Microbial Community Development in a Dynamic Gut Model Is Reproducible, Colon Region Specific, and Selective for *Bacteroidetes* and *Clostridium* Cluster IX[∇][#]

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Dynamic, multicompartment *in vitro* gastrointestinal simulators are often used to monitor gut microbial dynamics and activity. These reactors need to harbor a microbial community that is stable upon inoculation, colon region specific, and relevant to *in vivo* conditions. Together with the reproducibility of the colonization process, these criteria are often overlooked when the modulatory properties from different treatments are compared. We therefore investigated the microbial colonization process in two identical simulators of the human intestinal microbial ecosystem (SHIME), simultaneously inoculated with the same human fecal microbiota with a high-resolution phylogenetic microarray: the human intestinal tract chip (HITChip). Following inoculation of the *in vitro* colon compartments, microbial community composition reached steady state after 2 weeks, whereas 3 weeks were required to reach functional stability. This dynamic colonization process was reproducible in both SHIME units and resulted in highly diverse microbial communities which were colon region specific, with the proximal regions harboring saccharolytic microbes (e.g., *Bacteroides* spp. and *Eubacterium* spp.) and the distal regions harboring mucin-degrading microbes (e.g., *Akkermansia* spp.). Importantly, the shift from an *in vivo* to an *in vitro* environment resulted in an increased *Bacteroidetes*/*Firmicutes* ratio, whereas *Clostridium* cluster IX (propionate producers) was enriched compared to clusters IV and XIVa (butyrate producers). This was supported by proportionally higher *in vitro* propionate concentrations. In conclusion, high-resolution analysis of *in vitro*-cultured gut microbiota offers new insight on the microbial colonization process and indicates the importance of digestive parameters that may be crucial in the development of new *in vitro* models.

The human gastrointestinal tract harbors a complex microbial ecosystem with a coding capacity exceeding that of the host genome by a factor of 100 (13). These gut microbes play a determining role in host health by converting otherwise indigestible compounds (14, 19), protecting against gut epithelial cell injury (46), regulating host fat storage (49), and inducing immunity (20, 48). Modulation of the composition and metabolic activity of these microbes to improve host health attracts a lot of attention and is referred to as gastrointestinal resource management (15, 37). Such new strategies are often evaluated during human trials or *in vivo* studies of animals associated with conventional or human microbiota (50).

Despite the physiological relevance, *in vivo* experimental setups are inherently associated with some drawbacks. First, apart from fecal analyses over time, most *in vivo* data are derived from endpoint measurements, thereby limiting the dynamic monitoring of the gut microbiota. Second, troublesome sampling of different gut regions makes it difficult to locate the effects of a treatment. For mechanistic reasons, a third drawback of an *in vivo* approach is the inability to focus solely on gut microbial activity, because there is always a host involved. For these reasons, different types of *in vitro* systems have been developed, ranging from simple nonstirred batch cultures without pH control (44) to more complex continuous models involving pH-controlled single (55) or dynamic multicompartment (2, 29, 32, 34) culture systems. Other advantages are the lack of ethical constraints and a higher reproducibility due to strict control of environmental factors that can influence the microbiota, such as retention time, pH, temperature, and food intake. Therefore, *in vitro* methods are widely used to elucidate the mechanism behind the degradation of prebiotics (17, 52), bioactivation of polyphenols (10, 36, 38), adhesion of microbes to mucins (51), or bioavailability of environmental contaminants (53, 54).

Dynamic *in vitro* gut models need to fulfil certain criteria before they can be used to monitor the modulating potency of specific treatments toward the microbiota. To ensure that effects are due solely to the treatment and not to the adaptation of microbes to the *in vitro* environment, steady-state conditions in terms of microbial community composition and metabolic activity need to be established prior to the actual start of the
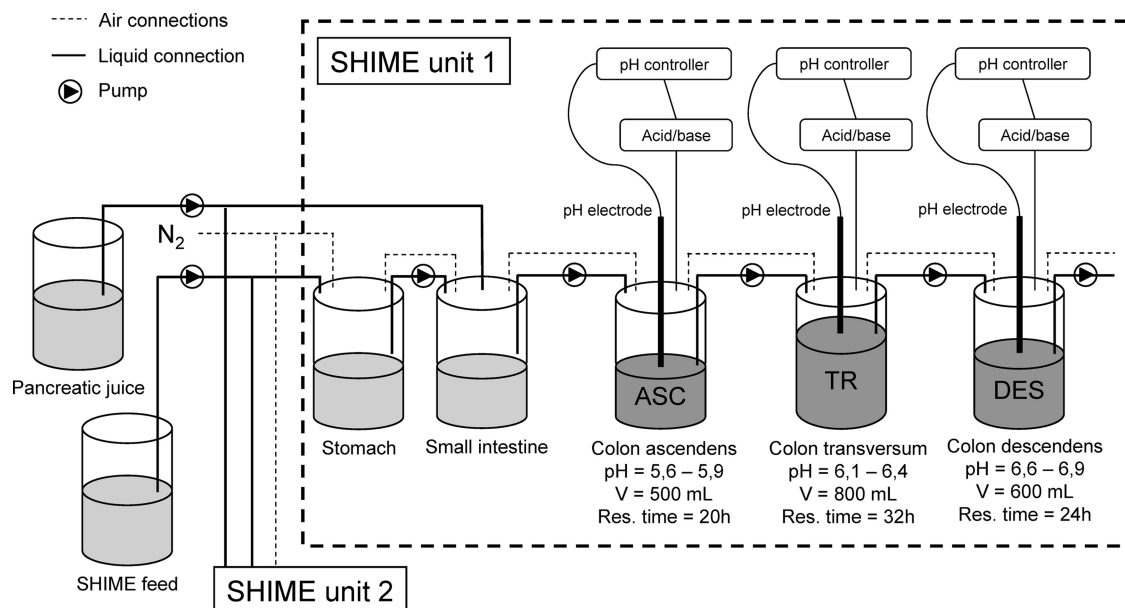

FIG. 1. Schematic representation of a Twin-SHIME which consists of two identical SHIME units, SHIME units 1 and 2. Liquid SHIME feed and pancreatic juice enter the compartments which simulate the stomach and small intestine, respectively. After a residence time of 4 h in these sterile compartments, the suspension goes to three consecutive colon compartments, the ascending (ASC), transverse (TR), and descending (DES) colon compartments, each characterized by distinct pHs and residence times. These compartments are inoculated with human fecal microbiota. All vessels are kept anaerobic by flushing the headspace with N_2, continuously stirred, and kept at 37°C.

experiment (39). Moreover, the stabilization of this *in vitro* microbiota needs to be reproducible, as comparison of different treatments requires identical starting communities. Former studies assumed but never fully substantiated this requirement (17, 38). Further, *in vitro* microbiota need to be gut region specific, be representative for the *in vivo* situation, and maintain a high diversity. The potency of *in vitro* models thus relies on a good characterization of its microbiota. Molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) (17, 39, 52), fluorescent *in situ* hybridization (FISH) (6), and quantitative real-time PCR (Q-PCR) (17, 29), provide useful information but do not provide direct phylogenetic information or target only a limited group of previously identified organisms, therefore limiting current knowledge. Recently, high-resolution techniques, such as microarrays (41, 42) and pyrosequencing (59), have provided access to phylogenetic and metagenomic analysis of the gut microbiota in unprecedented detail.

In this study, we performed conventional metabolic analysis, applied existing molecular techniques (DGGE), and for the first time provided an in-depth phylogenetic analysis on the simulator of the human intestinal microbial ecosystem (SHIME) *in vitro* microbiota using the recently developed human intestinal tract chip (HITChip) microarray (41, 42). We evaluated the microbial colonization process in two parallel *in vitro* simulators (Twin-SHIME) simultaneously inoculated with the same human fecal microbiota. The aims of this study were (i) to determine when the microbial community composition and metabolic activity reach steady-state conditions, (ii) to assess the reproducibility of the stabilization process in two identical *in vitro* simulators, (iii) to obtain a high-resolution characterization of the colon region specificity of the residing

communities, and (iv) to evaluate how the *in vivo* fecal inoculum changes to the *in vitro* colon microbial communities.

MATERIALS AND METHODS

Twin-SHIME experiment. The SHIME is a dynamic *in vitro* model of the human gastrointestinal tract that is composed of five double-jacketed vessels, simulating the stomach, small intestine, and the three colon regions (ascending, transverse, and descending colon), with a total retention time of 72 h (Fig. 1). Three times per day, 140 ml SHIME feed and 60 ml pancreatic juice were added to the stomach and small intestine compartments, respectively. The SHIME feed contained (in grams/liter) arabinogalactan (1.0), pectin (2.0), xylan (1.0), starch (4.0), glucose (0.4), yeast extract (3.0), peptone (3.0), mucin (1.0), and cystein (0.5). The pancreatic juice contained (in grams/liter) $NaHCO_3$ (12.5), bile salts (6.0) (Difco, Bierbeek, Belgium), and pancreatin (0.9) (Sigma, Bornem, Belgium). All vessels were kept anaerobic by flushing them with N_2, and they were continuously stirred and kept at 37°C. A Twin-SHIME setup comprises two SHIME units in parallel (17, 38) in order to attain identical environmental conditions for both units. In this experiment, the three colon regions of each SHIME unit of a Twin-SHIME were inoculated with human fecal microbiota from a healthy male volunteer (22 years old) who had no history of antibiotic treatment 6 months prior to the study. More details on reactor setup and inoculum preparation were as previously described (34, 39). A specific feature of the SHIME setup is the initial 2-week stabilization period, which allows the microbiota to adapt to the imposed *in vitro* conditions and to evolve from a fecal microbial community to one representative of a specific colon region. During the first 26 days after inoculation, samples were collected and DGGE was applied to investigate the stabilization of the microbial communities in terms of microbiota composition. Metabolic parameters were analyzed to evaluate this stabilization also in terms of metabolic activity. Between days 19 and 26, samples were taken to characterize the metabolite production and enzyme activity of the stabilized microbial communities. Samples of the inoculum from days 19 and 26 were used for in-depth community composition analysis with the HITChip.

Microbial community analysis: DGGE and HITChip. Total DNA extractions were performed according to Boon et al. (4). Denaturing gradient gel electrophoresis (DGGE) was applied to separate PCR products of 16S rRNA genes obtained with the general bacterial primers 338F-GC and 518R. Gels had a
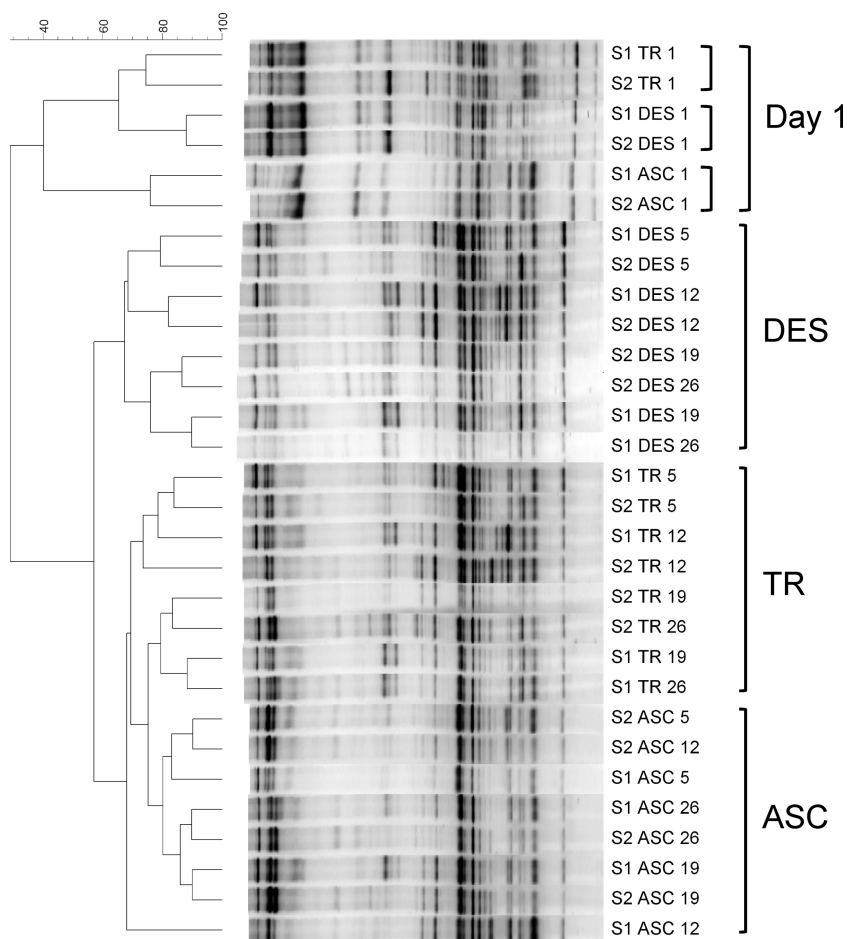
FIG. 2. Clustering tree based on Pearson and UPGMA correlations of the DGGE profiles in the ascending (ASC), transverse (TR), and descending (DES) colon compartments of a Twin-SHIME (S1, SHIME 1; S2, SHIME 2) at different times after inoculation (days 1, 5, 12, 19, and 26).

denaturing gradient ranging from 45% to 60% (52) and were run on an Ingeny PhorU apparatus (Ingeny International, Goes, Netherlands). Normalization and further analysis of the gels were carried out using BioNumerics software, version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Extracted data were processed according to Marzorati et al. (30) for ecological interpretation, including calculation of the range-weighted richness (RR) (approximate environmental carrying capacity for microbial diversity), the dynamics (Dy) (approximate changes within the community over a fixed time frame), and a functional organization (FO) parameter (approximate structure of the microbial community in terms of its evenness).

Microbiota composition was determined using the human intestinal tract chip (HITChip), a gastrointestinal tract-specific phylogenetic microarray, as described by Rajilic-Stojanovic et al. (41, 42). Using 4,800 oligonucleotide probes, this microarray targets 1,140 unique microbial phylotypes (with <98% identity), providing data from 131 genus-like groups. Briefly, 16S rRNA genes were amplified and subsequently *in vitro* transcribed, labeled with the Cy3 and Cy5 dyes, and hybridized to the array. After the arrays were washed and scanned, data were extracted using the Agilent Feature Extraction software, version 7.5 (Agilent Technologies), and normalized using a set of R-based scripts (http://www.r-project.org/). Analysis of the microarray was performed in a custom relational database which runs under the MySQL database management system (MySQL) using a series of custom R scripts, as previously described (41, 42). These robust statistical packages allow the processing of data for comparative analysis of microbiota patterns, various forms of microbiota composition visualization (e.g., microbial grouping), and semiquantitative microbiota reconstruction at different phylogenetic levels, from phylum level to bacterial group level. Quantitative analyses at the bacterial group level were validated with Q-PCR (41). To provide an ecolog-

ical interpretation of these data, the Simpson reciprocal index was calculated as a measure of diversity (27). An increase in the Simpson reciprocal index reflects a diversity increase, with 1 being the lowest possible number and the number of bacterial species present in the sample being the maximal number.

Metabolic activity analysis: SCFAs, lactate, succinate, ammonium, and enzymatic assays. Short-chain fatty acid (SCFA) and ammonium ion analyses were performed as reported by Nollet et al. (35) and De Boever et al. (10), respectively. Lactate and succinate were measured using a D-lactate/L-lactate and succinate kit (R-Biopharm, Mannheim, Germany), according to the manufacturer's protocols. β-Glucosidase, β-glucuronidase, α-L-arabinofuranosidase, β-D-xylosidase, and endo-1,4-β-xylanase activities were determined with p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-α-l-arabinofuranoside, p-nitrophenyl-β-D-xylopyranoside (Sigma-Aldrich, Bornem, Belgium), and azo-wheat-arabinoxylan (Megazyme, Bray, Ireland), respectively (17, 52). One unit is the enzyme activity needed to release 1 μmol of p-nitrophenol from the appropriate substrate (2.5 mM) in 1 min under the assay conditions.

Enzyme assays of azoreductase and nitroreductase were optimized for measurement in SHIME suspensions based on earlier protocols (18, 57). For azoreductase activity, 0.05 ml of an amaranth solution (1.5 mM, dissolved in anaerobic phosphate buffer) was added to 1.5 ml of SHIME suspension. After anaerobic incubation for 4 h at 37°C, the mixture was centrifuged for 2 min (3,000 × g). Discoloration of the medium was monitored by measuring the absorbance of the supernatant at 520 nm with a multiwell spectrophotometer (PowerWave 340 [Bio-Tek Instruments Inc., Winooski, VT]). Nitroreductase activity was determined by adding 0.5 ml of 1.5 mM p-nitrobenzoic acid to 1 ml SHIME suspension. After anaerobic incubation for 4 h at 37°C, the amount of p-aminobenzoic acid was determined by using the method of Bratton and Marshall, as changed
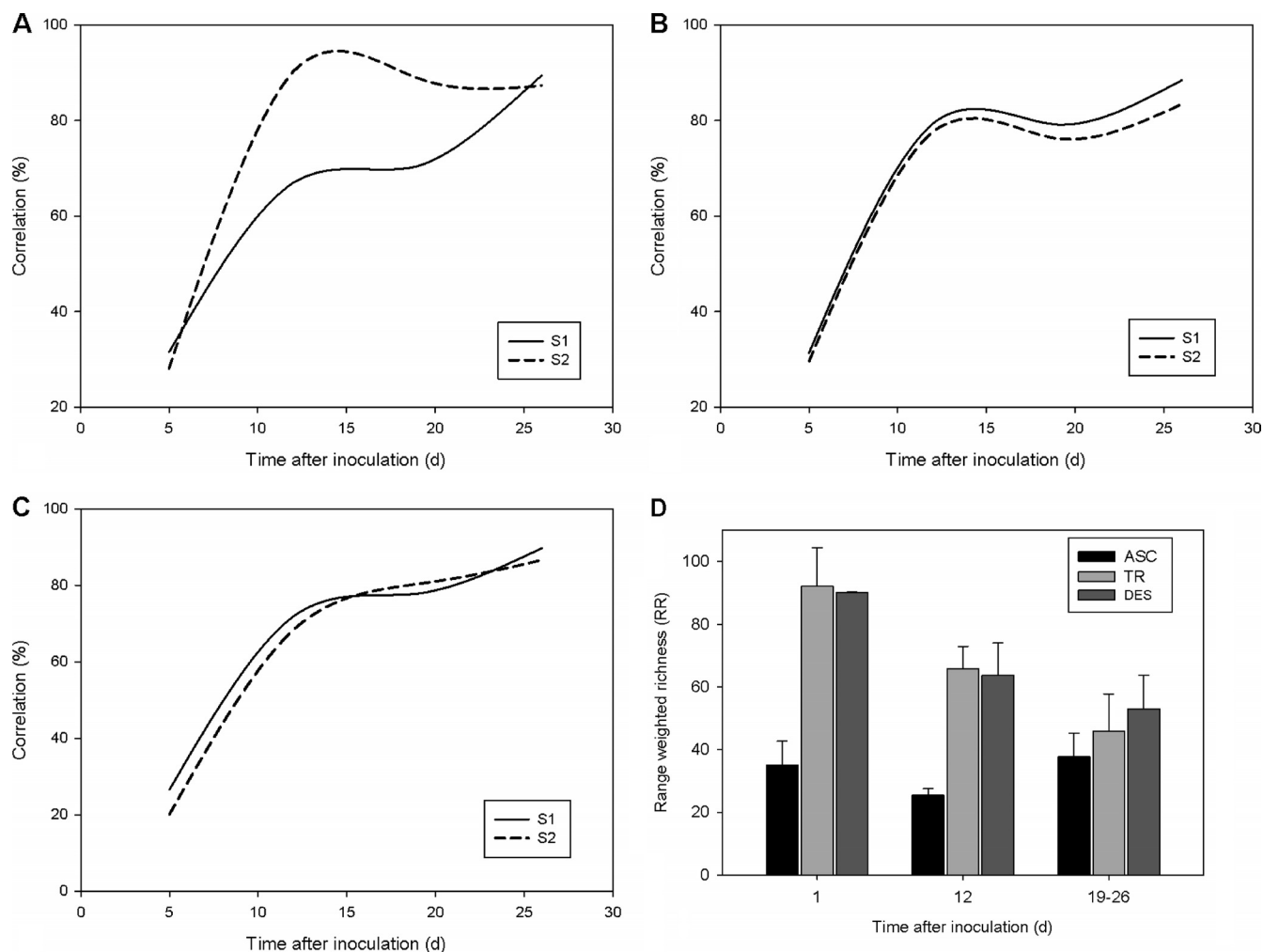
FIG. 3. Correlation coefficients (percentages) between DGGE profiles of consecutive time points of the Twin-SHIME (SHIME 1 and 2) during the stabilization period in the ascending (A), transverse (B), and descending (C) colon compartments. (D) Average range-weighted richness (RR) of SHIME 1 and 2, based on the DGGE patterns of the ascending (ASC), transverse (TR), and descending (DES) colon at different time points after inoculation, specifically, day (d) 1 ($n = 2$), day 12 ($n = 2$), and during the control period (days 19 to 26 [$n = 4$]) (n indicates the number of replicate experiments).

by Goldbarg and Rutenburg (16). This included addition of the following solutions: 1 ml NaNO$_2$ (0.1%), 1 ml NH$_4$ sulfamate (0.5%), and, after intensive mixing, 2 ml N-(1-naphthyl)ethylenediamine dihydrochloride (0.05%) dissolved in 100% ethanol. After 10 min of incubation at 25°C, the absorbance was measured at 550 nm. Both assays were also performed using a positive and a negative control (boiled sample), so that results are expressed as percentages of amaranth and p-nitrobenzoic acid conversion.

Statistics. All data were analyzed using SPSS 16 software (SPSS Inc., Chicago, IL). Before investigating the probability of intergroup differences, the normality and homogeneity of variances were studied with Kolmogorov-Smirnov and Levene tests, respectively. Under normal distribution and homogeneity of variances, an analysis of variance (ANOVA) with a (post hoc) Bonferroni test was performed; otherwise, a Kruskal-Wallis with a Mann-Whitney U test was applied. To investigate differences between both SHIME units of the Twin-SHIME, a paired t test was applied when inter-SHIME differences were normally distributed; otherwise, a Wilcoxon signed-rank test was applied. Differences were considered significant at P values of <0.05.

The Pearson correlation coefficient and unweighted-pair group method using average linkages (UPGMA) clustering algorithm were used to create dendrograms of DGGE profiles from the stabilization period, taking into account both band position and band density. Cosine correlation coefficients were calculated on the basis of normalized values for metabolic parameters during the stabilization period (ammonium, acetate, propionate, butyrate, and branched SCFAs).

To assess the correlation of microbial groups detected by the HITChip with specific colon regions, redundancy analysis (RDA) was used as implemented in Canoco for Windows 4.5 (22). Average signal intensities for 131 bacterial groups were used as species data, and diagrams were plotted using the CanoDraw for Windows utility. The Monte Carlo permutation procedure (21) was used to assess statistical significance of the variation in data sets in relation to sample origin, i.e., the ascending, transverse, or descending colon compartment.

RESULTS

Stabilization of microbial community composition in a Twin-SHIME. DGGE was applied to assess the microbiota composition of the stabilizing communities in both SHIME units after inoculation of the human fecal sample (Fig. 2). Colon samples from day 1 were more similar to one another than colon samples from later time points. From day 5 onwards, samples from the different colon compartments clustered separately, independently of the time point of sampling. Interestingly, the microbial communities of both SHIME units developed similarly during the stabilization period, with day 26
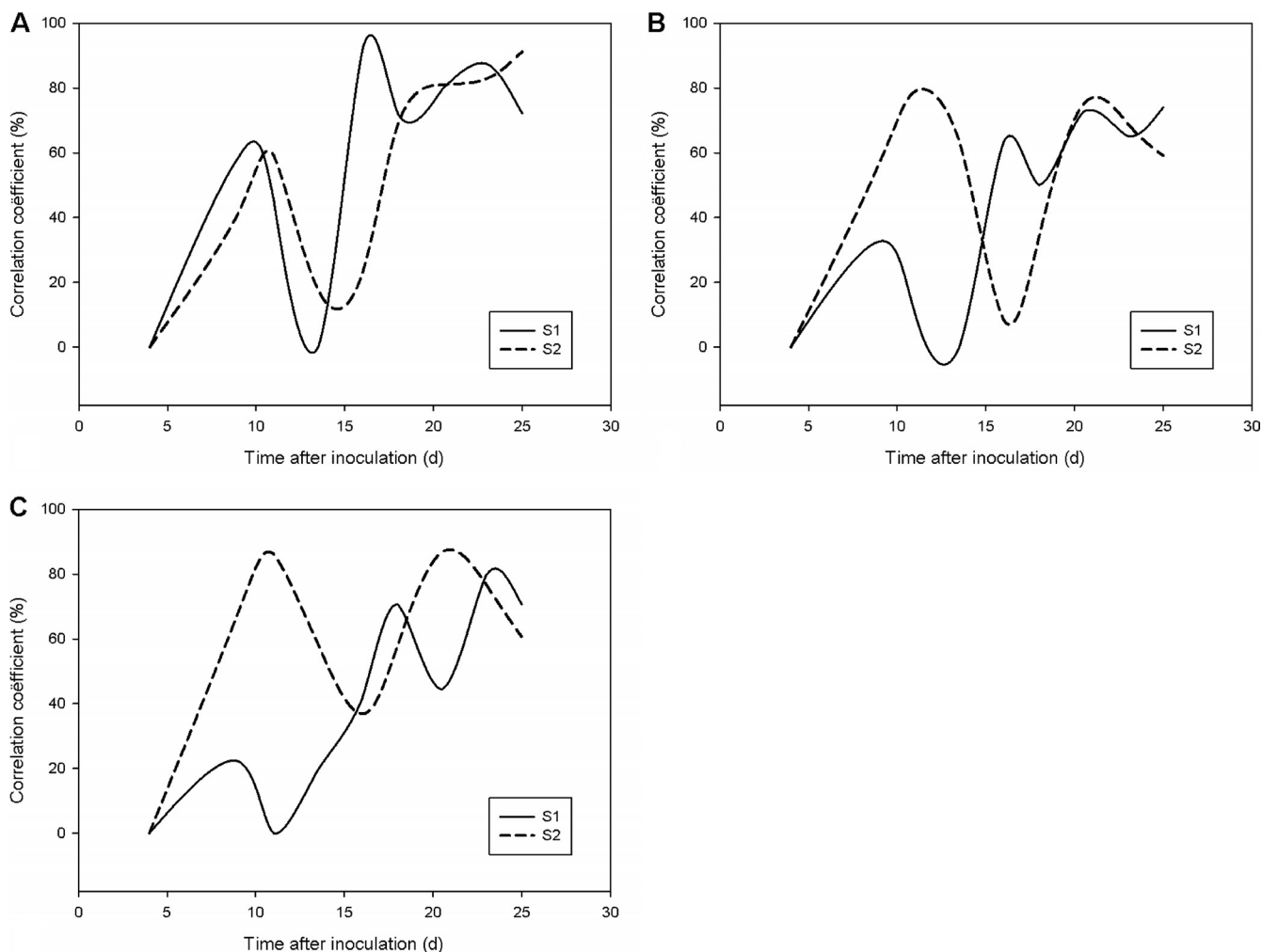
FIG. 4. Correlation coefficients (percentages) between the metabolic profiles of consecutive time points (ammonium, acetate, propionate, butyrate, and branched SCFAs) of the Twin-SHIME (SHIME 1 and 2) during the stabilization period in the ascending (A), transverse (B), and descending (C) colon compartments.

displaying correlations of 90%, 82%, and 76% in the ascending, transverse, and descending colon compartments, respectively.

The initial weekly rates of change (100% − percent correlation) were around 70% in all colon regions of both SHIME units. After 14 days, rates of change dropped below 20% for each colon compartment, indicating steady-state microbial compositions (Fig. 3A to C). The range-weighted richness (RR; approximate diversity) of the ascending colon was low on the first day after inoculation, while both the transverse and descending colons were characterized by initially high RR values that decreased during further stabilization (Fig. 3D). Finally, microbial communities from all colon compartments were highly structured, as the functional organization (FO) parameter (ranging between 0.40 and 0.48) was stable throughout the stabilization period; i.e., species abundances were distributed among dominant and subdominant members.

Stabilization of microbial community activity in a Twin-SHIME. The metabolic fingerprints (ammonium, acetate, propionate, butyrate, and branched SCFAs) were variable in all

colon regions of both SHIME units during the stabilization, reaching a steady-state level after approximately 17 to 20 days (Fig. 4A to C).

In-depth analysis of microbial community composition in a stabilized Twin-SHIME. The HITChip provided detailed characterization of the stable microbial communities (samples from days 19 and 26) at the phylum and bacterial group levels in the respective colon compartments of the SHIME. Corresponding colon regions of both SHIME units showed similar microbiota abundances, so the averages from both units were calculated per colon compartment (Tables 1 and 2 and Fig. 5). There was a large similarity between the different colon parts at the phylum level (Table 1). Three phyla dominated the microbial communities, with *Bacteroidetes* being much more abundant than *Firmicutes* (*Clostridium* clusters IV, IX, and XIVa) and *Proteobacteria*. There were trends of higher proportions of *Clostridium* clusters IX and XIVa in the ascending colon and of *Clostridium* cluster IV and *Proteobacteria* in the descending colon. *Verrucomicrobia* was the only phylum for which the numbers of organisms significantly differed between the colon

TABLE 1. Abundances of higher taxonomic groups[a]

Higher taxonomic group	% abundance in:			
	ASC	TR	DES	Human feces
Actinobacteria	1.11	0.47	0.71	0.27
Bacteroidetes	75.50	80.59	75.60	52.49
Firmicutes				
Bacilli	0.38	0.34	0.36	1.12
Clostridium cluster I	0.12	0.11	0.11	0.05
Clostridium cluster III	0.06	0.04	0.05	0.07
Clostridium cluster IV	1.54	1.19	2.41	26.07
Clostridium cluster IX	4.65	2.71	3.14	0.38
Clostridium cluster XI	0.14	0.12	0.12	0.20
Clostridium cluster XIII	0.03	0.03	0.03	0.01
Clostridium cluster XIVa	9.44	5.63	6.46	15.95
Clostridium cluster XV	0.04	0.04	0.08	0.12
Clostridium cluster XVI	0.06	0.06	0.06	0.14
Clostridium cluster XVII	<0.01	<0.01	<0.01	<0.01
Clostridium cluster XVIII	0.05	0.04	0.05	0.13
Uncultured *Clostridiales*	0.29	0.26	0.37	0.33
Total	16.81	10.56	13.23	44.57
Cyanobacteria	<0.01	<0.01	<0.01	<0.01
Fusobacteria	0.03	0.03	0.02	0.01
Proteobacteria	5.90	7.69	9.83	2.36
Spirochaetes	0.01	0.01	0.01	<0.01
Tenericutes				
Asteroleplasma species	<0.01	<0.01	<0.01	<0.01
Uncultured *Mollicutes*	0.07	0.07	0.07	0.22
Verrucomicrobia	0.02 A	0.17 B	0.11 B	0.01

[a] Abundances of groups (approximate phylum level) are based on the HIT-Chip analysis of the ascending (ASC) ($n = 4$), transverse (TR) ($n = 4$), and descending (DES) ($n = 4$) colon compartments of a stabilized Twin-SHIME (days 19 and 26) and the human fecal microbiota that was used as the inoculum for the *in vitro* model ($n = 2$) (n indicates the number of replicate experiments). Corresponding colon regions of both SHIME units showed similar abundances, so the averages from both units were calculated. The Simpson reciprocal index reflects the diversity within these communities, i.e., species richness and evenness. Values with different letters are significantly different between the different colon regions of the SHIME. The Simpson reciprocal indexes for ASC, TR, DES, and human feces are 90 ± 15, 100 ± 3, 106 ± 7, and 193 ± 11, respectively.

regions: the ascending colon was hardly colonized with *Verrucomicrobia*, whereas the transverse and descending colons possessed much higher numbers ($P = 0.02$). The Simpson reciprocal index tended to increase from the ascending (90 ± 15) to the transverse (100 ± 3) and descending (106 ± 7) colon compartments. At a lower phylogenetic level, HITChip analysis elucidated the presence of numerous bacterial groups in the *in vitro* microbiota (see Table S1 in the supplemental material). Redundancy analysis (RDA) at the bacterial group level revealed that the colon compartments were significantly different ($P = 0.008$) (Fig. 5). The RDA highlighted that 20 bacterial groups could be allocated to a specific colon compartment. The percentage of variation exhibited by these 20 bacterial groups was four times higher along the *x* axis of the RDA than along the *y* axis. Nonparametric statistical tests also indicated a significant correlation between most of these 20 groups and a colon compartment (Table 2). Bacteria that were characteristic for the ascending colon included *Actinomycetaceae*, *Mitsuokella multacida*, and relatives and groups belonging to the *Bacteroidetes* and *Clostridium* cluster XIVa. *Akkermansia* spp. and

four *Bacteroidetes* groups were correlated to the transverse colon. Finally, *Bacteroidetes*, *Clostridium* clusters IV and XVI, and *Proteobacteria* displayed an increased abundance in the descending colon.

To compare the data of the *in vitro* microbiota with the initially present microbial community, the human fecal inoculum was also analyzed with the HITChip. The microbial community of the inoculum was dominated by different *Clostridium* clusters (especially clusters IV and XIVa) and *Bacteroidetes*. Compared to the organisms in the SHIME, *Firmicutes* were enriched (especially *Clostridium* clusters IV and XIVa) in the fecal inoculum, whereas the abundance of *Bacteroidetes*, *Proteobacteria*, and *Clostridium* cluster IX declined (Table 1). The Simpson reciprocal index was higher for the fecal inoculum than for the SHIME compartments (193 ± 11).

Microbial community activity in a stabilized Twin-SHIME. Corresponding colon regions of both SHIME units showed similar concentrations of metabolites and enzyme activities ($P = 0.680$), so the averages from both units were calculated per colon compartment (Table 3). The concentrations of these metabolic parameters were colon specific, with significant differences between the respective compartments. While succinate and lactate tended to decrease in the distal colon compartments, the other parameters increased along the different colon regions. The average acetate/propionate/butyrate ratio was 68/25/6, 65/27/8, and 66/25/9 for the ascending, transverse, and descending colon, respectively. Likewise, no significant differences in enzymatic activities were found between both SHIME units. Several enzyme activities were characteristic of specific colon regions: while glucosidase and glucuronidase activities were highest in the transverse and descending colons, nitroreductase and azoreductase activities were highest in the ascending colon. Xylosidase, arabinofuranosidase, and xylanase activities were comparable between the different regions.

DISCUSSION

Multistage dynamic *in vitro* models of the human gastrointestinal tract are frequently used when evaluating strategies to steer the intestinal microbial community in a health-promoting direction (24). To compare treatments, reproducible starting points in terms of steady-state microbiota composition and metabolic activity are required. Therefore, the microbial colonization in the colon compartments of two identical multistage SHIME units was monitored for 26 days after inoculation. Analysis of the microbial community dynamics revealed an initial peak of change, due to the transit from *in vivo* to *in vitro* conditions (Fig. 3). Whereas the microbial community's composition was stable after 12 days (Fig. 3), its metabolic activity required 17 to 20 days before reaching a steady state (Fig. 4). This confirms that a minimum of 2 weeks is required for stabilization (39). Importantly, not only the final (Fig. 2 and Table 1) but also the transient microbial community compositions in the corresponding colon regions were highly similar between the two SHIME units (Fig. 2). In addition, the colon compartments of the two identical SHIME units were similar from ecological (Table 1 and Fig. 3D) and metabolic (Table 3) perspectives. We therefore conclude that multistage *in vitro* simulators inoculated with the same microbiota can be repro-

TABLE 2. Abundances of bacterial groups belonging to higher taxonomic groups[a]

Higher taxonomic group	Bacterial group	P value (Kruskal-Wallis)	% abundance in:		
			ASC	TR	DES
Actinobacteria	*Actinomycetaceae*	0.026	**0.03 A**	0.02 B	0.02 B
Bacteroidetes	*Bacteroides fragilis*-like	0.015	**8.41 A**	5.97 B	5.42 B
	Bacteroides uniformis-like	0.023	**11.35 A**	7.64 B	8.12 B
	Alistipes-like	0.039	2.80 A	**4.54 B**	**4.79 B**
	Bacteroides intestinalis-like	0.023	4.55 A	**8.65 B**	6.74 AB
	Bacteroides plebeius-like	0.024	1.92 A	**4.25 B**	4.02 B
	Bacteroides vulgatus-like	0.059	4.08 A	**8.95 B**	7.18 AB
	Bacteroides splanchnicus-like	0.873	2.74 A	3.08 A	2.95 A
Firmicutes					
Clostridium cluster IV	*Clostridium cellulosi*-like	0.016	0.03 A	0.03 A	**0.05 B**
	Clostridium orbiscindens-like	0.009	0.03 A	0.03 A	**0.11 B**
	Oscillospira guilliermondii-like	0.016	0.03 A	0.04 A	**0.16 B**
	Subdoligranulum variabile-like	0.012	0.04 A	0.11 AB	**0.24 B**
Clostridium cluster IX	*Mitsuokella multacida*-like	0.058	**0.24 A**	0.13 B	0.15 AB
Clostridium cluster XIVa	*Clostridium colinum*-like	0.017	**0.06 A**	0.03 B	0.03 B
	Eubacterium hallii-like	0.015	**0.14 A**	0.04 B	0.05 B
	Eubacterium rectale-like	0.035	**0.08 A**	0.04 B	0.04 B
Clostridium cluster XVI	*Eubacterium biforme*-like	0.090	0.03 AB	0.02 A	**0.03 B**
Proteobacteria	*Oxalobacter formigenes*-like	0.029	0.09 A	0.18 AB	**0.29 B**
	Burkholderia	0.053	1.28 A	3.21 AB	**5.88 B**
	Sutterella wadsworthensis-like	0.138	1.10 A	2.14 A	2.68 A
Verrucomicrobia	*Akkermansia*	0.019	0.05 A	**0.41 B**	0.27 B

[a] Abundance is based on the HITChip analysis for the ascending (ASC) (*n* = 4), transverse (TR) (*n* = 4), and descending (DES) (*n* = 4) colon compartments of a stabilized Twin-SHIME (days 19 and 26) (*n* indicates the number of replicate experiments). Corresponding colon regions of the SHIME units showed similar abundances, so the averages from both units were calculated. Nonparametric statistical tests were applied to determine the significance of the correlation of each bacterial group to a certain colon region. Boldface represents colon regions characterized by the highest abundance of a certain bacterial group. Values with different letters are significantly different.

duced to contain similar microbial communities that reach compositional and functional stability after approximately 2 weeks.

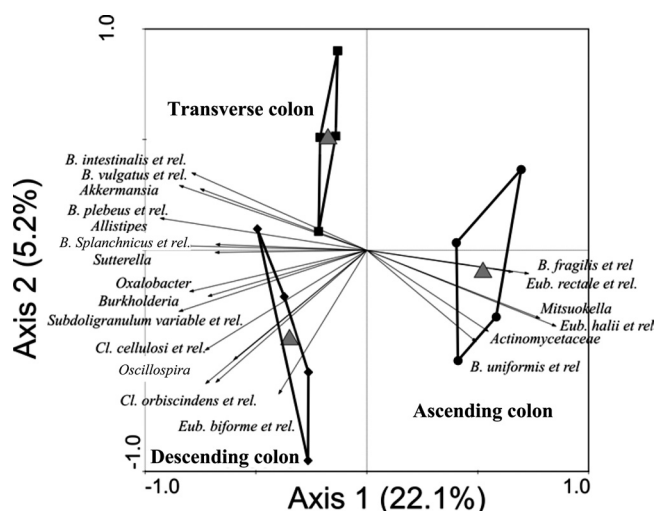Ecological analysis of the colon microbial community com-

FIG. 5. Redundancy analysis of HITChip data at the bacterial group level of the SHIME in its three colon parts, the ascending (ASC) (*n* = 4), transverse (TR) (*n* = 4), and descending (DES) (*n* = 4) colon compartments (*P* = 0.008) (*n* indicates the number of replicate experiments). et rel., and related species.

position showed strong differences between the ascending colon on the one hand and the transverse/descending colon on the other hand. From the first day after inoculation, the ascending colon compartments deviated from the transverse and descending colon compartments through an immediate loss of species richness (Fig. 3). Moreover, the Simpson reciprocal index indicated that the species richness was consistently lower throughout the stabilization period (Table 1). This can be explained by the fact that the imposed conditions in the ascending colon (a lower pH and a higher bile salt concentration) strongly differ from fecal conditions. In contrast with the species richness and clustering of DGGE profiles, the functional organization of the microbial community was independent of time and colon regions. The values were indicative of well-adapted communities with an adequate species distribution, enabling the counteraction of stress and preservation of metabolic functionality (58). Despite the lack of direct phylogenetic information from DGGE profiles, the derived ecological descriptors provided a useful tool to deliver additional information on community dynamics, structure, and functioning. These items tend to be overlooked with conventional culture-based detection methods (2, 25, 34).

In vivo validation of *in vitro* microbial community development has been a common objective of many research studies (25, 32, 33), yet the applied molecular tools have often had insufficient resolving power. High-resolution HITChip analysis showed that *in vitro* microbial populations are very diverse (Table 1) but still less diverse than the inoculum, indicating a

TABLE 3. Metabolite concentrations in a Twin-SHIME[a]

Metabolite or enzyme	Concn or % conversion in:		
	ASC	TR	DES
Ammonium (mM)	16.4 ± 0.9 A	22.4 ± 1.2 B	26.2 ± 1.4 C
Acetate (mM)	31.7 ± 2.2 A	34.5 ± 2.8 B	38.4 ± 2.1 C
Propionate (mM)	11.6 ± 1.3 A	14.1 ± 0.6 B	14.6 ± 1.1 B
Butyrate (mM)	3.0 ± 0.8 A	4.3 ± 0.7 B	5.2 ± 0.7 C
Branched SCFA (mM)	0.6 ± 0.1 A	2.2 ± 0.3 B	2.6 ± 0.1 C
Total SCFA (mM)	46.7 ± 3.0 A	55.2 ± 3.7 B	61.3 ± 2.6 C
Lactate (mM)	1.0 ± 0.3 A	0.5 ± 0.2 B	0.3 ± 0.3 B
Succinate (mM)	3.2 ± 1.6 A	2.7 ± 1.0 B	2.2 ± 0.6 B
β-Glucosidase (U/liter)	8.6 ± 7.3 A	25.2 ± 3.0 B	23.0 ± 2.1 B
β-Glucuronidase (U/liter)	1.8 ± 2.1 A	8.4 ± 3.7 B	8.7 ± 2.0 B
β-D-Xylosidase (U/liter)	64.7 ± 30.3 A	49.4 ± 8.1 A	36.2 ± 4.9 A
α-L-Arabinofuranosidase (U/liter)	22.7 ± 21.4 A	48.4 ± 7.2 A	42.7 ± 7.6 A
Endo-1,4-xylanase (U/liter)	2.4 ± 0.9 A	3.0 ± 1.0 A	3.2 ± 1.8 A
Nitroreductase (% conversion of p-nitro benzoic acid)	31.6 ± 0.5 A	9.6 ± 2.4 B	8.4 ± 1.7 B
Azoreductase (% conversion of amaranth)	94.4 ± 5.4 A	76.9 ± 6.0 B	76.6 ± 18.1 AB

[a] Concentrations are of ammonium, acetate, propionate, butyrate, branched SCFAs, total SCFA, lactate, and succinate in the ascending (ASC), transverse (TR), and descending (DES) colon compartments (10 repetitions). Average enzymatic activities are of β-glucosidase ($n = 8$), β-glucuronidase ($n = 4$), β-D-xylosidase ($n = 4$), α-L-arabinofuranosidase ($n = 8$), endo-1,4-xylanase ($n = 4$), nitroreductase ($n = 8$), and azoreductase ($n = 8$) in the ASC, TR, and DES colon compartments of a stabilized Twin-SHIME (days 19, 21, 23, and 26). Corresponding colon regions of the SHIME units showed similar abundances, so averages from both units were calculated. The averages ± standard deviations are shown and are within one metabolite/enzyme. Values with different letters are significantly different.

certain degree of specialization under *in vitro* conditions. First, an *in vitro* enrichment of *Clostridium* cluster IX (propionate producers) (56) but not of clusters IV and XIVa (butyrate producers) (23) was observed. This finding was supported by *in vitro* molar SCFA ratios, which were similar to *in vivo* ratios (57/21/22, acetate-propionate-butyrate) (9) but displayed higher propionate than butyrate levels. Such mild changes in SCFA profile have been observed previously (2). Second, the *in vitro* microbiota was enriched in *Proteobacteria* and *Bacteroidetes* but not in *Firmicutes*. This corresponds to earlier findings with the HITChip, with which a loss of *Firmicutes* as opposed to *Bacteroides* groups was observed during the initial *in vivo-in vitro* shift in the dynamic TNO intestinal model (TIM) (41). Moreover, more conventional molecular tools, such as culture-based techniques (2, 25), Q-PCR (17, 29), and DGGE (39), already determined the *in vitro* dominance of *Bacteroidetes*. There are several reasons for the discrepancy in *Firmicutes*/*Bacteroidetes* ratios between the *in vitro* gut model and the inoculum. First, *in vitro* systems may contain higher micromolar levels of oxygen, which could potentially favor aerotolerant microbes, such as *Bacteroides* species (5, 43) or *Proteobacteria*. Second, the high carbohydrate content of the *in vitro* SHIME nutritional medium may promote the proliferation of *Bacteroidetes*, which possess a larger glycobiome than *Firmicutes* (28). This opens perspectives to restore the *Bacteroidetes*/*Firmicutes* ratio by alteration of the SHIME feed. Third, *in vitro* simulators often lack the simulation of mucosal adhesion sites that are considered important for prolonged colonization. Recently, it has been proposed that the mucous layer is a protected microenvironment, containing a backup of microbes that can colonize the lumen and restore associations after luminal perturbations (P. Van den Abbeele, S. Possemiers, W. Verstraete, and T. Van de Wiele, submitted for publication). In contrast to *Firmicutes*, *Bacteroides* spp. are less capable of mucosal adhesion (31). The absence of adhesion as a selective advantage may therefore result in their higher abundance *in vitro* than in an *in vivo* gut environment. *In vitro*

models could therefore be improved by including mucous layer simulations, such as mucin-agar tubes (26) or mucin beads (40). Finally, the selective pressure of the host on the microbiota is only partly simulated *in vitro*. Both retention time (6, 25) and pH (56), in addition to a variety of more specific defense mechanisms, can significantly change microbial communities, with the innate and adaptive immunity being most important (1, 7).

Application of the HITChip provided detailed characterization of the microbiota in the respective colon compartments of the SHIME. Overall, no significant differences at the phylum level were noted between colon regions (Table 1). Yet, RDA at the bacterial group level revealed that microbiota in the proximal (ascending) and distal (transverse/descending) colon compartments cluster separately (Fig. 5). From the 131 bacterial groups detected, 20 groups explained around 20% of the differences between colon regions, which is a higher percentage than expected from a flowthrough setup (Table 2). Interestingly, the colon specificity of some bacterial groups could be related to the metabolic activities of the bacteria. Although the lack of an absorption module in the SHIME gut model leads to accumulation of SCFAs in the distal colon compartment, calculation of the net SCFA production resulted in the highest values in the ascending colon (Fig. 5). This indicates it to be the main site of carbohydrate fermentation. The bacterial groups that correlated to the ascending colon should thus typically be involved in carbohydrate fermentation (e.g., *Bacteroides* spp. and *Eubacterium* spp.). Rapid fermentation by these groups could deplete essential growth factors and thus negatively affect microbes that grow on more difficult substrates, for instance, mucins. *Akkermansia muciniphila*, a recently isolated mucin degrader (12), is indeed virtually absent in the ascending colon yet abundantly present in the transverse and descending colon compartments (Table 2), where its presence could relate to the distally increased glucuronidase activity (11). In addition, four *Bacteroides* groups specifically correlated with the transverse colon. As *Bacteroides* species are also potent mucin

degraders (8, 45, 47), one may therefore assume that mucin degradation may be a more important process in the transverse colon than in the ascending colon. Alternatively, distally occurring microbiota could participate in the distal fermentation of arabinose- and/or xylose-containing polysaccharides (xylan or arabinogalactan), as arabinofuranosidase and xylanase also increased in the distal regions (3, 17). These data illustrate the potency of HITChip analysis in revealing relationships between microbial community phylogenetics and specific metabolic activities.

In conclusion, our study shows that well-controlled multi-compartment gastrointestinal *in vitro* models display a reproducible microbial colonization and community development upon inoculation. Moreover, phylogenetic microarray analysis evidenced the colon region specificity of the colonization process, with the proximal regions harboring saccharolytic microbes and the distal regions harboring mucin-degrading microbes (e.g., *Akkermansia* spp.). As a consequence, once the respective microbial populations have reached functional steady state, such gut models will thus enable placebo-controlled *in vitro* studies or direct comparison of the modulatory effects of two different treatments for the gut microbiota. Importantly, the shift from an *in vivo* to an *in vitro* environment resulted in an increased *Bacteroidetes*/*Firmicutes* ratio, while *Clostridium* cluster IX (propionate producers) was enriched compared to clusters IV and XIVa (butyrate producers), supported by proportionally higher *in vitro* propionate concentrations. These findings illustrate the potency of high-resolution microbial analytical techniques in revealing the importance of hitherto less explored gut parameters, such as the presence of a mucosal surface, that may need to be incorporated when new or modified dynamic gastrointestinal simulators are developed.

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