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Running headline: Bifidobacteria enumeration by qPCR and FISH

Abstract

Aims: To detect and enumerate bifidobacteria in faeces with a new quantitative multiplex real-time PCR (qPCR) method and to compare the results obtained with fluorescence in *situ* hybridation (FISH) methods.

Methods and Results: A multiplex qPCR assay was developed which enabled the enumeration of *Bifidobacterium* spp. by targeting the bifidobacterial xylulose-5-phosphate/fructose-6-phosphate phosphoketolase gene (*xfp*) and total bacteria using universal Eub-primers targeting 16S rRNA gene from the domain Bacteria. The qPCR assay showed high sensitivity and specificity and a low detection limit of about $2{\cdot}5 \times 10^3$ bifidobacterial cells per g of faeces. The qPCR results were compared with fluorescence *in situ* hybridization (FISH) combined with microscopy or flow cytometry. No statistical difference among bifidobacterial counts averages measured with the three methods was observed. Total bacteria count averages were higher with the FISH method coupled with microscopic analyses compared to FISH with flow cytometry, whereas total cell numbers estimated by qPCR lied in between.

Conclusions: The new qPCR assay was shown to be sensitive, rapid and accurate for enumerating bifidobacteria in faeces.

Significance and Impact of the Study: This method is a valuable alternative for other molecular methods for detecting faecal bifidobacteria, especially when their counts are below the detection limit of the FISH methods.

Introduction

The intestinal microbiota is a complex bacterial community which affects the host in many ways and has considerable influences on the host biochemistry, physiology and immunology (Gill *et al.* 2006). Bifidobacteria are predominant members of the faecal microbiota in newborns, representing 60 to 90% of the total microbiota in breast-fed infants and approximatively 50% in formula-fed infants. They often become subdominant after the weaning period (representing 3-4% of the total adult faecal microbiota) (Rigottier-Gois *et al.* 2003; Vaughan *et al.* 2005). It is assumed that bifidobacteria by promoting or restoring a beneficial balance in the intestinal microbiota are implicated in the healthy status of breast-fed babies, less susceptible to intestinal disorders (Mata *et al.* 1976). Therefore many attempts have been made to increase their relative proportions in the intestinal tract, either by supplying bifidobacterial probiotic strains or prebiotic compounds stimulating their growth (Cummings and Macfarlane 2002; Picard *et al.* 2005).

To understand the role of the intestinal microbiota on health and well being of the host, a specific, sensitive and reliable characterization and enumeration method is required (Bartosch *et al.* 2004). A broad range of molecular and culture-independent techniques, using the 16S rRNA or its gene as a molecular fingerprint, are now available for identifying and enumerating specific populations present in the intestinal microbiota (Vaughan *et al.* 2005). Fluorescence *in situ* hybridization (FISH) is one of the most prevalent techniques for the quantitative assessment of the intestinal microbiota, however it is laborious and time-consuming when combined with detection by microscopy. This drawback has been improved by combining FISH with flow cytometry (Rigottier-Gois *et al.* 2003). However, the FISH technique still presents some problems such as low signal intensity due to low permeability and low activity of cells of certain species frequently associated with low amounts of RNA, as well as low sensitivity with a detection limit of approximatively 10^7 cell number g^{-1} of faeces

(dry weight) (Schwiertz *et al.* 2000). Quantitative real-time PCR (qPCR) is considered a valuable alternative to FISH for its high sensitivity. qPCR has been successfully applied for quantification of bacterial DNA in various environments and is increasingly used for detection and quantification of bacterial populations in the large intestine, including bifidobacteria. Several qPCR methods for the quantification of *Bifidobacterium* spp. in faecal samples have been described already (Gueimonde *et al.* 2004; Haarman and Knol 2005; Malinen *et al.* 2003; Matsuki *et al.* 2004; Penders *et al.* 2005). However, in most systems, 16S rRNA gene is used as the target gene, which can be problematic for quantification since it can be present in several copies in one single bacterial cell. Moreover 16S rRNA gene is associated with contamination risks and is shear sensitive during DNA isolation (von Wintzingerode *et al.* 1997). Therefore we developed a new qPCR assay targeting the *xfp* gene for the D-xylulose 5-phosphate / D-fructose 6-phosphate (X5P/F6P) phosphoketolase (Xfp), which is the key enzyme of the F6P -phosphoketolase pathway in bifidobacteria. Xfp has been widely used to characterized bifidobacteria (Orban and Patterson 2000). To date, a single copy of this gene has been detected in *Bifidobacterium longum* NCC2705 (Schell *et al.* 2002) and *Bifidobacterium longum* DJO10A (Lee *et al.* 2008). This new multiplex qPCR method, performed with a quencher oligonucleotide, enabled accurate and sensitive enumeration of *Bifidobacterium* spp. and total bacteria in human faeces when compared with the FISH method combined with microscopy or flow cytometry.

Materials and methods

Bidfidobacteria cultures

Bifidobacterium lactis DSM 10140 and *Bifidobacterium longum* DSM 20219 used in this study were cultivated in de Man Rogosa and Sharp (MRS, Biolife, Milan, Italy) broth incubated overnight at 37°C in anaerobic jars with an atmosphere generation system (Oxoid

AnaeroGenTM, Oxoid, Basel, Switzerland). Viable cell counts were measured in duplicate after appropriate dilution of the sample in peptonized water (8·5 g l^{-1} NaCl, 1 g l^{-1} peptone, pH 7·0), by spotting 20 μl of 10-fold diluted samples on MRS agar and incubating for 48 h at 37°C in anaerobic jars.

Faecal samples and bacterial cell fixation

Faecal samples were collected from 10 healthy people; two four-month-old infants (a formula-fed [FF]) and a breast-fed [BF]) infant), two two-year-old children and six (aged 26-51 year-old) adults. None had received antibiotic treatment within three months before faecal collection. Infants and children faecal samples were immediately placed in anaerobic jars with an atmosphere generation system (Oxoid AnaeroGenTM, Oxoid, Bâle, Switzerland) by the mothers and delivered to the laboratory within two hours after defecation. Adult faecal samples were directly collected in the laboratory in sterile tubes, placed in an anaerobic jar and processed within one hour after defecation. One part (4 g) of the faecal samples was diluted (1:10) in PBS (137 mmol l^{-1} NaCl, 2·7 mmol l^{-1} KCl, 10·1 mmol l^{-1} Na_2HPO_4, 1·8 mmol l^{-1} KH_2PO_4, pH 7·4) reduced with L-cystein (0·05%) and vortexed with four glass beads for three minutes for homogenization. The suspension was centrifuged at 300 ***g*** for one minute at 4°C to remove large particles, and one volume of this faecal suspension was added to one volume of ice-cold ethanol, and stored at -20°C until use. The second part of the faecal sample was aseptically aliquoted in Eppendorf tubes (200 mg per tube) and frozen at -25°C until DNA extraction.

Oligonucleotides

FISH probes, TaqMan probes (TM-probes) and primers used in this study are listed in Table 1 and were purchased from Microsynth (Balgach, Switzerland). The EUB338 probe, targeting a

conserved region of the bacterial 16S rRNA, was used as a positive control for total bacteria hybridization. The NON-EUB338 probe was used as control for non-specific oligonucleotide binding in flow cytometry, and Bif164 was used to quantify bifidobacteria. These three FISH probes were either labelled at their 5'- end with indodicarbocyanin (Cy5: Ex/Em 646/662 nm) for flow cytometry, or with indocarbocyanin (Cy3: Ex/Em 552/570 nm) for microscopic detection. Two DNA stains, DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich; Buchs, Switzerland) (Ex/Em 358/461 nm) and SYBR Green I (Invitrogen, Basel, Switzerland) (Ex/Em 497/520 nm) were used for detection of total bacteria by microscopy and flow cytometric analysis, respectively.

The TM-probe (Eub515r TMP) and Eub-primers (Eub340 F and Eub781 R) designed by Nadkarni *et al.* (2002) for detection of the domain Bacteria, were used for total bacteria detection in qPCR. The newly designed TM-probe (xfp-T) and xfp-primers (xfp-fw and xfp-rv, amplicon of 235 pb) used for the bifidobacterial *xfp* system detection with qPCR were designed using Primer 3 software (Rozen and Skaletsky 2000) on a partial alignment (497 bp) of known sequences encoding the D-xylulose 5-phosphate / D-fructose 6-phosphate (X5P/F6P) phosphoketolase gene (*xfp* system) specific for bifidobacteria (positions 2099 to 2595 of the *xfp* sequence of *B. lactis* [Acc. no. AJ293946]) (Meile *et al.* 2001). These new xfp-primers and TM-probes were aligned with available bacterial nucleotide sequences coding for the *xfp* gene present in the GenBank database by using Clustal W (Thompson *et al.* 1994) and with the BLAST function of the National Center for Biotechnology Information (GenBank) (http:www.ncbi.nlm.nih.gov/, last access in June 2008) for checking their specificity and cross-reactivity (Fig. 1). Primer and TM-probe concentrations used in the multiplex fluorescent qPCR for simultaneous detection of total and bifidobacterial populations were optimized as individual assays. However, because the 16S rRNA gene is more abundant than the bifidobacterial *xfp* gene within the intestinal ecosystem, we checked

different TM-probe and Eub-primer concentrations in the optimization assay and chose the lowest ones giving the best qPCR efficiency to be applied in the multiplex assay. Both TM-probes (xfp-T and Eub515r TMP) were labelled at their 5' end with 6-carboxyfluorescein (FAM) and 6-carboxy-1,4-dichloro-2,7-dichlorofluorescein (TET), respectively, and with 6-carboxytetramethyl-rhodamine (TAMRA) at their 3' end (Table 1).

Fluorescence *in situ* hybridization (FISH)

The same hybridization conditions (temperatures, lysozyme treatment, hybridization and washing buffers) already described by Rigottier-Gois *et al.* (2003) were used for all probes and for both flow cytometry and microscopic analysis to allow method comparisons. Each hybridization was done three times independently.

FISH analysis by microscopy

FISH analyses on glass slides were performed as described by Cinquin *et al.* (2006). Briefly, 10 μl of diluted (1/10 to 1/400 depending on sample and oligonucleotide probe tested) fixed faecal samples were spotted on 3-aminopropyl-triethoxysilane (APES, Sigma, Buchs, Switzerland) treated glass slides, dried at 37°C for 15 min, and dehydrated by successive immersions in ethanol series (50, 70 and 96% [v/v]) for three minutes. The samples were treated with 15 μl of lysozyme buffer (100 mmol l^{-1} Tris-HCl [pH 8], 50 mmol l^{-1} EDTA, lysozyme 105000 U ml^{-1} [Sigma, Buchs, Switzerland]), incubated 10 min at room temperature and dehydrated again. Ten microliters of hybridization buffer (0·9 mol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl [pH 8], 0·1% sodium dodecyl sulfate (SDS), 30% formamide) containing 5 ng μl^{-1} of Cy3-labeled oligonucleotides were added on each well and slides were incubated overnight at 35°C. After hybridization, the slides were rinsed in 50 ml of washing buffer (6·4 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl [pH 8], 5 mmol l^{-1} EDTA, 0·1% SDS) for 20

min at 37°C, rinsed with water and air dried. The slides were then mounted with citifluor AF1 (Citifluor Ltd, London, United Kingdom) to prevent fading of fluorescence and supplemented with DAPI (1 μg ml^{-1}) for total cell counts.

A radial cell concentration gradient is typically observed in wells. To minimize the counting error due to this specific distribution, bacterial concentration was calculated from the bacterial density corresponding to 15 annular regions as already described (Cinquin *et al.* 2006). Each assay was performed in triplicate.

FISH analysis by flow cytometry

Hybridization on microtubes was done according to the method described by Zoetendal *et al.* (2004) for flow cytometry with some modifications. Briefly, 100 μl of fixed faecal samples were centrifuged at 9000 ***g*** for three minutes. The pellet was washed once in Tris-EDTA buffer (100 mmol l^{-1} Tris-HCl [pH 8], 50 mmol l^{-1} EDTA), resuspended in Tris-EDTA containing lysozyme (105000 U ml^{-1}) and incubated for 10 min at room temperature. After removing the lysozyme solution and washing the cells with fresh hybridization buffer (0·9 mol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl [pH 8], 0·1% SDS, 30% formamide), the cells were resuspended in 160 μl of preheated hybridization buffer, thoroughly vortexed and divided into six aliquots of 25 μl. Aliquots (except the negative controls) were hybridized with 5 ng μl^{-1} of the appropriate labeled probe overnight at 35°C. After hybridization, 980 μl of warm (37°C) washing buffer (6·4 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl [pH 8], 5 mmol l^{-1} EDTA, 0·1% SDS) was added and the bacterial cells incubated for 20 min at 37°C. Bacterial cells were finally centrifuged at 9000 ***g*** for three minutes and the pellet resuspended in 2 ml of Tris-EDTA buffer supplemented with potassium citrate (100 mmol l^{-1} Tris-HCl [pH 7·4], 50 mmol l^{-1} EDTA, 30 mmol l^{-1} potassium citrate) (Lebaron *et al.* 1998) to obtain a final bacterial concentration of about 10^7 -10^8 bacteria per ml. To differentiate bacteria from non-bacterial

material, 0·5 μl of diluted (1:100) SYBR Green I was added per ml of sample and incubated at room temperature for at least 15 min.

In order to determine bacterial cell numbers, 100 μl of Flow-Count^{TM} Fluorospheres beads (Beckman Coulter International SA, Nyon, Switzerland) at known concentrations (1012 beads μl^{-1}) were added to 100 μl of diluted sample and 800 μl of Tris-EDTA buffer supplemented with potassium citrate, for each analysis, according to the manufacturer's instructions. Measurements were performed with a Cytomics FC 500 (Beckman Coulter International SA, Nyon, Switzerland) equipped with an air-cooled argon ion laser emitting 20 mW at 488 nm and a Red Solid State Diode laser emitting 25 mW at 633 nm. The 633 nm laser was used to detect red fluorescence of bacteria hybridized with Cy5-labeled probes (PMT4 in a 655 nm long pass filter) and the 488 nm laser was used to measure the forward angle light scatter (FSC), the side angle light scatter and the green fluorescence conferred by SYBR Green I (PMT1 in a 525 nm band pass filter). The minimum possible acquisition threshold on the machine was set in the forward scatter channel. The flow rate was set at 1000-3000 events per second and 100000 events were stored in list mode files. Data were analyzed using the CXP software (Beckman Coulter International SA, Nyon, Switzerland). A PMT1 histogram (green fluorescence) was used to evaluate the total number of bacteria stained with SYBR Green I. In this histogram, a gate which included the total number of bacterial cells in the sample was designed and used to make a PMT4 histogram (red fluorescence). This PMT4 histogram was then used to determine the *Bifidobacterium* cells marked with Cy5-labelled Bif164 probe. To quantify bifidobacteria and total cells, a correction was made to eliminate background fluorescence, measured using the negative control NON-EUB338-Cy5 probe, as described by Rigottier-Gois *et al.* (2003). Analyses were performed in triplicate.

Extraction of faecal DNA

DNA was extracted from 200 mg of faecal material using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before using the kit, preliminary experiments were done showing its low cross-contamination risk and the high quality and purity of isolated DNA (data not shown). To test the effect of faecal matrix on bifidobacterial DNA extraction, autoclaved and sonicated (15 min at high power) faecal samples were spiked prior to DNA isolation with ten-fold serial dilutions from $3{\cdot}9 \times 10^8$ CFU g^{-1} of *B. longum* DSM 20219 grown in MRS broth, a bifidobacterial species commonly detected in human faeces (Vaughan *et al.* 2002). DNA extracts were frozen at -20°C until analysis. The copy number of the *xfp* gene present in DNA isolated from spiked and autoclaved faeces without DNA spiking was then measured by qPCR. The efficiency of bifidobacterial DNA isolation in faeces was calculated from the ratio of cell numbers estimated by bifidobacterial *xfp* copy number using qPCR in spiked faeces after subtracting the bifidobacterial *xfp* copy number obtained in autoclaved faeces (background bifidobacteria DNA in autoclaved and sonicated faeces) to *B. longum* concentration spiked in faeces.

Quantitative multiplex fluorescent real-time PCR

A quantitative multiplex qPCR system was developed to simultaneously detect the bifidobacterial *xfp* gene and the conserved region of the 16S rRNA gene belonging to the domain Bacteria. Therefore, 1 μl of total DNA (concentration ranging from 1 to 10 ng μl^{-1}) extracted from 200 mg faecal sample was analyzed in 25 μl amplification reactions consisting of 1 × qPCR MasterMix (Eurogentec, Seraing, Belgium), 2% Tween 20, 0·9 μmol l^{-1} xfp-fw and xfp-rv primers, 0·06 μmol l^{-1} Eub340 F and Eub781 R primers, 0·225 μmol l^{-1} xfp-T TM-probe and 0·2 μmol l^{-1} Eub515r TMP (Table 1). To overcome bias caused by inhibitory compounds in faecal samples, DNA samples were at least diluted tenfold before amplification. The amplification conditions consisted of an initial cycle of 50°C for two

minutes, 95°C for 10 min, 45 cycles of 95°C for 15 sec and 60°C for one minute. The qPCR reactions were performed in MicroAmp optical plates sealed with optical adhesive covers (Applied Biosystems, Forster City, CA, USA). Thermal cycling, fluorescent data collection and data analysis were carried out with ABI Prism 7700 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's instructions.

Standard curves were made by plotting cycle threshold (C_T) values against dilutions of the quantitative standard (*xfp* PCR fragment or pLME21 plasmid DNA, see below) for which the number of gene copies was known. For total bacteria, cell numbers were directly estimated by the copy number of the 16S rRNA gene with no correction for the different rRNA-operon copy number (*rrn*), which is not realizable with the complex metabolic structure of intestinal microbiota, as described in Ott *et al*., (2004). For bifidobacteria, which harbor a single copy of *xfp* gene per cell, the measured C_T value was directly proportional to log *xfp* gene copy number and consequently to $\log_{10}$ cell number. The amplification efficiency was calculated from the slope of the standard curve using the following formula: $E = [(10^{-1/\text{slope}}/2) \times 100]$.

For the detection of total bacteria 16S rRNA gene in the qPCR assay, the plasmid pLME21 (Meile *et al.* 1997) containing the 16S rRNA gene from *B. lactis* was used as a quantitative standard. Therefore, the plasmid pLME21 (4·1 kb) was isolated from *Escherichia coli* JM109 using the FlexiPrep Kit (Amersham Biosciences, Otelfingen, Switzerland) and photometrically quantified using a Biophotometer (Eppendorf, Basel, Switzerland) as recommended by the manufacturer. Dilution series were done with 625 ng, 625 pg, 6250 fg, 62·5 fg, 0·625 fg, 0·312 fg, 0·156 fg, 0·0625 fg, 0·042 fg and 0·0042 fg plasmid DNA and used as template for the qPCR, one ng pLME21 plasmid DNA corresponding to $2{\cdot}4 \times 10^8$ 16S rRNA gene copies.

For bifidobacteria quantification, a PCR fragment of the *xfp* gene was used as an internal standard. Therefore, DNA was extracted from a pure culture of *B. lactis* using the methods of

Leenhouts *et al.* (1989). Amplification of this DNA was performed with primers xfp-fw and xfp-rv in a Biometra Gradient PCR apparatus (Biometra, Göttingen, Germany). One microliter DNA was used as template in a 50 μl reaction mixture composed of 1 × PCR Buffer (Euroclone, Milano, Italia), 0·2 mmol l^{-1} each of dATP, dCTP, dGTP, dTTP (Amersham Biosciences, Otelfingen, Switzerland), 1·5 mmol l^{-1} $MgCl_2$ (Euroclone), one μmol l^{-1} for each primers and 2·5 U TaqPolymerase (Eurogentec, Seraing, Belgium). The conditions for PCR consisted of an initial cycle of 95°C for three minutes, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for one minute, and a final polymerization step of seven minutes at 72°C. The PCR product (235 bp) was then purified using the GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Otelfingen, Switzerland) according to the manufacturer's instructions and photometrically quantified. Different dilutions of purified PCR product (625 ng, 625 pg, 6250 fg, 62·5 fg, 0·625 fg, 0·312 fg, 0·156 fg, 0·0625 fg, 0·024 fg and 0·00024 fg DNA) were used as a template for the standard curve. One nanogram of *xfp* PCR product corresponded to 4·2 × 10^9 *xfp* copies. The qPCR assays were replicated three times independently.

Statistics

A one-way analysis of variance (ANOVA) was performed using SPSS 13·0 for Windows (SPSS Inc., Chicago, IL, USA) to analyze the effects of the quantification method on bifidobacteria and total bacterial counts in faecal samples (qPCR, FISH coupled with microscopy or flow cytometry). Mean bacterial counts (n = 3) were compared using the student test with a probability level of $P < 0·05$. The effects of DNA-stains (DAPI and SYBR Green I) on total bacteria counts were compared using a paired-samples T-Test ($P < 0·05$). Data from different methods were compared by calculating intra-assay coefficients of variation (CV_{assay}), defined as standard deviation from triplicates divided by the mean and

expressed in percent. A coefficient of variation (CV_{total}, expressed in percent) including both the intra-assay error and differences for bacterial faecal composition among samples within an age group or for all tested faecal samples, was also calculated.

Results

Specificity, sensitivity and linearity of qPCR assays

Among 29 bifidobacterial species tested, 20 showed *xfp* gene sequences with a perfect alignment with the xfp-fw and xfp-rv primers and TM-probe, whereas one mismatch was observed for six species and two to four mismatches were observed in three species (Fig. 1). Among these three last species, *B. lactis* and *B. pseudolongum* exhibited three and four mismatches, respectively, but none were situated at the 3' end of the xfp-primers. Two closely related species *Parascardovia denticolens* and *Scardovia inopinata* presented two and five mismatches respectively. The alignment of the four bifidobacterial complete genomes (*B. longum* DJO10A, *B. longum* NCC2705, *B. adolescentis* ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697) in the GOLD database (http://genomesonline.org/, last access in December 2008) with the *xfp* gene sequence using the BLASTn function of NCBI confirmed the presence of one single hit per genome.

To maximize sensitivity and reproducibility of the assay, xfp-primer and TM-probe concentrations used in the *xfp* qPCR assay were optimized with pure cultures of *B. longum*. A concentration of 900 nmol l^{-1} for the primers xfp-fw and xfp-rv and 225 nmol l^{-1} for the TM-probe xfp-T gave the lowest C_T values and highest fluorescence (ΔR_n-values). The sensitivity and linearity of the assay were tested using different concentrations of *xfp* gene DNA fragment (produced by PCR) and pLME21 plasmid containing the 16S rDNA of *B. lactis*. Using the *xfp* DNA fragment of *B. lactis* as standard, the qPCR assay detection range was

between 0·0625 fg DNA (corresponding to 260 *B. lactis* cells) and 6250 fg DNA (corresponding to $2{\cdot}6 \times 10^7$ *B. lactis* cells). This assay for bifidobacteria was linear for C_T values from 16 to 32. The assay with the primer Eub340 F and Eub781 R showed a detection range from 62·5 fg to 625 pg DNA and was linear for C_T values between 16 and 31, corresponding to $1{\cdot}5 \times 10^4$ and $1{\cdot}5 \times 10^8$ copies of the 16S rRNA gene, respectively. Both standard curves (Fig. 2) used to quantify bifidobacteria and total bacteria showed a high linear correlation (R^2) coefficient of 0·997 and 0·999 for the *xfp* gene and pLME21 plasmid, respectively. The slopes of the standard lines for *xfp* and total bacteria were very similar, with -3·627 and -3·637, for calculated amplification efficiencies of 94·3 and 94·2%, respectively.

Bifidobacterium DNA recovery and qPCR sensitivity in faecal samples

Only small matrix-dependent effects were observed when comparing the mean *xfp* gene copy number in spiked autoclaved faecal samples and pure cultures of *B. longum*. After substracting the background bifidobacterial cell numbers measured in autoclaved faeces ($2{\cdot}5 \times 10^3$ *xfp* copies g^{-1}), the mean recovery rate of bifidobacterial DNA in spiked faecal samples was 78·2 ± 6·6% (*n*=3), regardless of the initial concentration of *B. longum*.

The sensitivity of the qPCR method for *Bifidobacterium* spp. was assessed by analyzing serial 10-fold dilutions of *B. longum* spiked in autoclaved faeces. The detection limits for the bifidobacteria and 16S rRNA gene (total bacteria) assay were $2{\cdot}5 \times 10^3$ and $2{\cdot}5 \times 10^5$ bacteria per g of faeces, respectively.

Enumeration of bifidobacteria and total bacteria using FISH coupled with microscopy

Significantly ($P < 0{\cdot}05$) higher total bacterial concentrations were obtained with DAPI staining (MS-DAPI) ranging from 10·8 to 11·7 $\log_{10}$ cell numbers g^{-1}, compared with EUB338 hybridization (MS-EUB338) ranging from 10·6 to 11·3 $\log_{10}$ cell numbers g^{-1} in all

age groups (Table 2). The mean proportion of bacterial cells hybridized with EUB338 (MS-EUB338) among DAPI-stained bacteria (MS-DAPI) calculated on the three age groups was 57%. The highest value (84%) was obtained for the BF infant.

The bifidobacterial populations (MS-Bif164) recovered in the BF infant faeces (10·8 $\log_{10}$ cell numbers g^{-1}) were much higher (plus 1·5 $\log_{10}$) than in adults (mean of 9·3 ± 0·3 $\log_{10}$ cell numbers g^{-1}) and children (mean of 9·2 ± 0·3 $\log_{10}$ cell numbers g^{-1}). In contrast the FF infant harbored a lower concentration of bifidobacteria (9·6 $\log_{10}$ cell numbers g^{-1}). The proportion of bifidobacteria (MS-Bif164) among total bacteria (MS-EUB338) ranged from 0·9 to 3·1% in adults; from 1·4 to 6·7% in children; and was 59 % in the BF infant and only 9% in the FF infant.

This microscopic detection method showed low intra-assay coefficients of variation (CV_{assay}, n = 3) ranging from 0·2 to 5·6% in MS-DAPI, MS-EUB338 and MS-Bif164 for all age groups. The highest values were found in children (3·8 and 5·6%) and FF infant (2·9 %) for bifidobacteria hybridization (MS-Bif164) (Table 2). The CV_{total} calculated for each age group were below 2·2% for total bacteria enumeration with MS-DAPI and MS-EUB338. Higher CV_{total} values were obtained with MS-Bif164 (ranging from 3 to 8·1%) due to the high inter-individual variation of bifidobacteria counts among individuals (especially with infants).

Enumeration of bifidobacteria and total bacteria using FISH coupled with flow cytometry

Typical flow histograms and dot-plots obtained for the BF infant faecal sample stained with SYBR Green I and hybridized with NONEUB338, EUB338, and Bif164 are shown in Fig. 3. Compared to the control measured with the NONEUB338 probe for non-specific hybridization, both probes (EUB338 and Bif164) as well as SYBR Green I gave a strong shift (more than 1·2 $\log_{10}$ units) in fluorescence intensity, allowing cells to be counted. About 85 to

95% of the total events were stained with SYBR Green I, whereas the NONEUB338 probe, used as negative control, generally gave non-specific binding below 1%. This value was then subtracted from the specific binding obtained with Bif164.

Data from flow cytometry quantification are presented in Table 2. The highest total bacteria concentrations analyzed with FISH coupled with flow cytometry were obtained with SYBR Green I (FCM-SYBR Green I) in the range 9·6 to 10·9 $\log_{10}$ cell numbers g^{-1}, whereas total bacteria enumerated with the EUB338 probe (FCM-EUB338) reached 9·2-10·7 $\log_{10}$ cell numbers g^{-1} and bifidobacteria concentrations (FCM-Bif164) ranged between 7·7 and10·6 $\log_{10}$ cell numbers g^{-1}. The mean proportion of total bacteria stained with SYBR Green I (FCM-SYBR Green I) among the ones hybridized with EUB338 (FCM-EUB338) was 58% for all age groups. Very high and low proportions were observed for BF infant (95%) and FF infant (10%), respectively.

In accordance to MS, the BF infant harbored a very high concentration of bifidobacteria (10·6 ± 0·2 $\log_{10}$ cell numbers g^{-1}), compared to adults (mean of 9·3 ± 0·4 $\log_{10}$ cell numbers g^{-1}) and children (mean 9·6 ± 0·2 $\log_{10}$ cell numbers g^{-1}). The bifidobacterial concentration (FCM-Bif164) obtained for the FF infant was particularly low (7·7 $\log_{10}$ cell numbers g^{-1}, 2 $\log_{10}$ below the value obtained with microscopic detection). The proportion of bifidobacteria (FCM-Bif164) among total bacterial populations (FCM-EUB338) represented 1·5 to 35% of cells in adults, compared to 91% in the BF infant, 47% and 69% in children and only 3% in the FF infant. The FF infant data showed high intra-assay variations (CV_{assay} of 2·7 % for total bacteria hybridization [FCM-EUB338] and 12·5% for bifidobacteria [FCM-Bif164]). For all other samples analysed with flow cytometry (FCM), CV_{assay} ranged from 0·1 to 6·4%. The CV_{total} in adults were similar for FCM-SYBR Green I (4·5%), FCM-EUB338 (4·1%) and FCM-Bif164 (4·8%). The CV_{total} for children were lower than that of adults for total bacteria and bifidobacteria.

Enumeration of bifidobacteria and total bacteria by qPCR

Total faecal bacterial population concentrations measured by the qPCR assay (qPCR Eub-primers) ranged from 10·4 to 10·9 $\log_{10}$ cell numbers g^{-1}, with the exception of one adult (A5) and the BF infant showing lower total bacterial concentrations (9·9 ± 0·1 $\log_{10}$ cell numbers g^{-1}). Total bacterial counts estimated with qPCR were lower (minus 0·5 in average) than MS-EUB338 counts, but higher than FCM-EUB338 values (plus 0·4 in average).

As for MS-Bif164, bifidobacteria concentrations measured with qPCR (qPCR xfp-primers) were similar for adults (mean of 9·7 ± 0·4 $\log_{10}$ cell numbers g^{-1}), children (mean of 9·6 ± 0·3 $\log_{10}$ cell numbers g^{-1}) and the FF infant (9·3 ± 0·5 $\log_{10}$ cell numbers g^{-1}). The BF infant harbored a much lower (8·6 ± 0·2 $\log_{10}$ cell numbers g^{-1}) bifidobacteria concentration estimated by qPCR than with MS-Bif164 and FCM-Bif164. The proportions of bifidobacteria (qPCR xfp-primers) among total bacterial population (qPCR Eub-primers) were 4% in BF infant, 8% in the FF infant, 9 and 12% in children, whereas they varied between 8% and 18% in adults.

The bifidobacterial xfp sequences were confirmed by sequencing a 235-bp fragment obtained by using both primers xfp-fw and xfp-rv with DNA extracted from the children faeces sample (as described in Materials and methods section). The DNA sequence had highest identities to the xfp gene of *Bifidobacterium pseudocatenulatum* strain DSM 20438 (97%) and *Bifidobacterium catenulatum* strain ATCC 27539 (96%). The *xfp* genes of other bifidobacteria showed identities in the range of 95-81% whereas xfp sequences of nonbifidobacteria (with high G+C contents in their genomes) had identities of less than 71% according to alignments with sequences in the GenBank. Except for the FF infants, results obtained with the xfp-primers showed low intra-assay coefficient of variations with values ranging from 0.6% to 2.7%.

Discussion

Bifidobacteria are one of the most important groups of the human intestinal microbiota. They are considered to be beneficial for the host and are therefore often used as probiotics in fermented foods and pharmaceutical products (Picard *et al.* 2005). However, a standard method for enumeration of this genus is still lacking and comparisons of results among different studies remain difficult.

In this study, a new qPCR method was developed for the detection of bifidobacteria in faecal samples, and the validity of the assay was assessed by comparison to FISH combined with microscopy and flow cytometry. Different to published qPCR methods for bifidobacteria quantification, we used the bifidobacterial *xfp* gene, which encodes the D-xylulose 5-phosphate / D-fructose 6-phosphate (X5P/F6P) phosphoketolase (Xfp). This gene is common to all previously described bifidobacteria and to date has only been detected as a single copy on the bifidobacterial genome. However, it is known that the *xfp* gene is not fully specific to bifdobacteria, as highly similar sequences have been found with the xylulose 5-phosphate phosphoketolase gene (*xpkA*) from *Lactobacillus plantarum* (Posthuma *et al.* 2002). The new xfp-primers and TM-probe were designed to target a partial sequence (positions 2099 to 2595) of the *xfp* gene of *B. lactis* [Acc no. AJ293946], which is highly specific to bifidobacteria. Alignment of new xfp-primers and TM-probe using the NCBI database showed high similarities for all bifidobacterial species and two other related species, *Parascardovia denticolens* and *Scardovia inopinata*, previously named *Bifidobacterium denticolens* and *B. inopinatum*, respectively (Jiang and Dong 2002). A multiplex qPCR assay was used, combining these newly designed xfp-primers and a previously published sequence of the 16S rRNA gene to detect total bacteria (Nadkarni *et al.* 2002). To perform two independent reactions in the same tube, xfp-primer concentrations were adjusted in order to obtain the most accurate threshold cycles for bifidobacteria detection. Therefore, Eub-primer

concentrations for total bacteria detection were minimized to avoid depletion of typical reactants available for amplifying minority species (Applied Biosystems 1998). The *xfp* qPCR assay was shown to be sensitive, detecting approximatively $2{\cdot}5 \times 10^3$ *Bifidobacterium* cells per g faeces, which is in the low range of detection limits (5×10^3 to 10^6 cell numbers g^{-1} faeces) reported in other studies targeting *Bifidobacterium* spp. in faecal samples (Gueimonde *et al.* 2004 ; Malinen 2003 ; Penders *et al.* 2005).

Faecal samples are problematic for PCR analysis since some bacterial cells are difficult to lyse and multiple substances can inhibit the polymerase enzyme (Matsuki *et al.* 2002). Recovery of *B. longum* in spiked autoclaved faecal samples was high (78%), indicating that the assay was not significantly influenced by potentially inhibitory components from stool samples and could specifically detect bifidobacterial DNA, even at low concentrations in a complex environment. However, the lysis efficiency of cells growing in MRS-medium may differ from that of commensal *B. longum* present in stool samples. The recovery rate of *B. longum* with the QIAamp DNA stool mini kit was 78·2% for all concentrations tested, in agreement with 78.8% reported by Ott *et al.* (2004) for the peqGOLD Stool DNA Kit. It seems that DNA was not completely destroyed by autoclaving and that low DNA fragments of the size of the amplicon (235 bp) remained since $2{\cdot}5 \times 10^3$ *xfp* copy g^{-1} were measured by qPCR in autoclaved faeces. Both standard curves developed to quantify the total bacterial population and *Bifidobacterium* spp. had slopes (-3·64 and -3·63, respectively) close to the optimal theoretical value of -3·32, which confirms a high efficiency for both assays.

When comparing total counts obtained for the two *in situ* hybridization methods and qPCR, several differences were observed. With microscopy, higher counts were obtained with DAPI compared with EUB338 probe, which targets *ca*. 60% of DAPI stained bacteria, as reported in other studies (Harmsen *et al.* 2002; Matsuki *et al.* 2004). This discrepancy is explained by different target molecules. DAPI stains chromosomal DNA, whereas EUB338 binds to

ribosomal RNA (Harmsen and Welling 2002). Moreover dead or metabolically inactive cells are not detected with EUB338 due to lack of rRNA, accessibility problems and limitations of the probe and hybridization techniques (Harmsen and Welling 2002; Porter and Pickup 2000). In addition, permeation treatments with lysozyme prior to hybridization with oligonucleotide probes might result in lysis of Gram-negatives preventing their detection (Harmsen and Welling 2002). The high detection rate with EUB338 among DAPI-stained cells in infants can be explained by a dominance of bifidobacteria in faecal microbiota of breast-fed infants, which are easily permeabilized and whose target rRNA is easily accessible. The high fluorescence intensity of SYBR Green I used with flow cytometry allowed a good discrimination between bacterial and non-bacterial material. Flow cytometry with EUB338 or SYBR Green I gave lower cell counts than microscopy. This can be explained by chains and cell clusters that produced a single event in flow cytometry analysis, whereas all individual cells were enumerated by microscopy (Amann *et al.* 1990a). Total cell counts obtained with qPCR were significantly higher than counts with FCM but lower than for microscopy (DAPI stained and EUB338 binding). This discrepancy with microscopy was likely due to several factors. First, the multiplex assay used in this study was optimized for the *xfp* qPCR assay and not for the total bacteria qPCR assay at the studied concentrations. Another possibility is that not all bacteria were equally lyzed during DNA isolation, or that a differential amplification of target sequence occurred, caused by differences in G+C composition of 16S rRNA gene. Genes with high G+C content dissociated less efficiently than genes with low G+C during the denaturation step in a previous study (von Wintzingerode *et al.* 1997). On the other hand, qPCR data ranged between the two FISH methods (EUB338), which in agreement with Ott *et al*. (2004) suggests that this method can be directly used for estimating total bacteria number with no correction of the results for *rrn* operon copy numbers which range from two

(*Lactobacillus acidophilus*) to 10-15 (*Clostridium difficile*) and are also related to the metabolic status and growth rate of the bacteria.

Mean group bifidobacterial concentrations with the three methods were no significantly different although few differences were observed on individual data, especially for the two infant faecal samples (BF and FF). Data obtained with Bif164 probe and microscopy showed good agreement with faecal levels of bifidobacteria reported by other authors using this method (Franks *et al.* 1998; Gueimonde *et al.* 2004; Harmsen *et al.* 1999; Langendijk *et al.* 1995; Rycroft *et al.* 2001) and were well-correlated with flow cytometry, except for the FF infant, which showed lower bifidobacterial concentration (< 2 log) with flow cytometry compared with microscopy. The significant difference between the two hybridization methods for the FF infant can be explained by difficulties in pelleting during sample preparation, causing a possible loss of bacteria. The bifidobacterial counts obtained with qPCR were in good agreement with results obtained in other studies and for most samples were not significantly different from FCM and microscopy. The higher counts obtained with qPCR for three subjects can be explained by targeting of extracted DNA, whereas FISH targets rRNA and its concentration depends on cell activity. In addition, the choice of probe is important. For hybridization, Bif164 was used, which failed to target all faecal *Bifidobacterium* species (Arrigoni *et al.* 2002), which is a major disadvantage compared to the *Bifidobacterium* genus specific hybridization probe lm3 (Kaufmann *et al.* 1997). The low detection of *Bifidobacterium* spp. and total bacteria in BF infant might be explained by a lower efficiency in DNA extraction or the presence of a qPCR-inhibiting substance in this sample.

All three culture-independent molecular methods used in this study were faster and more reliable than culture methods used to enumerate bacteria in a complex ecosystem such as faecal samples (Welling *et al.* 1997). The qPCR method has several advantages: it is rapid and sensitive, it measures very low levels of bifidobacteria compared to hybridization

techniques; and it can be automated. The PCR products can be quantified with better reproducibility while eliminating the need for post-PCR processing, thus preventing carry-over contamination (Fukushima *et al.* 2003). This method is therefore highly suitable for high throughput analyses, although it still remains expensive (Mangin *et al.* 2006). However, inaccuracies due to DNA isolation from population samples might introduce error and PCR-based methods are known to amplify DNA sequences from mixed populations especially in infants with different efficiencies which can lead to biases in bacterial counts (Requena *et al.* 2002). Hybridization techniques also have limits due to differences in target region accessibility, cell permeability and intracellular ribosome content. Moreover, the hybridization methods with microscopy and flow cytometry have a low sensitivity (Rinttila *et al.* 2004).

In this study, we quantified bifidobacteria in human faeces using a multiplex real time PCR assay targeting the *xfp* gene of bifidobacteria and a highly conserved region of the 16S rRNA gene. The TM-probes and primer developed to enumerate *Bifidobacterium* spp. by qPCR showed high sensitivity and specificity and can therefore be used to enumerate faecal bifidobacteria. Percentages of bifidobacteria present in faecal samples were markedly different by the three methods applied in this study, mainly due to differences in total bacterial counts. The bifidobacterial concentrations were similar in all three methods except for the infant samples which showed difficulties in pelleting during centrifugation steps prior to the flow cytometry analysis (FF) and a high inhibition of the qPCR (BF). Total counts obtained with flow cytometry and qPCR were lower than for microscopy, leading to higher bifidobacterial percentages. This emphasizes the importance of comparing cell concentration data (in $\log_{10}$ cell numbers g^{-1}) in addition to percentages when studying the composition of intestinal microbiota.

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List of figures

Figure 1 Multiple sequence alignment of a 235 bp region of *xfp* gene from *B. lactis* [Acc. No. AJ293946] with 29 bifidobacterial species and closest relatives.

Figure 2 Linear relation between threshold cycle (C_T) and serial dilution of *B. lactis xfp*-PCR-product and pLME21 plasmid DNA determined by qPCR (n = 3). The CT values obtained for the xfp gene and pLME21 plasmid were plotted against the base 10 logarithm of initial copy number of the gene and plasmid in the sample, respectively.

Figure 3 Flow cytometry dot-plots and histograms obtained by FISH coupled to flow cytometry analysis of the BF infant faecal sample. Fixed faecal samples were hybridized in (a) with NON-EUB338-Cy5 ; (b) with a combination of NON-EUB338-Cy5 and SYBR Green I ; (c) with a combination of Bif164-Cy5 and SYBR Green I; and (d) with a combination of EUB338-Cy5 and SYBR Green I. PMT1 histograms show the green fluorescence intensities conferred by SYBR Green I. The events within the bar correspond to bacterial cells stained with SYBR Green I. This region was designed according to the background level when NON-EUB338-Cy5 was used. PMT4 histograms show red fluorescence intensities conferred by the Cy5 probes. The events within the bar represent the proportion of bacterial cells hybridized with different group-species probes within the total bacterial cells stained with SYBR Green I. For proportion of cells, background fluorescence, measured with NON-EUB338-Cy5 was eliminated.

Table 1 Oligonucleotide primers and probes used in this study

Primer/Probes	Method	Sequence (5'- to 3'-end)	Target	Reference
EUB 338	FISH	GCTGCCTCCCGTAGGAGT	Total bacteria	(Amann *et al.* 1990b)
NONEUB 338	FISH	ACATCCTACGGGAGGC	Non specific binding	(Wallner *et al.* 1993)
Bif164	FISH	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk *et al.* 1995)
Eub340 F	qPCR	TCCTACGGGAGGCAGCAGT	Total bacteria	(Nadkarni *et al.* 2002)
Eub781 R	qPCR	GGACTACCAGGGTATCTAATCCTGTT	Total bacteria	(Nadkarni *et al.* 2002)
Eub515r TMP	qPCR	*TET*[a]-CGTATTACCGCGGCTGCTGGCAC-*TAMRA*[b]	Total bacteria	(Nadkarni *et al.* 2002)
xfp-fw	qPCR	ATCTTCGGACCBGAYGAGAC	*Bifidobacterium* spp.	This study
xfp-rv	qPCR	CGATVACGTGVACGAAGGAC	*Bifidobacterium* spp.	This study
xfp-T	qPCR	*FAM*[c]-ACGGCATCTGGAGCTCCTA-*TAMRA*[b]	*Bifidobacterium* spp.	This study

[a]TET: reporter stain for 16S system.

[b]TAMRA: quencher.

[c]FAM: reporter stain for *xfp* system.

Table 2 Total bacterial and *Bifidobacterium* counts (log_{10} cell number g^{-1} faeces) in faecal samples determined by FISH coupled with microscopy (MS) or flow cytometry (FCM), and quantitive PCR (qPCR). For each sample, means and intra assay coefficient of variation (CV_{assay} in %, in brackets) are shown for triplicate analyses. Means and total coefficient of variation (CV_{total} in %, in brachets) are reported for each group (adults, infants and children)

	Total bacteria (DNA-stains)			Total bacteria (probes / primers)			Bifidobacteria (probes / primers)		
Faeces[a]	MS	FCM	qPCR	MS	FCM	qPCR	MS	FCM	qPCR
	DAPI	SYBR Green I		EUB338	EUB338	Eub-primers[c]	Bif164	Bif164	xfp-primers
A1	11•7a (0•5)	10•9b (0•8)	-	11•3a (0•9)	10•5b (0•3)	10•7b (0•8)	9•3a (1•0)	9•8b (0•7)	9•9b (1•0)
A2	11•5a (0•5)	10·8a (4•0)	-	11•3a (0•5)	10•5b (1•2)	10•7c (0•5)	9•0a (0•8)	8•7a (1•2)	9•8b (1•2)
A3	11•5a (0•6)	10•6b (0•2)	-	11•2a (1•1)	10•6b (0•1)	10•7b (0•6)	9•6a (1•2)	9•6a (0•6)	9•8a (2•1)
A4	11•5a (0•3)	10•4b (0•8)	-	11•2a (0•4)	10•3b (1•2)	10•9c (0•9)	9•3a (1•6)	9•0b (1•0)	10•2c (0•6)
A5	11•0a (1•0)	9•6b (0•9)	-	10•7a (0•2)	9•5b (0•8)	9•9c (0•5)	8•9a (1•3)	9•0a (4•9)	8•9a (2•6)
A6	11•3a (1•1)	10•6a (6•4)	-	11•1a (0•7)	10•0b (1•5)	10•7b (0•8)	9•5a (1•8)	9•6a (2•5)	9•7a (1•2)
Means[b]	**11•4a (2•1)**	**10•5b (4•5)**	**-**	**11•1a (2•1)**	**10•2b (4•1)**	**10•6c (3•1)**	**9•3a (3•0)**	**9•3a (4•8)**	**9•7a (4•6)**
C1	11·1a (1·7)	10·2b (0·8)	-	10•8a (1•2)	10•1b (0•6)	10•7a (0•2)	8•9a (3•8)	9•7b (1•4)	9•8b (2•1)
C2	10·8a (0·4)	10·0b (0·8)	-	10•6a (1•6)	9•6b (4•8)	10•4a (0•4)	9•4a (5•6)	9•4a (2•7)	9•4a (2•7)
Means	**11·0a (1·7)**	**10·1b (1·7)**	**-**	**10•7a (1•7)**	**9•8b (3•4)**	**10•6a (3•1)**	**9•2a (3•2)**	**9•6a (2•2)**	**9•6a (3•4)**
BF	11·0a (0·4)	10·7a (1·5)	-	10·9a (1·8)	10•7a (1•5)	9•9b (1•3)	10•8a (1•1)	10•6a (1•7)	8•6b (2•4)
FF	11·0a (1·5)	10·2a (0·6)	-	10·6a (1•5)	9•2b (2•7)	10•4a (1•6)	9•6a (2•9)	7•7b (12•5)	9•3a (5•7)

[a]A : adult, BF : breast-fed infant, FF : formula-fed infant, C : child. [b]Means with different letters in a row significantly differ (effect of quantification method for each bacterial population tested) with the ANOVA test ($P < 0.05$). [c]Log of copy 16S rRNA gene number

1