
ECOLOGY OF THE GASTROINTESTINAL TRACT**

A.L. Engelbrektson¹, J.R. Korzenik², M.E. Sanders³, B.G. Clement⁴ T.R.

Klaenhammer⁵, and C.L. Kitts¹

¹*Environmental Biotechnology Institute, California Polytechnic State University, San
Luis Obispo, CA*

²*Massachusetts General Hospital, Harvard Medical School, Boston, MA*

³*Dairy and Food Culture Technologies, Centennial, CO*

⁴*Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA*

⁵*North Carolina State University, Raleigh, NC*

Abstract

A large number of studies have been performed investigating gastrointestinal flora and changes in the gastrointestinal community. However, a concern in these studies is how best to assess changes in gastrointestinal community structure. This paper presents two different human trials where the fecal TRFLP data sets were analyzed to search for treatment effects. Principle Components Analysis and Cluster analysis based on grouped data are compared with analysis of data by subject using distance coefficients. Comparison to baseline within an individual before grouping by treatment provided a clearer indication of treatment effects than an evaluation of data grouped before analysis. In addition, a large within-subject sample size and multiple baseline samples are necessary to accurately analyze treatment effects.

Introduction

The gut represents the largest mucosal surface in the body and the microbial community present can have a large effect on immune function. This community consists of a large number of different species and the species present differ between the mucosa and the feces [1]. However, most human studies utilize fecal samples due to the ease of sample collection. It is believed that a healthy intestinal flora can protect against certain types of infection and studies have shown that a healthy intestinal flora is a good barrier against potentially pathogenic microorganisms [2].

Antibiotics are known to disrupt the normal intestinal flora. They can cause disturbance in normal bowel function, disruption of mucosal integrity, and symptoms including diarrhea, bloating, flatulence, and intestinal pain. Antibiotic therapy can also result in *Clostridium difficile* colitis, which can cause severe symptoms [3]. Other treatments that are hypothesized to affect the gastrointestinal flora include the ingestion of probiotics and prebiotics. Probiotics are defined as live microbial feed supplements that can benefit a host by improving its intestinal microbial balance [4] and prebiotics are non-digestible substances that when consumed provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria [5]. Probiotic treatment can facilitate gut normalization after antibiotic treatment and probiotic bacteria can produce a variety of health benefits including reduction in the intensity and duration of diarrheal illness, improvement in immune system function, alleviation of lactose intolerance, anticarcinogenic effects, and antihypertensive effects [6, 7]. Probiotics, primarily *Lactobacillus* and *Bifidobacterium*

species, are found in many dairy foods and supplements, including over 80% of commercially available yogurt. However, when fed to healthy subjects, probiotics only modestly affect fecal flora and the strains used do not tend to be permanent colonizers [8, 9].

A large number of studies have been performed investigating fecal communities and changes in fecal communities. However, it is difficult to assess changes in fecal community structure. A number of different methods have been used including standard plating on selective media [10, 11] and different rRNA gene-based molecular methods including the use of group and species specific primers or probes [12-16], denaturing gradient or temperature gradient gel electrophoresis (DGGE or TGGE) analysis [17-19], cloning and sequencing [20, 21], and terminal restriction fragment pattern analysis (TRFLP) [22-24]. PCR methods are generally thought to be superior to culture methods in that many fecal species are not culturable using standard techniques. Cloning can provide very detailed phylogenetic information, but is costly and labor intensive and thus not realistic for processing large numbers of samples. DGGE and TRFLP are the best methods for the rapid high throughput comparison of bacterial communities. However, DGGE primarily provides presence/absence information and is not easily digitized [25]. TRFLP patterns are good for characterization of bacterial communities because they can be used to determine both species dominance and species richness within samples and they are automatically digitized. Different phylotypes of bacteria present in each sample can also be tentatively identified by comparison with a database [26].

TRFLP patterns are generally analyzed using multivariate statistical techniques such as canonical correspondence analysis [27], principle components analysis (PCA)

[28, 29] or cluster analysis [26, 30, 31]. Distance or similarity coefficients are implicit in all these methods for analysis of TRFLP data. Euclidean distance is commonly used and is an implicit part of PCA, but is not appropriate for data with a large number of zeros [32]. Jaccard, Dice, Sorensen's and Simpson's coefficients are appropriate for presence/absence analysis but ignore species abundance. Bray-Curtis similarity is superior to other coefficients for analysis of TRFLP data because it can better handle the large amounts of zeros present in TRFLP data and has high statistical power and robustness with species abundance data [32, 33]. Culture data can also be treated as multivariate data and Euclidean distance is then an appropriate coefficient of similarity.

Any statistical analysis of fecal community structure needs to take into account that human subjects tend to have individually unique intestinal communities that may or may not be stable over time [34-36]. This makes analysis of the data difficult because traditionally, data from multiple subjects are analyzed as a group using either univariate statistics with culture data or PCA/cluster analysis with multivariate (TRFLP) data. Grouping of fecal community data before analysis can result in loss of statistical significance or false negative results. The approach outlined in this study involves the use of Bray-Curtis similarity to measure an individuals' divergence from baseline after treatment. This can overcome the problem of subject-to-subject variability because subjects are analyzed individually before treatment group differences are assessed. This paper presents two different human trials where the fecal TRFLP data sets were analyzed to search for treatment effects. Analyses based on grouped data are compared to individual data. Culture data from one of the studies was also analyzed.

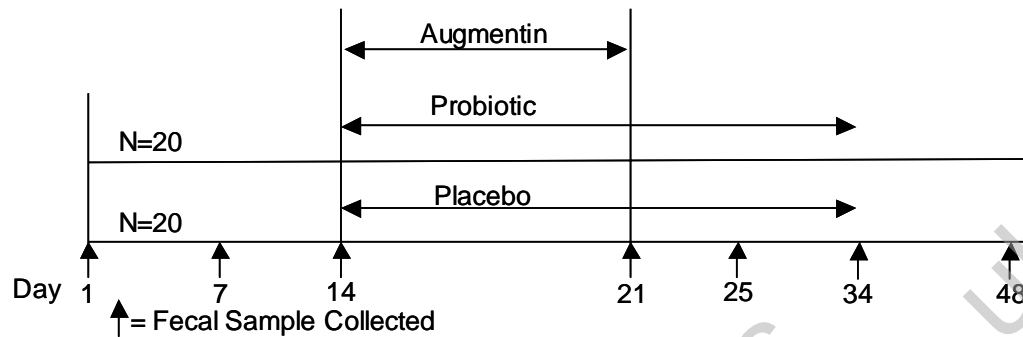
Methods

Probiotic-Prebiotic study design

This randomized, double blind, placebo-controlled, parallel group dietary study was comprised of two independent blocks of 32 healthy subjects. The delivery device was 200 mL of pasteurized whole yogurt, consumed twice daily, at least 8 hours apart. The probiotics consisted of *Lactobacillus rhamnosus* strain 271 (Probio AB, Lund, Sweden), *Lactobacillus acidophilus* strain NCFM (Danisco, Madison, WI), *Lactobacillus paracasei* ssp. *paracasei* strain DN114001 (Danisco, Madison, WI), and *Bifidobacterium* sp. strain DN BIO 173010 (Le Plessis, Robinson, France). The prebiotic was Frutafit[®], a type of inulin. All subjects were given control yogurt for the first 3 weeks, and were then randomized into 4 groups and allocated to different experimental yogurts. Group 1 was fed a yogurt with added probiotic cultures (10^5 - 10^6 CFU of each culture per mL). Group 2 was fed a yogurt with both added probiotic cultures (10^7 - 10^8 CFU of each culture per mL) and the prebiotic (5% w v⁻¹). Group 3 was fed a yogurt supplemented with only the prebiotic (5% w v⁻¹). Group 4, the control, was kept on the control yogurt with no additives (All yogurt products were manufactured by Leatherhead Food RA, Surrey, UK). At the end of week 9, all groups were then given the control yogurt for a further 3 weeks. Fecal samples were collected into sterile bags (weeks 3, 9 and 12) and were immediately placed on ice and stored at -20°C until analysis. By the end of the study, 45 people had complete sample sets from all 3 sampling times and were used to assess the effects of probiotics and probiotics on fecal bacterial communities.

Antibiotic-Probiotic Study Design

Healthy individuals were recruited who agreed to a one week antibiotic treatment for study purposes only, receiving the antibiotic Augmentin (GlaxoSmithKline, Brentford London), a mixture of amoxicillin and clavulanic acid, 875 mg orally twice a day. This antibiotic was selected because of a high rate of antibiotic-associated diarrhea. Patients were then randomized (1:1) to either the placebo or the probiotic test product consisting of a capsule containing a dried bacterial preparation of probiotic bacteria in the genera, *Lactobacillus* and *Bifidobacterium*. The following strains and amounts were fed to individuals in the probiotic group: *Bifidobacterium bifidum* Bb-02 (5×10^8), *Bifidobacterium lactis* B1-04 (5×10^9), *Bifidobacterium lactis* Bi-07 (5×10^9), *Lactobacillus acidophilus* NCFM (5×10^9), and *Lactobacillus paracasei* Lpc-37 (5×10^9). The total dose of probiotic was 2×10^{10} bid (4×10^{10} daily). The other group received a placebo consisting of the same filler used in the bacterial preparation, maltodextran, without the bacteria. The study was conducted over 48 days. Three baseline (no treatment) fecal samples were obtained at days 1, 7, and 14, followed by the 7-day course of Augmentin. Fecal samples were then collected on days 21, 25, 34, and 48. Probiotic or Placebo treatment began on day 14 and continued until day 34 (Fig. 1).

Fig. 1. Schematic of the Antibiotic-Probiotic experimental design.

Forty subjects were recruited with enrollment criteria permitting only patients over eighteen without significant acute or chronic illnesses. Permitted medications included those that were constant throughout the study and only if they had no established or suspected impact on gut flora. Individuals were excluded if they were pregnant, breastfeeding, had a penicillin allergy, a history of gastrointestinal illness or had been on any antibiotics in the preceding four weeks. Fermented foods or any probiotic preparations were prohibited for four weeks before entry into the study and throughout the duration of the protocol.

Fecal samples were obtained for TRF analysis by adding approximately 1 gram of feces to a 2 mL screw-cap tube and freezing at -80°C until shipment. For culture analyses, five grams of each fecal sample were placed into 16 ml Cary Blair Transport Medium (Difco, Franklin Lakes NJ) with indicator (Remel, Lenexa KS) resulting in a 1:4.2 dilution factor. The sample was then shaken/vortexed briefly to disperse and frozen at -80°C until shipment.

Bacterial culturing (Probiotic/Antibiotic Study)

Frozen samples received from the clinical study, were stored at -80°C until enumeration, and thawed at 37°C immediately prior to plating. Duplicate serial dilutions of the samples were prepared (10^{-2} - 10^{-8}) in sterile, pre-reduced 1 % yeast extract. According to Summanen *et al.* [37], liquid media were boiled for five minutes to drive off dissolved oxygen and used within the same day. Yeast extract diluent was autoclaved for fifteen minutes at 121°C and allowed to cool prior to being placed within the anaerobic chamber and dispensed into sterile tubes. All fecal samples were thawed, diluted and plated in an anaerobic chamber (Coy Laboratory Products, Grass Lake MI), maintained at 37°C for 3 to 5 days with an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide.

Dilutions were plated onto duplicate plates. Bifidobacteria were enumerated using BIM-25 [38-40], which is a reinforced clostridial agar base containing polymixin B, tetrazolium red, iodoacetate, kanamycin, and naladixic acid (Sigma-Aldrich, St. Louis MO). Lactobacilli were enumerated using LBS Agar (Difco, Franklin Lakes NJ) plus 200 ml per liter tomato juice from concentrate (Campbell Soup, Camden NJ) [41, 42]. Organisms in the *Bacteroides fragilis* group were enumerated with Bacteroides Bile Esculin Agar (BBE; Difco, Franklin Lakes NJ). *Clostridium* species were enumerated with Egg Yolk Agar (EYA; Difco, Franklin Lakes NJ). For selection of *Clostridium* species, aliquots of each dilution were treated at 80°C for 10 minutes to kill vegetative cells, leaving spore-formers for enumeration as described by Summanen *et al.* [37]. *Enterobacteriaceae* were enumerated with MacConkey Agar (MAC; Difco, Franklin Lakes NJ).

Creation and normalization of TRF pattern data

In the Probiotic-Prebiotic study, DNA was isolated according to Clement and Kitts [43]. 16S rRNA gene TRFLP patterns were created and data was normalized following the protocol in Kaplan et al [23] with TaqGold® (Applied Biosystems, Foster City CA) as the polymerase and *DpnII* (New England Biolabs; Beverly, MA) as the digesting enzyme.

In the Antibiotic-Probiotic study, samples were extracted in triplicate using the MoBio Ultraclean® soil DNA kit (MoBio Laboratories, Carlsbad CA) following manufacture's protocol with the addition of 5 extra washes with S4. Success of each extraction was determined by measuring DNA concentration in the extraction product with a Spectramax® spectrophotometer (Molecular Devices, Palo Alto CA).

PCR was performed using primers homologous to conserved regions on the Bacterial 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 (D4; GenSet, La Hoya CA), were used for each reaction. 50 µL reactions were carried out using 1 µL of undiluted extraction product, 5 µL of 10x Buffer, 3 µL of 10 mM dNTP, 2 µL of 20 mg mL⁻¹ BSA, 7 µL of 25 mM MgCl₂, 1 µL of each primer, and 0.3 µL of 5 U µL⁻¹ TaqGold® (Applied Biosystems, Foster City CA). Reaction temperatures and times were 96°C for 10 min; 35 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 a MoBio Ultraclean® PCR Cleanup Kit (MoBio Laboratories, Carlsbad CA) following manufacturer's protocol. PCR products were quantified using a fluorometer tuned to the labeling dye. An

enzyme digest was performed on 75 ng of cleaned PCR product using the restriction endonuclease *HaeIII* (New England Biolabs; Beverly, MA). Each 40 μ L digestion used 75 ng of DNA, 1 U of enzyme, and 4 μ L of buffer. The samples were digested for 4 h at 37°C and inactivated for 20 min at 65°C. The digestion products were ethanol precipitated and resuspended in 20 μ L of formamide and 0.25 μ L of CEQ 600 base pair standard. Terminal restriction fragment profiles were obtained using a Beckman Coulter CEQ8000X DNA analysis system.

Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQ8000 into Excel (Microsoft, Seattle, WA). To standardize the data for comparison between samples, the area under each TRF peak was normalized to total amount of DNA analyzed and expressed as parts per million (ppm). Peaks with an area of less than 5,000 ppm (<0.5% of the total for that sample) were excluded from analysis to reduce noise.

Analysis of TRF pattern data

For both studies, normalized TRF data sets were transformed by taking the square root of the area under each TRF peak to de-emphasize large TRF peaks while still taking relative abundance into account [44]. Transformed data were analyzed by Principal Components Analysis (PCA), Cluster Analysis with Euclidean distance, and Bray-Curtis similarity was performed using Minitab 14 (Minitab Inc., State College PA) and Excel.

Results

Analysis of the Probiotic-Prebiotic Study TRF Data

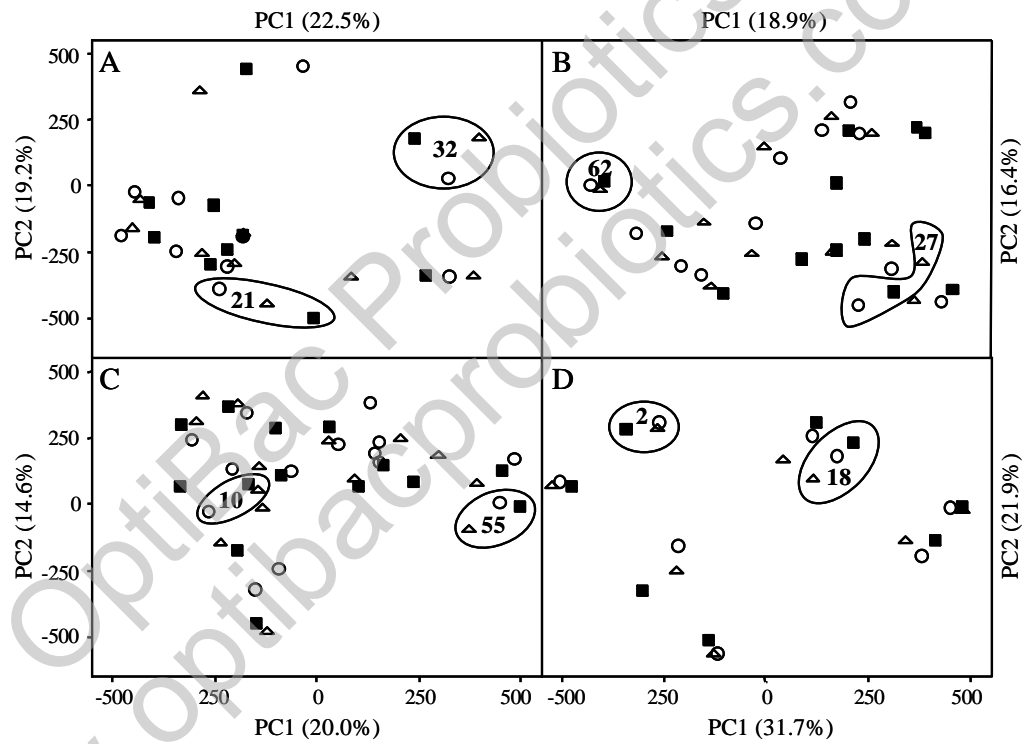
The goal of the Probiotic-Prebiotic study was to describe the changes in human fecal microbial communities caused by the ingestion of probiotic bacteria or prebiotic

fructooligosaccharides in yogurt. All subjects were given a control yogurt for three weeks and then placed into four groups and fed yogurt containing probiotic bacteria (4 strains), prebiotics (Frutafit[®]; Sensus, Roosendal NL), both probiotics and prebiotics, or control yogurt until week 9. All subjects were then fed the control yogurt until week 12. Each subject gave one fecal sample at weeks 3, 9, and 12.

The initial analysis followed a standard approach for TRF data, where PCA score plots were created for each treatment group (Fig. 2). The samples clearly did not cluster by week, indicating a large amount of subject-to-subject variability. Between-subject variation in TRF patterns was clearly greater than within-subject variability, as indicated by an obvious grouping by subject.

OptiBac Probiotics
www.optibacprobiotics.co.uk

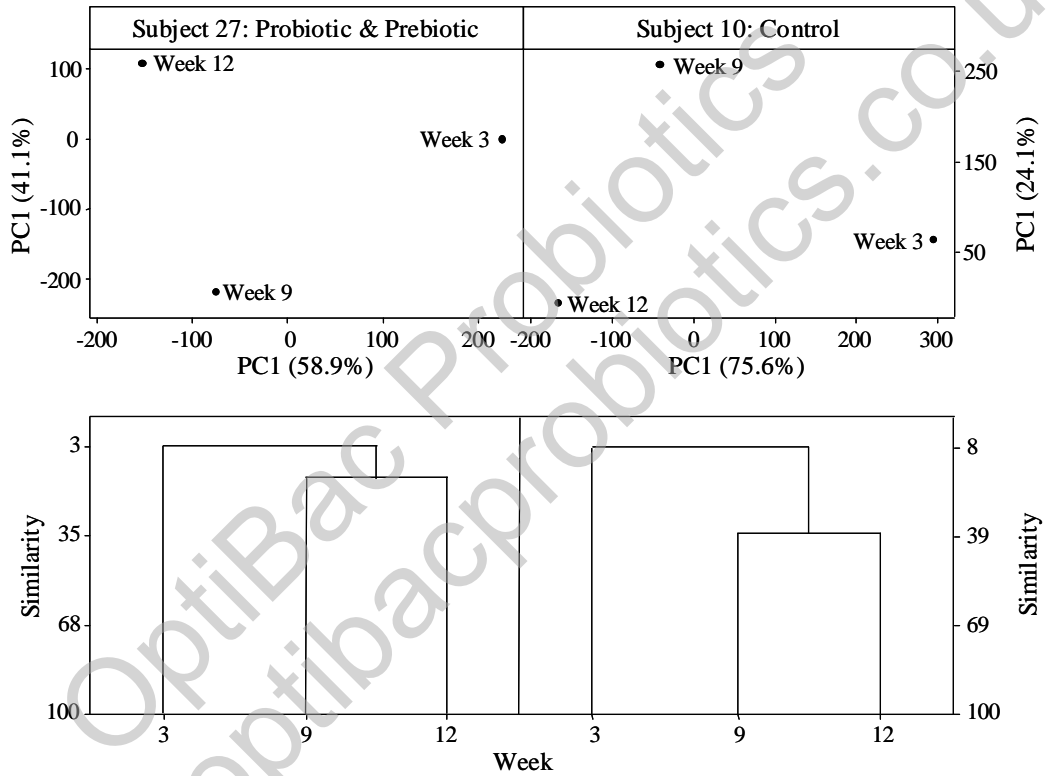
Fig. 2. PCA score plots of all subjects in each treatment group. Panel A, probiotics only; panel B, probiotics and prebiotics; panel C, control yogurt; panel D, prebiotics only. Circles, week 3 samples; squares, week 9; triangles, week 12. Samples from eight subjects (two per group) are circled and numbered by subject. Percent variation covered by each principal component is indicated in parentheses in the axis titles.



Since the PCA score plots grouped by treatment could not identify any treatment effect, another common approach was attempted. PCA score plots and Euclidean distance Cluster analysis dendrograms were created for each individual subject. For example, PCA plots for both subject 27 (treated with both probiotics and prebiotics) and subject 10 (control) indicated that weeks 9 and 12 are more similar to each other than they are to the sample from week 3 (Fig. 3). The dendrograms showed that weeks 9 and 12 were between 20-40% similar while week 3 was 3-8% similar to weeks 9 and 12. Unfortunately, there is really no way to evaluate the significance of this apparent difference in flora by sample week. In fact, these differences may be entirely random.

OptiBac Probiotics
www.optibacprobiotics.co.uk

Fig. 3. PCA score plots and cluster analysis dendrograms of two subjects from different treatment groups. Percent variation covered by each principal component is indicated in parentheses in the axis titles of the PCA score plots. Percent similarity based upon Euclidean distance is indicated on the right and left axis of the dendrograms.



It is tempting to categorize subject's response to treatment based on these analyses. A treatment would have an effect if the week 3 (pretreatment sample) were different from the other two samples. Although sample-to-sample differences are visible in these individual analyses (Fig. 3), the problem is how to assess the statistical significance of these differences (i.e. no way to obtain a p-value) and there is very little statistical power because there are more than 200 variables (TRFLP peaks) and only three samples for each subject. As a result each sample has a 33% chance of being the most different.

In addition, both PCA and this Cluster analysis method required the use of Euclidean distance, which is not the best choice for calculating distances with this type of data [32]. Bray-Curtis similarity was specifically designed for use with species abundance data sets [45] and is less susceptible to bias introduced by large numbers of zero abundance data (Fig. 4). Bray-Curtis similarity takes the relative abundance of each TRFLP peak into account when comparing two patterns. TRFLP data often contains zero values (a peak present in one pattern that is not seen in another) and can cover as much as 2.5 orders of magnitude variation in TRFLP peak area. Bray-Curtis similarity is therefore, a preferable similarity measure when comparing TRFLP patterns.

In the Probiotic-Prebiotic study, Bray-Curtis similarity was calculated between weeks 3 and 9, 3 and 12, and 9 and 12 and interval plots of those values were created for each different treatment group (Fig. 5). With this method, it should be very easy to see changes due to treatment; the similarity between week 3 and week 9 samples should be the least of all three comparisons across all treated subjects.

No within-baseline comparison was possible with this sampling regime, but it was possible to estimate the reproducibility of the TRFLP method with fecal samples. Pair-wise comparisons were made of five replicate TRF patterns from the same sample (replication from the DNA extraction step) resulting in an average similarity of 83%. Intriguingly, the variation from week to week in the probiotic treatment group was the same as what was seen in the reproducibility experiment (Fig. 5). Therefore, there was no change due to treatment. The week-to-week variation for the prebiotic groups was less than was seen in the reproducibility experiment but all three pair-wise similarities were the same, as indicated by overlapping standard error bars. Once again, this indicates there was no change due to treatment. The similarity of week 9 to week 12 samples was clearly higher than the other pair-wise similarities, but this cannot be explained by any particular treatment.

The negative results of this study were most clear after using Bray-Curtis pair-wise similarity. This made it possible to visualize the lack of change in fecal bacterial communities given the sampling regime in this study, where no statistical measure with any power could be used. This study also made it clear that a set of baseline samples must be included in investigations of human fecal flora.

Fig. 4. Formulas for the two distance measures used for TRF and culture analysis.

This case is set up to compare TRF peak area for TRFs from 60 to 600 nucleotides in one subject from sample d1 to sample d7.

Euclidean Distance

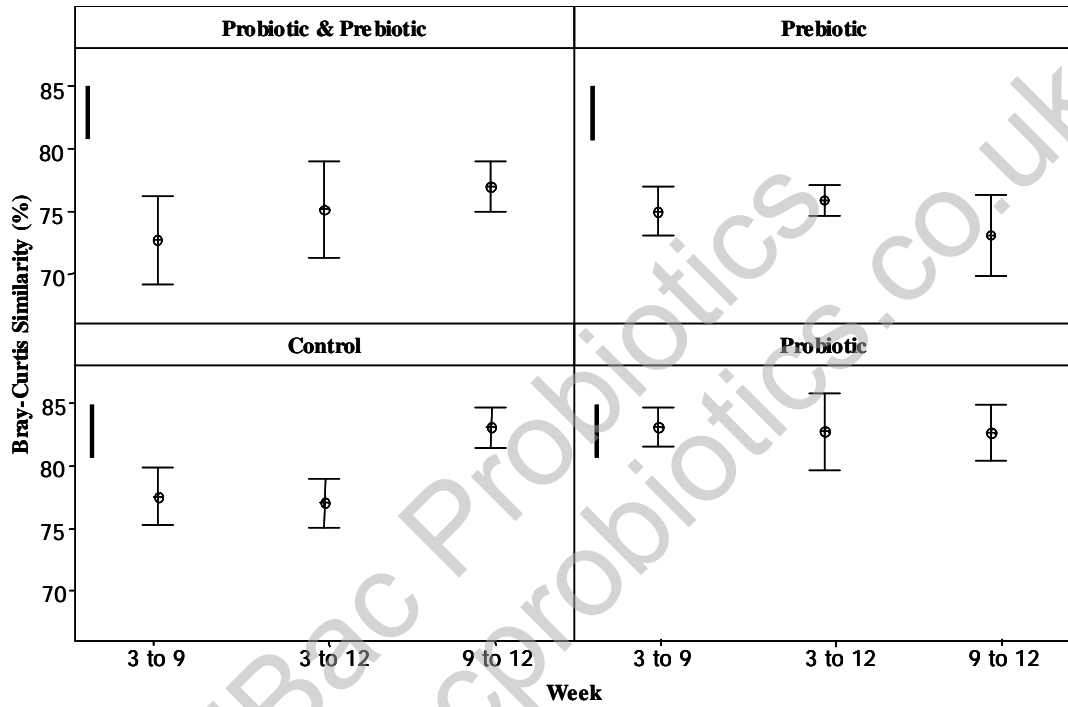
$$\sqrt{\sum_{TRF=60}^{600} (Area_{TRF,d1} - Area_{TRF,d7})^2}$$

Bray-Curtis Similarity

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

OptiBac Probiotics
www.optibacprobiotics.co.uk

Fig. 5. Interval plots of Bray-Curtis similarities of weeks 3 to 9, 3 to 12, and 9 to 12 for each treatment group. Error bars represent one standard error. The large black bar represents the standard error of replicate TRF patterns from a single sample.

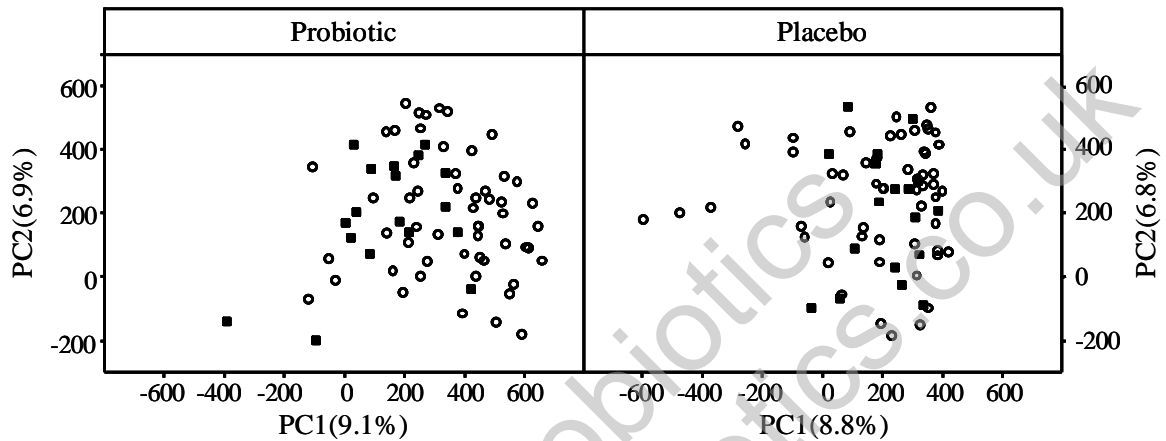


Analysis of the Antibiotic-Probiotic Study TRF Data

The goal of the Antibiotic-Probiotic study was to observe the effects of probiotic treatment concurrent with antibiotic therapy on fecal communities in humans. Three baseline (pre-treatment) fecal samples were collected at days 1, 7, and 14 and then the subjects were randomized into one of two groups (probiotic and placebo). Both groups were given a one-week course of antibiotics and the probiotic group was concurrently given a pill containing a mixture of probiotic bacteria until day 34. (Fig.1). Additional samples were collected at days 21, 25, 34, and 48.

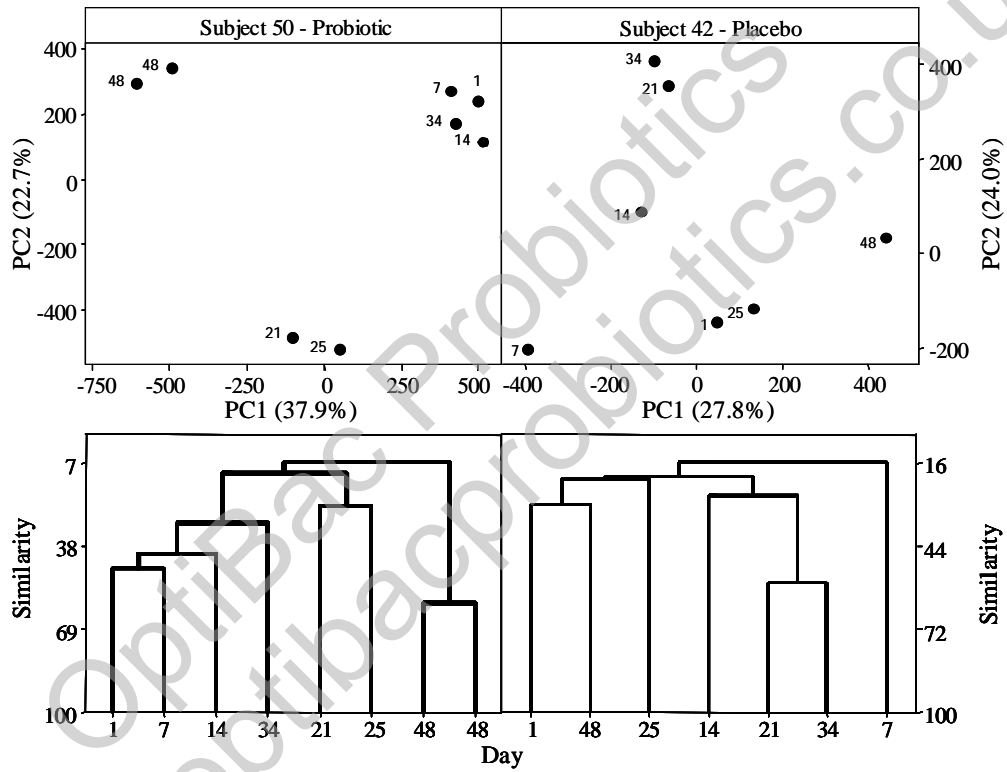
PCA score plots of all subjects' TRF data collected over days 1-21 were used to elucidate the effect of antibiotics on the fecal flora. However, subject-to-subject variation obscured the effect of antibiotic treatment (Fig. 6). In addition, PC1 and PC2 combined only represented a small fraction of the total variation in TRF data and thus may under-represent any changes induced by antibiotic consumption.

Fig. 6. PCA score plots of all subjects' days 1-21. Circles represent days 1, 7, and 14 and squares represent day 21. Percent variation covered by each principal component is indicated in parentheses in the axis titles.



Because of the subject-to-subject variability seen in Fig. 6, PCA and Cluster analysis were performed on each subject and it became clear that subjects fell into two major categories: those with stable baseline flora (days 1, 7 and 14) and those whose baseline flora varied significantly. For example, subject 50 (Fig. 7) showed a clear antibiotic effect on the fecal bacterial community structure at days 21 and 25 that appeared to be gone by day 34. After the subject stopped taking probiotics the fecal community changed again (day 48). Subject 42 (Fig. 7) showed a smaller effect on the fecal bacterial community structure from antibiotic treatment because variation in the baseline data made it difficult to tell if the shift at day 21 was significant. Although the antibiotic effect for subject 50 appears obvious in the cluster analysis, there is no way to determine statistical significance and the similarities are actually very low.

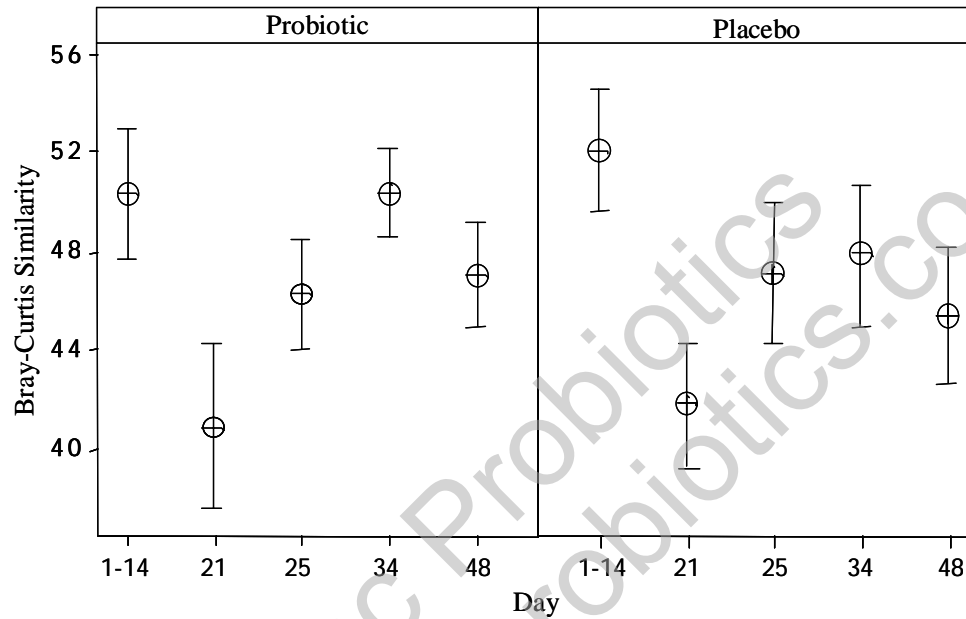
Fig. 7. PCA score plots and cluster analysis dendrograms of two subjects from different treatment groups. Percent variation covered by each principal component is indicated in parentheses in the axis titles of the PCA score plots. Percent similarity based upon Euclidean distance is indicated on the right and left axis of the dendrograms. Subject 50 had replicate data for day 48.



Since the standard methods of PCA and Cluster analysis could not give statistical significance, Bray-Curtis similarity to baseline (days 1-14) was calculated for each day after antibiotic treatment. Similarity within baseline was also calculated for each subject. By comparing within baseline similarity to similarity from day 21 to baseline it was clear that the antibiotics had a significant effect on fecal flora across all subjects (ANOVA, $p < 0.001$). The average similarity of baseline samples compared to the first day after antibiotics (day 21) was 42% while average similarity within baseline (days 1-14) was 51%. Average similarity to baseline at day 25 increased to 47% and was not significantly different from within baseline at a 95% confidence level ($p = 0.078$). At day 34 the average similarity reached 49% and at day 48 it decreased to 46%. The significance of the change at day 48 is not clear, especially since it occurred in both the probiotic and placebo treatment groups and thus cannot be solely attributed to the cessation of probiotic ingestion at day 34.

To assess the effect of probiotic ingestion on changes brought about by antibiotic treatment, the Bray-Curtis similarity data were analyzed separately for probiotic and placebo groups (Fig. 8). While both groups exhibited a similar trend toward increased similarity to baseline over days 21 through 34, the probiotic group exhibited a larger increase in similarity at day 34. The difference between the two groups was not significant when all four post-antibiotic treatment days were analyzed (MANOVA, $p = 0.135$). However, when the anomalous results of day 48 were removed from analysis a trend revealing a difference could be detected at a 90% confidence level ($p = 0.066$).

Fig. 8. Average Bray-Curtis similarity from base line (days 1-14) for each day after treatment. Error bars represent one standard error. Bray-Curtis similarity in percent is indicated on the left axis.



Analysis of the Probiotic-Prebiotic Study Culture Data

The culture data were analyzed for antibiotic effects using interval plots as a standard univariate method to look for significant differences with treatment (Fig. 9).

Increasing trends were visible in *Bacteroides* and enterics at day 21. However, the large

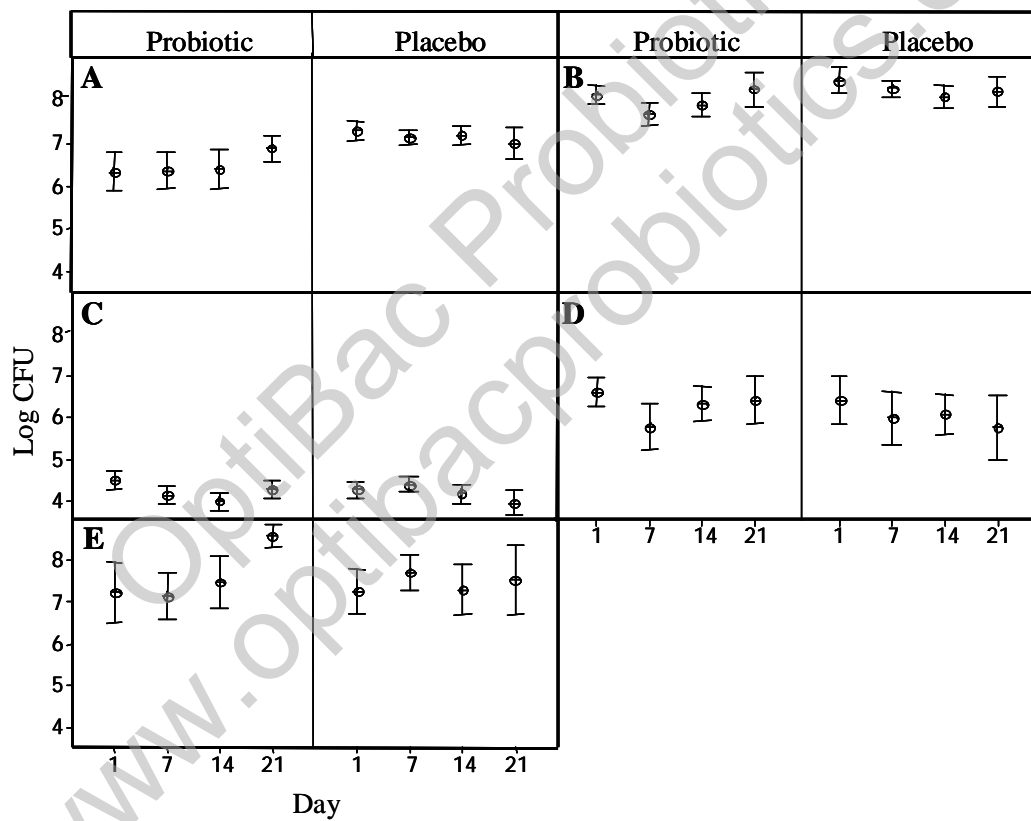
variation in counts (~1 log standard error) across all subjects precluded detecting significant differences (ANOVA). There was no trend seen for *Clostridium*,

Bifidobacterium, and *Lactobacillus*. MANOVA of data from all 5 culture media again

showed no statistical difference between bacterial counts before antibiotic treatment

compared to the days after antibiotic treatment (MANOVA, $p = 0.34$).

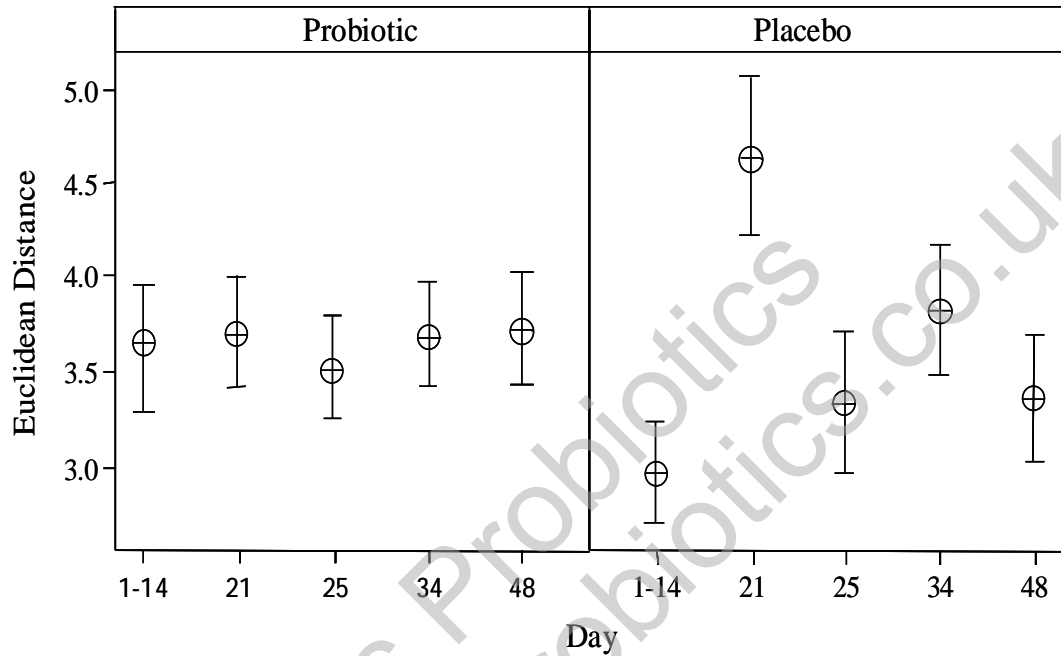
Fig. 9. Interval plots of log colony-forming units (CFU) for all subjects' days 1-21 for each type of media. Error bars represent one standard error. Panel A, BBE agar for organisms in the *Bacteroides fragilis* group; panel B, BIM-25 agar for bifidobacteria; panel C, egg yolk agar for *Clostridium*; panel D, LBS agar for lactobacilli; panel E, MacConkey agar for *Enterobacteriaceae*.



Euclidian distance was then used to compare the average distance of baseline samples (days 1-14) to the first day after antibiotics (day 21) for each subject. Euclidian distance was used in this case because the standard assumptions of normality and non-zero data are not violated with culture data as they are with TRF data. When the effect of antibiotics was evaluated on an individual subject basis in the same way as with the TRF data, a significant effect was detected (ANOVA, $p = 0.003$). The average pair-wise distance at baseline was 3.3 compared to an average distance to baseline of 4.2 after antibiotic treatment. At day 25 the distance to baseline decreased to 3.5, not significantly different from within baseline ($p = 0.56$). At day 34 the average distance to baseline was 3.7 and at day 48 it was 3.5.

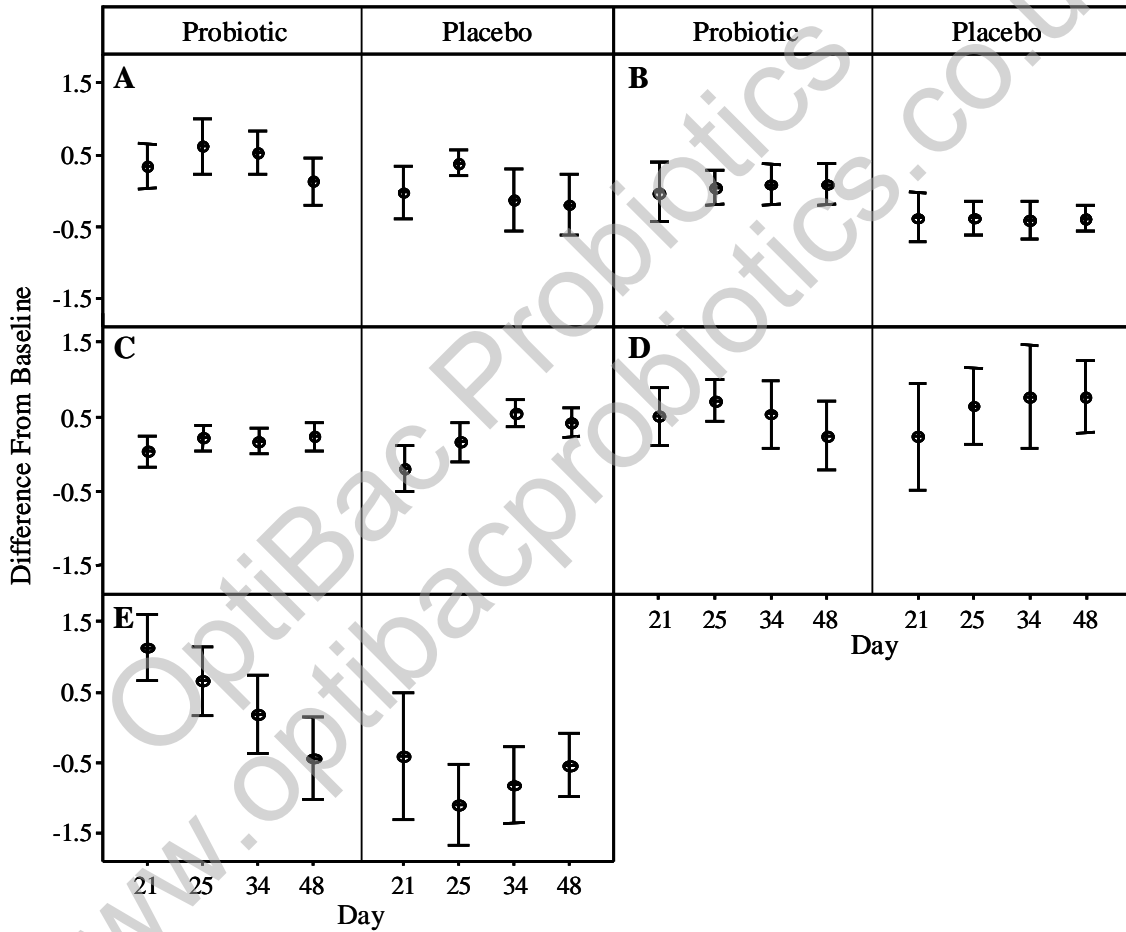
To assess the effect of probiotic ingestion on changes brought about by antibiotic treatment the Euclidian distance data were analyzed separately for probiotic and placebo groups (Fig. 10). Here the culture data showed a much greater difference than the TRF data with the probiotic group maintaining an average distance to baseline of around 3.6 to 3.7 throughout the study. In contrast, the placebo group showed a large shift from baseline at day 21 and a return to near baseline thereafter. The difference between the two groups was quite significant when all four post-antibiotic treatment days were analyzed (MANOVA, $p = 0.004$). When day 48 was removed from analysis there was still a significant difference between groups ($p = 0.046$).

Fig. 10. Average Euclidian distance from base line (days 1-14) for each day after treatment. Error bars represent one standard error.



To account for individual variation and still determine which media showed the largest change in fecal flora, the average baseline counts for each subject were subtracted from the counts for that subject on each day subsequent to antibiotic treatment. While these data could not be used to show an antibiotic effect, they still reflected the significant difference between the probiotic and placebo treatment groups (MANOVA, $p = 0.049$) over the four post-antibiotic treatment days (Fig. 11). Follow up ANOVA's indicated that probiotic effect was significant for bifidobacteria ($p=0.030$) and *Enterobacteriaceae* ($p = 0.006$), but not significant for the *Bacteroides fragilis* group (ANOVA, $p = 0.104$), *Clostridium* ($p = 0.601$), and lactobacilli ($p = 0.772$).

Fig. 11. Interval plots of difference from base line (days 1-14) for each day after treatment for each different medium. Error bars represent one standard error. Panel A, BBE agar for organisms in the *Bacteroides fragilis* group; panel B, BIM-25 agar for bifidobacteria; panel C, egg yolk agar for *Clostridium*; panel D, LBS agar for lactobacilli; panel E, MacConkey agar for *Enterobacteriaceae*.



Discussion

There are a variety of ways to collect quantitative data for the assessment of treatment effects on intestinal communities, including both culture and molecular methods. Culturing is a common approach, but can give an incomplete picture of the intestinal microflora since feces contain large numbers of unculturable or difficult to culture organisms. Media choice and sample handling can also skew the data. Analysis of culture data generally involves univariate statistics, but multivariate statistics can be applied if multiple media were investigated. In fact, multivariate statistics provide a more accurate way to test for treatment effects. Molecular profile methods, such as DGGE and TRFLP, may represent a broader view of the intestinal microflora [8, 46, 47]. However, the large number of variables generated by these methods makes the use of multivariate statistics essential. Generally, pair-wise similarity or distance measures are used and are implicit in both PCA and Cluster analysis. Molecular profile data can be analyzed as presence-absence data [19, 24, 46] or relative abundance data [22, 23, 47], although when only presence-absence data are analyzed, information that may be critical to assess treatment effect is lost.

Assessment of treatment effects on individual subjects, especially human subjects, can be problematic because subject-to-subject variation can outweigh treatment effects, thus analysis of data grouped by treatment may not show any effects. For culture data especially, the large variation in counts results in a loss of power for statistical analyses, unless many subjects are included in the study. In the antibiotic-probiotic study presented here, univariate analysis of culture data showed no significant treatment effect

(Fig. 9). Sullivan et al. [11] presented univariate analyses of culture data and could only discuss trends since no statistically significant effects could be reported. Madden et al. [10] artificially reduced error by excluding uncountable plates from their analyses. However, this approach is inappropriate since it will bias the analysis, possibly leading to false conclusions. The problem of subject-to-subject variation is compounded with molecular profile data since many more variables are introduced. In some cases, significant results can be obtained when grouping individual data. For example, Sakata et al. [24] evaluated the effect of infant breast-feeding by grouping all subjects together, but the high sample number in the study allowed for some significant results. However, multivariate analyses of molecular data in the studies presented here only served to emphasize subject-to-subject variability (Fig. 2 and 6). Both Simpson et al and Wang et al also discussed subject-to-subject variation in human intestinal microflora, while others [34, 36] emphasized that individual humans have a unique intestinal microflora. Therefore, if possible, treatment effects must be evaluated separately for each individual.

The large reduction in sample number when analyzing the data by individual instead of grouping by treatment results in a drastic loss of statistical power, invalidating multivariate hypothesis tests, such as MANOVA. Some recent studies have relied on a simple visual comparison of raw molecular data to show treatment effects [8, 48, 49]. Unfortunately, data analyzed in this way could be quite misleading and may overlook subtle differences in community structure. Therefore, analyses of treatment effects within individuals have generally involved multivariate data reduction methods such as PCA or Cluster analysis [1, 22, 47]. As shown in both studies presented here, these methods can give a good visual representation of treatment effects in an individual, clearly showing

which samples cluster together (Fig. 3 and 7). Unfortunately, it is not possible to extract statistical significance from these clusters, which only allows for a discussion of trends. However, the distance measures that are used in PCA and Cluster analysis can be used for statistical hypothesis testing.

Distance measures are, by definition, pair-wise comparisons. Thus, assessment of treatment effects requires comparison of pre-treatment samples (baseline) with post-treatment samples. The natural variation in native microflora can potentially mask treatment effects. Prior studies of stability in intestinal microflora assert that individuals are generally stable [34, 36, 46]. However, since these studies did not quantify stability, it is difficult to identify a subject as having unstable intestinal microflora. Clearly, a single baseline sample does not allow for an assessment of natural variation. Therefore, the collection of multiple baseline samples per subject is advisable. A comparison of Fig. 5 (1 baseline sample per subject in the prebiotic-probiotic study) and Fig. 8 (3 baseline samples per subject in the antibiotic-probiotic study) highlights the advantages of having multiple baseline samples.

Euclidean distance is most commonly used in the literature with molecular profile data and is implicit in PCA. However, Rees et al. [32] argue that Bray-Curtis distances are superior to Euclidean distances due to the large blocks of zeros present in molecular profile data sets. Conversely, Euclidean distance is more appropriate for culture data sets because this type of data does not usually include large blocks of zeros. Whichever measure is used, however, the resulting data can be grouped by treatment for univariate hypothesis testing since the comparison to baseline implicitly takes subject-to-subject variation in intestinal microflora into account. A good way to visually represent

the pairwise distances is with interval plots (Figs. 5, 8, and 10). These interval plots clearly show the significant changes in community structure. For culture data, the use of univariate analyses can be appropriate to identify individual population effects, but only when they are used after an appropriate multivariate analysis. Instead of using the log CFU measurements (Fig. 9), however, the proper univariate method uses the difference between a subject's own individual baseline and each post-treatment measurement (Fig. 11).

The method of comparison to baseline within an individual before grouping by treatment provides both a good visual representation of the data and a clearer indication of treatment effects on intestinal microflora than an evaluation of data grouped before analysis. This applies whether the study has a positive result (antibiotic-probiotic study) or negative result (prebiotic-probiotic study). It is also apparent from analyzing these two data sets that a large within-subject sample size and multiple baseline samples are necessary to accurately analyze treatment effects on intestinal microflora. In addition, it is important to use Bray-Curtis distances for molecular profile data and Euclidean distances for culture data and univariate analysis of culture data is significant only if it follows the appropriate multivariate method.

Acknowledgements

The antibiotic-probiotic project was funded by Danisco USA and Dairy Management Incorporated funded the prebiotic-probiotic project. We would like to thank Greg Leyer from Danisco USA , Rosemary Dawes from North Carolina State University for the culturing work, and Raphael Jimenez-Flores and Raul Cano for editing this publication.

References

- [1] Zoetendal, E. G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A. D., de Vos, W. M. (2002) Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl. Environ. Microbiol.* 68, 3401-3407.
- [2] Fuller, R. (1991) Probiotics in human medicine. *Gut.* 32, 439-442.
- [3] Bergogne-Berezin, E. (2000) Treatment and prevention of antibiotic associated diarrhea. *Int. J. Antimicrob.* 16, 521-6.
- [4] Fuller, R. (1989) Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365-378.
- [5] Gibson, G. R. and Roberfroid, M. B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr.* 125, 1401-12.
- [6] Gill, H.S. and Guarner, F. (2004) Probiotics and human health: a clinical perspective. *Postgrad. Med. J.* 80, 516-526.
- [7] Sanders, M.E. (1999) Probiotics. *Food Technol.* 53, 67-77.
- [8] Tannock, G. W., Munro, K., Harmsen, H. J., Welling, G. W., Smart, J., Gopal, P. K. (2000) Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl. Environ. Microbiol.* 66, 2578-2588.
- [9] Chen, R. M., Wu, J. J., Lee, S. C., Huang, A. H., and Wu, H. M. (1999) Increase of intestinal *Bifidobacterium* and suppression of coliform bacteria with short-term yogurt ingestion. *J. Dairy Sci.* 82, 2308-2314.

- [10] Madden, J. A., Plummer, S. F., Tang, J., Garaiova, I., Plummer, N. T., Herbison, M., Hunter, J. O., Shimada, T., Chang, L., Shirakawa, T. (2005) Effect of probiotics on preventing disruption of the intestinal microflora following antibiotic therapy: A double-blind, placebo-controlled pilot study. *Int. Immunopharmacol.* 5, 1091-1097.
- [11] Sullivan, A., Barkholt, L. and Nord, C.E. (2003) *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus* F19 prevent antibiotic-associated ecological disturbances of *Bacteroides fragilis* in the intestine. *J. Antimicrob. Chemo.* 52, 308-311.
- [12] Kok, R.G., De Waal, A., Schut, F., Welling, G.W., Weenk, G. and Hellingwerf, K.J. (1996) Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant feces. *Appl. Environ. Microbiol.* 62, 3668-3672.
- [13] Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., and Tanaka, R. (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 68, 5445-5451.
- [14] Silvi, S., Verdenelli, M.C., Orpianesi, C. and Cresci, A. (2003) EU project Crownalife: functional foods, gut microflora and healthy ageing. Isolation and identification of *Lactobacillus* and *Bifidobacterium* strains from faecal samples of elderly subjects for a possible probiotic use in functional foods. *J. Food Eng.* 56, 195-200.
- [15] Song, Y., Kato, N., Liu, C., Matsumiya, Y., Kato, H. and Watanabe, K. (2000) Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex

- PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiol. Lett.* 187, 167-173.
- [16] Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., Hammes, W. P. (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces using group specific PCR primers and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67, 2578-2585.
- [17] Heilig, H.G., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D. and de Vos, W.M. (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* 68, 114-123.
- [18] Satokari, R.M., Vaughan, E.E., Akkermans, A.D. and Saarela, M. (2001) Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67, 504-513.
- [19] Simpson, J.M., McCracken, V.J., Gaskins, H. R. and Mackie, R.I. (2000) Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Appl. Environ. Microbiol.* 66, 4705-4714.
- [20] Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., Moller, K. (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl. Environ. Microbiol.* 68, 673-690.

- [21] Wilson, K.H. and Blichington, R.B. (1996) Human colonic biota studied by ribosomal DNA sequence analysis. *Appl. Environ. Microbiol.* 62, 2273-2278.
- [22] Jernberg, C., Sullivan, A., Edlund, C. and Jansson, J.K. (2005) Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. *Appl. Environ. Microbiol.* 71, 501-506.
- [23] Kaplan, C.W., Astaire, J.C., Sanders, M.E., Reddy, B.S. and Kitts, C.L. (2001) 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 67, 1935-1939.
- [24] Sakata S., Tonooka, T., Ishizeki, S., Takada, M., Sakamoto, M., Fukuyama, M. and Benno, Y. (2005) Culture-independent analysis of fecal microbiota in infants, with special reference to *Bifidobacterium* species. *FEMS Microbiol. Letters.* 243, 417-423.
- [25] Forney, L.J., Zhou, X. and Brown, C.J. (2004) Molecular microbial ecology: land of the one eyed king. *Curr. Opin. Microbiol.* 7, 210-220.
- [26] Kitts, C. L. (2001) Terminal restriction fragment patterns: A tool for comparing microbial communities and assessing community dynamics. *Curr. Issues Intest. Microbiol.* 2, 17-25.
- [27] Ayala-del-Rio, H. L., Callister, S. J., Criddle, C. S., and Tiedje, J. M. (2004) Correspondence between community structure and function during succession in phenol-plus-trichloroethene-fed sequencing batch reactors. *Appl. Environ. Microbiol.* 70, 4950-4960.

- [28] Kaplan, C. W., and Kitts, C. L. (2004) Bacterial succession in a petroleum land treatment unit. *Appl. Environ. Microbiol.* 67, 1935-1939.
- [29] Clement, B.G., Kehl, L.E., DeBord, K.L. and Kitts, C.L. (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J. Microbiol. Methods.* 31, 135-142.
- [30] Hiraishi, A., Iwasaki, M. and Shinjo, H. (2000) Terminal restriction pattern analysis of 16S rRNA genes for the characterization of bacterial communities of activated sludge. *J. Biosci. Bioeng.* 90,148-156.
- [31] Urakawa, H., Yoshida, T., Nishimura, M. and Ohwada, K. (2000) Characterization of depth-related population variation in microbial communities of a coastal marine sediment using 16S rDNA-based approaches and quinone profiling. *Environ. Microbiol.* 2, 542-554.
- [32] Rees, G.N., Baldwin, D.S., Watson, G.O., Perryman, S. and Nielson, D.L. (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Ant. Van Leeuw.* 86, 339-347.
- [33] Faith, D.P., Humphrey, C.L. and Dostine, P.L. (1991) Statistical power and BACI designs in biological monitoring: comparative evaluation of measures of community dissimilarity based on benthic macroinvertebrate communities in Rockhole Mine Creek, Northern Territory, Australia. *Aust. J. Mar. Freshwat. Res.* 42, 589-602.

- [34] Vanhoutte, T. Huys, G., De Brandt, E. and Swings, J. (2004) Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. *FEMS Microbiol. Ecol.* 48, 437-446.
- [35] Vaughan, E.E., Schut, F., Heilig, H.G., Zoetendal, E.G., de Vos, W.M. and Akkermans, A.D. (2000) A molecular view of the intestinal ecosystem. *Curr. Issues Intest. Microbiol.* 1, 1-12.
- [36] Zoetendal, E.G., Akkermans, A.D. and de Vos, W.M. (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.* 64, 3854-3859.
- [37] Summanen, P.E., Baron, J., Citron, D.M., Strong, C., Wexler, H.M. and Finegold, S.M. (1993) *Wadsworth Anaerobic Bacteriology Manual Fifth Ed.* Star Publishing, Los Angeles, CA.
- [38] Lapiere, L., Undeland, P. and Cox, L.J. (1992) Lithium chloride-sodium propionate agar for the enumeration of *Bifidobacterium* in fermented dairy products. *J. Dairy Sci.* 75, 1192.
- [39] Muñoa, F.J. and Pares, R. (1988) Selective medium for isolation and enumeration of *Bifidobacterium* spp. *Appl. Environ. Microbiol.* 54, 1715-1718.
- [40] Rada, V., Sirotek, K. and Petr, J. (1999) Evaluation of selective media for *Bifidobacteria* in poultry and rabbit caecal samples. *J. Vet. Med. Ser.* 46, 369-373.
- [41] Rogosa, M., Mitchell, J.A. and Wiseman, R.F. (1951) A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J. Bacteriol.* 62, 132-133.

- [42] Sabine, D.B. and Vaselekos, J. (1965) Isolation of *Lactobacillus acidophilus* from faecal material. *Nature*. 206, 960-961.
- [43] Clement, B.G. and Kitts, C.L. (2000) Isolating PCR-quality DNA from human feces with a soil DNA kit. *Biotechniques*. 28, 640-646.
- [44] Blackwood, C.B., Marsh, T., Kim, S. and Paul, E.A. (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Appl. Environ. Microbiol.* 69, 926-932.
- [45] Beals, EW. (1984) Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. In: *Advances in ecological research* (A. Macfadyen), v14, pp. 1-49. Academic Press Inc., London.
- [46] Donskey, C. J., Hujer, A. M., Das, S. M., Pultz, N. J., Bonomo, R. A., Rice, L. B. (2003) Use of denaturing gradient gel electrophoresis for analysis of the stool microbiota of hospitalized patients. *Microbiol. Methods*. 54, 249-256.
- [47] Wang, M., Ahrne, S., Antonsson, M. and Molin, G. (2004) TRFLP combined with principle component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. *J. Microbiol. Methods*. 59, 53-69.
- [48] Sakamoto, M., Hayashi, H., Benno, Y. (2003) Terminal restriction fragment length polymorphism analysis for human fecal microbiota and its application for analysis of complex Bifidobacterial communities. *Microbiol. Immunol.* 47, 133-142.

- [49] Hayashi, H., Sakamoto, M., Benno, Y. (2002) Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. *Microbiol. Immunol.* 46, 819-831.

OptiBac Probiotics
www.optibacprobiotics.co.uk

**CHAPTER 2: THE EFFECT OF PROBIOTICS ON HEALTHY SUBJECTS
UNDERGOING ANTIBIOTIC THERAPY**

Final Report

Submitted to Danisco by:

Anna Engelbrektson and Christopher Kitts, Cal Poly State University

Joshua Korzenik, Massachusetts General Hospital

Todd Klaenhammer, North Carolina State University

Mary Ellen Sanders, Dairy and Food Culture Technologies

OptiBac Probiotics
www.optibacprobiotics.co.uk

Executive Summary

Probiotic bacteria of the species *Lactobacillus* and *Bifidobacterium* have been demonstrated to produce health benefits ranging from reduction in the length and severity of diarrheal incidence to anti-carcinogenic effects. This has resulted in the use of probiotics for intestinal disease becoming an area of intense research. One area of particular interest is whether probiotics can lessen the adverse intestinal symptoms that result from antibiotic disruption of intestinal flora. The purpose of this study was to investigate the role of probiotics in the normalization of gut flora after disturbance by antibiotic therapy. Fecal samples were collected from 40 individuals at seven time points over a 48-day period. Individuals were given a course of antibiotics, with half of the subjects concurrently consuming probiotics and the other half receiving a placebo. Bacterial DNA was extracted from each fecal sample and PCR was performed using 16S rRNA gene targeted Eubacterial primers. A restriction endonuclease was used to digest the PCR product and terminal restriction fragment (TRF) pattern analysis was performed using capillary gel electrophoresis. Bacterial culturing was performed using five different selective media for Bifidobacteria, *Lactobacillus*, members of the *Bacteroides fragilis* subgroup, *Clostridium* species, and *Enterobacteriaceae*.

Baseline culture counts of bacteria varied by four orders of magnitude from subject to subject. In addition, application of a statistical technique, Principle Components Analysis, to TRF patterns showed a high level of subject-to-subject variation in fecal bacterial community structure that masked the effects of antibiotics. Therefore, an individual subject analysis was used comparing each post-antibiotic time point to a subject's own baseline data (days 1, 7, 14). This required the use of

distance/similarity measures since both TRF data and culture data were multivariate. TRF data were assessed using Bray-Curtis similarity and culture data were assessed using Euclidian distance. When the data were analyzed in this way, the effects of antibiotics on fecal flora were clear at day 21 in both TRF and culture data.

Culture data showed an immediate and significant effect of probiotic treatment. *Enterobacteriaceae* increased in the probiotic group and decreased in the placebo group. *Bifidobacterium* counts were unchanged in the probiotic group and decreased in the placebo group. TRF data showed a stabilizing effect of probiotic treatment at day 34. In addition, TRF data showed a further shift in fecal flora at day 48, two weeks after probiotic feeding had ceased.

Baseline stability was an important factor for observing changes in fecal flora. If fecal flora from a subject's baseline samples (days 1, 7, 14) were extremely dissimilar then it was highly likely that an antibiotic effect could not be identified, preventing the detection of a probiotic effect. A specific group of organisms identified by TRF analysis appeared to be related to the stability of a subject's fecal flora. Although TRF analysis could not give an exact identification of these organisms, species of *Clostridium* and *Bifidobacterium* were associated with stable communities while other species of *Clostridium* and *Bacteroides* were associated with unstable communities. Only individuals with stable microflora in their baseline TRF data were useful to determine the types of organisms affected by antibiotics. Several types of bacteria represented by TRF peaks changed in relative abundance with antibiotic treatment, including *Enterobacteriaceae*, *Bifidobacterium*, *Clostridium*, *Ruminococcus*, *Bacteroides* and *Porphyromonas*.

The overall conclusion from this study was that the specific probiotic mixture used had a stabilizing effect on the fecal flora of subjects treated with Augmentin. Additionally, TRF and culture data proved complementary in this study.

OptiBac Probiotics
www.optibacprobiotics.co.uk

Background

Probiotics, primarily *Lactobacillus* and *Bifidobacterium* species, are found in some dairy foods and supplements. Research shows that probiotics can help produce health benefits ranging from reduction in the symptoms of diarrheal illness to anticarcinogenic effects [1]. The goal of this study was to investigate the impact of probiotics on the fecal flora of individuals taking antibiotics, which are known to disturb the normal fecal flora [2]. Since many fecal bacteria are not easily culturable using traditional plating methods, this study used a PCR-based method known as Terminal Restriction Fragment (TRF) Length Polymorphism analysis in addition to standard culture techniques to track changes. This TRF method can overcome biases introduced by culture methods and accurately track many species that might be missed using standard culture methods [3].

Terminal Restriction Fragment (TRF) patterns are a practical way to characterize bacterial communities because they can be used to identify both species dominance and species richness within the samples. Different phylotypes of bacteria present in each sample can also be tentatively identified using a database. Generation of a TRF pattern involves extraction of DNA from a sample, PCR using a labeled primer, digestion with a restriction endonuclease, ethanol precipitation to clean up the digestion product, and use of a capillary gel electrophoresis system to generate the TRF pattern. The TRF pattern is then analyzed using a variety of statistical techniques.

More formally, the specific primary aim of the study was to determine the effect of probiotic therapy during and after antibiotic therapy on levels of lactobacilli and bifidobacteria (as determined by culture techniques) in the feces of healthy adults.

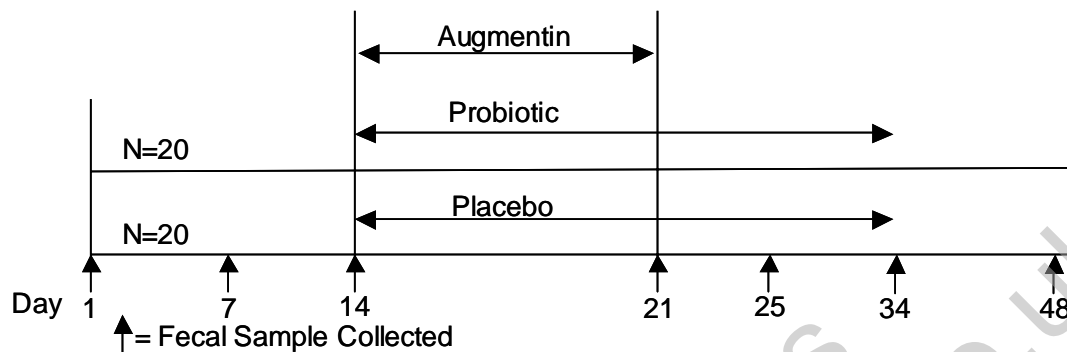
Secondary objectives were to determine the effect of probiotic therapy during and after antibiotic therapy on levels of *Enterobacteriaceae*, *Bacteriodes* and *Clostridium* (as determined by culture techniques) in the feces of healthy adults.

Study Design

Healthy individuals were recruited who agreed to be on antibiotics for one week for study purposes only, receiving one-week of the antibiotic Augmentin (amoxicillin and clavulanic acid) 875 mg orally twice a day. This antibiotic was selected because of a high rate of antibiotic-associated diarrhea identified with this antibiotic. Patients were then randomized (1:1) to either placebo or the probiotic test product consisting of a capsule containing a dried bacterial preparation of probiotic bacteria in the genera, *Lactobacillus* and *Bifidobacterium* as follows:

- a. *Bifidobacterium bifidum* Bb-02 (5×10^8)
- b. *Bifidobacterium lactis* B1-04 (5×10^9)
- c. *Bifidobacterium lactis* Bi-07 (5×10^9)
- d. *Lactobacillus acidophilus* NCFM (5×10^9)
- e. *Lactobacillus paracasei* Lpc-37 (5×10^9)

The total dose of probiotic was 2×10^{10} bid (4×10^{10} daily). The other group received a placebo consisting of the same filler used in the bacterial preparation, maltodextran, without the bacteria. The study was conducted over 48 days. Three baseline (no treatment) fecal samples were obtained at days 1, 7, and 14, followed by the 7-day course of Augmentin. Fecal samples were then collected on days 21, 25, 34, and 48. Probiotic/Placebo treatment began on day 14 and continued until day 34 (Fig. 1).

Fig. 1. Schematic of the experimental design

Methods

Subject selection and sampling protocol

Forty subjects were enrolled with the aim of 36 patients completing the study, anticipating a 10% dropout rate. Subjects were recruited from hospital staff through email solicitation to the community as well as signs posted in the hospital. Enrollment criteria permitted only patients over eighteen without significant acute or chronic illnesses. Permitted medications included those that were constant throughout the study and only if they had no established or suspected impact on gut flora. Individuals were excluded if they were pregnant, breastfeeding, had a penicillin allergy, a history of gastrointestinal illness or had been on any antibiotics in the preceding four weeks. Fermented foods or any probiotic preparations were prohibited for four weeks before entry into the study and throughout the duration of the protocol. Subjects completed a diary of stool frequency, gastrointestinal symptoms and overall well being throughout the protocol. The Washington University School of Medicine institutional review board approved the protocol. Written consent was obtained from each individual.

Fecal samples were obtained for TRF analysis by adding approximately 1 gram of feces to a 2 mL screw-cap tube and freezing at -80°C until shipment. For culture analyses, five grams of each fecal sample were placed into 16 ml Cary Blair Transport Medium (CBTM, BBL) with indicator, (Remel, Lenexa Ks 66215) resulting in a 1:4.2 dilution factor. The sample was then shaken/vortexed briefly to disperse and frozen at -80°C until shipment. Three lots of samples (approximately 100 each) were shipped on dry ice to Todd Klaenhammer at NCSU (for culture analysis) and Christopher Kitts at CPSU (for TRF analysis).

Bacterial culturing

Frozen samples received from the clinical study, were stored at -80°C until enumeration, and thawed at 37°C immediately prior to plating. Serial dilutions of the samples were prepared (10^{-2} - 10^{-8}) in duplicate, in sterile, pre-reduced 1 % yeast extract in water. According to Summanen *et al.* [4], liquid media were boiled for five minutes to drive off dissolved oxygen and used within the same day. For this study, yeast extract diluent was autoclaved for fifteen minutes at 121°C and allowed to cool prior to being placed within the anaerobic chamber and dispensed into sterile tubes. All fecal samples were thawed, diluted and plated in an anaerobic chamber (Coy Environmental Chamber, Coy Laboratory Products, MI), maintained at 37°C for 3 to 5 days with an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Dilutions (10^{-2} - 10^{-8}) were plated onto duplicate plates of nonselective media itemized below.

- a. BIM-25 for enumeration of Bifidobacteria [5-7] for cultivation from human feces (sewage, wastewater). This is a reinforced clostridial agar base with polymixin B, tetrazolium red, iodoacetate, kanamycin, and naladixic acid (Sigma).

- b. LBS Agar (LBS; Difco) plus 200 ml per liter tomato juice from concentrate (Campbell's) for enumeration of lactobacilli [8, 9].
 - c. Bacteroides Bile Esculin (BBE, Difco) Agar for enumeration of the *Bacteroides fragilis* group
 - d. Egg Yolk Agar (EYA, Difco) for enumeration of *Clostridium* species. For selection of *Clostridium* species, aliquots of each dilution were treated at 80°C for 10 minutes to kill vegetative cells, leaving spore-formers for enumeration as described by Summanen *et al.* [4].
 - e. MacConkey (MAC, Difco) agar for enumeration of *Enterobacteriaceae*
- Culture data were presented in the form of colony forming units (CFU) per gram feces. Because the variation from subject to subject sometimes spanned several orders of magnitude the data were log transformed before analysis.

TRF patterns

Fecal samples were collected healthy adult subjects divided into two groups of probiotic and placebo. Samples were collected and delivered to the hospital within 8 hours of collection, and aliquots of 3 grams were stored at -80° C. Samples were extracted in triplicate using the MoBio Ultraclean® soil DNA kit following manufacture's protocol with the addition of 5 additional washes with S4. Success of each extraction was determined by measuring DNA concentration in the extraction product with a Spectramax spectrophotometer. 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 mL of undiluted extraction product, 5 mL of 10x Buffer, 3 mL of 10 mM DNTP, 2 mL 20 mg/mL BSA, 7 mL 25 mM MgCl₂, 1 mL K₂R, 1 mL Ba₂FD₄, 29.7 mL water, and 0.3 mL 5 U/mL TaqGold®. Reaction temperatures and times were 96 °C for 10 min; 35 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 a MoBio Ultraclean® PCR Cleanup Kit following manufacturer's protocol. Amounts of DNA in each sample were determined using a fluorometer. An enzyme digest was performed on each PCR cleanup product using the restriction endonuclease *Hae*III (New England Biolabs; Beverly, Massachusetts). Additional digestions for the multienzyme analysis were performed using the restriction endonucleases *Alu*I and *Hpa*II (New England Biolabs; Beverly, Massachusetts). Each 40 mL digestion used 75 ng of DNA, 1 U of enzyme, and 4 mL of buffer. The samples were digested for 4 h at 37 °C and inactivated for 20 min at 65 °C. The digestion products were ethanol precipitated and resuspended in 20 mL of formamide and 0.25 mL of CEQ 600 base pair standard. Terminal restriction fragment profiles were obtained using a Beckman Coulter CEQ8000X DNA analysis system.

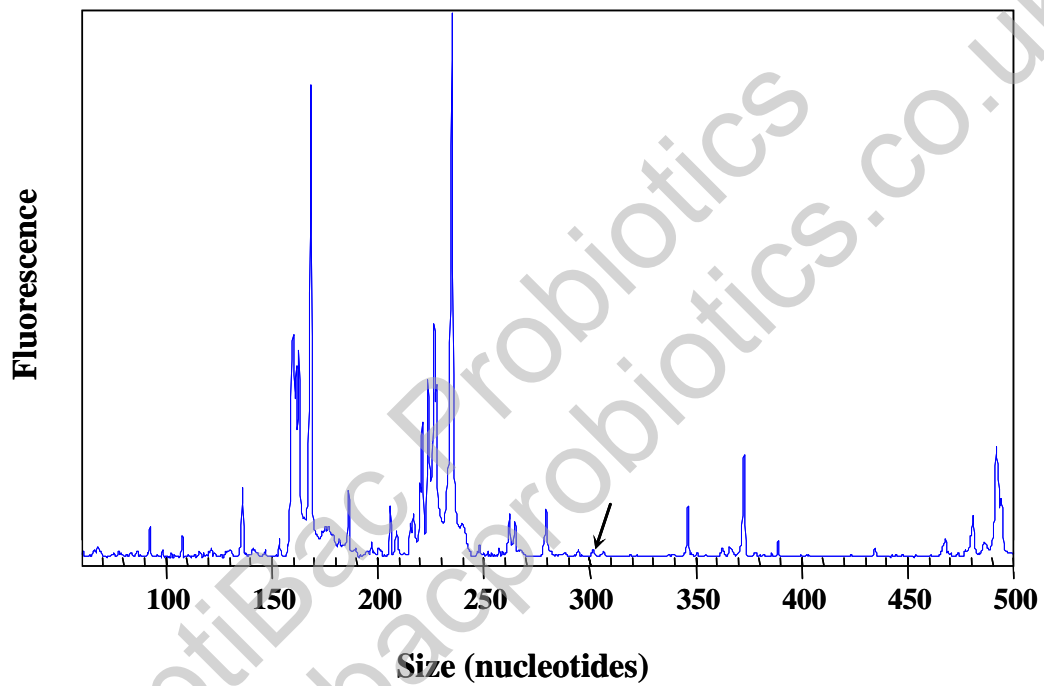
TRF data are gleaned from an electropherogram of the labeled DNA fragments (Fig. 2) and consists of TRF peak sizes (length in nucleotides of the DNA fragments detected) and area under each TRF peak (a measure of abundance for each DNA fragment). Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQ8000 into Excel. To standardize the data for comparison between samples the area under each TRF peak was normalized to total amount of DNA analyzed and expressed as parts per million (ppm). Peaks with an area of less than 5,000

ppm (<0.5% of the total for that sample) were excluded from analysis to reduce noise.

The TRF data were transformed by taking the square root of each TRF peak area to de-emphasize large TRF peaks while still taking relative abundance into account in Principle Components and Bray-Curtis analyses. Statistical analyses were performed using SAS, Minitab 14 and Microsoft Excel.

OptiBac Probiotics
www.optibacprobiotics.co.uk

Fig. 2. Electropherogram of TRFs from a single sample showing TRF peak size in nucleotides and area under the peak. Note the arrow indicating a TRF peak with area less than 0.5% of the total area as an example of the noise cut off.



The database for TRF matching was created by obtaining all the 16S rRNA gene sequences from GenBank. These sequences were trimmed using in silico (with an Excel macro) PCR with the Ba2F and K2R primers. In silico digestion was performed using several restriction endonucleases. Observed TRF peaks were compared to predicted TRF peaks found in the database. Observed TRF peaks were allowed to be within ± 1 bp of the predicted TRFs.

Results

Fifty subjects were enrolled, with 40 subjects completing the protocol. No patient discontinued because of any adverse event associated with the antibiotics or study medications. Of those completing the study and counted as part of the evaluated data set, nine subjects were male and 31 female (four men and 16 women in the placebo group; 5 men and fifteen women in the active group). The average age was 37.2 of all subjects completing the study (39.5 years mean in the placebo group and 36.5 years mean in the active treatment group). Stools samples were complete in 33 subjects and were missing one sample in seven subjects. No patient discontinued due to adverse events during the study. All patients who discontinued did so because of logistical reasons (transportation difficulties) or because they chose not to continue participation in the study.

Adherence to the protocol was excellent for both the antibiotic and placebo/probiotic administration. Only eight individuals took less than 100 % of the antibiotics: four in the probiotic group (92%, 71%, 79%, 93%) and three in the placebo (79%, 93%, 64%). In the placebo group, only four individuals took less than 85% of the placebo product (23%, 68%, 75%, 81%). In the active treatment group, only one

individual took less than 85% of the test product (58%). Both the placebo and active treatment were well tolerated. Only four patients developed diarrhea lasting more than three days during the study (one in placebo and three in active treatment). No patient developed severe or persistent diarrhea. Other adverse events included yeast infection (four in active treatment; one in placebo) and abdominal cramping (three in active treatment and three in placebo).

First Level of Analysis for TRF data – Principal Components Analysis

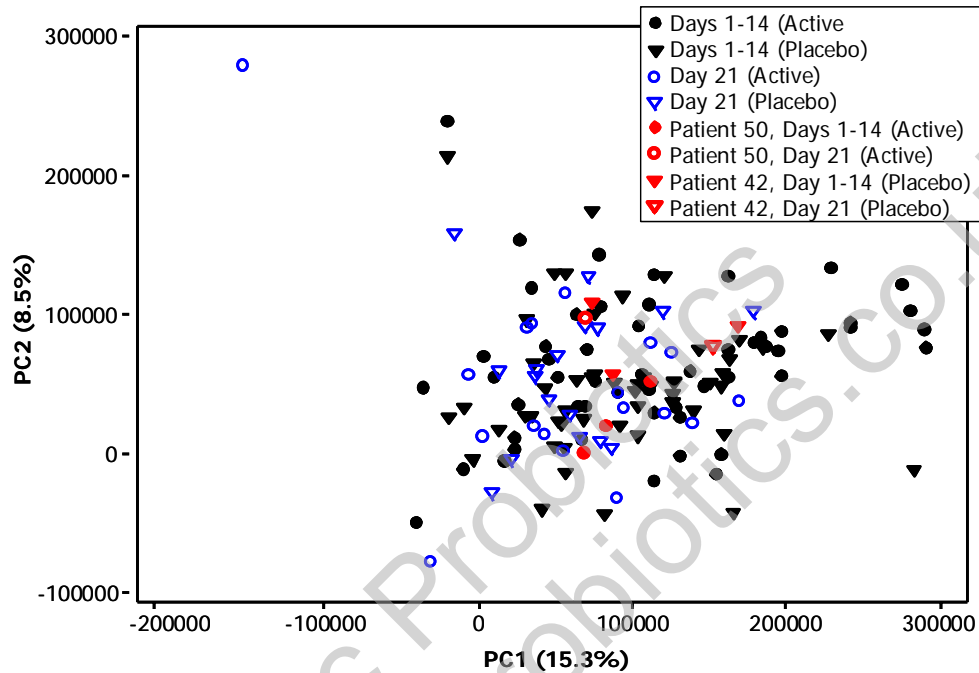
Principle Components Analysis (PCA) provides a rapid way to assess variation with multidimensional data such as those provided by TRF patterns. PCA is a method of data reduction that transforms the data into new variables (the principal component scores), which are constructed to represent the greatest variation in the data set. The first principal component (PC) accounts for as much of the variability in the data as possible, and each succeeding PC accounts for as much of the remaining variability as possible. PC scores are generated by assigning a loading factor to each variable and summing the total of all variables multiplied by their loadings. Plotting the first PC scores on the x-axis and the second PC scores on the y-axis creates a score plot. A good result would be obvious grouping of samples within the plot. Plotting the first PC loadings on the x-axis and the second PC loadings on the y-axis creates a loading plot. A loading plot indicates which variables are important to each PC score. Score plots and loading plots can be overlaid to increase the information content.

A PCA score plot of all subjects' TRF data collected over days 1-21 indicated a large amount of subject-to-subject variation that obscured the overall effect of antibiotics on fecal flora (Fig. 3). Although a t-test of the PC1 scores for days 1-14 (baseline) versus

day 21 indicated a significant difference ($p < 0.001$), this difference was not clear in the PCA score plot. In addition, PC1 only represented 15% of the total variation in TRF data and thus may under-represent any changes induced by antibiotic consumption.

Differences in fecal flora can be seen on an individual subject level, for example, subject 50 (red circles) showed a clear shift at day 21 while subject 42 (red triangles) showed high variation in the baseline data masking a possible shift at day 21. When PCA was performed on data from individual subjects the shift in fecal flora became clearer.

OptiBac Probiotics
www.optibacprobiotics.co.uk

Fig. 3. Principle Components Analysis score plot of all subjects days 1-21

PCA was performed on each subject and it became clear that subjects fell into two major categories: those with stable baseline flora (days 1, 7 and 14) and those whose baseline flora varied significantly. For example, subject 50 (Fig. 4) showed a clear antibiotic effect on the fecal bacterial community structure at days 21 and 25 that appeared to be gone by day 34. After the subject stopped taking probiotics the fecal community changed again (day 48). Subject 42 (Fig. 5) showed a smaller effect on the fecal bacterial community structure from antibiotic treatment because variation in the baseline data made it difficult to tell if the shift at day 21 was significant.

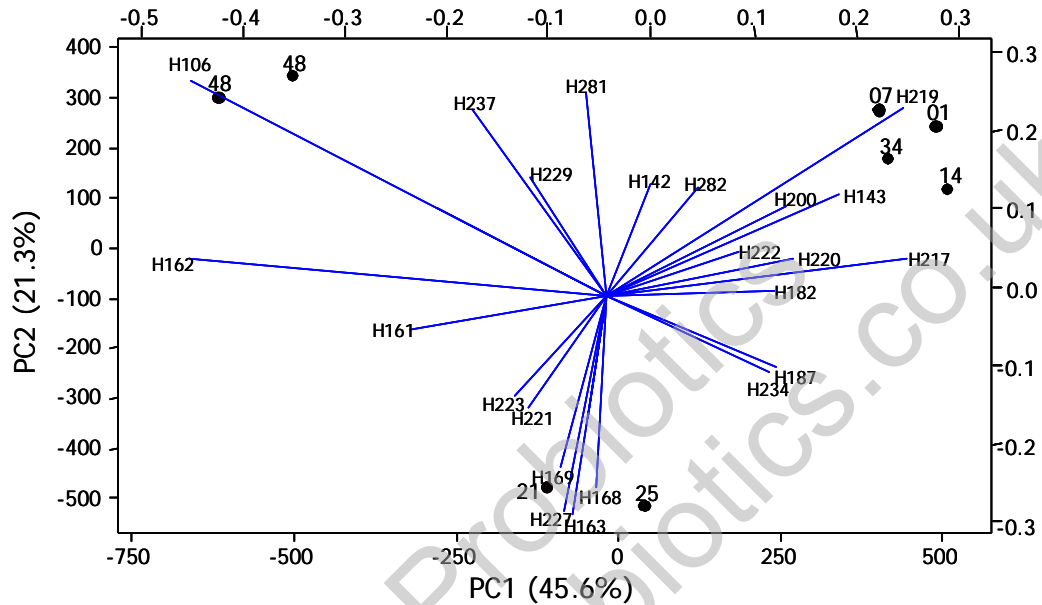
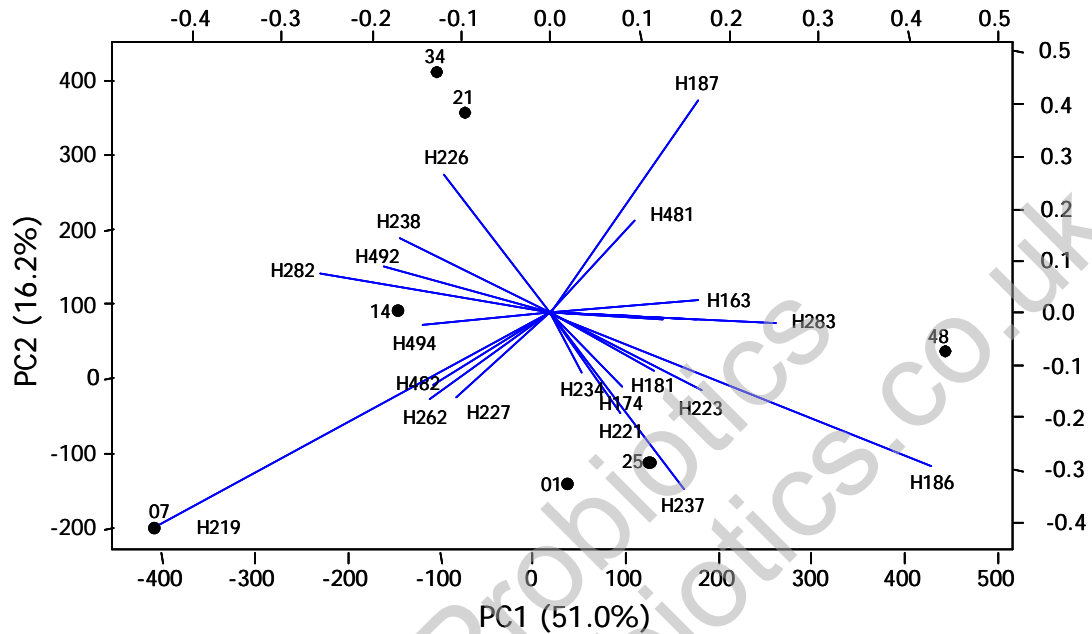
Fig. 4. Principle Components Analysis score and loading plots for subject 50

Fig. 4 and 5 illustrate the utility of PCA because the contributions of different types of bacteria to shifts in community structure become clear. For example, the lines marked H168 and H169 (representing *Enterobacteriaceae*) pointing to days 21 and 25 for subject 50 indicate that these TRF peaks increased markedly in this subject on days 21 and 25. However, PCA cannot be used to judge the statistical significance of changes in TRF patterns because with seven observations and over 100 variables several of the key assumptions (eg. data fitting a normal distribution) cannot be confirmed. Some other form of data reduction is necessary to evaluate changes in community structure. Several measures for comparing patterns exist that return a single number, the similarity or distance between two patterns, may work better.

Fig. 5. Principle Components Analysis score and loading plots for subject 42

Distance Measures

Two methods for estimating pattern similarity; Euclidean distance and Bray-Curtis similarity (Fig. 6) were used with TRF data and culture data to evaluate changes in a subject's fecal flora after antibiotic treatment. Both Euclidean Distance and Bray-Curtis Similarity take the relative abundance of each TRF peak into account when comparing two patterns. Euclidian distance is a standard method for evaluating two data sets but it is prone to bias by large numbers and can give anomalous results when data sets contain large numbers of zero values. 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. Therefore, the Bray-Curtis similarity measure was also investigated. Bray Curtis similarity was specifically designed for use with species abundance data sets [10]

and is less susceptible to bias introduced by large numbers of zero abundance data. Bray-Curtis similarity is therefore a preferable similarity measure when comparing TRF patterns. When the culture data were analyzed, Euclidian distance was used because there were fewer variables (five different media) and very few zero abundance data points. The baseline data (days 1, 7, 14) were used to calculate the distance from (or similarity to) baseline for each post-antibiotic treatment day. Using these methods it was possible to look for changes in fecal flora specific to an individual subject.

Fig. 6. Formulas for the three distance measures 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)

Euclidean Distance

$$\sqrt{\sum_{TRF=60}^{600} (Area_{TRF,d1} - Area_{TRF,d7})^2}$$

Bray-Curtis Similarity

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

Identifying Changes in TRF Peaks

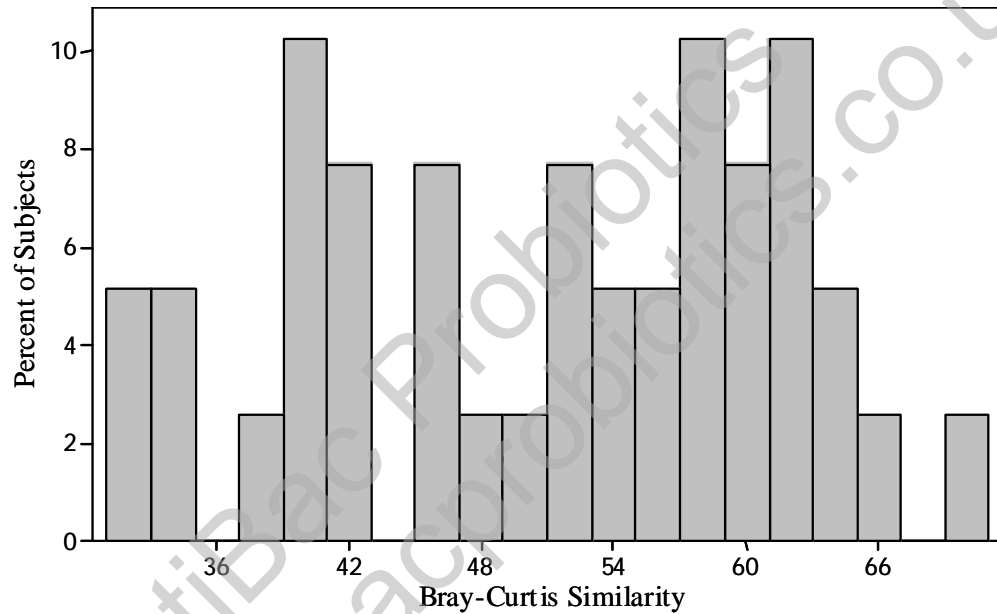
The advantage of PCA is that construction of the PC scores keeps track of the contribution of individual TRFs so that changes in fecal flora could be tracked to individual types of bacteria. When using similarity measures to identify changes in fecal

flora one loses the contribution of individual TRF peaks to the overall similarity score. We were thus forced to search for another method to identify TRF peaks (representing bacterial types) responsible for the shifts in fecal flora. TRF data from the first three days was compared separately to each day after antibiotic therapy and TRF peaks that fell outside a 90% confidence level were noted. These TRF peaks were then collected for subjects showing changes in fecal flora by similarity analysis and the most common TRF peaks were used to search a database for organism identification.

Stability of Fecal Flora

Large amounts of variation in fecal flora were discovered in the fecal samples from individual subjects. This was visible in culture and TRF data analyses (Fig. 7 and 8). TRF data separated subjects into two categories in a bimodal distribution: those with stable microflora (22 subjects) and those with unstable microflora (17 subjects). Stability appeared to be randomly distributed (ANOVA $p=0.615$) across probiotic treatment groups (active and placebo) when using the TRF based categories collected from Fig. 7. As might be expected however, stability had a significant effect on the detection of differences in TRF patterns after antibiotic treatment (MANOVA $p=0.047$).

Fig. 7. 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.



Analysis of TRF data using TRF-based stable/unstable designation provided a basis for determining which TRF peaks helped define stable versus unstable flora. Discriminant analysis was performed on both presence/absence data (TRF peak present or absent – P/A) and square root of TRF peak area data (a method to de-emphasize large TRF peaks but still take relative abundance into account – Sqrt). Several TRF peaks were detected which helped to discriminate between subjects with stable and unstable flora and had significant differences between the two categories (Table 1). TRF analysis did not

give a confirmed species identification for these peaks given the limits of identification possible with only 1 enzyme digest.

Table 1. TRF peaks discriminating between “Stable and Unstable” categories with TRF data.

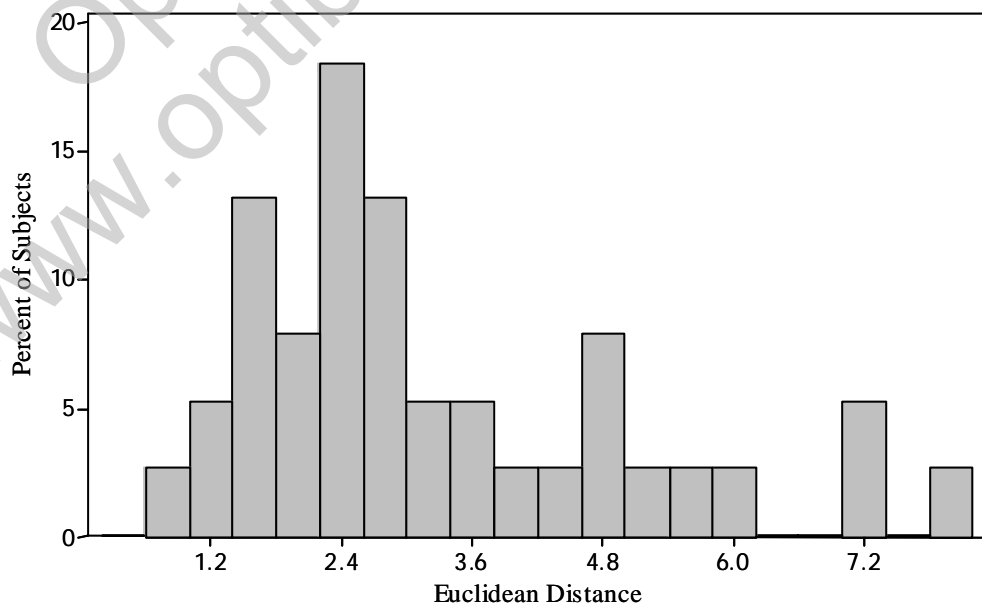
Peak	Category	p-value	Genera represented by TRF peak (+/- 1 bp)	Data set*
182	Stable	0.001	<i>Clostridium, Cytophaga</i>	Sqrt
219	Stable	0.011	<i>Bifidobacteria, Clostridium, Ruminococcus</i>	P/A
223	Stable	0.000	<i>Bacteroides, Bifidobacteria</i>	P/A
278	Stable	0.001	<i>Enterococcus, Lactobacillus, Clostridium</i>	P/A
87	Unstable	0.003	<i>Procabacter, Planctomyces, Microbacterium</i>	P/A
199	Unstable	0.023	<i>Clostridium</i>	P/A
285	Unstable	0.004	<i>Lactobacillus, Clostridium</i>	P/A
287	Unstable	0.007	<i>Lactobacillus, Clostridium</i>	Sqrt
487	Unstable	0.007	<i>Bacteroides, Cytophaga</i>	P/A
487	Unstable	0.004	<i>Bacteroides, Cytophaga</i>	Sqrt

* P/A, TRF peak present or absent; Sqrt, square root of TRF peak area.

Bacterial Culture Analysis

Euclidian Distance was used to repeat a stability analysis with the culture data (Fig. 8). Subjects fell into a skewed distribution with only 10 subjects clearly outside a normal distribution and thus categorized as unstable. Unfortunately, only 5 subjects were classified as unstable by both TRF and culture data so the existence of a subset of subjects with a truly unstable fecal flora seems tenuous at best. It is plausible that variation in data between the first three fecal samples from one subject could reflect changes in bacterial numbers brought about by variation in sample handling and storage.

Fig. 8. Average Euclidian distance analysis of plate count patterns from each subject across the three initial samples (days 1, 7, 14). Note the skewed distribution. An estimated cut-off for Stable/Unstable categories was assigned at a distance of 4 based on a projected normal distribution. Subjects with average distance < 4 were deemed stable.



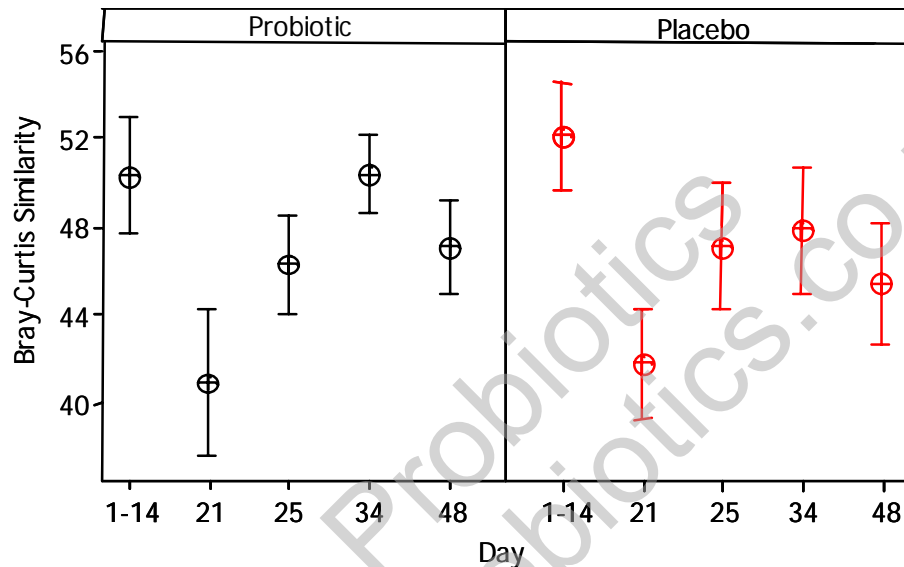
Effects of Antibiotics and Probiotics

Effects seen with TRF data

Bray-Curtis similarity to baseline (days 1-14) was calculated for each subject and each day after antibiotic treatment. By comparison to similarity within baseline it was clear that the antibiotics had a significant effect on fecal flora (ANOVA, $p < 0.001$). The average similarity of baseline samples compared to the first day after antibiotics was 42% while average similarity in a single subject at baseline was 51%. Average similarity to baseline at day 25 increased to 47% and was no longer statistically significant at a 95% confidence level ($p = 0.078$). At day 34 the average similarity reached 49% and at day 48 it decreased to 46%. The significance of the change at day 48 is not clear, especially since it occurred in both the probiotic and placebo treatment groups and thus cannot be solely attributed to the cessation of probiotic ingestion at day 34.

To assess the effect of probiotic ingestion on changes brought about by antibiotic treatment the Bray-Curtis similarity data were analyzed separately for probiotic and placebo groups (Fig. 9). While both groups exhibited a similar trend toward increased similarity to baseline over days 21 through 34, the probiotic group exhibited a larger increase in similarity at day 34. The difference between the two groups was not significant when all four post-antibiotic treatment days were analyzed (MANOVA, $p = 0.135$). When day 48 was removed from analysis a trend revealing a difference could be detected at a 90% confidence level ($p = 0.066$).

Fig. 9. Average Bray-Curtis similarity from base line (days 1-14) for each day after treatment. Black symbols are probiotic treatment and red symbols are placebo. Error bars represent one standard error.



TRF Peaks Changing with Antibiotic Treatment

TRF peaks that increased after antibiotic treatment were identified using an analysis of the 15 subjects (8 placebo and 7 probiotic) that showed a noticeable change in TRF pattern on day 21 (subjects 4, 6, 7, 9, 11, 12, 13, 24, 27, 29, 33, 35, 46, 48, 50) and were in the “stable baseline” category. In an initial assessment, PCA loadings of data from days 1 to 21 for each subject were used to identify TRF peaks that were predominantly associated with day 21. This method was corroborated by the 90% confidence cut-off method described above. A total of 14 TRF peaks showed large changes in abundance in at least 10 of the 15 subjects evaluated (Table 2). Several of these TRF peaks appeared to cluster, representing classes of organisms. For example,

TRFs of 167 and 168 nucleotides, most likely representing *Enterobacteriaceae*, increased in almost all subjects. Similarly, the relative abundance of TRFs from 265 to 267 nucleotides in length, most likely representing the anaerobic *Ruminococcus* or *Clostridium* genera, increased in almost all subjects. To help obtain a better identification of these TRF peaks additional TRF data were collected using an additional two restriction enzymes.

OptiBac Probiotics
www.optibacprobiotics.co.uk

Table 2. TRF peaks associated with day 21 in patients that showed an antibiotic effect.

TRF Peak	Count	Pos/Neg *	Placebo	Probiotic	Tentative Identification (genus level or higher)
167	10	10/0	5	3	β -, γ -, δ - proteobacteria, most commonly <i>Enterobacteriaceae</i>
168	13	12/1	9	4	β -, γ -, δ - proteobacteria, most commonly <i>Enterobacteriaceae</i>
200	13	4/9	11	2	<i>Eubacterium</i> , <i>Clostridium</i> , Fecal Streptococci, <i>Enterobacteriaceae</i>
221	10	10/0	7	3	<i>Bifidobacterium</i> , <i>Bacteroides</i> , <i>Ruminococcus</i> , <i>Clostridium</i>
231	11	11/0	6	5	<i>Heliobacter</i> , <i>Clostridium</i>
242	10	6/4	5	5	<i>Lactobacillus</i> , <i>Eubacterium</i> , <i>Clostridium</i>
248	12	12/0	7	5	<i>Bifidobacterium</i> , <i>Eubacterium</i> , <i>Enterococcus</i> , <i>Clostridium</i>
265	14	14/0	11	3	<i>Ruminococcus</i> , <i>Clostridium</i>
266	13	9/4	11	2	<i>Ruminococcus</i> , <i>Clostridium</i>
267	11	7/4	8	3	<i>Ruminococcus</i> , <i>Clostridium</i>
372	11	11/0	5	6	<i>Vibrio</i> , <i>Rahnella</i> , <i>Cytophaga</i> , <i>Shewanella</i> , <i>Bacteroides</i> , <i>Chlamydia</i>
488	11	8/3	5	4	<i>Bacteroides</i> , <i>Cytophaga</i> , <i>Porphyromonas</i>
492	17	15/2	7	9	<i>Porphyromonas</i>
494	11	11/0	6	4	Unidentified

* Pos refers to an increase in the relative abundance of this TRF peak after antibiotic treatment, Neg refers to a decrease in relative abundance.

The TRF peaks identified in Table 2 were further investigated across all subjects (stable baseline notwithstanding) to determine the behavior of these TRF peaks with respect to probiotic treatment. The average baseline TRF peak area (days 1-14) for each subject was subtracted from the peak area for that subject on each day subsequent to antibiotic treatment. These data were then visualized with an interval plot. Eight of the 14 TRF peaks in Table 2 showed some visible difference between treatment groups. TRFs 167-8, 221, 231 and 494 showed a more rapid trend toward baseline in the probiotic group (Fig. 10). TRFs 242, 372, and 492 showed a more rapid trend toward baseline in the placebo group (Fig. 11).

OptiBac Probiotics
www.optibacprobiotics.co.uk

Fig. 10. Difference in TRF peak area from base line (days 1-14) for each day after treatment. TRF peaks that returned to baseline faster in the probiotic treatment group are presented. Black symbols are probiotic treatment and red symbols are placebo. Error bars represent one standard error.

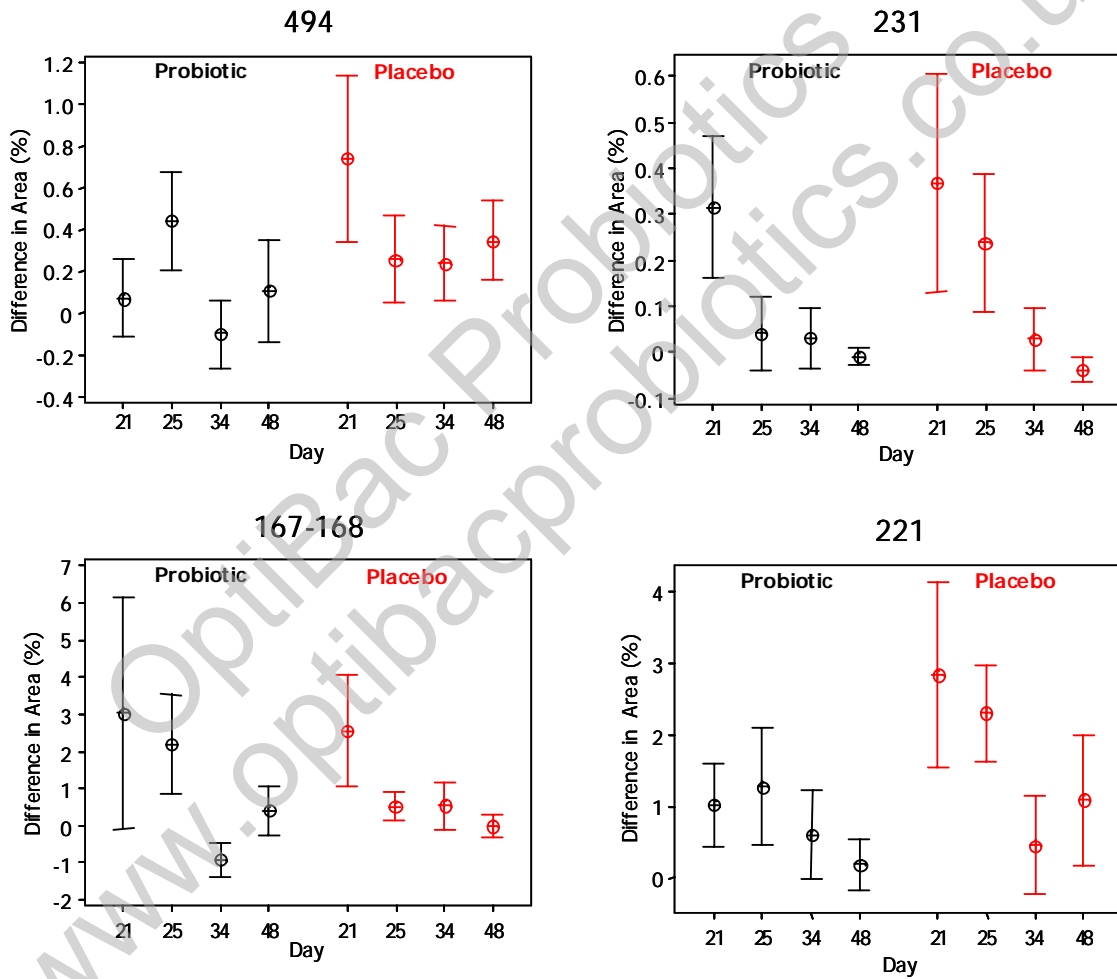
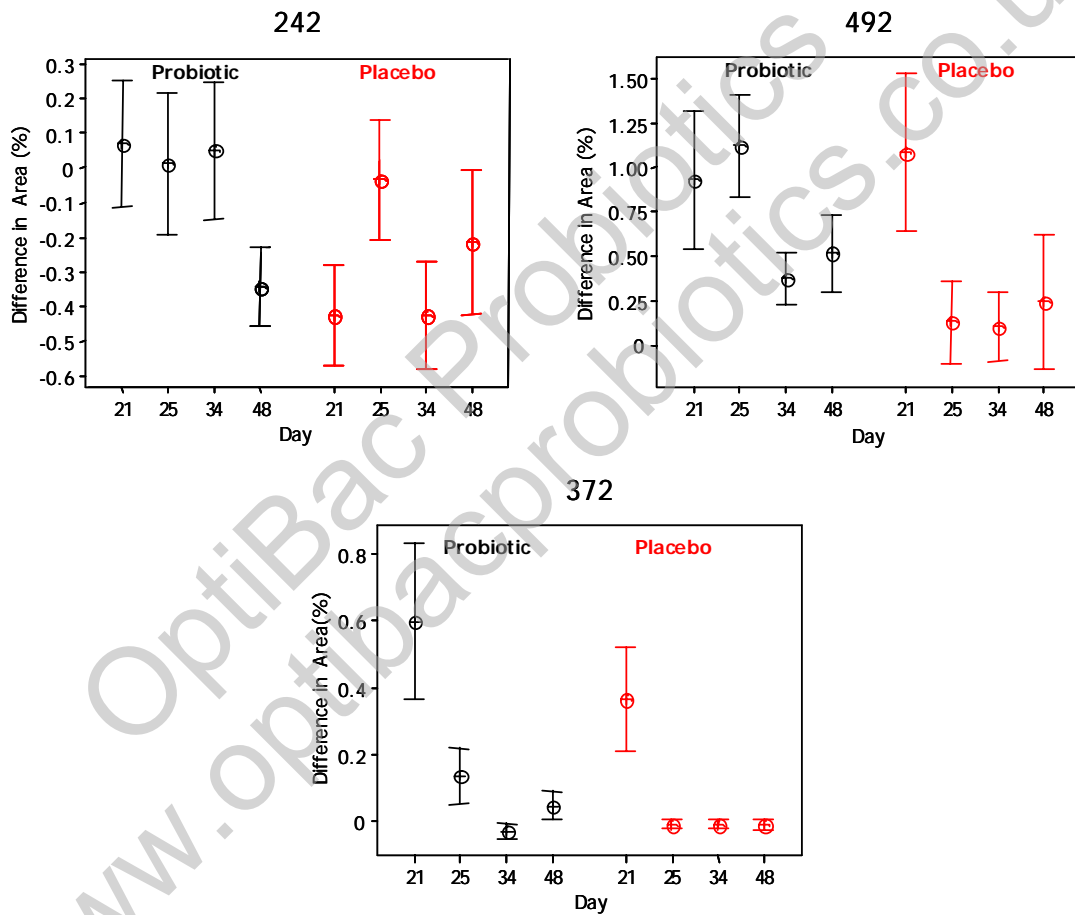


Fig. 11. Difference in TRF peak area from base line (days 1-14) for each day after treatment. TRF peaks that returned to baseline faster in the placebo treatment group are presented. Black symbols are probiotic treatment and red symbols are placebo. Error bars represent one standard error.



Multi enzyme analysis

In order to better identify TRF peaks, samples from 10 subjects (6, 13, 17, 24, 27, 29, 33, 46, 48, 50) were digested with two additional enzymes (*HpaII* and *AluI*). Hae, Hpa, and Alu peaks were matched to one another by comparing the trend in relative abundance of each peak over the study period. Peaks with a similar trend in relative abundance were likely the result of the same organism or group of organisms.

This type of analysis is difficult when working with a collection of human subject because different subjects appear to have different species/strains of the same type of organism, each with slightly different DNA sequences, and therefore different cut sites for the restriction endonucleases. This results in organisms appearing across subjects over a range of peaks in the data that matches a range of peaks in the database (Table 3).

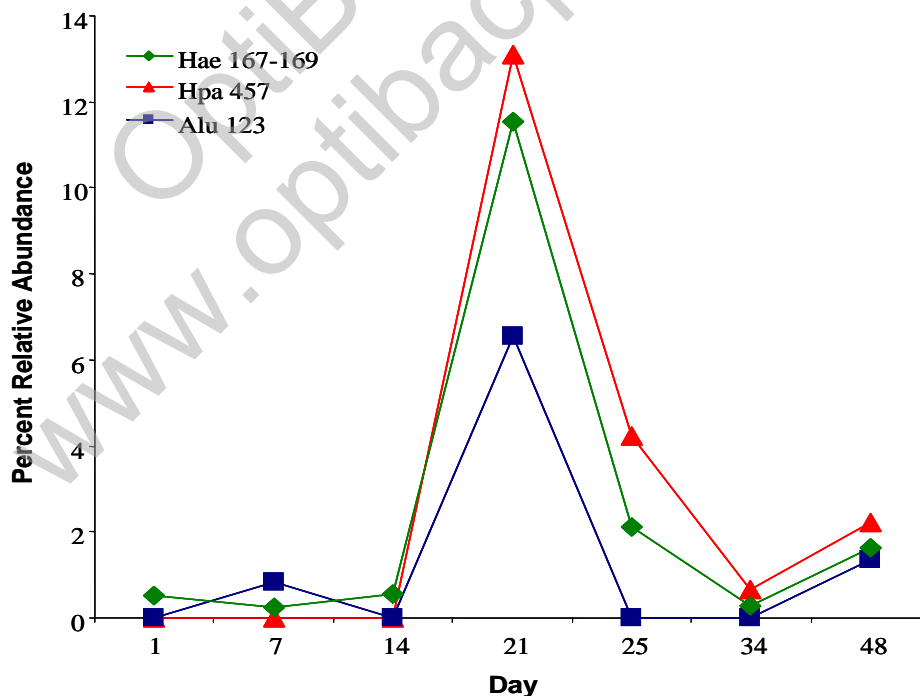
Table 3. TRF peaks associated with enteric organisms in multiple subjects indicating the variation in bacterial strains from subject to subject.

Subject	Hae	Hpa	Alu
6	169	457	NP
17	168	457	NP
24	167, 168	457	122, 123
27	168, 169	457	121, 122
29	168, 169	457	NP
46	169	457	NP
50	169,168	457	NP

Many enteric organisms (*Enterobacteriaceae*) are found in the database at Hae 166-168, Hpa 457-458, and Alu 36 or 121-123. The database, in concert with trends in relative abundance, indicated that enteric organisms, represented by Hae 167-169, Hpa 457 and Alu 123, were present and effected by antibiotic treatment (Fig. 12). Average abundance for the Alu 123 peak is lower than for the Hae and Hpa peaks, likely because enteric organisms can also be represented by a peak at Alu 36 base pairs, which is below the detection limit of the CEQ 8000 instrument.

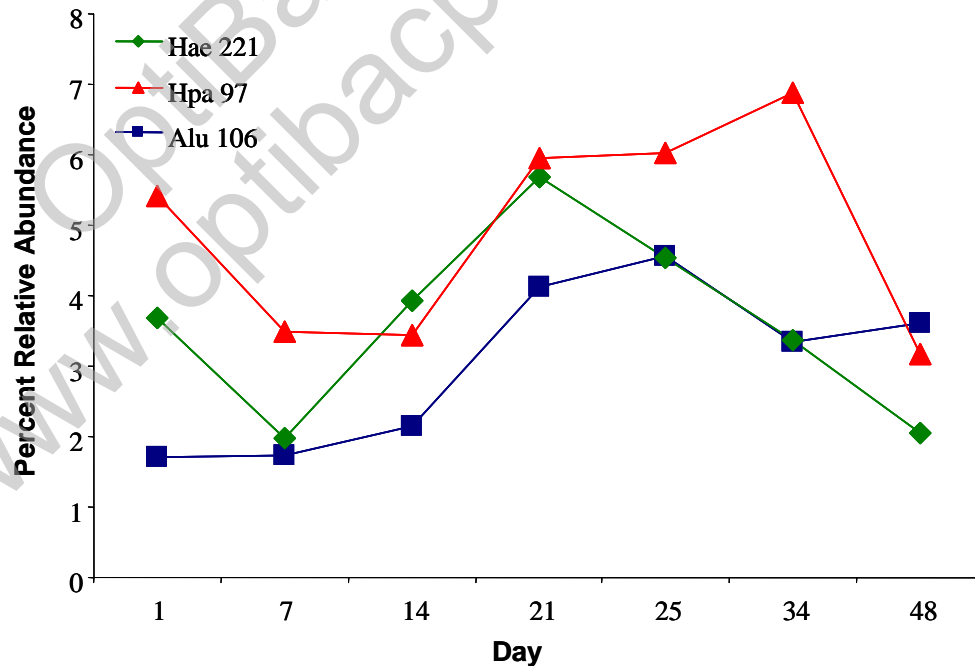
Fig. 12. Comparison of multiple enzyme data for peaks representing enteric organisms.

The Y-axis presents average relative abundance across all 10 subjects when the peak was present.



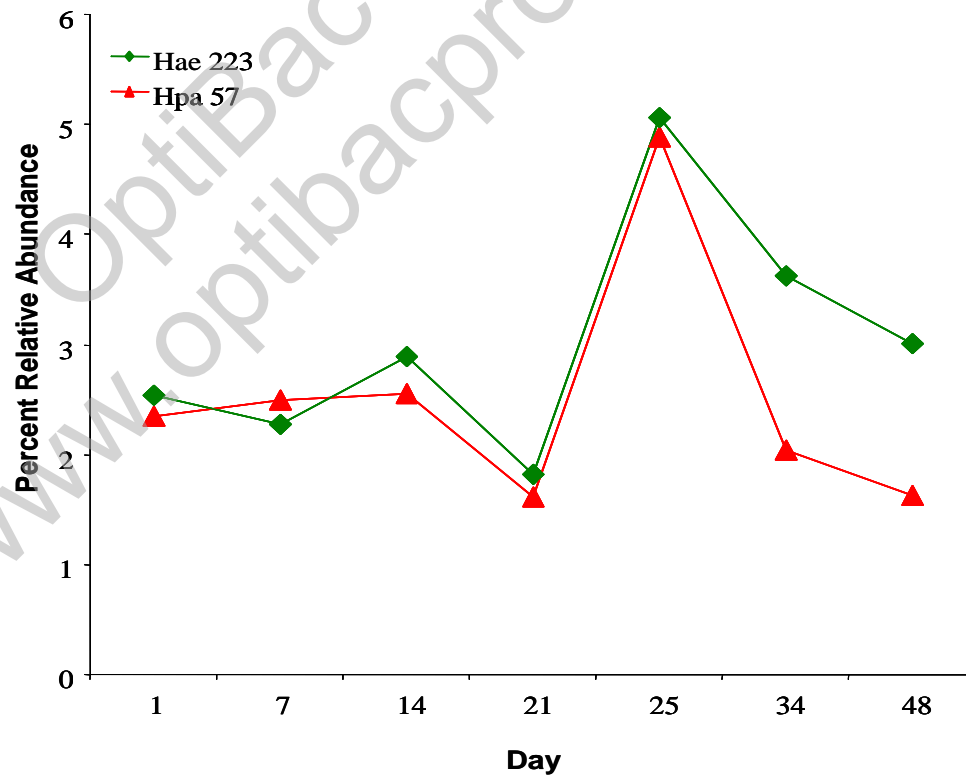
Many *Bifidobacterium* species are found in the database at Hae 219-221, Hpa 95-97, and Alu 104-106. The database, in concert with trends in relative abundance, indicated that *Bifidobacterium*, represented by Hae 221, Hpa 97 and Alu 106, increased as a result of antibiotic treatment and then decreased slowly as the study period went on (Fig. 13). The reasons that the peaks do not show a more similar trend is likely because the database shows that *Bifidobacterium* and *Bacteroides* species may have overlapping representative TRFs in the data (see below).

Fig. 13. Comparison of multiple enzyme data for peaks representing *Bifidobacterium*. The Y-axis presents average relative abundance across all 10 subjects when the peak was present.



Many *Bacteroides* and *Prevotella* species are found in the database at Hae 221-226, Hpa 56-60, and Alu less than 50. The database, in concert with trends in relative abundance, indicated that *Bacteroides* and *Prevotella*, represented by Hae 223, Hpa 57, were present in affected by antibiotic treatment, initially decreasing, but increasing significantly on day 25 (Fig. 14). Alu peaks for these organisms are predicted to be less than 50, which is below the detection limit and therefore not seen with this enzyme.

Fig. 14. Comparison of the multiple enzymes for peaks corresponding with *Bacteroides* or *Prevotella*. The Y-axis presents average relative abundance across all 10 subjects when the peak was present.

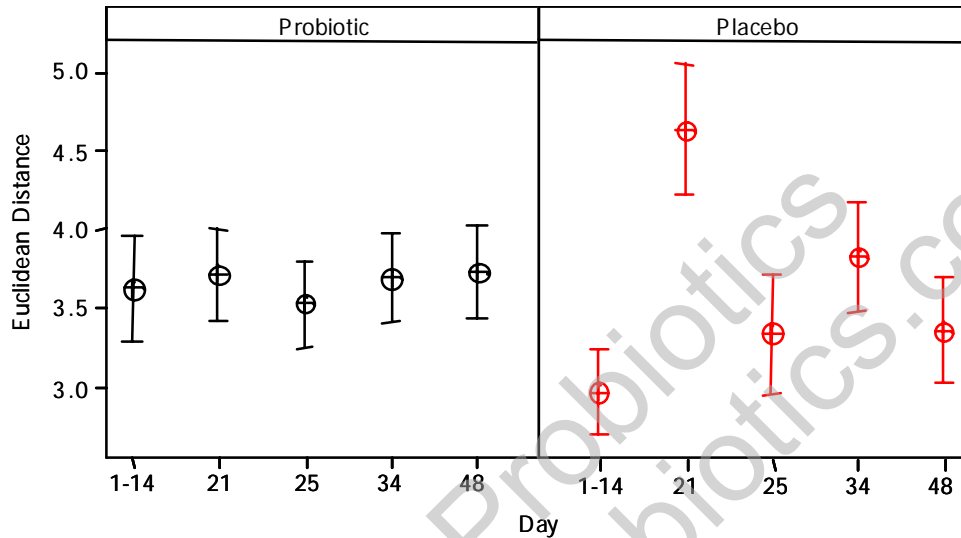


Effects Seen with Culture Data

Because the culture data were gathered with only 5 culture media it was possible to assess changes in the bacterial counts without using similarity or distance measures as was necessary with TRF data. However, because of a very large subject-to-subject variation in counts, there was no statistical difference between the average bacterial counts before antibiotic treatment compared to the days after antibiotic treatment (MANOVA, $p = 0.34$). When Euclidian distance was used to assess changes in a similar manner as applied to the TRF data, it was clear that the antibiotics had a significant effect on day 21 (ANOVA, $p = 0.003$). The average distance of baseline samples compared to the first day after antibiotics was 4.2 while the average baseline distance in a single subject was 3.3. Average distance to baseline at day 25 decreased to 3.5 and was no longer statistically significant ($p = 0.56$). At day 34 the average distance to baseline was 3.7 and at day 48 it was 3.5.

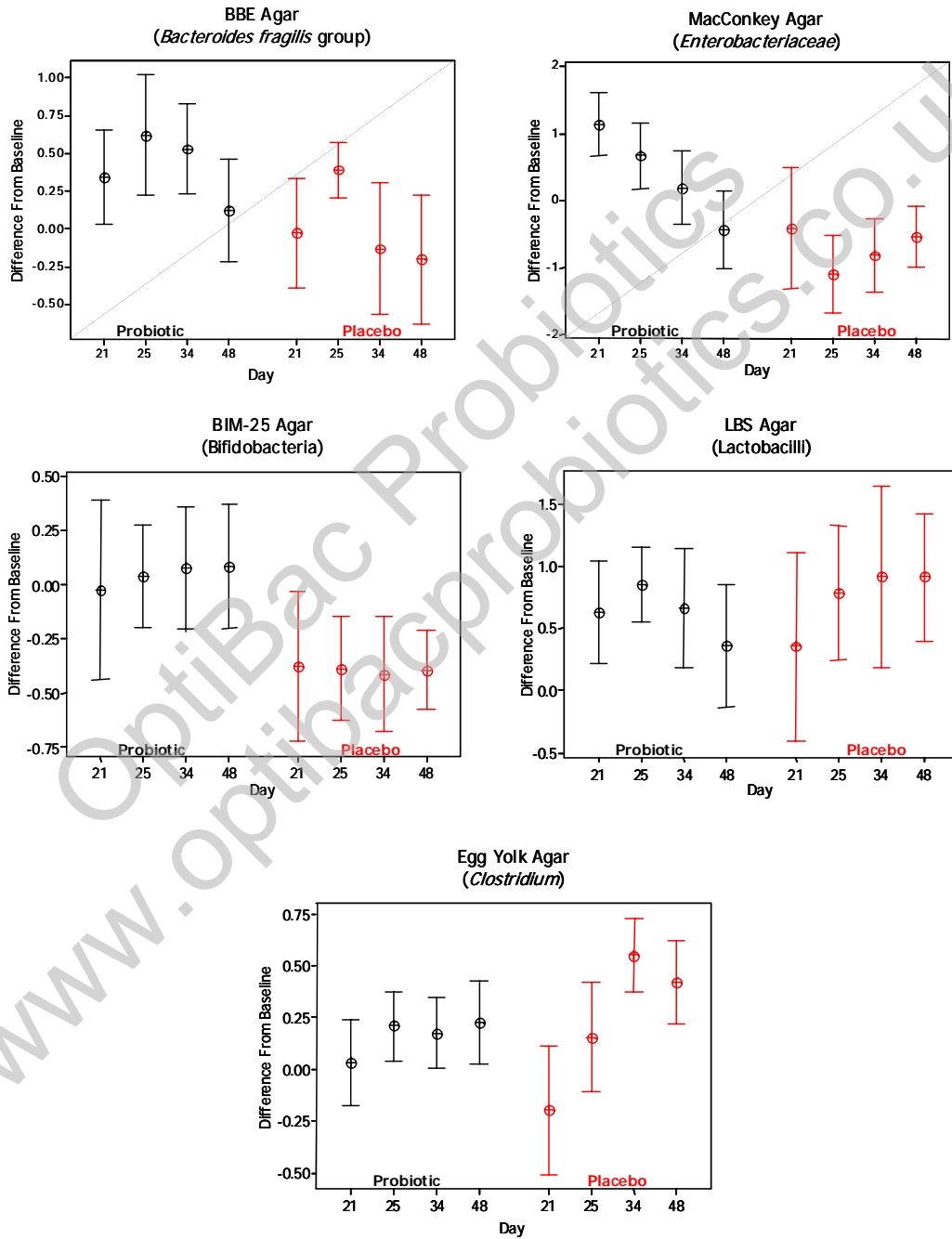
To assess the effect of probiotic ingestion on changes brought about by antibiotic treatment the Euclidian distance data were analyzed separately for probiotic and placebo groups (Fig. 15). Here the culture data showed a much greater difference than the TRF data with the probiotic group maintaining an average distance to baseline of around 3.6 to 3.7 throughout the study. In contrast, the placebo group showed a large shift from baseline at day 21 and a return to near baseline thereafter. The difference between the two groups was quite significant when all four post-antibiotic treatment days were analyzed (MANOVA, $p = 0.004$). When day 48 was removed from analysis there was still a significant difference between groups ($p = 0.046$).

Fig. 15. Average Euclidian distance from base line (days 1-14) for each day after treatment. Black symbols are probiotic treatment and red symbols are placebo. Error bars represent one standard error.



To account for individual variation and still determine which media showed the largest change in fecal flora the average baseline counts (days 1-14) for each subject were subtracted from the counts for that subject on each day subsequent to antibiotic treatment. While these data could not be used to show an antibiotic effect, they still reflected the significant difference between the probiotic and placebo treatment groups (MANOVA, $p = 0.049$) over the four post-antibiotic treatment days (Fig. 16). Intriguingly, counts on MacConkeys agar (*Enterobacteriaceae*) were significantly different between groups (ANOVA, $p = 0.006$), corroborating the results seen with TRF data (Table 2, Fig. 10). Similarly, counts on BIM agar (*Bifidobacterium*) were significantly different between groups (ANOVA, $p = 0.030$), and the relative abundance of *Bifidobacterium* was also affected by antibiotics according to the TRF data (Table 2).

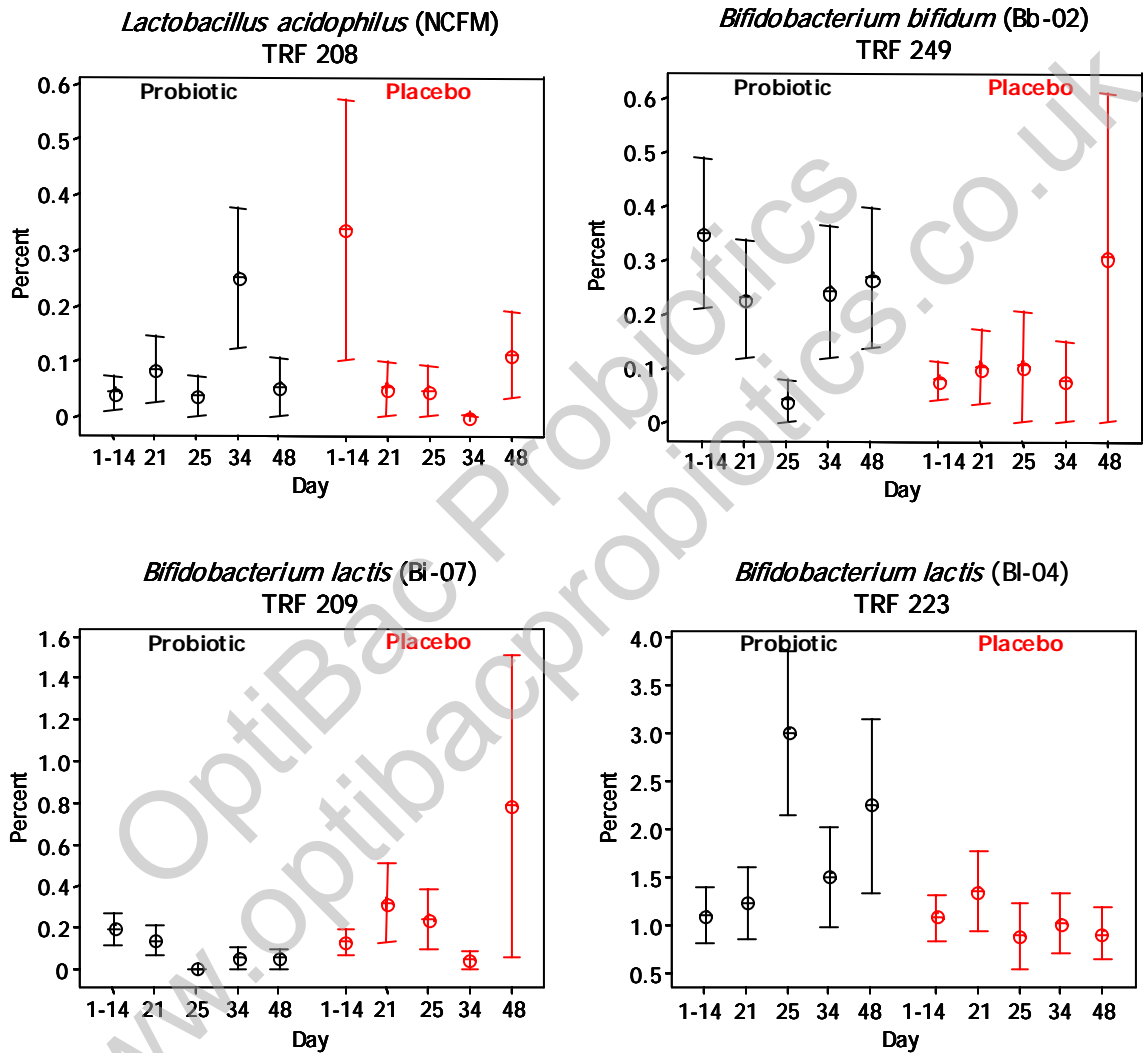
Fig. 16. Interval plots of difference from base line (days 1-14) for each day after treatment for each different medium. Black symbols are probiotic treatment and red symbols are placebo. Error bars represent one standard error.



Tracking Probiotic Strains with TRF data

The TRF peaks corresponding to the five strains of probiotic bacteria were observed and used to track the appearance of these bacteria in subjects' fecal samples. *Bifidobacterium bifidum* Bb-02 was characterized by a TRF peak of 249 nucleotides; *Bifidobacterium lactis* B1-04 by 223; *Bifidobacterium lactis* Bi-07 by 209; *Lactobacillus acidophilus* NCFM by 208; *Lactobacillus paracasei* Lpc-37 by 290 (Fig. 17 & 18). Two different views of the probiotic tracking effort are presented. Fig. 17 is an average of the TRF peak area across all subjects in each group, including subjects where no TRF peak was detected. This view will tend to underestimate the average abundance of the TRF as well as overestimating the error in the average (larger error bars). Fig. 18 is an average of the TRF peak area across all subjects where a TRF peak was detected in each group. This view shows the average abundance for the TRF when it was detected. It is possible that many different native organisms could produce the same TRF peaks as those described for the treatment probiotics.

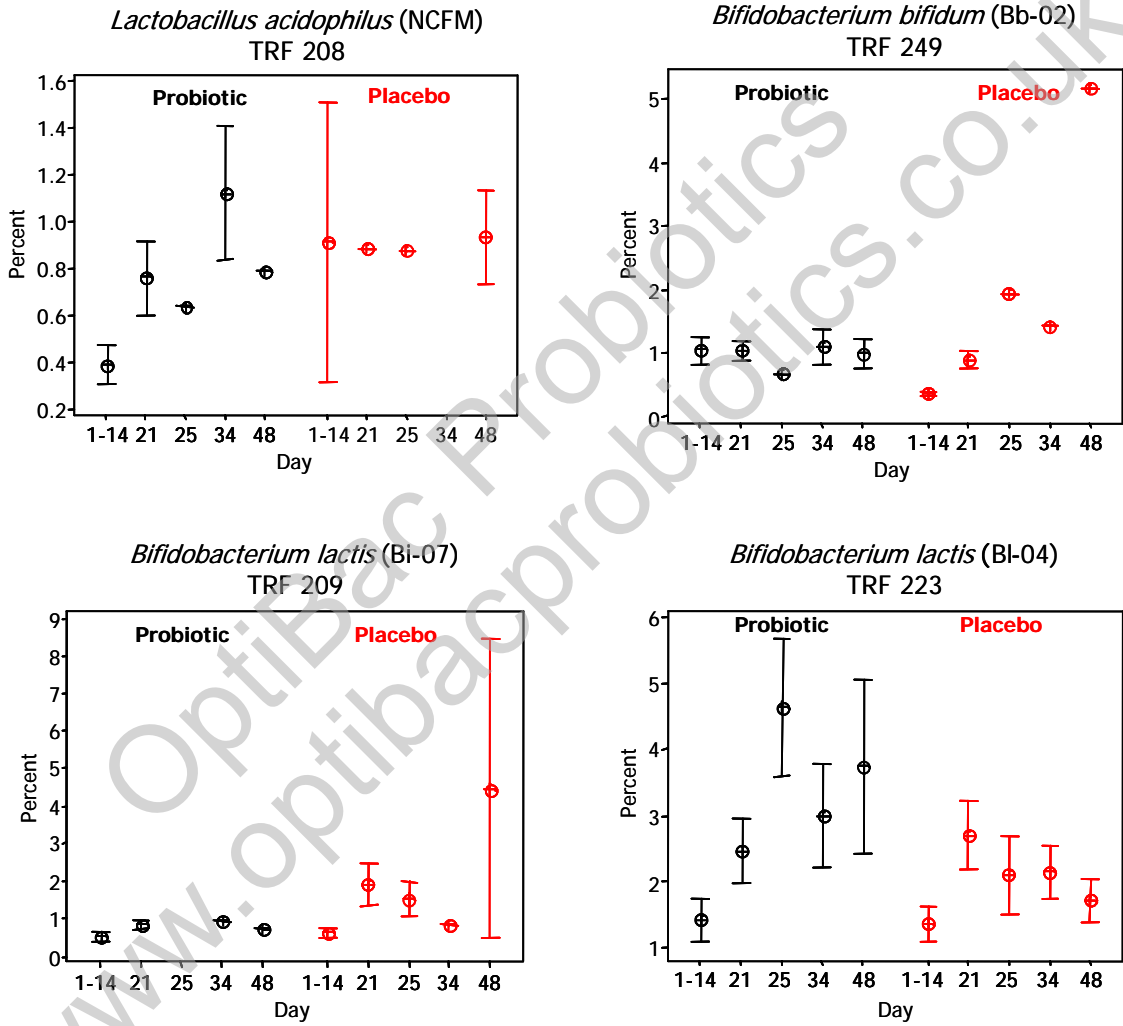
Fig. 17. Interval plots of TRF peak area (including zeros) for each peak that corresponds to a probiotic strain. Black symbols are probiotic treatment and red symbols are placebo. Error bars represent one standard error.



No evidence for the presence of Lpc-37 was found in any subject. This implies that the organism was always less abundant than 0.5% of the total fecal population ($< 10^9/g$). The TRF peak representing NCFM appeared to be lower in probiotic treated subjects initially, although there was a great deal of variation in placebo subjects. Only BI-04 showed an increased abundance in the probiotic subjects. This was true for both the overall average (Fig. 17) and the average abundance only when the peak was present (Fig. 18).

OptiBac Probiotics
www.optibacprobiotics.co.uk

Fig. 18. Interval plots of TRF peak area (excluding zeros) for each peak that corresponds to a probiotic strain. Black symbols represent probiotic treatment and red symbols represent placebo. Error bars represent one standard error.



Conclusions

Overall the results of this study proved that the mixture of probiotics administered had a stabilizing effect on the fecal flora of subjects treated with Augmentin.

Several other conclusions can also be made from this investigation including insight into the way in which culture and TRF data complemented each other.

1. Antibiotic effects on fecal flora were immediately clear (day 21) with both TRF and culture data. Both TRF and culture data showed an immediate and significant antibiotic effect on the fecal bacterial community.
 - a. Several types of bacteria represented by TRF peaks changed in relative abundance with antibiotic treatment, including *Enterobacteriaceae*, *Bifidobacterium*, *Clostridium*, *Ruminococcus*, *Bacteroides* and *Porphyromonas*.
 - b. Culture data showed significant changes in counts for *Enterobacteriaceae* and *Bifidobacterium* and a trend for *Bacteroides*.
2. Both TRF and culture data showed a stabilizing effect of probiotic treatment after antibiotics.
 - i. Culture data showed a significant difference between treatment groups while TRF data showed a clear trend with significance at 90% confidence.
 1. TRF data describe a complex system in more detail than the culture methods used. TRF patterns cover >200 variables while the culture data were restricted to counts on five media. It is therefore not surprising that a larger amount of

noise was present in the TRF data, resulting in less discriminating power.

Finally, several key procedural issues should be mentioned because future studies would be much improved if these issues were addressed.

1. Individual variation in fecal flora was so large that analysis required the use of distance/similarity measures with both TRF and culture data.
 - a. Change from baseline also provided a good way to deal with individual variation.
 - b. The collection of multiple baseline samples was therefore critical to obtaining significant results.
2. Baseline stability was an important factor for observing changes in fecal flora.
 - a. Subjects with poor baseline stability served to increase the level of random noise in the data.
 - b. An increased number of baseline samples might have improved the power of the methods used to detect changes in fecal flora.
3. The addition of day 48 in the sampling design, after the cessation of probiotic treatment, was problematic.
 - a. The effect of probiotic treatment cessation cannot be ascertained without multiple samples.
 - b. The major antibiotic effect took place in the first two weeks after treatment. More sampling times nearer to the end of antibiotic treatment would have provided better data on the effect of probiotic treatment.

- c. A suggested sampling scheme would be 5 samples two days apart before antibiotics and five samples two days apart afterwards.
4. Sample handling procedures may have had an effect on the variation in the data.
- a. Different samples from the same subject were subjected to different lengths of freezer storage.
 - b. Several sample labeling issues affected analysis and some subjects with complete data sets had to be discarded.

References

- [1] Gill, H.S. and Guarner, F. (2004) Probiotics and human health: a clinical perspective. *Postgrad. Med. J.* 80, 516-526.
- [2] Beaugerie L., and Petit J.C. (2004) Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. *Best Pract. Res. Clin. Gastroenterol.* 18, 337-52.
- [3] Kitts C.L. (2001) Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Curr Issues Intest Microbiol.* 2, 17-25.
- [4] Summanen, P.E., Baron, J., Citron, D.M., Strong, C., Wexler, H.M., and Finegold, S.M. (1993) *Wadsworth Anaerobic Bacteriology Manual Fifth Ed.* Los Angeles: Star Publishing.
- [5] Muñoa, F.J. and Pares, R. (1988) Selective medium for isolation and enumeration of *Bifidobacterium* spp. *Appl. Environ. Microbiol.* 54, 1715-1718.
- [6] Lapiere, L., Undeland, P. and Cox, L.J. (1992) Lithium chloride-sodium propionate agar for the enumeration of *Bifidobacterium* in fermented dairy products. *J. Dairy Sci.* 75, 1192.
- [7] Rada, V., Sirotek, K. and Petr, J. (1999) Evaluation of selective media for *Bifidobacteria* in poultry and rabbit caecal samples. *J. Vet. Med. Ser.* 46, 369-373.
- [8] Rogosa, M., Mitchell, J.A. and Wiseman, R.F. (1951) A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J. Bacteriol.* 62, 132-133.
- [9] Sabine, D.B. and Vaselekos, J. (1965) Isolation of *Lactobacillus acidophilus* from faecal material. *Nature.* 206, 960-961.

- [10] Beals, E.W. (1984) Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. In: Advances in ecological research (A. Macfadyen), v14, pp. 1-49. Academic Press Inc., London.

OptiBac Probiotics
www.optibacprobiotics.co.uk

**CHAPTER 3: THE EFFECT OF PROBIOTICS ON THE SPECIES
DISTRIBUTION OF *BIFIDOBACTERIUM* AND *LACTOBACILLUS* IN
HEALTHY SUBJECTS UNDERGOING ANTIBIOTIC THERAPY**

Final report submitted by:

Anna Engelbrektson, Hailinh Nguyen, Dominic Lung, and Christopher Kitts
Cal Poly State University, Environmental Biotechnology Institute, San Luis
Obispo, CA

Joshua Korzenik

Massachusetts General Hospital, Harvard Medical School, Boston, MA

Todd Klaenhammer

North Carolina State University, Raleigh, NC

Greg Leyer

Danisco, Madison, WI

Mary Ellen Sanders

Dairy and Food Culture Technologies, Centennial, CO

Executive Summary

Antibiotic therapy is known to disrupt the intestinal microbial community, sometimes with unpleasant (diarrhea) or even dangerous (*Clostridium difficile* colitis) consequences. Oral consumption of live, beneficial bacteria (probiotics) has been suggested as a means to normalize intestinal communities after antibiotic use. Strains of *Bifidobacterium* and *Lactobacillus* are commonly used. Physiologic effects of probiotics are not clearly understood, but the significance can be inferred by observing changes in the fecal flora after probiotic consumption.

The goal of this study was to evaluate the effect of *Bifidobacterium* and *Lactobacillus* probiotics in volunteers administered antibiotics. Half the subjects were given a mixture of probiotic bacteria, while the other half were given a placebo. Fecal samples were collected 7 times during the 48-day study. The antibiotic Augmentin was administered from days 14 to 21 and the probiotic/placebo from days 14 to 34. DNA was extracted from the fecal samples and amplified with *Bifidobacterium* and *Lactobacillus* specific PCR primers. Species distribution for each genus was analyzed by Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of the PCR products. This analysis did not show significant, Augmentin-induced changes in the *Bifidobacterium* community, but probiotic feeding did moderate the effect of antibiotic treatment on the *Lactobacillus* community. Additionally, TRF could not specifically detect the administered probiotic strains of *Bifidobacterium* and *Lactobacillus* due to overlapping TRF signals from native strains.

Introduction

The gut represents the largest mucosal surface in the body and the microbial community present can have a large effect on immune function. This community consists of a large number of different species, although only a few account for most of the bacteria present. It is believed that a healthy intestinal flora can protect against certain types of infection and studies have shown that a healthy intestinal flora is a good barrier against potentially pathogenic microorganisms [1]. Antibiotics are known to disrupt this normal intestinal flora. They can cause disturbance in normal bowel function, disruption of mucosal integrity, and symptoms including diarrhea, bloating, flatulence, and intestinal pain [2].

It has been hypothesized that probiotics, live microbial feed supplements that can benefit a host by improving its intestinal microbial balance [3], can facilitate gut normalization after antibiotic treatment. They can produce a variety of health benefits including reduction in the intensity and duration of diarrheal illness, improvement in immune system function, alleviation of lactose intolerance, anticarcinogenic effects, and antihypertensive effects [4, 5]. Probiotics, primarily *Lactobacillus* and *Bifidobacterium* species, are found in many dairy foods and supplements, including over 80% of commercially available yogurt. However, when fed to healthy subjects, probiotics only modestly affect fecal flora and the strains used do not tend to be permanent colonizers.

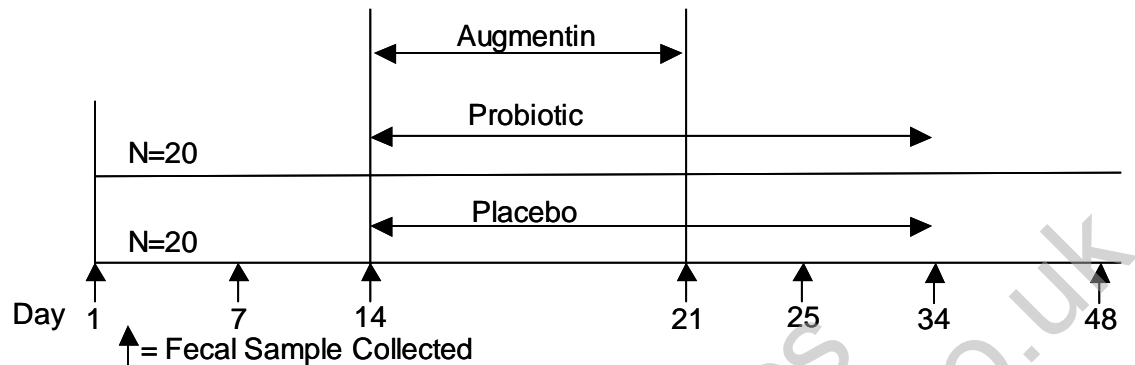
Lactobacilli and Bifidobacteria represent only a fraction of the normal healthy intestinal community. However, changes in their population structure could potentially have important health effects. TRFLP analysis is a rapid way of estimating population structure and because PCR primers specific to *Lactobacillus* and *Bifidobacterium* groups

are available, TRFLP was used to follow changes in *Lactobacillus* and *Bifidobacterium* populations

Materials and Methods

Study Design

Healthy individuals were recruited who agreed to be on antibiotics for one week for study purposes only. Each subject received a one-week course of the antibiotic Augmentin (GlaxoSmithKline, Brentford London), a mixture of amoxicillin and clavulanic acid, 875 mg orally twice a day. This antibiotic was selected because of a high rate of antibiotic-associated diarrhea. Patients were then randomized (1:1) to either the placebo or the probiotic test product consisting of a capsule containing a dried bacterial preparation of probiotic bacteria in the genera, *Lactobacillus* and *Bifidobacterium*. The following strains and amounts were fed: *Bifidobacterium bifidum* Bb-02 (5×10^8), *Bifidobacterium lactis* B1-04 (5×10^9), *Bifidobacterium lactis* Bi-07 (5×10^9), *Lactobacillus acidophilus* NCFM (5×10^9), and *Lactobacillus paracasei* Lpc-37 (5×10^9). The total dose of probiotic was 2×10^{10} bid (4×10^{10} daily). The other group received a placebo consisting of the same filler used in the bacterial preparation, maltodextran, without the bacteria. The study was conducted over 48 days. Three baseline (no treatment) fecal samples were obtained at days 1, 7, and 14, followed by the 7-day course of Augmentin. Fecal samples were then collected on days 21, 25, 34, and 48. Probiotic/Placebo treatment began on day 14 and continued until day 34 (Fig. 1).

Fig. 1. Schematic of the Antibiotic-Probiotic experimental design.

Forty subjects recruited with enrollment criteria permitting only patients over eighteen without significant acute or chronic illnesses. Permitted medications included those that were constant throughout the study and only if they had no established or suspected impact on gut flora. Individuals were excluded if they were pregnant, breastfeeding, had a penicillin allergy, a history of gastrointestinal illness or had been on any antibiotics in the preceding four weeks. Fermented foods or any probiotic preparations were prohibited for four weeks before entry into the study and throughout the duration of the protocol.

Isolation of bacterial DNA

Fecal samples were obtained for TRF analysis by adding approximately 1 gram of feces to a 2 mL screw-cap tube and freezing at -80°C until shipment. Samples were then extracted in triplicate using the MoBio Ultraclean® soil DNA kit following manufacture's protocol with the addition of 5 additional washes with S4. Success of each extraction was determined by measuring DNA concentration in the extraction product with a Spectramax spectrophotometer.

PCR Amplification

PCR was performed using primers homologous to highly conserved regions on the 16S rRNA gene. For *Bifidobacterium* sp., the reverse primer was Bifid-R (5'-ggTgTT CTT CCC gAT ATg CTA CA-3') [6], and the fluorescently labeled forward primer was Bif164-F (5'-ggg Tgg TAAT gCC ggATg-3') [7]. 50 μL reactions were carried out using 5 μL of 10x Buffer, 3 μL of 10 mM dNTP, 2 μL 20 $\mu\text{g mL}^{-1}$ BSA, 7 μL 25 mM MgCl_2 , 1 μL of each primer, and 0.3 μL 5 U μL^{-1} TaqGold®. Reaction temperatures and times were 92°C for 10 min; 30 cycles of 94°C for 20 sec, 57.4°C for 20 sec, and 72°C for 30 sec; and a final extension of 72°C for 10 min.

For *Lactobacillus* sp., the reverse primer was Lab677 (5'CACCgTACACATgg Ag-3') [8], and the fluorescently labeled forward primer was Ba2F (5' gCY TAA CAC ATg CAA gTC gA-3') – a universal Bacterial primer [9]. The 50 μL reactions were carried out using 5 μL of 10x Buffer, 3 μL of 10 mM dNTP, 2 μL 20 $\mu\text{g mL}^{-1}$ BSA, 7 μL 25 mM MgCl_2 , 1 μL of each primer, and 0.3 μL 5 U μL^{-1} TaqGold®. Reaction temperatures and times were 92°C for 10 min; 30 cycles of 94°C for 20 sec, 56°C for 20 sec, and 68°C for 40 sec; and a final extension of 72°C for 10 min.

All reactions were performed in triplicate and then combined using a MoBio Ultraclean® PCR Cleanup Kit following manufacture's protocol. Amounts of amplified PCR product DNA in each sample were determined using a fluorometer.

Enzyme Digest and TRF Pattern Generation

An enzyme digest was performed on each cleaned PCR product using New England Biolabs restriction endonucleases *HhaI* (*Bifidobacterium*) and *HaeIII* (*Lactobacillus*). Each 40 μL *HhaI* digestion used 75 ng of DNA, 1 U of enzyme, 0.4 μL of BSA, and 4 μL of buffer. The samples were digested for 4 hours at 37°C and inactivated

for 20 min at 65°C. Each 40 µL *Hae*III digestion used 75 ng of DNA, 1 U of enzyme, and 4 mL of buffer. The samples were digested for 4 h at 37°C and inactivated for 20 min at 80°C. 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 profiles were obtained using CEQ 8800 DNA Analysis system (Beckman Coulter, Fullerton, CA). TRF peaks were identified by matching to TRFs generated using sequences from the Ribosomal Database Project and GenBank.

Data Preparation and Statistical Analysis

Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQ 8800 into Excel (Microsoft, Seattle, WA). To standardize the data for comparison between samples, the area under each TRF peak was normalized to total amount of DNA analyzed and expressed as parts per million (ppm). Peaks with an area of less than 10,000 ppm (<1% of the total for that sample) were excluded from analysis to reduce noise.

Normalized TRF data sets were transformed by taking the square root of the area under each TRF peak to de-emphasize large TRF peaks while still taking relative abundance into account [10]. Transformed data were analyzed using Bray-Curtis similarity [11] using Minitab (Minitab Inc., State College PA) and Excel.

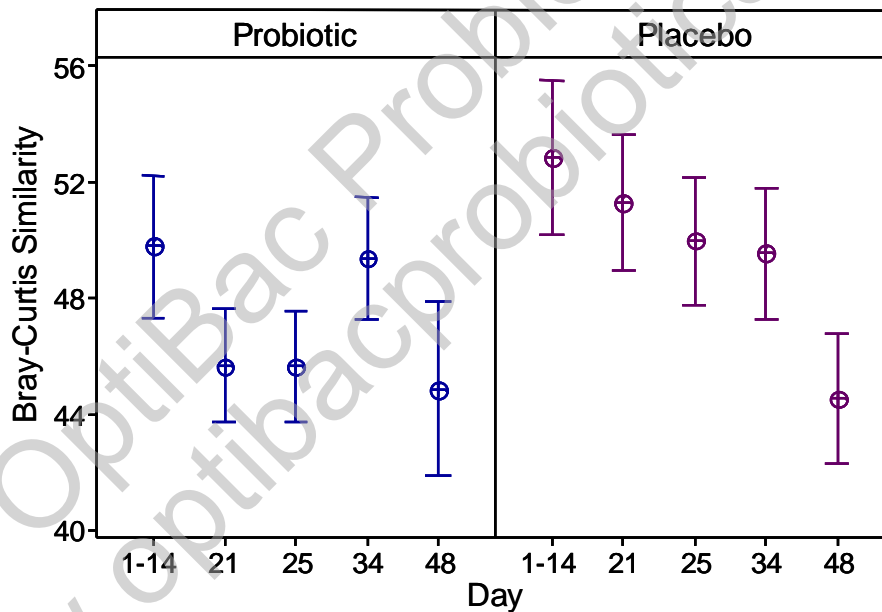
Results

Bifidobacterium

256 samples, from 36 subjects (18 in each treatment group) fit the criteria necessary to provide meaningful data on the effects of probiotics on fecal bifidobacterial communities. Antibiotic effect was determined by calculating Bray-Curtis similarities to

baseline (days 1-14) for day 21 individually for each subject (Fig. 2). Within baseline similarity compared to similarity of day 21 to baseline resulted in no significant difference in either treatment group (T-test, probiotic, $p = 0.189$; placebo, $p = 0.663$). There appeared to be a trend of return to baseline by day 34 in the probiotic group, but not in the placebo treatment group (Fig. 2). However, this trend could not be statistically significant because there was not a significant antibiotic effect.

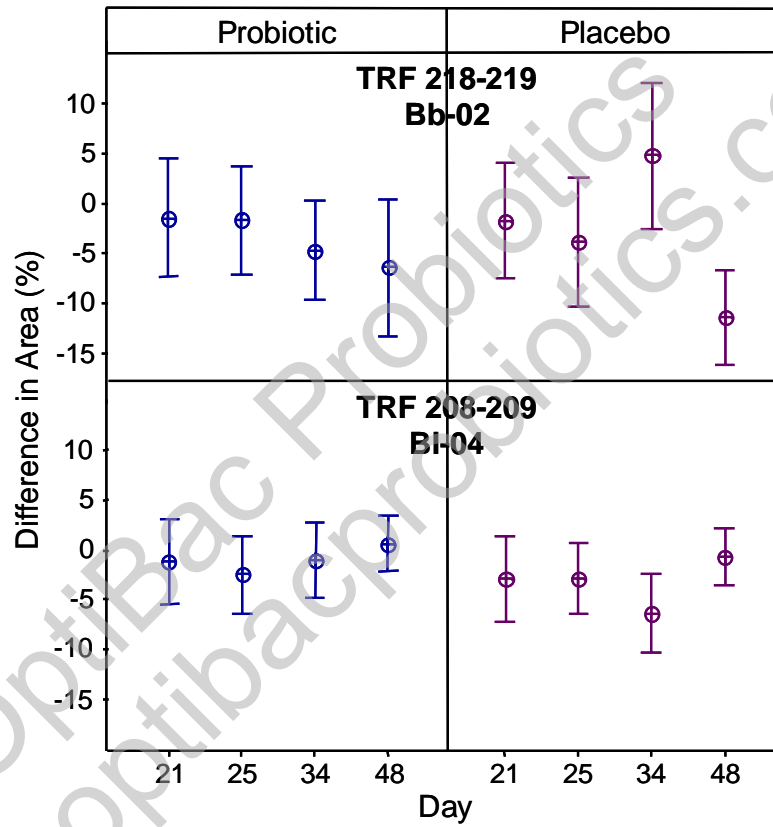
Fig. 2. Average Bray-Curtis similarity from baseline (days 1-14) for each day after treatment. Error bars represent one standard error.



The three different strains of probiotic bifidobacteria fed to the subjects (probiotic treatment group) were tracked in the TRF patterns. There was no apparent trend for either *Bifidobacterium bifidum* Bb-02 (TRFs 218-219) or *Bifidobacterium lactis* B1-04 (TRFs 208-209) (Fig. 3). In fact, there appeared to be no change from baseline values.

Bifidobacterium lactis Bi-07 (TRF 106) was not found in any subject, before or after probiotic treatment.

Fig. 3. Difference in TRF peak area from baseline (days 1-14) for each day after treatment.

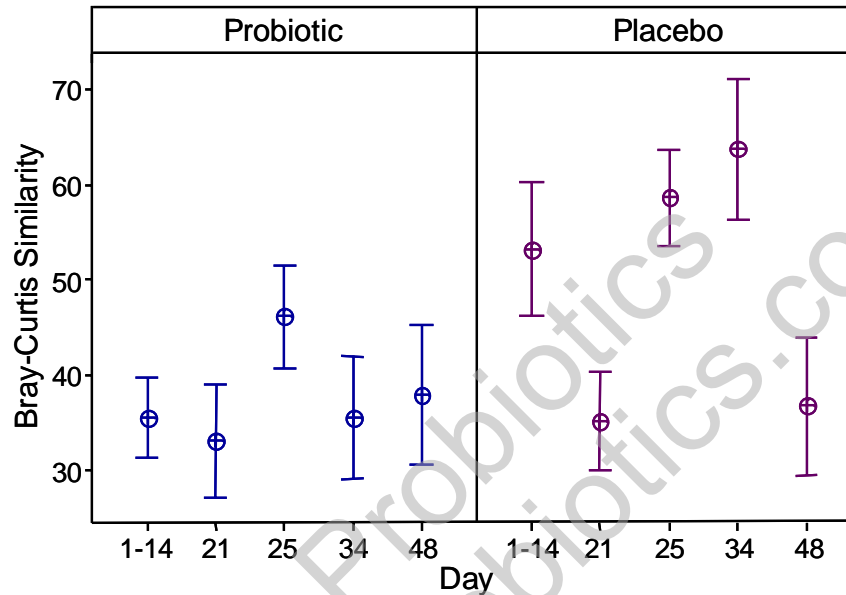


Lactobacillus

There were very low numbers of *Lactobacillus* present in most of the fecal samples. Lactobacilli were only detected by PCR in about 40% of the samples. The other 60% of the fecal samples either had *Lactobacillus* numbers that were below the detection limit for the PCR primers or had PCR inhibitors present that inhibited amplification of *Lactobacillus* DNA.

Antibiotic effect was determined by calculating Bray-Curtis similarities to baseline (days 1-14) for day 21 individually for each subject with data for at least two baseline samples and at least one after treatment sample (Fig. 4). Therefore, 7 subjects from the probiotic treatment group and 6 subjects from the placebo group were included in the analysis. T-tests of within baseline (days 1-14) similarity to similarity of day 21 to baseline resulted in a significant effect in the placebo group ($p = 0.047$) and no effect in the probiotic group ($p = 0.751$). Therefore, probiotics did not appear to significantly affect *Lactobacillus* community structure (Fig. 4) but may have stabilized the *Lactobacillus* community against an antibiotic challenge.

Fig. 4. Average Bray-Curtis similarity from baseline (days 1-14) for each day after treatment. Error bars represent one standard error.



Discussion

Overall results from the *Lactobacillus* and *Bifidobacterium* specific primers were mixed. Probiotic strains could not be accurately tracked, most likely because of too many overlapping TRF's from native flora. For *Bifidobacterium*, an antibiotic effect on community structure was observed, but was not statistically significant. A probiotic effect was also observed, but was not significant. Therefore, the probiotic strains did not appear to significantly alter community structure but, may have stabilized the *Bifidobacterium* community against an antibiotic challenge. For lactobacilli, cell numbers were often below the PCR detection limit, but in the PCR positive samples, it appeared as if community structure was significantly altered by antibiotic therapy in the placebo group,

but not the probiotic group. Once again, probiotic treatment may have stabilized the *Lactobacillus* community against an antibiotic challenge.

OptiBac Probiotics
www.optibacprobiotics.co.uk

References

- [1] Fuller, R. (1991) Probiotics in human medicine. *Gut*. 32, 439-442. Bergogne-Berezin, E. (2000) Treatment and prevention of antibiotic associated diarrhea. *Int. J. Antimicrob.* 16, 521-6.
- [3] Fuller, R. (1989) Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365-378.
- [4] Gill, H.S. and Guarner, F (2004) Probiotics and human health: a clinical perspective. *Postgrad. Med. J.* 80, 516-526.
- [5] Sanders, M.E. (1999) Probiotics. *Food Technol.* 53, 67-77. Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., and Tanaka, R. (2002) Development of 16s rRNA-Gene-Targeted Group-Specific Primers for the Detection and Identification of Predominant Bacteria in Human Feces. *Appl. Environ. Microbiol.* 68, 5445-5451.
- [7] Satokari, R., Vaughan, E., Akkermans, A., Saarela, M., and de Vos, W. (2001) Bifidobacterial Diversity in Human Feces Detected by Genus-Specific PCR and Denaturing Gradient Gel Electrophoresis. *Appl. Environ. Microbiol.* 67, 504-513.
- [8] Heilig, H., Zoetendal, E., Vaughan, E., Marteau, P., Akkermans, A., and de Vos, W. (2002) Molecular Diversity of *Lactobacillus* spp. and Other Lactic Acid Bacteria in the Human Intestine as Determined by Specific Amplification of 16S Ribosomal DNA. *Appl. Environ. Microbiol.* 68, 114-123.
- [9] Kaplan, C.W., Astaire, J.C., Sanders, M.E., Reddy, B.S. and Kitts, C.L. (2001) 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 67, 1935-1939.

- [10] Blackwood, C.B., Marsh, T., Kim, S., and Paul, E.A. (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Appl. Environ. Microbiol.* 69, 926-932.
- [11] Rees, G.N., Baldwin, D.S., Watson, G.O., Perryman, S., and Nielson, D.L. (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Ant. Van Leeuw.* 86, 339-347.

OptiBac Probiotics
www.optibacprobiotics.co.uk