

# Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy

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A novel combination of culturing and DNA-based terminal restriction fragment length polymorphism (TRFLP) analysis was used to investigate the effect of probiotics on antibiotic-induced gut microbiota alterations to determine if a probiotic preparation containing bifidobacteria and lactobacilli, taken during and after antibiotic therapy, can minimize antibiotic disturbance of faecal microbiota. Healthy subjects administered amoxicillin/clavulanate were randomized and concomitantly received a placebo or probiotic mixture. The primary end point was similarity of faecal microbiota as determined by culturing and TRFLP from subjects taking probiotics compared to those taking a placebo measured by comparing data from baseline to post-treatment for each subject. TRFLP analysis revealed a high subject to subject variation in the baseline faecal microbiota. The most common antibiotic-induced disturbance was a relative increase in *Clostridium*, *Eubacterium*, *Bacteroides* and *Enterobacteraceae*. The mean similarity to the baseline increased over time in both treatment groups, although the probiotic group was less disturbed according to both TRFLP and culture data. The culture method revealed that post-antibiotic faecal microbiota in probiotic-consuming subjects were more similar to the baseline microbiota than the control group ( $P=0.046$ ). Changes in *Enterobacteraceae* ( $P=0.006$ ) and *Bifidobacterium* ( $P=0.030$ ) counts were significantly different between the groups. Analysis of TRFLP data reinforced the trend between groups but was not statistically significant ( $P=0.066$ ). This study indicates this mixture of probiotics promotes a more rapid return to pre-antibiotic baseline faecal bacterial microbiota.

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## INTRODUCTION

Antibiotic use is often associated with adverse gastrointestinal effects. Attempts to minimize these side effects have included the use of probiotics with the aim of stabilizing intestinal bacterial communities and minimizing possible alterations in microbial community structure.

The microbial environment of the intestine is highly diverse and complex, with an estimated population of over  $10^{14}$  bacteria, of which perhaps 60–80% are not culturable

by conventional microbiology methods (Eckburg *et al.*, 2005). Recent advances in techniques using 16S rRNA sequences, which permit the identification of unique bacterial subspecies, have begun to yield new insights into the diversity, organization and dynamics of gut microbiota in health and disease (Backhed *et al.*, 2005). A recently established method, terminal restriction fragment length polymorphism (TRFLP) analysis, is a practical way to characterize microbiota because species richness and relative abundance are measured rapidly and reproducibly (Blackwood *et al.*, 2003; Kitts, 2001). Because the data from TRFLP are automatically digitized the technique is well

Abbreviations: MANOVA, multivariate ANOVA; TRF, terminal restriction fragment; TRFLP, terminal restriction fragment length polymorphism.

suited to high throughput analysis of a large numbers of samples. Different phylotypes of bacteria present in each sample can also be identified using an established database (Kitts, 2001).

Probiotics have been studied as therapeutic agents for limiting antibiotic-associated symptoms (Katz, 2006). Presumably, probiotics exert this effect by minimizing the disruption to the baseline gut bacterial community. The availability of DNA-based techniques such as TRFLP allows an encompassing perspective when investigating the stabilizing effect of probiotics.

This study tested if a probiotic supplement could minimize antibiotic-induced disruption of faecal microbiota and facilitate its return to the baseline in healthy volunteers. Augmentin (GlaxoSmithKline) was selected because of its high frequency of use and high rate of antibiotic-associated diarrhoea. Alterations in gut microbiota were evaluated using conventional microbiology techniques and TRFLP analysis, permitting a comparison and cross-validation of these techniques.

## METHODS

**Study design.** The study design was described previously in a report that analysed statistical methods for data analysis (Engelbrekton *et al.*, 2006). Because the molecular methods used in this study are relatively new, a separate treatment investigating analysis was necessary. This report presents the clinical aspects of the study. Briefly, healthy subjects were requested to maintain their diet without significant alterations during the study period. The study was conducted over 48 days. Three baseline (prior to therapy) faecal samples were obtained at days 1, 7 and 14, followed by treatment of the subjects with a 7 day course of Augmentin (amoxicillin and clavulanic acid) at 875 mg orally twice daily. Faecal samples were then collected on days 21 (at end of the antibiotic treatment), 25, 34 and 48. Probiotic/placebo treatment began on day 14 and continued until day 34. Subjects completed a diary recording stool frequency, gastrointestinal symptoms and overall well-being throughout the protocol. The primary end points were similarity of gut microbiota of subjects on probiotics versus those on a placebo measured by comparing data from the baseline days (1, 7 and 14) to the post-treatment days (21, 25, 34 and 48) for each subject.

Subjects were randomized (1:1) to either the placebo group or the probiotic test product group. The test product consisted of a capsule containing a dried bacterial preparation of *Bifidobacterium lactis* Bl-04 ( $5 \times 10^9$  c.f.u.), *B. lactis* Bi-07 ( $5 \times 10^9$  c.f.u.), *Lactobacillus acidophilus* NCFM ( $5 \times 10^9$  c.f.u.), *Lactobacillus paracasei* Lpc-37 ( $5 \times 10^9$  c.f.u.) and *Bifidobacterium bifidum* Bb-02 ( $5 \times 10^8$  c.f.u.), used at a total dose of  $2.05 \times 10^{10}$  c.f.u. twice daily ( $4.1 \times 10^{10}$  c.f.u. daily). The placebo consisted solely of maltodextran, the same filler material used in the probiotic preparation. The Washington University School of Medicine institutional review board approved the protocol and written consent was obtained from each individual. Subjects were provided financial remuneration for their participation.

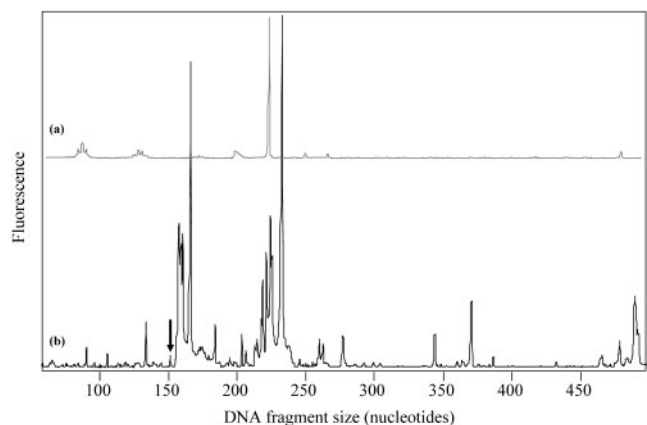
**Microbial analysis.** Faecal samples were delivered to and processed by the study coordinator within 8 h of collection. For samples destined for TRFLP analysis, approximately 3 g faeces was placed in a 2 ml screw-cap tube and frozen at  $-80^\circ\text{C}$  until shipment. Samples were prepared for culture analyses as follows. Faeces (5 g) was placed into 15 ml Cary-Blair transport medium with indicator (Remel). The

samples were then shaken/vortexed briefly to disperse them and frozen at  $-80^\circ\text{C}$  until shipment. Samples were shipped on dry ice for both culture and TRFLP analysis.

**Probiotic viability analysis.** Probiotic capsules (hydroxypropyl-methylcellulose) were stored with a desiccant pack in bottles at  $4^\circ\text{C}$ . Total cell viability was monitored over a 12 month span, which covered the effective consumption timelines of all participants in the study. At monthly intervals, a new bottle was opened and the contents of the capsules emptied out to achieve 11 g in total. The material was rehydrated using De Man, Rogosa and Sharpe (MRS) broth (Difco) mixed for 30 s using a Stomacher blender (Seward Instruments), allowed to stand for 30 min at room temperature for complete rehydration, serially diluted in 0.1% peptone dilution blanks, and pour plated into MRS agar containing 0.05% cysteine.HCl. Plates were incubated at  $37^\circ\text{C}$  under anaerobic conditions (an  $\text{H}_2/\text{CO}_2$  gas pack in an anaerobic jar) for 72 h and the total cell count reported.

**Creation and normalization of TRFLP data.** Terminal restriction fragment (TRF) patterns were obtained by extraction of sample DNA, PCR with a labelled primer to amplify 16S rRNA genes, digestion with a restriction endonuclease to generate phylotype specific fragments, and fragment separation by capillary gel electrophoresis as described previously (Engelbrekton *et al.*, 2006). TRF data were gleaned from the resulting electropherogram (Fig. 1) and consisted of TRF peak sizes (the length in nucleotides of the DNA fragments detected) and the area under each TRF peak (a measure of abundance for each DNA fragment). TRFLP data were produced using three different primer sets. The first primer set was used to amplify DNA from as many bacterial species as possible as described previously (Engelbrekton *et al.*, 2006) and the other two primer sets were genus-specific for *Bifidobacterium* or *Lactobacillus*.

For *Bifidobacterium*, the reverse primer Bifid-R (5'-GGT GTT CTT CCC GAT ATG CTA CA-3') (Matsuki *et al.*, 2002) and the fluorescently labelled forward primer Bif164-F (5'-GGG TGG TAA TGC CGG ATG-3') (Satokari *et al.*, 2001) were used. For *Lactobacillus*, the reverse primer Lab677 (5'-CAC CGT ACA CAT GGA G-3') (Heilig *et al.*, 2002) and the fluorescently labelled forward primer Ba2F (5'-GCY TAA CAC ATG CAA GTC GA-3')



**Fig. 1.** Electropherograms from two TRF patterns showing TRF peak size in nucleotides and the area under each peak. (a) Trace pattern produced by *B. bifidum* Bb-02 showing a single peak at 223 nt. (b) Trace pattern produced from a single faecal sample; the arrow in (b) indicates a TRF peak with an area less than 0.5% of the total area as an example of random noise from a single sample.

(Engelbrekton *et al.*, 2006) were used. For both genus-specific primers 50 µl reactions were carried out in triplicate and then combined using an UltraClean PCR cleanup kit (Mo Bio Laboratories) following the manufacturer's protocol. The reaction components for *Bifidobacterium* sp. were as follows: 5 µl 10× buffer, 3 µl 10 mM dNTP, 2 µl 20 µg BSA ml<sup>-1</sup>, 7 µl 25 mM MgCl<sub>2</sub>, 1 µl each primer and 0.3 µl 5 U µl<sup>-1</sup> *Taq*Gold (Applied Biosystems). Reaction temperatures and times were: 92 °C for 10 min; 30 cycles of 94 °C for 20 s, 57.4 °C for 20 s and 72 °C for 30 s; and a final extension of 72 °C for 10 min. The reaction components for *Lactobacillus* sp. were as follows: 5 µl 10× buffer, 5 µl 10 mM dNTP, 2 µl 20 µg BSA ml<sup>-1</sup>, 5 µl 25 mM MgCl<sub>2</sub>, 1 µl each primer and 0.25 µl 5 U µl<sup>-1</sup> *Taq*Gold. Reaction temperatures and times were: 94 °C for 10 min; 30 cycles of 94 °C for 30 s, 56 °C for 20 s and 68 °C for 40 s; and a final extension of 68 °C for 7 min. An enzyme digestion was performed on each genus-specific PCR cleanup product using *Hha*I (*Bifidobacterium*) and *Hae*II (*Lactobacillus*) (New England Biolabs). Each 40 µl digestion used 75 ng DNA, 1 U enzyme and 4 µl 10× buffer (0.4 µl 20 µg BSA ml<sup>-1</sup> were added to *Hha*I digestions). Samples were digested for 4 h at 37 °C and inactivated for 20 min at 65 °C (80 °C for *Hae*III). Digestion products were ethanol precipitated and resuspended in 20 µl formamide and 0.25 µl CEQ 600 base pair standard. TRF profiles were obtained using a CEQ 8800 genetic analysis system (Beckman Coulter).

**PCR analysis for *L. acidophilus* NCFM.** Faecal samples from eight subjects were plated on lactobacillus selective (LBS) agar (Difco) containing 20% tomato juice. Forty presumptive *Lactobacillus* colonies were streaked for isolation on LBS+ tomato juice agar. Purified colonies were picked and the DNA was extracted using an UltraClean microbial DNA kit according to the manufacturer's instructions. DNA from the colonies was used in PCRs with a multiplex set of primers optimized for detection of *L. acidophilus* NCFM (Table 1) (Souther, 2004). Each 25 µl reaction contained 100 ng DNA, 2.5 µl 10× buffer (100 mM Tris/HCl, 15 mM MgCl<sub>2</sub>, 500 mM KCl, pH 8.3), 4 µl dNTP mixture (1 mM each), 1.25 µl IS1272 (20 µM), 1.25 µl p\_77 (20 µM), 2.5 µl IS150 (20 µM), 1.25 µl p\_1954 (20 µM), 1.25 µl IS605 (2 µM), 1.25 µl p\_614 (2 µM), 1.25 µl p\_1569 (2 µM), 1 µl *Taq* polymerase (1U µl<sup>-1</sup>). Reaction temperatures and times were: 94 °C for 10 min, after which *Taq* polymerase was added; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 4 min; and a final extension of 72 °C for 7 min. Positive controls using colonies recovered from a pure NCFM culture yielded four bands of 960, 1700, 2200 and 3100 bp (data not shown). In some experiments, faecal samples in Cary–Blair medium were inoculated (1 ml) directly into LBS broth (10 ml) and incubated for 48 h to enrich for lactobacilli. Genomic DNA was extracted from 1 ml culture using the Mo Bio kit and then analysed by multiplex PCR.

**Bacterial culturing.** *Bifidobacterium*, *Lactobacillus*, *Bacteroides fragilis* group, *Clostridium* species and *Enterobacteriaceae* were enumerated as previously described (Engelbrekton *et al.*, 2006). No attempt was made to confirm the identity of bacteria growing on the specified media.

**Statistical analyses.** Two pairwise methods were used to estimate pattern similarity and to evaluate changes in subjects' faecal microbiota after antibiotic treatment, Euclidean distance (culture data) and Bray–Curtis similarity (TRFLP data) (Engelbrekton *et al.*, 2006). Stability was assessed by averaging the Euclidean distances (culture data) or Bray–Curtis similarities (TRFLP data) of days 1 to 7, 1 to 14, and 7 to 14 to create a within-baseline measure for each individual. Statistical analyses were performed using SAS (SAS Institute), Minitab (Minitab) and Excel (Microsoft) software.

## RESULTS

A total of 51 subjects were enrolled in the study, with 40 subjects completing the protocol. Of those completing the study, 9 subjects were male and 31 female (4 men and 16 women in the placebo group, 5 men and 15 women in the probiotic group). The mean age of all subjects completing the study was 37.2 years (39.5 years mean age in the placebo group and 36.5 years mean age in the probiotic treatment group). Stool samples were complete for 33 subjects and were missing 1 sample for 7 subjects. No subject discontinued due to adverse events during the study; all subjects who discontinued did so because of logistical reasons (transportation difficulties) or because they chose not to continue participation.

Adherence to the protocol (pill count) was good for both groups. Seven individuals took less than 100% of the antibiotics: four in the probiotic group (93, 71, 79 and 93%) and three in the placebo (79, 93 and 64%). In the placebo group, four individuals took less than 85% of the placebo (23, 68, 75 and 81%). In the probiotic treatment group, only one individual took less than 85% of the test product (58%). Both the placebo and probiotic treatment were well tolerated. Four subjects developed diarrhoea (three subjects had diarrhoea for 4 days, and one for 7 days with the final 3 days described in the diary as soft stools and not as diarrhoea) during the study (three subjects in

**Table 1.** Primer sets used in multiplex PCR

Conserved primer	Conserved primer sequence	Variable primer	Variable primer sequence	Expected product size
IS1272	5'-GAT AAG CAG GGC GGC CTG TTT TAG CAG TTT-3'	p_77	5'-GAT TCT GAT TTT GCG TTA ATC TAA CAG TGA-3'	~3100 bp
IS150	5'-AAT TAT ACA AGA TAC CGT TGC TTC TTA GTC C-3'	p_614	5'-CTG AAA ATG ACT TAT CTC GTG CCA TTA TTT C-3'	~960 bp
IS150	5'-AAT TAT ACA AGA TAC CGT TGC TTC TTA GTC C-3'	p_1954	5'-AAG CTG AAC CAT TAT TCT GTT CTT CAA CAT A-3'	~2200 bp
IS605	5'-GCC TTT ATT CCA GCA GTA TCG CCG ATA GTC-3'	p_1569	5'-ACT GAC GAG ATG GCT TAT GAA GCC GGT AGT-3'	~1700 bp

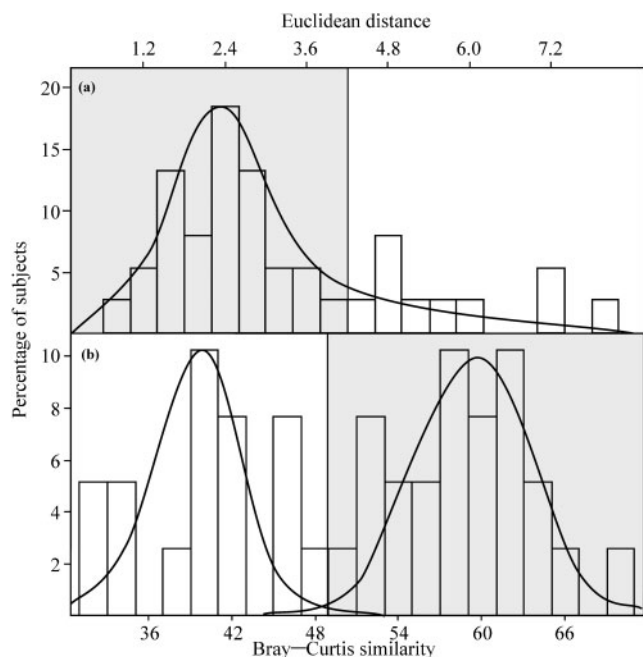
the probiotic treatment group and one subject in the placebo group), and no subject developed severe or persistent diarrhoea. The difference in rates of diarrhoea between the two groups was not statistically significant ( $P>0.60$ ). Other adverse events included vaginal yeast infections (four subjects in the probiotic treatment group and one subject in the placebo group) and abdominal cramping (three subjects in the probiotic treatment group and three subjects in the placebo group). Analysing the data for all adverse events, there was no difference between the two groups ( $P=0.32$ ).

### Probiotic product analysis

The targeted daily dose was  $4 \times 10^{10}$  c.f.u. per day of the probiotic mixture. Shelf-life evaluation indicated that the cell count of the probiotic mixture started at  $4.3 \times 10^{10}$  (108 % of the daily dose) and remained not less than  $3.6 \times 10^{10}$  (90 % of the targeted dose) throughout a 12 month product evaluation trial.

### Stability of faecal microbiota

A high degree of variation in the baseline faecal microbiota was detected in some individuals. This was observed for both culture (Fig. 2a) and TRFLP (Fig. 2b) data. Stability



**Fig. 2.** Analysis of the baseline faecal bacterial community stability for all subjects. Shaded areas indicate subjects that were considered stable, all other subjects were considered unstable. (a) Mean Euclidean distance of culture data (days 1, 7 and 14). The curve emphasizes the positively skewed distribution. (b) Mean Bray-Curtis similarity of TRFLP data from the three initial sampling times. The curves emphasize the bimodal distribution.

analysis of the culture data (Fig. 2a) resulted in a positively skewed distribution. Subjects within a normal distribution (29 subjects) were classified as stable and those to the right (10 subjects) as unstable. TRFLP data, however, resulted in a bimodal distribution (Fig. 2b). Those individuals with a Bray-Curtis similarity of 50 % or higher were categorized as having a stable bacterial community (22 subjects) and those with a similarity of less than 50 % were categorized as having an unstable bacterial community (17 subjects). Five subjects were classified as unstable by both TRFLP and culture data while 14 subjects were classified as stable by both methods.

An antibiotic effect was observed less often in subjects with an unstable baseline bacterial community after antibiotic treatment on day 21 (MANOVA  $P=0.047$ ). However, stability was randomly distributed across treatment groups (probiotic and placebo) for the TRFLP data (ANOVA  $P=0.615$ ) and the culture data (ANOVA  $P=0.171$ ). **Therefore, subjects with unstable baseline microbiota did not directly influence assessment of probiotic treatment effects, although the overall statistical power of this analysis was diminished.**

TRF peaks associated with either a stable or an unstable baseline bacterial community were further investigated by stepwise discriminant analysis of TRF peak presence using the stability designations from Fig. 2(b). This provided a basis for defining significant differences between stable and unstable microbiota (Table 2). The genus *Bifidobacterium* correlated with a stable microbiota. *Clostridium*, *Bacteroides* and *Lactobacillus* were implicated in both stable and unstable baseline microbiota. This indicates the possibility of species-level response differences in these genera, although genus-level identification is preliminary. Digestion with a second enzyme would provide greater confidence in phylotype identification (Kitts, 2001).

### Effect of antibiotics

The mean Bray-Curtis similarity for individual subjects within baseline TRFLP data was 51 %. Significant decreases in similarity to the baseline at days 21 (42 % ANOVA  $P<0.001$ ) and 25 (47 %  $P=0.003$ ) clearly established that antibiotics had a significant effect on faecal microbiota. By day 34 there was no longer a significant effect (49 %  $P=0.184$ ). However, at day 48 similarity to the baseline decreased to 46 % ( $P=0.001$ ). The significance of this 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.

Antibiotic effects were assessed by colony enumeration on five different culture media using both absolute numerical changes and Euclidean distance. No statistical difference between the mean bacterial counts before antibiotic treatment compared to the days after antibiotic treatment ( $P=0.340$ ) was observed due to large subject-to-subject

**Table 2.** TRF peaks that discriminate significantly (stepwise discriminant analysis) between the stable ( $n=29$ ) and unstable ( $n=10$ ) categories of subjects

The subject categories for this analysis were defined based solely on TRFLP data from pretreatment (baseline) faecal samples. Representative genera were obtained by comparison to a database of TRF lengths.

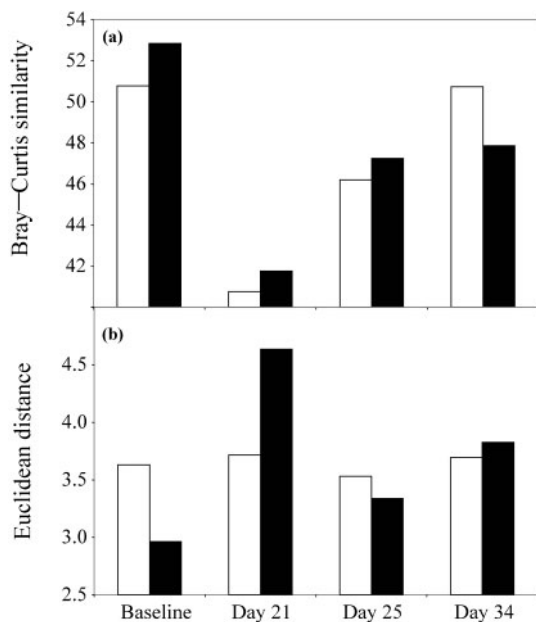
TRF peak (nt)	Category	<i>P</i> value	Genera represented by TRF peak ( $\pm 1$ bp)
219	Stable	0.011	<i>Bifidobacterium</i> , <i>Clostridium</i> , <i>Ruminococcus</i>
223	Stable	0.000	<i>Bacteroides</i> , <i>Bifidobacterium</i>
278	Stable	0.001	<i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Clostridium</i>
87	Unstable	0.003	<i>Procabacter</i> , <i>Planctomyces</i> , <i>Microbacterium</i>
199	Unstable	0.023	<i>Clostridium</i>
285	Unstable	0.004	<i>Lactobacillus</i> , <i>Clostridium</i>
487	Unstable	0.007	<i>Bacteroides</i> , <i>Cytophaga</i>

variations in colony counts. However, analysis of pairwise Euclidian distance with culture data resulted in a significant difference from the baseline at day 21 ( $P=0.003$ ). A difference from the baseline was not observed at day 25 ( $P=0.564$ ) or day 34 ( $P=0.171$ ).

### Effect of probiotics

To assess probiotic effects with TRFLP, Bray–Curtis similarity data were analysed separately for probiotic and

placebo groups, excluding day 48 when probiotics were not administered (Fig. 3a). While both groups exhibited a similar trend toward increased similarity to the baseline over days 21 through 34, the probiotic group exhibited a larger increase in similarity to the baseline by the time probiotic treatment ended at day 34 (MANOVA  $P=0.066$ ). To assess probiotic effect using the culture data, these data were also analysed separately for probiotic and placebo groups using Euclidian distance from the baseline (Fig. 3b), and showed a significant difference between treatment groups ( $P=0.046$ ) with almost no change in distance to the baseline for the probiotic group, while the placebo group showed a marked shift away from the baseline.



**Fig. 3.** Changes in bacterial community structure before and after antibiotic treatment as assessed by TRFLP and culture data. (a) Mean Bray–Curtis similarity within baseline samples (days 1–14) and to the baseline for each day after treatment using TRFLP data. (b) Mean Euclidean distance between baseline samples and to the baseline for each day after treatment using culture data. Adapted from Engelbrekton *et al.* (2006). Black bars, placebo control group; white bars, probiotic treatment group.

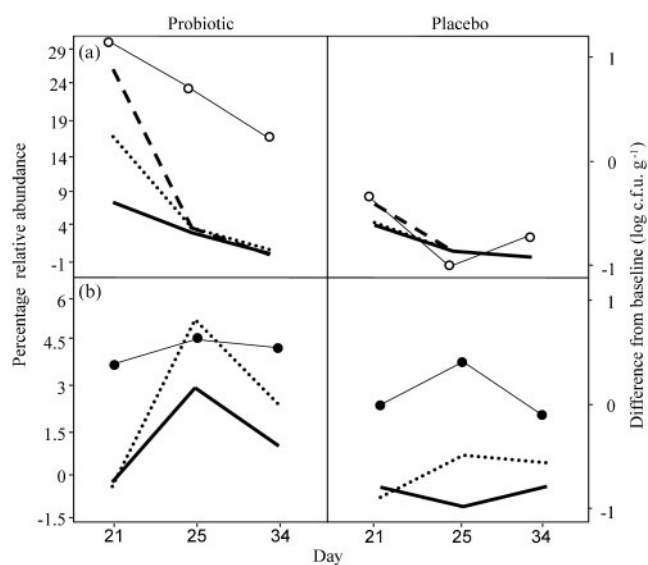
### Probiotic effect on specific bacteria

To account for individual variation and still determine which of the media showed the largest change in faecal microbiota, mean 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 post-antibiotic treatment days. Counts on MacConkey agar (*Enterobacteriaceae*) were significantly higher in the probiotic group (ANOVA  $P=0.006$ ). Similarly, the counts on bifidobacterium iodoacetate medium agar (*Bifidobacterium*) were significantly higher in the probiotic group ( $P=0.030$ ), with the placebo group showing a decrease in mean counts after antibiotics. Differences between groups were not significant for the other media.

To determine the genera represented by the TRF peaks with greater certainty, two additional enzymes (*HpaII* and *AluI*) were used to create TRFLP data from samples from ten subjects (five placebo, five probiotic, all stable). *HaeIII* (original enzyme), *HpaII* and *AluI* 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.

Many enteric organisms (*Enterobacteriaceae*) are found in the database at *HaeIII* 166–168, *HpaII* 457–458 and *AluI* 36 or 121–123. The database, in concert with trends in relative abundance, indicated that enteric organisms, represented by *HaeIII* 167–169, *HpaII* 457 and *AluI* 123, were affected by antibiotic treatment (Fig. 4a) and showed a large increase in relative abundance on day 21, decreasing by day 25. This trend is confirmed by the plate count data (Fig. 4a) and was pronounced in the probiotics treatment group as opposed to the placebo group. Mean abundance for the *AluI* 123 peak was lower than for the *HaeIII* and *HpaII* peaks, likely because enteric organisms can also be represented by a peak at *AluI* 36 bp, which is below the detection limit for TRF length.

Many *Bacteroides* and *Prevotella* species are found in the database at *HaeIII* 221–226, *HpaII* 56–60 and *AluI* <50. The database, in concert with trends in relative abundance, indicated that *Bacteroides* and *Prevotella*, represented by *HaeIII* 223 and *HpaII* 57, were affected by antibiotic treatment, initially decreasing, but increasing significantly



**Fig. 4.** Comparison of multi-enzyme TRF signatures to bacterial counts in 10 subjects after antibiotic therapy. (a) Comparison of the multi-enzyme TRF peaks corresponding to *Enterobacteriaceae* (*HaeIII* 167–169, *HpaII* 457 and *AluI* 123) and bacterial colony counts for *Enterobacteriaceae* (○) on MacConkey agar for both treatment groups. (b) Comparison of the multi-enzyme TRF peaks corresponding to *Bacteroides/Prevotella* (*HaeIII* 223 and *HpaII* 57) and bacterial colony counts for organisms in the *Bacteroides fragilis* group (●) on bacteroides bile aesculin agar for both treatment groups. The solid line represents *HaeIII*, the dotted line represents *HpaII*, and the dashed line represents *AluI*. The left-hand y-axis presents mean relative abundance for a TRF peak subtracted from the baseline (days 1–14) across the 10 selected subjects when the peak was present. The right-hand y-axis represents the log of bacterial counts subtracted from the baseline (days 1–14).

on day 25 (Fig. 4b). This trend is confirmed by the plate count data, although the difference between placebo and probiotics groups is not significant. *AluI* peaks for these organisms are predicted to be <50 nt, which is below the detection limit for TRF length.

### Tracking probiotic strains

TRF peaks corresponding to the five strains of probiotic bacteria were confirmed (e.g. Fig. 1) and used to track the appearance of these bacteria in subjects' faecal samples. *B. bifidum* Bb-02 was characterized by a TRF peak of 249 nt; *B. lactis* Bl-04 by 223 nt; *B. lactis* Bi-07 by 209 nt; *L. acidophilus* NCFM by 208 nt; *L. paracasei* Lpc-37 by 290 nt. 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 faecal population (<10<sup>9</sup> cells g<sup>-1</sup>). The TRF peaks representing all the other probiotic strains mentioned above showed a great deal of variation in all subjects, even in the baseline data, indicating that native bacteria may have produced the same TRF peaks.

A set of nested PCR primers was used to specifically identify NCFM in faecal samples from eight different individuals. All 40 presumptive *Lactobacillus* colonies isolated from these samples failed to show the characteristic multiplex banding pattern typical of NCFM (Vanhoutte *et al.*, 2004). In addition, DNA extracted directly from these samples failed to produce the characteristic NCFM banding pattern. Therefore, NCFM was not present in these samples at high enough concentrations to be detected with this method.

### Genus-specific PCR primers

Data from 36 subjects (18 in each treatment group) were used to assess the effects of antibiotics and probiotics on faecal *Bifidobacterium* communities. Within-baseline similarity compared to the similarity of day 21 to the baseline showed no significant antibiotic effect on the distribution of bifidobacterial types in either treatment group (probiotic  $P=0.189$ , placebo  $P=0.663$ ).

The three different strains of probiotic bifidobacteria fed to the subjects (probiotic treatment group) were tracked in the bifidobacteria TRFLP data. All of the fed bifidobacteria strains produced a TRF peak at 219–220 nt. About 55% of the baseline samples had TRF peaks at 219–220 nt, indicating the presence of native bifidobacteria with the same TRF peaks. The percentage of samples with TRF 219–220 nt did not significantly change after probiotic treatment. Similarly, the difference from the baseline in relative abundance of TRF 219–220 nt did not change significantly in either treatment group. *Lactobacillus* was frequently present at low levels as determined by cell count [ $<10^7$  c.f.u. (g faeces)<sup>-1</sup> in 50% of samples] and not detected in 60% of samples using *Lactobacillus*-specific PCR.

## DISCUSSION

This study investigated the capacity of a probiotic mixture to minimize disruption of the intestinal microbiota in individuals undergoing antibiotic therapy. Antibiotic-induced alterations in gut microbiota were identified and the probiotic mixture minimized the antibiotic disturbances. The reestablishment of the baseline microbiota occurred more rapidly in the probiotic-treated group, with a pronounced effect particularly on concentrations of *Enterobacteriaceae*, *Bifidobacterium* and *Bacteroides*. This was demonstrated most clearly by the culture data.

In other clinical trials, Augmentin was associated with diarrhoea in over 20% of subjects (Block *et al.*, 2003; Caron *et al.*, 1991; Ferreira *et al.*, 2006). The numbers of subjects developing diarrhoea in this study would be expected from the antibiotic, and since symptoms did not continue throughout the course of the probiotic administration, there is no clear link to probiotic ingestion. The rate of vaginal yeast infection would also be expected, given that 75% of the participants were female.

A secondary aim was to validate the use of TRFLP techniques compared with standard culturing techniques to assess changes in gut microbiota. Molecular techniques based on 16S rRNA, including TRFLP, provide a broad approach to describing the complex microbial environment of the intestines (Jernberg *et al.*, 2005; Kitts, 2001). TRFLP analysis is limited in that it is only semiquantitative and does not detect organisms with an abundance of less than 0.5% of the total community. However, TRFLP is well suited to identify relative changes between samples and yields a more comprehensive fingerprint of the faecal microbiota. Although culture techniques have lower detection limits, their scope is more focused and they do not assess broad changes in the bacterial species present, many of which are not culturable. This study demonstrated that the findings from each technique are complementary.

One limitation to this study was the large variability in the three baseline samples. The faecal microbiota has been shown to reflect a compilation of both mucosal and luminal microbes (Ley *et al.*, 2005). The bacterial community of the lumen reflects changes in diet and ingestion of transient microbes, which may have contributed to differences detected in the baseline samples. Furthermore, although the mucosal bacterial community in the gastrointestinal tract of the healthy human is considered by some to be relatively stable (Mättö *et al.*, 2005; Vanhoutte *et al.*, 2004; Zoetendal *et al.*, 1998), this conclusion may need to be further examined with additional evaluation of the impact of diet and longer term sampling (Dethlefsen *et al.*, 2006). It is noteworthy for this work that few human studies have obtained more than one baseline sample when investigating probiotic treatment effects. Gopal *et al.* (2003) collected three baseline samples, although only one sample was used in subsequent analyses. In the current study, three baseline samples were collected over 2 weeks to ensure an accurate

estimation of the baseline bacterial community and as such represents the first report analysing the baseline microbiota in a large group of healthy individuals. Some subjects displayed more baseline variation than anticipated, which may be due to true intra-individual variability, or may represent an artefact of sample handling and processing despite efforts to standardize these measures. A change in diet is unlikely to account for this variation since baseline samples were taken over a short period of time. In addition, subjects were instructed not to take in foods with significant probiotics components (i.e. yogurt, kefir) and not to introduce new foods or change their diet during the study. Minor fluctuations in diet would be unlikely to influence the findings of this study.

Variability was more apparent by TRFLP than by culture data. The microbiota of more subjects was deemed stable by culture data (29 subjects) than by TRFLP data (22 subjects). This finding is not surprising since culture methods tend to show a limited view of microbial diversity because only five different media were used, each medium selected for a relatively broad group of organisms and specific taxonomies were not investigated. In addition, the sample-handling procedures used in this study may have affected culture results. This study suggests that there may indeed be a subset of healthy individuals with a truly unstable faecal bacterial community, especially since five subjects were classified as unstable by both TRFLP and culture analysis. As this finding contradicts some other studies, it should be interpreted cautiously and may be explained by sample processing, though no differences were apparent between subjects.

The findings of this study are suggestive of a possible probiotic benefit in limiting antibiotic-associated disturbances in gut microbiota. The probiotic mixture used was selected because it represents strains of the two most common probiotic genera, *Bifidobacterium* and *Lactobacillus*. While these probiotic species have a variety of identified physiological health benefits, the hypothesis of this study was that these bacteria function in a central ecological niche in the intestine to maintain a stable intestinal bacterial community. The specific strains used here were chosen based on prior work involving their health benefits, tolerance to industrial manipulation and shelf life. This probiotic mixture appeared to minimize the antibiotic-associated disruption of gut microbiota, thus permitting a more rapid return to the baseline microbiota. This probiotic effect was observed with TRFLP results significant at only a 90% confidence level. However, the TRFLP data in this study tracked more than 200 phylotypes of bacteria. Additionally, each individual subject's post-treatment samples can only be compared to her/his own individual baseline samples, resulting in only three pairwise comparisons per post-treatment day. Therefore, achieving 90% confidence with these types of data may actually indicate large differences for subpopulations of bacteria. In fact, the culture data showed no apparent antibiotic effect in the probiotic treatment group. Thus, it appears that the

dominant microbes assessed in this study may be those strongly affected by probiotic treatment. According to the culture data, *Enterobacteriaceae*, *Bifidobacterium* and *Bacteroides* showed the largest probiotic effect. This was partially confirmed by closer inspection of a subset of the TRFLP data. When TRFLP data from multiple enzyme digestions were analysed, *Enterobacteriaceae* and *Bacteroides* again showed a large probiotic effect. Thus, culturing in this study focused on bacteria that were highly affected by the probiotic treatment.

A mechanism of action for this effect was not investigated as part of this study but we would speculate that a probiotic effect may be due to the metabolism of the supplemented bacteria. The probiotics may metabolize luminal components to generate a substrate that may be a preferred fuel source or create a preferred physical environment, such as localized pH, for the critical bacteria that are central to the broader stability of the microbiota.

This study does identify a benefit of probiotics, in part through increasing *Bifidobacterium* that may limit the disruption of gut microbiota by antibiotics, stabilizing concentrations of *Enterobacteriaceae* and *Bacteroides* in particular. While this does not represent a clinical end point in itself, this study provides important insight into the nature of the disruption of gut microbiota by antibiotics and a possible mechanism whereby probiotics limit gastrointestinal adverse events associated with antibiotics. Further studies are necessary to assess the clinical relevance of these observations.

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