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New Real-Time Quantitative PCR Procedure for Quantification of Bifidobacteria in Human Fecal Samples

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The application of a real-time quantitative PCR method (5′ nuclease assay), based on the use of a probe labeled at its 5′ end with a stable, fluorescent lanthanide chelate, for the quantification of human fecal bifidobacteria was evaluated. The specificities of the primers and the primer-probe combination were evaluated by conventional PCR and real-time PCR, respectively. The results obtained by real-time PCR were compared with those obtained by fluorescent in situ hybridization, the current gold standard for intestinal microbiota quantification. In general, a good correlation between the two methods was observed. In order to determine the detection limit and the accuracy of the real-time PCR procedure, germfree rat feces were spiked with known amounts of bifidobacteria and analyzed by both methods. The detection limit of the method used in this study was found to be about 5×10^4 cells per g of feces. Both methods, real-time PCR and fluorescent in situ hybridization, led to an accurate quantification of the spiked samples with high levels of bifidobacteria, but real-time PCR was more accurate for samples with low levels. We conclude that the real-time PCR procedure described here is a specific, accurate, rapid, and easy method for the quantification of bifidobacteria in feces.

The human gastrointestinal tract hosts a rich and complex microbiota which is specific for each person, depending on environmental and genetic factors (33, 34). This microbiota provides a barrier against harmful food components and pathogenic bacteria (3, 8, 17), and it has also been shown to have a direct impact on the morphology of the gut (5). Therefore, the intestinal microbiota is an important factor for the health and well-being of the human host. Major dysfunctions of the gastrointestinal tract are thought to be related to disturbances of intestinal microbiota (9). Understanding the role of intestinal microbiota in these dysfunctions has been limited by the lack of data on its development and normal composition. The major restricting factor has been the laboriousness and poor accuracy of traditional plate culture methods, which have limited our understanding of the role of the gut microbiota in health and disease.

Advances in molecular biology have led to the development of alternative, culture-independent methods. One of the most widely used approaches for intestinal microbiota assessment has been the use of 16S rRNA and its genes as target molecules. Specific PCR primers can be designed based on the variable regions of this molecule to detect certain species or groups of bacteria predominant in the gastrointestinal tract (23, 24, 35, 36). These primers have been a useful tool for qualitative intestinal microbiota (species composition) assessment but unfortunately do not report quantitative data. The development of 16S rRNA-targeted hybridization probes has permitted the development of some quantitative culture-independent methods, such as fluorescence in situ hybridization (FISH) (7, 10, 18, 31), which is one of the current key techniques for assessment of intestinal microbiota. Unfortunately, this technique is laborious and time-consuming, and difficulties in visually counting the samples have limited the wide use of this method in research assessing the significance of intestinal microbiota in human health in food and nutrition research and clinical monitoring of gut microbiota in intestinal diseases. Therefore, new quantitative methods for intestinal microbiota assessment facilitating rapid and objective counting of a large number of samples are needed.

It has been shown that bifidobacteria are one of the predominant fecal organisms, especially in infants and also in adults (3, 13, 29). Significant qualitative and quantitative differences in the bifidobacterial microbiota composition of allergic and healthy children have been reported (15, 27), and several health-promoting properties have been attributed to the microorganisms of this genus. On the other hand, bifidobacteria are the main target of nondigestible carbohydrates (prebiotic compounds) that are not absorbed by the intestinal epithelium but can be metabolized, selectively stimulating bifidobacterial growth. A number of genus- and species-specific primers and probes have been developed for bifidobacteria; therefore, this genus could be a good target for the initial development of quantitative culture-independent methods for intestinal microbiota assessment.

Real-time PCR is a promising tool to study the composition of such complex communities as the gastrointestinal tract. The development of real-time quantitative PCR methods could lead to a thorough knowledge about gastrointestinal community composition, relation with the diet, and its role in health and disease and would provide a useful tool for clinical assessment of probiotics and prebiotic efficacy. The TaqMan (12, 19) (Applied Biosystems) assay has mainly been used for intestinal microbiota assessment. This procedure has been used for the quantification of bifidobacteria (28) and some bifidobacterial groups (20) in fecal samples and *Escherichia coli* and *Bacte-*

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TABLE 1. Specificity of the primers and the primer-probe combination used in this study, tested by qualitative PCR and quantitative real-time PCR, respectively

Strain[a]	Specificity	
	Qualitative PCR	Quantitative real-time PCR
Bifidobacterium adolescentis JCM 1275^T	+	+
Bifidobacterium angulatum JCM 7096^T	+	+
Bifidobacterium bifidum JCM 1254^T	+	+
Bifidobacterium breve JCM 1192^T	+	+
Bifidobacterium catenulatum JCM 7130	+	+
Bifidobacterium denticolens DSM 10105^T	+	+
Bifidobacterium dentium DSM 20436^T	+	+
Bifidobacterium infantis DSM 20088^T	+	+
Bifidobacterium longum JCM 1217^T	+	+
Bifidobacterium pseudocatenulatum JCM 1200^T	+	+
Bacteroides vulgatus DSM 1447^T	–	–
Clostridium butyricum DSM 10702^T	–	–
Clostridium coccoides DSM 935^T	–	–
Enterobacter aerogenes DSM 30053^T	–	–
Enterococcus faecalis DSM 20478^T	–	–
Enterococcus faecium Gaio	–	–
Escherichia coli K-12	–	–
Eubacterium cylindroides DSM 3983^T	–	–
Eubacterium hallii DSM 3353^T	–	–
Lactobacillus acidophilus La-5	–	–
Lactobacillus gasseri DSM 20077	–	–
Lactobacillus jensenii DSM 20557^T	–	–
Lactobacillus casei DSM 20011^T	–	–
Lactobacillus paracasei DSM 20244	–	–
Lactobacillus plantarum 299v	–	–
Lactobacillus rhamnosus GG	–	–
Peptostreptococcus anaerobius DSM 2949^T	–	–
Anaerococcus prevotii DSM 20548^T	–	–
Ruminococcus hansenii DSM 20583^T	–	–
Streptococcus thermophilus DSM 20617^T	–	–
Veillonella dispar DSM 20735^T	–	–

[a] JCM, Japan Collection of Microorganisms, Saitama, Japan; DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; ^{T}, type strain.

roides vulgatus in gastrointestinal mucosa (14). SYBR Green I (Molecular Probes)-based assays have also been used for PCR quantification of intestinal bifidobacterial species or groups (20, 22).

In this work we used a different quantitative real-time PCR assay based on the use of environmentally sensitive lanthanide chelates and the 5′-3′ exonucleolytic activity of the DNA polymerase (25). In addition, the quantitative real-time PCR was conducted with a quencher oligonucleotide and a dual-temperature assay (26).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. Strains were grown in GAM broth or agar (Nissui Pharmaceutical Co, Tokyo, Japan) and incubated at 37°C in a Concept 400 anaerobic chamber (Ruskinn Technology, Leeds, United Kingdom) with the exception of *E. coli* K-12, which was grown in Trypticase soy broth (Pronadisa, Madrid, Spain) and incubated at 37°C in aerobic conditions.

Fecal samples. A total of 24 human fecal samples, 14 from 1-year-old infants, four from adults, and six from elderly (>65 years old) persons, were included in this study. Fecal specimens were cooled to 4°C immediately after collection, delivered within 24 h, and frozen at −75°C directly on receipt until analysis.

Oligonucleotides. The oligonucleotide primers and probes used for quantitative real-time PCR (Table 2) were purchased from Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany). To check for specificity, the sequences of the selected PCR primers and probes were compared to the sequences available at both the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) (1) and with the Probe Match application at the Ribosomal Database Project II (www.rdp.cme.msu.edu/html) (4). Two bases that do not hybridize with the target sequence were added to the 3′ end of the real-time PCR probe to make sure that it could not act as a primer during the PCR. The bifidobacteria detection probe (Bifidoprobe) was labeled postsynthetically with an isothiocyanate-modified, stable, fluorescent europium chelate as described by Nurmi and coworkers (26).

Testing of the oligonucleotide primers by qualitative PCR. In order to test the specificity of the primers, DNA was extracted from pure cultures of the different intestinal and food microorganisms indicated in Table 1 as follows: 1 ml of culture ($A_{600} \approx 1$) was harvested, washed twice with TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8]), resuspended on 50 μl of TE saline buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8], 0.9% [wt/vol] NaCl), heated at 98°C for 10 min, immediately cooled, and centrifuged at 16,000 × *g* for 10 min, and the supernatant was stored at −20°C until its use.

Amplification of the DNA was performed with primers Bifido5 and Bifido3 in a PCR iCycler apparatus (Bio-Rad, Espoo, Finland). The total volume of each reaction mixture was 50 μl, employing 4 μl of DNA extract as a template. The PCR mixture was composed of 1× PCR buffer II (Applied Biosystems, Foster City, Calif.) with 2.5 mM $MgCl_2$, 0.2 μM each primer, 200 μM each of the four deoxynucleoside triphosphates (Amersham Biosciences, Helsinki, Finland), and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermal cycle program consisted of an initial cycle of 95°C for 10 min for denaturation and polymerase activation, 30 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 45 s, and a final extension step of 10 min at 72°C. Amplified products were subjected to gel electrophoresis in 1% agarose gels and visualized by ethidium bromide staining.

Quantitative real-time PCR. We weighed 1 g of fecal material and homogenized it with 9 ml of phosphate-buffered saline buffer in a Stomacher 400 (Seward Ltd., London, United Kingdom) at full speed for 2 min; 200 μl of the homogenate was used for the DNA extraction with the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For standard curves, different dilutions (200 μl) of microbial cultures of three predominant human intestinal bifidobacteria, such as *B. infantis*, *B. longum*, and *B. adolescentis* (cell numbers ranging from 1.5×10^4 to 3.2×10^9 cells/ml), were used for the DNA extraction with the QIAamp DNA stool mini kit (Qiagen). To test the different strains by real-time PCR, 200 μl of a culture ($A_{600} \approx 1$) of the microorganism was used. The DNA extracts were frozen at −20°C until analysis.

Samples (1 μl) were analyzed in 50-μl amplification reactions consisting of 1× PCR buffer II, 3.5 mM $MgCl_2$, 0.2 μM each primer, 200 μM each deoxynucleoside triphosphate, 0.024 μM europium-labeled Bifidoprobe, 0.166 μM quencher probe, and 1.25 U of AmpliTaq Gold DNA polymerase. All reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems). Thermal cycling (iCycler) consisted of an initial cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and 61°C for 45 s, and 35°C for 15 s. Europium fluorescence measurements were performed in real time at the end of each cycle with a Wallac Victor 1420 multilabel counter (Perkin Elmer, Turku, Finland) at 35°C to determine the threshold cycles (C_t) of individual reactions. The C_t was defined as the PCR cycle at which the europium signal-to-noise ratio crosses a threshold value of 1.5. Standard curves were made by plotting the C_t values obtained for the standard cultures (different dilutions from cultures of *B. longum* JCM 1217^T, *B. infantis* DSM 20088^T, and *B. adolescentis* JCM 1275^T, 25 reactions in total) as a linear function of the base 10 logarithm of the initial number of bifidobacteria in the culture determined by plate counting. The number of cells of bifidobacteria in the fecal samples was deter-

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′-3′)[a]	Derivation (reference)
Bifido5′	GATTCTGGCTCAGGATGAACGC	LM26 (16)
Bifido3′	CTGATAGGACGCGACCCCAT	Bif228 (21)
Bifidoprobe	^{E}CATCCGGCATTACCACCCGTTT CCTC	Bif164 (18)
Bifido quencher	GTGGTAATGCCGGATG^D	

[a] Bold letters indicate bases that are not complementary to the target. ^{E}, 2,2′,2″,2‴-{{6,6′-{4″-[2-(4-isothiocyanatophenyl)ethyl]-1H-pyrazole-1″,3″-diyl}bis(pyridine)-2,2′-diyl}bis(methylenenitrilo)}tetrakis (acetato) europium(III); ^{D}, dabcyl.

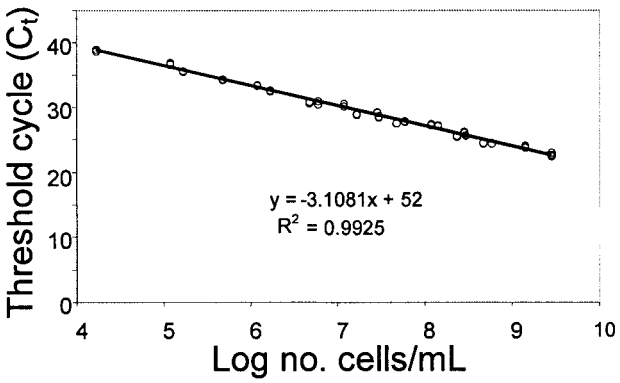

FIG. 1. Calibration curve obtained by plotting C_t values as a linear function of the base 10 logarithm of the initial number of bifidobacteria (*B. infantis* DSM 20088^T, *B. longum* JCM 1217^T, and *B. adolescentis* JCM 1275^T) in the culture determined by plate counting.

mined by comparing the C_t values obtained to the standard curve. DNA extracts from the different samples were analyzed in duplicate in each PCR in two independent PCR runs.

FISH. FISH was performed as previously described (15) with the fluorophore indocarbocyanine (Cy3)-labeled oligonucleotide probe BIF164 (5′-CATCCGG CATTACCACCC) (18). Bifidobacteria were counted visually with an Olympus BX51 epifluorescence microscope.

RESULTS

Specificity of the assay. Table 1 shows the results of the test for the specificity of the primers used in this work and the results obtained by analyzing the different strains with the quantitative real-time PCR procedure. In addition to this specificity test, the sequences of the primers and probe were compared with the target sequences of the nonbifidobacteria test species included in this study (Table 1) showing one mismatch of the primer Bifido5′ with species of the genus *Eubacterium*, *Lactobacillus casei*, and *Lactobacillus paracasei* and two or more mismatches with the rest of the test organisms. With regard to the primer Bifido3′ and the probe (Bifidoprobe), all the nonbifidobacteria test species had at least four or five mismatches, respectively (data not shown).

The PCR primers (Bifido5′and Bifido3′) and the real-time PCR conditions were specific for the bifidobacteria species tested. All nonbifidobacterial strains tested showed no amplification during PCR and no increase in fluorescence during real-time PCR.

Quantification of bifidobacteria in feces. Standard curves obtained for the strains *B. infantis* DSM 20088^T, *B. longum* JCM 1217^T, and *B. adolescentis* JCM 1275^T were in good agreement (data not shown). For the standard curve, the results obtained (C_t values) for the three strains were plotted together against the initial number of cells in the cultures (Fig. 1). Results were found to be linear over the range of cell concentrations tested (10^4 to 10^9 cells/ml).

To evaluate the applicability of the assay to the quantification of bifidobacteria in fecal samples, 24 samples were analyzed by quantitative real-time PCR and FISH. The results showed, in general, a good correlation between the two methods (Table 3), but in samples with small numbers of bifidobacteria (samples I6, I12, and A3), the results of FISH appear to overestimate the number of bifidobacteria with respect to the real-time PCR by about two logarithmic units. The presence of PCR inhibitors in those samples was tested by conducting a real-time PCR with a control sample in the presence of 1 μl of DNA extract from the problem sample; the fluorescent signal was the same for the control sample in the presence or absence of DNA extract from the problem samples, indicating that there were no PCR inhibitors in those DNA extracts.

The inter-PCR reproducibility of the quantification was found to be very high based on the C_t values obtained for two DNA extracts (a pure culture of *B. infantis* DSM 20088^T and a fecal specimen) in five replicate runs. The average C_t values obtained for those samples were 23.35 (range, 23.0 to 23.5) and 26.9 (26.8 to 27.0). Similar results were observed with other samples. On the other hand, by analyzing duplicate DNA extracts from five fecal samples, the interextract variability was found to be less than 10% (mean, 9.82%; standard deviation, 5.3%) (data not shown).

Quantification of bifidobacteria in spiked germ-free feces. In order to determine the detection limit of the real-time PCR method and to compare the accuracy of real-time PCR and FISH, feces from germfree *Agus* rats were spiked with different amounts of *B. infantis* DSM 20088^T and analyzed by the two methods (Fig. 2). Both methods led to accurate quantification in the spiked samples with high levels of bifidobacteria, but real-time PCR was more accurate than FISH for samples with low levels of analyte.

On the other hand, the detection limit of the real-time PCR procedure with the DNA extract from feces and the PCR conditions described in this paper was found to be about 5×10^4 cells/g of feces.

TABLE 3. Results obtained by real-time PCR and FISH for different fecal samples

Sample[a]	Mean log no. of cells/g of feces ± SD	
	Real-time PCR	FISH
I1	9.18 ± 0.08	8.39 ± 0.34
I2	9.32 ± 0.02	9.36 ± 0.08
I3	8.00 ± 0.00	8.40 ± 0.06
I4	8.33 ± 0.03	8.31 ± 0.27
I5	8.05 ± 0.02	8.63 ± 0.30
I6	5.25 ± 0.03	7.45 ± 0.48
I7	8.59 ± 0.10	9.02 ± 0.02
I8	8.57 ± 0.07	8.82 ± 0.12
I9	9.82 ± 0.07	9.60 ± 0.15
I10	9.08 ± 0.06	8.70 ± 0.01
I11	8.37 ± 0.08	8.60 ± 0.12
I12	5.98 ± 0.02	7.70 ± 0.02
I13	8.96 ± 0.01	8.50 ± 0.13
I14	9.15 ± 0.00	8.30 ± 0.10
A1	8.66 ± 0.00	8.00 ± 0.16
A2	9.10 ± 0.04	9.40 ± 0.08
A3	<4.70	7.50 ± 0.28
A4	9.18 ± 0.05	8.90 ± 0.12
E1	9.25 ± 0.02	9.41 ± 0.22
E2	9.21 ± 0.01	9.25 ± 0.04
E3	9.04 ± 0.01	9.32 ± 0.12
E4	7.88 ± 0.04	7.86 ± 0.16
E5	10.33 ± 0.00	9.68 ± 0.22
E6	8.01 ± 0.02	8.08 ± 0.07

[a] I, infant sample; A, adult sample; E, elderly sample.

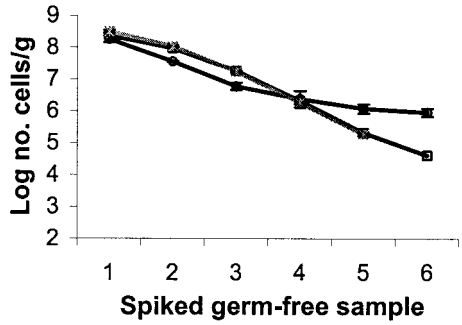

FIG. 2. Results of the analysis of germfree rat feces spiked with known amounts of *B. infantis* DSM 20088. □, cells added; ○, cells detected by FISH; ×, cells detected by real-time PCR.

DISCUSSION

It has been shown, by comparative analysis of 16S rRNA sequences amplified from human feces, that less than 25% of the molecular species identified correspond to known organisms and more than 60% of the intestinal bacteria cannot usually be cultivated (32). With regard to bifidobacteria, important differences have been observed when comparing different culture media and culture-independent methods (2), indicating the limitations of culture-based approaches for the study of the intestinal microbiota. The microbiota of each individual has a unique pattern and is partly dependent on host genomics (33). Over time, the microbiota has been shown to be less complex and more unstable in children and especially unstable in developing infants (6, 38). With regard to the bifidobacteria population, in adults *B. adolescentis* and *B. catenulatum* are most common, while *B. infantis*, *B. longum*, and *B. breve* are most often found in infants (24, 29). This high variability in the intestinal microbiota between individuals requires the analysis of large numbers of samples to obtain reliable conclusions. Thus, easy, accurate, and rapid methods are urgently needed.

FISH has been widely used for intestinal microbiota assessment. However, the technique is laborious, there are difficulties in visual counting of the bacteria, and it is time-consuming, limiting its further applicability. Moreover, variation in FISH can be caused by differences in target region availability, cell permeability, and, when 16S rRNA-targeted probes are used, differences in the ribosome content of the cells. Low fluorescence levels in positively hybridized cells can also significantly overlap the high signals of the negative controls (18). In addition, coaggregation of bacteria, remains of broken cells, and contaminating substances can make counting difficult.

In this study, an easy, rapid, and accurate culture-independent real-time PCR method combining the high specificity of fluorescent oligonucleotides and the sensitivity of PCR was used for the quantification of bifidobacteria in fecal samples. A good correlation has been shown between the results obtained by quantitative real-time PCR (TaqMan) and by culture (28). The results obtained by the real-time PCR method were compared with those obtained by FISH, considered the "gold standard" for intestinal bacteria quantification. Our results obtained by both methods are in agreement with the fecal levels of bifidobacteria reported by other authors by means of FISH (11, 18, 21).

Quantitative real-time PCR and FISH showed, in general, a good correlation, but differences were found between these methods in samples with small amounts of bifidobacteria. The analyses of germ free feces showed that the real-time PCR method is more accurate in those samples, indicating that in some cases FISH could lead to overestimation of the number of bifidobacteria in fecal samples with low bifidobacteria contents.

The specificity of the assay was shown both by testing the specificity of the primers with a collection of intestinal and food bacteria and by testing the assay itself. Taking into account that a probe specific for intestinal bifidobacteria (18) was used together with the *Bifidobacterium*-specific primers, high specificity can be expected. Furthermore, the low detection limit (5×10^4 cells/g of feces) obtained with the DNA extraction and PCR conditions described in this study, and taking into account that it could possibly be improved by adding more PCR cycles or concentrating the extracted DNA, seems to indicate that this method has the potential for very high sensitivity. A high reproducibility (>99%) was found for our quantitative real-time PCR assay, and this is in accordance with the results obtained by other authors with the TaqMan assay (14).

The 16S rDNA genes are mainly being used as target molecules, but as more bacterial sequences are becoming available, new specific primers and probes targeting other genes will be available in the near future to be used when the 16S ribosomal DNA is not an adequate target. It must be taken into account that bacterial quantification by real-time PCR can be influenced by differences in the number of rRNA operons between the quantified species or groups, sequence heterogeneity between different operons in the same species, and differential amplification of different DNA molecules (30, 37). Requena and coworkers (28) compared the accuracy of quantitative real-time PCR (TaqMan) with 16S rRNA- and transaldolase-targeted probes for quantification of fecal bifidobacteria, finding that, in samples from adults, the two methods correlated well, but in samples from infants, the real-time PCR targeting the 16S rRNA was more accurate.

We conclude that the quantitative real-time PCR described here is an accurate, rapid, and easy method for the quantification of bifidobacteria in human feces. This method will facilitate rapid and objective counting of large numbers of samples, contributing to the efficient use of intestinal bacterial assays in research, food, and pharmaceutical development as well as in the assessment of dietary management of diseases. Further studies applying this quantitative real-time PCR procedure to other intestinal microorganisms are warranted.

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