Microbiology (2010), **156**, 3270–3281 DOI 10.1099/mic.0.042044-0

Evaluating the microbial diversity of an *in vitro* model of the human large intestine by phylogenetic microarray analysis

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Received 8 June 2010
Revised 13 August 2010
Accepted 10 September 2010

A high-density phylogenetic microarray targeting small subunit rRNA (SSU rRNA) sequences of over 1000 microbial phylotypes of the human gastrointestinal tract, the HITChip, was used to assess the impact of faecal inoculum preparation and operation conditions on an *in vitro* model of the human large intestine (TIM-2). This revealed that propagation of mixed faecal donations for the production of standardized inocula has only a limited effect on the microbiota composition, with slight changes observed mainly within the Firmicutes. Adversely, significant shifts in several major groups of intestinal microbiota were observed after inoculation of the *in vitro* model. Hierarchical cluster analysis was able to show that samples taken throughout the inoculum preparation grouped with microbiota profiles observed for faecal samples of healthy adults. In contrast, the TIM-2 microbiota was distinct. While members of the Bacteroidetes and some groups within the Bacilli were increased in TIM-2 microbiota, a strong reduction in the relative abundance of other microbial groups, including *Bifidobacterium* spp., *Streptococcus* spp., and *Clostridium* clusters IV and XIVa, was observed. The changes detected with the HITChip could be confirmed using denaturing gradient gel electrophoresis (DGGE) of SSU rRNA amplicons.

INTRODUCTION

The composition and functionality of the human intestinal microbiota is intimately linked to the nutrition and health of its host (Bäckhed *et al.*, 2005; Collins *et al.*, 2009; Ley, 2010; Zoetendal *et al.*, 2006). Hence, considerable interest has focused on studying the intestinal microbiota, and over the years more than 1000 different microbial phylotypes have been reported to inhabit the human intestinal tract (Rajilić-Stojanović *et al.*, 2007), providing an overwhelming catalogue of metabolic functions to their host, as was recently shown by metagenome analysis of over 100 individuals (Qin *et al.*, 2010). To better understand the composition and functionality of this complex microbial ecosystem, a number of methodologies have been developed (Zoetendal *et al.*, 2008), among which *in vitro* model systems have been introduced to bypass obvious restrictions with respect to the accessibility of the intestine for frequent sampling (Egert *et al.*, 2006; Kovatcheva-Datchary *et al.*, 2009b). Currently available models of the large intestine range from simple batch fermentation systems to more or less sophisticated, well-controlled, single- and multi-vessel continuous bioreactor systems (for a recent overview and discussion of the different models, see Macfarlane & Macfarlane, 2007). In contrast to most of the models, the TIM-2 model of the large intestine is characterized by a physiological water content and constant removal of metabolites via a dialysis system, allowing the recording of metabolite production kinetics and the prevention of product inhibition of fermentation processes (Egert *et al.*, 2006; Minekus *et al.*, 1999). This model has been used to investigate several microbiota-mediated processes that occur in the colon, such as the fermentation of a variety of potentially prebiotic carbohydrates (van Nuenen *et al.*, 2003; Venema *et al.*, 2003, 2005). Recently, stable isotope probing of microbial RNA has proven to be a useful instrument for the characterization of microbial populations responsible for the degradation of carbohydrates in the TIM-2 model (Egert *et al.*, 2007; Kovatcheva-Datchary *et al.*, 2009a).

Abbreviations: DGGE, denaturing gradient gel electrophoresis; PCA, principal component analysis; SCFA, short-chain fatty acid; SSU rRNA, small subunit rRNA.

Two supplementary figures are available with the online version of this paper.

Intestinal models have, in general, been validated based on how representative their metabolite production is in comparison with the *in vivo* situation obtained from sudden death victims (Macfarlane *et al.*, 1992, 1998; Minekus *et al.*, 1999). Nevertheless, only limited information is available with respect to the diversity and stability of the microbiota present in such model systems. The reproducibility and compositional stability of *in vitro* model systems have been evaluated using various approaches, such as group-specific cultivation on selective media (Macfarlane *et al.*, 2005; Minekus *et al.*, 1999; Venema *et al.*, 2000, 2003), and cultivation-independent small subunit rRNA (SSU rRNA)-based approaches, including fluorescent *in situ* hybridization (FISH) (Child *et al.*, 2006; Macfarlane *et al.*, 2005; Probert *et al.*, 2004), dot-blot hybridization (Hopkins *et al.*, 2003), quantitative real-time PCR (qPCR; van de Wiele *et al.*, 2004), and microbiota fingerprinting by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified SSU rRNA gene fragments (Possemiers *et al.*, 2004; van de Wiele *et al.*, 2004; Venema *et al.*, 2000, 2003). However, such approaches are in general not suitable to simultaneously provide data at sufficient phylogenetic and spatiotemporal resolution, as they allow either analysis of only a few samples at high phylogenetic resolution by clone library analysis, or profiling of multiple samples for only a restricted number of organisms (qPCR, FISH) or without any direct phylogenetic information (DGGE). Such information can now be generated by barcoded 454 pyrosequencing of PCR-amplified SSU rRNA gene fragments (Andersson *et al.*, 2008; Claesson *et al.*, 2009), as well as by phylogenetic microarrays, which are in general based on SSU rRNA-targeted diagnostic oligonucleotide probes that allow for the detection of micro-organisms at different levels of taxonomic resolution (Guschin *et al.*, 1997; Loy & Bodrossy, 2006; Smoot *et al.*, 2005; Wagner *et al.*, 2007; Zhou, 2003). Phylogenetic microarrays have now been developed for a wide range of complex microbial ecosystems, including the human intestine and the oral cavity (Palmer *et al.*, 2006; Rajilić-Stojanović *et al.*, 2009; Smoot *et al.*, 2005). The recently developed Human Intestinal Tract Chip (HITChip) offers the possibility of following the microbiota composition in intestinal model systems at unprecedented phylogenetic resolution to elucidate shifts in community composition that result from the operation procedures of intestinal models (Rajilić-Stojanović *et al.*, 2009). Its application here with the microbiota of the TIM-2 model reveals a highly diverse microbial community that contains some specific community shifts that can be avoided if specific precautions are taken.

METHODS

The TIM-2 model of the human colon: inoculum preparation, operating conditions and sampling. The TNO *in vitro* model of the human large intestine, TIM-2, represents the conditions found in the proximal colon. Standard operation procedures for inoculum preparation and model operation have previously been established and validated (Minekus *et al.*, 1999; van Nuenen *et al.*, 2003). The inoculum used in this study was derived from faecal donations of 10 healthy volunteers. For the purpose of this study, two slightly different procedures of inoculum preparation were used. For procedure IN1, 80 g fresh mixed faecal material was mixed in a 2 l fermenter with 670 ml of a complex medium that represents terminal ileal chyme [modified from Gibson *et al.* (1988) as described by van Nuenen *et al.* (2003)] and incubated at pH 5.8 and 37 °C. Additional medium was added after an initial 4 h batch incubation over the course of 40 h to reach a final volume of 2000 ml. For procedure IN2, 80 g faecal material was mixed with 670 ml dialysis liquid (van Nuenen *et al.*, 2003). Medium as described above, but concentrated 1.5-fold and amended with 1.7 % pig gastric mucin (type II, Sigma-Aldrich), was added over the course of 44 h of incubation, yielding the same final volume as described for IN1. Aliquots of 1000 ml were removed from the reactor, mixed with 10 % (w/v) glycerol, divided into aliquots inside an anaerobic cabinet, frozen in liquid N_2 and stored at −80 °C. For both procedures, samples were taken at 0, 4, 20, 24, 28 and 44 h of incubation, and stored at −80 °C until further analysis.

Duplicate modules of the fully computer-controlled TIM-2 model were inoculated with material obtained by procedure IN1 after thawing for 1 h at 37 °C, and were operated at three different pH values, 5.8, 6.4 and 7.0, as previously described (van Nuenen *et al.*, 2003). In brief, the model consisted of glass units with a flexible wall inside. By pumping warm water into the space between the glass jacket and the flexible walls at regular intervals, peristaltic movements were achieved. In this way, the contents of the lumen were mixed and transported through the system, and the warm water allowed the lumen in the model to be at body temperature (37 °C). The pH was set at 5.8, 6.4 or 7.0, and controlled by using a pH sensor in combination with NaOH addition. A dialysis system consisting of semi-permeable hollow fibres was run through the lumen. Due to the constant flow of dialysate through the dialysis system, water and fermentation products were constantly removed and thereby physiological concentrations of small molecules, such as electrolytes, were maintained and product inhibition of enzymes due to accumulation of microbial metabolites was prevented. A constant volume of the luminal content was maintained by water absorption controlled by a level sensor. The system was kept anaerobic by flushing with gaseous nitrogen. This allowed the growth of a dense, complex microbiota, comparable with that found in the first part of the human colon (Minekus *et al.*, 1999; Venema *et al.*, 2000).

At the start of each experiment, the model was inoculated with approximately 30 ml of the standardized microbiota and 80 ml of 2× diluted standard ileal efflux medium (SIEM; described below). The microbiota was allowed to adapt to the model conditions and SIEM for 16 h. Then a 3-day experimental period was started, in which SIEM was added at a flow rate of 60 ml per day or 2.5 ml h^{-1}. Samples were taken from the lumen and from the dialysate (~2 ml each) starting after the adaptation period (set as time point 0), and after 24, 48 and 72 h of addition of the test compound. After 24 and 48 h, a total lumen sample of 25 ml was removed from the system to simulate passage of material from the proximal to the distal colon.

SIEM and dialysate. SIEM simulates material passing the ileo-caecal valve in humans, or in other words material that reaches the colon. It was slightly modified for experiments in TIM-2 compared with the medium described by Gibson *et al.* (1988), concerning the following components (g l^{-1}): pectin (9.4), xylan (9.4), arabinogalactan (9.4), amylopectin (9.4), casein (47.0), starch (78.4), Tween 80 (34.0), Bacto Peptone (47.0) and ox bile (0.8). Dialysis liquid contained (per litre): 2.5 g $K_2HPO_4.3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4.7H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.45 g $CaCl_2.2H_2O$, 0.05 g bile and 0.4 g cysteine-HCl, plus 1 ml of a vitamin mixture containing (per litre): 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg *p*-aminobenzoic acid and 4 mg thiamine.

The pH was adjusted to 5.8. All medium components were provided by Tritium Microbiology.

Concentrations of the short-chain fatty acids (SCFAs) acetate, propionate and n-butyrate, and branched-chain fatty acids isobutyrate and isovalerate, were determined by GC, as described previously (van Nuenen *et al.*, 2003).

For evaluation of the microbial diversity during *in vitro* preparation, faecal samples from healthy human volunteers that had been analysed in a separate study were used (Rajilić-Stojanović *et al.*, 2009).

DNA extraction, PCR amplification and DGGE analysis. Samples were thawed at room temperature, and DNA was extracted from 200 µl using the FastDNA SPIN Kit for Soil (Qbiogene) and a FastPrep disruptor (FP120, Savant Instruments). DNA yield was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

DGGE of PCR-amplified 16S rRNA gene fragments was used to initially compare overall bacterial diversity in samples taken throughout inoculum preparation and model operation. To this end, primers GC-968-f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3′) and 1401-r (5′-GCG TGT GTA CAA GAC CC-3′) were used to amplify the V6–V8 region of the 16S rRNA gene, as described by Nübel *et al.* (1996). Amplicons were then separated by DGGE (Muyzer *et al.*, 1993), using a DCode system (Bio-Rad). Electrophoresis was done as described previously (Heilig *et al.*, 2002). Briefly, samples were loaded onto 8 % polyacrylamide gels with a denaturant gradient of 30–60 % (100 % was defined as 40 % formamide and 7 M urea), pre-run for 5 min at 200 V, and subsequently electrophoresed at 85 V for 16 h at 60 °C. Gels were developed by silver staining by the method of Sanguinetti *et al.* (1994), scanned at 400 d.p.i., and further analysed by BioNumerics 4.5 software (Applied Maths). Pearson product-moment correlation based on densitometric curves was used to assess the similarity of DGGE patterns obtained for different samples (Fromin *et al.*, 2002; Häne *et al.*, 1993; Zoetendal *et al.*, 2001).

HITChip analysis

PCR. The SSU rRNA gene was amplified from faecal DNA using the primers *T7prom*-Bact-27-for (5′-TGA ATT GTA ATA CGA CTC ACT ATA GGG GTT TGA TCC TGG CTC AG-3′) and Uni-1492-rev (5′-CGG CTA CCT TGT TAC GAC-3′), which ensured the introduction of a T7 promoter sequence at the 5′ terminus of the rRNA gene amplicon. PCRs were carried out in a final volume of 50 µl, and 10 ng DNA samples were used as template. Samples were initially denatured at 94 °C for 2 min, followed by 35 cycles of 94 °C (30 s), 52 °C (40 s) and 72 °C (90 s), and a final extension at 72 °C for 7 min. The PCR products were purified using the DNA Clean & Concentrator kit (Zymo Research) according to the manufacturer's instructions. The final DNA concentration was determined by using a NanoDrop spectrophotometer as described above.

RNA production and labelling. *In vitro* transcription of the T7 promoter carrying the SSU rRNA gene was performed according to the manufacturer's protocol using the Riboprobe System (Promega), 500 ng of the T7-16S rRNA gene amplicon, and in addition rATP, rGTP, rCTP, a 1 : 1 mix of rUTP and aminoallyl-rUTP (Ambion). The transcription reaction was performed at room temperature for 2 h, the template DNA was digested using the Qiagen RNase-free DNase kit, and RNA was purified using the RNeasy MiniElute kit (Qiagen). RNA yield was measured as described above.

Amino-allyl-modified nucleotides were coupled with CyDye using the Post-Labelling Reactive Dye pack (Amersham Biosciences), which had been dissolved in 84 µl DMSO. The labelling reaction was performed in a 25 mM sodium bicarbonate buffer (pH 8.7) by adding 20 µl dissolved CyDye to 2 µg purified RNA in a final volume of 40 µl. Samples were incubated in the dark at room temperature for 90 min. The reaction was stopped by adding 15 µl 4 M hydroxylamine and incubating in the dark for 15 min. RNase-free water was added to a total volume of 100 µl, and labelled RNA was purified and quantified as described above.

Microarray production, hybridization and data extraction. The HITChip microarrays were produced by Agilent Technologies. The oligonucleotide probes were extended at the 3′ end (at the array support side) by 10 nt T spacers and were printed on the array using *in situ* surface-attached oligonucleotide probe synthesis (Blanchard *et al.*, 1996). The arrays used in this study were of the 2 × 11K format, with two arrays per glass slide. Each array was hybridized with two samples, labelled with Cy3 and Cy5. Combined Cy3- and Cy5-labelled target mixtures were fragmented by adding 1 µl Ambion 10 × fragmentation reagent (Ambion) and incubation at 70 °C for 20 min, according to the manufacturer's instructions. Fragmentation was stopped by adding 1 µl Ambion stop solution. Hybridization mix was prepared by adding to the RNA mixture 31.5 µl 20 × saline sodium citrate (SSC) (Sambrook *et al.*, 1989), 6.3 µl 10 % SDS (Sambrook *et al.*, 1989), 25 µl Agilent Control Target mix and RNase-free water to a total volume of 210 µl. Hybridization was carried out at 62.5 °C in a rotation oven (Agilent) for 16 h. Slides were washed at room temperature in 2 × SSC, 0.3 % SDS for 10 min, and at 38 °C in 0.1 × SSC, 0.3 % SDS for 10 min. SDS was completely removed by washing the slides in 0.06 × saline-sodium phosphate-EDTA (SSPE) (Sambrook *et al.*, 1989) for 5 min, followed by a quick dry with compressed nitrogen.

Data were extracted from microarray images using the Agilent Feature Extraction software, version 7.5 (http://www.agilent.com). Data normalization was performed using a set of R-based scripts (http://www.r-project.org/), while the further microarray analysis was performed in a custom-designed relational database which runs under the MySQL database management system (http://www.mysql.com/) using a series of custom-made R scripts, as previously described (Rajilić-Stojanović *et al.*, 2009). The similarity of the total microbiota composition based on the HITChip profiles was assessed by calculating Pearson's product-moment correlation (Pearson's correlation), which reflects the degree of linear relationship between analysed datasets. Ward's minimum variance method was used for the generation of hierarchical clustering of probe profiles by calculating a distance matrix between the samples based on the squared difference between each pair of profiles (squared Euclidian distance; E^2) (Carter *et al.*, 1989).

Statistical analysis. To assess correlations of microbial groups detected by HITChip analysis with sample characteristics, principal component analysis (PCA) was used as implemented in Canoco for Windows 4.5 (Lepš & Šmilauer, 2003). Mean signal intensities for 131 bacterial groups defined on the basis of the SSU rRNA gene sequence similarity were used as species data. PCA was performed focusing on inter-species correlations, and diagrams were plotted by using the CanoDraw for Windows utility. For the evaluation of the statistical significance of observed correlations in the entire dataset, the Monte Carlo permutation procedure (Hope, 1968), as implemented in the Canoco package, was used, and for the individual phylogenetic groups, Student's *t* test was used. Calculated *P* values were two-tailed and assumed equal variance. Because of the large number of simultaneous analyses, obtained Student's *t* test *P* values were corrected into q values (Storey, 2003). Correlations were considered significant at a *P* or q value lower than 0.05.

The diversity of the microbial community assessed by the HITChip analysis was expressed as Simpson's reciprocal index of diversity (1/*D*) (Simpson, 1949), which was calculated using the equation $\lambda = 1/\Sigma \bullet P_i^2$, where P_i is the proportion of the *i*th taxon. The

proportion of each taxon was calculated as a proportion of each probe signal compared with the total signal obtained per sample. Simpson's reciprocal index of diversity takes into account both the number of taxa present in a sample and their abundance in the community. A higher value of the Simpson's index corresponds to a more diverse community.

RESULTS

Production of standardized faecal inocula

Differences in feeding regime between the two procedures, i.e. initial batch addition of 30 % of the medium in the case of IN1 and linear addition of a more concentrated medium for IN2, were reflected by different profiles of SCFA production during the 44 h of inoculum propagation. Acetate, propionate and n-butyrate were more readily produced in the initial phase of IN1, in line with the initial batch addition, while final concentrations reached in IN1 and IN2 were comparable (80 mM acetate, 30 mM propionate, 25 mM n-butyrate). Reproducibility between metabolite measurements obtained with two technical replicates was very high (99.9 %), with a relative standard deviation of measurements of 1.25 %. Similar kinetics were observed for the production of isobutyrate and isovalerate, although these branched-chain fatty acids were produced at 10-fold lower concentrations. DGGE profiling, as well as HITChip analysis based on the signal intensity of 3700 distinct oligonucleotide probes, revealed that neither of the procedures, IN1 or IN2, dramatically affected community composition. The microbiota present after 44 h was for IN1 85.0 % (SD 2.1 %) and for IN2 79.2 % (SD 4.0 %), similar to the initial mixture of the faecal donations based on Pearson product-moment correlations of DGGE profiles (Fig. 1).

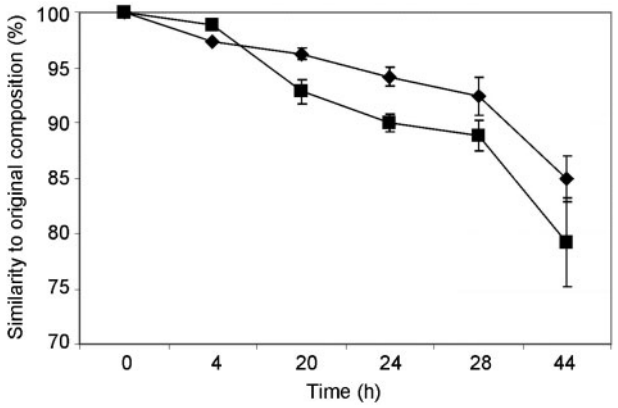

Fig. 1. Similarity indices based on Pearson product-moment correlation coefficients for DGGE fingerprints generated from the initial inoculum compared with those taken at 0, 4, 20, 24, 28 and 44 h during the propagation of faecal inocula for *in vitro* intestinal models by two different procedures, IN1 (◆) and IN2 (■). Mean data from duplicate DGGE gels are shown with their respective standard error.

The similarity between the initial microbiota composition and the outcome of the inoculum preparation using both procedures, assessed by HITChip analysis, was comparable with the results obtained by the analysis of the DGGE gels. The calculated similarity for IN1 was 82.5 % (85.0 % based on DGGE), whereas for IN2 it was 74.9 % (79.2 % based on DGGE). The same trend of the results, and the slightly lower values produced by the HITChip, showed the good agreement between the two profiling techniques and indicated the greater sensitivity of the HITChip profiles. Moreover, during propagation of the two inocula, the bacterial community composition seemed, based on the DGGE analysis, to be similarly affected, as the same change in the relative intensity of in most cases identical DGGE bands was observed for IN1 and IN2 (Supplementary Fig. S1). Analysis of the phylogenetic fingerprints obtained using the HITChip provided a more detailed insight into subtle differences between faecal mix, and inocula IN1 and IN2 (Fig. 2). It appeared that both procedures affected individual populations within the phylum Firmicutes in a similar manner, while procedure IN2 had an additional pronounced impact on members of the Bacteroidetes (Fig. 2). This strong stimulation of the Bacteroidetes subpopulation during the IN2 preparation procedure resulted in a shift in the relative contributions of Gram-positive and Gram-negative bacteria to the total community. In the initial faecal mix, the relative contribution to the hybridization signal of all HITChip probes targeting Gram-negative bacteria, which include members of the phyla Bacteroidetes, Cyanobacteria, Fusobacteria, Proteobacteria, Spirochaetes and Verrucomicrobia, was 18.7 %, while after 44 h of propagation it reached 25.1 and 66.1 % for IN1 and IN2 procedures, respectively.

DGGE analysis of samples obtained before and after freezing of the inocula indicated that material generated by procedure IN1 was less prone to detectable freeze–thaw-induced changes in community composition. Similarity values were 96.5 and 67.1 % for IN1 and IN2, respectively. Consequently, inoculum prepared by procedure IN1 was chosen for subsequent experiments with the TIM-2 model, as described below.

Impact of *in vitro* model operation on the microbiota composition

IN1-derived material was used to inoculate duplicate modules of the TIM-2 model of the proximal colon, and samples taken at regular 24 h intervals were analysed for production of metabolites, as well as composition and stability of the microbiota. The total amount of SCFA produced after 72 h of the TIM-2 model run was notably lower for pH 5.8, at 167 mmol compared with 207 mmol produced at pH 6.4 and 210 mmol at pH 7.0. Acetate, propionate and n-butyrate were the main metabolites observed, but the molar ratios differed among runs at the three pH values, and for pH 6.4 only they reached ratios of 3 : 1 : 1, similar to values found during inoculum

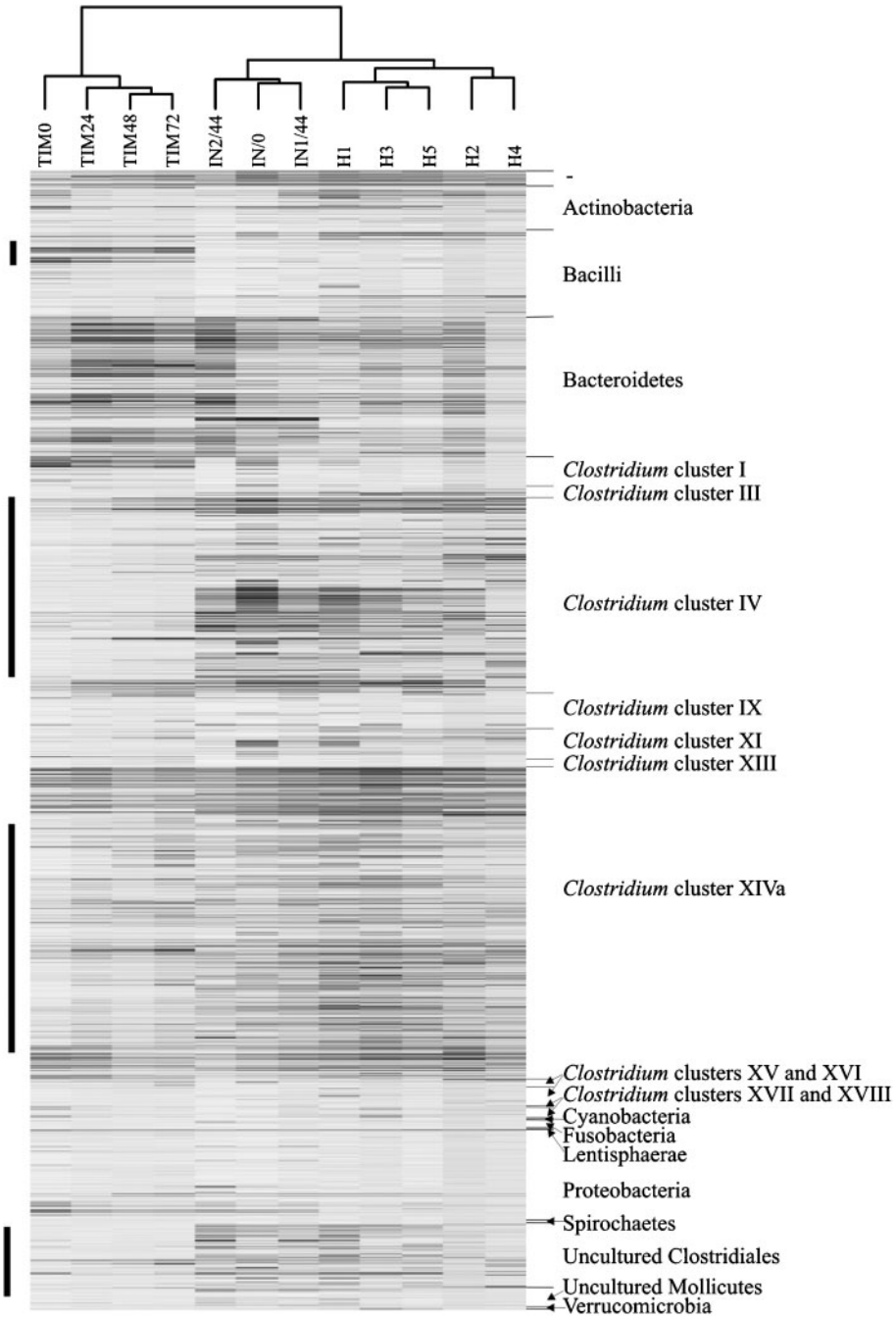

Fig. 2. Hierarchical cluster analysis of phylogenetic HITChip fingerprints generated from a faecal mix sample (IN/0), and after a sample was taken after 44 h of inoculum preparation using both procedures (IN1/44 and IN2/44), as well as samples taken at 0, 24, 48 and 72 h of TIM-2 operation after an initial 16 h stabilization of inoculum IN1/44 (TIM0–TIM72), and faecal samples of five randomly selected healthy adult volunteers (H1–H5). The highest phylogenetic ranks of probe specificity are given to the right of the figure. Vertical bars at the left indicate microbial groups most affected by *in vitro* model operation.

propagation and ratios found normally in the human colon (Weaver *et al.*, 1988) (Fig. 3a). At the other two pH values (pH 5.8 and 7.0), the relative contribution of acetate was higher, and observed ratios were 3.9 : 1 : 1.5 and 4.5 : 1.5 : 1, respectively (data not shown).

DGGE profiles for microbiota development during *in vitro* model operation were generated for duplicate runs for all three analysed pH values (Supplementary Fig. S2). At pH 6.4 and 7.0, microbiota profiles were diverse and showed similar development with time, as evaluated by a

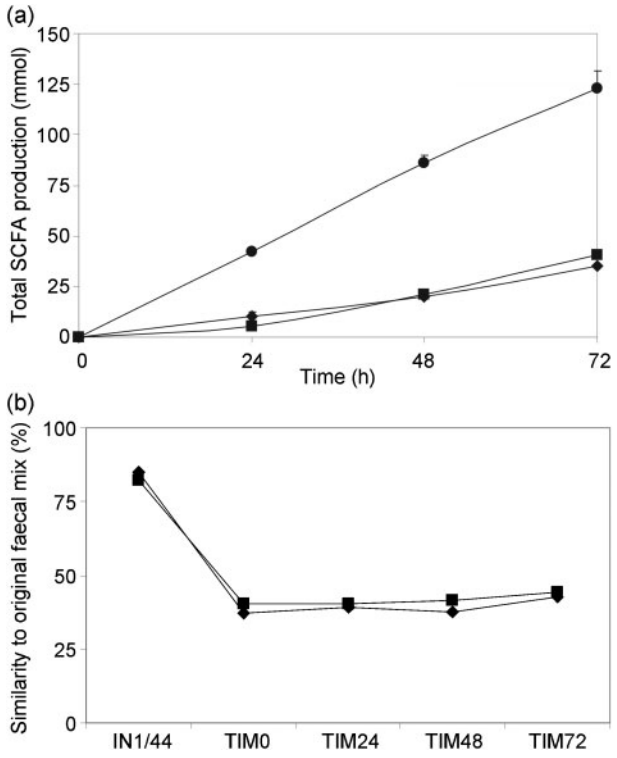

Fig. 3. (a) Cumulative production of the SCFAs acetate (●), propionate (■) and n-butyrate (◆) during TIM-2 operation at pH 6.4 and 37 °C. Mean data from duplicate TIM-2 runs are shown with their respective standard error. (b) Similarity of the TIM-2 model microbiota to that of the initial faecal mixture used for inoculum preparation, based on DGGE (◆) and HITChip (■) analyses. IN1/44, inoculum prepared according to procedure IN1, after 44 h of cultivation; TIM0, -24, -48, -72, samples taken from the TIM-2 model at 24 h intervals, starting after an initial adaptation for 16 h of the inoculum IN1/44.

comparable, albeit moderate, similarity to the starting community composition at different time points. The observed trends were strongly positively correlated for both pH values (95.8 %). In contrast, the total microbiota profiles obtained at pH 5.8 appeared to be less diverse, and remained more similar to those of the starting community (Supplementary Fig. S2).

Bearing in mind that TIM-2 is the *in vitro* model of the proximal colon, further phylogenetic analyses were performed using the samples collected while the model was operated at pH 6.4. Similarity indices, calculated on the bases of DGGE and HITChip profiles, between the microbiota of the starting faecal mix and the samples taken during the course of the *in vitro* model operation at pH 6.4, showed similar trends (Fig. 3b). A notable shift of the microbiota composition during the initial 16 h of stabilization of the inoculum was observed, as similarity to the original faecal mix dropped to values of around 40 %, which were only slightly increased throughout the subsequent model operation for 72 h.

The mean similarity between the total microbiota profiles of the faecal mixure (IN/0) and two propagated inoculum samples (IN1/44 and IN2/44) analysed on the HITChip, and faecal samples was 71.7 %, which is very close to the inter-individual similarity of the faecal microbiota of different individuals (mean value for the five analysed faecal samples was 76.8 %). In contrast, the similarity between the total microbiota of the TIM-2 model samples and the selected faecal samples appeared to be lower (mean 50.0 %) and outside of the 2SD confidence range for the inter-individual similarity of the microbiota of healthy subjects.

Hierarchical clustering of HITChip profiles obtained for samples taken from TIM-2 with those taken throughout the preparation of inocula, as well as randomly selected HITChip profiles generated from faecal samples of healthy adults, confirmed that the microbiota selected by TIM-2 operation conditions deviated considerably from normal human colonic microbiota (Fig. 2), whereas samples taken during inoculum preparation still clustered with the profiles of the human faecal microbiota.

Visual interpretation of the total microbiota profiles indicated that after an initial drop in the microbial diversity, the microbial community became more complex during the running of the TIM-2 model (Fig. 2). This was confirmed by assessing the Simpson's index of diversity, as it was found that the diversity index of the microbial community dropped from 148 in the inoculum (IN1/44) to 55 at the beginning of the TIM-2 operation. During the run of the TIM-2 model, the diversity index increased again, reaching a value of 117 after 72 h of *in vitro* model operation. For comparison, the mean diversity index of the five faecal samples presented in this study was 175.

To identify those microbial populations responsible for the observed deviation of TIM-2 from the *in vivo* situation, Student's *t* tests were performed. This revealed that a total of 23 out of the 131 bacterial groups were significantly different between TIM-2 samples and human faecal profiles (Table 1). The most pronounced changes could be attributed to an increase in the abundance of several groups within the order Bacilli (*Enterococcus*, *Granulicatella*), while typical anaerobes belonging to the phylum Firmicutes were significantly suppressed in the TIM-2 mode. A significant change of the hybridization signal of a large proportion of the intestinal microbial groups suggested that overall the microbiota was significantly affected in the TIM-2 model. This was confirmed by multivariate redundancy analysis (RDA), which showed that the composition of the total microbial community in the TIM-2 *in vitro* model of the human colon was significantly different from the microbial community present in the inoculum of the model or in healthy adults (P=0.001) (Fig. 4).

DISCUSSION

The composition and stability of the intestinal microbiota maintained in the TIM-2 *in vitro* model of the human colon was evaluated by bacterial SSU rRNA gene-targeted

Table 1. Microbial groups significantly affected by TIM-2 operation as compared with faecal microbiota of healthy volunteers, assessed by mean HITChip signal intensities for 131 phylogenetic groups based on their SSU rRNA sequence similarity

Mean signal intensities and *P* values are shown.

Higher taxonomic group/group	*P* value	Mean signal intensity	
		TIM	Faeces
Bacteroidetes			
Prevotella tannerae-like	0.030	237	37
Bacteroides intestinalis-like	0.050	309	74
Actinobacteria			
Bifidobacterium	0.041	31	180
Firmicutes			
Bacilli			
Enterococcus	<0.001	304	24
Granulicatella	0.002	121	16
Streptococcus bovis-like	0.002	28	189
Streptococcus mitis-like	0.002	30	95
Streptococcus intermedius-like	0.006	32	74
Aneurinibacillus	0.027	21	74
***Clostridium* cluster III**			
Clostridium stercorarium-like	0.002	11	45
***Clostridium* cluster IV**			
Ruminococcus callidus-like	0.001	15	57
Oscillospira guillermondii-like	0.007	56	177
Clostridium orbiscindens-like	0.009	31	119
***Clostridium* cluster XI**			
Anaerovorax odorimutans-like	0.005	28	57
***Clostridium* cluster XIVa**			
Anaerostipes caccae-like	0.001	17	228
Eubacterium hallii-like	0.001	63	368
Ruminococcus gnavus-like	0.010	45	158
Coprococcus eutactus-like	0.021	37	147
Clostridium symbiosum-like	0.032	63	97
Dorea	0.034	54	174
Lachnospira pectinoschiza-like	0.036	19	69
Ruminococcus obeum-like	0.042	147	456
Outgrouping *Clostridium* cluster XIVa	0.049	18	68

DGGE and phylogenetic microarray analysis using the HITChip. To this end, a number of steps during the preparation and preservation of standardized inocula, as well as the actual operation of the model system, were investigated. In addition to the monitoring of the inoculum production process, we also compared two methods, which differed (i) in the presence or absence of mucin, and (ii) with respect to the conditions of medium addition to the cultivation vessel. The intestinal mucus layer provides an ecological niche, and mucus can be seen as a rich carbon and energy source for intestinal microbiota (Derrien *et al.*, 2004). Remarkably, it has been shown that not only are mucin beads added to a two-stage intestinal model system readily colonized by bacterial biofilms, but also that mucin is largely degraded by luminal microbiota, reinforcing the notion that mucin can serve as substrate for a variety of intestinal micro-organisms (Leitch *et al.*, 2007; Macfarlane *et al.*, 2005).

To provide a standardized inoculum for a large number of experiments, allowing for high reproducibility and comparability between runs, a protocol had previously been developed in which faecal donations from 10 healthy volunteers were mixed, propagated and preserved by freezing in the presence of glycerol as a cryo-protectant (van Nuenen *et al.*, 2003; Venema *et al.*, 2000). The extent to which this procedure affected the microbial composition was readdressed using the HITChip, although it had previously been assessed by DGGE and selective plating, because of the limitations of the two latter techniques. DGGE analysis does not directly provide any information about the phylogenetic affiliation of populations represented by bands in diversity profiles, while plating is laborious and seriously impacted by our inability to cultivate the majority of intestinal micro-organisms with currently available media (Zoetendal *et al.*, 2004).

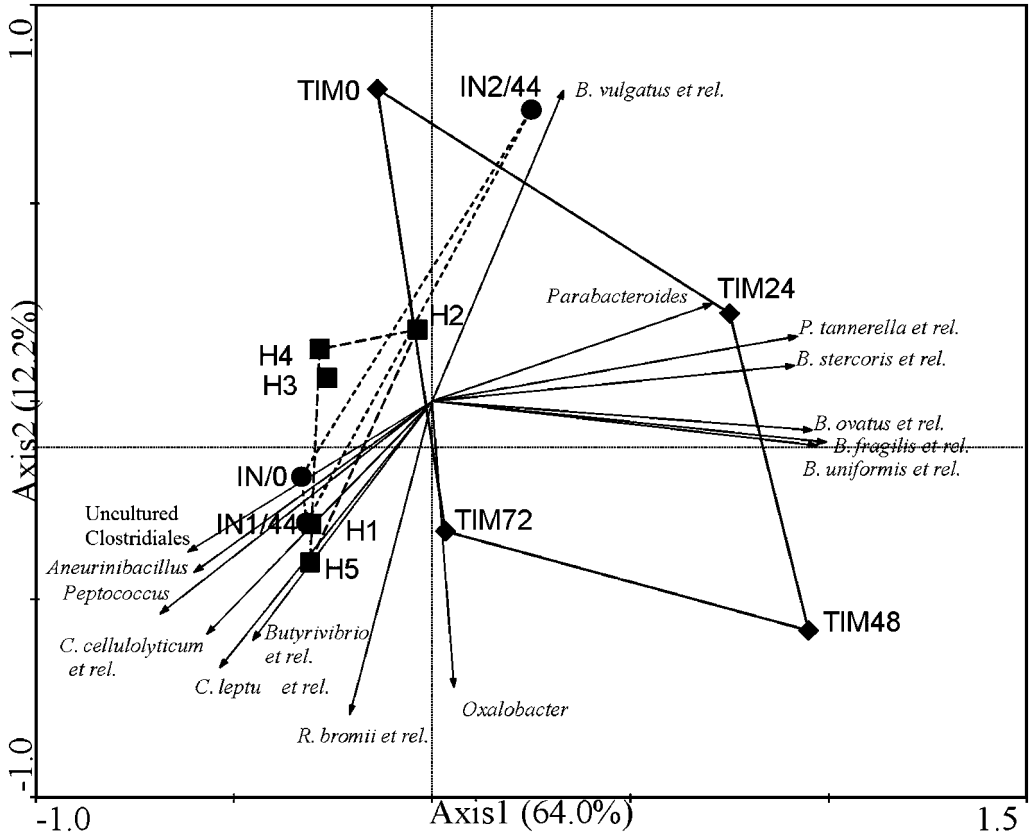

Fig. 4. PCA of the microbiota composition as measured by the mean hybridization signals for 131 phylogenetic groups in samples taken during inoculum preparation (●; IN/0, IN1/44, IN2/44), samples taken at 0, 24, 48 and 72 h of TIM-2 operation after an initial 16 h stabilization of the inoculum IN1/44 during TIM-2 model operation (◆; TIM0–TIM72), and five randomly selected faecal samples of healthy adults (■; H1–H5). Microbial groups that contributed at least 75 % to the first two principal components used as explanatory axes in the plot are shown as vectors. For the details about coding see Fig. 2. Monte Carlo permutation procedure (MCPP) revealed that the environmental variables 'inoculum', 'human' and 'TIM' were highly significantly correlated with the variation of the microbiota composition (P=0.001).

The results of the comprehensive HITChip analysis showed that the two inoculum preparation procedures affected individual populations within the phylum Firmicutes in a similar manner. These changes can be explained as stabilization of a novel ecosystem, which was made by combining the 10 independent ecosystems present in the faecal samples used. Furthermore, procedure IN2 had a pronounced impact on members of the phylum Bacteroidetes, which made its composition more deviant from that present in healthy adults. The specific enrichment of primarily *Bacteroides vulgatus*-like organisms was most likely induced by the addition of mucus, since *B. vulgatus* was found to be the predominant mucus colonizer of two out of four analysed subjects in a recent study (Leitch *et al.*, 2007). The ability to extract these data directly from the microbiota profiles emphasizes the advantage of phylogenetic microarray-based fingerprints in comparison with those obtained using other, well-established fingerprinting techniques such as DGGE, and reinforces earlier evidence for the superiority of phylogenetic microarrays (Neufeld *et al.*, 2006). The stimulation of the members of the phylum Bacteroidetes caused a strong shift in the ratio between Gram-negative and Gram-positive bacteria after propagation of IN2, compared with the faecal mixture. A higher proportion of the more sensitive Gram-negative bacteria could explain the more pronounced sensitivity to the freeze–thaw procedure of the IN2 inoculum compared with the IN1 inoculum.

Based on the high similarity between the two inoculum samples with randomly selected faecal samples, reported shifts in the microbiota composition of the IN2, and the evaluated effect of the thawing procedure on the microbial community in the standard inoculum samples, the previous procedure for inoculum preparation (IN1) was recognized as optimal (van Nuenen *et al.*, 2003).

Considerable changes in the composition of the microbial community were observed after the stabilization of the inoculum, which could have their origin in any of the steps that precede the actual *in vitro* model experiment, i.e.

freezing and thawing of the standardized inoculum, traces of oxygen during −80 °C storage of faecal samples, oxygen entering the system during the actual inoculation of the model, and the adaptation period of 16 h. In addition, the decreased similarity between the TIM-2 samples and the faecal donor samples, as compared with the initial inoculation samples, as well as the reduced diversity of the TIM-2 samples, could be due to the wash-out of dead cells that are present in the initial inoculum and are not degraded during the first 44 h of fermentation. After the initial change of the microbial community, during the run of the TIM-2 model, the microbial community was developing, which resulted in increasing microbial diversity, and increasing similarity with the inoculum microbiota and microbiota of faecal samples. The diversity index after 72 h of TIM-2 model operation was lower than that of faecal samples, but at a level similar to that of the diversity index of luminal samples of the small intestine (Booijink, 2009). Furthermore, it should be noted that although similarity indices indicated that samples of the TIM-2 model were statistically different from faecal samples, the microbiota of the TIM-2 model was overall more similar to that of the faecal samples (50.0 %) than it was to the microbiota of the small intestine (25.6 %) or the faecal microbiota of infants (41.4 %; unpublished data).

Groups that significantly decreased during model operation included several populations within *Clostridium* clusters IV (*Clostridium leptum* group) and XIVa (*Clostridium coccoides* group), as well as species related to *Clostridium stercorarium* (Table 1, Figs 2 and 4). Some of these groups that were negatively affected in the present study represent fastidious anaerobes, which in the past have largely eluded cultivation. This holds specifically for members of the Firmicutes, such as the *Clostridium* cluster IV and XIVa groups (Konstantinov *et al.*, 2005; Suau *et al.*, 1999; Tannock, 1995). Despite the fact that the changes in the microbiota composition of the TIM-2 model were not global, the effect appeared major and the microbiota was found to be significantly different from that of healthy subjects. Interestingly, in a previous study that employed the TIM-2 model, where only the active microbiota (represented by RNA samples) was analysed, the TIM-2 model samples, although different, could not be statistically distinguished from true faecal samples (Kovatcheva-Datchary *et al.*, 2009a). This might partly be explained by differences in inoculation procedure, as for the latter study, fresh faecal material from each of seven individuals was mixed in an anaerobic glove box and used to inoculate the TIM-2 system, and also partly by the fact that in the TIM-2 model, only the active part of the microbiota can be stimulated. DNA-based methods allow for detection of both live and dead bacterial cells (Josephson *et al.*, 1993). Novel approaches for specific detection of only viable subpopulations by flow cytometry- and DNA-based analyses of microbiota communities have recently been developed (Ben-Amor *et al.*, 2005; Nocker *et al.*, 2006). Such analysis of the viable fraction would enable us to truly evaluate the effect of the TIM-2 model operational procedure on the initial microbial community and would be a suggested approach for future studies.

In a previous study, in which stable isotope probing of bacterial RNA was used to identify major players in colonic carbohydrate fermentation using glucose as a model substrate for methods development, terminal restriction fragment length polymorphism (T-RFLP) fingerprinting and SSU rRNA clone library analysis revealed that the microbiota that was enriched in the TIM-2 model resembled more the small rather than the large intestinal situation (Egert *et al.*, 2007). As all identified bacterial species in the study of Egert and colleagues were oxygen-tolerant, the presence of oxygen traces seems to be a highly relevant factor that induces shifts in the microbiota composition of the TIM-2 *in vitro* model. Since the extent of the effect of oxygen on the microbial community in the TIM-2 model appears to be much larger in the study of Egert and colleagues, it can be speculated that the microbial community of the same *in vitro* model, but in different runs, can be substantially different. This result enforces the need for (re-)evaluation of the actual microbial diversity for each study based on this and other *in vitro* models, as only a well-defined starting community in an *in vitro* model would enable reliable interpretation of the results obtained.

Specific changes of the microbial community caused by the TIM-2 model operation procedure reported in this study were not observed in another *in vitro* model of the human colon (Child *et al.*, 2006). This can be explained by the fact that different technologies were used for the evaluation of the microbial diversity in the *in vitro* models, and also by specific effects of the standard operating procedures for both models. Because of the substantial differences in the design and operation procedures of different *in vitro* models of the human large intestine, it is rational to expect that the effect on the microbiota differs between different models, as has been shown for *in vitro* models of other ecosystems (Oomen *et al.*, 2002). It would be of value to confirm this hypothesis by comparing the microbiota of the TIM-2 model with that of other *in vitro* models. Recently, the dynamics and composition in the SHIME *in vitro* model (Molly *et al.*, 1993), which contains three individual compartments that simulate the ascending, transverse and descending colon, have been assessed by DGGE and HITChip analysis (Van den Abbeele *et al.*, 2010). In line with observations described in the present study for the TIM-2 model, the microbiota in the SHIME model is also characterized by a reduced diversity when compared with the corresponding faecal inoculum. Also, similar differences were observed with respect to changes in the relative abundance of specific microbial groups. In line with data presented here, SHIME operation has been shown to selectively stimulate members of the Bacteroidetes and *Clostridium* cluster IX, whereas *Clostridium* clusters IV and XIVa are reduced (Van den Abbeele *et al.*, 2010).

In conclusion, two SSU rRNA gene-based techniques were employed to identify shifts in the faecal microbiota composition during the preparation of standardized inoculum and during the operation of the TIM-2 *in vitro* model of the human large intestine. The results obtained using DGGE and the HITChip were consistent, while more detail was provided with the HITChip phylogenetic microarray analyses. The phylogenetic insight into the microbiota composition showed that despite small changes during the preparation procedure, the microbiota of the standardized faecal inoculum resembles that of fresh faecal samples. However, the initial adaptation of the faecal inoculum in the TIM-2 *in vitro* model introduces considerable shifts in the microbiota composition, which affect the relative abundance of several microbial groups. As a result of these shifts, the stimulated microbial community in the *in vitro* model appears to be significantly different from the microbial community of healthy adults. This can at least partially be attributed to the fact that DNA-based microbiota profiles represent the entire microbial community independent of its viability, while in *in vitro* models, only the viable fraction of the microbiota can be stimulated. Nevertheless, the results reported in this study provide new leads for adjustments in standard operating procedures of the TIM-2 *in vitro* model that will ensure a more accurate representation of the diversity of the gastrointestinal microbiota of healthy adults. Such studies are currently under way, focusing on the effects of individual steps during model operation, including initial propagation of a mixed faecal sample, freezing and thawing, inoculation of the model, and stabilization of the inoculum under the *in vitro* model conditions. To this end, the results presented here also indicate that the pH values chosen for individual steps should be considered as well. Finally, because of the different responses of the microbial community to different runs of the analysed *in vitro* model, even with standardized operational procedures, we strongly recommend evaluation of the microbial diversity in comparison with that in the *in vivo* situation for all studies, as only acknowledging the initial microbial diversity will allow appropriate interpretation of the results.

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Edited by: H. J. Flint