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ORIGINAL ARTICLE

Butyrate-producing *Clostridium* cluster XIVa species specifically colonize mucins in an *in vitro* gut model

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The human gut is colonized by a complex microbiota with multiple benefits. Although the surface-attached, mucosal microbiota has a unique composition and potential to influence human health, it remains difficult to study *in vivo*. Therefore, we performed an in-depth microbial characterization (human intestinal tract chip (HITChip)) of a recently developed dynamic *in vitro* gut model, which simulates both luminal and mucosal gut microbes (mucosal-simulator of human intestinal microbial ecosystem (M-SHIME)). Inter-individual differences among human subjects were confirmed and microbial patterns unique for each individual were preserved *in vitro*. Furthermore, in correspondence with *in vivo* studies, Bacteroidetes and Proteobacteria were enriched in the luminal content while Firmicutes rather colonized the mucin layer, with *Clostridium* cluster XIVa accounting for almost 60% of the mucin-adhered microbiota. Of the many acetate and/or lactate-converting butyrate producers within this cluster, *Roseburia intestinalis* and *Eubacterium rectale* most specifically colonized mucins. These 16S rRNA gene-based results were confirmed at a functional level as butyryl-CoA:acetate-CoA transferase gene sequences belonged to different species in the luminal as opposed to the mucin-adhered microbiota, with *Roseburia* species governing the mucosal butyrate production. Correspondingly, the simulated mucosal environment induced a shift from acetate towards butyrate. As not only inter-individual differences were preserved but also because compared with conventional models, washout of relevant mucin-adhered microbes was avoided, simulating the mucosal gut microbiota represents a breakthrough in modeling and mechanistically studying the human intestinal microbiome in health and disease. Finally, as mucosal butyrate producers produce butyrate close to the epithelium, they may enhance butyrate bioavailability, which could be useful in treating diseases, such as inflammatory bowel disease.

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Introduction

The human colon is colonized by a complex microbiota, mostly (>90%) consisting of Bacteroidetes and Firmicutes (Eckburg *et al.*, 2005; Claesson *et al.*, 2011; Walker *et al.*, 2011). Beneficial contributions of this microbiota to human health include the breakdown of otherwise indigestible food compounds (Koropatkin *et al.*, 2012) and regulation of host metabolism (Backhed *et al.*, 2005). Furthermore, the importance of the intestinal mcrobiota follows from the number of diseases that have been correlated with a dysbiosed microbial composition, such as inflammatory bowel diseases (IBDs; Willing *et al.*, 2009; Walker *et al.*, 2011) or obesity (Turnbaugh *et al.*, 2006). A novel focus in gut microbiology is to not only study microbes in the intestinal content but also those that colonize the mucus layer (Swidsinski *et al.*, 2008; Van den Abbeele *et al.*, 2011c; Belzer and de Vos, 2012). The rationale is that mucosal microbes can interact more closely with the epithelium than their luminal counterparts, which may be crucial for achieving immunomodulatory effects. Moreover, by locally excreting antimicrobials or competing with

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pathogens, mucosal microbes more effectively limit pathogen translocation. Besides host immune effectors (Lievin-Le Moal and Servin, 2006), microbial properties, such as mucus adhesion (Roos and Jonsson, 2002) or the ability to degrade host-derived glycans (Derrien *et al.*, 2004), also impact the distinct surface-attached microbial composition, generally characterized by an enrichment of Firmicutes (especially *Clostridium* cluster XIVa) over Bacteroidetes (Eckburg *et al.*, 2005; Frank *et al.*, 2007; Hill *et al.*, 2009; Shen *et al.*, 2010; Willing *et al.*, 2010; Hong *et al.*, 2011; Nava *et al.*, 2011). Moreover, the mucus layer is persistently colonized by all types of hydrogenotrophs: methanogenic archaea, sulphate-reducing bacteria and acetogenic bacteria (Nava *et al.*, 2012).

Despite their physiological relevance, human studies are often limited to faecal samples, which do not provide information on this mucosal microbiota. For this purpose, biopsies need to be collected, but given the invasive sampling procedures, these are often only taken at the end of an experiment, preventing dynamic monitoring or detailed mechanistic studies (Zoetendal *et al.*, 2002). By contrast, *in vitro* studies have the advantage that they are well-suited to perform mechanistic research. However, the current models generally only provide short-term information and often ignore the interaction between luminal and mucosal microbes. Recently, a long-term dynamic *in vitro* model was developed, which accounts for both the luminal and mucosal microbiota (mucosal-simulator of human intestinal microbial ecosystem (M-SHIME); Van den Abbeele *et al.*, 2011b). The simulated mucosal environment consisted of carrier material coated with commercial pig gastric mucins. When focussing on Lactobacilli, it was found that, in correspondence with *in vivo* data, this *in vitro* mucosal environment was colonized by specific *Lactobacillus* species (*L. mucosae* and *L. rhamnosus* GG). However, the overall microbial community shifts remain to be elucidated.

Therefore, the aim of this study was to perform an in-depth analysis of the *in vitro* mucosal M-SHIME microbiota using the human intestinal tract chip (HITChip), a recently developed and widely used phylogenetic micro-array (Jonkers *et al.*, 2009; Rajilic-Stojanovic *et al.*, 2009; Van den Abbeele *et al.*, 2011a). Each experiment was conducted with samples from different human subjects to account for the inter-individual variability. The aims were to determine whether the inter-individual differences among human subjects can be preserved in the model, to determine the distinct composition of the mucosal microbiota compared with the luminal one and to assess the main metabolic activities of mucosal microbes. By comparing these results with recent *in vivo* data and results obtained with conventional *in vitro* models without surface-attached bacteria, the novel M-SHIME model was validated.

Materials and methods

Chemicals and preparation of growth media

Unless stated otherwise, chemicals were obtained from Sigma (Bornem, Belgium). The experiments were conducted using sugar-depleted nutritional medium containing (in $g\,l^{-1}$) yeast extract (3.0), peptone (1.0), commercial pig gastric mucin (4.0) and cystein (0.5). Pancreatic juice contained (in $g\,l^{-1}$) $NaHCO_3$ (12.5), bile salts (6.0; Difco, Bierbeek, Belgium) and pancreatin (0.9). Mucin agar was prepared by boiling dH_2O containing 5% commercial pig gastric mucin and 1% agar. The pH was adjusted to 6.8 with 10 M NaOH.

Dynamic in vitro gut model for the luminal and mucosal microbiota (M-SHIME)

Although the conventional luminal (**L**)-SHIME (registered trademark, Ghent University-Prodigest, Ghent, Belgium) only simulates luminal microbes (Van den Abbeele *et al.*, 2010), the **M**-SHIME also contains a niche for surface-attached microbes (Figure 1a; Van den Abbeele *et al.*, 2011b). Briefly, microcosms (K1-carrier, AnoxKaldnes AB, Lund, Sweden) were submerged in mucin agar and combined in a polyethylene netting (Zakkencentrale, Rotterdam, The Netherlands). At the start of each experiment, 500 ml nutritional medium and 100 mucin-covered microcosms were added to each colon unit, followed by inoculation with 40 ml of a 1:5 dilution of fresh stools of a healthy human volunteer (Possemiers *et al.*, 2004). After an initial incubation of 18 h, 140 ml nutritional medium and 60 ml pancreatic juice were supplied to each colon compartment three times per day. The M-SHIME was at 37 °C and kept anaerobic by flushing twice per day for 15 min with N_2.

Based on earlier studies, several factors were modified to optimally study mucin-adhered microbes. (i) Long-term M-SHIME studies have demonstrated that regular replacement of microcosms, results in wash-out of mucin-adhered microbes, probably due to limited cross-contamination between old and new microcosms (unpublished results). Therefore, we performed short-term experiments (3 days), which avoids renewal of microcosms. (ii) Because such short-term studies are confronted by a strong build-up of gasses and acids due to fermentation of sugar-rich nutritional medium, we used sugar-depleted medium, which allowed a more moderate carbohydrate fermentation upon inoculation. (iii) Thirdly, instead of operating two SHIME units which each consist of an ascending, transverse and descending colon (Van den Abbeele *et al.*, 2010), we used the six colon vessels as separate units allowing to test more conditions. (iv) Finally, the pH of each unit was maintained between 6.15 and 6.4 (~transverse colon).

Experimental design

In the first/main experiment, the overall mucin-adhered microbiota composition was characterized.

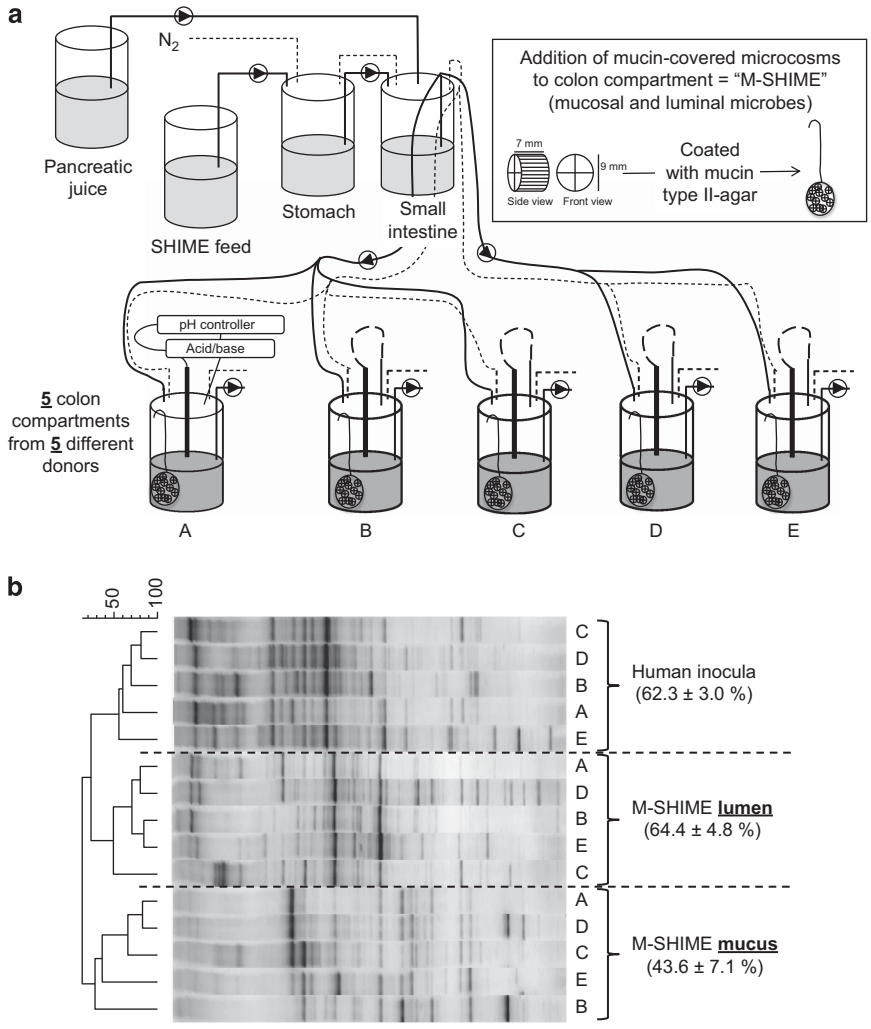

Figure 1 (**a**) Schematic representation of the main/first experiment: five colon compartments with a simulated mucosal environment (M-SHIME) were each inoculated with a different human faecal sample (donor A, B, C, D or E). (**b**) Dendrogram for the total bacterial DGGE profiles of the luminal and mucosal microbiota of these five M-SHIMEs, 3 days after inoculation with faecal samples of five human donors. The average inter-individual similarity between different donors (± s.e.m.) is shown for each type of sample: inoculum, lumen and mucin layer.

Therefore, five M-SHIME units were inoculated with stools of different human subjects: donor A (male, 26 years), B (male, 33 years), C (male, 32 years), D (male, 24 years) and E (female, 32 years; Figure 1a). Samples for short-chain fatty acid (SCFA) analysis were collected after 18, 42 and 68 h. Samples of the inocula together with mucosal and luminal samples of the final time point (68 h) were snap-frozen in liquid nitrogen for microbial characterization (denaturing gradient gel electrophoresis (DGGE), HITChip) and a clone library for the butyryl-CoA:acetate-CoA transferase gene.

To validate results of this main experiment, two additional experiments were performed. In the second experiment, the contribution of mucosal microbes to the overall microbial activity and composition was evaluated. Therefore, three M-SHIMEs (with mucosal environment) and three L-SHIMEs (without mucosal environment) were simultaneously inoculated with faecal inocula of donors A, B and D (Supplementary Figure S1A). In the third experiment, the impact of the presence of mucins in the mucosal environment on mucosal microbial activity and composition was evaluated. Therefore, microcosms were coated with mucin-containing or mucin-free agar. In this way, two colon units with a mucosal environment containing mucins (M(+)-SHME), two colon units with a mucosal environment free of mucins (M(−)-SHME) and two units without a mucosal environment (L-SHIME) were inoculated with faecal samples of donors A and D (Supplementary Figure S2A). Samples for SCFA analysis were collected after 18, 42 and 68 h. Samples of the inocula together with mucosal and luminal samples of the final time point were subjected to DGGE.

Microbial community (DGGE and HITChip) and metabolic activity analysis (SCFA)

The most prominent shifts within the microbiota were monitored via DGGE. After DNA extraction (Boon *et al.*, 2003) and PCR (Muyzer *et al.*, 1993), gels were run using an Ingeny PhorU apparatus (Ingeny International, Goes, The Netherlands). Pearson correlation and UPGMA (Unweighted Pair Group Method using Arithmetic Mean) clustering were used to calculate dendrograms using BioNumerics v5.10 (Applied Maths, Sint-Martens-Latem, Belgium).

The HITChip was used for deep characterization of the microbiota during the first/main experiment (Rajilic-Stojanovic *et al.*, 2009). The array contains duplicated sets of 4800 16S rRNA oligonucleotide probes targeting 1140 intestinal phylotypes (<98% identity) and 131 genus-like groups (<90% identity). Quantification of these groups has been validated with fluorescence *in situ* hybridization, quantitative PCR and 454 pyrosequencing (Rajilic-Stojanovic *et al.*, 2009; Claesson *et al.*, 2011; van den Bogert *et al.*, 2011). Briefly, 16S rRNA genes were amplified, *in vitro* transcribed, labelled with Cy3/Cy5 and hybridized to the microarray, washed and scanned. Spot intensities were extracted using Agilent Feature Extraction software v9.5 and normalised using R-based scripts (http://www.r-project.org/). Analysis were performed in a custom-designed relational database, which runs under MySQL database management system (http://www.mysql.com/) using a series of custom R-scripts as described previously (Rajilic-Stojanovic *et al.*, 2009).

Acetate, propionate, butyrate, valerate, caproate and branched SCFA (isobutyrate, isovalerate and isocaproate) were measured as described previously (De Weirdt *et al.*, 2010).

Clone library for butyryl-CoA:acetate CoA-transferase gene

DNA was amplified with primers BCoATscrF/BCoATscrR (2.5 μM) (Louis and Flint, 2007). PCR products were cut from a gel, purified (QIAquick Gel Extraction Kit, QIAGEN, Antwerp, Belgium) and cloned with a TOPO TA Cloning Kit with pCR2.1-TOPO Vector (Invitrogen, Carlsbad, CA, USA). Clones were amplified with primers M13F/M13R and sequenced (AGOWA, Berlin, Germany). Sequences (~480 bp) were manually inspected and compared with databases at the NCBI website (http://blast.ncbi.nlm.nih.gov/blast.cgi). Further, they were aligned using the ClustalW algorithm with the ClustalW 1.6 weight matrix and a neighbour-joining tree with 2500 bootstrap iterations and the Kimura 2-parameter substitution model was constructed (MEGA5), using the 4-hydroxybutyrate CoA-transferase sequence from *A.caccae* L1-92 as outgroup. Sequences with <98% similarity to the 32 operational taxonomic units (OTUs) described by Louis *et al.* (2010) were considered as novel OTUs. Sequences have been submitted to the European Nucleotide Archive under accession numbers HE984158–HE984296.

Statistics

All data were analyzed using SPSS16 (SPSS Inc., Chicago, IL, USA). Normality and homogeneity of variances were studied with a Kolmogorov–Smirnov and Levene test, respectively. If so, an analysis of variance with Bonferroni test was performed to investigate intergroup differences, otherwise a Kruskal–Wallis with Mann–Whitney test was applied.

A singular value decomposition was performed to identify which factors most strongly determined the microbial differences measured with the HITChip. These factors consisted of three locations (faecal inocula, lumen, mucin layer) and five sources (donor A/B/C/D/E). The initial matrix X consisted of 131 ($=s$) rows representing abundances of 131 genus-like groups and 15 ($=t$) columns representing the 15 samples, being organized as follows: five inocula, five luminal M- and five mucosal M-SHIME samples, each time for donor A–E. Each column was converted to ranks and this rank-transformed matrix, X_R, was decomposed in 15 terms each consisting of a singular value (λ_j), vector u_j (length s) and vector v_j^t (length t): $X_R = \sum_{j=1}^{15} \lambda_j u_j v_j^t$. The 15 terms are ordered so that $\lambda_1 \geqslant \lambda_2 \geqslant \ldots \geqslant \lambda_{15}$. Hence, the first term is the most important in the approximation of X_R, the second term the second most important, and so on. The significance of the contribution of location and source was verified by applying analysis of variance F-tests. Corresponding p-values (pL and pS) were computed based on a Monte Carlo approximation of the exact permutation null distributions.

To assess correlation of microbial groups detected by HITChip with human inocula, mucin layer or lumen, redundancy analysis was used as implemented in Canoco for Windows 4.5. Average signal intensities for 131 genus-like groups were used as microbial data, and diagrams were plotted using the CanoDraw for Windows utility. The Monte Carlo Permutation Procedure was used to assess statistical significance of the variation in data sets in relation to sample origin.

Results

In vitro mucosal environment enhances butyrate production

SCFA were analyzed in the luminal content of M-SHIME units of five different human subjects (Figure 1a). As observed from the low standard errors, SCFA levels were similar among individuals (Table 1). Sixty-eight hours after inoculation, the acetate/propionate/butyrate ratio of the M-SHIME was 65/15/20, while for conventional models without surface-attached bacteria such as the L-SHIME, ratios of around 68/25/6 have been reported (Van den Abbeele *et al.*, 2010). Simulating the mucosal environment thus seems to enhance butyrate levels.

Table 1 Average (±s.e.m.) absolute (mM) SCFA levels in the luminal content of M-SHIME units during the first 3 days (18 h, 42 h and 68 h) after inoculation with faecal samples of five different human donors ($n=5$)

Time after inoculation	*0 h*	*18 h*	*42 h*	*68 h*
Absolute values (mM)				
Acetate	7.7 ± 1.5	36.5 ± 1.4	31.1 ± 1.1	31.3 ± 0.5
Propionate	2.5 ± 0.4	8.3 ± 0.7	7.0 ± 0.4	7.1 ± 0.2
Butyrate	2.9 ± 1.1	7.8 ± 1.7	11.3 ± 1.1	9.9 ± 0.7
Valerate	0.6 ± 0.1	1.9 ± 1.1	2.4 ± 0.9	1.6 ± 0.6
Caproate	0.5 ± 0.2	0.3 ± 0.3	0.5 ± 0.5	0.3 ± 0.3
Branched SCFA	1.2 ± 0.1	3.0 ± 0.7	3.7 ± 0.9	2.5 ± 0.7
Total SCFA	15.4 ± 3.2	57.9 ± 3.7	56.0 ± 3.2	52.9 ± 1.7
Proportional values (mol%)				
Acetate	50.8 ± 1.2	63.6 ± 2.2	56.0 ± 2.4	59.5 ± 2.1
Propionate	16.6 ± 0.8	14.3 ± 0.8	12.6 ± 0.4	13.5 ± 0.5
Butyrate	17.1 ± 2.4	13.3 ± 2.6	20.1 ± 1.2	18.8 ± 1.2
Valerate	4.0 ± 0.4	3.0 ± 1.7	4.1 ± 1.4	2.9 ± 1.1
Caproate	2.7 ± 0.8	0.4 ± 0.4	0.8 ± 0.8	0.6 ± 0.6
Branched SCFA	8.8 ± 1.7	5.4 ± 1.4	6.5 ± 1.3	4.7 ± 1.1

Abbreviations: M-SHIME, mucosal-simulator of human intestinal microbial ecosystem; SCFA, short-chain fatty acid.

Compared with the conventional L-SHIME, the novel M-SHIME not only differed in the incorporation of a simulated mucosal environment but also in a shorter incubation time, sugar-depleted nutritional medium and a single-stage fermentation. To eliminate influence of the latter and straightforwardly evaluate the impact of a mucosal environment, three M-SHIME and three L-SHIME units were simultaneously inoculated with faeces of three donors (Supplementary Figure S1A). It followed that from the second day after inoculation onwards, the mucin layer induced a proportional shift from acetate (−6.3%) to butyrate (+3.9%) (Supplementary Table S1). In a third experiment, the importance of the presence of mucins in the mucosal environment was evaluated (Supplementary Figure S2A). It followed that the shift from acetate to butyrate was more profound when the microcosms were coated with mucin-containing as opposed to mucin-free agar (only −2.6% acetate and +0.9% butyrate; Supplementary Table S2).

Distinct microbial composition of human faeces, lumen and mucin layer (DGGE)

To observe the main microbial shifts upon inoculation of the M-SHIME, DGGE was performed on 16S rRNA amplicons of the total bacterial community (Figure 1b). The profiles were grouped as three separate clusters according to sample origin: human inocula, lumen and mucin layer. Based on the Pearson correlation coefficients, it followed that the mucin-adhered microbiota was very different from the luminal microbiota (only 19 ± 5% similar). Furthermore, although similar shifts were observed for the different human donors (Supplementary Figure S3), there was significant inter-individual variability among their faecal inocula (62 ± 3% similar), which continued to exist in the luminal (64 ± 5% similar) and mucin-adhered (44 ± 7% similar) M-SHIME microbiota.

Also the DGGE profiles of the second (Supplementary Figure S1B) and third (Supplementary Figure S2B) experiment revealed clustering according to sample origin (inocula, lumen and mucin layer) rather than to the donor (Supplementary Figures S4 and S5). Both experiments confirmed the distinct nature of the luminal and mucosal communities (only 14 ± 6% similar), together with the inter-individual variability among human inocula and resulting luminal and mucosal microbiota. Both experiments also showed that the luminal microbiota of the L- and M-SHIME are highly similar (90 ± 4% similar), suggesting that the mucosal environment does not majorly affect microbial composition in the luminal content (Supplementary Figures S1B and S2B). From the third experiment, it followed that the presence of mucins in the mucosal environment is crucial for the mucosal microbiota development. Although the microbiota that colonized the mucin-free microcosms was different from the luminal microbiota (63 ± 1% similar), the microbiota that colonized the mucin-containing microcosms was much more distinct from the luminal microbiota (only 25 ± 5% similar; Supplementary Figure S2B).

In vitro gut model preserves the specific inter-individual differences (HITChip)

In order to decipher the factors that influence microbial colonization in the M-SHIME, a singular value decomposition was applied on the high-resolution phylogenetic HITChip data of the five human inocula and five luminal and mucosal M-SHIME samples. The first two terms in the decomposition explained 39.6% and 28.4% of the variation in the microbial dataset, caused by the distinct microbial composition of the human inocula, lumen and mucin layer (Table 2 and Supplementary Figure S6). The next four terms also explained a significant part of the microbial changes (in total, 21.6%). Interestingly, these changes were independent of the location but attributed to the inter-individual differences among human subjects, indicating that the individual-specific microbial patterns are preserved in both the luminal and mucosal M-SHIME environment.

Detailed characterization of luminal and mucosal M-SHIME microbiota (HITChip)

Not only did the HITChip data confirm the distinct microbial composition of the human inocula as opposed to the lumen and mucin layer of the M-SHIME (Supplementary Figure S7), it also provided a detailed phylogenetic characterization (Figure 2 and Supplementary Table S3). The

Table 2 The singular value decomposition of the matrix containing abundances of 131 genus-like groups (as determined with the HITChip) of the human inocula, luminal and mucosal M-SHIME samples ($n=5$) resulted in six terms, which significantly explained the variation in the data set caused by either the location (=human inoculum, lumen M-SHIME or mucin layer M-SHIME) or source of the sample (=human donor A, B, C, D and E)

Term	P-*value location*	P-*value source*	*% Variation explained*	*Cause of variation*
1	0.0002	0.9492	39.6	Inoculum↔M-SHIME lumen/mucin layer
2	0.0000	0.9931	28.4	M-SHIME lumen↔ M-SHIME mucin layer
3	0.8400	0.0008	8.4	Interindividual variability human inocula
4	0.9858	0.0205	5.6	Interindividual variability human inocula
5	0.6273	0.0371	4.5	Interindividual variability human inocula
6	0.9637	0.0242	3.1	Interindividual variability human inocula

Abbreviations: HITChip, human intestinal tract chip; M-SHIME, mucosal-simulator of human intestinal microbial ecosystem.

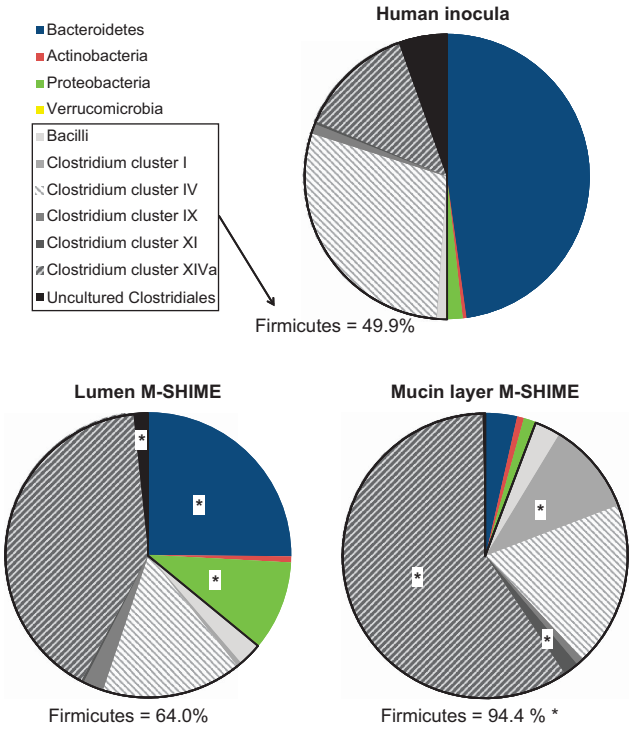

Figure 2 The average abundance (%) of higher taxonomic groups (~ phylum level) based on the HITChip analysis of the human faecal inocula and the resulting luminal and mucosal environment of the M-SHIME, 3 days after inoculation ($n=5$). An asterisks indicates a significant difference in the abundance between the lumen and mucin layer of the M-SHIME. The averages and s.e. can be found in Supplementary Table S3.

Firmicutes phylum was significantly enriched in the mucin layer (94% of the total community) as opposed to the lumen (64%), attributed to increased levels of Bacilli (3%) and bacteria belonging to the *Clostridium* clusters I (10%), IV (19%), XI (2%) and especially *Clostridium* cluster XIVa (59%). By contrast, the mucin layer was virtually devoid of Bacteroidetes (4%) and Proteobacteria (1%), which rather colonized the luminal content (25% and 10%, respectively).

Butyrate-producing bacteria from Clostridum cluster XIVa colonize the mucin layer (HITChip)

A redundancy analysis of the HITChip data at the bacterial group level (~131 genus-like groups) confirmed the distinct microbiota of inocula, lumen and mucin layer ($P=0.02$; Figure 3a). Furthermore, it specified the microbial changes as 68 genus-like groups correlated with specific locations (Figure 3b and Supplementary Table S4). Of these 68 groups, 15 specifically colonized the mucin layer (all belonging to the Firmicutes), while 29 specifically correlated to the lumen (21 belonging to the Proteobacteria or Bacteroidetes). Finally, 24 bacterial groups were equally abundant between lumen and mucin layer but differed in abundance compared with the inocula.

Several butyrate producers belonging to *Clostridium* clusters IV and XIVa, but particularly the latter, specifically colonized the mucosal M-SHIME environment (Table 3). They include (expressed as ratio of mucin-adhered/lumen) *Roseburia intestinalis* (84.3), *Eubacterium rectale* (38.9), *Papillibacter cinnamivorans* (16.0), *Eubacterium ventriosum* (15.3), *Butyrivibrio crossotus* (12.8), *Faecalibacterium prausnitzii* (3.9), *Clostridium orbiscidens* (2.9) and *Coprococcus eutactus* (2.9). By contrast, few butyrate-producing groups specifically colonized the lumen (expressed as ratio of lumen/mucin-adhered): *A. caccae* (4.9), *Subdoligranulum variabile* (2.7), *Anaerotruncus colihominis* (2.3) and *Clostridium symbiosum* (1.4).

Clone library for butyryl-CoA:acetate CoA-transferase gene confirms specific mucosal butyrate producers

Overall, 45% of the sequences of the butyryl-CoA:acetate CoA-transferase gene belonged to uncultured bacteria, with 16% of the sequences belonging to novel butyrate-producing species (OTU33–39). The 16S rRNA gene-derived results (HITChip) were confirmed at a functional level as the genes responsible for butyrate production were carried by different microbes in the mucin-adhered as opposed to the luminal microbiota (Figure 4). Although *Roseburia* species and an unknown species (OTU39) dominated the mucin-adhered microbiota (*R. intestinalis*, *R. hominis* and *R. inulinvorans*), the luminal butyrate producers mainly consisted of strain M62-1 and related unknown species (OTU24, 25 and 38). Five other uncultured butyrate producers, (distantly) related to

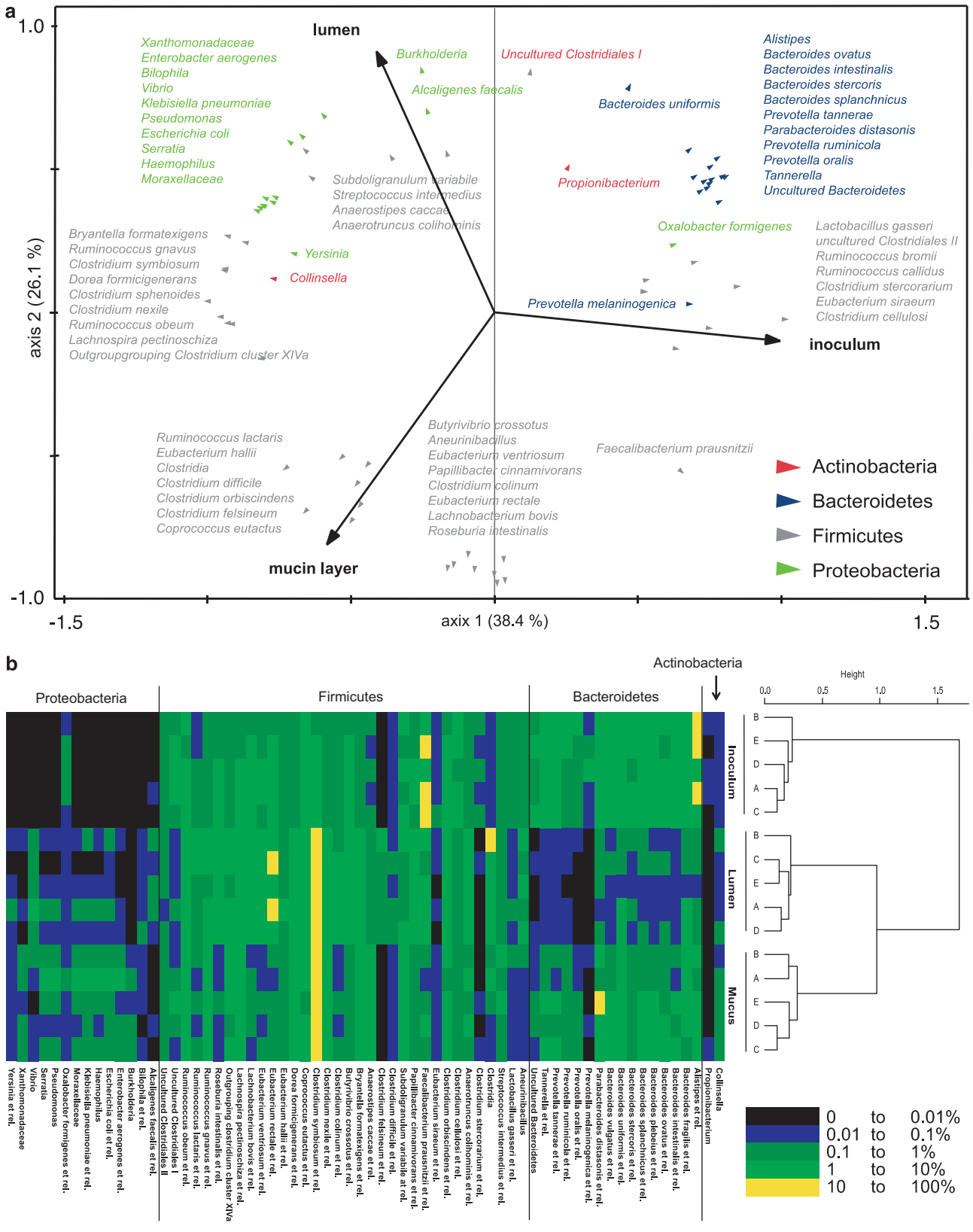

Figure 3 (**a**) Redundancy analysis at genus-like group-level based on the HITChip data of the inoculum, lumen and mucin layer of M-SHIMEs inoculated with faecal samples of five human subjects ($n=5$; $P=0.02$). (**b**) Dendrogram of the same samples based on the HITChip data for 131 genus-like groups, including a heat map which shows the inter-individual distribution of microbial groups that differed among inocula, lumen and/or mucin layer.

Table 3 The abundance (%) of butyrate-producing genus-like groups belonging to *Clostridium* clusters IV and XIVa (<Firmicutes) that were significantly different between the luminal or mucosal M-SHIME environment, as determined with the HITChip ($n=5$)

Bacterial group	*Inoculum*	*Lumen*	*Mucin layer*
Clostridium cluster IV			
Anaerotruncus colihominis	0.47 ± 0.09^{a}	1.94 ± 0.30^{b}	0.86 ± 0.25^{a}
Clostridium orbiscindens	1.49 ± 0.21^{a}	1.76 ± 0.39^{a}	5.17 ± 0.86^{b}
Faecalibacterium prausnitzii	10.92 ± 1.20^{a}	0.56 ± 0.25^{b}	2.20 ± 0.50^{c}
Papillibacter cinnamivorans	1.00 ± 0.21^{a}	0.27 ± 0.07^{b}	4.31 ± 0.73^{c}
Subdoligranulum variabile	$1.61 \pm 0.63^{a,b}$	2.53 ± 0.43^{a}	0.94 ± 0.15^{b}
Clostridium cluster XIVa			
Anaerostipes caccae	0.15 ± 0.05^{a}	1.66 ± 0.14^{b}	0.34 ± 0.06^{c}
Butyrivibrio crossotus	1.58 ± 0.53^{a}	0.35 ± 0.08^{b}	4.48 ± 1.00^{c}
Clostridium symbiosum	1.83 ± 0.32^{a}	15.54 ± 0.98^{b}	10.95 ± 0.22^{c}
Coprococcus eutactus	1.73 ± 0.21^{a}	1.65 ± 0.25^{a}	4.83 ± 0.72^{b}
Eubacterium rectale	2.33 ± 0.56^{a}	0.22 ± 0.06^{b}	8.55 ± 1.47^{c}
Eubacterium ventriosum	0.78 ± 0.21^{a}	0.11 ± 0.04^{b}	1.68 ± 0.27^{c}
Roseburia intestinalis	1.62 ± 0.49^{a}	0.08 ± 0.01^{b}	6.74 ± 0.59^{c}

Abbreviations: HITChip, human intestinal tract chip; M-SHIME, mucosal-simulator of human intestinal microbial ecosystem.
[a]The location (inoculum, lumen or mucin layer) with highest abundance was shaded with a darker background. Values with a different superscript are significantly different (a, b or c).

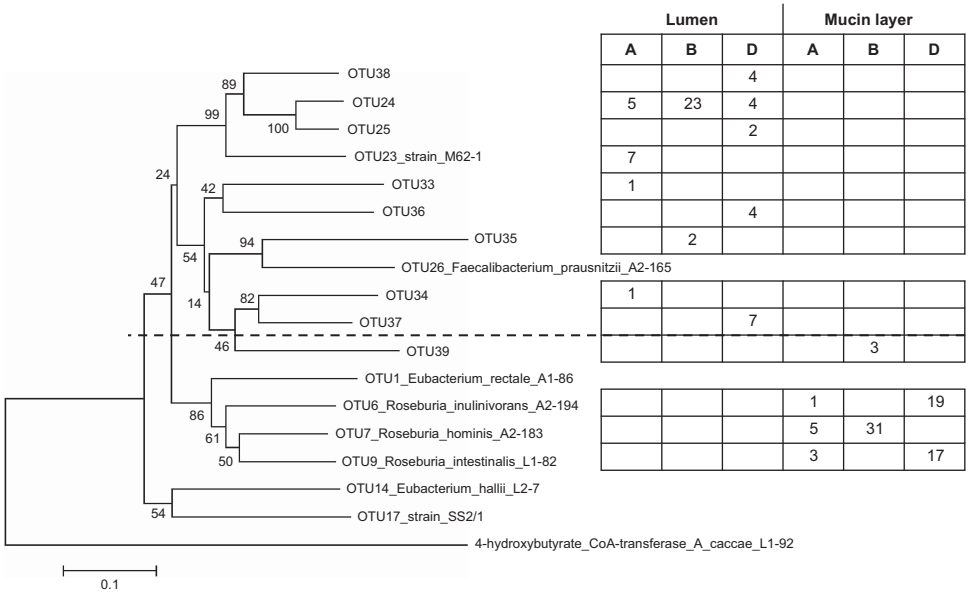

Figure 4 Phylogenetic tree and the amount of butyryl-CoA:acetate-CoA transferase gene sequences (~480 bp) identified in the luminal content and mucin layer of the M-SHIME 68 h after inoculation with human faecal samples of different donors (A, B and D; first experiment). Sequences with at least 98% sequence identity were grouped together under the same OTU number of which one sequence was chosen to build the phylogenetic tree. OTU numbers >32 indicate novel butyrate-producing species identified during the current study.

F. prausnitzii, were also detected in the luminal microbiota (OTU33–37).

Simulating a mucosal environment avoids wash-out of surface-attached bacteria

The previously published HITChip data of a conventional L-SHIME without mucosal environment (Van den Abbeele *et al.*, 2010), showed that this setup results in an enrichment of Bacteroidetes and Proteobacteria and wash-out of Firmicutes (Supplementary Figure S8). As the mucosal environment of the M-SHIME was specifically colonized by Firmicutes members, especially belonging to the *Clostridium* cluster XIVa, wash-out of these surface-attached, mucosal Firmicutes is avoided (Figure 2).

This was further confirmed at the bacterial group level, where HITChip analysis of the M-SHIME microbiota also allowed characterizing bacterial groups that rather colonized the mucin layer or

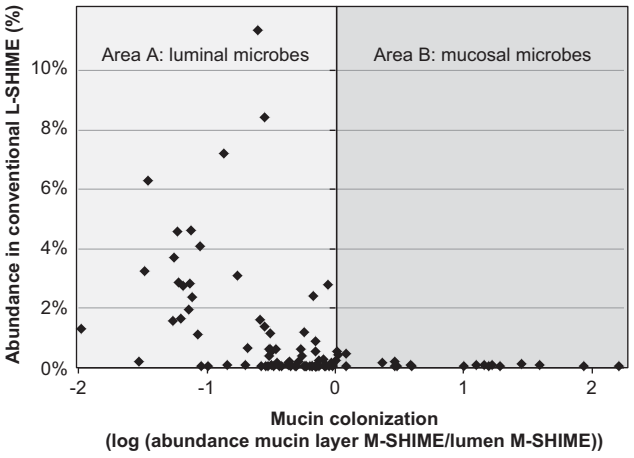

Figure 5 Average abundance (%) of genus-like groups as determined with the HITChip in a conventional L-SHIME without simulation of the mucosal environment, as reported previously (Van den Abbeele *et al.*, 2010) ($n=4$), as a function of the mucin colonization of these microbes in the novel M-SHIME ($n=5$). Mucin colonization represents the preference of a bacterial group to colonize the *in vitro* mucosal environment and is expressed as the logarithm of the abundance in the mucosal environment versus the abundance in the luminal content of the M-SHIME. A value <0 indicates that the microbial group rather colonizes the lumen (area A), a value >0 indicates that this group rather colonizes the simulated mucosal environment (area B).

lumen. Although bacteria characterized as luminal in the M-SHIME (Figure 5: area A) were maintained at high levels in the conventional L-SHIME, those characterized as mucosal in the M-SHIME (Figure 5: area B) disappeared from the conventional L-SHIME. Simulating a mucosal environment is thus crucial for many Firmicutes species in order to persist in an *in vitro* gut model.

Discussion

We recently developed the M-SHIME, an *in vitro* gut model, which not only provides a niche for luminal but also for surface-attached, mucosal microbes (Van den Abbeele *et al.*, 2011b). In this study, we confirm the inter-individual differences in microbiota composition among different human stool and mucosal samples (Zoetendal *et al.*, 1998, 2002; Eckburg *et al.*, 2005) and demonstrate that these microbial patterns unique to each individual are preserved in the luminal and mucosal *in vitro* microbiota. A microarray with high phylogenetic resolution revealed the unique composition of the *in vitro* mucosal microbiota in great detail.

The distinct microbial composition of the mucin layer, lumen and faeces was the most determining factor within the HITChip dataset accounting for 68% of the microbial differences, which was higher than the variation caused by the inter-individual differences among human subjects (21.6%; Table 2). The Firmicutes phylum largely dominated the mucosal M-SHIME microbiota (>90%), with *Clostridium* cluster XIVa species accounting for 60% of the total mucosal microbiota (Figure 2). Concordant with the fact that this cluster includes many acetate- and/or lactate-converting butyrate producers, the simulation of a mucosal environment induced a shift from acetate towards butyrate, independent of the inoculum. Despite the fact that, in living animals, the continuous desquamation of mucus into the luminal content obscures the distinction between luminal and mucosal microbes, recent *in vivo* studies also show an enrichment of Firmicutes (especially *Clostridium* cluster XIVa> Lachnospiraceae family), over Bacteroidetes in biopsies compared with luminal or faecal samples, both in rodents (Hill *et al.*, 2009; Nava *et al.*, 2011) and humans (Eckburg *et al.*, 2005; Frank *et al.*, 2007; Shen *et al.*, 2010; Wang *et al.*, 2010; Willing *et al.*, 2010; Hong *et al.*, 2011). This *in vivo* enrichment of Firmicutes in mucus, although sometimes less strong as during our *in vitro* study, suggests that similar forces may drive the mucosal microbiota composition *in vivo* and *in vitro*, likely to include selection of specific groups that adhere to mucins (Leitch *et al.*, 2007) or insoluble substrates in general (Walker *et al.*, 2008). Furthermore, as opposed to the luminal content where the pH was maintained constant, local accumulation of acids in mucus may cause a lower pH, selecting for Firmicutes over Bacteroidetes and Proteobacteria (Duncan *et al.*, 2009). In addition, mucins may also serve as a growth substrate for butyrate-producing Firmicutes, possibly via cross-feeding with mucin-degrading microbes that deliver partial breakdown products, acetate and/or lactate (Belzer and de Vos 2012), similar as reported for fructo-oligosaccharides (Belenguer *et al.*, 2006; Falony *et al.*, 2006). Independent of the underlying reason, the mucus colonization by specific butyrate-producing Firmicutes (especially *Clostridium* cluster XIVa) species provides novel insight in the ecology of these abundant human gut colonizers.

Although bacteria belonging to *Clostridium* cluster XIVa, and to a lesser extent *Clostridium* cluster IV, were enriched in the *in vitro* mucosal environment, some butyrate-producing bacterial groups belonging to these clusters had higher abundances in the luminal content (Table 3). The most specific mucosal colonizers, as identified with the HITChip, included *R. intestinalis* and *E. rectale*, while *A. caccae* rather colonized the lumen. In contrast to *A. caccae* that is non-motile, *R. intestinalis* and *E. rectale* possess flagella, which may allow for penetration into the mucus layer (Louis and Flint, 2009). Moreover, another difference is that the mucosal butyrate producers solely require acetate and no lactate for butyrate production (Louis and Flint, 2009), indicating that cross-feeding with other microbes might relate to the specific colonization of mucus.

The selective mucus colonization by specific butyrate-producing Firmicutes members may be

interconnected with processes that affect the intestinal mucus as illustrated by two examples: consumption of prebiotic compounds and IBD. Firstly, prebiotics often enhance mucus production (Ten Bruggencate *et al.*, 2004; Van den Abbeele *et al.*, 2011a) and, according to our findings, this may stimulate the release of mucosal butyrate producers towards the lumen. This hypothesis is supported by a study in which humanized rats were treated with long-chain arabinoxylans or inulin (Van den Abbeele *et al.*, 2011a). Long-chain arabinoxylans increased caecal mucin levels threefold, led to higher butyrate levels and higher abundances of butyrate producers, identified as mucosal butyrate producers during this study (*E. rectale* and *R. intestinalis*). Remarkably, inulin increased caecal mucin levels by sixfold and corresponded with even higher butyrate levels and higher abundances of the same mucosal butyrate producers. Besides the selective degradation of prebiotics by specific butyrate producers (Duncan *et al.*, 2002; Schwiertz *et al.*, 2002), stimulating mucus secretion may be a mechanism by which prebiotics increase butyrate levels. A second example is IBD, a disease in which the mucus layer becomes thinner and more discontinuous (Strugala *et al.*, 2008). Recent *in vivo* (Swidsinski *et al.*, 2005; Sokol *et al.*, 2008; Walker *et al.*, 2011) and *in vitro* (Vermeiren *et al.*, 2012) studies revealed that this disease correlates with lower levels of mucosal butyrate producers, including *Roseburia* and *Faecalibacterium*, indicating that a damaged mucus layer may lower the ecological fitness of specific butyrate producers. *In vivo* validation of the preference of butyrate producers to reside in mucus together with better understanding of factors that drive these processes may allow to develop novel therapies. As mucosal butyrate producers release butyrate close to the epithelium, they may enhance butyrate bioavailability for the host, which may be particularly useful for IBD patients where transport of butyrate to colonocytes is impaired (Thibault *et al.*, 2010).

The mucosal environment of the M-SHIME prevents wash-out of microbes that disappeared from conventional *in vitro* models (Figure 5). The enrichment of Firmicutes over Bacteroidetes and Proteobacteria in the mucosal M-SHIME environment and the fact that this contributes to higher butyrate levels in the *in vitro* model further indicate that the M-SHIME is a significantly improved simulation of the intestinal microbiota compared with conventional *in vitro* models. The latter models are indeed confronted with lower abundances of Firmicutes, especially butyrate-producing species belonging to the *Clostridium* cluster XIVa and IV (Rajilić-Stojanović *et al.*, 2010; Van den Abbeele *et al.*, 2010), whereas Bacteroidetes and Proteobacteria are very abundant (Allison *et al.*, 1989; Macfarlane *et al.*, 1998; Mäkeläinen *et al.*, 2009; Rajilić-Stojanović *et al.*, 2010; Van den Abbeele *et al.*, 2010), typically resulting in lower butyrate levels (Allison *et al.*, 1989; Van den Abbeele *et al.*, 2010). By avoiding washout of mucosal microbes, the M-SHIME allows mechanistic studies that not only focus on planktonic but also on surface-attached microbes, increasing the relevance of *in vitro* research.

Although meaningful results have been obtained with the M-SHIME, several adaptations may further enhance its value. Firstly, in order to obtain a large surface area, one is currently limited to use commercially available pig gastric mucins. These mucins have lower molar mass averages and contain more impurities than freshly prepared gastric mucins (Jumel *et al.*, 1996). Moreover, the composition of these gastric mucin (mainly MUC1, MUC5AC and MUC6) differs from colonic mucins (mainly MUC2) and changes in gastrointestinal diseases (Reis *et al.*, 1999; Corfield *et al.*, 2000). In order to improve the mucosal simulation in the M-SHIME, more relevant mucins may be used in future experiments. Ideally, human colonic mucins would be coated on the carrier materials using an agar-independent method. Next, *Akkermansia muciniphila,* the only known Verrucomicrobia representative that is a marker for mucin degradation (Belzer and de Vos, 2012), did not attain high densities in the current setup. Recent studies demonstrated that *Akkermansia* degrades mucins in (distal) colon regions characterized by depleted nutrient levels and long residence times (Van den Abbeele *et al.*, 2010, 2011a). As colonization of mucus by different mucolytic species may be crucial with respect to human health (Png *et al.*, 2010), the M-SHIME may be improved by adding a distal colon region. Further, for performing long-term experiments, a setup needs to be designed in which mucin-covered microcosms can be regularly renewed without disturbing the stability of the mucosal and luminal microbiota, particularly without opening the vessel and exposing the micro-organisms to oxygen. Finally, microbes are confronted with an oxygen gradient *in vivo* as oxygen is continuously released from the blood towards the mucus layer. Accounting for this gradient would also add to the relevance of the M-SHIME.

In conclusion, the recently developed M-SHIME simulates not only planktonic but also surface-attached gut microbes. In correspondence with *in vivo* studies, the simulated mucosal environment was specifically colonized by Firmicutes members, including many butyrate producers of the *Clostridium* cluster XIVa. Compared with conventional *in vitro* models that do not account for surface-attached microbes, wash-out of the latter species was avoided resulting in a more *in vivo*-like microbial composition and activity, allowing for more relevant mechanistic *in vitro* studies to unravel the importance of mucosal gut microbes in health and disease. The M-SHIME may thus be an excellent tool to isolate novel mucosal microbes and to

study factors that drive health-promoting or disease-causing microbes to reside in the mucosal microbiota from where they can closely interact with the host epithelium. The potential to isolate novel microbes was supported by the finding that 45% of the butyrate producers in the M-SHIME belonged to uncultured species. In this context, discovery of mucosal butyrate producers may lead to a novel therapy for diseases such as IBD, which are characterized by an impaired butyrate transport to the colonocytes. Further, as the microbial patterns unique to the five tested human subjects were preserved in the *in vitro* model, the M-SHIME may be used in future studies using larger cohorts to focus on these inter-individual differences.

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