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PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem

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Abstract

Investigating the role of intestinal microbial populations significantly relies on the assumption of stability. Therefore, the microbial community composition of the simulator of the human intestinal microbial ecosystem was qualitatively, quantitatively and functionally characterised during reactor start-up to evaluate its capacity to produce a stable bacterial community, representative for the human intestinal tract. Using moving window correlation, a stability criterion was introduced to analyse the stability over time of the PCR-DGGE, plate counts, short chain fatty acids and ammonium results. A community was regarded stable when minimum 80% correlation was measured over at least one cell residence time. Species composition stability was reached after about 2 weeks, while it took some 3 weeks to reach functional stability. The combination of PCR-DGGE with moving window correlation proved to be an efficient approach to quantitatively evaluate the stability of the in vitro cultured intestinal microbial community.

Keywords: *Bacteroides*; Intestinal bacteria; *Lactobacillus*; Moving window correlation; PCR-DGGE; Community stability; SHIME

1. Introduction

The intestinal tract is one of the most complex and diverse ecosystems, comprising up to 10^{14} cells of 300–500 different microbial species, many of which have never been cultured [1]. The intestinal microbial community plays a significant role in processes of food digestion, bioconversion of endogenous or exogenous compounds, immunomodulation, and prevention from infection by intestinal pathogens [2,3]. Because of this role, the human gastrointestinal tract has frequently been studied and in the last decades several gastrointestinal simulators were developed to facilitate research in this field [4–10]. These systems offer robust control of parameters and easy sampling, thus allowing mechanistic studies. Moreover, the lack of ethical constraints when using in vitro model systems relative to human or animal subjects is a major advantage. However, because of the simplified nature of such reactors, final valorisation with in vivo data remains essential. Simulators have been used to study a wide variety of topics concerning gastrointestinal health and ecology such as the effects of pre- and probiotics [11,12], and more recently bioaccessibility and bioconversion of soil pollutants [13] and the stability and activity of bacteriophages [14].

In the present study, we used the simulator of the human intestinal microbial ecosystem (SHIME), a five-stage sequential continuous reactor system simulating the different parts of the gastrointestinal tract, based on the model as developed by Molly et al. [10] and optimised by De Boever et al. [11]. The most important control parameters are feed composition, pH control and retention time. Based on plate counts and functional measurements, such as fermentation patterns, gas analysis and enzymatic activities, it has been assumed that the SHIME harbours a stable microbial community, representative of

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that in the human intestinal tract [15]. From the results of De Boever et al. [11], where standard deviation values of plate count data of various bacterial groups ranged between 0.30 and 1.45 log (CFU g^{-1}) during a control period of 14 days, it can be derived that significant changes in microbial community composition occurred during the first weeks after the start-up of the SHIME. After this control period, significant changes in population numbers and functional performances were only detected after a perturbation, e.g. the addition of a prebiotic product.

Recent developments in molecular analysis of bacterial communities have offered new tools for the exploration of highly diverse intestinal ecosystem [16]. While selective plating techniques give an estimate of the number of culturable bacteria, molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (PCR-DGGE) or fluorescence in situ hybridisation (FISH) give additional information about the bacterial species composition, including non-culturable strains. Comparison of plate counts with microscopic and FISH data revealed that only 50–80% of the intestinal microbial community could be enumerated using conventional culture-based techniques [17–19]. Furthermore, it has been shown that the selectivity of selective culture media is often limited [20,21]. In this way, culture data lack reliability and molecular techniques have gained more and more attention. So far, however, molecular data on the microbial ecology of in vitro models of the gastrointestinal system are very scarce.

When experiments are designed to monitor the effect of a specific treatment on the composition of the in vitro microbial ecosystem, the reliability of the results strongly depends on the assumption of stability. Ecologists now generally agree that ecosystem stability is a dynamic process with functional stability but continuous population variability [22,23]. However, when using in vitro simulators, before influencing the microbial ecosystem, it is crucial to start from a stable community, which is more or less representative to the in vivo human situation. Otherwise specific shifts can't be related to a specific in vitro treatment. As DGGE patterns offer a more accurate view on the species distribution and composition in a microbial community, PCR-DGGE was applied to monitor changes in the in vitro microbial community composition during the start-up period of the SHIME reactor system. To evaluate the necessary assumption of stability, we propose a novel statistical analysis of DGGE patterns by combining the results with the principle of moving window correlation. As opposed to principal component analysis (PCA), multidimensional scaling (MDS), cluster analysis and diversity indices, this technique includes a time-based analysis of the DGGE data. The capacity of this DGGE analysis to monitor the stabilisation of the SHIME microbial community over time was evaluated by comparing the results with the measured variability of plate counts and metabolic data. Furthermore, the microbial community composition of the SHIME inoculum and faecal matter were compared to evaluate whether this inoculum, and the SHIME, harboured a community representative of in vivo situations.

2. Materials and methods

2.1. Simulator of the human intestinal microbial ecosystem

The reactor setup was adapted from the SHIME, representing the gastrointestinal tract of the adult human as described by Molly et al. [10]. The SHIME consists of a succession of five reactors that represent the different parts of the human gastrointestinal tract (Table 1). The first two reactors were of the fill-and-draw principle to simulate different steps in food uptake and digestion, with peristaltic pumps adding a defined amount of SHIME feed and pancreatic and bile liquid to the stomach and duodenum compartment and emptying the respective reactors after specified intervals. The last three compartments were continuously stirred reactors with constant volume and pH control. Retention time and pH of the different vessels were chosen in order to resemble in vivo conditions in the different parts of the gastrointestinal tract (Table 1). The overall residence time of the last three vessels, simulating the large intestine, was 76 h. Reactor feed consisted of the following components: 1 $g\,l^{-1}$ arabinogalactan, 2 $g\,l^{-1}$ pectin, 1 $g\,l^{-1}$ xylan, 3 $g\,l^{-1}$ potato starch, 0.4 $g\,l^{-1}$ glucose, 3 $g\,l^{-1}$ yeast extract, 1 $g\,l^{-1}$ pepton, 4 $g\,l^{-1}$ mucin, 0.5 $g\,l^{-1}$ cystein. The pH of the feed was set to 2 and the feed was stored at 4 °C before administration to reactor one. The passage of food in the small intestine was simulated in reactor two by the addition of 60 ml artificial pancreatic and bile liquid [6 $g\,l^{-1}$ oxgall (Difco, Bierbeek, Belgium), 1.9 $g\,l^{-1}$ pancreatin (Sigma, Bornem, Belgium) and 12.5 $g\,l^{-1}$ $NaHCO_3$]. The temperature of the system was kept at 37 °C by a thermostat and the system was kept anaerobic by flushing it with N_2 for 15 min every day. Inoculum was prepared from faecal matter, collected from a 25 year-old male person, as described in De Boever et al. [11]. Reactor three, four and five were filled with nutritional medium and pH was ad-

Table 1
Setup of the simulator of the human intestinal microbial ecosystem (SHIME) with reactor volumes and retention times listed

Reactor	Volume (ml)	Residence time (h)	pH
R1: stomach	200	4	
R2: small intestine	200	4	
R3: ascending colon	500	20	5.6–5.9
R4: transverse colon	800	32	6.1–6.4
R5: descending colon	600	24	6.6–6.9

justed to the respective pH range (Table 1). Finally, 50 ml inoculum was added to the last three reactors.

2.2. Plate counting

The bacterial groups that were analysed and the specific media that were used are given in Table 2. Because of the short retention time and the fill-and-draw principle, a microbial ecosystem cannot be established in reactor one or two. Therefore, these reactors were not sampled. Liquid samples were withdrawn from the culture system on days 1, 3, 5, 8, 12, 17 and 24 after the start-up of the SHIME and diluted serially in salt solution (8.5 $g\,l^{-1}$ NaCl). Three plates were inoculated with 0.1 ml sample of three dilutions and incubated at 37 °C using the conditions given in Table 2. Anaerobic incubation of plates was performed in jars with a gas atmosphere (84% N_2, 8% CO_2 and 8% H_2) adjusted by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium). To evaluate community stability, percentage variability between day x and day $x-2$ retention times was calculated and plotted as function of time.

2.3. Short chain fatty acid (SCFA) determination

Liquid samples (10 ml) from each colon reactor were collected and frozen at −20 °C for subsequent analysis. The SCFA were extracted from the samples with diethyl ether and determined with a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands). The GC was equipped with a capillary free fatty acid packed column [EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 m × 0.53 mm; film thickness 1.2 μm], a flame ionisation detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as the carrier gas at a flow rate of 20 $ml\,min^{-1}$. The column temperature was set at 130 °C and the temperature of the injector and detector was set at 195 °C [24]. Stability of SCFA production was monitored by plotting correlation coefficients per two retention times (6 days).

2.4. Ammonium determination

Liquid samples (10 ml) from each colon reactor were frozen at −20 °C for subsequent analysis. Using a 1026 Kjeltec Auto Distillation (FOSS Benelux, Amersfoort, The Netherlands), ammonium in the sample was liberated as ammonia by the addition of an alkali (MgO). The released ammonia was distilled from the sample into a boric acid solution [25]. The solution was back-titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm). Stability of ammonium production was monitored by plotting correlation coefficients per two retention times (6 days).

2.5. DNA extraction and PCR

From day 1 after the start-up, samples were analysed every 2 days until day 27. Total DNA extractions of 1 ml liquid sample were performed using the method adapted from Griffiths et al. [26] and Kowalchuk et al. [27] as described in Boon et al. [28]. General Bacterial DNA as well as specific DNA from *Lactobacillus* sp. and *Bacteroides/Prevotella* was amplified using specific primer sets and temperature–time programs (Table 3). Before amplification DNA was diluted ten times, to dilute possible PCR-inhibiting compounds. The 16S rRNA genes for all members of the Bacteria were amplified by PCR using the forward primer PRBA338f and the reverse primer P518r, and a GC-clamp of 40 bp was added to the forward primer. To amplify the 16S rRNA genes of the *Bacteroides/Prevotella* group and *Lactobacillus* sp., a nested PCR approach was used. In the first PCR round, group specific primer sets were used, and in the second PCR round, primers PRBA338fGC and P518r were used. After the first PCR round, one clearly visible band was present on agarose gel, which suggested that no non-specific amplification was expected in the second round. Before the nested PCR, DNA was diluted five times.

2.6. DGGE analysis

DGGE based on the protocol of Muyzer et al. [29] was performed using the Bio-Rad D gene system (Bio-Rad, Hercules, CA). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1× TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). To separate the amplified DNA fragments, the polyacrylamide gels were made with denaturing gradients ranging from 45% to 60%. On each gel, a homemade marker of different PCR

Table 2
Analysed microbial groups together with the isolation media used and incubation conditions

Bacterial group	Medium	Condition	Time (h)
Total aerobes	Brain heart infusion agar	Aerobic	24
Total anaerobes	Brain heart infusion agar + 0.5 $g\,l^{-1}$ cystein	Anaerobic	72
Lactobacillus sp.	Rogosa agar	Anaerobic	72
Total coliforms	Mc. Conkey agar	Aerobic	24
Enterococcus sp.	*Enterococcus* agar	Aerobic	48
Staphylococcus sp.	Mannitol salt agar	Aerobic	48

Table 3
Primers and temperature–time programs used in this study

Primers	Reference	Temperature–time program	Specificity
PRBA338fGC P518r	[52]	(1) 5′; 94 °C (2) 1′; 95 °C/1′; 53 °C/2′; 72 °C (30×) (3) 10′; 72 °C	Bacteria
SGLAB0159f SGLAB0667r	[35]	(1) 7′; 95 °C (2) 1′; 94 °C/1′; 56 °C/2′; 72 °C (35×) (3) 10′; 72 °C	*Lactobacillus* sp.
FD1 RbacPre	[34]	(1) 5′; 94 °C (2) 2′; 94 °C/30″; 60 °C/2′; 72 °C (30×) (3) 10′ 72 °C	*Bacteroides/Prevotella*

fragments was loaded, which was required for processing and comparing the different gels. The electrophoresis was run for 16 h at 60 °C and 38 V. Staining and analysis of the gels were performed as described previously [30].

The obtained DGGE patterns were subsequently normalised and analysed with the BioNumerics software version 2.0 (Applied Maths, Kortrijk, Belgium). During this processing, the different lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalisation, and the correlation matrix was calculated. Clustering was done with Pearson correlation and the UPGMA method. The correlation between day x and day $x-2$ was plotted for day 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 to evaluate the community stability within the groups of Bacteria, *Lactobacillus* and *Bacteroides/Prevotella* in reactor three, four and five using the principle of moving window correlation.

3. Results

3.1. Establishment of a threshold as stability criterion

To evaluate the stability of the obtained DGGE, plate count, SCFA and ammonium results, a stability criterion was introduced based on the intrinsic variability within the different techniques. In case of DGGE analysis, within-gel variability between the marker lanes reached up to 20%, plate counts, SCFA and ammonium analysis showed up to 8%, 5% and 5% variation, respectively. Because DGGE showed highest variability, a threshold of 20% variability or 80% similarity was set as stability criterion to evaluate the stability of the different results.

3.2. Inoculum preparation

To evaluate the suitability of the SHIME inoculum, DGGE analysis was performed on duplicate samples of this inoculum and the faeces from which it was prepared, using primers specific for Bacteria, *Lactobacillus* sp., and the *Bacteroides/Prevotella* group. Fig. 1 shows that no differences were observed between the faeces and inoculum, using these three different primer sets. With different primer sets a minimum of 88% correlation was found between the SHIME inoculum and the faecal material it was derived from. Hence, it can be concluded that the inoculum harbours a bacterial community that is representative for that of the faeces.

3.3. Stability of the microbial community in the SHIME reactor

3.3.1. Plate count data

Plating was done in triplicate for total aerobes, total anaerobes, *Lactobacillus* sp., total coliforms, *Staphylococcus* sp. and *Enterococcus* sp. on selected days between day 1 and day 23 after the start-up of the SHIME. The

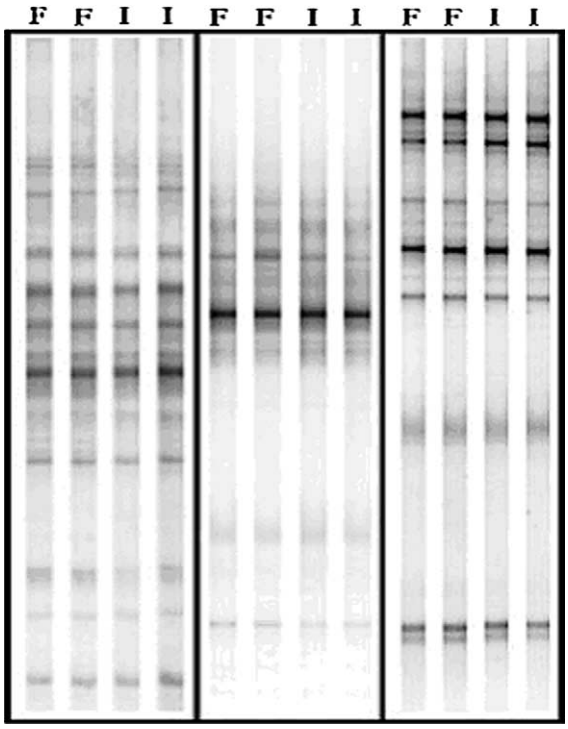

Fig. 1. Composite DGGE patterns from duplicate samples of the inoculum (I) and the faeces (F) that was used to prepare the inoculum, using primers specific for Bacteria (left), *Lactobacillus* sp. (middle), and the *Bacteroides/Prevotella* group (right). Minimum 88% correlation was found between the inoculum and the initial faecal matter.

retention time of the last three SHIME reactors was set on 76 h, which is close to the normal in vivo situation. To evaluate the stability of the microbial community, the percentage variability (calculated as the variation coefficient) per two retention times was plotted and compared with the introduced stability criterion of 20% variability per period of two retention times (Fig. 2). Total aerobes and anaerobes, which are in fact a large denominator of several different species, showed little variation when measured with this parameter. The variability decreased almost immediately to below 20%. The same pattern was detected for total coliforms. From day 12 on, the variability was very low. However, the groups of *Lactobacillus* sp., *Staphylococcus* sp. and *Enterococcus* sp. showed much more variation. For *Lactobacillus* sp. most variability was detected in reactor four; *Staphylococcus* sp. showed most variation in reactor three and four and for *Enterococcus* sp. this was seen in reactor five. Within these groups, variability only passed the threshold after 17 days. The mean concentration of the different groups in the SHIME from day 12 to day 23 (Table 4) corresponded well with in vivo data. However, for *Staphylococcus* sp. and *Enterococcus* sp. the concentrations were somewhat lower compared to in vivo.

3.3.2. Bacterial metabolism

Short chain fatty acids analysis and ammonium production are often used to characterise intestinal microbial populations. In this way, functional stability instead of cell number stability can be evaluated (Fig. 3). The acetic, propionic and butyric acids, comprised over 90% of the total SCFA production. The other SCFA present were low concentrations of isobutyric, isocapronic, isovaleric and valeric acids. The total SCFA concentration changed until week 3, with the highest production towards the distal end of the SHIME. In reactor three, almost 98% of total SCFA production consisted of acetic acid. The small production of butyric acid that was seen in the beginning disappeared completely and acetic acid production dominated after stabilisation. A similar pattern was seen in reactor four and five. The percentage acetic acid was 79% and 60%, respectively. This decrease was mainly due to an increase of propionic acid, because butyric acid production declined to remain stable at low levels (<200 mg l^{-1}). It

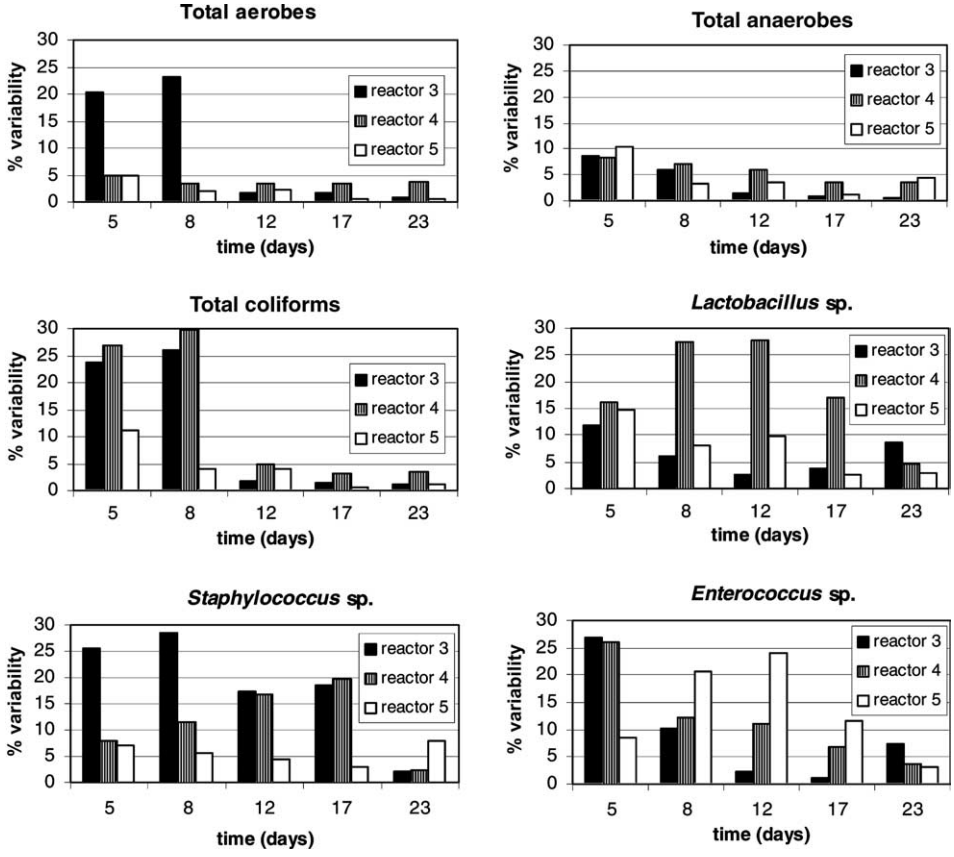

Fig. 2. Percentage variability per two cell retention times for reactor three, four and five, as calculated from the plating results between day 1 and day 23 after the startup of the SHIME. The cell retention time of the SHIME was set on 76 h. After the start-up, the mean variability was calculated between day x and day $x-2$ retention times to evaluate stability within the monitored groups. Variability was compared with the stability criterion of 20% variation per two cell retention times.

Table 4
Plate count data for reactor three (R3), reactor four (R4) and reactor five (R5) of the SHIME representing the ascending colon, transverse colon and descending colon, respectively

	Concentration [log(CFU ml^{-1})]			
	Reactor 3	Reactor 4	Reactor 5	In vivo
Total aerobes	7.88 ± 0.08	7.82 ± 0.29	7.67 ± 0.05	7–9
Total anaerobes	8.85 ± 0.04	9.27 ± 0.34	8.76 ± 0.39	7–11
Lactobacillus sp.	5.77 ± 0.50	5.16 ± 0.07	5.35 ± 0.15	5–9
Total coliforms	7.78 ± 0.09	7.79 ± 0.27	7.53 ± 0.08	7
Staphylococcus sp.	5.08 ± 0.11	5.08 ± 0.12	6.05 ± 0.49	7
Enterococcus sp.	3.93 ± 0.29	3.96 ± 0.15	4.12 ± 0.13	5–6

Average and standard variation of the log(CFU ml^{-1}) values were calculated from culture data between day 12 and day 23. In vivo data are presented as the range of the average composition of the adult ecosystem [log(CFU g^{-1})] [10,50].

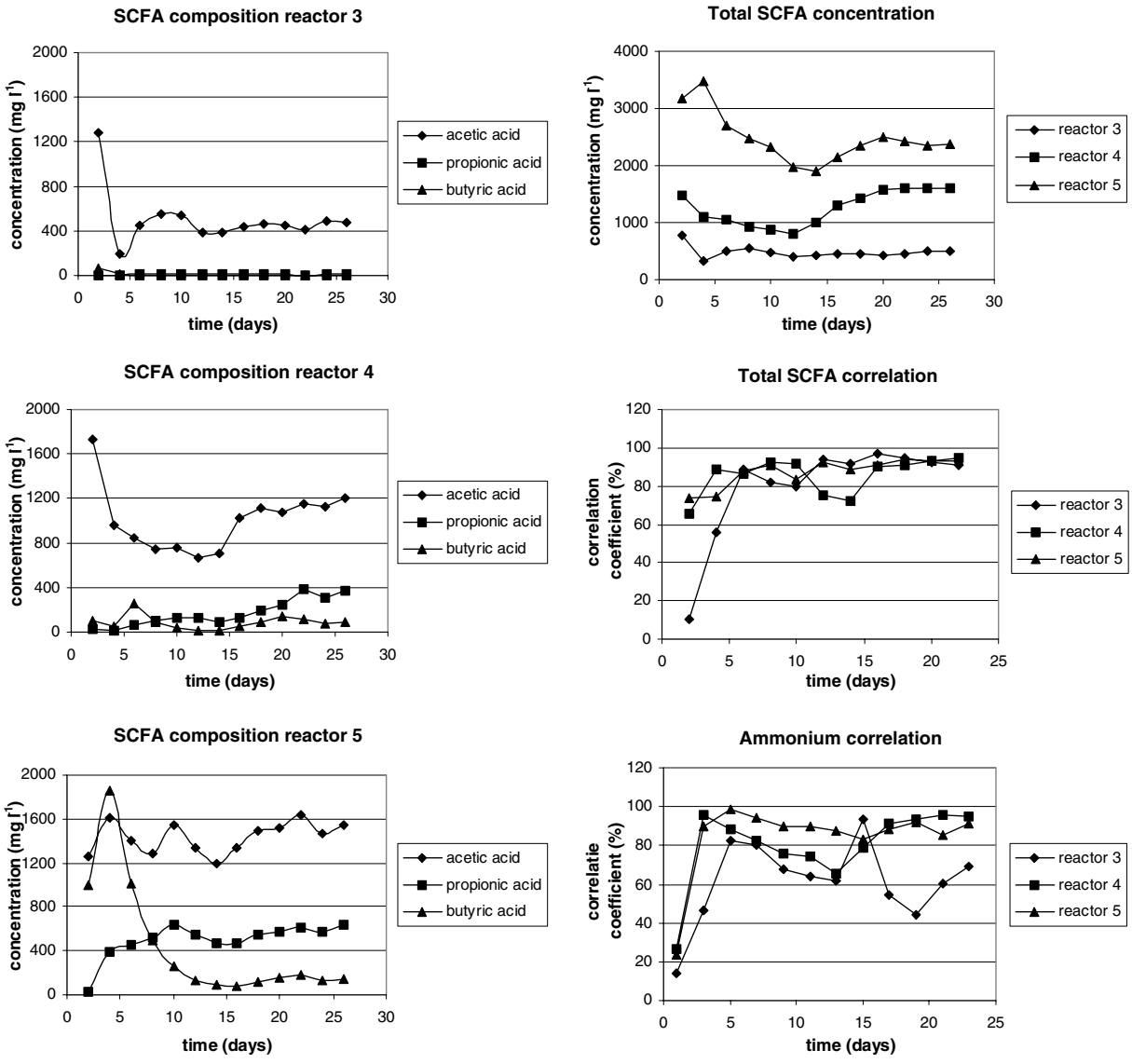

Fig. 3. Metabolic activity of the microbial community in reactor three, four and five from day 1 until day 27 after start-up of the SHIME. Total as well as individual (acetate, propionate and butyrate) short chain fatty acid (SCFA) and ammonium production were monitored for the different reactors. Variability of total SCFA and ammonium concentration was calculated as correlation during two retention times.

took 17 days to reach the stability criterion of 20% variability per two retention times for all three colon reactors.

An increase in ammonium production over time was noted for all three reactors (results not shown). Like SCFA production, the formation of ammonium in-

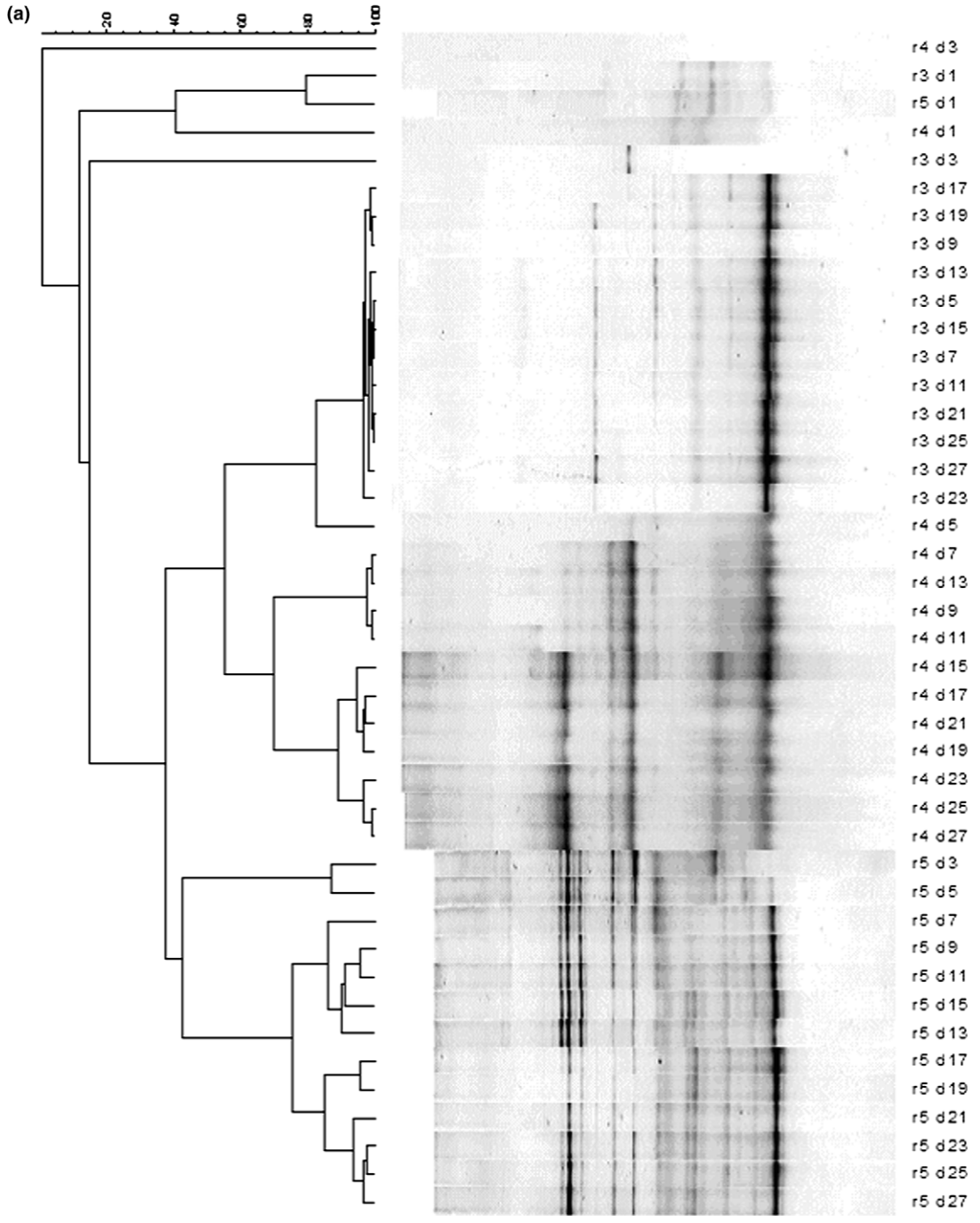

Fig. 4. Cluster analysis of the DGGE profiles of the general Bacteria (a), the *Lactobacillus* sp. (b), and *Bacteroides*/*Prevotella* group (c) in the simulated ascending colon (R3), transverse colon (R4), and descending colon (R5) at days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 after reactor start-up.

creased towards the last SHIME reactor (the descending colon). Although the pattern never became completely stable in reactor three, the same trend occurred as for the SCFA production. In reactor four and five, the stability criterion was reached after 3 weeks. From that time on, the ammonium production remained constant at a level of about 120 mg l^{-1} in reactor three, 230 mg l^{-1} in reactor four and 330 mg l^{-1} in reactor five.

3.3.3. PCR-DGGE

DGGE profiles of the general Bacteria, *Lactobacillus* sp. and the *Bacteroides*/*Prevotella* group were constructed for reactor three, four and five and analysed using cluster analysis and the principle of moving window correlation. As before, 80% similarity was set as threshold to analyse the DGGE clusters (Fig. 4). For the general Bacteria, the different reactors showed different

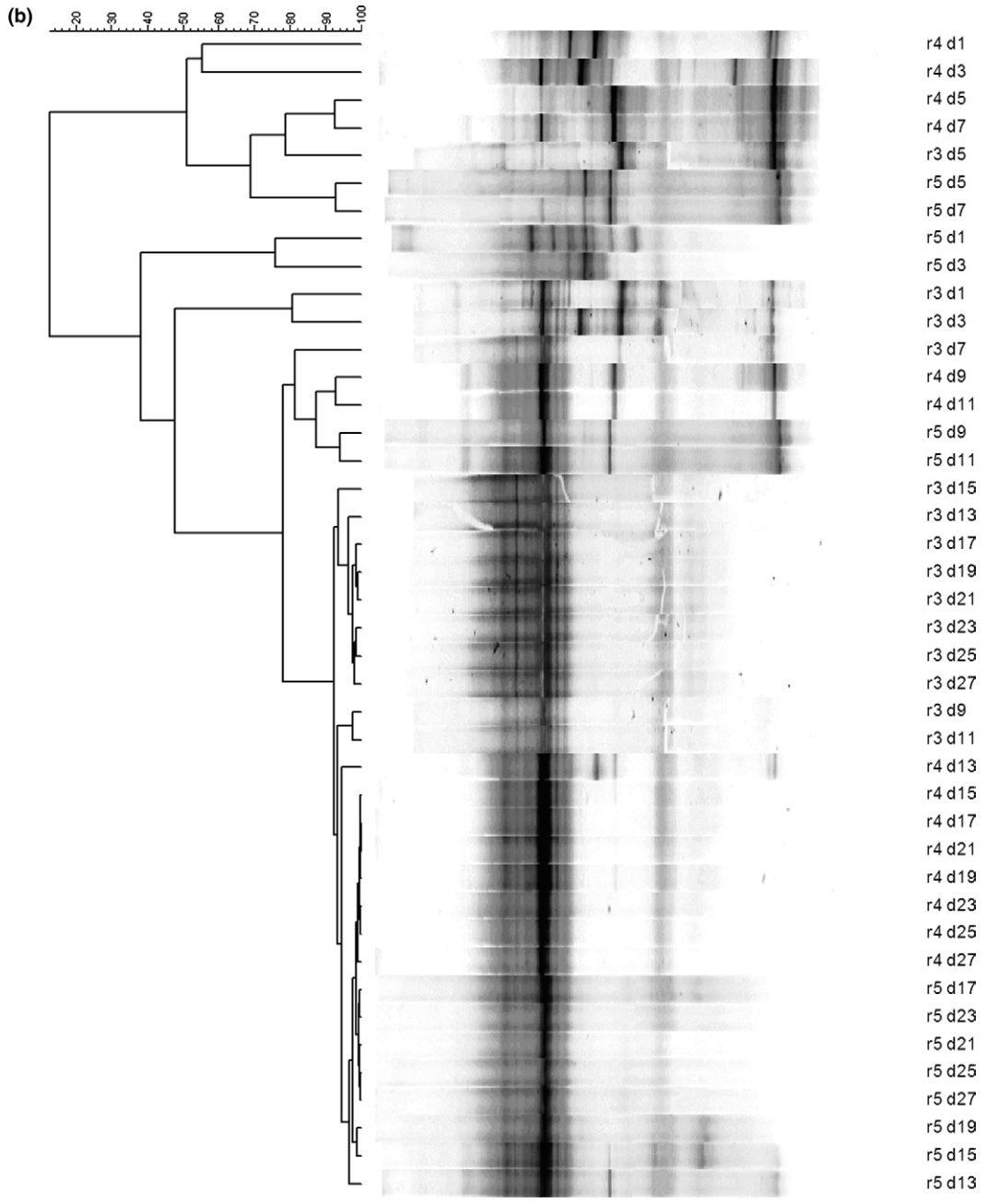

Fig. 4. (*continued*)

patterns. Due to the dilution of the inoculum during the start-up of the SHIME, only a few bands were present the first days. While the profiles of reactor three and four remained similar until day 15, the pattern of reactor five clustered separately from day one on. In reactor three, the overall pattern remained stable from day five onwards but in the profiles of reactor four and five, two or three distinct groups were formed due to shifts in band patterns. Within the group of *Lactobacillus* sp. several changes appeared during the first period. Until day 13, daily changes in DGGE patterns were observed, which indicated that the community was unstable. It was only from day 13 on that 80% similarity was reached. In contrast with the profile of the Bacteria, the profiles of the three reactors were very similar with only 8% variability among them. The only difference between the reactors was a difference in relative abundance of the different species, as indicated by the different intensities of the bands. For the *Bacteroides*/*Prevotella* group three clusters were formed, corresponding to the three SHIME reactors. Due to the dilution effect, only a few bands were present after the start-up, but the profiles remained constant after 11 days for reactor three, 3 days for reactor five and up to 15 days for reactor four. The

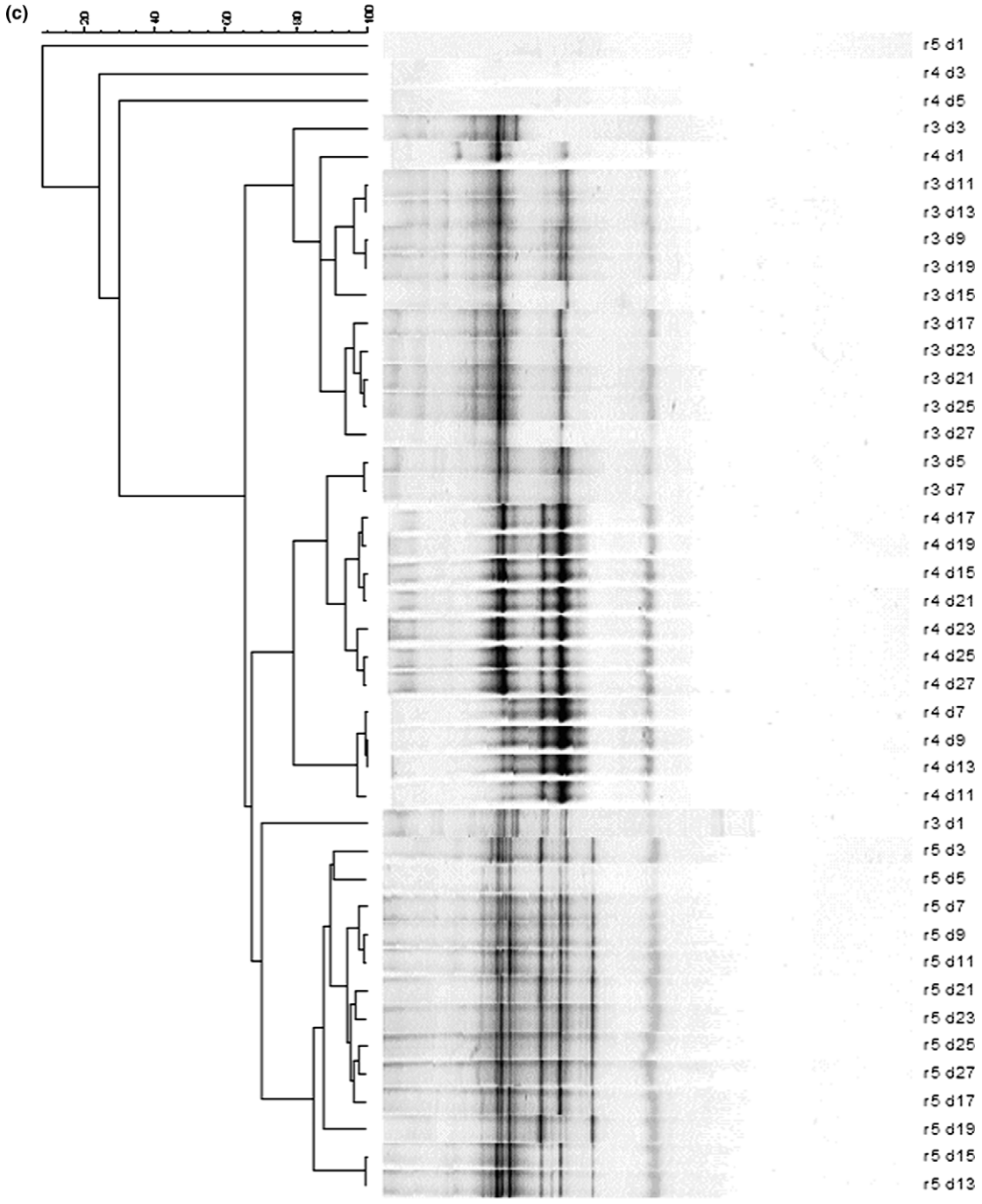

Fig. 4. (*continued*)

fact that the samples named r3d5 and r3d7 showed higher similarity to r4 instead of visually expected r3 samples was inherent to the applied protocol during analysis, based on peak intensities and not on visually assigned bands. This is a more objective method but sometimes peak overlap occurs and two bands are seen as one with unexpected clustering as result.

In the moving window correlation approach, the evolution of the similarity over time is depicted. When the correlation coefficients between day x and day $x-2$ for each reactor are plotted against the time, a graphical representation of the evolution of stability over time for each bacterial group in the three different reactors is obtained (Fig. 5). Again, the highest variability was seen for the *Lactobacillus* sp. For the latter, it took 11 days to reach the threshold of 80% similarity, whereas the *Bacteroides*/*Prevotella* group stabilised after 9 days. For the general Bacteria, stabilisation occurred at day 7.

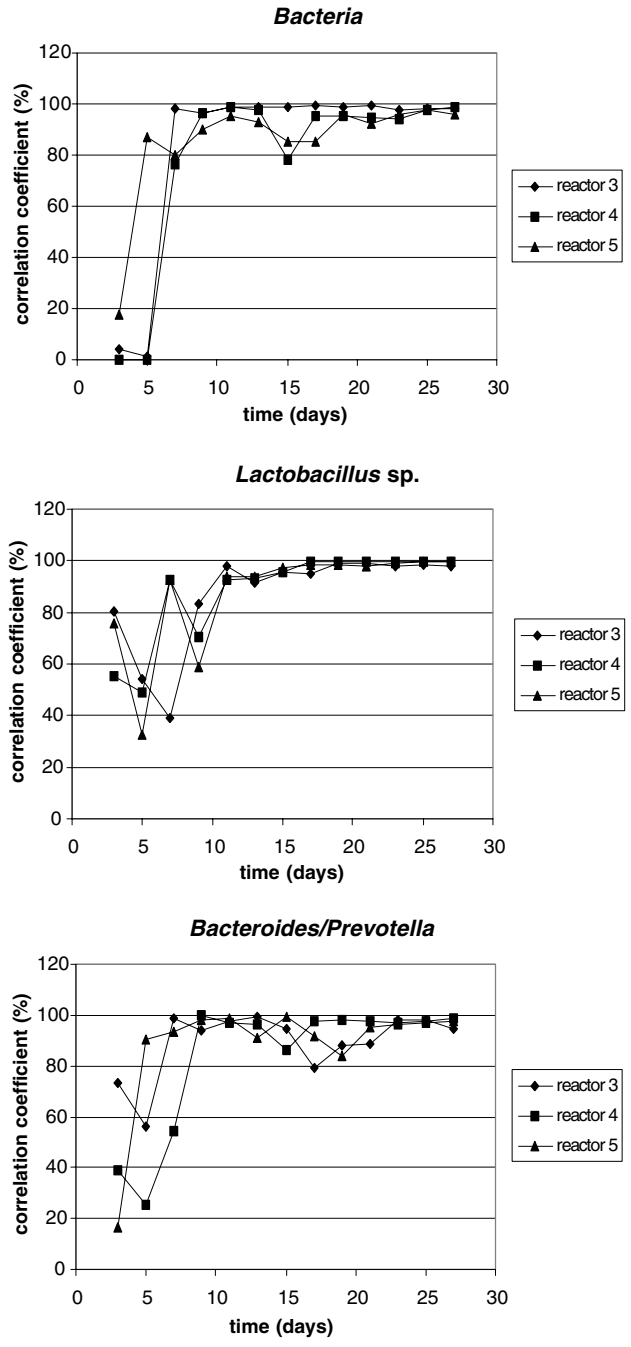

Fig. 5. Moving window correlation for reactor three, four and five of the SHIME, representing the ascending colon, transverse colon, and descending colon, respectively. Variability between day x and day $x-2$ was calculated from DGGE patterns of the general Bacteria, the *Bacteroides/Prevotella* group and *Lactobacillus* sp. using the BioNumerics software.

4. Discussion

In vitro systems such as the SHIME can mimic in vivo situations to a certain degree and have the major advantage that they can easily be operated and offer a reproducible way to investigate the effect of specific perturbations on the intestinal microbial ecosystem [11–13,24,31–33]. Still they are only a simulation of reality. For instance, interaction with the human host, i.e. specific absorption and host specific secretions, cannot be simulated. Therefore one has to be careful with the extrapolation of these results to in vivo and it is very important to simulate all conditions as good as possible. In this context, one of the most crucial aspects is the assumption of stability of the SHIME microbial community before the start of an experiment, because otherwise the shifts observed may not be due to the added compound. Because former studies never proved this assumption, we introduced a technique to quantitatively characterise the stability of the SHIME microbial community. The application of the molecular PCR-DGGE method to study the SHIME microbial community and the analysis of the results according to the principle of moving window correlation created a new and reliable way to analyse these microbial community shifts and proved that the assumption of stability was fulfilled after 2 weeks following start-up of the SHIME.

PCR-DGGE is a powerful technique for examination of the diversity within microbial communities [29]. Taking into account the fact that, when using bacterial primers, only the most abundant species are detected, two sets of group specific primers were applied to monitor the stabilisation period. The *Bacteroides/Prevotella* group comprises the most dominant anaerobic bacteria present in the intestinal tract [34]. *Lactobacillus* sp. are a well studied group because of their possible health beneficial properties [35]. For these two bacterial groups, validated primers are readily available. Many molecular techniques can be applied to monitor population stability such as PCR-DGGE [36], restriction fragment length polymorphism (RFLP), FISH [37] and quantitative membrane hybridisation [38]. But most of the time these data are qualitatively analysed by visual comparison or cluster analysis. Here we propose the principle of moving window correlation, a more quantitative measure to evaluate diversity and evolutionary shifts over time, for which a defined stability criterion can easily be used. This latter threshold was based on the within-gel variability of DGGE analysis and a stability criterion of 80% was applied to evaluate the results.

Based on the correlation coefficients between day x and $x-2$, the stability profiles (Fig. 5) for the different reactors and primer sets could be quantitatively evaluated and compared with the plating results. As for the plating results, DGGE profiles from *Lactobacillus* sp. showed more variation than the general Bacteria. It took 11 days to reach the stability criterion, while Bacteria and *Bacteroides/Prevotella* stabilised after 7–9 days. Both the plating technique and the DGGE analysis gave a stabilisation period of 2 weeks; hence both techniques are able to characterise microbial popula-

tions based on the quantitative or qualitative variation in the community composition. As mentioned before, the major drawback of conventional plating techniques is the lack of culturability of an important fraction of intestinal populations, which creates a considerable bias. Harmsen et al. [39] reported ten times higher counts of *Lactobacillus* sp. using FISH compared with plating. Furthermore, while total counts can remain constant, it is possible that the relative importance of specific species within the enumerated group still changes. These variations cannot be evaluated with plate counting but they can by using DGGE and moving window correlation.

As very little variation was seen in the plate counts for total aerobes and anaerobes, the specific groups that were monitored showed far more variation. For the latter, except for total coliforms, it took 12 days to reach the stability threshold. This means that the general bacterial concentration, as counted with the total (an)aerobic plates, quantitatively reached its steady-state level within few days, while the qualitative community composition, as evidenced by the evolution of specific groups, changed up to day 12. Even after the latter day, the variation within the specific groups remained 5–10%, while total variation decreased below 5%. The same phenomenon was seen during the analysis of the DGGE clusters (Fig. 4). For the general Bacteria and the dominant *Bacteroides*/*Prevotella* group, little variation over time was seen within each reactor. In contrast to this, *Lactobacillus* sp. showed daily changes within each reactor during the first 10 days with many transiently appearing bands, indicating major community shifts. The observation that more variation occurs in the less abundant group of *Lactobacillus* sp., was also seen in vivo. Indeed, although it was found that the composition of the predominant intestinal species remained stable over time [40], this was not always the case for smaller groups like *Lactobacillus* sp. [41]. In a study over a 12-month period, McCartney et al. [42] saw shifts of 2 log(CFU g^{-1}) in the *Lactobacillus* concentration in some individuals, while *Bacteroides* sp. and *Bifidobacterium* sp. almost did not show any shifts. From a survey with ten human subjects, Kimura et al. [43] found that *Lactobacillus* numbers not only varied greatly between individuals (10^4–10^8 g^{-1}) but also between samples from the same subject over a 3- to 9-month period. This was also noted by Tannock et al. [44].

While the latter techniques investigate composition stability of microbial communities, studies on microbial metabolism reveal another aspect of a stable ecosystem, namely functional stability. While composition stability occurred after 12 days, it took 17 days before total SCFA concentration reached steady state. In an in vivo experiment with germ free rats that were inoculated with cecum content, it took 30 days to get a fully active intestinal ecosystem [45]. It is thus important to keep this difference in mind. When one is interested in changes in microbial activity after the setup of an experiment [24,46], functional stability has to be reached, while if one wants to look at microbial composition changes, composition stability is more important.

Another very important factor which influences the effectiveness of the SHIME in simulating intestinal conditions is the suitability of the initial bacterial inoculum and the formation of a representative microbial community after stabilisation. As it is being prepared from faecal matter, the microbial community in this inoculum has to have the same composition as the one in the starter material. DGGE analysis for the general Bacteria, *Lactobacillus* sp., and the *Bacteroides*/*Prevotella* group showed that in all cases the profiles of the inoculum and the initial faecal matter showed minimum 88% correlation. Therefore it can be concluded that with the type of inoculum used, a microbial community was introduced in the SHIME, which is representative for the initial one in the faecal matter. Nevertheless, the question remains to what extent the faecal ecosystem (and thus the ecosystem introduced in the SHIME) is a reliable representation of the one in the different parts of the intestine. Although community composition of faecal matter was shown not to be significantly different from the one in mucosal biopsies, bacteria present in faecal matter mainly originate from the lumen of the distal colon. Therefore it can be argued whether faecal bacteria are adapted to the conditions in the different colon regions [47,48]. So, although using faecal matter to inoculate the SHIME is the only practical possibility, one has to keep these aspects in mind. The plate counts of the steady-state microbial community (see Table 4) were in good concordance with previous reports of SHIME experiments [10,11,49] except for the *Lactobacillus* sp. For this group, concentrations from 3 [11] to 8 log(CFU ml^{-1}) [49] were reported. When compared with in vivo results [50], counts for *Staphylococcus* sp. and *Enterococcus* sp. were ten times lower than expected. This means that, except for some less abundant groups, the SHIME community composition was representative for the in vivo situation.

The SHIME system is valuable in maintaining a complex microbial community, representative in microbial composition and function of that of the human intestinal system [11–13,15,24,31–33]. Microbial diversity and ecosystem composition are determined by many conditions of physical, biochemical, and physiological origin. As in vitro models are designed to approach those conditions, it is important to control these parameters as close as possible. The utmost important factor that has to be controlled in SHIME is the stable microbial community composition. From the obtained results, at least 2 weeks of stabilisation of the SHIME community are necessary to form a representative microbial ecosystem that can be used for in vitro investigations. Because of the time-consuming

nature of performing SHIME experiments, stabilisation of only one SHIME run was completely analysed, but results were in good concordance with preceding experiments [11]. Other researchers who use in vitro models apply stabilisation periods of 24 h [4], 14 days [5] or 48 days [51], but a motivation for the choice of this period is not apparent. Here we propose an easy-to-use DGGE-based method to investigate formation and composition stability of microbial communities of in vitro systems. Of course, also other important microbial communities, for example those in neonates [36] or bioreactors [38], could be analysed in a similar way. Because it has been reported earlier that healthy intestinal ecosystems remain more or less stable over time [40], the principle of moving window correlation can generally be used to monitor the effect of disturbances such as antibiotics on population variability within these systems. In this way the stability criterion could essentially be used to monitor the disturbances and health of a microbial community.

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