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ORIGINAL ARTICLE

Quantitative assessment of faecal bifidobacterial populations by real-time PCR using lanthanide probes

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Abstract

Aim: To develop real-time quantitative PCR methods, based on the use of probes labelled with a stable fluorescent lanthanide chelate, for the quantification of different human faecal bifidobacterial populations.

Methods and Results: The designed quantitative PCR assays were found to be specific for the corresponding *Bifidobacterium* species or groups (*Bifidobacterium longum* group, *Bifidobacterium catenulatum* group, *Bifidobacterium adolescentis*, *Bifidobacterium breve*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum* and *Bifidobacterium dentium*). The detection limits of the methodologies used ranged between 2×10^5 and 9×10^3 cells g^{-1} of faeces. The applicability of the developed assays was tested by analysing 20 human faecal samples. *Bif. longum* group was found to be the qualitatively and quantitatively predominant bifidobacterial group.

Conclusions: The real-time PCR procedures developed here are specific, accurate, rapid and easy methods for the quantification of *Bifidobacterium* groups or species in human faecal samples.

Significance and Impact of the Study: The developed procedures will facilitate rapid and objective counting of large numbers of samples increasing our knowledge on the role of gut bifidobacterial microbiota in health and disease. This will contribute 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.

Introduction

The human gastrointestinal tract hosts a very rich and complex microbiota. Major dysfunctions of the gastrointestinal tract are related to intestinal microbiota aberrancies (Guarner and Malagelada 2003). Such disturbances indicate potential targets for further research and clinical applications. However, understanding the role of the microbiota in these dysfunctions has been limited because of the laboriousness and poor accuracy of the traditional culture-dependent techniques. The application of molecular biology has allowed the development of alternative culture-independent methods. The utilization of 16S rRNA-targeted hybridization probes has permitted the culture-independent quantitative analysis of the microbiota by means of fluorescence in situ hybridization (FISH) (Franks *et al.* 1998; Harmsen *et al.* 2002). However, this technique is very laborious and there are some difficulties for the visual counting of the samples, limiting its application. Therefore, other quantitative methods facilitating rapid and objective counting of a large number of samples are needed. In this context, 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 gut microbiota, relation with the diet and its role in health and disease, and would provide a useful tool for clinical assessment of probiotics/prebiotics efficacy.

Bifidobacterium is one of the predominant human faecal organisms and several health-promoting properties have

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been attributed to this genus. Several culture-dependent techniques have been used for the identification and typification of bifidobacterial isolates (Gueimonde *et al.* 2004a; Kwon *et al.* 2005; Krizova *et al.* 2006). Qualitative and quantitative differences in the *Bifidobacterium* microbiota composition of allergic and healthy children have been reported (Kalliomäki *et al.* 2001; Ouwehand *et al.* 2001). A number of real-time PCR assays have been developed for the quantification of members of the genus *Bifidobacterium* (Gueimonde *et al.* 2004b; Matsuki *et al.* 2004; Haarman and Knol 2005; Lahtinen *et al.* 2005). The TaqMan quantitative PCR assay has been used for the quantification of *Bifidobacterium* (Requena *et al.* 2002; Penders *et al.* 2005) and some bifidobacterial groups or species (Malinen *et al.* 2003; Haarman and Knol 2005) in faecal samples. Also SYBR Green based assays have been used for PCR quantification of bifidobacterial species or groups (Malinen *et al.* 2003; Matsuki *et al.* 2004).

In this work we have developed real-time PCR assays for the quantification of different intestinal *Bifidobacterium* species or groups by using a different real-time PCR approach. The developed assays are based on the use of environmentally sensitive lanthanide chelates and the 5′-3′ exonucleolytic activity of the DNA polymerase. This methodology has previously been proved to be very reproducible and accurate for the quantification of the total number of bifidobacteria in faeces (Gueimonde *et al.* 2004b).

Materials and methods

Bacterial strains and growth conditions

The strains used in this study include the *Bifidobacterium* strains shown in Table 1 as well as an array of another 21 intestinal and food related bacteria, including members of the genera *Bacteroides*, *Clostridium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Eubacterium*, *Lactobacillus*, *Peptostreptococcus*, *Anaerococcus*, *Ruminococcus*, *Streptococcus* and *Veillonella* (data not shown). 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, UK).

Oligonucleotides

PCR primers targeting different *Bifidobacterium* species or groups were used (Table 2). The specificity of these primers has been previously reported (Rinne *et al.* 2005). In addition, in order to run quantitative PCR assays based on the 5′-3′ exonucleolytic activity of the DNA polymerase internal oligonucleotide probes were designed (Table 2). To check the specificity of the designed probes

Table 1 Strains used in this study and specificity of the *Bifidobacterium* species-specific PCR procedures

Strain	*Bifidobacterium longum*	*Bifidobacterium catenulatum*	*Bifidobacterium dentium*	*Bifidobacterium bifidum*	*Bifidobacterium angulatum*	*Bifidobacterium adolescentis*	*Bifidobacterium breve*
Bif. adolescentis JCM 1275^T	–	–	–	–	–	+	–
Bif. angulatum JCM 7096^T	–	–	–	–	+	–	–
Bifidobacterium animalis JCM 1190^T	–	–	–	–	–	–	–
Bif. bifidum JCM 1254^T	–	–	–	+	–	–	–
Bif. breve JCM 1192^T	–	–	–	–	–	–	+
Bif. catenulatum JCM 7130	–	+	–	–	–	–	–
Bifidobacterium denticolens DSM 10105^T	–	–	–	–	–	–	–
Bif. dentium DSM 20436^T	–	–	+	–	–	–	–
Bifidobacterium gallicum DSM 20093^T	–	–	–	–	–	–	–
Bifidobacterium infantis DSM 20088^T	+	–	–	–	–	–	–
Bifidobacterium inopinatum DSM 10107^T	–	–	–	–	–	–	–
Bifidobacterium lactis Bb12	–	–	–	–	–	–	–
Bif. longum JCM 1217^T	+	–	–	–	–	–	–
Bifidobacterium pseudocatenulatum JCM 1200^T	–	+	–	–	–	–	–

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Table 2 Oligonucleotides and annealing temperatures (T^a) used in this study

Oligo	Sequence (5′ → 3′)*	T^a	Specificity
Primer 5′	TTCCAGTTGATCGCATGGTCTTCT	65	*Bifidobacterium longum* group
Primer 3′	GGCTACCCGTCGAAGCCACG		
Probe	[E]GCGACCCCATCCCATACCGCGA**TT**		
Quencher	ATGGGATGGGGTCGC[D]		
Primer 5′	GGATCGGCTGGAGCTTGCTCCG	63	*Bifidobacterium adolescentis*
Primer 3′	CCCCGAAGGCTTGCTCCCAGT		
Probe	[E]CTCCAGTTGGATGCATGTCCTTCTGG**CT**		
Quencher	TGCATCCAACTGGAG[D]		
Primer 5′	AATGCCGGATGCTCCATCACAC	62	*Bifidobacterium breve*
Primer 3′	GCCTTGCTCCCTAACAAAAGAGG		
Probe	[E]CATGCCGCAAAGGCTTTCCCAACAC**TG**		
Quencher	AGCCTTTGCGGCATG[D]		
Primer 5′	TGACCGACCTGCCCCATGCT	61	*Bifidobacterium bifidum*
Primer 3′	CCCATCCCACGCCGATAGAAT		
Probe	[E]TGTTCCACATGATCGCATGTGATTGTG**CC**		
Quencher	CGATCATGTGGAACA[D]		
Primer 5′	GCCGGATGCTCCGACTCCT	64	*Bifidobacterium catenulatum* group
Primer 3′	ACCCGAAGGCTTGCTCCCGAT		
Probe	[E]TACCGATGAAATCTTTCCCGACACCC**GT**		
Quencher	AAGATTTCATCGGTA[D]		
Primer 5′	GGATCGGCTGGAGCTTGCTCCG	66	*Bifidobacterium angulatum*
Primer 3′	TCACCCGAAGGCTTGCTCCCCAA		
Probe	[E]ATCTTTCCCAGACCACCATGCGATGGAC**AC**		
Quencher	TGGTCTGGGAAAGAT[D]		
Primer 5′	ATCCCGGGGGTTCGCCTCC	62	*Bifidobacterium dentium*
Primer 3′	ATACCGATGGAACCTTTCCCGG		
Probe	[E]TGCTCCGGTTGGATGCATGTCCTTCC**CC**		
Quencher	CATCCAACCGGAGCA[D]		

*Bold letters indicate bases that are not complementary to the target. [E]2,2′,2″,2‴-[2-(4-Isothiocyanatophenyl) ethylimino]bis (methylene)bis4-[4-(α-galactopyranoxy)phenyl] ethynylpyridine-6,2-diylbis (methylene–nitrilo)tetrakis(acetato)europium (III). [D]Dabcyl.

the sequences were compared with the sequences available in both the BLAST database search program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.* 1997) and the Ribosomal Database Project II (http://www.rdp.cme.msu.edu/html) (Cole *et al.* 2003). Oligonucleotide primers and probes used were purchased from Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany). The probes were labelled postsynthetically with an isothiocyanate-modified stable, fluorescent europium chelate as described by Nurmi *et al.* (2002). In this way sets of specific primers and probes were developed for *Bifidobacterium longum* group, *Bifidobacterium catenulatum* group, *Bifidobacterium dentium*, *Bifidobacterium bifidum*, *Bifidobacterium angulatum*, *Bifidobacterium adolescentis* and *Bifidobacterium breve* (Table 2).

Bifidobacterium species-specific real-time PCR assays

To test the specificity of the primers-probes sets, DNA was extracted from pure cultures of the different microorganisms as previously described (Gueimonde *et al.* 2004b) and stored at −20°C until its use.

Amplification of the DNA was performed in a PCR iCycler apparatus (BioRad, Espoo, Finland). The PCR reaction mixture was composed of 50 μl amplification reactions consisting of 1 × PCR buffer II, 3·5 mmol l^{-1} $MgCl_2$, 0·2 μmol l^{-1} of each primer, 200 μmol l^{-1} of each dNTP, 0·024 μmol l^{-1} europium-labelled probe, 0·166 μmol l^{-1} quencher probe and 1·25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Espoo, Finland). 2 μl of DNA extract (≈30 ng) was used as the template. Thermal cycling (iCycler) consisted of the following time and temperature profile: an initial cycle of 95°C 10 min, 40 cycles of 95°C 15 s, 90 s at the corresponding annealing temperature (Table 2) and 35°C 15 s. Europium fluorescence measurements were performed in real-time at the end of each cycle, using a Wallac Victor 1420 Multilabel Counter (Perkin Elmer, Turku, Finland) at 35°C.

Analysis of faecal samples by quantitative real-time PCR

A total of 20 human faecal samples, corresponding to eight infants (<2 years), eight adults (20–40 years) and four elderly people (>70 years) were included in this

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study. Faecal specimens were immediately cooled to 4°C after collection, delivered within 24 h, and frozen at −75°C directly on receipt until analysis. One gram of faecal material was weighted and homogenized with 9 ml of PBS buffer in a Stomacher 400 (Seward Ltd., London, UK) at full speed for 2 min. 200 μl of the homogenate were used for the DNA extraction by using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For standard curves, different dilutions of microbial cultures of the appropriate bifidobacterial species shown in Table 1 (cell numbers ranging from $1{\cdot}5 \times 10^4$ to $9{\cdot}8 \times 10^9$ cells ml^{-1}) were used for the DNA extraction with the QIAamp DNA stool Mini Kit (Qiagen). Standard curves were made by plotting the C_t values obtained for the standard cultures as a linear function of the base-10 logarithm of the initial number of cells, of the corresponding bifidobacterial species (Table 1), in the culture determined by plate counting.

Samples (2 μl) were analysed in 50 μl amplification reactions with the conditions described above. Europium fluorescence measurements were performed in real-time at the end of each cycle 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 (S/N) crosses a threshold value of 1·2. The numbers of cells of the different *Bifidobacterium* species or groups in the faecal samples were determined by comparing the C_t values obtained to the standard curve. DNA extracts from the different samples were analysed in duplicate in each PCR in at least two independent PCR runs. Levels of total bifidobacteria were determined by using the genus-specific methodology previously described (Gueimonde *et al.* 2004b).

Statistical analysis

Statistical analysis was carried out using the SPSS 11·0 software (SPSS Inc., Chicago, IL, USA). Data were subjected to one-way ANOVA using the subject group as factor with three categories; infants, adults and elderly. The least significant difference (LSD) test was used for comparison of means.

Results

Bifidobacterium species-specific quantitative PCR assays

Table 1 shows the results of the test of the specificity of the developed assays obtained by using the primers, probes and annealing temperatures shown in Table 2. All non-bifidobacterial strains tested showed no amplification with any of the bifidobacterial species-specific assays (data not shown) and all the assays were specific for the *Bifidobacterium* species for which they were developed, including, *Bif. longum* group, *Bif. catenulatum* group, *Bif. dentium*, *Bif. bifidum*, *Bif. angulatum*, *Bif. adolescentis* and *Bif. breve*.

For the standard curves, the results obtained (C_t values) for each species or group were plotted against the initial number of cells in the corresponding culture (data not shown). In all the assays the results were found to be linear over the range of cell concentrations tested (10^4–10^{10} cells ml^{-1}). The coefficients of determination (R^2) obtained for the different standard curves ranged from 0·991 to 0·999 and the slopes varied between −3·28 and −3·60, corresponding with PCR efficiencies of between 99% and 91%, respectively. On the other hand, the detection limits of the different specific real-time PCR procedures, following the DNA extraction and PCR conditions described here, ranged from 2×10^5 to 9×10^3 cells g^{-1}. A high reproducibility was observed for our quantitative PCR assays, which is in accordance with the results obtained by other researchers (Huijsdens *et al.* 2002; Gueimonde *et al.* 2004b).

Quantification of bifidobacterial species or groups in faecal samples

To test the applicability of the developed methods human faecal samples were analysed (Table 3). The presence of PCR inhibitors in the negative samples was tested by conducting a real-time PCR with a DNA extract positive for the corresponding species or group in the presence of 2 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.

In the samples containing *Bifidobacterium* the average number of different bifidobacterial species or groups detected was 2·15 ± 0·76. Table 4 shows the prevalence and levels of the different bifidobacterial species or groups in infants, adults and elderly subjects. Although the number of samples analysed does not allow us to establish firm conclusions, *Bif. longum* group was the most widely found and the most numerous in 11 of 19 samples harbouring bifidobacteria, representing a 27·2% ± 22% of the total bifidobacterial population. *Bifidobacterium catenulatum* was the most numerous group in four samples, *Bif. breve* was the predominant species in two samples whilst *Bif. bifidum* and *Bif. dentium* were predominant in one sample each (Table 3).

Interestingly, the levels of total bifidobacteria and members of *Bif. longum* group were significantly higher ($P < 0{\cdot}05$) in infants than in adults or elderly faeces. No statistically significant differences were found for any of the other groups analysed.

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Table 3 Faecal levels (Log *n* cells g^{-1}) of the different *Bifidobacterium* species/groups tested as determined by the quantitative PCR procedures

Sample	Genus	*Bifidobacterium longum*	*Bifidobacterium bifidum*	*Bifidobacterium breve*	*Bifidobacterium catenulatum*	*Bifidobacterium adolescentis*	*Bifidobacterium angulatum*	*Bifidobacterium dentium*
I1	10·04 ± 0·20	9·30 ± 0·10	8·91 ± 0·07	8·67 ± 0·04	–	–	–	–
I2	9·97 ± 0·06	8·72 ± 0·05	8·13 ± 0·09	8·94 ± 0·09	–	–	–	–
I3	9·98 ± 0·06	9·57 ± 0·29	–	–	5·98 ± 0·02	–	–	–
I4	9·64 ± 0·11	8·86 ± 0·17	–	–	9·49 ± 0·17	–	–	–
I5	9·69 ± 0·09	9·52 ± 0·06	–	–	8·32 ± 0·04	–	–	–
I6	9·97 ± 0·11	8·74 ± 0·08	–	–	–	–	–	–
I7	9·31 ± 0·07	7·68 ± 0·5	–	–	–	6·24 ± 0·30	–	–
I8	9·23 ± 0·06	8·95 ± 0·04	7·43 ± 0·12	–	8·38 ± 0·01	6·93 ± 0·09	–	–
A1	9·10 ± 0·05	8·14 ± 0·11	–	–	8·19 ± 0·12	–	–	–
A2	9·18 ± 0·03	8·19 ± 0·10	–	–	7·92 ± 0·02	–	–	–
A3	8·66 ± 0·14	7·21 ± 0·03	–	–	7·83 ± 0·04	–	–	–
A4	–	–	–	–	–	–	–	–
A5	8·78 ± 0·09	8·52 ± 0·10	–	–	–	–	–	–
A6	9·12 ± 0·06	8·40 ± 0·02	–	–	–	7·45 ± 0·18	–	–
A7	8·96 ± 0·05	8·71 ± 0·06	8·14 ± 0·08	–	–	–	–	–
A8	8·31 ± 0·06	8·03 ± 0·07	–	8·70 ± 0·14	–	–	–	–
E1	8·37 ± 0·02	7·95 ± 0·03	–	–	7·58 ± 0·03	–	–	–
E2	9·80 ± 0·03	–	–	–	7·72 ± 0·07	–	8·5 ± 0·12	9·22 ± 0·05
E3	8·68 ± 0·01	7·69 ± 0·10	7·17 ± 0·02	–	7·88 ± 0·04	–	–	–
E4	6·60 ± 0·11	–	6·72 ± 0·10	–	–	–	–	–

Table 4 Prevalence and levels (Log *n* cells g^{-1}) of the different bifidobacterial species and groups analysed in the studied subjects

Bifidobacteria		Infants	Adults	Elderly
Genus	Prevalence	8/8	7/8	4/4
	Mean (SD)	9·74 (0·30)^{b}	8·88 (0·31)^{a}	8·37 (1·31)^{a}
Bifidobacterium longum group	Prevalence	8/8	7/8	2/4
	Mean (SD)	8·92 (0·60)^{b}	8·17 (0·48)^{a}	7·82 (0·18)^{a}
Bifidobacterium bifidum	Prevalence	3/8	1/8	2/4
	Mean (SD)	8·16 (0·74)	8·14 (0·00)	6·93 (0·33)
Bifidobacterium breve	Prevalence	2/8	1/8	0/4
	Mean (SD)	8·80 (0·19)	8·4 (0·00)	–
Bifidobacterium catenulatum group	Prevalence	4/8	3/8	3/4
	Mean (SD)	8·04 (1·47)	7·98 (0·19)	7·73 (0·15)
Bifidobacterium adolescentis	Prevalence	2/8	1/8	0/4
	Mean (SD)	6·58 (0·49)	7·45 (0·00)	–
Bifidobacterium angulatum	Prevalence	0/8	0/8	1/4
	Mean (SD)	–	–	8·50 (0·00)
Bifidobacterium dentium	Prevalence	0/8	0/8	1/4
	Mean (SD)	–	–	9·22 (0·00)

Means with different superscripts in the same row differ significantly ($P < 0·05$).

By using the seven species/group specific real-time PCR procedures described here it was possible to quantify almost 50% (average, 49%) of the total number of bifidobacteria detected with the genus specific quantitative PCR method, indicating that probably there are still other bifidobacterial species or groups present in these samples.

Discussion

It has previously been shown that more than 60% of the intestinal bacteria cannot usually be cultivated (Suau *et al.* 1999). With regard to bifidobacteria, important differences have been observed when comparing culture-dependent and culture-independent methods (Apajalahti *et al.* 2003), indicating the limitations of the former for the study of the intestinal microbiota and the need of easy, accurate and rapid culture-independent methods.

In this study easy, rapid and accurate culture-independent real-time PCR methods, based on the use of lanthanide chelates, combining the high specificity of fluorescent oligonucleotides and the sensitivity of PCR, were developed and used for the quantification of *Bifido-*

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bacterium species or groups in faecal samples. A good correlation has been previously shown between results obtained by this quantitative real-time PCR methodology and those obtained by FISH, the considered golden standard for intestinal bacteria quantification (Gueimonde *et al.* 2004b).

The specificity of the assays was shown by testing them with a collection of intestinal and food bacteria. Taking into account that specific probes were used together with the *Bifidobacterium* species-specific primers, a high specificity can be expected. Furthermore, the low detection limits ($\leq 10^5$) obtained by using the DNA extraction and PCR conditions described in this study, and taking into account that they could be improved by adjusting the number of PCR cycles or concentrating the extracted DNA, seems to demonstrate that these methods have potential for a very high sensitivity.

To test the applicability of these methods 20 human faecal samples from infants, adults and elderly persons were analysed. In general, the levels of total *Bifidobacterium* as well as those of the different bifidobacterial species or groups were in the range of those previously reported (Gueimonde *et al.* 2004b; Matsuki *et al.* 2004; Haarman and Knol 2005; Hopkins *et al.* 2005). The *Bifidobacterium* levels observed in infants were significantly higher than those found in adults. Differences between infants and adults were also found in the level of members of *Bif. longum* group. In accordance with previous reports (Matsuki *et al.* 1999; Haarman and Knol 2005) our study demonstrates that micro-organisms belonging to *Bif. longum* group (including *Bif. longum* and *Bifidobacterium infantis*) are the most widely found and the ones showing higher levels in infant faeces. Contrary to this Matsuki *et al.* (2004) found that in adults *Bif. adolescentis*, *Bif. catenulatum* and *Bif. longum* are the predominant species. In agreement with our results they reported *Bif. longum* as the most widely distributed species but *Bif. adolescentis* as the numerically predominant species. In our study *Bif. longum* and *Bif. catenulatum* were the most frequently found species in adults but *Bif. adolescentis* was present in only one sample. 16S rDNA sequence heterogeneity has been reported in *Bif. adolescentis* (Matsuki *et al.* 2004). Sequence divergences in the regions targeted by our oligonucleotides could lead to a failure of the DNA amplification and this could explain the lower prevalence and levels of *Bif. adolescentis* observed in our study. Bacterial quantification by real-time PCR can be influenced by differences in the number of rRNA operons, sequence heterogeneity or differential amplification of different DNA molecules (Schmalenberger *et al.* 2001). Mainly the rDNA genes are being used as the 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 in cases in which the 16S rDNA is not an adequate target.

The seven species/group specific real-time PCR procedures reported here allowed the identification and quantification of about half (average, 49%) of the total number of bifidobacteria detected with the genus specific quantitative PCR method. This coverage is in good agreement with the results obtained by Haarman and Knol (2005) using primers specific for the same species but targeting the rDNA intergenic region. These results appear to indicate that there is still an important proportion of bifidobacteria that cannot be detected and other *Bifidobacterium* species may be present in human faecal samples. In this regard, *Bifidobacterium lactis* has been reported to be more common in human faeces than previously expected, indicating that certain species could have been overlooked in the traditional culture studies (Bartosch *et al.* 2005).

We conclude that the quantitative real-time PCR methodologies described are accurate, rapid and easy methods for the quantification of bifidobacterial groups or species in human faeces. These methodologies 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.

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