RESEARCH ARTICLE

The composition and metabolic activity of child gut microbiota demonstrate differential adaptation to varied nutrient loads in an *in vitro* model of colonic fermentation

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Abstract

The extent to which the dietary loads of simple sugars, carbohydrates, protein, and fiber impact colonic fermentation in children is unknown. This study assessed the impact of dietary energy on gut microbial communities and metabolism using a three-stage *in vitro* continuous fermentation model. Two separate models, replicating the proximal, transverse, and distal colon regions, were inoculated with immobilized fecal microbiota from one of two female children. Three different fermentation media were designed to examine the effects of prevalent Western dietary trends on gut microbiota. Media compositions reflected obese (high energy), normal weight (normal energy), and anorectic (low energy) child dietary intakes and were alternately supplied to each microbiota during separate fermentation periods. Gut microbiota demonstrated differential metabolic and compositional adaptation to varied substrate availability. High energy medium was strongly butyrogenic, resulting in significant stimulation of butyrate-producing members of clostridia cluster XIVa, whereas members of cluster IV demonstrated greater adaptive variability. Normal and low energy nutrient loads induced significantly less metabolic activity in both microbiota, with low energy medium inducing a broad reorganization of the commensal community structure. These results suggest a concerted metabolic adaptation in response to nutrient load, exercised by different microbial populations, indicating substantial redundancy in gastrointestinal metabolic pathways.

Introduction

A dichotomy in eating behavior, exemplified on one hand by increasing obesity and conversely by calorie-restrictive diets and eating disorders, is on the rise in Western societies. Seventeen per cent of U.S. children aged 2–19 are currently classified as overweight or obese representing a threefold rise since 1980 (Eberhardt *et al.*, 2005). High fructose corn syrup, refined carbohydrates, and dietary fats, all major dietary components not predominating the preagricultural hominin age, are considered hallmarks of the 'Western' diet (Kopelman, 2000; Cordain *et al.*, 2005). The significant nutrient load of these components coupled to reduced physical activity has been associated with the development of type-2 diabetes mellitus, chronic cardiovascular disease, hyperlipidemia, and hyperinsulinemia in children and young adults (Berenson *et al.*, 1998). On the other hand, it is estimated that 10% of adolescents have at some point struggled with an eating disorder (Academy for Eating Disorders, http://www.aedweb.org). Extreme dietary restriction is the characteristic of anorexia nervosa and may include refusal of food intake, avoidance of certain foods, extreme exercise, and purging behaviors (Fernstrom *et al.*, 1994; Affenito *et al.*, 2002). Clinical studies aimed at dissecting anorectic eating behavior have observed severe caloric restriction ranging from 80% to as little as 40% of normal daily energy intakes (Fernstrom *et al.*, 1994; Affenito *et al.*, 2002).

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Despite the abundance of data depicting the impact both obese and anorectic lifestyles impart upon host physiology, there is increasing interest in understanding the impact these highly variable nutrient loads confer upon the resident gut microbiota.

Commensal microorganisms create a beneficial symbiotic relationship with the human host and are primarily responsible for dietary energy extraction as well as attenuation of infection and inflammation (Cummings & Macfarlane, 1997; Backhed *et al.*, 2005). 16S rRNA gene-based sequencing techniques have implied changes within the commensal community structure may potentially contribute to the development of multiple pathologies and metabolic diseases including inflammatory bowel disease and obesity (Elson *et al.*, 1995; Sartor, 1997; Ley *et al.*, 2005). Several studies have highlighted a correlation between the gut microbial community structure and obesity (Eckburg *et al.*, 2005; Ley *et al.*, 2005, 2006; Turnbaugh *et al.*, 2006; Nadal *et al.*, 2009), while other attempted correlations of obesity with changes in individual gut microbial populations have not succeeded (Duncan *et al.*, 2008; Schwiertz *et al.*, 2010). A counter hypothesis suggests that metabolic activity and not composition of the gut microbiota might be more relevant to the development of obesity, and higher levels of short chain fatty acids (SCFA) have been reported in obese vs. normal-weight adult subjects (Schwiertz *et al.*, 2010). Similarly, higher levels of propionate and butyrate were detected in feces of obese children, whereas normal-weight children harbored higher concentrations of intermediate metabolites, suggesting exhaustive substrate utilization by obese microbiota (Payne *et al.*, 2011b). SCFA production resulting from microbial fermentation of hydrolyzed dietary starches, fibers, and sugars indeed provide an additional 10% daily dietary energy to the host, which may be used for *de novo* hepatic triglyceride and glucose synthesis (McNeil, 1984; Scheppach, 1994; Backhed *et al.*, 2004; Flint *et al.*, 2008). As such, a mere increase of 1% in metabolic activity could provide an additional 20 kcal day^{-1} based on a 2000 kcal day^{-1} diet, resulting in nearly 1 kg of weight gain annually. Comparably, little knowledge exists regarding gut microbial fermentation in starved populations with most studies directed at elderly and aging populations (Macfarlane *et al.*, 2004; van Tongeren *et al.*, 2005; Tiihonen *et al.*, 2010). While higher levels of methanogens have been observed in anorexic patients, description of the commensal flora during anorectic feeding and nutrient deprivation remains at large (Armougom *et al.*, 2009).

The aim of this study was to employ a three-stage *in vitro* continuous gut fermentation model to investigate the impact highly variable nutrient loads confer upon the composition and metabolic activity of gut microbiota in children. A major feature of this *in vitro* gut fermentation model is the use of fecal microbiota immobilized within a porous, nonbiodegradable polysaccharide matrix (Cinquin *et al.*, 2006a, b). The resulting fecal beads are used to inoculate a proximal colon-replicating bioreactor (R1) that subsequently supplies the in series-connected transverse (R2) and distal (R3) colon reactors during continuous fermentation (Cinquin *et al.*, 2004, 2006a, b; Zihler *et al.*, 2010). Two continuous intestinal fermentation models were separately inoculated with immobilized feces from one of two female subjects, one of which was categorized as obese based on gender- and age-specific BMI percentiles from the Centers for Disease Control (http://ww.cdc.gov). The impact of variable nutrient loading was assessed by supplying the different fermentation media during separate fermentation periods. A re-stabilization period was performed between each medium switch in each system to attenuate any experimentally induced changes to the gut microbiota prior to introducing new experimental conditions. Gut microbiota compositions and metabolic activities were measured during pseudo-steady-state conditions of each model, consistent with data reporting of *in vitro* gut fermentation systems (Payne *et al.*, 2011a).

Materials and methods

Bacterial strains and culture conditions

Roseburia intestinalis (DSM 14610) and *Blautia hansenii* (DSM 20583) were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Escherichia coli* (ATCC 25288), *Bacteroides fragilis* (ATCC 25285T), *Bifidobacterium longum* (ATCC 15707), and *Lactobacillus rhamnosus GG* (ATCC 53103) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Anaerobic culture methods were used with O_2-free CO_2 sparged Hungate tubes sealed with butyl-rubber stoppers (Dutscher SAS, Brumath, France) for cultivation of *R. intestinalis*, *B. hansenii*, *B. fragilis*, *B. longum*, and *L. rhamnosus GG* (Bryant, 1972). Strains were grown at 37 °C in 10 mL yeast extract-casitone-fatty acid (YCFA) media supplemented with 2% each D-glucose and soluble starch (Sigma, Buchs, Switzerland) as previously described (Duncan *et al.*, 2002a). *Escherichia coli* was grown aerobically overnight at 37 °C in Luria-Bertani (LB) broth.

Fecal sample collection

Single fecal samples came from two female children, ages 8 and 11. Based on gender- and age-specific BMI percentiles from the Centers for Disease Control (http://www.cdc.gov), the younger child was classified as obese (child

A; BMI = 21) and the older as normal weight (child B; BMI = 18). Neither child had been exposed to antibiotic treatment for the past 3 months. Children and parents gave written consent and both consumed occidental diets. Fresh fecal samples were deposited in sterile plastic containers (Dutscher SA). Containers were maintained under anaerobiosis by use of anaerobic sacks (Anaerocult A min; Merck KGaA, Darmstadt, Germany). Fecal samples were delivered to the laboratory per courier within 2 h of defecation and immediately processed for immobilization.

Immobilization procedure

Fecal immobilization was performed under strict anaerobic conditions in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI) containing 5% (v/v) hydrogen and balance N_2. Fecal samples were maintained under anaerobiosis and immobilized in 1–2 mm gel beads composed of gellan (2.5% w/v) and xanthan gums (0.25% w/v) and sodium citrate (0.2% w/v) as previously described (Cinquin *et al.*, 2004, 2006a, b).

Fermentation media preparation

HE, NE, and LE media were based on the medium of Macfarlane *et al.* with slight modifications: addition of granular amylopectin maize starch and D-fructose (Sigma) (Gibson & Wang, 1994; Macfarlane *et al.*, 1998). Dry matter compositions and rational design of each medium are given in Supporting Information, Table S1. pH was adjusted to 5.5 using 5 M HCl prior to autoclaving. Media were placed overnight in an anaerobic chamber (Coy Laboratories) prior to usage.

Batch fermentation

Batch fermentations were performed for 48 h under strict anaerobic conditions in two separate custom-stirred flat bottom glass bioreactors (Sixfors; Ismatec, Glattbrugg, Switzerland) as previously described (Cleusix *et al.*, 2008). Briefly, each R1 (working volume 150 mL) was inoculated with 30% (v/v) freshly prepared fecal beads from one child donor resulting in two replicate fermentations, designated A and B. To facilitate the best nutritional environment for establishment and colonization of each individual microbiota *in vitro*, each replicate fermentation model was initially supplied the medium complementary to the native host environment. Fresh HE medium was supplied to R1 containing fecal beads from the obese child (A) and NE medium supplied to R1 inoculated with fecal beads from the normal-weight child (B). Temperature was set to 37 °C and pH maintained at 5.5 to simulate the adolescent proximal colon (Hernandez *et al.*, 1979).

Continuous fermentation

The switch from batch to continuous fermentation proceeded by connecting the transverse (R2) and distal (R3) reactors, half-filled with sterile fermentation medium, in series to R1. R1 was continuously fed fermentation medium using a peristaltic pump at a flow rate of 37.5 mL^{-1} h. Fermented medium from R1 was transferred to R2 and R3 (working volume 300 mL, respectively) using a multi-head peristaltic pump. Total retention time of each system was 20 h with individual reactor retention times of R1: 4 h, R2: 8 h, and R3: 8 h, representing an average of reported colonic retention times of the adolescent large intestine (Casasnovas *et al.*, 1991; Sutcliffe *et al.*, 2006). Reactor volumes were controlled by adjusting the heights of harvesting tubes. Temperatures were set at 37 °C and pH maintained at 5.5 (R1) and 6.2 (R2) by the addition of 5 M NaOH. pH was monitored but control was deemed unnecessary for R3 as it normalized to distal colon physiological conditions of 6.8. Each 42-day fermentation was divided into four periods of 10 days each (Fig. 1a and b). Media changes occurred after pseudo-steady-state conditions were reached, indicated by < 20 mM change in total SCFA production over 3 days (Fig. S1). Effluent samples (10 mL) were collected daily for metabolite, TGGE (temperature gradient gel electrophoresis), and quantitative PCR (qPCR) analysis.

Metabolite analysis by high-performance liquid chromatography (HPLC)

SCFA were determined by HPLC as previously described (Cleusix *et al.*, 2008). SCFA were analyzed in duplicate daily to check system stability (Fig. S1). Mean metabolite concentrations were calculated from duplicate samples of the last

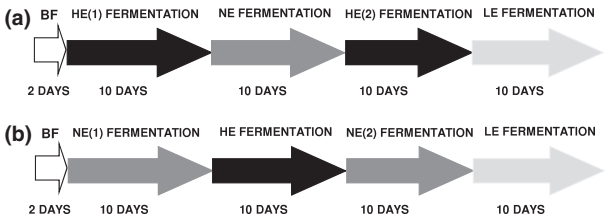

Fig. 1. Overview of intestinal fermentation experiments during different fermentation periods. (a) Fermentation model A. (b) Fermentation model B. BF: batch fermentation; HE(1) high energy medium fermentation; NE: normal energy medium fermentation; HE (2): re-stabilization period with high energy medium; LE: low-energy medium fermentation. (b) Fermentation model B inoculated with normal-weight microbiota: BF: batch fermentation; NE(1) normal energy medium fermentation; HE: high energy medium fermentation; NE(2): re-stabilization period with normal energy medium; LE: low-energy medium fermentation.

3 days of each fermentation period, representing pseudo-steady-state conditions, except where noted (Fig. S1).

Nucleic acid extraction

Genomic DNA was isolated from cultivated strains using the Wizard® Genomic DNA Purification Kit (Promega AG, Dübendorf, Switzerland). The FastDNA Spin Kit for Soil (Qbiogene AG, Basel, Switzerland) was used for fecal and fermentation effluent DNA extractions. DNA was extracted from 250 mg fresh feces and 1 mL fermentation effluent and quantified using the Nanodrop® ND-1000 Spectrophotometer (Witec AG, Littau, Switzerland) at 260 nm.

qPCR analysis

Amplification and detection of DNA by qPCR were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems Europe BV, Zug, Switzerland) using optical-grade 96-well plates. Duplicate sample analysis was routinely performed in a total volume of 25 μL using SYBR® Green PCR Master Mix (Applied Biosystems) containing standardized 100 ng μL^{-1} template DNA diluted either 1 : 10 or 1 : 100 depending upon target species and 200 nM of both forward and reverse primers (Table 1). Total bacteria were quantified using plasmid pLME21 containing a 16S rRNA gene fragment of *Bifidobacterium lactis* aligning to position numbers 15–1432 of the *E. coli* 16S rRNA gene (Brosius *et al.*, 1978; Meile *et al.*, 1997). Standard curves were routinely performed for each qPCR run using serial dilutions of control standard amplicons generated using the primer sets and target species listed in Table 1. PCR conditions consisted of initial activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 60 °C for 30 s. Data from duplicate samples were analyzed using the SEQUENCE DETECTION Software Version 1.4 (Applied Biosystems). Mean copy numbers of populations were calculated from the last 3 days of each fermentation period, representing pseudo-steady-state conditions, except where noted (Fig. S1).

PCR amplification of 16S rRNA gene

DNA (100 ng μL^{-1}) was used to PCR amplify the variable V2-V3 16S rRNA gene sequence using 200 nM universal primers HDA-1GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGGG AC TCC TAC GGG AGG CAG CAG T) and HDA-2 (GTA TTA CCG CGG CTG CTG GCA C) and a modified protocol of Ogier *et al.* (Ogier *et al.*, 2002). PCR reactions consisted of 2× Fermentas PCR Mastermix (1× PCR Buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP and 1 U *Taq* polymerase) diluted 1 : 1 with sterile ultra-pure water (Milipore AG). Samples were amplified on a Biometra Personal CyclerTM (Biometra, Châtel-St-Denis, Switzerland): 94 °

Table 1. Group and species-specific 16S rRNA gene-targeted primers used in this study

Target organism	5′–3′ sequence	Primer	Standard	References
Total bacteria	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	Eub 338F Eub 518R	Plasmid pLME21, containing *Bifidobacterium lactis* 16S rDNA	Meile *et al.* (1997)
Bacteroides	GAA GGT CCC CCA CAT TG CGC KAC TTG GCT GGT TCA G	Bac303F Bfr-Femrev	*B. thetaiotaomicron* 16S rRNA	Ramirez-Farias *et al.* (2009)
Firmicutes	GGA GYA TGT GGT TTA ATT CGA AGC A AGC TGA CGA CAA CCA TGC AC	Firm934F Firm1060R	*R. intestinalis* 16S rRNA	Guo *et al.* (2008)
Roseburia/E. rectale	GCG GTR CGG CAA GTC TGA CCT CCG ACA CTC TAG TMC GAC	RrecF Rrec630mR	*R. intestinalis* 16S rRNA	Ramirez-Farias *et al.* (2009)
E. hallii	GCG TAG GTG GCA GTG CAA GCA CCG RAG CCT ATA CGG	EhalF EhalR	*E. hallii* 16S rRNA	Ramirez-Farias *et al.* (2009)
F. prausnitzii	GGA GGA AGA AGG TCT TCG G AAT TCC GCC TAC CTC TGC ACT	FPR-2F Fprau645R	*F. prausnitzii* 16S rRNA	Ramirez-Farias *et al.* (2009)
Lactobacillus	AGC AGT AGG GAA TCT TCC A CGC CAC TGG TGT TCY TCC ATA TA	F_Lacto 05 R_Lacto 04	*L. delbrueckii* 16S rRNA	Furet *et al.* (2009)
Bifidobacterium phosphoketolase	ATC TTC GGA CCB GAY GAG AC CGA TVA CGT GVA CGA AGG AC	xfp-fw xfp-rv	xfp amplicon	Cleusix *et al.* (2010)
Enterobacteriaceae	CAT TGA CGT TAC CCG CAG AAG AAG C CTC TAC GAG ACT CAA GCT TGC	Eco1457F Eco1652R	*E. coli* 16S rRNA	Frank *et al.* (2007)
Sulfate-reducing bacteria	CGG CGT TGC GCA TTT YCA YAC VVT GCC GGA CGA TGC AGH TCR TCC TGR WA	dsrA_290F dsrA_660R	*D. piger* dsrA	Pereyra *et al.* (2010)
Veillonella spp.	TGC TAA TAC CGC ATA CGA TCT AAC C GCT TAT AAA TAG AGG CCA CCT TTC A	Vpa_X84005_F Vpa_X84005_R	*Veillonella parvula* 16S rRNA	Leung *et al.* (2007)

C for 5 min, 35 cycles of 94 °C for 3 min, 58 °C for 30 s, 68 °C for 1 min and finally 68 °C for 7 min.

TGGE analysis of PCR amplicons

TGGE gels (16 cm × 16 cm × 1 mm) were composed of 6% acrylamide/bis-acrylamide 37.5 : 1 (Sigma), 7 M urea (Sigma), and 1.5× Tris Acetate EDTA (TAE) buffer (Muyzer *et al.*, 1993). TGGE was performed with 50 ng PCR amplicons using a Dcode universal mutation system (Bio-Rad, Reinach, Switzerland). A custom marker was created by mixing equal concentrations of PCR amplicons of *B. fragilis*, *E. coli*, *R. intestinalis*, *B. hansenii*, *B. longum*, and *L. rhamnosus GG*. Electrophoresis conditions included a prerun of 20 min at 20 V followed by 16 h at 70 V in 1.5× TAE buffer. Temperature was increased from 66 to 70 °C using a ramp rate of 0.4. Gels were stained for 30 min in ethidium bromide and destained for 1 h in dH_2O prior to imaging.

TGGE band cloning and sequencing

TGGE bands were excised and stored overnight at 4 °C in 10 mM Tris-EDTA (TE) buffer. DNA was precipitated overnight at −20 °C by the addition of 0.1 volume 3 M sodium acetate (pH 5.5) and 3 volumes 100% ethanol. PCR amplification conditions of precipitated DNA were identical to those described above except the forward primer HDA-1 lacked the GC-clamp. Amplicons were ligated into the pGEM-T Easy Vector (Promega). One microlitre of ligation product was mixed with 40 μL XL-1 blue electrocompetent cells (Stratagene, Amsterdam, the Netherlands) on ice. Electroporation was performed at 2500 V. Cells were immediately transferred to 960 μL 37 °C SOC media and incubated at 37 °C for 1 h. 100 μL of serial dilutions were plated on LB agar plates (1.5% w/v) containing 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside [X-gal; final concentration of 80 μg mL^{-1}, prepared in dimethylformamide (DMF)] and isopropyl-1-thio-β-D-galactopyranoside (IPTG, final concentration of 20 mM, prepared in sterile dH_2O). Plates were incubated aerobically overnight at 37 °C. Multiple clones per TGGE band were grown overnight in 3 mL LB broth supplemented with 6% ampicillin. Plasmids were isolated using the GeneJETTM Plasmid Miniprep Kit (Fermentas, Le Mont-sur-Lausanne, Switzerland). Insert size and presence were verified by PCR. Plasmid inserts were sequenced (Microsynth AG, Balgach Switzerland) using the T7 sequencing primer. Sequences were compared to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) using BLAST (Altschul *et al.*, 1997). Sequences with percentage identity of 97% or higher were considered to represent the same species.

Statistical analysis

Results are presented as means ± SE. Mean copy numbers were transformed to their base 10 logarithmic values for variance homogeneity. One-way ANOVA was performed to test the effects of varying nutrient loads on bacterial populations and SCFA production using JMP 8.0 (SAS Institute Inc., Cary, NC). When significant differences were found below $P < 0.05$, treatment means were compared using the Tukey–Kramer HSD test. Principal component analysis of mean-centered copy number data and metabolite concentrations were performed with the *prcomp* function of the *stats* package of the R statistical language, version 2.13.1 (http://www.r-project.org/).

Results

Microbial diversity of fecal microbiota

qPCR results of ten microbial populations plus total bacteria present in feces of both child donors are given in Table 2. With the exception of lactobacilli in child A (obese) and *Veillonella* in child B (normal weight), both of which were not detected presumably due to population numbers below the 3 $\log_{10}$ copies g^{-1} detection limit of the qPCR assay, qualitative diversity of both microbiota was highly similar. Quantitative differences between the two microbiota were observed for only a few evaluated populations. Higher populations of *Firmicutes*, *Eubacterium hallii*, and *Roseburia/Eubacterium rectale* were observed in feces from child A and higher sulfate-reducing bacteria (SRB), *Bifidobacteria* in child B (Table 2).

Effect of high energy nutrient loading on the microbial community structure

The impact of HE medium fermentation was examined for the adaptation of the microbial community structure to high energy availability. Samples from the last 3 days of each fermentation period were routinely chosen for all community analyses as pseudo-steady-state fermentation conditions had been established (Fig. S1). Results were compared to those obtained during NE medium fermentation. The qualitative community structure remained similar to feces with neither lactobacilli nor *Veillonella* detected in models A and B, respectively, likely a function of population numbers below the assay detection limit of 3 $\log_{10}$ copies g^{-1} (Table 2). *Bacteroides* were reproducibly and significantly lower in proximal colon reactors (6.1 and 7.8 $\log_{10}$ copies 16S rRNA gene g^{-1} fermenta-

Table 2. Bacterial populations measured by qPCR in effluent samples in each reactor of replicate *in vitro* fermentation models during HE, NE, and LE media fermentation

Fermentation period	R1[a]			R2[a]			R3[a]			FECES[b]	
Bacterial population	A	B	SE[c]	A	B	SE[c]	A	B	SE[c]	A	B
HE											
Total bacteria	11.1	11.1	±0.1	11.3	11.3	±0.2	11.3	11.2	±0.2	11.0	11.1
Bacteroides	6.1	7.8	±0.3	10.1	9.9	±0.3	10.2	10.0	±0.2	9.7	9.6
Firmicutes[d]	9.8	9.9	±0.1	9.9*	10.1	±0.3	9.9**	9.7	±0.1	10.0	9.8
Roseburia sp./*E. rectale*[d]	7.6	9.5**	±0.2	7.5	9.7**	±0.3	7.3	9.0*	±0.2	9.8	8.4
E. halii[d]	8.1**	7.9	±0.3	8.8**	9.0	±0.1	8.7***	8.8	±0.2	7.4	7.1
F. prausnitzii[d]	4.8	3.8	±0.3	7.6***	7.1*	±0.1	7.6***	7.2	±0.2	9.1	9.0
Lactobacillus	ND	6.8	±0.3	ND	7.3	±0.2	ND	7.3	±0.1	ND	7.5
Bifidobacterium[d]	9.5**	8.7	±0.2	9.5**	9.1	±0.3	9.4**	9.3	±0.2	7.3	8.1
Enterobacteriaceae	6.9	7.8	±0.2	8.6	9.1*	±0.3	8.9**	8.1	±0.2	5.3	5.3
Sulfate-reducing bacteria	3.7	3.7	±0.01	3.1	3.1	±0.4	3.5	3.5	±0.2	5.6	6.6
Veillonella spp.	ND	ND		3.1	ND	±0.1	3.0***	ND	±0.08	9.0	ND
NE											
Total bacteria	10.9	11.1	±0.3	11.2	11.1	±0.3	11.3	11.1	±0.2		
Bacteroides[e]	7.6‡‡	8.4‡	±0.2	10.4‡‡	9.9	±0.07	10.4	10.0	±0.2		
Firmicutes	9.8	9.9	±0.5	9.3	10.0	±0.1	9.4	9.9	±0.2		
Roseburia sp./*E. rectale*[e]	9.8‡‡	9.1	±0.2	9.0‡‡‡	9.1	±0.1	8.9‡‡‡	8.9	±0.1		
E. hallii[e]	7.4	8.7‡‡	±0.5	8.2	9.3‡	±0.2	8.3	9.1‡	±0.3		
F. prausnitzii	5.0	3.7	±0.3	7.1	7.0	±0.2	7.3	7.1	±0.2		
Lactobacillus	ND	7.5	±0.2	ND	6.9	±0.1	ND	7.3	±0.2		
Bifidobacterium[e]	8.7	9.3‡	±0.2	9.3	9.2	±0.1	9.2	9.3	±0.1		
Enterobacteriaceae	6.8	7.9	±0.5	8.8	8.5	±0.2	9.6	7.7	±0.8		
Sulfate-reducing bacteria	3.8	3.6	±0.1	3.1	3.7	±0.1	3.5	3.2	±0.5		
Veillonella spp.	ND	ND		3.4	ND	±0.07	ND	ND			
LE											
Total bacteria	10.3	10.6	±0.08	10.5	10.7	±0.04	10.3	10.6	±0.1		
Bacteroides[f]	9.4§§§	9.7§§§	±0.2	10.1	10.2	±0.1	10.4	10.2	±0.1		
Firmicutes[f]	10.6§§§	10.6	±0.1	10.1§	10.5§§	±0.2	9.4	10.2§	±0.2		
Roseburia sp./*E. rectale*[f]	10.1§	10.2	±0.3	9.5§§§	10.3§§§	±0.2	9.1§§	9.8§§§	±0.3		
E. hallii	7.4	8.1	±0.3	7.6	8.0	±0.3	7.2	7.9	±0.2		
F. prausnitzii[f]	4.6	4.7§§§	±0.3	7.0	6.6	±0.3	7.3	6.5	±0.3		
Lactobacillus[f]	7.9§§§	8.3	±0.2	8.9§§§	7.9	±0.5	8.5§§§	7.9	±0.5		
Bifidobacterium	7.5	9.1	±0.3	7.9	8.6	±0.3	7.9	8.2	±0.3		
Enterobacteriaceae[f]	7.1	8.9	±0.1	9.1	9.1§§	±0.2	9.6	8.8§	±0.4		
Sulfate-reducing bacteria[f]	3.1	3.2	±0.2	4.1§§	3.5§§§	±1.0	6.5	7.0§	±0.4		
Veillonella spp.[f]	5.0§§§	ND		4.8§§§	ND	±0.1	ND	ND	±0.2		

R1, proximal colon reactor; R2, transverse colon reactor; R3, distal colon reactor.

[a]Data are mean $\log_{10}$ copies 16S rRNA gene g^{-1} fermentation effluent; ND: not detected.

[b]Data are mean $\log_{10}$ copies 16S rRNA gene g^{-1} feces; ND: not detected.

[c]SE: standard errors of the mean of replicates A and B.

[d]Bacterial populations that were significantly higher during HE vs. NE medium fermentation are denoted by significance level: *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$.

[e]Bacterial populations that were significantly higher during NE vs. HE medium fermentation are denoted by significance level: ‡$P < 0.05$, ‡‡$P < 0.001$, ‡‡‡$P < 0.0001$.

[f]Bacterial populations significantly higher during LE vs. NE medium fermentation are denoted by significance level: §$P < 0.05$, §§$P < 0.01$, §§§$P < 0.005$.

tion effluent, model A and B, respectively) and is a direct result of the low pH (5.5) maintained in R1 for proximal colon simulation. *Bacteroides* numbers rebounded in both R2 and R3 where circumneutral pH conditions (e.g. pH 6–7.5) favorable for *Bacteroides* growth prevailed; however, high nutrient loading produced no significant effect on this population (Table 2; HE). A significant increase in *Bifidobacteria* ($P < 0.005$) was observed in all three reactors of model A. *Firmicutes* demonstrated a similar response to HE medium fermentation. In particular, *Firmicutes* in R2 and R3 of model A demonstrated a preference for high energy conditions (Table 2; HE). Contributing to the increase in *Firmicutes* during HE medium fermentation was *Roseburia*/*E. rectale* and *E. hallii*, both

butyrate-producing members of clostridia cluster XIVa. Significant stimulation within this clostridia cluster was observed with individual subpopulation differences between replicate models. *Roseburia/E. rectale* were significantly higher (*P*-values ranging from < 0.05 to < 0.005) in model B and *E. hallii* (*P*-values ranging from < 0.005 to < 0.0001) in model A (Table 2; HE). Conversely, *F. prausnitzii*, a butyrate-producing member of clostridia cluster IV, demonstrated a pH-specific response to high energy conditions. *F. prausnitzii* were significantly elevated in R2 and R3 ($P < 0.05$ model B and $P < 0.0001$ model A, respectively) however remained significantly low in R1. This observation is analogous to the low *Bacteroides* populations observed in R1 and is also presumably a function of the low pH maintenance in this reactor.

Effect of normal energy nutrient loading on the microbial community structure

NE medium supplementation produced a favorable response by *Bacteroides*, resulting in significantly higher populations in R1 ($P < 0.05$ to $P < 0.001$) and also in R2 in comparison with high energy conditions (Table 2; NE). Clostridia cluster XIVa again demonstrated individual subpopulation differences between replicate models. A significant increase in *Roseburia/E. rectale* during normal nutrient availability was observed in all three reactors of model A (9.8, 9.0, and 8.9 $\log_{10}$ copies 16S rRNA gene g^{-1} fermentation effluent, respectively), with this increase corresponding to nearly 2 $\log_{10}$ in comparison with the HE medium populations (7.6, 7.5, and 7.3 $\log_{10}$ copies 16S rRNA gene g^{-1} fermentation effluent, respectively). *Eubacterium hallii* were more abundant during normal energy conditions in all three reactors of model B (8.7, 9.3, and 9.1 $\log_{10}$ copies 16S rRNA gene g^{-1} fermentation effluent, respectively) vs. the HE medium population (7.9, 9.0, and 8.8 $\log_{10}$ copies 16S rRNA gene g^{-1} fermentation effluent, respectively). Neither lactobacilli nor *Veillonella* were detected during NE medium fermentation in models A and B, respectively. *Veillonella* populations in model A demonstrated a mixed and pH-dependent response to both high and normal energy conditions, particularly in R3 (Table 2; HE and NE). Furthermore, *Veillonella* remained relatively stable in R2 but were not detected in R1 during either nutrient load, analogous to the absence of low pH-intolerant *Bacteroides* and *F. prausnitzii* in this proximal colon reactor.

High energy nutrient loading confers a butyrogenic effect on metabolic activity

The effect of high energy nutrient loading was investigated for alterations to the commensal metabolic activity. SCFA concentrations in fermentation effluent samples from all three reactors were analyzed during pseudo-steady-state conditions (Table 3). SCFA concentrations gradually increased from R1 to R3 because of the accumulation of product in the system, consistent with operation of this *in vitro* model design (Cinquin *et al.*, 2004, 2006a, b). Propionate production was mostly unaffected by nutrient load with both HE and NE media resulting in similar propionate levels (Table 3). HE medium fermentation resulted in significantly higher acetate production in model A. Butyrate production was most significantly stimulated (*P*-value range of < 0.05 to < 0.005) across all three reactors during high energy conditions (Table 3). Interestingly, increased butyrate production in model B during HE medium fermentation occurred in the presence of similar acetate production during both NE and HE medium fermentation suggesting preferential conversion of acetate to butyrate during high energy availability. Overall, metabolic activities during HE medium fermentation were increased by approximately 30% in comparison with normal energy conditions, which is in agreement with the 25% increase of fermentable carbohydrate added to the NE medium in creating the HE medium (Table S1).

Low substrate availability produces a distinct response in both microbial community adaptation and metabolic activity

The response of individual gut microbial populations during nutrient deprivation was investigated using a low energy medium consisting of approximately 40% less available fermentable substrate vs. NE medium (Table S1). Quantitative results demonstrated a surprising number of populations thriving during low energy conditions (Table 2; LE). Significant increases in *Firmicutes*, *Roseburia/E. rectale*, and SRB were all observed, although the level of significance was highly varied and dependent upon reactor conditions (Table 2; LE). Interestingly, R1 demonstrated increased populations in *Bacteroides*, *F. prausnitzii*, and *Veillonella*, all pH-sensitive populations previously demonstrating repressed number under both normal and high nutrient conditions (Table 2; LE). *Roseburia/E. rectale* increased on average half a $\log_{10}$ in comparison with normal energy conditions. SRB represented the most responsive population to reduced energy availability with > 2 $\log_{10}$ increases in R2 and R3 (Table 2; LE). A similar trend was observed for *Veillonella* in model A with significant increases (*P*-values ranging from < 0.005 to < 0.001) in all three reactors. A surprising and unexpected lactobacilli bloom was observed in model A were population numbers went from below detection in feces, HE, and NE nutrient loads to approximately 8 $\log_{10}$ copies 16S rRNA

Table 3. SCFA concentrations and ratios in effluent samples of each reactor of replicate *in vitro* fermentation models during HE, NE, and LE medium fermentation

Fermentation period	R1		% SCFA			R2		% SCFA			R3		% SCFA			FECES		% SCFA	
Metabolite[a]	A	B	A	B	SE[b]	A	B	A	B	SE[b]	A	B	A	B	SE[b]	A	B	A	B
HE																			
Total SCFA	124.8*	128.7	100	100	±3.3	167.9**	173.6	100	100	±5.9	191.8**	177.9	100	100	±4.1	77.2	108.4	100	100
Acetate	70.7*	52.9	56.6	41.1	±1.4	96.8**	94.1	57.6	54.2	±2.1	114.5**	99.4	59.7	55.9	±1.6	41.8	56.4	54.1	52.0
Butyrate	51.5*	73.1**	41.3	56.8	±1.5	60.5**	64.3**	36.0	37.0	±3.2	62.8**	62.2*	32.7	34.9	±2.1	25.5	28.1	33.0	25.9
Propionate	2.6	2.7	2.1	2.1	±0.4	10.7	15.3	6.4	8.8	±0.6	14.4	16.2	7.6	9.2	±0.5	9.9	23.9	12.9	22.1
NE																			
Total SCFA	102.1	116.6	100	100	±6.5	147.1	154.5	100	100	±2.3	165.8	149.1	100	100	±1.2				
Acetate	56.1	72.2	54.8	70.0	±4.1	76.8	97.1	52.2	62.8	±1.0	93.9	91.4	56.6	61.3	±7.6				
Butyrate	43.1	41.6	42.2	35.6	±0.6	53.8	40.6	36.6	26.3	±0.9	53.9	44.1	32.5	29.6	±1.6				
Propionate	3.1	2.8	3.0	2.4	±1.8	16.5	16.8	11.2	10.9	±0.4	17.9	13.7	10.9	9.1	±2.4				
LE																			
Total SCFA	48.0†††	48.6††††	100	100	±2.5	64.0†††	69.4††††	100	100	±3.2	74.1†††	78.8††	100	100	±2.7				
Actetate	27.4†††	19.8†	57.1	40.7	±1.7	36.8††	39.2†††	57.5	56.5	±2.4	46.1†††	46.7††	62.2	59.2	±1.9				
Butyrate	18.8†	24.9	39.1	51.2	±0.6	21.6†††	22.8††††	33.8	32.9	±0.7	21.5†††	23.5†††	29.0	29.8	±0.7				
Propionate	1.8	3.9††††	3.8	8.1	±0.2	5.6††	7.4††††	8.7	10.6	±0.1	6.5†††	8.6††	8.8	11.0	±0.2				

R1, proximal colon reactor; R2, transverse colon reactor; R3, distal colon reactor.

SCFA concentrations significantly higher during HE vs. NE medium fermentation are denoted by significance level: *$P < 0.05$ and **$P < 0.005$.

SCFA concentrations significantly reduced during LE vs. NE medium fermentation are denoted by significance level: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, $^{\dagger\dagger\dagger}P < 0.001$, and $^{\dagger\dagger\dagger\dagger}P < 0.0001$.

[a]Data are expressed as mM and are mean values calculated for the last 3 days of each fermentation period.

[b]SE: standard errors of the mean of replicates A and B.

gene g^{-1} fermentation effluent, reaching numbers consistently observed in model B (Table 2).

Metabolic activity of commensal microbiota demonstrated a concerted adaptation in response to reduced nutrient availability. Total SCFA, acetate, butyrate, and propionate were all negatively affected with significant (P-values of < 0.0001) production decreases ranging from 35% to 50% in comparison with both high and normal nutrient loads, in agreement with the 40% reduction in nutrient availability (Table 3).

Qualitative assessment of biodiversity by TGGE

TGGE profiles of pseudo-steady-state HE, NE, and LE fermentation periods in each reactor of models A and B are illustrated in Figs 2a–c and 3a–c, respectively. TGGE was selected to qualitatively demonstrate changes to the biodiversity as a function of varied substrate availability. Bands displaying significant changes in relative intensity were chosen for cloning and sequence analysis to identify species impacted by each fermentation medium as a complement to quantitative qPCR assessment. Changes in nutrient loading resulted in highly visible shifts within the biodiversity in all three reactors of both replicate models, with the most evident changes in R1 (Figs 2a and 3a). Sequence analysis of clones from the HE vs. NE fermentation period in both R1 identified members of the *Firmicutes* as predominately impacted, qualitatively confirming results obtained with qPCR. *Enterococcaceae* and *Clostridiaceae* (Table 4; model A clones a–c) and *Eubacterium eligens* ATCC 27750 and *E. rectale* ATCC 33656 (Table 4; model B clones c–d) were stimulated during high- vs. normal energy nutrient loading. Analysis of clones obtained during NE medium fermentation confirmed quantification of higher populations of *E. hallii* and *Bifidobacterium* in model B (Table 4; model B clones a, and b, respectively) and supported the qPCR data for *Roseburia* sp./*E. rectale* in model A (Fig. 2a–c and Table 4; model A clones d–e). Furthermore, the decrease in *Bifidobacterium* in R1 of model A during NE medium fermentation was substantiated by cloning and sequencing a band with decreased relative intensity corresponding to *Bifidobacterium adolescentis* L2-32 in model A (Fig. 2a and Table 4; model A clone f). TGGE profiling of the LE medium vs. NE medium also supports the qPCR quantified increase of *Bacteroides*, with the appearance of a band corresponding to species with high homology to *Bacteroides ovatus* ATCC 8483 in R1 of model A (Fig. 2a; clone g). It should be noted that all sequence data reported are partial and the 200 bp sequence should be treated with some caution and used primarily as a guide. However, the region of sequence used for analysis includes the variable V3 region of the bacterial 16S rRNA gene, previously

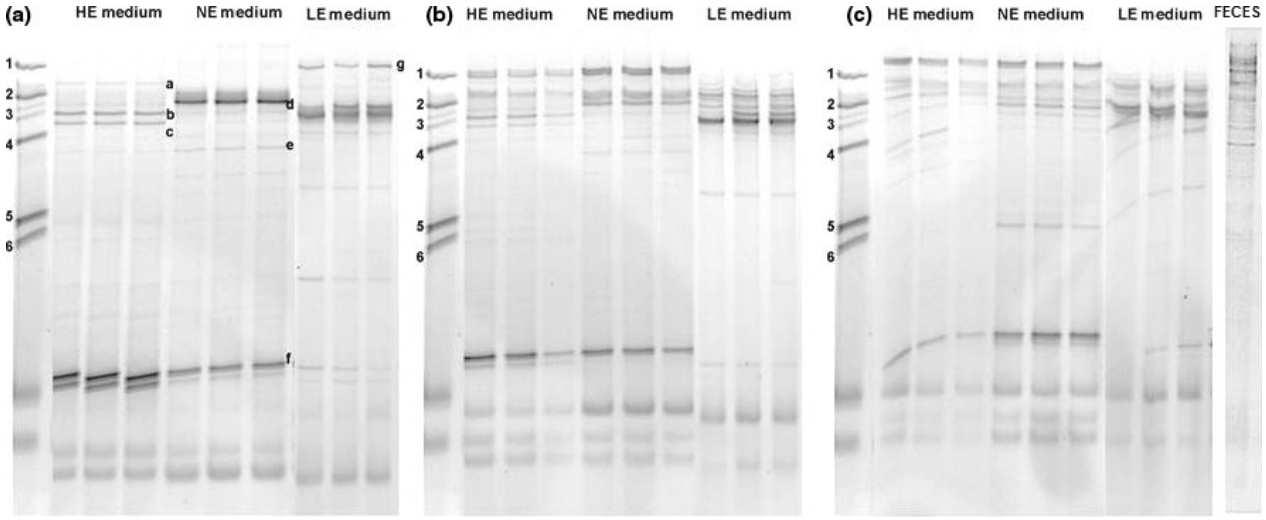

Fig. 2. TGGE profiles of fermentation model A during each nutrient load. (a) R1; (b) R2; (c) R3. HE medium: high energy fermentation; NE medium: normal energy fermentation; LE medium: low energy fermentation. FECES: fecal TGGE profiles. Marker strains denoted by numbers on right: 1: *Bacteroides fragilis*; 2: *Roseburia intestinalis*; 3: *Blautia hansenii*; 4*: Escherichia coli*; 5: *Bifidobacterium longum*; 6*: Lactobacillus rhamnosus GG*. Bands excised for cloning denoted by lower case letters.

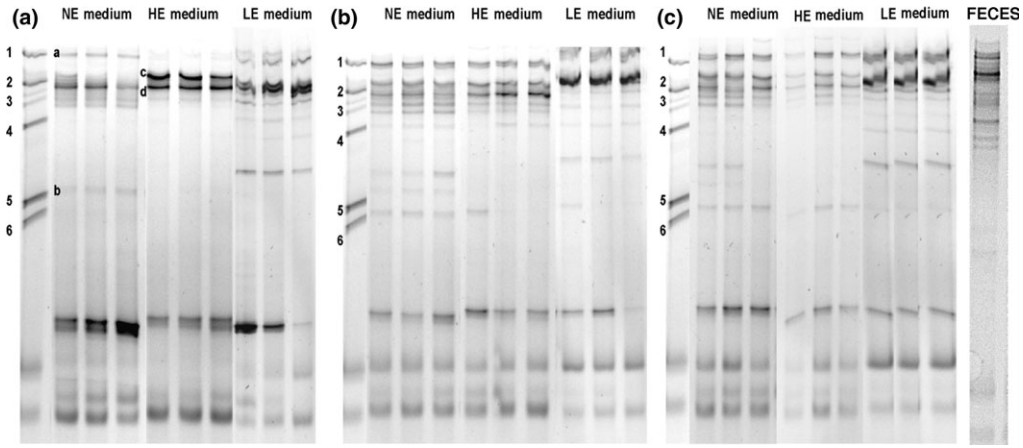

Fig. 3. TGGE profiles of fermentation model B during each nutrient load. (a) R1; (b) R2; (c) R3. NE medium: normal energy fermentation; HE medium: high energy fermentation; LE medium: low energy fermentation. FECES: fecal TGGE profiles. Marker strains denoted by numbers on right: 1: *Bacteroides fragilis*; 2: *Roseburia intestinalis*; 3: *Blautia hansenii*; 4: *Escherichia coli*; 5: *Bifidobacterium longum*; 6*: Lactobacillus rhamnosus GG*. Bands excised for cloning denoted by lower case letters.

demonstrated to be an excellent indicator of phylogeny (McCaig *et al.*, 2001; Jensen *et al.*, 2004).

Multivariate analysis of nutrient loading on community structure and metabolic activity

Principal component analysis (PCA) was applied to mean-centered copy number data and metabolite concentrations to reveal intrinsic treatment patterns within data. Lactobacilli and *Veillonella* were excluded from analysis as their populations were not detected in feces of both child A and B, respectively. PCA demonstrated a replicative robustness of each model, highlighted by the clustering of the separately simulated transverse and distal colon region (Figs 4a and 5a). R1 consistently failed to demonstrate any specific clustering ability, a likely influence of the low population numbers observed for *Bacteroides* and *F. prausnitzii* as function of low pH maintenance in this reactor. Nutrient loading resulted in a differential impact on community structure and metabolic activity. A concerted

Table 4. Closest nucleotide matches of GenBank sequences to excised TGGE bands

Clone	Model	Fermentation period	Maximum similarity to organism	GenBank accession number	% Similarity	Phylogenetic group
a	A	HE	*Enterococcus faecalis* S613	ADDP00000000	99	*Enterococcaceae*
b	A	HE	*Clostridium* sp. M62/1 1	ACFX00000000	99	*Clostridiaceae*
c	A	HE	*Clostridium sp*. SS2/1	ABGC00000000	99	*Clostridiaceae*
d	A	NE	*Eubacterium rectale* ATCC 33656	NC 012781	99	*Eubacteriaceae*
e	A	NE	*E. rectale* ATCC 33656	NC 012781	100	*Eubacteriaceae*
f	A	NE	*Bifidobacterium adolescentis L2-32*	AAXD00000000	99	Actinobacteria
g	A	LE	*Bacteroides ovatus* ATCC 8483	AAXF00000000	99	*Bacteroidaceae*
a	B	NE	*Eubacterium hallii* DSM 3353	ACEP00000000	100	*Eubacteriaceae*
b	B	NE	*B. adolescentis* ATCC 15703	NC 008618	100	Actinobacteria
c	B	HE	*Eubacterium eligens* ATCC 27750	NC 012780	98	*Eubacteriaceae*
d	B	HE	*E. rectale* ATCC 33656	NC 012781	98	*Eubacteriaceae*

and distinct community clustering pattern was observed for LE medium fermentation only (Fig. 5a). Conversely, R2 and R3 of both NE and HE medium fermentation aggregated together, suggesting that both normal and overabundant substrate loads impose a similar impact upon circumneutral microbial community structures. Random aggregation of R1, regardless of nutrient load, was observed (Fig. 4). PCA of SCFA production during variable substrate availability demonstrated a significant nutrient load-specific effect (Fig. 5a). With the exception of R1 supplied HE medium, several distinctive clustering patterns were identified. Clusters aggregated as a function of the metabolic response to HE, NE, and LE nutrient supply and simulated colon region (Fig. 5a).

Discussion

This study represents the first description of the impact highly variable nutrient loads confer upon the commensal microbiota in children using a three-stage *in vitro* model of colonic fermentation. A major challenge of this study was designing fermentation media to accurately simulate the various dietary conditions and respective nutrient loads. The composition of each medium was designed after thorough investigation of published dietary records of obese, normal-weight, and anorexic children. While high levels of fat intake are generally associated with the 'Western' diet and obesity, *in vitro* fermentation models are ill-equipped for testing diets with fat content, as aggregation of fat globules results in consistent blockage of the fermentation pumps. However, dietary investigation revealed major differences in protein, fiber, and sugar consumption between obese and normal-weight children, justifying the decision to focus on these nutritional compounds (Fernstrom *et al.*, 1994; Ludwig *et al.*, 1999; Nicklas *et al.*, 2001; Affenito *et al.*, 2002; Aeberli *et al.*, 2007a, b; Wright *et al.*, 2007). Significantly, more protein ($P < 0.05$), lower fiber ($P < 0.05$), and twice the amount of fructose were identified as major constituents of obese vs. normal-weight child dietary loads (Beyer *et al.*, 2005; Aeberli *et al.*, 2007a, b). Anorectic diets, characterized by severe caloric restriction (40–80% of total daily energy intake), demonstrated increased carbohydrate/protein ratios (Fernstrom *et al.*, 1994; Affenito *et al.*, 2002). Based on this information, we decided to utilize a previously published and validated fermentation medium, supplemented with D-fructose and amylopectin maize starch, as NE medium from which the HE and LE media were subsequently based (Macfarlane *et al.*, 1998). Media compositions and justifications are accurately described in Table S1.

Feces used in this study came from two female children, highly similar in composition with the exception of lactobacilli and *Veillonella* (Table 2). As one of the two subjects was deemed to be obese, each replicate *in vitro* fermentation model was initially supplied the fermentation medium containing the nutrient load most similar to the corresponding host gastrointestinal environment. The rationale behind this experimental design was to provide the microbiota the best opportunity to effectively quantitatively and qualitatively establish their populations *in vitro*. Comparison of initial fermentation periods (Fig. 1a and b, HE(1), and NE(1), respectively) with fecal microbiota demonstrates the effectiveness of this approach, as population numbers were highly comparable (Table 2). A re-stabilization period was performed between switching experimental nutrient loads to attenuate any experimentally induced changes to the microbiota composition (Table S2) and metabolic activity (Table S3).

High energy nutrient loading was highly butyrogenic with butyrate production significantly higher (P-values ranging from < 0.05 to < 0.005) in comparison with normal and low energy conditions (Table 3). *Roseburia/E. rectale* and *E. hallii*, both subpopulations of clostridia cluster XIVa, responded favorably to increased nutrient availability. Surprisingly, different relative contributions

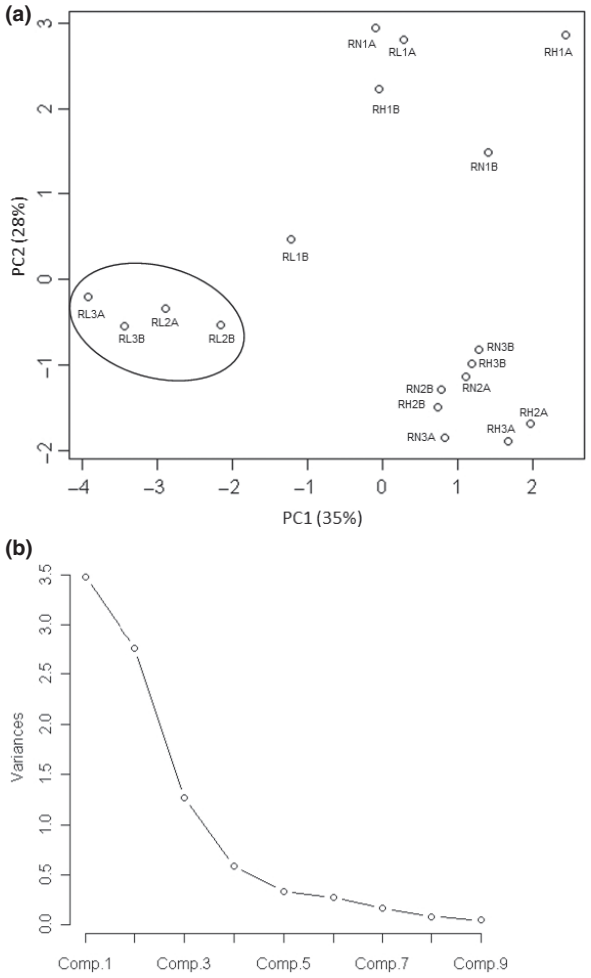

Fig. 4. (a) PCA plot of the effect of nutrient load on community structure and (b) Scree plot of PCA component variances. RN1A: NE medium R1 model A; RN2A: NE medium R2 model A; RN3A: NE medium R3; model A; RH1A: HE medium R1 model A; RH2A: HE medium R2 model A; RH3A: HE medium R3 model A; RL1A: LE medium R1 model A; RL2A: LE medium R2 model A; RL3A: LE medium R3 model A; RN1B: NE medium R1 model B; RN2B: NE medium R2 model B; RN3B: NE medium R3; model B; RH1B: HE medium R1 model B; RH2B: HE medium R2 model B; RH3B: HE medium R3 model B; RL1B: LE medium R1 model B; RL2B: LE medium R2 model B; RL3B: LE medium R3 model B. Circles denote nutrient load-dependent clusters.

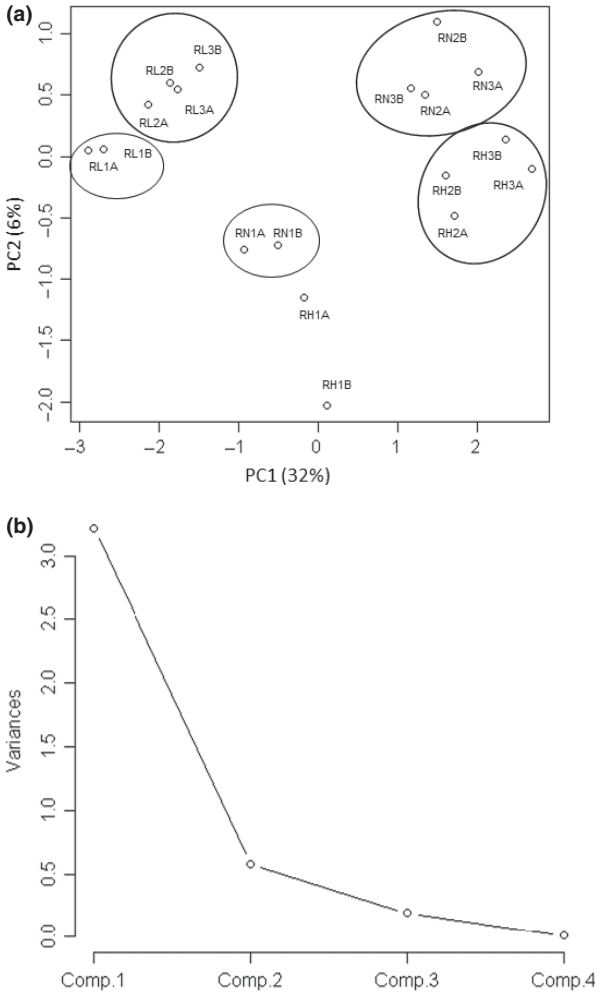

Fig. 5. (a) PCA plot of the effect of nutrient load on metabolic activity and (b) Scree plot of PCA component variances. RN1A: NE medium R1 model A; RN2A: NE medium R2 model A; RN3A: NE medium R3; model A; RH1A: HE medium R1 model A; RH2A: HE medium R2 model A; RH3A: HE medium R3 model A; RL1A: LE medium R1 model A; RL2A: LE medium R2 model A; RL3A: LE medium R3 model A; RN1B: NE medium R1 model B; RN2B: NE medium R2 model B; RN3B: NE medium R3; model B; RH1B: HE medium R1 model B; RH2B: HE medium R2 model B; RH3B: HE medium R3 model B; RL1B: LE medium R1 model B; RL2B: LE medium R2 model B; RL3B: LE medium R3 model B. Circles denote nutrient load-dependent clusters.

of each subpopulation were observed between replicates. *Roseburia*/*E. rectale* were primarily stimulated and likely responsible for the increased butyrate production in model B during high energy conditions, whereas *E. hallii* predominated in model A (Table 2). Normal nutrient conditions resulted in the opposite effect on both subpopulations with *E. hallii* responding favorably in model B and *Roseburia*/*E. rectale* in model A. However, *F. prausnitzii*, a butyrate-producing member of clostridia cluster IV, demonstrated a pH-dependent response to high energy load with low growth observed in R1. Despite rebounding population numbers in R2 and R3, *F. prausnitzii* produced a concerted and lesser response to HE medium in comparison with both *Roseburia*/*E. rectale* and *E. hallii* (Table 2). Gram-positive anaerobes demonstrate great variability in their ability to thrive at pH < 6.7 (Duncan *et al.*, 2009). As such the demonstrated differences between clostridia cluster XIVa and IV in thriving at low pH suggest variable species-specific adaptive capacities. Furthermore, these results suggest a

potential struggle between intrinsic microbiota regulatory factors and nutrient availability in determining population growth and metabolic activity. Nevertheless, despite differences observed within clostridia cluster XIVa, the capacity for subtle inter-population shifts in inducing the same reproducible metabolic effect are effectively demonstrated. The majority of butyrate produced in the gut occurs via the butyryl CoA/acetate CoA pathway; however, butyrate production via butyrate kinase activity has also been reported (Louis *et al.*, 2004). Fermentation of various nonstarch polysaccharides was shown to differentially impact butyrate production by inducing either butyryl CoA/acetate CoA transferase or butyrate kinase in the gastrointestinal tract of weaned pigs (Metzler-Zebeli *et al.*, 2010). Sequence analysis of clones excised from TGGE profiles during the HE fermentation period predominately identified butyrate-producing *Firmicutes*, further substantiating the observed qPCR results (Tables 2 and 4). While most cluster XIVa isolates possess either butyryl CoA/acetate CoA transferase or butyrate kinase activity, some species of *Lachnospiraceae* and *Eubacteriaceae* exhibit both (Duncan *et al.*, 2002a, b). It is possible that the differences observed within the different clostridia cluster XIVa subpopulations of *E. hallii* and *Roseburia*/*E. rectale* in the two models during NE and HE medium fermentation was a function of different butyrate production pathways, induced by intermediate metabolite (e.g. lactate) availability. *Eubacterium hallii* are known lactate-utilizing, butyrate-producing species, whereas no significant ability to use lactate has been detected in *R. intestinalis* or *E. rectale* (Duncan *et al.*, 2004). Furthermore, *E. hallii* may produce butyrate from acetate, lactate as well as carbohydrates (Duncan *et al.*, 2004; Sato *et al.*, 2008).

Nutrient deprivation produced a concerted and distinct metabolic and community restructuring effect. Metabolic activities were significantly lower and represented 35–50% of normal energy metabolic activity, consistent with the 40% reduction in fermentable energy (Table 3). SRB demonstrated the most substantial response to low energy conditions (Table 2; LE). SRB have been implicated in the pathology of inflammatory bowel disease, and it is widely postulated that SRB-mediated production of hydrogen sulfide may be culprit (Macfarlane *et al.*, 2009a, b). SRB have also been described as efficient mucin-utilizers owing to the release of sulfate during mucin degradation (Gibson *et al.*, 1988). Although the nutrient load, including micronutrients, was reduced by 40% in the low energy medium, mucin concentrations remained the same for each medium. Hence, it is possible that SRB are able to outcompete other commensal organisms during periods of nutrient starvation by the ability to utilize sulfate resulting from mucin degradation. A significant lactobacilli bloom was observed, particularly in model A and was a surprising observation as lactobacilli remained below detection during both HE and NE medium fermentation and in feces (Table 2). It is possible that lactobacilli are capable of maintaining growth through scavenging a wide variety of complex substrate under nutrient limitation. As such, lactate could become a central key intermediate metabolite under starvation conditions. Previous results have also demonstrated an inhibitory effect of SRB on butyrate production by *E. hallii* (Marquet *et al.*, 2009). Taken together, this hypothesis could explain the simultaneous blooms of lactobacilli and lactate-utilizing SRB and *Veillonella* and subsequent reduction in lactate-utilizing, butyrate-producing *E. hallii* but not acetate-utilizing, butyrate-producing *Roseburia*/*E. rectale* during low energy conditions. Succinate production and utilization could also possibly account for the ecological reshuffling during nutrient deprivation and should be included in future metabolic analyses. While the metabolism and ecology of SRB are well documented in environmental microbiology and *Veillonella* are recognized as early colonizers of Western infants, their exact roles in the healthy gut remain poorly understood (Adlerberth & Wold, 2009). These results however suggest a complex hierarchy and network of cross-feeding mediated by numerically smaller populations, which may be poised to outcompete populations accustomed to sufficient nutrient availability.

PCA of normal, high, and low energy loading on community structure and metabolic activity demonstrated the reproducibility and robustness of this fermentation model (Figs 4a and 5a). Community structure clusters were predominately a function of simulated colonic conditions. Normal and high energy-dependent community structures of the transverse and distal colon regions formed one distinct cluster, suggesting that community structures are relatively robust with little substantial change during normal and overabundant nutrient supply (Fig. 4a). Conversely, LE medium produced a distinct cluster (Fig. 4a). No specific intrinsic effect of high and normal energy loading on the community structures in R1 of both models could be demonstrated and is likely a function of the low population numbers of circumneutral pH favoring *Bacteroides*, *F. prausnitzii*, SRB, and *Enterobacteriaceae* (Duncan *et al.*, 2009) in this reactor owing to low pH. Similar results were observed using PCA to assess the effects of nutrient load on metabolic activity (Fig. 5a). In this case, metabolic activity in each simulated colonic region clustered according to the nutrient load supplied. Thus, nutrient load appears to be a greater determinant of metabolic activity. While these observations are consistent with previous reports of diet-induced changes to the gut microbiota (Ley *et al.*, 2006; Duncan *et al.*, 2008; Santacruz *et al.*, 2009; Schwiertz *et al.*, 2010), our results

highlight the impact nutrient deprivation imparts upon the gut microbiota. Furthermore, despite changes to microbial community structures, microbiota demonstrated a concerted metabolic adaption suggesting overlapping and redundant metabolic pathways, exercised by different population members.

A frequent misconception of *in vitro* gut fermentation modeling is that each model attempts to be an exact 1 : 1 replicate of intestinal and host physiology. However, host physiology cannot be unequivocally reproduced in this *in vitro* model design and results generated represent a microbial response independent of host modulatory mechanisms (Payne *et al.*, 2011a, b). Furthermore, the robustness of the model is dependent upon compositional and metabolic stability of the microbiota, so that any observed effects on community structure and metabolic activity are indeed a function of experimental treatment and not an artifact of adaptation of the microbiota to *in vivo* simulated conditions (Payne *et al.*, 2011a, b). Nevertheless, the robustness of this model was demonstrated both quantitatively and by PCA and is consistent with the effects of nutrient loading on the gut microbiota *in vivo* (Jumpertz *et al.*, 2011). Although microbiota-specific effects on the clostridia cluster XIVa community structure were observed as a function of varied substrate availability, the use of a single microbiota each precludes the ability to ascertain the significance of these effects with the difference in host weight status. Multiple repetitions of this study using obese and normal-weight microbiota are necessary in definitively attributing any of these results to weight status. In conclusion, this study identified several interesting and novel trends regarding the impact dietary nutrient loads may confer upon the resident gut microbiota, suggesting the need to better elucidate the relationship between community membership and metabolic pathways encoded by the commensal gut community.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Daily total SCFA concentrations measured during the entire 40 day continuous fermentation.
Table S1. HE, NE and LE fermentation media dry matter compositions.
Table S2. Bacterial populations measured by qPCR in effluent samples in each reactor of replicate *in vitro* fermentation models during the re-stabilization period.
Table S3. SCFA concentrations and ratios in effluent samples of each reactor of replicate *in vitro* fermentation models during the re-stabilization period.

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