Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers

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Abstract

According to the current insights, the predominant bacterial community in human feces is considered to be stable and unique for each individual over a prolonged period of time. In this study, the temporal stability of both the predominant population and a number of specific subpopulations of the fecal microbiota of four healthy volunteers was monitored for 6–12 weeks. For this purpose, a combination of different universal (V₃ and V₆–V₈) and genus- or group-specific (targeting the *Bacteroides fragilis* subgroup, the genera *Bifidobacterium* and *Enterococcus* and the *Lactobacillus* group, which also comprises the genera *Leuconostoc*, *Pediococcus* and *Weissella*) 16S rRNA gene primers was used. Denaturing gradient gel electrophoresis (DGGE) was used to analyze the 16S rRNA gene amplicons generating population fingerprints which were compared visually and by numerical analysis. DGGE profiles generated by universal primers were relatively stable over a three-month period and these profiles were grouped by numerical analysis in subject-specific clusters. In contrast, the genus- and group-specific primers yielded profiles with varying degrees of temporal stability. The *Bacteroides fragilis* subgroup and *Bifidobacterium* populations remained relatively stable which was also reflected by subject-specific profile clustering. The *Lactobacillus* group showed considerable variation even within a two-week period and resulted in complete loss of subject-grouping. The *Enterococcus* population was detectable by DGGE analysis in only half of the samples. In conclusion, numerical analysis of 16S rRNA gene-DGGE profiles clearly indicates that the predominant fecal microbiota is host-specific and relatively stable over a prolonged time period. However, some subpopulations tended to show temporal variations (e.g., the *Lactobacillus* group) whereas other autochthonous groups (e.g., the bifidobacteria and the *Bacteroides fragilis* subgroup) did not undergo major population shifts in time.

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1. Introduction

The human gastrointestinal (GI) tract is one of the most complex ecosystems known in microbial ecology usually containing 10¹⁰–10¹¹ bacteria. These organisms may belong to at least 400 different bacterial species, although it is thought that 99% of the total community consists of only 30–40 species [1]. The microbial activity

of this community has an important metabolic and protective function in the GI-tract and thus plays a major role in the nutrition and health of the host [2,3]. The genera that are considered to be predominant in the large bowel include *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Bifidobacterium* and *Fusobacterium* [4]. For many years, descriptions of bacterial diversity in the GI tract were based mainly on the use of anaerobic culture techniques which are usually very labor-intensive and time-consuming. Moreover, comparisons with molecular methods have indicated that culture-dependent approaches underestimate bacterial diversity in the GI

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tract as only 10–50% of this population is culturable with currently available methods [5–7].

Molecular methods rely on culture-independent approaches such as PCR amplification or fluorescent in situ hybridization (FISH) and allow a more complete and rapid assessment of microbial diversity, especially of complex ecosystems like the colon [8–10]. To analyze the composition and changes of the intestinal microbiota, population fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are effective tools. These methods rely on the sequence-specific separation of equally sized PCR products amplified from 16S rRNA gene or other universal markers [11]. Studies in which DGGE or TGGE were applied to analyze the intestinal microbiota indicated that the predominant bacterial community was stable and host-specific in human subjects [3,12] as well as in animals [13,14]. In addition, subpopulations of *Bifidobacterium* [15] and *Lactobacillus* [16,17] were also analyzed although none of these studies reported on the combined use of universal and specific primers.

The primary goal of the present study was to check the temporal stability of the human fecal microbiota of four healthy individuals including both the predominant microbial populations and a number of specific subpopulations. The predominant fecal microbiota was analyzed by using two universal primers targeting the V₃ and V₆–V₈ regions of the 16S rRNA gene [3,11]. Specific subpopulations were studied with genus- and group-specific primers: the *Bacteroides fragilis* subgroup (this study), the genus *Bifidobacterium* [4], the *Lactobacillus Leuconostoc Pediococcus Weisella* complex [16] and the genus *Enterococcus* (this study). Due to the extreme bacterial complexity of the human colon, the use of specific primers can complement the analysis and interpretation of the results obtained with the universal primers by focusing on subpopulations of a bigger entity.

2. Materials and methods

2.1. Collection and processing of fecal samples

Fresh fecal samples were obtained from four healthy volunteers (B–E; one female and three males; subject A was excluded after antibiotic intake) who were between 22 and 55 years old. Four samples were collected from each subject with a 14-day interval over a six-week period and an additional fifth sample was collected from two volunteers 3 months after the start of the study. All participating subjects were asked not to take any antibiotics from one month before the start until the end of the test period. Upon collection of the fecal samples in sterile plastic containers, 1.4 g (wet weight) was homogenized in 18.6 ml PBS buffer (1% [wt:vol] peptone

[catalog no. L37; Oxoid], 0.5% [wt:vol] NaCl, 0.35% [wt:vol] Na₂PO₄, 0.15% [wt:vol] NaH₂PO₄), and immediately stored at –20 °C. Samples were processed within 48 h of collection.

2.2. Total DNA extraction

Three DNA extraction protocols, applied on eight fecal samples, were compared to select the method with the best overall results, i.e., the QIAamp DNA Stool Mini Kit (catalog no. 51504, QIAgen), the method of Zoetendal et al. [3] and a modified version of the method of Pitcher et al. [18] as described below. From the fecal sample suspension, 1 ml was centrifuged at 20,000g for 5 min. After removal of the supernatant, the pellet was resuspended in 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and was again centrifuged at 20,000g for 5 min. The pellet was resuspended in 150 µl enzyme solution (6 mg lysozyme powder [catalog no. 28262, Serva] and 40 µl mutanolysine [catalog no. M4782, Sigma] dissolved in 110 µl TE (1×) per sample) followed by incubation at 37 °C for 40 min. Next, 500 µl GES reagent (Guanidiumthiocyanate–EDTA–Sarkosyl; 600 g l^{–1} guanidiumthiocyanate [catalog no. G-6639, Sigma], 200 ml l^{–1} 0.5 M EDTA, 10 g l^{–1} sarkosyl) was added to complete all lysis, after which the solution was put on ice for 10 min. In the following step, 250 µl ammonium acetate (7.5 M) was added and the mixture was put on ice for 10 min. Subsequently, two chloroform–iso-amylalcohol extractions were performed with 500 µl chloroform/iso-amylalcohol solution (24/1). Finally, DNA was precipitated by adding 0.54 volumes of ice-cold isopropanol. After centrifugation at 20,000g for 5 min, the pellet was washed twice with 150 µl 70% EtOH, air dried and allowed to dissolve overnight in 150 µl TE (1×) buffer. The remaining RNA was removed by adding 7.5 µl RNase (2 mg ml^{–1}; catalog no. 34390, Serva) after which samples were incubated for 1.5 h at 37 °C. Purified DNA extracts were stored at –20 °C. For comparison between the three extraction methods, DNA integrity was checked electrophoretically by loading 6 µl DNA solution on a 1% agarose gel stained with ethidium bromide. The quality and concentration of the DNA extracts were determined by spectrophotometric measurements at 260, 280 and 234 nm.

2.3. Primer design and PCR amplification for DGGE

The KodonTM (version 1.0) software (Applied Maths, St-Martens-Latem, Belgium) was used to develop a *Bacteroides fragilis* subgroup-specific primer and an *Enterococcus* genus-specific primer, with the *Bacteroides fragilis* subgroup comprising *B. fragilis*, *B. acidifaciens*, *B. caccae*, *B. eggerthii*, *B. ovatus*, *B. stercoris*, *B. thetaiotaomicron*, *B. uniformis* and *B. vulgatus* [19,20]. A total of 7000 16S rRNA gene sequences of 113 known GI

Table 1
Results of 16S rRNA gene primer specificity tests

Species	Strain no.	Expected amplicon with primers ^a					
		V ₃	V ₆ –V ₈	Bact	g-Bifid	Lac	Ent
<i>Anaerostipes caccae</i>	DSM 14662 ^T	+	+	–	–	–	–
<i>Bacillus cereus</i>	LMG 6923 ^T	+	+	–	–	+	–
<i>Bacillus fumarioli</i>	LMG 19448 ^T	+	+	–	–	+	–
<i>Bacillus oleronius</i>	LMG 17952 ^T	+	+	–	–	+	–
<i>Bacteroides coagulans</i>	LMG 8206 ^T	+	±	–	–	–	–
<i>Bacteroides distansoni</i>	DSM 20701 ^T	+	±	–	–	–	–
<i>Bacteroides eggerthii</i>	DSM 20697 ^T	+	+	+	–	–	–
<i>Bacteroides fragilis</i>	LMG 10263 ^T	+	+	+	–	–	–
<i>Bacteroides ovatus</i>	DSM 1896 ^T	+	±	+	–	–	–
<i>Bacteroides splanchnius</i>	LMG 8202 ^T	+	+	–	–	–	–
<i>Bacteroides thetaiotaomicron</i>	DSM 2079 ^T	+	+	+	–	–	–
<i>Bacteroides ureolyticus</i>	LMG 6451 ^T	+	–	+	–	–	–
<i>Bacteroides vulgatus</i>	LMG 17767 ^T	+	±	+	–	–	–
<i>Bifidobacterium species</i> ^b		+	+	–	+	–	–
<i>Clostridium species</i> ^c		+	+	–	–	–	–
<i>Enterobacter aerogenes</i>	LMG 2094 ^T	+	+	–	–	–	–
<i>Enterococcus avium</i>	LMG 10744 ^T	+	+	–	–	–	+
<i>Enterococcus casseliflavus</i>	LMG 10745 ^T	+	+	–	–	–	+
<i>Enterococcus cecorum</i>	LMG 12902 ^T	+	+	–	–	–	–
<i>Enterococcus columbae</i>	LMG 11740 ^T	+	+	–	–	–	–
<i>Enterococcus dispar</i>	LMG 13521 ^T	+	+	–	–	–	+
<i>Enterococcus durans</i>	LMG 10746 ^T	+	+	–	–	–	+
<i>Enterococcus flavescens</i>	LMG 13518 ^T	+	+	–	–	–	+
<i>Enterococcus raffinosus</i>	LMG 12588 ^T	+	+	–	–	–	+
<i>Enterococcus saccharolyticus</i>	LMG 11427 ^T	+	+	–	–	–	+
<i>Enterococcus solitarius</i>	LMG 12890 ^T	+	+	–	–	–	–
<i>Dorea formicigenerans</i>	DSM 3992 ^T	+	+	–	–	–	–
<i>Escherichia coli</i>	LMG 2092 ^T	+	+	–	–	–	–
<i>Eubacterium species</i> ^d		+	+	–	–	–	–
<i>Fusobacterium nucleatum</i>	LMG 13131 ^T	+	+	–	–	–	–
<i>Lactobacillus species</i> ^e		+	+	–	–	+	–
<i>Leuconostoc lactis</i>	LMG 8894 ^T	+	+	–	–	+	–
<i>Megashaera elsdennii</i>	DSM 20460 ^T	+	+	–	–	–	–
<i>Mitsuokella multiacida</i>	DSM 20544 ^T	+	+	–	–	–	–
<i>Pediococcus pentosaceus</i>	LMG 11488 ^T	+	+	–	–	+	–
<i>Peptostreptococcus anaerobius</i>	LMG 15865 ^T	+	+	–	–	–	–
<i>Prevotella melaninogenica</i>	DSM 7089 ^T	+	+	–	–	–	–
<i>Proteus mirabilis</i>	LMG 3257 ^T	+	+	–	–	–	–
<i>Ruminococcus productus</i>	LMG 21654 ^T	+	+	–	–	–	–
<i>Staphylococcus aureus</i>	LMG 8064 ^T	+	+	–	–	–	–
<i>Streptococcus salivarius</i>	LMG 11489 ^T	+	+	–	–	–	–
<i>Vagococcus fluvialis</i>	LMG 9464 ^T	+	+	–	–	–	–
<i>Veillonella parvula</i>	DSM 2008 ^T	+	+	–	–	–	–
<i>Weissella confusa</i>	LMG 9497 ^T	+	+	–	–	+	–

^a +, positive; –, negative; ±, fuzzy band on DGGE gel.

^b *Bifidobacterium* strains tested: *B. adolescentis* LMG 10502^T, *B. angulatum* LMG 10503^T, *B. bifidum* LMG 11041^T, *B. breve* LMG 11042^T, *B. catenulatum* LMG 11043^T, *B. dentium* LMG 11045^T, *B. gallicum* LMG 11596^T, *B. infantis* LMG 8811^T, *B. longum* LMG 13197^T, *B. pseudocatenulatum* LMG 10505^T.

^c *Clostridium* strains tested: *C. bifermentans* LMG 3029^T, *C. beijerinckii* LMG 5716^T, *C. butyricum* LMG 1217^T, *C. clostridioforme* DSM 933^T, *C. innocuum* DSM 1286^T, *C. nexile* DSM 1787^T, *C. paraputrificum* DSM 2630^T, *C. perfringens* LMG 11264^T, *C. spiroforme* DSM 1552^T, *C. sporogenes* LMG 8421^T, *C. sporosphaeroides* DSM 1294^T, *C. symbiosum* DSM 934^T, *C. tyrobutyricum* LMG 1285^T.

^d *Eubacterium* strains tested: *Eub. cylindroides* DSM 3983^T, *Eub. dolichum* DSM 3991^T, *Eub. eligens* DSM 3376^T, *Eub. limosum* DSM 20543^T, *Eub. ventriosum* DSM 3988^T.

^e *Lactobacillus* species tested: *L. acidophilus* LMG 9433^T, *L. amylovorus* LMG 9496^T, *L. brevis* LMG 6906^T, *L. casei* LMG 6904^T, *L. crispatus* LMG 9479^T, *L. gasseri* LMG 9203^T, *L. johnsonii* LMG 9437^T, *L. reuteri* LMG 9213^T, *L. plantarum* LMG 6907^T, *L. ruminis* LMG 10756^T, *L. salivarius* LMG 9477^T.

tract species and of 740 related organisms were retrieved from the EMBL database (<http://srs6.ebi.ac.uk>) and imported in a KodonTM database. This software allows

multiple alignments of selected sequences and searching for potential primer target sites. Validation of the developed primers was performed in silico and with DNA

Fig. 1. Comparison of SYBR[®] Gold (A) and EtBr (B) as staining agents for DGGE gels. On both gels, identical samples were loaded in the same order (1–6); R, reference lane.

staining in comparison with SYBR[®] Gold. Furthermore, visual inspection of inverted DGGE profiles following data processing with the BioNumerics software allowed detection of multiple additional bands in SYBR[®] Gold stained profiles not visible in the corresponding EtBr profiles.

3.2. Evaluation of different DNA extraction methods

The commercially available QIAamp DNA Stool Mini Kit (QIAGEN) and the widely used method of Zoetendal et al. [3] were compared with a modified version of the method of Pitcher et al. [18] for the isolation of total bacterial DNA from fecal samples.

First, some adjustments were made to optimize the performance of the QIAGEN Kit. In this regard, an increase in temperature of the chemical lysis step from 70

to 95 °C and the addition of a preliminary enzymatic lysis step with lysozyme and mutanolysine (37 °C, 40 min) resulted in a higher DNA yield and the visualization of more bands in the DGGE pattern (Fig. 2B). Likewise, the introduction of a preliminary enzymatic lysis step in the method of Zoetendal and co-workers led to visualization of a higher number of band fragments. However, no difference in DGGE profile complexity could be observed with or without the use of bead beating (Fig. 2B).

These two methods were compared with the modified method of Pitcher and co-workers. Electrophoretic evaluation of the DNA integrity showed an intense band at the top of the agarose gel for the modified method of Pitcher and co-workers whereas for both other methods only a weak band was visible (Fig. 2A). The spectrophotometric value ranges of eight fecal samples indicating the DNA concentration and quality are shown in Table 3. The highest DNA yields were observed with the modified method of Pitcher and co-workers and also the A_{260}/A_{280} and A_{234}/A_{260} ratios of the DNA extracts obtained with this method indicated the highest purity. Spectrophotometric analysis, DNA integrity and DGGE pattern quality indicated that the modified protocol of Pitcher and co-workers gave the highest performance.

3.3. Validation of 16S rRNA gene-DGGE primers

Specificity of the *Bacteroides fragilis* subgroup-specific primer and the *Enterococcus* genus-specific primer was tested in silico with Kodon to check if the primers anneal with one of the 7000 other 16S rRNA gene

Fig. 2. (A) DNA integrity check after extraction of the same sample with QK + enz (lane 1), MZ + enz (lane 2) or MMP (lane 3) M molecular marker. (B) Comparison of DGGE profiles of V₃ PCR amplicons from one fecal sample using DNA extracts obtained with different methods. QK: QIAamp DNA Stool Mini Kit, MZ: method of Zoetendal and co-workers, MMP: modified method of Pitcher and co-workers, + enz: with enzymatic lysis step with lysozyme and mutanolysine.

Table 3
Spectrophotometric analysis of fecal DNA extracts

DNA extraction protocol	OD ₂₆₀	A_{260}/A_{280}	A_{234}/A_{260}
QIAamp DNA Stool Mini Kit	0.5–2.31	1.37–3.25	0.25–2.51
Method of Zoetendal	2.69–13.92	1.25–1.93	0.53–1.07
Method of Zoetendal + enz.	4.6–11.71	1.56–2.05	0.51–1.07
Modified method of Pitcher	8–25	1.62–2.02	0.52–1.21

Value ranges of eight samples. The DNA was considered to be of sufficient quality if the ratio A_{260}/A_{280} was in the range 1.8–2.2 and the ratio A_{234}/A_{260} was in the range 0.5–0.8.

sequences retrieved from the EMBL database encompassing intestinal species and related organisms. Primer specificity was also assessed in vitro with DNA extracts of a subset of human intestinal species (Table 1). No species other than members of the *B. fragilis* subgroup showed a perfect match with the Bact. 596F/Bact. 826R primers at an annealing temperature of 60 °C. The *in silico* specificity check showed annealing of the Ent. 1017F/Ent. 1263R primers only with *Enterococcus* species when annealing temperature was set at 62 °C and these results were also confirmed by in vitro evaluation (Table 1).

Finally, the specificity of previously described universal and group-specific primers used in this study was also tested (Table 1). In contrast to the primers targeting the V₃ region, not all *Bacteroides* species could be detected by PCR amplification using the universal V₆–V₈ primers. With *B. ureolyticus* the V₆–V₈ primers yielded no PCR product whereas for the species *B. coagulans*, *B. distansoni*, *B. ovatus* and *B. vulgatus* a smear instead of a clear band was visible on DGGE. Also, it was found that the Lac primers yielded amplicons with the non-LAB species *Bacillus cereus*, *Bacillus fumarioli* and *Bacillus oleroni*. However, none of these three organisms has so far been recognized as an inhabitant of the human fecal microbiota.

3.4. Temporal stability of DGGE patterns

All fecal samples were analyzed with the PCR-DGGE approach to monitor the temporal stability of the predominant fecal microbiota and some specific subgroups.

Visual comparison of the DGGE banding patterns obtained with the different universal primers showed that the V₃ primer profiles are more complex (between 24 and 33 bands) than the V₆–V₈ primer profiles (between 12 and 19 bands). Overall, both profile types exhibited very little or no detectable variation within one individual (Fig. 3). Furthermore, the patterns differed between each individual both in the number of bands as well as in the positions of these bands. The uniqueness and the stability of the patterns of each individual were also demonstrated by numerical analysis (Fig. 3). All profiles of each individual subject formed a separate cluster with similarity of the Dice band-based coefficient values ranging from 82.6% to 92.5% for the V₃ primer and from 88.1% to 95.7% for the V₆–V₈ primer profiles. Similar grouping was observed when clustering with the Pearson product-moment correlation coefficient and UPGMA but similarity values were lower (data not shown).

In addition to the use of universal primers, a number of group-specific primers were used to study the temporal stability of several subpopulations. The *Bacteroides fragilis* subpopulation, visualized in DGGE with the Bact. primers, showed relatively stable patterns over the test period within the same individual (Fig. 4). The different sample profiles from each individual clustered closely together (Dice/UPGMA) with similarity values ranging from 85.2% to 96.0% whereas the variability between the individuals was less pronounced compared to the grouping of the V₃ and V₆–V₈ profiles.

The *Bifidobacterium* populations detected by the g-Bifid primers were stable over the entire test period

Fig. 3. Clustering of DGGE profiles obtained with universal primers V₃ (a) or V₆–V₈ (b) of four individuals (B–E) using Dice's coefficient and UPGMA. Samples 1–4 were collected over a six week period (14 day interval). Of individuals C and E, a fifth sample was collected 3 months after the start of the study.

Fig. 4. DGGE profiles of the *Bacteroides fragilis* subgroup population from four individuals (B–E) obtained with the Bact primers. Samples C1–C4 were collected over a six-week period (14-day interval) and C5 was collected 3 months after the start of the study. For individuals B, D and E, only one sample was shown because the other sample profiles within a given individual were highly similar if not identical ($\geq 91.2\%$ similarity). Clustering was performed using Dice's coefficient and UPGMA.

(Fig. 5), except for sample E5 which differed from the other samples of subject E. The DGGE patterns also appeared to be host-specific although some common bands could be observed across different subjects. Clustering analysis (Dice/UPGMA) showed close profile grouping of each subject with similarity values ranging from 81.4% to 100% except for the profiles of subject E that displayed a very low similarity value mainly due to variation in the DGGE profile of sample E5.

DGGE analysis of the PCR amplicons revealed relatively high variation and low host-specificity in the population profiles of the *Lactobacillus* group within each of the four subjects, even within a two-week interval (Fig. 6). Subjects B and C displayed profiles with a low complexity that appeared to be more stable in comparison to the more complex profiles of subjects D

Fig. 5. DGGE profiles of the *Bifidobacterium* population from four individuals (B–E) obtained with the g-Bifid primers. Samples E1–E4 were collected over a six-week period (14-day interval) and E5 was collected 3 months after the start of the study. For individuals B, C and D, only one sample was shown because the other sample profiles within a given individual were highly similar if not identical ($\geq 90.0\%$ similarity). Clustering was performed using Dice's coefficient and UPGMA.

Fig. 6. DGGE profiles of the *Lactobacillus* group population from four individuals (B–E) obtained with the Lac primers. Samples 1–4 were collected over a six-week period (14-day interval) whereas sample 5 was collected 3 months after the start of the study. Clustering was performed using Dice's coefficient and UPGMA.

and E. In the case of subject E, it was striking that the profiles of samples E1 and E3 were very similar (92.31%) but very different from samples E2, E4 and E5.

Visualization of the *Enterococcus* population required the inclusion of a nested PCR since only one sample yielded PCR product when the Ent. primers were used directly on the fecal sample DNA. But even with the nested PCR approach, only half of the investigated samples were *Enterococcus* positive in DGGE analysis. Moreover, positive samples displayed only two different band positions in their DGGE profiles and only one band could be detected per sample (data not shown).

4. Discussion

During the past decade, various studies based on TGGE or DGGE profiling showed that gastro-intestinal bacterial populations of the same subjects were remarkably stable over a long time period when universal primers were used for analysis of animal (15,16) and human (4,14) fecal samples. The objective of the present study was to investigate this temporal stability by the use of different universal and group-specific primers that have the potential to provide a more in-depth view of different subpopulations of the gastro-intestinal ecosystem in healthy humans. Following optimization of the

DGGE analysis protocol in terms of reproducibility and detection capacity, the modified protocol of Pitcher and co-workers and the SYBR[®] Gold dye was selected and used in this study.

Through seeding of a fecal sample with a pure culture of *Bacillus*, the detection limit of the DNA extraction-PCR-DGGE method used in this study was determined at 4×10^5 – 4×10^6 bacteria g⁻¹ feces (wet weight), which is in agreement with previous findings [22]. However, it should be kept in mind that the detection limit is a relative value that may strongly depend on the total number of bacteria present in the human stool samples.

The DGGE profiles obtained with the universal primers (V₃ and V₆–V₈) were relatively stable and unique for each subject. Based on V₆–V₈ profiles, Zoetendal and colleagues [3] likewise concluded that the composition of the predominant fecal microbiota of humans does not alter over a short period of time. The temporal stability and host-specificity of the predominant fecal community was also confirmed by numerical analysis of digitized DGGE fingerprints. It appeared that the similarity values of the V₆–V₈ primer were usually higher than those of the V₃ primer which is probably due to the higher number of bands in the V₃ primer profiles. This difference probably reflects the fact that the V₆–V₈ primer is less efficient as a universal primer than the V₃ primer, as evidenced by some difficulties of the V₆–V₈ primer to generate an amplicon from some type strains of *Bacteroides* species (Table 1). Indeed, when checking the annealing sites of the V₆–V₈ primer for *Bacteroides*, several mismatches were found for the forward ($n = 0$ – 6) and the reverse ($n = 0$ – 1) primer.

Members of the genus *Bacteroides* are considered to constitute one of the most abundant bacterial groups in the human colon, representing approximately 30% of all culturable fecal bacteria, most of which belong to the *Bacteroides fragilis* cluster [23]. The Bact. primers used in this study were designed for the detection of all species of the *Bacteroides fragilis* cluster. In addition, *Prevotella heparinolytica* and *P. zoogloformans* are situated in the *B. fragilis* subgroup and show only two mismatches with the Bact. 596F primer and may also be detected with the primers. Using the Bact. primers, *B. fragilis* subgroup-specific DGGE patterns were found to be relatively stable for each subject over the test period (Fig. 4) indicating that this subpopulation is not subjected to dramatic temporal shifts. Because of their predominance in colon microbiota, no abrupt shifts are expected in the *Bacteroides* community of a given subject. Since all reference strains of the tested *Bacteroides* species yielded multiple bands in DGGE, it was not possible to identify all detected species reliably.

Bifidobacterium is the third most common genus in the human intestinal microbiota after *Bacteroides* and *Eubacterium* [24], and some species have been used as

probiotics because of their claimed health promoting properties [25]. In this study, the *Bifidobacterium* genus-specific primer described by Matsuki et al. [4] showed a stable and host-specific population of bifidobacteria for all four subjects (Fig. 5) which is consistent with previous results [15,26]. Recently, a method for identifying bifidobacteria in different environments is described, based on a nested-PCR-DGGE application [27]. The primers used in our study did not allow identification of the detected bifidobacteria because identical band positions were observed for several species.

The genus *Lactobacillus* makes up less than 1% (10^4 – 10^8 CFU g⁻¹) of the fecal microbial community [28]. Lactobacilli are intensively marketed in fermented foods and probiotic products because of the health promoting properties of some species [24]. In the current study, a group-specific primer was used for the detection of lactobacilli in conjunction with *Leuconostoc*, *Pediococcus* and *Weissella* [16]. From our results, a clear temporal variation within the *Lactobacillus* group DGGE profiles could be observed in all subjects. In numerical analysis, the lack of a stable and host-specific *Lactobacillus* group population resulted in a complete loss of subject-grouping. In a number of studies [24,29,30], it has been reported that approximately half of the investigated subjects harbored a relatively simple *Lactobacillus* population in which one or two strains were numerically predominant. In the present study, a similar tendency could be observed with subjects B and C that displayed a relatively simple profile compared to the more complex and variable profiles of subjects D and E. At least half of the LAB detected in feces are associated with foods and/or used as food fermentation starters [16]. For this reason, many *Lactobacillus* species should probably be considered as transient, allochthonous species in the intestinal tract. The presence of such transient species in food could explain why some bands reappeared in the DGGE profiles of certain subject samples whereas they were absent in other samples of the same subject (Fig. 6). The genus *Enterococcus* is also autochthonous to the human gut but represents an even less abundant community in human feces and is also used in fermented foods and probiotic products. The developed Ent. primers detected all *Enterococcus* species except for *E. solitarius*, *E. cecorum* and *E. columbae* (Table 1). Their exclusion probably only has limited consequences for the detection of fecal enterococci since these species are generally not encountered in human feces. Because in only half of the samples the *Enterococcus* population was detectable, it was impossible to draw conclusions about the stability of the *Enterococcus* population in the fecal samples investigated. The introduction of a nested-PCR step already improved detection and further optimization could make it possible to lower the detection limit and visualize the *Enterococcus* population in all samples.

In conclusion, the study reinforces the current belief that the fecal microbiota is host-specific and relatively stable over time within each individual when only universal primers are used in 16S rRNA gene-DGGE population fingerprinting. However, in-depth analysis with group-specific primers indicates that some populations tend to show strong temporal variations (e.g., the *Lactobacillus* group) whereas other autochthonous groups (e.g., *Bifidobacterium* and the *Bacteroides fragilis* subgroup) do not undergo major population shifts. Clearly, these observations need further confirmation in future studies using a higher number of subjects. Although our knowledge of microbial diversity within the human colon is continually increasing with the description of new species [31–33], many of the factors influencing the establishment and consistency of these gut communities are still poorly understood. In this regard, the further extension of our current knowledge on the metabolic, genetic and immunological interactions in the human GI tract will certainly benefit the development of functional ‘health-improving’ foods.

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