Characterization of human intestinal bifidobacteria using competitive PCR and PCR-TTGE

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Abstract

In this study, a competitive PCR was developed to estimate the quantity of bifidobacteria in human faecal samples using two 16S rRNA gene *Bifidobacterium* genus-specific primers, Bif164f and Bif662r. A PCR-temporal temperature gradient gel electrophoresis (TTGE) with the same primers also allowed us to describe the *Bifidobacterium* species present in these faecal samples. The PCR product obtained from the competitor had 467 bp, and was 47 bp shorter than the PCR products obtained from *Bifidobacterium* strains. The number of bifidobacterial cells was linear from 10 to 10^8 cells per PCR assay. Taking into account the dilutions of the extracted DNA, the linear range was over 8×10^5 bifidobacteria g^{-1} of faeces. Reproducibility was assessed from 10 independent DNA extractions from the same stool and the coefficient of variation was 0.5%. When the competitive PCR was compared with the culture method, a similar count of seven out of nine *Bifidobacterium* pure cultures were obtained, or had a difference inferior or equal to 1 $\log_{10}$. In faecal samples, the enumeration of *Bifidobacterium* genus in most cases gave higher results with competitive PCR than with culture on selective Columbia–Beerens agar pH 5 ($P < 0.05$). In conclusion, this competitive PCR allows a rapid, highly specific and reproducible quantification of *Bifidobacterium* genus in faecal samples. TTGE fragments co-migrating with *B. longum* CIP64.63 fragment were found in 10 out of 11 faecal samples. *Bifidobacterium adolescentis* and *B. bifidum* were detected in five out of 11 subjects. Thus, cPCR and PCR-TTGE can be associated in order to characterize human faecal bifidobacteria.

Introduction

The human large intestine contains a substantial and diverse population of bacteria that carry out important functions for human nutrition and health. Bifidobacteria from dominant microflora are present in 74% of adult subjects with an average count of 1.58×10^{10} cells g^{-1} of faeces (Finegold *et al.*, 1983). These micro-organisms are considered beneficial for the host (Gibson *et al.*, 1995), as they produce lactic and acetic acids that decrease pH and inhibit the growth of potential pathogenic bacteria (Modler *et al.*, 1990). Bifidobacteria predominate in the gastrointestinal tract of breast-fed infants until weaning (Mackie *et al.*, 1999). During the last decade, there has been major interest in influencing the composition of intestinal microflora, in a seemingly positive way, by increasing the relative proportion of bifidobacteria. One method has been through the intake of probiotic strains (Fuller, 1991; Bouhnik *et al.*, 1992) and another through the addition of prebiotic oligosaccharides to the diet (Gibson *et al.*, 1995; Bouhnik *et al.*, 1997; Kleessen *et al.*, 1997). Recently, it was shown that bifidobacteria supplementation could modulate the composition of gut microflora during weaning, resulting in fewer cutaneous symptoms in allergic subjects (Kirjavainen *et al.*, 2002). However, *Bifidobacterium* species were also significantly associated with an increased risk of colon cancer (Moore & Moore, 1995). Therefore, quantification of the *Bifidobacterium* genus in the human intestinal microflora is of great concern. Numerous studies are based on their culture using selective media (Beerens, 1990; Nebra & Blanch, 1999). Nevertheless, biases can occur and the results also depend on the species or strains present in the faecal sample (Apajalahti *et al.*, 2003). Molecular

techniques founded on a comparative analysis of 16S rRNA sequences provide a powerful culture-independent tool for the reliable identification of microorganisms from various ecosystems (Borneman *et al.*, 1996; Godon *et al.*, 1997; Kroes *et al.*, 1999; Suau *et al.*, 1999). These methods have permitted the detection of uncultivated intestinal species (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Mangin *et al.*, 2004) and the subsequent development of oligonucleotide probes to monitor and quantify specific species or groups of micro-organisms. Fluorescent *in situ* hybridization (FISH) and dot-blot hybridization with specific 16S rRNA-targeted oligonucleotide probes have been widely used to quantify bifidobacteria or other specific groups from faecal microflora (Franks *et al.*, 1998; Mangin *et al.*, 2002). In contrast to culture counting, some of these molecular methods may be performed on frozen samples, avoiding the immediate processing of stools and allowing a more reliable quantification of this genus. At the present time, real-time PCR provides a good quantitative tool for monitoring this group (Requena *et al.*, 2002; Bartosch *et al.*, 2004; Gueimonde *et al.*, 2004; Matsuki *et al.*, 2004), but the equipment required for this technique remains expensive for many laboratories. Competitive PCR (cPCR) also has the ability to count targeted bacteria with a high sensitivity and has been used to analyse various environmental samples (Phillips *et al.*, 2000; Koike & Kobayashi, 2001; Leloup *et al.*, 2004; Qiu *et al.*, 2004). Furthermore, this method is reliable and simple to perform.

To obtain a qualitative characterization of the microbial communities, and specifically those bifidobacterial ones, methods such as PCR coupled with temporal temperature gradient gel electrophoresis (PCR-TTGE) or denaturing gradient gel electrophoresis based on the sequence-specific separation of 16S rRNA gene amplicons, have been developed and validated (Zoetendal *et al.*, 1998; Satokari *et al.*, 2001).

In this study, we report the development of a competitive PCR for quantification of the *Bifidobacterium* genus from faecal samples. We also compared bifidobacterial counts obtained from 14 faecal samples using cPCR and a culture method. The PCR-TTGE was then carried out on the previously extracted DNAs to obtain a qualitative characterization of the bifidobacterial communities present in these faecal samples.

Materials and methods

Bacterial strains and culture conditions

The competitor was constructed from DNA extracted from *Bifidobacterium pseudocatenulatum* strain H12 (GenBank accession no. AY856700) isolated from human stools (Suau, unpubl. data). The collection strains used as positive controls were *Bifidobacterium adolescentis* CIP64.59^T, *Bifidobacterium angulatum* CIP104167^T, *Bifidobacterium bifidum* CIP56.7^T, *Bifidobacterium breve* CIP64.69^T, *Bifidobacterium gallicum* CIP103417^T, *Bifidobacterium infantis/longum* CIP64.67, *Bifidobacterium lactis* CIP105265^T, *Bifidobacterium longum* CIP64.62^T, *B. longum* CIP64.63 and *Bifidobacterium pseudocatenulatum* CIP104168^T. The negative controls were *Bacteroides uniformis* ATCC8492^T, *Clostridium perfringens* ATCC13124^T, *Collinsella aerofaciens* ATCC25986^T, *Fusobacterium nucleatum* sp. *nucleatum* CIP101130^T, *Lactobacillus acidophilus* ATCC4356^T, *Ruminococcus flavefaciens* ATCC19208^T and *Ruminococcus productus* ATCC27340^T.

The nine *Bifidobacterium*-type strains were grown at 37 °C under anaerobic conditions (Anaerogen^{TM}, Oxoid SA, Dardilly, France) in 10 mL of overnight culture in M20 broth (Institut Pasteur, Paris, France). They were inoculated to be counted on Columbia/Beerens agar (Columbia-agar acidified with propionic acid; Favier *et al.*, 1997) at pH 5 in order to be under the same conditions as *Bifidobacterium* isolates recovered from human faecal samples. Bifidobacterial cells from each culture were also enumerated with a Malassez counting chamber. Human faecal samples were processed by suspending 1 g (wet wt) in 9 mL of quarter-strength cysteinated Ringer solution. Tenfold serial dilutions were carried out, after which 0.1 mL was inoculated on Columbia–Beerens agar pH 5 plates and incubated for 1 week at 37 °C under anaerobic conditions. The colonies were then counted, subcultured (about 20 per sample), and identified by morphological and biochemical characteristics (glucose +, lactose +, indole production−, nitrate reduction−, and variable fermentations of sucrose, mannitol and starch (Biavati *et al.*, 1991)).

Extraction of total DNA from faecal samples or centrifuged cells

Total DNA was extracted from 125 mg of frozen faecal samples or centrifuged cells as previously described (Godon *et al.*, 1997). For the purification, an equivalent volume of phenol was added and gently mixed before the two phases were separated by centrifugation at 12 000 ***g*** for 5 min. The aqueous layer was transferred to a new Eppendorf tube and an equivalent volume of chloroform–isoamyl alcohol (24 : 1) was added, mixed by inversion and centrifuged at 12 000 ***g*** for 5 min. The aqueous phase was retrieved and this step was repeated once more. Total DNA was precipitated for 2 h at −20 °C with 3 M sodium acetate (0.1 volume) and isopropanol (1 volume). Dry pellets were suspended in 200 μL of sterile water after a 70% ethanol wash.

Competitor construction and evaluation of amplification efficiency

The competitor was constructed by deleting a portion of the 16S rRNA gene from the strain H12 using PCR with a

modified forward primer Bif164/228 [(5′-GGG TGG TAA TGC CGG ATG **ATG GGG TCG CGT CCT ATC**-3′, which resulted from the combination of S-G-Bif-164-a-S-18 (Langendijk *et al.*, 1995) and Bif-228-a-S-18 (Mangin *et al.*, 2002)], and a reverse primer S-G-Bif-662-a-A-18 (Langendijk *et al.*, 1995) (5′-CCA CCG TTA CAC CGG GAA-3′), as previously described (Celi *et al.*, 1993; Phillips *et al.*, 2000). PCR amplification was carried out with a standard PCR mix (0.5 U of *Taq* DNA polymerase (Ampli*Taq* Gold; Perkin-Elmer Corporation, Foster City, CA, USA), 1× reaction buffer II, 2.5 mM $MgCl_2$, 200 μM of each dNTP and 0.4 μM of each primer in a final volume of 20 μL). Initial denaturation of template DNA and *Taq* activation were carried out at 94 °C for 10 min, followed by 35 cycles consisting of 97 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min 30 s and a final extension at 72 °C for 10 min. The PCR product was purified and concentrated using a QIAquick spin PCR Purification Kit (QIAGEN S.A., Courtaboeuf, France). Its concentration and size were estimated using 1% (w/v) agarose gel electrophoresis containing ethidium bromide (0.1 ng mL^{-1}) in TBE 1× buffer. The purified product was cloned into pGEM® -T (Promega Corp., Madison, WI, USA) as recommended by the manufacturer. Plasmids of a single clone were extracted using QIAGEN Plasmid Maxi Kit (QIAGEN, S.A.) and eluted with 200 μL of pure water. Plasmid concentration was then determined spectrophotometrically using absorbances at 260.0 nm (A_1) and 280.0 nm (A_2) according to the formula: Absorbance rate = A_1/A_2, DNA concentration = $(62.9 \times A_1 - 36.0 \times A_2)$ (Shimadzu Corp). Plasmid copy number = DNA concentration/plasmid mass with plasmid mass = 3.82×10^{-18} g.

The PCR product obtained from competitor with primers S-G-Bif-164-a-S-18 (Bif164f) and S-G-Bif-662-a-A-18 (Bif662r) had 467 bp, and it was 47 bp shorter than the PCR product obtained from the DNA of isolate H12. Both products could be separated on a 2% (w/v) agarose gel containing ethidium bromide (0.1 ng mL^{-1}) in TBE 1× buffer, at constant voltage (100 V) for 2 h.

Optimization and calibration

The number of PCR cycles was optimized to ensure that the amplification remained in the exponential phase. Twenty, 25, 30, 32, 35, 37 and 40 cycles of PCR were compared on DNA of isolate H12 using the primer set Bif164f and Bif662r, and the products were run on a 2% agarose gel.

cPCR and data processing

The competitor was serially diluted 10-fold and co-amplified using PCR with a constant amount of target DNA from each pure culture or human faecal sample (a first PCR allowed us to determine the optimal dilution). A 2-μL sample of competitor and 2 μL of the target DNA were mixed in a final volume of 20 μL. The standard PCR mix described above was used with the forward and reverse primers Bif164f and Bif662r. Initial denaturation of the template DNA was carried out at 94 °C for 10 min, followed by 35 cycles of 97 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min 30 s and a final extension at 72 °C for 10 min. The competitor dilution which resulted in an equal amplification with the target sample or which corresponded to the shift from the competitor band to the target band was used as a reference point for making twofold serial dilutions of the competitor. cPCR products were separated on a 2% agarose gel containing ethidium bromide (0.1 ng mL^{-1}) and then scanned using Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA) (Fig. 1A). Relative intensities from the individual bands were measured using Quantity One software (Bio-Rad Laboratories) which provided the data for the corresponding calibration curve (Fig. 1B). The number of 16S rRNA genes amplified from the target DNA was interpolated using a calculated regression equation and assuming a mass of 3.82×10^{-18} g for the competitor plasmid carrying a single copy of the target sequence. The number of 16S rRNA gene copies that had previously been determined in *Bifidobacterium* species (Bourget *et al.*, 1993; Mangin *et al.*, 1994, 1996; Schell *et al.*, 2002) was then used to convert the copy numbers into a cell equivalent value. The assay value was divided by the number of copies of 16S rRNA gene present in the *Bifidobacterium* strain when pure cultures were used, or by a mean value of 4 when faecal

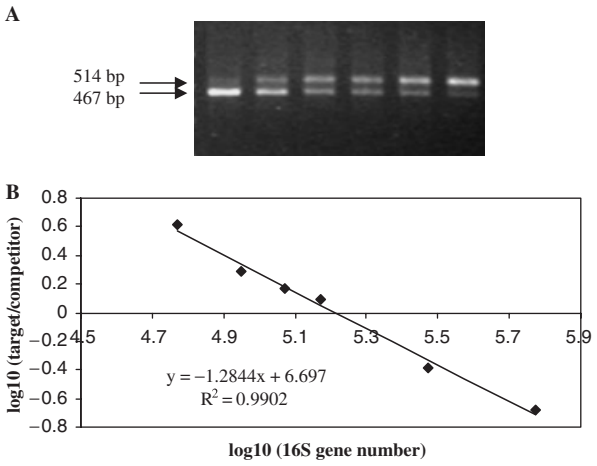

Fig. 1. Molecular quantification of the 16S rRNA genes using competitive polymerase chain reaction (cPCR). (A) Dilutions from 6×10^4 to 6×10^5 molecules of plasmid containing the competitor mixed with 1.6×10^5 copies of *Bifidobacterium* 16S rRNA gene from a faecal sample were amplified. (B) Relative intensities of PCR bands were used to construct the calibration curve. The number of 16S rRNA genes amplified from *Bifidobacterium* was deduced assuming a mass of 3.82×10^{-18} g for the competitor plasmid, which carries a single copy of the target sequence.

samples were analysed. The number of 16S rRNA gene known for the collection strains were: two for *B. bifidum* CIP56.7^T; three for *B. bifidum* CIP64.64, *B. bifidum* CIP64.65, *B. breve* CIP64.66, *B. breve* CIP64.68, *B. breve* CIP64.69^T, *B. breve* CIP64.70; four for *B. adolescentis* CIP64.58, *B. adolescentis* CIP64.60, *B. adolescentis* CIP64.61, *B. angulatum* CIP104167^T, *B. infantis/longum* CIP64.67, *B. lactis* CIP105265^T, *B. longum* CIP64.62^T, *B. longum* CIP64.63; and at least five for *B. adolescentis* CIP64.59^T and *B. catenulatum* ATCC27539^T.

Reproducibility

Competitive PCR reproducibility was checked using 10 independent serial dilutions of the target DNA, and then with 10 aliquots of the same stool.

cPCR limit of quantification in faecal samples

The limit of quantification in the faecal samples was determined by adding 10^8–0 cells of *B. longum* CIP64.62^T (from 10-fold serial dilutions) to identical faecal sample aliquots devoid of bifidobacteria (controlled using cPCR). The DNA extracted from each aliquot was co-amplified with serial 10-fold dilutions of competitor DNA and quantified as previously described.

Quantification of bifidobacteria in culture or in faecal samples

The number of bifidobacteria contained in 10 mL of overnight cultures in M20 broth of nine *Bifidobacterium* type strains and in 14 faecal samples from healthy adults was determined using cPCR and compared with results obtained using the colony-forming unit (CFU) counting method.

PCR amplification for bifidobacterial TTGE

The forward primer Bif164f and reverse primer Bif662r linked to a GC clamp [*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G*CC ACC GTT ACA CCG GGA A] (Satokari *et al*., 2001) were used to amplify 16S rDNAs of the *Bifidobacterium* genus from the samples. A PCR amplification of 35 cycles was done under the conditions previously described, with a lower hybridization temperature of 52 °C, because of the GC clamp. The PCR products were separated on TTGE, using a Dcode^{TM} system (Bio-Rad Laboratories). Electrophoresis was performed in a 9% polyacrylamide gel (160 × 160 × 1 mm) (37.5 : 1 acrylamide–bisacrylamide) and 8.5 M urea (Interchim, Montluçon, France). Premigration was realized at 20 V and 66 °C over a 15-min period. Gels were run overnight at 80 V with the temperature increasing at 0.2 °C h^{-1} from 66 to 70 °C. The DNA fragments were visualized using SYBR Green I staining (Interchim) and the gel was scanned using Gel Doc 2000 (Bio-Rad). The gel patterns were analysed using Diversity Database 2.1 software, which is part of the Discovery Series (Bio-Rad). Four TTGE bands were excised from the gel and stored overnight at 4 °C in 50 μL of sterile water. DNA was then amplified with primers Bif164f and Bif662rGC and the products checked on a TTGE gel. Next amplifications were carried out using the primers Bif164f and Bif662r, and the products were sequenced, without cloning, using primer Bif662r (Sequentia, Clermont-Ferrand, France). These sequences were manually corrected using Chromas ver. 1.45. A search of the GenBank nucleotide database was conducted using the BLAST algorithm to determine the closest relative of the partial 16S rRNA gene sequences.

Data analysis

Quantifications of cPCR and culture counting results were expressed as $\log_{10}$ cell equivalent values per gram. Reproducibility results were expressed as mean ± standard deviation of the mean. The coefficients of variation were calculated as the standard deviation divided by the mean multiplied by 100. Statistical comparison of populations was analysed using the Wilcoxon *T*-test for two paired samples.

Results

Competitor concentration

Plasmid concentration was determined spectrophotometrically. Finally, the calculated DNA concentration was 564 ng μL^{-1}, with A_1 and A_2 corresponding to 0.26 and 0.141 at dilution of 1 : 50, respectively. In this study, the competitor copy number was 1.48×10^{11} μL^{-1}.

Optimization of PCR conditions

To maintain the amplification within the exponential phase, 20, 25, 30, 32, 35, 37 and 40 PCR cycles were used for quantification of bifidobacterial control. Signals were not obtained with 20 and 25 PCR cycles. Signals were not saturated when a 35 PCR-cycle was carried out (data not shown). Primer specificity in our conditions was controlled with DNA extracted from species often retrieved from faecal samples. At 62 °C, the 514-bp PCR amplicon was observed for all *Bifidobacterium* strains, while no amplification was observed with any other bacterial species tested (data not shown).

Reproducibility

The coefficient of variation for 10 independent assays from serial dilutions of the target DNA ($9.8\ \log_{10}$ cells $g^{-1} \pm 0.07$) was 0.7%. The coefficient of variation for 10 independent DNA extractions from the same stool ($11.7\ \log_{10}$ cells $g^{-1} \pm 0.05$) was 0.5%.

Quantification of bifidobacteria in culture medium

The number of bifidobacteria obtained using cPCR, the Malassez counting chambers and the CFU counting method are presented in Table 1. Statistical analysis showed that the populations recovered using these methods differed significantly ($P < 0.05$). However, the difference between cPCR and CFU in most cases was inferior to $1\ \log_{10}$ for type strains of *B. adolescentis*, *B. angulatum*, *B. breve*, *B. gallicum*, *B. lactis*, *B. longum* and *B. pseudocatenulatum*. The population of *B. bifidum* was not recovered on Columbia–Beerens agar pH 5, although it represented 2×10^9 cells mL^{-1} with cPCR. For this strain, the difference between the cPCR and Malassez results was equal to $0.8\ \log_{10}$. The number of *B. infantis/longum* CIP64.67 cells determined using the cPCR method was $2\ \log_{10}$ higher than the culture method. In contrast, the difference between the cPCR and Malassez results was only equal to $0.2\ \log_{10}$.

Quantification limit of cPCR in faecal samples

The number of bifidobacterial cells was found to be linear up to 10 cells per PCR assay mixture ($R^2 = 0.99$). The threshold corresponding to 10 cells may be only seen when 15 μL of PCR product is deposited on the gel. However, this threshold was not detected each time as it depended on the sampling for the PCR assay. Serial dilutions of DNA achieved the same results. These results indicate that the linear range was over 8×10^5 *Bifidobacterium* cells g^{-1} (wet wt) of faeces when the dilutions of extracted DNA were taken into account.

Quantification of bifidobacteria in faecal samples

The number of bifidobacteria obtained using cPCR and the CFU counting methods are presented in Table 2. Statistical analysis showed that the quantification of the populations using the two methods differed significantly ($P < 0.05$). However, the difference between cPCR and CFU was inferior to $1\ \log_{10}$ for six out of 14 samples. For four other samples, the difference was inferior or equal to $2\ \log_{10}$, while another sample was near the detection limit ($5.8\ \log_{10}$ with cPCR vs. $3.1\ \log_{10}$ with culture). *Bifidobacterium* counts based on cPCR for the three last samples were much higher than those determined when using a culture on Columbia–Beerens agar pH 5.

Identification of bifidobacterial species from faeces using PCR-TTGE

The distribution of bifidobacterial species in faecal samples was analysed using PCR-TTGE. Two samples consisting of a mixture of PCR products from identified species were run alongside the faecal samples (Fig. 2). Samples 2, 8 and 11 had low bifidobacterial counts ($< 10^6$ cells g^{-1}), therefore no amplification was obtained. Most of the dominant bands from the other faecal samples co-migrated with bands corresponding to those obtained from the collection strains. Co-migration with the *B. longum* CIP64.63 band was found

Table 1. Comparison of bifidobacterial populations as determined using genus-specific competitive PCR (cPCR), Malassez counting chambers and culture method in Columbia–Beerens broth pH 5 (CFU)

Strain	$\log_{10}$ bifidobacteria mL^{-1}		
	cPCR	Malassez	CFU
Bifidobacterium adolescentis CIP64.59^T	9.2	8.8	8.2
Bifidobacterium angulatum CIP104167^T	8.9	8.7	8.8
Bifidobacterium bifidum CIP56.7^T	9.3	8.5	–
Bifidobacterium breve CIP64.69^T	9.2	9.1	9.2
Bifidobacterium gallicum CIP103417^T	8.9	8.6	8.8
Bifidobacterium infantis/ longum CIP64.67	9.1	8.9	7.1
Bifidobacterium lactis CIP105265^T	9.1	8.5	8.5
Bifidobacterium longum CIP64.62^T	9.3	8.9	8.8
Bifidobacterium pseudocatenulatum CIP104168^T	9.4	8.7	8.7

–, not detected.

Table 2. Comparison of competitive PCR (cPCR) and culture for detection and quantification of bifidobacteria in faecal samples of 14 adults

Sample	$\log_{10}$ bifidobacteria g^{-1}	
	cPCR	CFU
1	9.4	9.5
2	5.4	4.5
3	10.1	8.3
4	8.0	7.1
5	9.6	8.7
6	9.5	4.6
7	9.9	8.2
8	5.8	3.1
9	9.7	4.8
10	9.8	6.4
11	5.8	5.5
12	9.6	8.3
13	9.2	9.2
14	10.7	8.7

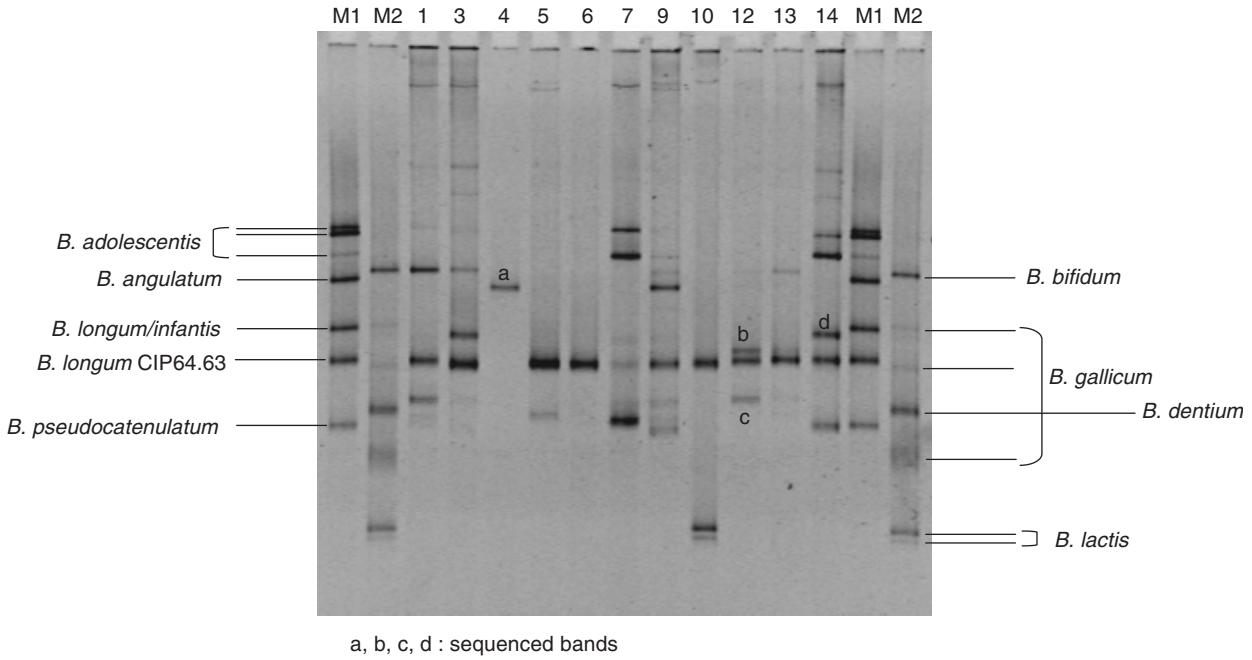

Fig. 2. Polymerase chain reaction-temperature gradient gel electrophoresis patterns of bifidobacteria from faecal samples of adults and from mixed PCR products from pure cultures (lanes M1 and M2).

in 10 out of 11 subjects. No sample produced a band co-migrating with the *B. longum*-type strain band. Co-migration with two of the three *B. adolescentis* bands occurred in two subjects (7 and 14) and low signals were also detected in subjects 1, 3 and 9. Co-migration with the *B. bifidum* fragment occurred in five subjects (1, 3, 9, 12 and 13). Subjects 5 and 7 displayed bands co-migrating with *B. dentium*, subjects 9 and 14 with *B. pseudocatenulatum*/*catenulatum*, and subject 10 with *B. lactis*. There was no band corresponding to *B. gallicum*, *B. angulatum* or *B. breve*. The latter usually gives a large band, which was not included in the marker as it migrated like one band of *B. gallicum*. Some bands migrated to different positions than those of the culture collection strains and could not be classified. Band a of sample 4, bands b and c of sample 12, and band d of sample 14 were sequenced (refer to Fig. 2) but only short sequences could be obtained. As previously determined, the sequences had a high similarity with many *Bifidobacterium* species and could not therefore be unambiguously identified at the species level (Satokari *et al*., 2001). The closest relative of band a (417 nucleotides) was *B. dentium* D86183 (99%). Bands b (198 nucleotides) and d (225 nucleotides) had the highest similarity with *B. adolescentis* clone nru-5 AF275882, which originated from one 16S rRNA gene copy of *B. adolescentis* type strain (99%), or its close phylogenetic relative *B. ruminantium* D86197 (b: 98% and d: 99%). The sequence from band c was too small to be discriminating. The distribution of species in faeces is shown in Table 3.

Discussion

The *Bifidobacterium* genus is usually quantified using culture methods. Molecular techniques provide an alternative approach for monitoring and quantifying intestinal

Table 3. Distribution of *Bifidobacterium* species in human faecal microflora of 11 adults

Species or combination	No. of positive samples (% of total)
Species	
B. adolescentis	5 (46)
B. angulatum	0 (0)
B. bifidum	5 (46)
B. breve	0 (0)
B. catenulatum group	2 (18)
B. lactis	1 (9)
B. longum	10 (91)
B. dentium	2 (18)
B. gallicum	0 (0)
Unclassified* a	2 (18)
Unclassified* b	1 (9)
Unclassified c	3 (27)
Unclassified* d	2 (18)
Combination of	
One species	2 (18)
Two species	3 (27)
Three to four species	5 (46)
Four to six species	1 (9)

*a, b, d: *B. adolescentis* or *B. ruminantium* or *B. dentium*, respectively.

microbiota, without the limitations of classical culture methods that involve anaerobic conditions, medium selectivity and immediate processing. In this study, a competitive PCR was developed to estimate the quantity of bifidobacteria in human faecal samples. The *Bifidobacterium* genus-specific primers used were previously designed as 16S rRNA-targeted oligonucleotide probes for specific FISH hybridizations (Langendijk *et al.*, 1995) and later used as primers in PCR reactions (Kok *et al.*, 1996; Satokari *et al.*, 2001).

Competitive PCR is simple and precise. The construction of a good competitor is a critical step in this method. Quantification is more accurate when the amplification efficiencies of the competitor and target are similar. Essential factors affecting the co-amplification efficiencies are competitor size and GC content, in addition to the sequence similarity between target DNA and competitor. The competitor we used was prepared by deleting a portion of the *Bifidobacterium* 16S rRNA gene so that the competitor and target sequences were very similar. Moreover, the size difference between target and competitor should allow for an easy separation on agarose gel. In our case, the PCR product obtained from the competitor had 467 bp and was 47 bp shorter than the PCR products obtained from *Bifidobacterium* strains. These products could be separated through a 2% agarose gel. Theoretically, competitive PCR should not be cycle dependent (Gilliland *et al.*, 1990). However, this is true only when the amplification efficiencies of target and competitor are identical. In this study, a PCR amplification of 35 cycles was performed to remain within the exponential phase. Since the coefficient of variation was only 0.5% for assays from 10 independent DNA extractions from the same stool, this method proved highly reproducible.

The number of rRNA genes previously determined in *Bifidobacterium* species (Bourget *et al.*, 1993; Mangin *et al.*, 1994, 1996; Schell *et al.*, 2002) was used to convert the assay values into cell equivalent values. The assay value was divided by the number of 16S rRNA gene copies present in the *Bifidobacterium* strain when pure cultures were used, or by a mean value of four with faecal samples. However, this figure could be 3.5 for infants because of the presence of *B. breve* species (only three copies of the 16S rRNA gene). This species was rarely recovered from adult faeces. The 16S rRNA gene copy number was recently evaluated using real-time PCR and Southern hybridization, and gave lower values than those above for species *B. longum* (two instead of four) and *B. breve* (two instead of three) (Candela *et al.*, 2004). In this last study, Southern hybridization using other restriction enzymes should be realized, as large-sized bands may contain more than one 16S rRNA gene.

Regarding competitive PCR targeting extracted DNA, the number of 16S rRNA genes and differential efficiency in DNA extraction may consequently influence any measurements made. For seven out of nine collection type strains, cell counts determined using cPCR were similar to those obtained using culture or had a difference lower or equal to $1 \log_{10}$. For one species, the cell counts using cPCR were $2 \log_{10}$ higher than determined with the culture method, whereas the Malassez counting chamber results were close to cPCR results (difference of $0.2 \log_{10}$). Such an underestimation may be explained by bifidobacterial cells lacking a colony-forming ability, oxygen sensitivity, occurrence of cell aggregation, and the selection bias of the medium, as was demonstrated for the *B. bifidum* type strain. Indeed this strain failed to grow under the culture conditions chosen for bifidobacterial selection from faecal samples, although this species is frequently present in adult faecal microflora (Mangin *et al.*, 1999; Matsuki *et al.*, 1999). For this strain, the difference between the cPCR and Malassez counting chamber results was equal to $0.8 \log_{10}$. The presence of cell aggregation may also explain this discrepancy. To avoid the growth of nontarget micro-organisms from faecal samples, a low pH of selective medium is essential. However, some strains may be more sensitive than others, but when the pH is increased to 5.5, lactobacilli or enterococci can also grow. Consequently, the enumeration of bifidobacteria in faecal samples often showed higher results with cPCR than with culture method at pH 5. Statistical analysis showed that the populations recovered by the two methods differed significantly ($P < 0.05$). Such results were in agreement with those in similar conditions using real-time PCR (Gueimonde *et al.*, 2004; Matsuki *et al.*, 2004). For example, the *B. bifidum* species was not recovered using culture from a faecal sample although it was estimated to represent 1.25×10^9 and 8×10^9 cells, using real-time PCR and FISH, respectively (Matsuki *et al.*, 2004). Similar results were also obtained from strains of *B. longum*, *B. catenulatum* or *B. angulatum* (Matsuki *et al.*, 2004).

FISH or dot-blot hybridizations quantified the predominant species present, i.e. over 10^8 cells g^{-1} (Franks *et al.*, 1998; Mangin *et al.*, 2002). Dot-blot hybridization measures the relative abundance of dominant groups (a percentage compared with total 16S rRNA) and not a cell number; whereas FISH counts cells but requires automatic counting for easier quantification. Furthermore, FISH is dependent of target accessibility, cell permeability and differences in the ribosome content of the cells. An improvement of this method was to combine FISH with flow cytometry which enabled large numbers of fixed faecal samples to be analysed (Rigottier-Gois *et al.*, 2003). Competitive PCR could detect target species when their concentrations were over 8×10^5 cells g^{-1} of faeces, and in contrast real-time PCR was able to detect, depending on the studies, 5×10^4 cells g^{-1} of faeces (Gueimonde *et al.*, 2004) or over 10^6 cells g^{-1} (Matsuki *et al.*, 2004). Thus, cPCR was equally sensitive.

To characterize the *Bifidobacterium* species present in faecal samples and those which could have difficulties in growing in our medium, a PCR-TTGE was carried out. No amplification was obtained for three samples which had very low bifidobacterial counts ($<8 \times 10^5$ cells g^{-1} wet wt faeces). Most species present in the samples co-migrated with strains from the culture collection but four bands migrated to different positions and could not be classified. Fragments which co-migrated with the *B. longum* CIP64.63 fragment were found in 10 of 11 faecal samples, suggesting that 91% of these 11 subjects were colonized with *B. longum*. This result was in agreement with other studies: *B. longum* (now associated with *B. infantis* (Sakata *et al*., 2002) was one of the most common species in adult microflora (Finegold *et al*., 1983; Mangin *et al*., 1999; Matsuki *et al*., 1999). However, no sample displayed a band co-migrating with the *B. longum*-type strain (as already observed in denaturing gradient gel electrophoresis (DGGE) profiles from five adult samples (Satokari *et al*., 2001)). In contrast to this earlier study, *B. adolescentis* and *B. bifidum*, two other dominant species in adult faeces (Finegold *et al*., 1983; Mangin *et al*., 1999; Matsuki *et al*., 1999), could be discriminated from each other and detected in at least five out of 11 subjects (46%). Satokari *et al*. (2001) showed that the five copies of the 16S rRNA gene from *B. adolescentis* type strain produced three bands in DGGE, the first corresponding to four copies, the second to the last copy and the third corresponding to an heteroduplex of previous fragments. In our study, only two bands co-migrated with the *B. adolescentis* bands. However, the 16S rRNA gene copy values known for the other *B. adolescentis* collection strains were four, not five. Whereas the *B. catenulatum* group was detected in 92% of 48 subjects in a preceding study (Matsuki *et al*., 1999), and in six out of six other subjects in another (Matsuki *et al*., 2004), this species was detected in only 2 of our 11 subjects. Depending on interindividual variability, fragments co-migrating with bands belonging to *B. dentium* and *B. lactis* were also detected. Our TTGE conditions also discriminated *B. breve* from *B. dentium*. *Bifidobacterium angulatum*, *B. breve* and *B. gallicum* were not detected in the samples analysed. These species represented low percentages in a previous study on bifidobacterial microflora from 48 adults (4.2%, 6% and 0%, respectively) (Matsuki *et al*., 1999). Some of the fragments of TTGE profiles from preterm infant faeces were sequenced (Magne *et al*., unpublished data): 20 bands co-migrating with the *B. longum* CIP64.63 fragment were excised from three different gels, cloned and sequenced (approximately 474 nucleotides). Its closest relatives were *B. longum* or *B. infantis* (99–100%). Moreover, six fragments co-migrating with the *B. breve* type strain and two fragments co-migrating with *B. pseudocatenulatum* were also cloned and sequenced (approximately 450 nucleotides). The closest relatives were *B. breve* (99–100%) and *B. pseudocatenulatum* (98–100%), respectively. Thus, band identification using TTGE profiles seems reliable. In our study, four unclassified bands were sequenced. The closest relatives of the first band were *B. dentium* (99%) and *B. adolescentis* (98%). Sequences of two other bands had the highest similarity with *B. adolescentis* (99%) or *B. ruminantium* (98–99%). In fact, these three species are phylogenetically very close (Miyake *et al*., 1998) and were already retrieved using excision and sequencing of unclassified bands in an earlier study on the bifidobacterial microflora (Satokari *et al*., 2001). An accurate identification cannot be obtained without cloning and sequencing the complete 16S rRNA gene copy, or at least a longer fragment. All these sequenced bands, often corresponding to *B. adolescentis*, should be entered into the TTGE *Bifidobacterium* Rf database as well as the culture collection strains, to allow for the identification of all bifidobacterial species.

The average number of species per sample was three to four, as previously found in other studies (Mangin et al., 1999; Matsuki *et al*., 1999). PCR-TTGE was also used to characterize strains which could not easily grow in Columbia–Beerens pH 5 medium. *Bifidobacterium bifidum*, a species especially sensitive to medium acidity, was recovered in several samples with an identical quantification from cPCR or culture methods. Therefore, strains from the same species could have differing sensitivities to pH. Three TTGE bands could correspond to species or strains which do not easily grow in our culture medium (band d, a band co-migrating with *B. pseudocatenulatum/catenulatum* group or a band co-migrating with *B. lactis* (samples 3, 9, 10, 14)). For sample 6, there was also a difference of 5 $\log_{10}$ between the cPCR and culture quantifications, but only one band co-migrating with the *B. longum* CIP64.63 fragment could be detected in the TTGE profile. Interestingly, several different strains from the same species can co-exist in the same microflora (Mangin *et al*., 1999). As anaerobic conditions were used when the bacteria were recovered from faecal samples, the culture bias may be explained by strain- and not species difficulties growing in Columbia–Beerens pH 5, as was previously demonstrated for *B. adolescentis* (Apajalahti *et al*., 2003). Indeed, the enumeration of *B. longum* cells in this study was independent of the analytical approach used (culture or molecular), whereas the recovery of *B. adolescentis* was highly dependent on method (Apajalahti *et al*., 2003). Variations could also be explained by the physiological conditions of the bacteria present in faeces. For instance, an individual suffering from constipation or from another intestinal disorder could harbour bacteria in different physiological states and thus of varying ability to grow on plates. Some species could be harmed during their passage along the colon and be recovered only with molecular methods. Moreover, *B. lactis* species originating from fermented yogurts and recovered in faeces could also be

stressed and thus lost on Columbia–Beerens agar pH 5. In addition to these hypotheses, another explanation could be that there is an overestimation in the cPCR results because of the detection of DNA from dead cells (Wollfs *et al*., 2005). Indeed, some strains from faecal samples may be dead but their DNA has not been completely degraded and is still amplifiable.

cPCR therefore provided an accurate and highly sensitive method for detecting and quantifying *Bifidobacterium*. The next step would be to develop specific primers to quantify each *Bifidobacterium* species as realized using real-time PCR (Matsuki *et al*., 2004). Nonetheless, the association with the PCR-TTGE analysis is already an appropriate approach for monitoring *Bifidobacterium* species over time, and is rapid to perform. An improved assessment of the gut microbiota composition in relation to the nutrition and health of a large number of samples will be an outcome of this method. Further studies applying a competitive PCR associated with the PCR-TTGE to other intestinal micro-organisms should be of great interest, notably the design of a competitor to quantify all bacteria.

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