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Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model

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ABSTRACT

In vitro gut models provide several advantages over *in vivo* models for the study of the human gut microbiota. However, because communities developed in these models are inevitably simplified simulations of the *in vivo* environment, it is necessary to broadly define the differences between *in vitro* consortia and the communities from which they are derived. In this study we characterized microbial community development in a twin-vessel single-stage chemostat model of the human distal gut ecosystem using both gel (Denaturing Gradient Gel Electrophoresis) and phylogenetic microarray (Human Intestinal Tract Chip) based techniques. Five different sets of twin-vessels were inoculated with feces from three different healthy adult donors and allowed to reach steady state compositions. We found that twin-vessel single-stage chemostats could develop and maintain stable, diverse, and reproducible communities that reach steady state compositions in all five runs by at most 36 days post-inoculation. As noted in other *in vitro* studies, steady state communities were enriched in *Bacteroidetes* but not *Clostridium* cluster XIVa, *Bacilli* or other *Firmicutes* relative to the fecal inocula. Communities developed within this model had higher within-run reproducibility than between-run repeatability when using consecutive fecal donations. Both fecal inocula and steady state chemostat communities seeded with feces from different donors had distinct compositions. We conclude that twin-vessel single-stage chemostat models represent a valid simulation of the human distal gut environment and can support complex, representative microbial communities ideal for experimental manipulation.

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1. Introduction

The human gut is the most densely inhabited ecosystem on Earth (Marchesi and Shanahan, 2007). Healthy gut microbial communities exert various protective, structural, and metabolic functions on the host gut epithelium and it is hypothesized that changes in the gut microbial ecosystem equilibrium can cause disease (Thompson-Chagoyan et al., 2005; O'Hara and Shanahan, 2006). Pure culture study of component species from a complex community only provides limited information about their role in the ecosystem; the function and behavior of the ecosystems are best studied as a whole.

Several *in vivo* and *in vitro* models have been used to study the response of the gut microbiota to different perturbations or treatments (Rycroft et al., 2001; Vulevic et al., 2004; Allison et al., 1989; Makelainen et al., 2009; Minekus et al., 1999; Molly et al., 1993; Macfarlane et al., 1998); however, while *in vivo* models have the advantage of biological significance, they also have several drawbacks. For example, because it is known that different subjects have unique, host-specific gut community profiles, comparison of the gut microbiota from subjects in different treatment groups is difficult (Zoetendal et al., 1998). Additionally, due to the inability of studies to focus on gut communities without host interference, mechanistic studies are often confusing and may also be limited to end-point measurements (Macfarlane and Macfarlane, 2007; Van den Abbeele et al., 2010). Issues of volunteer compliance, expense and ethical approval also restrict experimental design (Macfarlane and Macfarlane, 2007).

The use of *in vitro* models can overcome many of these limitations. Several *in vitro* models of the human gut microbiota have been developed, ranging from simple batch culture to complex continuous culture or 'chemostat' systems (Macfarlane and Macfarlane, 2007). Using chemostats, communities seeded from fresh feces can reach an equilibrium resembling the *in vivo* distal gut community from which they were sourced (Macfarlane et al., 1998; Van den Abbeele et al., 2010). Being host-free systems, chemostats supporting the gut microbiota make ideal vessels in which to study microbial perturbations resulting from the addition of exogenous stimuli, since microbial changes can be measured in isolation of any concurrent effects on the host (Macfarlane and Macfarlane, 2007). Other advantages of *in vitro* models include controllability (and thus high reproducibility), economy (most models are less expensive to run than *in vivo* assays), and the ability to sample as frequently as necessary without ethical constraints.

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To represent a valid model of the human distal gut the communities that develop within these vessels must be reproducible and stable, must retain the high level of diversity seen in their native counterparts, and must also share some similarity to the fecal inocula from which they were derived (Van den Abbeele et al., 2010). In this study we used denaturing gradient gel electrophoresis (DGGE) and the human intestinal tract chip (HITChip; Rajilic-Stojanovic et al., 2009) as complementary tools to characterize communities developed in a 'twin-vessel' (independent, identical) single-stage chemostat model of the healthy human distal gut. The aims of this study were (i) to determine the length of time required for microbial communities to reach 'steady state' compositions; (ii) to evaluate the within-run reproducibility (technical replicates) as well as the between-run repeatability (biological replicates) of communities developed in our twin vessel system; (iii) to assess how communities seeded with feces sourced from different donors develop within our system (alternative biological replicates); (iv) to obtain phylogenetic information on the communities developed and maintained, while also assessing the comparability between DGGE and HITChip-calculated parameters.

2. Materials and methods

2.1. Preparation of single-stage chemostats

An Infors Multifors bioreactor system was used for this work (Infors, Switzerland; Fig. S1). Conversion from a fermentation system into a chemostat was accomplished by blocking off the condenser vent and gently bubbling nitrogen gas through the culture to displace oxygen and maintain a constant culture volume (400 mL) through positive pressure (Allison et al., 1989; Fooks and Gibson, 2003). Vessels were maintained at 37 °C and pH 6.9–7.0 (Allison et al., 1989) through automated addition of acid (5% (v/v) HCl) or base (5% (w/v) NaOH).

Growth medium was based on previous studies (Macfarlane et al., 1998; Lesmes et al., 2008) with several modifications. Medium was prepared as outlined in Table 1 and sterilized, following which 2.5 mL of antifoam B silicone emulsion (J.T. Baker; Center Valley, Pennsylvania) was added per liter of prepared media. Growth medium was continuously fed at a rate of 400 mL/day (retention time of 24 h; Allison et al., 1989; Duncan et al., 2003).

Table 1
Composition of the growth medium used to culture microbial communities within the twin-vessel single-stage chemostat model (per liter of media). Numbers in superscripts denote chemical suppliers as follows: (1) Sigma-Aldrich (Oakville, Ontario); (2) Thermo Fisher Scientific (Ottawa, Ontario); (3) BD (Franklin Lakes, New Jersey); (4) Alfa Aesar (Ward Hill, Massachusetts); (5) BDH (Radnor, Pennsylvania).

Reagent	Weight (g)
Peptone water[2]	2
Yeast extract[3]	2
$NaHCO_3^2$	2
$CaCl_2^1$	0.01
Pectin (from citrus)[1]	2
xylan (from beechwood)[1]	2
Arabinogalactan[1]	2
Starch (from wheat, unmodified)[1]	5
Casein[4]	3
Inulin (from Dahlia tubers)[4]	1
NaCl[1]	0.1
$K_2HPO_4^1$	0.04
$KH_2PO_4^2$	0.04
$MgSO_4^5$	0.01
Hemin[5]	0.005
Menadione[1]	0.001
Bile salts[1]	0.5
L-cysteine HCl[2]	0.5
porcine gastric mucin (type II)[1]	4

2.2. Collection and preparation of fecal inocula

The Research Ethics Board of the University of Guelph approved this study (REB#09AP011). Three healthy donors provided fresh fecal samples: donor A (male, 44 years-old), donor B (female, 42 years-old) and donor C (male, 26 years-old). None of these donors had a recent history of antibiotic treatment prior to this study (within 9 months). Donors A and B provided fresh stool on two separate occasions. Donor A feces were used to inoculate chemostat runs A1 and A2 (A denotes the donor and 1 or 2 denotes the run number), while donor B feces were used to inoculate runs B3 and B4. Donors A and B provided 2 samples each, 5 and 6 months apart respectively.

Fresh fecal samples were immediately placed into an anaerobic chamber (90% N_2, 5% CO_2, 5% H_2) within 5–10 min of voiding. A 10% (w/v) fecal slurry was prepared by homogenizing 5 g of feces in 50 mL of pre-reduced growth medium for 1 min using a stomacher (Tekmar Stomacher Lab Blender, Seward; Worthing, West Sussex, UK). The resulting slurry was centrifuged for 10 min at 175 ×*g* to remove large food residues (De Boever et al., 2000). The resulting post-spin supernatant slurry was used as the inoculum for this study.

2.3. Inoculation, operation and sampling

100 mL of inocula was added to 300 mL of sterile media in each vessel; pairs of vessels were set up using identical parameters ('twin vessels') and inoculated with identical material. Cultures were gently agitated and vessel pH was adjusted to 6.9–7.0. Medium feed was started 24 h post-inoculation.

Each chemostat vessel was sampled daily by removing 4 mL of culture aseptically; samples were archived at −80 °C. Communities from A1 were maintained for 48 days while communities from A2, B3, B4, and C5 were maintained for 36 days.

2.4. DNA extraction

DNA was extracted from archived samples using bead beating and modified protocols from commercially available kits. Briefly, 200 μL of each sample was added to 300 μL of SLX buffer (Omega Bio-Tek E.Z.N.A.® Stool DNA kit; Norcross, Georgia), 10 μL of 20 mg/mL proteinase K and 200 mg of glass beads, and bead beat for 3 min. Samples were incubated at 70 °C for 10 min, 95 °C for 5 min and on ice for 2 min. The E.Z.N.A. protocol was followed to the inhibitor removal step, and then transferred into Maxwell®16 DNA Purification Kit cartridges (Promega; Madison, Wisconsin). The remainder of the extraction protocol was carried out according to the Maxwell kit instructions.

2.5. DGGE analysis

DNA was extracted from each vessel every 2 days, starting immediately following inoculation ("day 0" samples) until the end of each run. The V3 region (339–539 bp, *Escherichia coli* numbering) of the 16S rRNA gene was amplified using primers HDA1 and HDA2-GC (Tannock et al., 2000). DNA amplification was achieved using Tsg DNA polymerase (Bio Basic; Markham, Ontario) and 1× Thermopol buffer (2 mM $MgSO_4$, NEB; Whitby, Ontario), with extracted DNA as a template. The cycling conditions were: 92 °C for 2 min (92 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min) × 35; 72 °C for 10 min.

A DGGE standard ladder was prepared using strains available in house from our extensive collection of human bacterial gut isolates. Strains used were as follows: *Coprobacillus* sp. (1/2/53), *Enterococcaceae* sp. (30/1), *Veillonella* sp. (5/2/43 FAA), *Clostridium* sp. (1/1/41 A1 FAA CT2), and *Propionibacterium* sp. (7/6/55B FAA). DNA was extracted from these strains using the method described by Strauss et al. (2008). The V3 region from all strains was amplified and the reactions were pooled in equal ratios. Identical ladder samples were run on the first and middle lanes of each DGGE gel to allow gel standardization.

Samples were mixed with loading dye (0.05% (v/v) bromophenol blue; 0.05% (v/v) xylene cyanol; 70% (v/v) glycerol in HPLC grade water; Bio-Rad DCode Manual), and loaded on a prepared DGGE gel (DCode System, Bio-Rad Laboratories, Hercules, California) using the method of Muyzer et al. (1993) and a 6% (v/v) polyacrylamide gel. Amplicons were separated using a denaturing gradient of 30–55%. Gels were stained with ethidium bromide (Sigma-Aldrich) and images were captured using a SynGene G-Box gel doc system running GeneSnap software (version 6.08.04, Synoptics Ltd; Cambridge, UK). Images were normalized for saturation prior to capture.

Gel images were analyzed using Syngene GeneTools software (version 4.01.03, Synoptics Ltd). Similarities between DGGE banding patterns were analyzed by calculating Pearson correlation coefficient values. Similarity index (SI) values ranged from 0 to 1 (0 indicated no bands in common; 1 indicated identical banding patterns). Correlation coefficients (% similarity index values, %SI) were calculated by multiplying the similarity index value by 100. Dendrograms were generated using Pearson correlation coefficient values and the unweighted pair group with mathematical averages (UPGMA) method.

Gel-specific vessel comparison "cut-off thresholds" were calculated by comparing the similarity of identical standard ladder profiles within the same DGGE gel. Sample profiles with correlation coefficient values higher than the cut-off threshold were considered identical, and if correlation coefficient values were within 5% of the cut-off value the correlation coefficient values were considered similar.

2.6. Community dynamics

Community changes were measured using moving window correlation analysis (Marzorati et al., 2008; Possemiers et al., 2004). Plots were used to assess the stability of these communities and the time required to reach steady state. Rate-of-change cut-off thresholds were calculated by subtracting vessel comparison cut-off threshold values from 100%.

2.7. Compensation for DGGE gel-to-gel variation

DGGE Shannon diversity index (H′), Shannon equitability index ($E_{H'}$), and richness (S) values were adjusted to illustrate general patterns of ecological change over the course of a run. Day 0 samples were run on each gel and, as well, the last time point from one gel was run as the first time point on the next gel to create overlapping time points. H′, $E_{H'}$, and S values for the day 0 samples were compared between all gels within a chemostat run, and the gel where these values were closest to the average was set as the 'reference gel' to which other gels were aligned (Fig. S2). A 'correction value' was determined by calculating the average difference between identical samples. Values for aligned gels were adjusted by adding (or subtracting) the correction value to all samples within the gel. This shifted all values to align overlapping values with the flanking gel. Parameter values for overlapping time points were averaged, and relative within-gel differences were maintained.

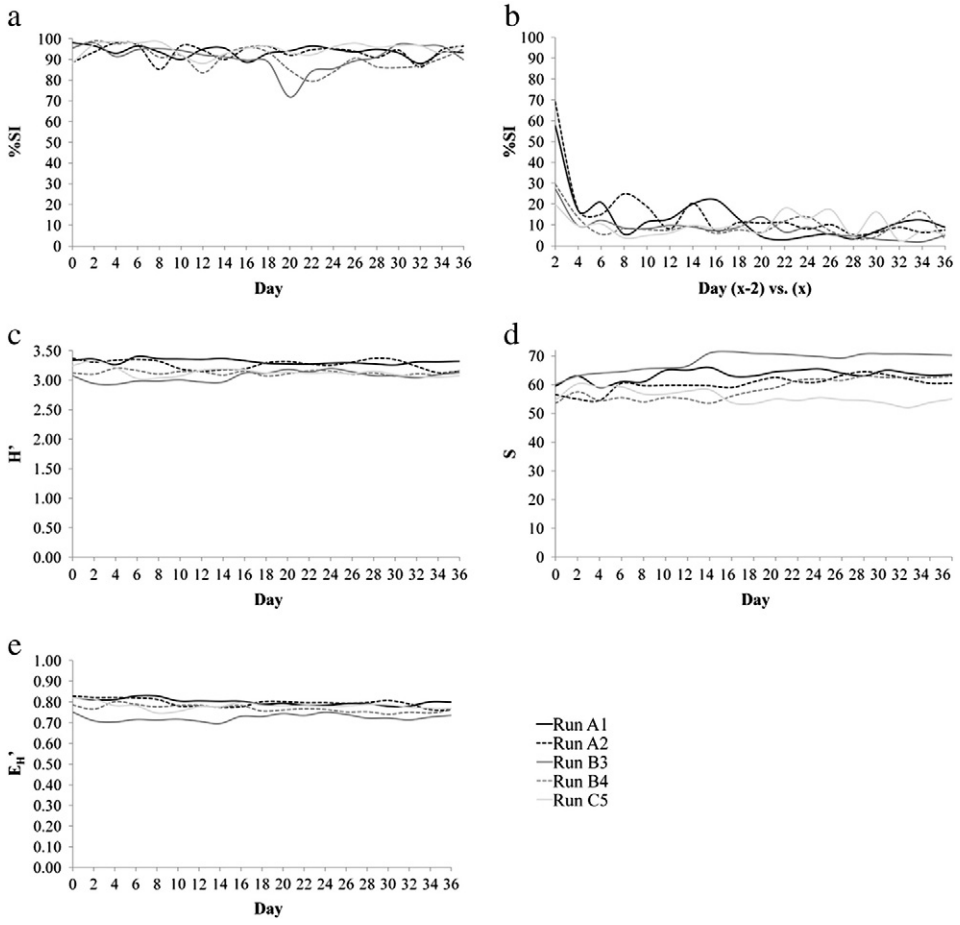

Fig. 1. DGGE community analysis of chemostat runs seeded with feces from three different healthy donors (donors A, B, and C). With the exception of the plot shown in panel a, each line represents the average values of samples taken from two identical chemostat vessels (where similar values were seen between both vessels). Samples were analyzed every two days until steady state conditions were met (days 0–36). Gel-specific cut-off values are not shown for simplification purposes. a) Correlation coefficients (expressed as percentages) comparing the profiles of each vessel at identical time points. b) Community dynamics calculated using moving window correlation analysis. c) Adjusted Shannon diversity index (H′). d) Adjusted band richness (*S*). e) Adjusted Shannon equitability index ($E_{H'}$).

2.8. Phylogenetic microarray (HITChip) analysis

The microbial community composition of 17 samples was evaluated using the Human Intestinal Tract Chip (HITChip; Rajilic-Stojanovic et al., 2009), including the fecal inoculum and steady state communities from each vessel (days 36 and 48). HITChip analyses were performed using standard protocols as previously described (Jalanka-Tuovinen et al., 2011). Hybridization and analysis procedure specific to this work is described in more detail in the Supplementary material.

2.9. Shannon diversity index, Shannon equitability index, richness

The Shannon diversity index (H′) was calculated according to previous studies (Magurran, 2004; Gafan et al., 2005):

$$H' = -\sum_{i=1}^{S}(p_i)(\ln p_i)$$

where, for DGGE profiles, S represented the total number of DGGE bands and p_i represented the proportion of the ith band (band peak height), and for HITChip data, S represented the total number of hybridized probe sequences and p_i represented the proportion of the ith hybridized probe sequence (hybridization signal intensity).

The Shannon equitability index ($E_{H'}$) was calculated as follows (Smith and Wilson, 1996; Pielou, 1975): $E_{H'} = H'/\ln S$ where H′ represented the Shannon diversity index and S represented the total number of bands (for DGGE profiles) or total number of hybridized probe sequences (for HITChip data).

Ecosystem richness (S) was approximated by enumerating the total number of bands present in a given DGGE profile, or the total number of hybridized probe sequences for HITChip data (Gafan et al., 2005; Fromin et al., 2002).

3. Results

3.1. Establishment of steady state microbial community compositions in a twin-vessel single stage distal gut model

Twin vessels from five different chemostat runs were analyzed by DGGE to determine the time required for communities to reach steady state compositions. The length of time required for correlation coefficients to rise above gel-defined cut-off thresholds varied depending on the chemostat run being analyzed; however these values met the cut-off thresholds in all five chemostats by at most 36 days post-inoculation (Fig. 1a, Table 2).

Moving window correlation analysis showed that rate of change (Δt) values were highest between days 0–18, but decreased and stabilized as steady state compositions were established. Rate of change values for twin vessels took varying lengths of time to drop below the gel-specific cut-off thresholds, but this occurred by 34-days post-inoculation in all five runs (Fig. 1b, Table 2).

Band richness values fluctuated in the beginning of each experiment, but stabilized by 34 days post-inoculation in all five runs (Fig. 1d, Table 2). Shannon diversity and equitability index values remained relatively stable compared to the other parameters measured, and were thus less informative of the time required for chemostat communities to reach steady state (Fig. 1c,e).

Table 2
Time (in days) to steady state compositions based on measured parameters from DGGE gels (bracketed numbers refer to values that met the 5% cut-off threshold by day 36).

Parameter	Run A1	Run A2	Run B3	Run B4	Run C5
Vessel comparison (%SI)	20	34	30	(36)	0
Rate of change (Δt)	18	(34)	28	(34)	30
Band richness (S)	34	34	28	30	30
Beginning of steady state	34	34	30	36	30

3.2. Reproducibility of fecal inocula and chemostat communities

3.2.1. Reproducibility of communities developed in twin vessels seeded with a single fecal donation (technical replicates)

Sets of twin vessels were compared to assess between-vessel reproducibility. We found that twin vessels were reproducible immediately following inoculation on day 0 (97.8 ± 1.9% similar by DGGE) and during steady state conditions on day 36 (92.6 ± 4.6% similar by DGGE, 96.7 ± 1.0% similar by HITChip; Fig. 1a). DGGE cluster tree and HITChip PCA analysis showed that twin vessel day 0 samples grouped together, as did twin vessel day 36 profiles (Figs. 2 and 3). At the end

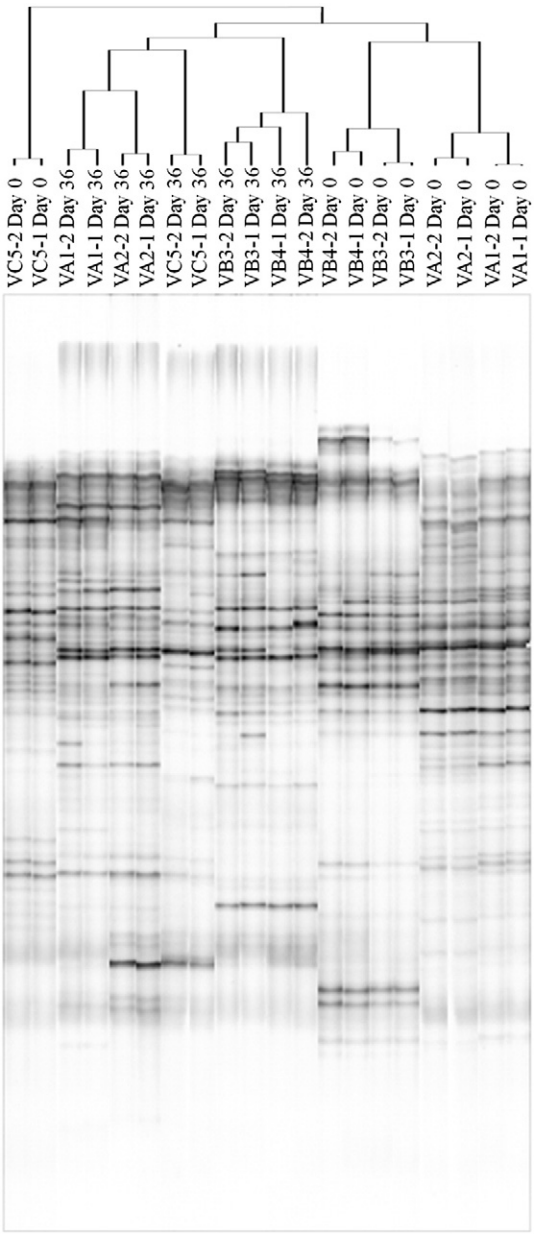

Fig. 2. DGGE dendrogram based on Pearson similarity coefficient and UPGMA correlation comparing communities present in twin-vessels for 5 different chemostat runs. DGGE profiles show samples taken from the vessel immediately following inoculation (day 0 samples) or during steady state conditions (day 36 samples). Runs A1, A2, B3, and B4 were used to assess biological repeatability and runs A1, B3, and C5 were used to compare between-donor differences.

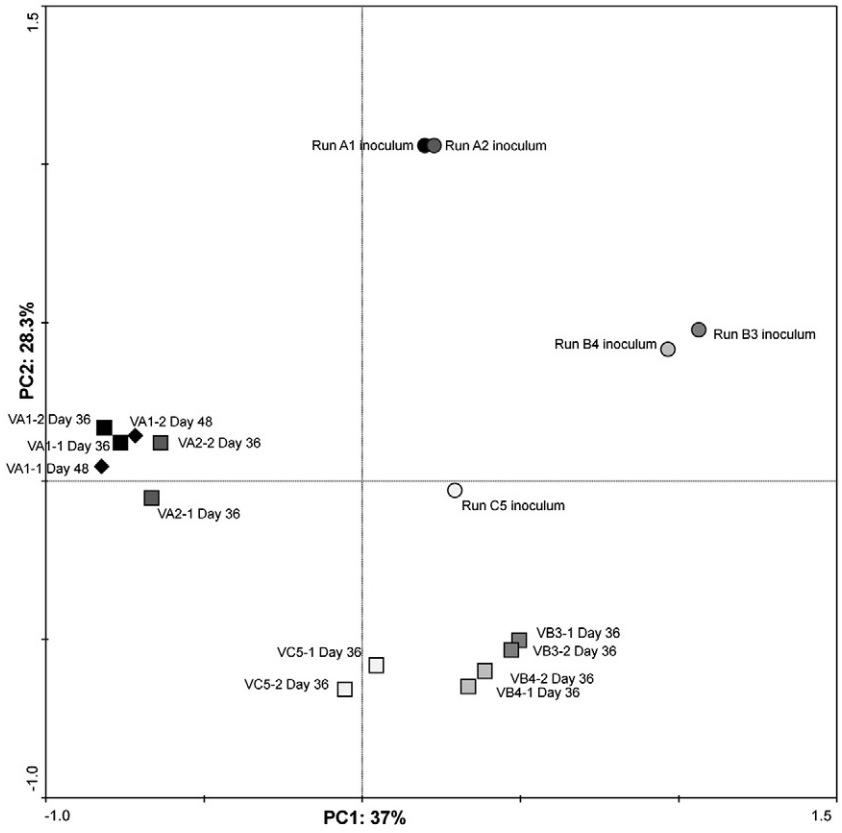

Fig. 3. Principal component analysis (PCA) at the oligonucleotide-level based on HITChip data of the fecal inocula (○) and steady state (days 36 and 48) chemostat communities (□). Samples from the same chemostat run are shaded similarly.

of the experiment (on day 48) VA1-1 and VA1-2 correlation coefficients were 81.7% similar by DGGE or 93.9% similar by HITChip.

Moving window correlation analysis showed that twin vessel communities developed in a similar manner for the duration of the run (Fig. 1b). Δt values were larger and more variable at the beginning of the run (during the stabilization period). Δt values decreased and stabilized as steady state conditions were established, dropping and remaining below the gel-defined cut-off thresholds by at most 36 days post-inoculation.

DGGE Shannon diversity and equitability index values were reproducible and relatively stable throughout the course of each chemostat run (Fig. 1c,e, Table S1). DGGE band richness was very reproducible throughout the duration of the experiment (Fig. 1d). Reproducible fluctuations in DGGE band numbers were seen toward the beginning of each chemostat run; however values were reproducible and stable as the communities reached steady state.

3.2.2. Repeatability of communities developed in twin vessels seeded with consecutive fecal donations from the same donor (biological replicates)

Consecutive chemostat runs seeded with different fecal donations from the same donor had higher within-run (between-vessel) reproducibility than between-run repeatability. Table S2 compares donor A profiles between runs A1 and A2 and donor B between runs B3 and B4. However, DGGE cluster tree analysis and HITChip PCA analysis showed that chemostat communities seeded with feces from the same donor clustered together, but not as tightly as communities seeded with an identical fecal inoculum (Figs. 2 and 3). H′, E_H′, and S values confirmed that within a given run twin-vessel communities were more reproducible than the communities from a consecutive chemostat run (Table S1).

3.3. Development of microbial communities seeded with feces from different donors

The steady state profiles from donors A, B, and C had distinct community structures and compositions throughout each run. Consecutive runs seeded with feces from the same donor were more similar to each other on days 0 or 36 than runs seeded with feces from different donors (Tables S2 and S3). This is illustrated in the DGGE cluster tree and HITChip PCA plot, which show that steady state samples from chemostat vessels seeded with feces from the same donor clustered more closely with each other than with samples from chemostat vessels seeded with feces from different donors (Figs. 2 and 3). It should be noted that differences seen at days 0 and 36 between each donor are not necessarily reflective of the initial inoculum. H′, E_H′, and *S* values indicated that vessels seeded with feces from different donors resulted in reproducible steady state communities with stable, biologically-relevant levels of diversity, evenness, and richness (Table S1).

3.4. Shifts in community composition following inoculation

Fecal inocula and steady state community profiles from each vessel were compared to examine how the community structure changed following the *in vivo* to *in vitro* transition. HITChip analysis of all five chemostat runs showed that steady state cultures were enriched in *Bacteroidetes* but not *Clostridium* cluster XIVa, *Bacilli* or other *Firmicutes* relative to the corresponding fecal inoculum (Table 3).

DGGE and HITChip analysis showed reproducible changes in microbial populations during steady state conditions relative to the fecal inoculum (Tables 4 and S4, Fig. S3). On average, fecal inocula (day 0) and steady state (day 36) chemostat communities were 45.4 ± 6.8% similar by DGGE or 67.3 ± 5.4% similar by HITChip. DGGE clustering tree and

Table 3
Comparison of bacterial groups (belonging to higher taxonomic groups) of average fecal inocula and average steady state (day 36) chemostat communities from all 5 chemostat runs. P values were calculated using the Wilcoxon signed-rank test corrected for multiple comparisons (applied at the genus-like level). Shaded p values remain significantly different after correction.

Level 1 (~phylum/class level)	Level 2 (genus-like level)	P values	Fecal inoculum abundance (%)	SD	Day 36 abundance (%)	SD
Bacteroidetes	Allistipes et rel.	0.153	4.00	1.11	5.71	1.20
	Bacteroides fragilis et rel.	0.006	1.03	0.73	6.59	2.70
	Bacteroides ovatus et rel.	0.006	1.49	1.15	8.52	1.72
	Bacteroides splachnicus et rel.	0.153	2.21	1.53	4.46	1.00
	Bacteroides stercoris et rel.	0.063	1.29	0.83	2.61	0.54
	Parabacteroides distasonis et rel.	0.010	3.12	1.63	7.58	2.34
	Prevotella ruminicola et rel.	0.006	0.55	0.36	2.10	0.41
	Tannerella et rel.	0.043	1.85	0.60	2.91	0.50
Bacilli	Aneurinibacillus	0.090	0.01	0.00	0.01	0.00
	Bacillus	0.121	0.01	0.00	0.02	0.01
	Streptococcus bovis et rel.	0.006	1.36	1.26	0.05	0.01
	Streptococcus intermedius et rel.	0.010	0.11	0.07	0.02	0.01
	Streptococcus mitis et rel.	0.010	0.65	0.59	0.04	0.02
Clostridium cluster III	Clostridium stercorarium et rel.	0.043	0.35	0.20	0.09	0.07
Clostridium cluster IV	Papillibacter cinnamivorans et rel.	0.006	0.65	0.25	0.07	0.04
Clostridium cluster IX	Peptococcus niger et rel.	0.121	0.03	0.01	0.01	0.00
Clostridium cluster XIVa	Bryantella formatexigens et rel.	0.063	1.87	0.74	0.68	0.89
	Clostridium nexile et rel.	0.019	2.48	0.57	0.71	0.64
	Coprococcus eutactus et rel.	0.006	1.56	1.12	0.13	0.05
	Dorea formicigenerans et rel.	0.090	3.93	1.03	1.68	1.33
	Eubacterium hallii et rel.	0.029	0.93	0.42	0.21	0.20
	Eubacterium ventriosum et rel.	0.006	1.29	0.24	0.30	0.19
	Lachnospira pectinoschiza et rel.	0.029	4.70	2.68	0.96	0.69
	Outgrouping Clostridium cluster XIVa	0.006	2.78	1.23	0.61	0.23
	Roseburia intestinalis et rel.	0.006	4.28	0.85	0.61	0.54
	Ruminococcus gnavus et rel.	0.006	1.02	0.17	0.32	0.14
	Ruminococcus lactaris et rel.	0.006	0.83	0.19	0.12	0.06
	Ruminococcus obeum et rel.	0.006	5.43	1.55	0.66	0.22
Uncultured Clostridiales	Uncultured Clostridiales II	0.006	0.18	0.11	1.24	0.29
Proteobacteria	Burkholderia	0.043	0.00	0.00	0.03	0.03
	Escherichia coli et rel.	0.029	0.02	0.00	0.03	0.01
Verrucomicrobia	Akkermansia	0.153	0.05	0.04	1.43	1.19

HITChip PCA analysis showed that, for each donor, steady state chemostat profiles clustered separately from fecal inocula profiles (Figs. 2 and 3).

4. Discussion

In this work, we set out to define the operational parameters of a single-stage chemostat model of the human distal gut, and to ascertain optimal conditions for this system justified by an analysis of a range of ecological parameters. We did not attempt to produce an exact copy of the human distal gut *in vitro*, but rather we aimed to culture stable, diverse communities representative of gut ecosystems that are suitable for the study of experimental perturbations. Indeed, chemostat communities developed in our twin-vessel single-stage model did not contain the initial quantitative composition of the fecal inocula as differences in the ratios of bacterial populations were noted (as expected; Van den Abbeele et al., 2010; Rajilic-Stojanovic et al., 2010). For example, HITChip analysis showed that steady state cultures were enriched in *Bacteroidetes* but not *Clostridium* cluster XIVa, *Bacilli* or other *Firmicutes* relative to the corresponding fecal inoculum. HITChip data obtained from a SHIME (multistage semi-continuous gut model) study (Van den Abbeele et al., 2010) and a TIM-2 (proximal colon gut model) study (Rajilic-Stojanovic et al., 2010) were consistent with our results. Reasons for the discrepancy in composition between communities developed in *in vitro* gut models and fecal inocula have been previously described (Van den Abbeele et al., 2010) and include variations in oxygen levels (Briukhanov and Netrusov, 2007; Rolfe et al., 1978), carbohydrate content of feed medium (Mahowald et al., 2009), lack of simulation of mucosal binding sites (Matsumoto et al., 2002), and lack of host selective pressures (Macfarlane et al., 1998; Child et al., 2006; Walker et al., 2005; Akira et al., 2006; Cooper and Alder, 2006). However, despite these differences, overall the compositions of steady state microbial communities developed within our model were in general agreement with findings from other *in vitro* models and representative of the corresponding human fecal communities.

In this study we carried out a total of five chemostat runs comprising 10 vessels and 3 donors overall. When all runs were considered, we found that compositional steady state was achieved by 36 days post-

Table 4
Percent abundance of higher taxonomic groups (~phylum/class level) based on HITChip analysis for the fecal inocula (inoc) and twin-vessel steady state (day 36) chemostat communities.

Higher taxonomic groups		Run A1 fecal inoc	Run A2 fecal inoc	Run A1 day 36 average	Run A2 day 36 average	Run B3 fecal inoc	Run B4 fecal inoc	Run B3 day 36 average	Run B4 day 36 average	Run C5 fecal inoc	Run C5 day 36 average
	Actinobacteria	1.71	1.31	0.09	0.12	9.38	4.83	0.19	0.22	5.27	0.25
	Bacteroidetes	19.07	9.65	55.55	54.04	17.11	34.87	54.00	53.55	47.57	67.37
Firmicutes	*Bacilli*	1.32	0.80	0.19	0.24	3.61	5.55	0.33	0.26	0.37	0.26
	Clostridium cluster I	0.20	0.55	0.05	0.06	0.05	0.05	0.07	0.08	0.06	0.08
	Clostridium cluster III	0.27	0.73	0.20	0.12	0.31	0.34	0.03	0.09	0.13	0.05
	Clostridium cluster IV	25.29	25.41	23.76	29.07	14.56	15.44	10.93	5.87	9.05	13.92
	Clostridium cluster IX	0.11	0.10	0.05	0.07	0.12	0.11	0.37	0.43	0.09	0.12
	Clostridium cluster XI	0.38	0.63	0.13	0.16	0.10	0.08	0.13	0.11	0.13	0.09
	Clostridium cluster XIII	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.02
	Clostridium cluster XIVa	50.10	57.24	16.21	12.93	54.08	37.88	26.51	30.97	36.51	15.61
	Clostridium cluster XV	0.02	0.02	0.07	0.18	0.02	0.02	0.71	0.73	0.02	0.02
	Clostridium cluster XVI	0.12	0.11	0.03	0.04	0.03	0.04	0.05	0.05	0.27	0.05
	Clostridium cluster XVII	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.01
	Clostridium cluster XVIII	0.02	0.03	0.02	0.03	0.12	0.21	0.03	0.03	0.07	0.04
	Uncultured *Clostridiales*	0.81	2.66	1.48	1.14	0.11	0.12	1.08	1.45	0.20	1.76
	Total *Firmicutes*	78.67	88.31	42.21	44.06	73.13	59.86	40.28	40.09	46.91	32.03
	Proteobacteria	0.40	0.55	1.15	1.13	0.27	0.34	2.77	3.07	0.19	0.26
Tenericutes	Uncultured *Mollicutes*	0.04	0.05	0.03	0.05	0.06	0.05	0.10	0.05	0.03	0.05
	Verrucomicrobia	0.08	0.11	0.95	0.59	0.02	0.03	2.62	2.99	0.01	0.01

inoculation in all vessels, but as soon as 30 days in some vessels. We found that steady state was most easily evaluated by examining correlation coefficients comparing duplicate vessels, moving-window correlation analysis, and band richness plots (Fig. 1a,b,d), and we considered communities at steady state at the point when a) the twin-vessel DGGE correlation coefficients rose and stayed above the cut-off threshold; b) the rate of change values dropped and remained below the cut-off threshold; c) band richness stabilized. Diversity and evenness were generally stable regardless of the time point. There is a general lack of consensus in the literature over the time required to reach steady state or stable community compositions (Allison et al., 1989; Macfarlane et al., 1998; Van den Abbeele et al., 2010; Lesmes et al., 2008; Possemiers et al., 2004; Gibson et al., 1988). While it is not unexpected that the time to steady state would vary using different chemostat models with different media compositions, operational parameters and fecal donors, it is important to establish the time it takes for a community to reach steady state before experimentation can begin. Although we do not report community metabolic output in this work, which can help to define steady state conditions, previous studies have shown that extremely dynamic bacterial communities (unstable composition) can still maintain a functionally stable ecosystem (Fernandez et al., 1999). We are currently applying analytical chemistry techniques to establish the functional capacity and stability of our chemostat-grown communities over time.

Similar to other *in vitro* gut models, we found that microbial richness declined following inoculation of the vessels (Van den Abbeele et al., 2010). Although this means that the dynamics of microbial communities must be assessed in *in vitro* models with the caveat that some microbial taxa will be missing, the development of stable, highly reproducible communities is more important when considering placebo-controlled or multi-treatment experiments on diverse communities simulating the human gut microbiota (Van den Abbeele et al., 2010). To this end, given our findings, we also suggest that it is important to operate a "test" vessel in parallel with a "control" vessel for such experiments, with identical communities set up in each; this would ensure that any changes in modeled complex communities were due to the applied treatment and not simply to microbial adaptation to the chemostat environment.

There is a general lack of information in the literature regarding the ability of individual continuous culture model systems to develop reproducible steady state communities seeded from consecutive fecal samples from the same donor (biological replicates) or different donors (alternative biological replicates). In this work, we compared consecutive fecal donations from 2 healthy donors (donors A and B) over a 5–6 month period. We found that, while technical replicates (a twin-vessel set) were very similar to each other using our experimental parameters, biological replicates for each donor varied to a greater degree. Temporal shifts of the fecal microbiota composition between fecal donations from a single donor have been previously reported (Payne et al., 2012), and it is possible that such shifts may become exaggerated when combined with an *in vivo* to *in vitro* transition, resulting in the development of alternative steady states, as described by the concept of stability landscapes (Relman, 2012). In our work communities from 3 different donors all showed varying degrees of compositional change as they transitioned from an *in vivo* to an *in vitro* environment. Donor-specific variations in diet, transit time, and other physiological conditions mean that *in vitro* models may more closely mimic the gut of one individual over another. Considering all these factors, our work shows that the best approach for minimizing experimental error is to work with multiple vessels seeded from a single fecal donation. Although technically challenging, such an approach is possible if an initial fecal inoculum is produced in a high enough volume to seed multiple vessels, and then frozen for a limited period of time (Feria-Gervasio et al., 2011). This is ultimately more biologically relevant than pooling fecal samples from multiple donors to produce an 'average' ecosystem, a practice which could lead to microbe–microbe interactions not normally seen within the gut microbiota of a single healthy individual, resulting in unstable communities not representative of a normal human gut ecosystem (Payne et al., 2012).

In this work, we used DGGE as a convenient molecular fingerprinting technique that allowed direct comparisons of community structure (between samples and over time). We also used the HITChip approach to profile a subset of samples, and this presented a further opportunity to compare HITChip and DGGE profiling to assess the usefulness of each. DGGE suffers from a lack of sensitivity that can be problematic when comparing complex communities (Gafan and Spratt, 2005). We found that %SI values for the same samples were higher for HITChip fingerprints than for DGGE fingerprints, as previously shown (Rajilic-Stojanovic et al., 2009), and in addition fecal inocula diversity was underrepresented in DGGE profiles relative to HITChip profiles (Table S1). Nevertheless, DGGE is a useful, rapid, low-cost alternative to the HITChip that can be used to target key samples for further analysis, and given these benefits, we also demonstrated that DGGE still provided useful information about a given community that was enhanced, but not contradicted, by HITChip analysis.

In conclusion, our study shows that twin-vessel, single stage chemostats can be inoculated with identical fresh fecal samples from a healthy donor to obtain and maintain stable, diverse, and reproducible

communities that reach steady state by at most 36-days post-inoculation. As expected, it was possible to discern a higher level of community similarity using consecutive donations from the same donor than from different donors. However, we demonstrate that care must be taken when using consecutive fecal samples from the same donor to inoculate successive runs as between-run repeatability may be compromised. A well-characterized distal gut model allows the exploration of the etiology of disease-associated distal gut microbiota in the absence of host involvement. Given the emerging importance of the role of the gut microbiota in the maintenance of health, as well as increasing evidence that gut microbial dysbiosis is associated with several disease states (DuPont and DuPont, 2011), modeling the gut ecosystem *in vitro* will undoubtedly become a valuable experimental tool; here we have attempted to define the limitations of the technique, and to recommend windows of appropriate experimentation.

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