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Quantification of Human Fecal *Bifidobacterium* Species by Use of Quantitative Real-Time PCR Analysis Targeting the *groEL* Gene

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Quantitative real-time PCR assays targeting the *groEL* gene for the specific enumeration of 12 human fecal *Bifidobacterium* species were developed. The housekeeping gene *groEL* (*HSP60* in eukaryotes) was used as a discriminative marker for the differentiation of *Bifidobacterium adolescentis*, *B. angulatum*, *B. animalis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, *B. longum*, *B. pseudocatenulatum*, *B. pseudolongum*, and *B. thermophilum*. The bifidobacterial chromosome contains a single copy of the *groEL* gene, allowing the determination of the cell number by quantification of the *groEL* copy number. Real-time PCR assays were validated by comparing fecal samples spiked with known numbers of a given *Bifidobacterium* species. Independent of the *Bifidobacterium* species tested, the proportion of *groEL* copies recovered from fecal samples spiked with 5 to 9 $\log_{10}$ cells/g feces was approximately 50%. The quantification limit was 5 to 6 $\log_{10}$ *groEL* copies/g feces. The interassay variability was less than 10%, and variability between different DNA extractions was less than 23%. The method developed was applied to fecal samples from healthy adults and full-term breast-fed infants. Bifidobacterial diversity in both adults and infants was low, with mostly ≤3 *Bifidobacterium* species and *B. longum* frequently detected. The predominant species in infant and adult fecal samples were *B. breve* and *B. adolescentis*, respectively. It was possible to distinguish *B. catenulatum* and *B. pseudocatenulatum*. We conclude that the *groEL* gene is a suitable molecular marker for the specific and accurate quantification of human fecal *Bifidobacterium* species by real-time PCR.

Microbial colonization of the initially sterile intestine starts with the exposure of the newborn to microbes from the mother and the environment. In this phase, *Bifidobacterium* species become predominant, representing 60 to 91% of fecal bacteria in breast-fed infants and 28 to 75% in formula-fed infants (17). At 2 years of age, when the complex gut microbiota is fully established, the proportion of fecal bifidobacteria decreases to 1 to 3% (41). Even lower levels or absence of bifidobacteria have been reported for the elderly (3, 15, 19, 38).

The establishment of the intestinal microbiota in early life is a critical phase that affects the maturation of the immune system. Numerous studies show that breast-fed infants have a lower incidence of gastrointestinal infections and atopic diseases than formula-fed infants (20, 46). It has been proposed that the health-promoting effect of breast milk is in part mediated by the intestinal microbiota, which is characterized by low diversity and a high proportion of bifidobacteria (11).

Common *Bifidobacterium* species of the human intestinal microbiota include *Bifidobacterium adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum*, and *B. pseudocatenulatum* (7). *B. gallicum* has rarely been detected (6, 7). Recently, *B. pseudolongum* and *B. thermophilum* were isolated from human adult and baby feces, respectively (44, 53). These species have previously been considered to be of animal origin (7). *B. animalis* subsp. *lactis* is commonly used as probiotic and thus may be detected in human feces.

Bifidobacteria are considered beneficial members of the gut microbiota. The assessment of their diversity and population size in the gastrointestinal tract is therefore important. However, only a few studies have enumerated bifidobacteria to the species level, and the majority of them used cultivation-based techniques (7). The latter are not only laborious but also hampered by partly fastidious growth conditions of bifidobacteria and by the lack of growth medium selectivity (2).

Since the advent of cultivation-independent methods, the 16S rRNA gene has been widely used as a valuable tool for bacterial identification (12). However, the resolution power of the 16S rRNA gene among closely related species is limited. Isolates displaying more than 97% 16S rRNA gene sequence identity are usually considered the same species. Since *Bifidobacterium* species reveal a relatively high 16S rRNA gene sequence identity (mean, 95% [31, 47]), more discriminative identification markers are needed.

Alternative target genes for the differentiation of *Bifidobacterium* species include housekeeping genes, such as *atpD* (49), *dnaK* (52), *groEL* (21, 27, 47, 50, 55), *groES* (50), *recA* (23, 51), *tal* (37), *tuf* (48, 51), and *xfp* (5, 47, 54). All these genes except *groES* were demonstrated to have similar or even higher discriminating power for bifidobacteria than the 16S rRNA gene. Unfortunately, only a limited number of sequences of these marker genes are available. The most sequences exist for the *groEL* gene. The Chaperonin Sequence Database (http://www.cpndb.ca; 18) currently contains more than 13,000 entries for prokaryotes, eukaryotes, and archaea, 121 of which belong to 27 *Bifidobacterium* species. The *groEL* gene encodes the chaperonin GroEL (synonyms are Cpn60, GroL, Hsp60, and MopA), which plays an essential role in the handling of cellular stress. For example, it promotes refolding of misfolded polypeptides. Southern blot experiments and sequence analysis of whole genomes have revealed that there is just one

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groEL copy per genome in bifidobacteria (14, 42, 50, 56). This facilitates quantitative analyses of bifidobacteria.

Here, we report the quantification of *Bifidobacterium* species in human feces with quantitative real-time PCR (qPCR) using *groEL* as a discriminative marker. Ninety-seven partial (~600-bp) or complete (~1,600-bp) *groEL* sequences of 12 *Bifidobacterium* species (Table 1) were used to design species-specific primers. They were applied to SYBR green I chemistry-based qPCR for quantification of *Bifidobacterium* species. The targeted species include members of the human gut microbiota and *B. animalis*, some strains of which are used as probiotics.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains listed in Table 1 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, and the American Type Culture Collection (ATCC), Manassas, VA. All bacteria were cultured anaerobically under a gas phase of N_2-CO_2 (80:20 [vol/vol]) in 15-ml Hungate tubes containing 10 ml ST medium, except for *Faecalibacterium prausnitzii*, which was grown on YCFA GSC medium (NCIMB Ltd., Aberdeen, Scotland) (9). The cultures were incubated overnight at 37°C and grown to a density of 9 to 10 $\log_{10}$ cells/ml.

The purity of bacterial cultures was checked by Gram staining and plating on Columbia agar with 5% sheep blood (bioMérieux, Vienna, Austria). The identities of the *Bifidobacterium* strains underlined in Table 1 (used to generate qPCR standards) were confirmed at the species level by sequencing either the 16S rRNA gene or the *groEL* gene (Eurofins MWG Operon, Ebersberg, Germany).

Subjects and fecal samples. Five healthy, full-term, exclusively breast-fed infants (4 males and 1 female) up to 3 months old with birth weights of 2,500 g to 4,500 g were included in the study. The infants had been born by caesarean section, except INF-4, who had been born by vaginal delivery. Infants undergoing antibiotic therapy during the first 14 days of life were excluded. Newborns were enrolled in a clinical study whose protocol was reviewed and approved by an independent ethics committee, and informed written consent was obtained from a legal representative(s). Fecal samples from infants were collected within 30 min after defecation and were kept in an anaerobic jar with a reduced atmosphere created by AnaeroGen (Oxoid, United Kingdom) at 4°C for up to 8 h until they were stored at −20°C. Ten healthy adults from our institute staff (5 males and 5 females), 20 to 40 years old, voluntarily provided fecal samples. They did not have any gastrointestinal disorders during sample collection and did not undergo antibiotic treatment in the 6 months prior to sampling. The fecal samples from the adults were stored at −20°C within 2 h after defecation.

Extraction of DNA from bacterial cultures. Genomic DNA of *Bifidobacterium* strains was extracted from 1-ml overnight cultures with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for Gram-positive bacteria. Since genomic DNA of *B. longum* subsp. *longum* ATCC 15707^T was used as a standard for quantification of total bifidobacteria by qPCR, high yields of DNA were required and were obtained with the RTP Invitek Bacteria Mini Kit (Invitrogen, Darmstadt, Germany). The manufacturer's protocol for Gram-positive bacteria was followed with some modifications. After enzymatic cell lysis, an additional mechanical lysis was performed in a 2-ml tube containing 0.75 g of sterile zirconium/silica beads (0.1 mm in diameter; Roth, Karlsruhe, Germany) by running the Fastprep FP120 Instrument (Thermo Electron Corp., Waltham, MA) at speed level 4 for 8 min. Genomic DNA of non-*Bifidobacterium* strains was extracted with the RTP Invitek Bacteria Mini Kit (Invitrogen, Darmstadt, Germany) following the manufacturer's instructions.

Extraction of bacterial DNA from fecal samples. Bacterial DNA was extracted from frozen fecal material using the QIAamp DNA stool kit (Qiagen, Hilden, Germany) with a modified protocol for cell lysis. After homogenization of 220 mg feces with 1.2 ml lysis buffer from the kit by vortexing for 2 min in a 2-ml tube containing 0.75 g of sterile zirconium/silica beads (0.1 mm in diameter; Roth, Karlsruhe, Germany), the final suspension was incubated at 95°C for 15 min with continuous shaking (1,400 min^{-1}; Thermomixer 5436; Eppendorf, Hamburg, Germany). The sample was allowed to cool on ice for 2 min. Cells were mechanically lysed by Fastprep treatment for 8 min and 15 s as described above. After cooling on ice for 2 min, coarse particles, cell debris, and the zirconium/silica beads were spun down by centrifugation (20,000 × *g*; 4°C; 1 min), and the supernatant was transferred to a 2-ml tube. The pellet was mixed with 350 μl lysis buffer from the kit, vortexed for 1 min, and incubated at 95°C for 5 min with continuous shaking as described above. After centrifugation at 20,000 × *g* and 4°C for 1 min, the supernatants were combined. DNA-damaging substances and PCR inhibitors present in the stool samples were removed by their adsorption to the InhibitEX matrix provided in the kit. The InhibitEX matrix was separated by centrifugation at 20,000 × *g* for 6 min, and the supernatant was collected and filled up to 1 ml with sterile phosphate-buffered saline (pH 7). DNA was purified with the QIAcube (Qiagen, Hilden, Germany) and eluted from the silica-based membrane with 200 μl ultrapure water.

***groEL* sequence analysis and design of species-specific primers.** *groEL* sequences belonging to target *Bifidobacterium* species (Table 2) and available in GenBank release 185.0 (4) were subjected to a multiple alignment with the program ClustalW2 version 2.1 (24) provided by the European Bioinformatics Institute (http://www.ebi.ac.uk). A region of approximately 600 bp located at positions ~250 to 840 of the complete *groEL* gene of ca. 1,600 bp was selected to identify discriminative target sites for the species-specific detection of bifidobacteria. Primer pairs for 12 *Bifidobacterium* species (Table 3) were manually designed on the basis of 97 partial and complete *groEL* sequences (Table 2).

Quantitative real-time PCR. (i) Standards. For quantification of the investigated *Bifidobacterium* species, represented by the strains underlined in Table 1, PCR products containing the target sequence were used as qPCR standards. Therefore, part of the genomic *groEL* gene was amplified with primers binding to a sequence at least 17 bp up- and downstream of the species-specific primer-binding sites to prevent their degradation during storage. The 50-μl PCR mixture consisted of 1× TopTaq PCR buffer (Qiagen, Hilden, Germany), 200 μM (250 μM for *B. pseudocatenulatum*) of each deoxynucleoside triphosphate (dNTP) (Invitek, Berlin, Germany), 200 nM each forward and reverse primer (Table 4), 1.25 U of TopTaq DNA polymerase (Qiagen, Hilden, Germany), and 1 μl genomic DNA (30 to 110 ng) of one of the representative strains (Table 1). The reactions were performed in a thermal cycler (Thermo Hybaid Multi-Block System, Ulm, Germany) under the conditions given in Table 4. The amplification products were purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The concentration of the amplified DNA (concentration$_{amplicon}$ [g/liter]) was determined spectrophotometrically at 260 nm (NanoDrop; Peqlab, Erlangen, Germany). The number of PCR products (or partial *groEL* gene copies) per volume was calculated as follows: (i) concentration$_{amplicon}$ (g/liter)/molecular mass$_{amplicon}$ (g/mol) = concentration$_{amplicon}$ (mol/liter); (ii) concentration$_{amplicon}$ (mol/liter) × N_A = concentration$_{amplicon}$ (molecules/liter), where N_A is the Avogadro constant (6.022×10^{23} molecules/mol). The molecular mass$_{amplicon}$ values are listed in Table 4.

The identities of the generated qPCR standards were confirmed by sequencing (Eurofins MWG Operon, Ebersberg, Germany). For the quantification of total bifidobacteria, genomic DNA from *B. longum* subsp. *longum* ATCC 15707^T was used as the qPCR standard. After extracting genomic DNA, its concentration was determined spectrophotometrically as described above. Assuming a mean genome size for *Bifidobacterium* of 2.25 Mb (GenBank [4]), the average mass of a single *Bifidobacterium* genome would be 2.47 fg: [2.25 Mb × average molecular mass$_{base\ pair}$ (660 g/mol)]/N_A = 2.47 fg/molecule. The number of bifidobacterial genomes per volume was calculated as follows: concentra-

TABLE 1 Bacterial strains used for testing the specificity of the developed primers by qPCR

Bifidobacterium strain(s)[b]	qPCR results with primers[a]:											
	B_ado-f/ B_ado-r	B_ang-f/ B_ang-r	B_ani-f/ B_ani-r	B_bif-f/ B_bif-r	B_bre-f/ B_bre-r	B_cat-f/ B_cat-r	B_den-f/ B_den-r	B_gal-f/ B_gal-r	B_lon-f/ B_lon-r	B_pcat-f/ B_pcat-r	B_plon-f/ B_plon-r	B_the-f/ B_the-r
B. adolescentis DSM 20083^T, DSM 20086	+	−	−	−	−	−	−	−	−	−	−	−
B. angulatum ATCC 27535^T, DSM 20225	−	+	−	−	−	−	−	−	−	−	−	−
B. animalis DSM 20105	−	−	+	−	−	−	−	−	−	−	−	−
B. animalis subsp. *animalis* DSM 20104^T	−	−	+	−	−	−	−	−	−	−	−	−
B. animalis subsp. *lactis* DSM 10140^T	−	−	+	−	−	−	−	−	−	−	−	−
B. bifidum DSM 20456^T, DSM 20215, DSM 20239	−	−	−	+	−	−	−	−	−	−	−	−
B. breve DSM 20213^T, DSM 20091	−	−	−	−[c]	+	−	−	−	−	−	−	−
B. catenulatum ATCC 27539^T, DSM 20224	−	−	−	−	−	+	−	−	−	−	−	−
B. dentium ATCC 27534^T, ATCC 27678, DSM 20084	−	−	−	−	−	−	+	−	−	−	−	−
B. gallicum DSM 20093^T	−	−	−	−	−	−	−	+	−	−	−	−
B. longum subsp. *longum* ATCC 15707^T	−	−	−	−	−	−	−	−	+	−	−	−
B. longum subsp. *suis* DSM 20211^T	−	−	−	−	−	−	−	−	+	−	−	−
B. pseudocatenulatum ATCC 27919^T, DSM 20439	−	−	−	−	−	−	−	−	−	+	−	−
B. pseudolongum subsp. *pseudolongum* ATCC 25526^T, DSM 20094	−	−	−	−	−	−	−	−	−	−	+	−
B. pseudolongum subsp. *globosum* DSM 20092^T	−	−	−	−	−	−	−	−	−	−	+	−
B. thermophilum DSM 20210^T, DSM 20209, DSM 20212	−	−	−	−	−	−	−	−	−	−	−	+

[a] In addition to the bifidobacterial strains listed, negative qPCR results were obtained for the following bacterial strains: *B. producta* DSM 2950^T, *B. thetaiotaomicron* DSM 2079^T, *B. vulgatus* DSM 1447^T, *Clostridium histolyticum* DSM 2158^T, *Escherichia coli* DSM 30083^T, *Faecalbacterium prausnitzii* DSM 17677, *Lactobacillus acidophilus* DSM 20079^T, *Lactobacillus gasseri* DSM 20243^T, *Ruminococcus albus* DSM 20455^T, and *Ruminococcus gauvreauii* DSM 19829^T. +, specific PCR product; −, no PCR product.

[b] The underlined strains served as representative strains for the respective species and were used to generate qPCR standards.

[c] In the case of *B. breve* DSM 20091, a nonspecific PCR product was observed.

TABLE 2 *groEL* sequences used for the design of *Bifidobacterium* species-specific primers

Bifidobacterium species	Strain	GenBank accession no.	Size (bp)
B. adolescentis	JCM 1275^T	AF210319	1,749[a]
	ATCC 15703^T	AP009256	1,617[a]
B. angulatum	JCM 7096^T	AF240568	590[b]
B. animalis	B83	AY166539	590[b]
	D1	AY166540	590[b]
	III-1	AY166541	590[b]
	III-6	AY166542	590[b]
subsp. *animalis*	JCM 1190^T	AY004273	590[b]
	ATCC 25527^T	AY488178	1,155[b]
	TEEV 1/10	HQ851043	587[b]
	TEEV 3/10	HQ851044	598[b]
	TEEV 1/11	HQ851047	586[b]
	TEEV 4/9 AG	HQ851048	608[b]
subsp. *lactis*	HN019	ABOT01000006	1,614[a]
	9952	AF286735	590[b]
	DSM 10140^T	AY004282	1,614[a]
	9950	AY004287	590[b]
	JB-1	AY166543	590[b]
	NCC 363	AY488176	1,155[b]
	NCC 402	AY488177	1,155[b]
	DSM 27674	AY488179	1,155[b]
	DSM 10140^T	AY488180	1,155[b]
	ATCC 27536	AY488181	1,155[b]
	NCC 239	AY488182	1,155[b]
	ATCC 27672	AY488183	1,155[b]
	LMG 18906	AY586539	1,614[a]
	AD011	CP001213	1,614[a]
	Bl-04 ATCC SD5219	CP001515	1,614[a]
	DSM 10140^T	CP001606	1,614[a]
	BB-12	CP001853	1,614[a]
	V9	CP001892	1,614[a]
	CNCM I-2494	CP002915	1,614[a]
	BLC1	CP003039	1,614[a]
B. bifidum	JCM 1255^T	AY004280	590[b]
	PRL2010	CP001840	1,626[a]
	S17	CP002220	1,626[a]
B. breve	DSM 20213^T	ACCG02000012	1626[a]
	DSM 20213^T	AF240566	584[b]
B. catenulatum	JCM 1194^T	AY004272	590[b]
	JCM 7130	AY166565	590[b]
B. dentium	ATCC 27679	AEEQ01000009	1,617[a]
	JCVIHMP022	AEHJ01000021	1,617[a]
	JCM 1195^T	AF240572	590[b]
B. gallicum	DSM 20093^T	ABXB03000002	1,611[a]
	JCM 8224^T	AF240575	592[b]
B. longum	N10	FJ577573	552[b]
	N103	FJ577576	552[b]
	N81	FJ577625	552[b]
	N86	FJ577629	552[b]
	N88	FJ577630	564[b]
subsp. *infantis*	JCM 1210	AF240576	591[b]
	JCM 1222^T	AF240577	591[b]
	ATCC 15697^T	AP010889	1,626[a]
	157F	AP010890	1,626[a]
	9901	AY004286	591[b]
	bb52	AY004288	591[b]
	6w-50	AY166564	590[b]
	D6-9-6	AY166566	590[b]
	D30-3-3	AY166567	590[b]
	D30-3-10	AY166568	590[b]
	D30-3-11	AY166569	590[b]
	L2	AY166570	590[b]
subsp. *longum*	ATCC 15697^T	CP001095	1,626[a]
	ATCC 55813	ACHI01000023	1,626[a]
	NCC 2705	AE014295	1,626[a]
	JCM 1217^T	AF240578	590[b]
	L4	AY166571	590[b]
	TJ	AY166572	593[b]
	JCM 7052	AY166573	590[b]
	JCM 7053	AY166574	590[b]
	ATCC 15707^T	AY835622	552[b]
	DJO10A	CP000605	1,626[a]
	JDM 301	CP002010	1,626[a]
	BBMN 68	CP002286	1,626[a]
	KACC 91563	CP002794	1,626[a]
	F8	FP929034	1,626[a]
	R0175	HM009034	1,626[a]
subsp. *suis*	JCM 1269^T	AY013248	591[b]
	JCM 7139	AY166575	590[b]
	TEEV 4/9	HQ851041	590[b]
B. pseudocatenulatum	DSM 20438^T	AY004274	589[b]
	JCM 7040	AY166552	590[b]
	T1-3-15	AY166553	590[b]
	T2-3-4	AY166554	590[b]
	Z2-3	AY166555	590[b]
B. pseudolongum subsp. *globosum*	JCM 5820^T	AF286736	591[b]
	1-23-3	AY166544	590[b]
	1-25-3	AY166545	590[b]
	02-2	AY166546	590[b]
	7#-3-7	AY166547	590[b]
	7#-10-11	AY166548	590[b]
	09-25-2	AY166549	590[b]
	Fb9	AY166550	590[b]
	MU8	AY166551	590[b]
subsp. *pseudolongum*	JCM 1205^T	AF240573	591[b]
B. thermophilum	JCM 1207^T	AF240567	591[b]
	RBL67	DQ340558	590[b]

[a] Complete coding sequence.
[b] Partial coding sequence.

tion$_{\text{genomic DNA}}$ (g/liter)/(2.47 fg/molecule) = concentration$_{\text{genomic DNA}}$ (molecules/liter). The standards were stored in 10 mM Tris-HCl (pH 8) at −20°C and subjected to 10-fold serial dilutions in ultrapure water prior to each measurement.

(ii) Conditions. qPCR based on the fluorescent dye SYBR green I was performed with 7500 Fast Real-Time PCR Systems using the 7500 software v2.0.5 (Life Technologies, Darmstadt, Germany). Reactions were measured in triplicate. Each reaction mixture of 25 μl contained 1× QuantiFast SYBR green PCR Master Mix (HotStarTaq *Plus* DNA polymerase, QuantiFast SYBR green PCR buffer, dNTP mixture, and the ROX

TABLE 3 *Bifidobacterium* species-specific primers based on the *groEL* gene[a]

Target species	Primer[b]	Sequence (5′ to 3′)[c]	Primer concn (nM)	Annealing temp (°C)	Amplicon	
					Size (bp)	Melting temp (°C)
B. adolescentis	B_ado-f	CTCCGCCGCTGATCCGGAAGTCG	300	75	268	88.0
	B_ado-r	AACCAACTCGGCGATGTGGACGACA				
B. angulatum	B_ang-f	CTGTCCTCCCAGCAGGACGTGGTC	300	80	97	85.5
	B_ang-r	GCGCTTCGCCGTCAACGTCTTCGG				
B. animalis	B_ani-f	CACCAATGCGGAAGACCAG	250	64	184	87.5
	B_ani-r	GTTGTTGAGAATCAGCGTGG				
B. bifidum	B_bif-f	CTCCGCAGCCGACCCCGAGGTT	300	64	233	88.5
	B_bif-r	TGGAAACCTTGCCGGAGGTCAGG				
B. breve	B_bre-f	GCTCGTCGTTGCCGCCAAGGACGTT	300	72	272	88.0
	B_bre-r	ACAGAATGTACGGATCCTCGAGCACG				
B. catenulatum	B_cat-f	GGCTATCGTCAAGGAGCTCA	300	64	188	87.0
	B_cat-r	AGTCCAGATCCAAACCGAAAC				
B. dentium	B_den-f	GGCCCAGTCTTTGGTGCATGAAGGCC	300	75	364	89.5
	B_den-r	GTCTTCGAGCACCGCGGTCTGGTCC				
B. gallicum	B_gal-f	AGCTCGTCAAGTCCGCCAAGC	300	68	188	87.5
	B_gal-r	CATACCTTCGGTGAACTCGAGG				
B. longum	B_lon-f	CGGCGTYGTGACCGTTGAAGAC	250	70	259	88.0
	B_lon-r	TGYTTCGCCRTCGACGTCCTCA				
B. pseudocatenulatum	B_pcat-f	AGCCATCGTCAAGGAGCTTATCGCAG	250	68	325	87.5
	B_pcat-r	CACGACGTCCTGCTGAGAGCTCAC				
B. pseudolongum	B_plon-f	CRATYGTCAAGGAACTYGTGGCCT	300	66	312	89.0
	B_plon-r	GCTGCGAMGAKACCTTGCCGCT				
B. thermophilum	B_the-f	ACTGGTCGCTTCCGCCAAGGATG	300	66	326	88.0
	B_the-r	CCARGTCAGCMAGGTGRACGATG				

[a] The target sites are located at positions ~250 to 840 of the corresponding nucleotide sequence.
[b] f, Forward primer; r, reverse primer.
[c] IUPAC ambiguity codes: M (A or C), K (G or T), R (A or G), Y (C or T).

passive reference dye; Qiagen, Hilden, Germany), 250 to 300 nM each forward and reverse primer (Table 3), and 1 μl template DNA. *Bifidobacterium* species were quantified with the following temperature program (Table 3): 95°C for 5 min, 40 cycles at 94°C for 15 s, 64 to 80°C for 15 s, 72°C for 15 s, and 83°C for 15 s. The fluorescence of SYBR green I was measured after each amplification cycle and during the last step at 83°C to get rid of nonspecific fluorescence signals caused by primer dimers or nonspecific minor products (36). Total bifidobacteria were quantified using the 16S rRNA gene-targeting primer pair g-Bifid-F (5′-CTC CTG GAA ACG GGT GG-3′)/g-Bifid-R (5′-GGT GTT CTT CCC GAT ATC TAC A-3′) with the corresponding temperature program as described elsewhere (29). Postamplification melting-curve analysis was performed by slowly increasing the temperature from 68°C to 95°C (increments of 1%, holding for 10 s), while fluorescence was measured continuously. Thus, the specific PCR product was verified based on the specific melting temperature (Table 3) and distinguished from possible nonspecific products. Background fluorescence was calculated during the initial stage of the qPCR corresponding to cycles 3 to 10. The threshold was set within the exponential phase of the amplification plot at 0.01 (single *Bifidobacterium* species) or 0.05 (total bifidobacteria) relative fluorescence. Threshold cycles (C_T) (the PCR cycle numbers at which the fluorescence exceeds the threshold above the calculated background) of less than 11 and more than 33 were excluded from the analysis. The number of *groEL* copies or bifidobacterial genomes in a fecal sample was calculated using a standard curve, which was generated by plotting the C_T values obtained for 10-fold serial dilutions of the qPCR standards as a linear function of the base 10 logarithm of known concentrations. All primers used in this study were commercially synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Spiking experiments. Fresh fecal samples were collected from two healthy human adults initially tested for the absence of the investigated *Bifidobacterium* species by the developed qPCR assays. Aliquots of 220 mg fecal material were spiked with 10-fold serial dilutions of known amounts of the representative strain of each *Bifidobacterium* species (Table 1) ranging from 4 to 9 $\log_{10}$ cells. The concentration of bacterial cells was estimated microscopically by using the Thoma-Zeiss counting chamber (chamber depth, 0.01 mm; small square, 0.0025 mm^2). Microscopic counts were determined at least in duplicate. Bacterial DNA from spiked feces was extracted as described above.

Statistics. Data analysis of the comparison of cell numbers determined with qPCR assays and the Thoma-Zeiss counting chamber was done with GraphPad Prism version 5.0 (San Diego, CA). Significance was tested by one-way analysis of variance (ANOVA) with Bonferroni's posttests. A P value of <0.05 was considered significant.

RESULTS

Specificities of primer pairs. The properties and specificity of the primer pair for each *Bifidobacterium* species were checked *in silico* with OligoAnalyzer 3.1, provided by Integrated DNA Technologies (35), and the Basic Local Alignment Tool (1), provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Each primer pair perfectly matched the *groEL* sequence of the targeted *Bifidobacterium* species but had several mismatches with those of the nontargeted *Bifidobacterium* species investigated in the study (Table 1). In the case of *B. thermophilum*, the designed primer pairs also matched the *groEL* of *B. thermacidophilum*, which, however, is not found in the human intestinal tract (7) and was therefore disregarded. The specificity of each primer pair was experimentally tested by performing qPCR on genomic DNA extracted from 28 *Bifidobacterium* strains and 10

TABLE 4 Primers used to generate PCR products containing *Bifidobacterium* species-specific primer-binding sites (qPCR standards)[a]

Target species	Primer[b]	Sequence (5′ to 3′)	PCR conditions	Amplicon	
				Size (bp)	Molecular mass (g/mol)[c]
B. adolescentis	B_ado-std-f	GATCAGATCGCTGCCACCGCAAC	94°C, 3 min; 30 times (94°C, 30 s; 69°C, 30 s; 72°C, 30 s); 72°C, 10 min	320	197,799.00
	B_ado-std-r	ATCAGCAGCGGCTTGCCGGTCTT			
B. angulatum	B_ang-std-f	AGTGCTCGAAGACCCGTACATCCT	94°C, 3 min; 30 times (94°C, 30 s; 65°C, 30 s; 72°C, 30 s); 72°C, 10 min	164	101,387.60
	B_ang-std-r	GGATGTTGTTCAGGATCAGGGTCG			
B. animalis	B_ani-std-f	GTCACCGTCGAAGACAACAACC	94°C, 3 min; 30 times (94°C, 30 s, 65°C, 30 s; 72°C, 30 s); 72°C, 10 min	299	184,812.60
	B_ani-std-r	CAGGACTTGAAGGTGCCACGG			
B. bifidum	B_bif-std-f	CAAGGACGTGGAGACCAAG	94°C, 3 min; 30 times (94°C, 30 s; 65°C, 30 s; 72°C, 30 s); 72°C, 10 min	391	241,687.40
	B_bif-std-r	CTTGTTCAGGATGAGGGTCG			
B. breve	B_bre-std-f	CCACCGAGGTCATCGTC	94°C, 3 min; 30 times (94°C, 20 s; 58°C, 20 s; 72°C, 20 s); 72°C, 10 min	322	199,032.80
	B_bre-std-r	TGCTGGGAGGAGACCTTG			
B. catenulatum	B_cat-std-f	GTCGTGGTATCGAGAAG	94°C, 3 min; 30 times (94°C, 30 s; 47°C, 30 s; 72°C, 30 s); 72°C, 10 min	244	150,819.60
	B_cat-std-r	TAGCCCTTGTCGAAACG			
B. dentium	B_den-std-f	CCACTACCGCAACTGTGCTG	94°C, 3 min; 30 times (94°C, 50 s; 62°C, 50 s; 72°C, 50 s); 72°C, 10 min	400	247,244.00
	B_den-std-r	GTCAGGAGGATGTACGGGTC			
B. gallicum	B_gal-std-f	TGAGAAGGCTTCCGACG	94°C, 3 min; 30 times (94°C, 30 s; 57°C, 30 s; 72°C, 30 s); 72°C, 10 min	242	149,590.80
	B_gal-std-r	GGGAGATGTAGCCCTTG			
B. longum	B_lon-std-f	CTGAGGCTCTGGACAAGGTCG	94°C, 3 min; 30 times (94°C, 30 s; 66°C, 30 s; 72°C, 30 s); 72°C, 10 min	323	199,651.20
	B_lon-std-r	GGTGCCACGGATGTTGTTCAGG			
B. pseudocatenulatum	B_pcat-std-f	CGTCGTGGTATCGAGAAGGCTTCCG	94°C, 3 min; 30 times (94°C, 50 s; 64°C, 50 s; 72°C, 50 s); 72°C, 10 min	434	268,244.60
	B_pcat-std-r	AAGGTCGGCAGAGCCTCGCCAT			
B. pseudolongum	B_plon-std-f	CCTCAAGAACGTTGTGGC	94°C, 3 min; 30 times (94°C, 30 s; 49°C, 30 s; 72°C, 30 s); 72°C, 10 min	420	259,607.00
	B_plon-std-r	CCGGTCTTCATCACGAG			
B. thermophilum	B_the-std-f	GAAGGCCTGAAGAACGTGGTCG	94°C, 3 min; 30 times (94°C, 40 s; 65°C, 40 s; 72°C, 40 s); 72°C, 10 min	449	277,522.60
	B_the-std-r	TCAGCCACGATCAGCAGCGGA			

[a] The target sites are located at least 17 bp up- and downstream of the *Bifidobacterium* species-specific primer-binding sites to prevent their degradation during storage.
[b] f, forward primer; r, reverse primer.
[c] Refers to the double-stranded amplicon.

non-*Bifidobacterium* strains representing dominant intestinal bacterial species (Table 1). At the appropriate annealing temperature and the chosen primer concentrations, the primer pairs were specific for their respective target species and yielded an amplicon of the expected size (data not shown). Application of the primer pairs to nontargeted bacterial species tested in this study never led to an amplification product. This conclusion was supported by the following observations: C_T values obtained for nontarget organisms were close to the C_T values of the no-template control, and there was no peak at the melting temperature of the specific amplicon as determined by postamplification melting-curve analysis. However, application of primer pair B_bif-f/B_bif-r to genomic DNA of *B. breve* DSM 20091 led to a PCR product that, based on the melting-curve analysis, could clearly be identified as nonspecific.

***Bifidobacterium* species qPCR assays.** For all *Bifidobacterium* species represented by the strains underlined in Table 1, 10-fold serial dilutions of qPCR standards (PCR products containing the respective partial *groEL* sequence) were used to generate a standard curve. The standard curves for all *Bifidobacterium* species were highly linear ($R^2 > 0.99$), at least in the range of 10 to 100,000 *groEL* copies per PCR, corresponding to 6 to 10 $\log_{10}$ *groEL* copies/g feces, except for *B. angulatum* and *B. pseudolongum*, for which linearity started at 100 *groEL* copies per PCR. The PCR efficiency (E) was calculated by the slope of the standard curve as follows: $E = 10^{(-1/\text{slope})} - 1$ (e.g., $E = 1$, or 100%). For *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, and *B. pseudocatenulatum*, E was at least 90%. For *B. angulatum*, *B. longum*, *B. pseudolongum*, and *B. thermophilum*, E was somewhat lower but at least 85%.

Reproducibility. The reproducibility of the qPCR assays was determined for DNA extracts from three adult fecal samples spiked with different cell numbers of the representative strain of each *Bifidobacterium* species (Table 1) in two replicate runs. The resulting coefficients of variation (CV) based on the *groEL* copy number were less than 10%, indicating that the developed qPCR assays are precise. The reproducibility between different DNA extracts was determined by extracting DNA three times from adult or infant fecal samples and quantifying *B. breve*, *B. longum*, and total bifidobacteria. The CV based on *groEL* copies varied less than 23%, demonstrating that the DNA extraction method was highly reproducible.

Quantification limit. To determine the lowest *groEL* copy number in feces detectable with the developed qPCR assays (quantification limit), adult fecal samples were spiked with the representative strain of each of the *Bifidobacterium* species under study. The quantification limit in feces was 1 to 10 *groEL* copies per PCR, corresponding to 5 to 6 $\log_{10}$ *groEL* copies/g feces (Table 5).

Comparison of cell numbers determined with qPCR assays and the Thoma-Zeiss counting chamber. Fecal samples were spiked with cells of six *Bifidobacterium* species (*B. adolescentis*, *B. angulatum*, *B. animalis*, *B. breve*, *B. pseudolongum*, and *B. thermophilum*). The number of *groEL* genes measured by the developed

TABLE 5 Quantification of *Bifidobacterium* species in feces of human adults and infants

Bifidobacterium species	Log_{10} *groEL* copies/g feces in sample[a]: Adults ($n = 10$)										Infants ($n = 5$)					
	AD-1	AD-2	AD-3	AD-4	AD-5	AD-6	AD-7	AD-8	AD-9	AD-10	INF-1 (wk 1)	INF-2 (wk 1)	INF-3 (wk 6)	INF-4 (mo 3)	INF-5 wk 6	INF-5 mo 3
B. adolescentis	9.7	9.7	−	9.9	9.8	−	8.9	9.3	9.3	−	−	−	−	−	−	−
B. angulatum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. animalis	−	7.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. bifidum	8.2	8.6	−	9.1	−	−	−	−	−	8.6	−	−	9.7	−	−	−
B. breve	−	−	−	−	−	−	−	−	−	−	−	6.2	10.4	10.1	−	8.3
B. catenulatum	−	8.1	−	−	8.1	−	−	−	−	−	−	−	−	−	−	−
B. dentium	−	6.4	6.7	−	7.1	−	−	−	−	−	−	−	8.8	−	−	−
B. gallicum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. longum	7.2	9.1	−	8.9	9.2	9.3	6.6	9.1	−	9.0	−	6.4	−	10.1	−	10.0
B. pseudocatenulatum	−	−	9.3	−	8.9	−	−	8.7	−	−	−	−	−	−	−	−
B. pseudolongum	−	−	8.0	−	−	−	−	−	−	−	−	−	−	−	−	−
B. thermophilum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sum of species	9.8	9.9	9.3	10.0	10.0	9.3	8.9	9.6	9.3	9.1	−	6.6	10.5	10.4	−	10.1
Total bifidobacteria[b] (log_{10} bifidobacterial genomes/g feces)	10.3	10.0	10.1	10.5	10.7	10.0	9.5	10.3	9.8	9.8	−	7.6	11.1	11.0	8.1	11.1

[a] Infants were around 1 week (wk 1), 6 weeks (wk 6), and 3 months (mo 3) old; adults were 20 to 40 years old. −, not quantified (the quantification limits were 5 log_{10} *groEL* copies/g feces for *B. adolescentis*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum*, *B. pseudocatenulatum*, and *B. thermophilum* and 6 log_{10} *groEL* copies/g feces for *B. angulatum*, *B. animalis*, *B. bifidum*, *B. gallicum*, and *B. pseudolongum*).

[b] The total number of bifidobacteria was determined with the 16S rRNA gene-targeting primer pair g-Bifid-F/R by Matsuki et al. (29). The quantification limit was reported to be 6 log_{10} cells/g feces.

qPCR assays was compared with the number of bifidobacteria added to the fecal sample and determined with the Thoma-Zeiss counting chamber prior to spiking. Independent of the *Bifidobacterium* species, the proportion of *groEL* copies recovered from fecal samples spiked with 5 to 9 log_{10} cells/g was 45 to 48% (Fig. 1A and B). These results indicated that the cell numbers of a given *Bifidobacterium* species present in a fecal sample and determined with the developed qPCR assays were comparable to those obtained by microscopy, suggesting sufficiently effective cell lysis. The recovery of *groEL* copies was considered in all calculations.

Presence of PCR inhibitors. To verify that PCR inhibitors present in feces had been completely removed during DNA extraction, *B. longum* was quantified with the developed qPCR assay using DNA obtained from four fecal samples (INF-4, INF-5 month 3, AD-5, and AD-6) at dilutions of 0 to −3 log_{10}. Since the cell numbers (*groEL* copies/g feces) calculated for a given sample were nearly the same for all four dilutions (10.09 ± 0.03 in INF-4, 9.86 ± 0.12 in INF-5 month 3, 9.17 ± 0.02 in AD-5, and 9.28 ± 0.05 in AD-6 [mean ± standard deviation {SD} log_{10}; $n = 4$]), it was concluded that PCR inhibitors had been completely removed.

Quantification of *Bifidobacterium* species in fecal samples. The designed and validated primers were subsequently applied to

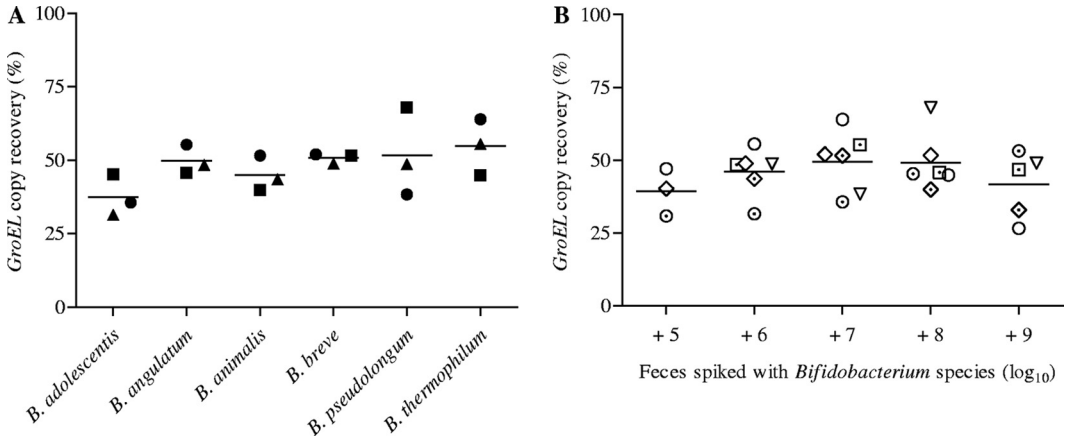

FIG 1 Comparison of cell numbers determined with qPCR assays and the Thoma-Zeiss counting chamber using human adult feces spiked with *Bifidobacterium* species cells. (A) Spiking was performed with 6 log_{10} (▲), 7 log_{10} (●), and 8 log_{10} (■) cells of six *Bifidobacterium* species. (B) Spiking was performed with 5 log_{10} ($n = 3$), 6 log_{10} ($n = 6$), 7 log_{10} ($n = 6$), 8 log_{10} ($n = 6$), and 9 log_{10} ($n = 5$) cells of *B. adolescentis* (⊙), *B. angulatum* (⊡), *B. animalis* (◈), *B. breve* (◇), *B. pseudolongum* (▽), and *B. thermophilum* (○). The values are means. Significance was estimated by one-way ANOVA with Bonferroni's posttests. Differences of means were not significant. *GroEL* copy recovery was 48% ± 6% (A) and 45% ± 6% (B) (means ± SD).

fecal samples from five healthy, full-term, breast-fed infants and 10 healthy adults (Table 5). Fecal samples from breast-fed infants contained no more than three *Bifidobacterium* species. *B. breve* was not detected in adult feces but was found in 4/6 infant samples at up to 10.4 $\log_{10}$ *groEL* copies/g feces. *B. bifidum* and *B. longum* were present at high concentrations (9.7 $\log_{10}$ *groEL* copies/g feces in INF-3 and 10.1 $\log_{10}$ *groEL* copies/g feces in INF-4, respectively). *B. adolescentis*, which is a typical *Bifidobacterium* species of the adult fecal microbiota, was not detected in infant feces. Most of the adult fecal samples contained between one and three *Bifidobacterium* species (one species, 2/10; two species, 2/10; three species, 4/10; five species, 1/10; and six species, 1/10). *B. adolescentis* and *B. longum* were frequently found in adults (7/10 and 8/10, respectively). Their concentrations were relatively high, with 8.9 to 9.9 $\log_{10}$ *groEL* copies/g feces for all but two subjects (AD-1 and AD-7), who harbored *B. longum* at much lower concentrations (7.2 $\log_{10}$ and 6.6 $\log_{10}$ *groEL* copies/g, respectively). *B. pseudocatenulatum*, even though detected in just 3/10 samples, also displayed high counts of 8.7 to 9.3 $\log_{10}$ *groEL* copies/g feces. In adult feces, the concentration of *B. bifidum*, a predominant *Bifidobacterium* species in the feces of infants, was in the range of 8.2 to 9.1 $\log_{10}$ *groEL* copies/g feces. *B. catenulatum* and *B. pseudolongum* were found rarely (2/10 and 1/10, respectively) at 8.0 to 8.1 $\log_{10}$ *groEL* copies/g feces. The lowest numbers, ranging from 6.4 to 7.1 $\log_{10}$ *groEL* copies/g feces, were measured for *B. dentium* and *B. animalis*. The latter was detected only once (AD-2). In adults, total bifidobacteria varied between 9.5 and 10.7 $\log_{10}$ bifidobacterial genomes/g feces. In contrast, 1-week-old infants harbored relatively low levels of total bifidobacteria (7.6 $\log_{10}$ bifidobacterial genomes/g feces in INF-2) or even none (INF-1). However, bifidobacteria were detected in higher numbers in 3-month-old infants than in adults (11.0 $\log_{10}$ bifidobacterial genomes/g feces in INF-4 and 11.1 $\log_{10}$ bifidobacterial genomes/g feces in INF-5). The sum of *groEL* copies of all targeted *Bifidobacterium* species was slightly lower than the number obtained with the genus-specific primer pair g-Bifid-F/R targeting the 16S rRNA gene (0.1 to 1.0 $\log_{10}$). The only exception was the sample collected from donor INF-5 at 6 weeks of age, in which none of the investigated *Bifidobacterium* species could be detected but the concentration of total bifidobacteria was 8.1 $\log_{10}$ bifidobacterial genomes/g feces. *B. angulatum*, *B. gallicum*, and *B. thermophilum* were not detected in any fecal sample from adults or infants.

DISCUSSION

The *groEL* gene is a powerful phylogenetic marker. The phylogenetic tree based on fragments of the *groEL* gene (538 bp, located at positions ~250 to 840 of the corresponding nucleotide sequence) or larger (~1,200-bp) or even complete (~1,600-bp) *groEL* sequences of *Bifidobacterium* species (27, 47, 50) was similar to the one based on 16S rRNA gene sequences (31). However, the genetic distances of the *groEL* sequences were greater than those of the 16S rRNA gene sequences (21, 55). The partial *groEL* sequence (~550 bp) identity among different *Bifidobacterium* species was 80 to 96%, providing considerable discriminative power at the interspecies level (55). The analysis of larger (~1,200-bp) and complete (~1,600-bp) *groEL* sequences even allowed the differentiation of *B. animalis* and *B. longum* at the subspecies level (27, 50). The ubiquitous GroEL-encoding gene nevertheless reveals a high degree of sequence conservation, in agreement with the central cellular function of the encoded chaperonin. All in all, the *groEL* gene fulfills several prerequisites to serve as a reliable alternative phylogenetic marker for *Bifidobacterium* species.

Advantages of the *groEL* gene compared to the 16S rRNA gene. Although the 16S rRNA gene, with a mean sequence identity of 95% (31, 47), allows the discrimination of most *Bifidobacterium* species, its discriminative power among closely related species is limited. Thus, it is not possible to distinguish *B. catenulatum* and *B. pseudocatenulatum*, which share 99.5% sequence identity in their 16S rRNA genes (31, 47). These species have therefore been detected together (16, 29, 33). As demonstrated here, *B. catenulatum* and *B. pseudocatenulatum* could easily be distinguished because the two organisms share only 91 to 93% sequence identity in the *groEL* sequence of ~550 bp (21, 55). Owing to more than 96% 16S rRNA gene sequence identity, it has not been possible to determine the evolutionary distance between strains belonging to *B. breve* and *B. longum* (25). This also applies to *B. adolescentis*/*B. dentium*, *B. breve*/*B. pseudolongum*, *B. catenulatum*/*B. dentium*, and *B. longum*/*B. pseudolongum* (25). In contrast, the selected region of the *groEL* gene used in this study revealed a considerably higher resolution power between these species than the 16S rRNA gene (21, 47, 55).

Limitations. Initially, phylogenetic analysis of bifidobacteria based on the *groEL* gene was constrained because the number of available *groEL* sequences was limited to 73 (21, 55). With increasing interest in the gene as a powerful phylogenetic marker and as a result of the sequencing of bifidobacterial genomes, the database of *groEL* sequences has continuously grown (18). Although *groEL* sequences are available for almost all 36 described *Bifidobacterium* species (http://www.dsmz.de), the majority of these sequences are incomplete; they consist of an approximately 600-bp region located at positions ~250 to 840 of the gene. For some *Bifidobacterium* species, only one or two sequences are available (e.g., *B. angulatum* and *B. catenulatum*, respectively).

Quantification of *Bifidobacterium* species in fecal samples. The colonization of the gastrointestinal tract starts at birth and is characterized by different microbial successions. Within the first days of life, facultative anaerobes, such as coliforms and enterococci, dominate the microbiota. They create a reduced environment, enabling the establishment of strict anaerobes, including bifidobacteria, by days 4 to 7 (26).

In our study, at 1 week after birth, *Bifidobacterium* species were absent or present at low concentrations. In contrast, at 3 months of age, bifidobacteria had advanced to become one of the dominant population groups in the infant gut. Differences in the patterns and sizes of bifidobacterial populations as reported here have been observed previously (19, 30, 38). Our study revealed low *Bifidobacterium* diversity and the presence of three or less than three species in a given sample from infants or adults (8/10). This result is similar to a previous study with higher numbers of subjects (30).

High cell counts of *B. bifidum*, *B. breve*, and *B. longum* (including *B. longum* subsp. *infantis*) were frequently found in feces from breast-fed infants, suggesting that these three species play a crucial role in gut colonization (16, 30, 39). In accordance with previous studies (15, 37, 38), *B. longum* was the most common species in human adult feces, followed by *B. adolescentis*, *B. bifidum*, and *B. pseudocatenulatum*. A high incidence of *B. longum*, *B. adolescentis*, *B. bifidum*, and members of the *B. catenulatum* group was observed in Japanese adults (29, 30). Although we were able to distinguish *B. catenulatum* and *B. pseudocatenulatum* in our study,

the prevalence of both species together was considerably lower than that reported for these Japanese subjects (40% versus 89% [29] and 92% [30], respectively).

As an inhabitant of the oral cavity (7) *B. dentium* may pass into the intestine, where it is infrequently detected at low cell numbers (29, 30, 39). In contrast to Matsuki et al. (29, 30), who occasionally detected *B. breve*, the species was mostly below the quantification limit in the adult samples we analyzed. The donor of sample AD-2 was not aware of having consumed a probiotic food prior to the collection of the sample. However, *B. animalis* subsp. *lactis* may be present in normal yogurts that are not explicitly labeled as containing bifidobacteria (3).

None of the few fecal samples tested in our study contained *B. angulatum*, *B. gallicum*, or *B. thermophilum*, all of which have rarely been detected. *B. angulatum* was occasionally found in infant and adult feces (29, 30), in the latter at relatively low concentrations (~6.6 $\log_{10}$ cells/g feces [29] and 7.5 to 8.3 $\log_{10}$ CFU/g [3]). *B. gallicum* was mostly absent (15, 30, 33), except for a baby delivered by caesarean section (6). *B. thermophilum*, first isolated from baby feces (53), was recently detected in the feces of a breast-fed infant at 6.7 $\log_{10}$ cells/g feces (28). Since *B. pseudolongum* has so far been associated with animals, it has rarely been investigated in humans. However, in agreement with our study, it was recently found in adults, but not in infants (44).

The numbers of *Bifidobacterium* species that we detected in adults and infants are essentially in agreement with previous findings (30). Matsuki et al. (30) mostly observed between one and four species in adults (29% harbored three and 31% harbored four species) and up to three species in infants (33% harbored one and 22% harbored three species). However, Matsuki et al. (30) were not able to further discriminate between members of the *B. catenulatum* group.

The total counts of bifidobacterial genomes determined with the genus-specific primers g-Bifid-F/R targeting the 16S rRNA gene were slightly higher than those resulting from the sum of *groEL* copies detected with the species-specific primers. It is well known that bifidobacteria possess two to five *rrn* loci (8, 10, 40). Differences in the 16S rRNA gene copy numbers are adequately considered when the qPCR standard curve uses genomic DNA from a bacterium that represents the predominant species in the sample (34). Taking this into account, *B. longum* subsp. *longum* ATCC 15707^T, with four 16S rRNA gene copies (13), was used as a standard for quantification of total fecal bifidobacteria in adults and infants. However, Candela et al. (10) have demonstrated that the *rrn* copies do not differ only at species level. Even different strains of *B. adolescentis* may harbor between two and five copies (10, 40). The genome size of bifidobacterial species varies between 1.92 and 2.83 Mb (GenBank; corresponding to 2.11 to 3.11 fg/cell). Assuming a mean genome size of 2.25 Mb (2.47 fg/cell) would result in overestimation of the cell number if the predominant *Bifidobacterium* strain in the sample has a genome size smaller than 2.25 Mb and/or a number of 16S rRNA gene copies higher than four. This would apply to *B. adolescentis* ATCC 15703^T (GenBank accession no. AP009256), *B. bifidum* PRL2010, and *B. bifidum* S17 (43, 56). These problems can be avoided by targeting a single-copy gene, such as *groEL*. However, a multiple alignment of all complete *groEL* sequences listed in Table 2 did not reveal consensus regions of sufficient length to design genus-specific primers. Quantification limits of qPCR with species-specific primers that target the single-copy *groEL* gene are comparable with the one targeting the multicopy 16S rRNA gene (Table 5), indicating that the qPCR assays developed in this study are highly sensitive.

During this study, two novel *Bifidobacterium* species were isolated from human feces, namely, *B. stercoris* (22) and *B. kashiwanohense* (32), for which at least partial *groEL* sequences are available. It is interesting that recent microbiomic analysis suggested the presence of previously undescribed *Bifidobacterium* species, which, however, have not been identified so far (45).

Conclusion. This study describes the use of the *groEL* gene as a target for the identification and quantification of human fecal *Bifidobacterium* species by qPCR. The method developed allows the distinction between closely related *Bifidobacterium* species, enabling monitoring of the bifidobacterial population in response to age, antibiotic treatment, pro- and prebiotic intake, or disease.

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REFERENCES

1. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Apajalahti JH, Kettunen A, Nurminen PH, Jatila H, Holben WE.** 2003. Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. Appl. Environ. Microbiol. **69**:5731–5735.
3. **Bartosch S, Woodmansey EJ, Paterson JC, McMurdo ME, Macfarlane GT.** 2005. Microbiological effects of consuming a synbiotic containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and oligofructose in elderly persons, determined by real-time polymerase chain reaction and counting of viable bacteria. Clin. Infect. Dis. **40**:28–37.
4. **Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW.** 2011. GenBank. Nucleic Acids Res. **39**:D32–D37.
5. **Berthoud H, Chavagnat F, Haueter M, Casey MG.** 2005. Comparison of partial gene sequences encoding a phosphoketolase for the identification of bifidobacteria. Lebensm. Wiss. Technol. **38**:101–105.
6. **Biasucci G, Benenati B, Morelli L, Bessi E, Boehm G.** 2008. Cesarean delivery may affect the early biodiversity of intestinal bacteria. J. Nutr. **138**:1796S–1800S.
7. **Biavati B, Mattarelli P.** 2006. The family *Bifidobacteriaceae*, p 322–382. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (ed), The prokaryotes, 3rd ed, vol 3. Springer, New York, NY.
8. **Bourget N, Simonet JM, Decaris B.** 1993. Analysis of the genome of the five *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome size estimation and *rrn* loci number. FEMS Microbiol. Lett. **110**:11–20.
9. **Braune A, Gütschow M, Engst W, Blaut M.** 2001. Degradation of quercetin and luteolin by *Eubacterium ramulus*. Appl. Environ. Microbiol. **67**:5558–5567.
10. **Candela M, Vitali B, Matteuzzi D, Brigidi P.** 2004. Evaluation of the *rrn* operon copy number in *Bifidobacterium* using real-time PCR. Lett. Appl. Microbiol. **38**:229–232.
11. **Edwards CA, Parrett AM.** 2002. Intestinal flora during the first months of life: new perspectives. Br. J. Nutr. **88**(Suppl. 1):S11–S18.
12. **Fox GE, et al.** 1980. The phylogeny of prokaryotes. Science **209**:457–463.
13. **Fukuda S, et al.** 2011. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature **469**:543–547.
14. **Garrigues C, Johansen E, Pedersen MB.** 2010. Complete genome sequence of *Bifidobacterium animalis* subsp. *lactis* BB-12, a widely consumed probiotic strain. J. Bacteriol. **192**:2467–2468.
15. **Gavini F, Cayuela C, Antoine JM, Lecoq CLB, Membré JM, Neut C.** 2001. Differences in the distribution of bifidobacterial and enterobacterial species in human faecal microflora of three different (children, adults, elderly) age groups. Microb. Ecol. Health Dis. **13**:40–45.
16. **Haarman M, Knol J.** 2005. Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. Appl. Environ. Microbiol. **71**:2318–2324.

17. **Harmsen HJ, et al.** 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. **30**:61–67.
18. **Hill JE, Penny SL, Crowell KG, Goh SH, Hemmingsen SM.** 2004. cpnDB: a chaperonin sequence database. Genome Res. **14**:1669–1675.
19. **Hopkins MJ, Sharp R, Macfarlane GT.** 2001. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut **48**:198–205.
20. **Howie PW, Forsyth JS, Ogston SA, Clark A, Florey CD.** 1990. Protective effect of breast feeding against infection. Br. Med. J. **300**:11–16.
21. **Jian W, Zhu L, Dong X.** 2001. New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. Int. J. Syst. Evol. Microbiol. **51**:1633–1638.
22. **Kim MS, Roh SW, Bae JW.** 2010. *Bifidobacterium stercoris* sp. nov., isolated from human faeces. Int. J. Syst. Evol. Microbiol. **60**:2823–2827.
23. **Kullen MJ, Brady LJ, O'Sullivan DJ.** 1997. Evaluation of using a short region of the *recA* gene for rapid and sensitive speciation of dominant bifidobacteria in the human large intestine. FEMS Microbiol. Lett. **154**:377–383.
24. **Larkin MA, et al.** 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23**:2947–2948.
25. **Leblond-Bourget N, Philippe H, Mangin I, Decaris B.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int. J. Syst. Bacteriol. **46**:102–111.
26. **Mackie RI, Sghir A, Gaskins HR.** 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. **69**:1035S–1045S.
27. **Masco L, Ventura M, Zink R, Huys G, Swings J.** 2004. Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov. Int. J. Syst. Evol. Microbiol. **54**:1137–1143.
28. **Mathys S, Lacroix C, Mini R, Meile L.** 2008. PCR and real-time PCR primers developed for detection and identification of *Bifidobacterium thermophilum* in faeces. BMC Microbiol. **8**:179.
29. **Matsuki T, et al.** 2004. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl. Environ. Microbiol. **70**:167–173.
30. **Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H.** 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. **65**:4506–4512.
31. **Miyake T, Watanabe K, Watanabe T, Oyaizu H.** 1998. Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. Microbiol. Immunol. **42**:661–667.
32. **Morita H, et al.** 2011. *Bifidobacterium kashiwanohense* sp. nov., isolated from healthy infant faeces. Int. J. Syst. Evol. Microbiol. **61**:2610–2615.
33. **Mullié C, Odou MF, Singer E, Romond MB, Izard D.** 2003. Multiplex PCR using 16S rRNA gene-targeted primers for the identification of bifidobacteria from human origin. FEMS Microbiol. Lett. **222**:129–136.
34. **Nadkarni MA, Martin FE, Jacques NA, Hunter N.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **148**:257–266.
35. **Owczarzy R, et al.** 2008. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. Nucleic Acids Res. **36**:W163–W169.
36. **Pfaffl MW.** 2001. Development and validation of an externally standardised quantitative insulin-like growth factor-1 RT-PCR using LightCycler SYBR Green I technology, p 281–292. *In* Meuer S, Wittwer C, Nakagawara K (ed), Rapid cycle real-time PCR: methods and applications. Springer, Heidelberg, Germany.
37. **Requena T, et al.** 2002. Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene. Appl. Environ. Microbiol. **68**:2420–2427.
38. **Reuter G.** 2001. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Curr. Issues Intest. Microbiol. **2**:43–53.
39. **Rinne MM, et al.** 2005. Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. FEMS Immunol. Med. Microbiol. **43**:59–65.
40. **Satokari RM, Vaughan EE, Akkermans AD, Saarela M, de Vos WM.** 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67**:504–513.
41. **Satokari RM, et al.** 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. Syst. Appl. Microbiol. **26**:572–584.
42. **Schell MA, et al.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. U. S. A. **99**:14422–14427.
43. **Turroni F, et al.** 2010. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc. Natl. Acad. Sci. U. S. A. **107**:19514–19519.
44. **Turroni F, et al.** 2009. Exploring the diversity of the bifidobacterial population in the human intestinal tract. Appl. Environ. Microbiol. **75**:1534–1545.
45. **Turroni F, et al.** 2009. Microbiomic analysis of the bifidobacterial population in the human distal gut. ISME J. **3**:745–751.
46. **van Odijk J, et al.** 2003. Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966–2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. Allergy **58**:833–843.
47. **Ventura M, et al.** 2006. Analysis of bifidobacterial evolution using a multilocus approach. Int. J. Syst. Evol. Microbiol. **56**:2783–2792.
48. **Ventura M, Canchaya C, Meylan V, Klaenhammer TR, Zink R.** 2003. Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. Appl. Environ. Microbiol. **69**:6908–6922.
49. **Ventura, M, Canchaya C, van Sinderen D, Fitzgerald GF, Zink R.** 2004. *Bifidobacterium lactis* DSM 10140: identification of the *atp* (*atpBEFHAGDC*) operon and analysis of its genetic structure, characteristics, and phylogeny. Appl. Environ. Microbiol. **70**:3110–3121.
50. **Ventura M, Canchaya C, Zink R, Fitzgerald GF, van Sinderen D.** 2004. Characterization of the *groEL* and *groES* loci in *Bifidobacterium breve* UCC 2003: genetic, transcriptional, and phylogenetic analyses. Appl. Environ. Microbiol. **70**:6197–6209.
51. **Ventura M, Zink R.** 2003. Comparative sequence analysis of the *tuf* and *recA* genes and restriction fragment length polymorphism of the internal transcribed spacer region sequences supply additional tools for discriminating *Bifidobacterium lactis* from *Bifidobacterium animalis*. Appl. Environ. Microbiol. **69**:7517–7522.
52. **Ventura M, Zink R, Fitzgerald GF, van Sinderen D.** 2005. Gene structure and transcriptional organization of the *dnaK* operon of *Bifidobacterium breve* UCC 2003 and application of the operon in bifidobacterial tracing. Appl. Environ. Microbiol. **71**:487–500.
53. **von Ah U, et al.** 2007. Classification of a moderately oxygen-tolerant isolate from baby faeces as *Bifidobacterium thermophilum*. BMC Microbiol. **7**:79.
54. **Yin X, Chambers JR, Barlow K, Park AS, Wheatcroft R.** 2005. The gene encoding xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) is conserved among *Bifidobacterium* species within a more variable region of the genome and both are useful for strain identification. FEMS Microbiol. Lett. **246**:251–257.
55. **Zhu L, Li W, Dong X.** 2003. Species identification of genus *Bifidobacterium* based on partial HSP60 gene sequences and proposal of *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov. Int. J. Syst. Evol. Microbiol. **53**:1619–1623.
56. **Zhurina D, et al.** 2011. Complete genome sequence of *Bifidobacterium bifidum* S17. J. Bacteriol. **193**:301–302.