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**Comparative study of a new quantitative real-time PCR targeting the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase bifidobacterial gene (*xfp*) in faecal samples with two fluorescence *in situ* hybridization methods**

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**Running headline:** Bifidobacteria enumeration by qPCR and FISH

1 **Abstract**

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

5 **Methods and Results:** A multiplex qPCR assay was developed which enabled the  
6 enumeration of *Bifidobacterium* spp. by targeting the bifidobacterial xylulose-5-  
7 phosphate/fructose-6-phosphate phosphoketolase gene (*xfp*) and total bacteria using universal  
8 Eub-primers targeting 16S rRNA gene from the domain Bacteria. The qPCR assay showed  
9 high sensitivity and specificity and a low detection limit of about  $2.5 \times 10^3$  bifidobacterial  
10 cells per g of faeces. The qPCR results were compared with fluorescence *in situ* hybridization  
11 (FISH) combined with microscopy or flow cytometry. No statistical difference among  
12 bifidobacterial counts averages measured with the three methods was observed. Total bacteria  
13 count averages were higher with the FISH method coupled with microscopic analyses  
14 compared to FISH with flow cytometry, whereas total cell numbers estimated by qPCR lied in  
15 between.

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

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

## 1 **Introduction**

2 The intestinal microbiota is a complex bacterial community which affects the host in many  
3 ways and has considerable influences on the host biochemistry, physiology and immunology  
4 (Gill *et al.* 2006). Bifidobacteria are predominant members of the faecal microbiota in  
5 newborns, representing 60 to 90% of the total microbiota in breast-fed infants and  
6 approximately 50% in formula-fed infants. They often become subdominant after the  
7 weaning period (representing 3-4% of the total adult faecal microbiota) (Rigottier-Gois *et al.*  
8 2003; Vaughan *et al.* 2005). It is assumed that bifidobacteria by promoting or restoring a  
9 beneficial balance in the intestinal microbiota are implicated in the healthy status of breast-fed  
10 babies, less susceptible to intestinal disorders (Mata *et al.* 1976). Therefore many attempts  
11 have been made to increase their relative proportions in the intestinal tract, either by  
12 supplying bifidobacterial probiotic strains or prebiotic compounds stimulating their growth  
13 (Cummings and Macfarlane 2002; Picard *et al.* 2005).

14 To understand the role of the intestinal microbiota on health and well being of the host, a  
15 specific, sensitive and reliable characterization and enumeration method is required (Bartosch  
16 *et al.* 2004). A broad range of molecular and culture-independent techniques, using the 16S  
17 rRNA or its gene as a molecular fingerprint, are now available for identifying and  
18 enumerating specific populations present in the intestinal microbiota (Vaughan *et al.* 2005).

19 Fluorescence *in situ* hybridization (FISH) is one of the most prevalent techniques for the  
20 quantitative assessment of the intestinal microbiota, however it is laborious and time-  
21 consuming when combined with detection by microscopy. This drawback has been improved  
22 by combining FISH with flow cytometry (Rigottier-Gois *et al.* 2003). However, the FISH  
23 technique still presents some problems such as low signal intensity due to low permeability  
24 and low activity of cells of certain species frequently associated with low amounts of RNA, as  
25 well as low sensitivity with a detection limit of approximately  $10^7$  cell number  $g^{-1}$  of faeces

1 (dry weight) (Schwiertz *et al.* 2000). Quantitative real-time PCR (qPCR) is considered a  
2 valuable alternative to FISH for its high sensitivity. qPCR has been successfully applied for  
3 quantification of bacterial DNA in various environments and is increasingly used for  
4 detection and quantification of bacterial populations in the large intestine, including  
5 bifidobacteria. Several qPCR methods for the quantification of *Bifidobacterium* spp. in faecal  
6 samples have been described already (Gueimonde *et al.* 2004; Haarman and Knol 2005;  
7 Malinen *et al.* 2003; Matsuki *et al.* 2004; Penders *et al.* 2005). However, in most systems,  
8 16S rRNA gene is used as the target gene, which can be problematic for quantification since it  
9 can be present in several copies in one single bacterial cell. Moreover 16S rRNA gene is  
10 associated with contamination risks and is shear sensitive during DNA isolation (von  
11 Wintzingerode *et al.* 1997). Therefore we developed a new qPCR assay targeting the *xfp*  
12 gene for the D-xylulose 5-phosphate / D-fructose 6-phosphate (X5P/F6P) phosphoketolase  
13 (Xfp), which is the key enzyme of the F6P -phosphoketolase pathway in bifidobacteria. Xfp  
14 has been widely used to characterized bifidobacteria (Orban and Patterson 2000). To date, a  
15 single copy of this gene has been detected in *Bifidobacterium longum* NCC2705 (Schell *et al.*  
16 2002) and *Bifidobacterium longum* DJO10A (Lee *et al.* 2008). This new multiplex qPCR  
17 method, performed with a quencher oligonucleotide, enabled accurate and sensitive  
18 enumeration of *Bifidobacterium* spp. and total bacteria in human faeces when compared with  
19 the FISH method combined with microscopy or flow cytometry.

20

## 21 **Materials and methods**

### 22 **Bifidobacteria cultures**

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

1 AnaeroGen™, Oxoid, Basel, Switzerland). Viable cell counts were measured in duplicate  
2 after appropriate dilution of the sample in peptonized water (8.5 g l<sup>-1</sup> NaCl, 1 g l<sup>-1</sup> peptone,  
3 pH 7.0), by spotting 20 µl of 10-fold diluted samples on MRS agar and incubating for 48 h at  
4 37°C in anaerobic jars.

5

## 6 **Faecal samples and bacterial cell fixation**

7 Faecal samples were collected from 10 healthy people; two four-month-old infants (a  
8 formula-fed [FF]) and a breast-fed [BF] infant), two two-year-old children and six (aged 26-  
9 51 year-old) adults. None had received antibiotic treatment within three months before faecal  
10 collection. Infants and children faecal samples were immediately placed in anaerobic jars with  
11 an atmosphere generation system (Oxoid AnaeroGen™, Oxoid, Bâle, Switzerland) by the  
12 mothers and delivered to the laboratory within two hours after defecation. Adult faecal  
13 samples were directly collected in the laboratory in sterile tubes, placed in an anaerobic jar  
14 and processed within one hour after defecation. One part (4 g) of the faecal samples was  
15 diluted (1:10) in PBS (137 mmol l<sup>-1</sup> NaCl, 2.7 mmol l<sup>-1</sup> KCl, 10.1 mmol l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 1.8  
16 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) reduced with L-cystein (0.05%) and vortexed with four glass beads  
17 for three minutes for homogenization. The suspension was centrifuged at 300 g for one  
18 minute at 4°C to remove large particles, and one volume of this faecal suspension was added  
19 to one volume of ice-cold ethanol, and stored at -20°C until use. The second part of the faecal  
20 sample was aseptically aliquoted in Eppendorf tubes (200 mg per tube) and frozen at -25°C  
21 until DNA extraction.

22

## 23 **Oligonucleotides**

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

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

10 The TM-probe (Eub515r TMP) and Eub-primers (Eub340 F and Eub781 R) designed by  
11 Nadkarni *et al.* (2002) for detection of the domain Bacteria, were used for total bacteria  
12 detection in qPCR. The newly designed TM-probe (xfp-T) and xfp-primers (xfp-fw and xfp-  
13 rv, amplicon of 235 pb) used for the bifidobacterial *xfp* system detection with qPCR were  
14 designed using Primer 3 software (Rozen and Skaletsky 2000) on a partial alignment (497  
15 bp) of known sequences encoding the D-xylulose 5-phosphate / D-fructose 6-phosphate  
16 (X5P/F6P) phosphoketolase gene (*xfp* system) specific for bifidobacteria (positions 2099 to  
17 2595 of the *xfp* sequence of *B. lactis* [Acc. no. AJ293946]) (Meile *et al.* 2001). These new  
18 xfp-primers and TM-probes were aligned with available bacterial nucleotide sequences  
19 coding for the *xfp* gene present in the GenBank database by using Clustal W (Thompson *et al.*  
20 1994) and with the BLAST function of the National Center for Biotechnology Information  
21 (GenBank) (<http://www.ncbi.nlm.nih.gov/>, last access in June 2008) for checking their  
22 specificity and cross-reactivity (Fig. 1). Primer and TM-probe concentrations used in the  
23 multiplex fluorescent qPCR for simultaneous detection of total and bifidobacterial  
24 populations were optimized as individual assays. However, because the 16S rRNA gene is  
25 more abundant than the bifidobacterial *xfp* gene within the intestinal ecosystem, we checked

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

6

### 7 **Fluorescence *in situ* hybridization (FISH)**

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

12

### 13 **FISH analysis by microscopy**

14 FISH analyses on glass slides were performed as described by Cinquin *et al.* (2006). Briefly,  
15 10  $\mu\text{l}$  of diluted (1/10 to 1/400 depending on sample and oligonucleotide probe tested) fixed  
16 faecal samples were spotted on 3-aminopropyl-triethoxysilane (APES, Sigma, Buchs,  
17 Switzerland) treated glass slides, dried at 37°C for 15 min, and dehydrated by successive  
18 immersions in ethanol series (50, 70 and 96% [v/v]) for three minutes. The samples were  
19 treated with 15  $\mu\text{l}$  of lysozyme buffer (100 mmol l<sup>-1</sup> Tris-HCl [pH 8], 50 mmol l<sup>-1</sup> EDTA,  
20 lysozyme 105000 U ml<sup>-1</sup> [Sigma, Buchs, Switzerland]), incubated 10 min at room  
21 temperature and dehydrated again. Ten microliters of hybridization buffer (0.9 mol l<sup>-1</sup> NaCl,  
22 20 mmol l<sup>-1</sup> Tris-HCl [pH 8], 0.1% sodium dodecyl sulfate (SDS), 30% formamide)  
23 containing 5 ng  $\mu\text{l}^{-1}$  of Cy3-labeled oligonucleotides were added on each well and slides were  
24 incubated overnight at 35°C. After hybridization, the slides were rinsed in 50 ml of washing  
25 buffer (6.4 mmol l<sup>-1</sup> NaCl, 20 mmol l<sup>-1</sup> Tris-HCl [pH 8], 5 mmol l<sup>-1</sup> EDTA, 0.1% SDS) for 20



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

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

8

### 9 **FISH analysis by flow cytometry**

10 Hybridization on microtubes was done according to the method described by Zoetendal *et al.*  
11 (2004) for flow cytometry with some modifications. Briefly, 100  $\mu\text{l}$  of fixed faecal samples  
12 were centrifuged at 9000  $g$  for three minutes. The pellet was washed once in Tris-EDTA  
13 buffer (100  $\text{mmol l}^{-1}$  Tris-HCl [pH 8], 50  $\text{mmol l}^{-1}$  EDTA), resuspended in Tris-EDTA  
14 containing lysozyme (105000  $\text{U ml}^{-1}$ ) and incubated for 10 min at room temperature. After  
15 removing the lysozyme solution and washing the cells with fresh hybridization buffer (0.9  
16  $\text{mol l}^{-1}$  NaCl, 20  $\text{mmol l}^{-1}$  Tris-HCl [pH 8], 0.1% SDS, 30% formamide), the cells were  
17 resuspended in 160  $\mu\text{l}$  of preheated hybridization buffer, thoroughly vortexed and divided into  
18 six aliquots of 25  $\mu\text{l}$ . Aliquots (except the negative controls) were hybridized with 5  $\text{ng } \mu\text{l}^{-1}$  of  
19 the appropriate labeled probe overnight at 35°C. After hybridization, 980  $\mu\text{l}$  of warm (37°C)  
20 washing buffer (6.4  $\text{mmol l}^{-1}$  NaCl, 20  $\text{mmol l}^{-1}$  Tris-HCl [pH 8], 5  $\text{mmol l}^{-1}$  EDTA, 0.1%  
21 SDS) was added and the bacterial cells incubated for 20 min at 37°C. Bacterial cells were  
22 finally centrifuged at 9000  $g$  for three minutes and the pellet resuspended in 2 ml of Tris-  
23 EDTA buffer supplemented with potassium citrate (100  $\text{mmol l}^{-1}$  Tris-HCl [pH 7.4], 50  $\text{mmol}$   
24  $\text{l}^{-1}$  EDTA, 30  $\text{mmol l}^{-1}$  potassium citrate) (Lebaron *et al.* 1998) to obtain a final bacterial  
25 concentration of about  $10^7$  - $10^8$  bacteria per ml. To differentiate bacteria from non-bacterial

1 material, 0.5  $\mu$ l of diluted (1:100) SYBR Green I was added per ml of sample and incubated  
2 at room temperature for at least 15 min.

3 In order to determine bacterial cell numbers, 100  $\mu$ l of Flow-Count<sup>TM</sup> Fluorospheres beads  
4 (Beckman Coulter International SA, Nyon, Switzerland) at known concentrations (1012 beads  
5  $\mu$ l<sup>-1</sup>) were added to 100  $\mu$ l of diluted sample and 800  $\mu$ l of Tris-EDTA buffer supplemented  
6 with potassium citrate, for each analysis, according to the manufacturer's instructions.

7 Measurements were performed with a Cytomics FC 500 (Beckman Coulter International SA,  
8 Nyon, Switzerland) equipped with an air-cooled argon ion laser emitting 20 mW at 488 nm  
9 and a Red Solid State Diode laser emitting 25 mW at 633 nm. The 633 nm laser was used to  
10 detect red fluorescence of bacteria hybridized with Cy5-labeled probes (PMT4 in a 655 nm  
11 long pass filter) and the 488 nm laser was used to measure the forward angle light scatter  
12 (FSC), the side angle light scatter and the green fluorescence conferred by SYBR Green I  
13 (PMT1 in a 525 nm band pass filter). The minimum possible acquisition threshold on the  
14 machine was set in the forward scatter channel. The flow rate was set at 1000-3000 events per  
15 second and 100000 events were stored in list mode files. Data were analyzed using the CXP  
16 software (Beckman Coulter International SA, Nyon, Switzerland). A PMT1 histogram (green  
17 fluorescence) was used to evaluate the total number of bacteria stained with SYBR Green I. In  
18 this histogram, a gate which included the total number of bacterial cells in the sample was  
19 designed and used to make a PMT4 histogram (red fluorescence). This PMT4 histogram was  
20 then used to determine the *Bifidobacterium* cells marked with Cy5-labelled Bif164 probe. To  
21 quantify bifidobacteria and total cells, a correction was made to eliminate background  
22 fluorescence, measured using the negative control NON-EUB338-Cy5 probe, as described by  
23 Rigottier-Gois *et al.* (2003). Analyses were performed in triplicate.

24

## 25 **Extraction of faecal DNA**

1 DNA was extracted from 200 mg of faecal material using the QIAamp DNA stool mini kit  
2 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before using the kit,  
3 preliminary experiments were done showing its low cross-contamination risk and the high  
4 quality and purity of isolated DNA (data not shown). To test the effect of faecal matrix on  
5 bifidobacterial DNA extraction, autoclaved and sonicated (15 min at high power) faecal  
6 samples were spiked prior to DNA isolation with ten-fold serial dilutions from  $3.9 \times 10^8$  CFU  
7  $g^{-1}$  of *B. longum* DSM 20219 grown in MRS broth, a bifidobacterial species commonly  
8 detected in human faeces (Vaughan *et al.* 2002). DNA extracts were frozen at  $-20^{\circ}C$  until  
9 analysis. The copy number of the *xfp* gene present in DNA isolated from spiked and  
10 autoclaved faeces without DNA spiking was then measured by qPCR. The efficiency of  
11 bifidobacterial DNA isolation in faeces was calculated from the ratio of cell numbers  
12 estimated by bifidobacterial *xfp* copy number using qPCR in spiked faeces after subtracting  
13 the bifidobacterial *xfp* copy number obtained in autoclaved faeces (background bifidobacteria  
14 DNA in autoclaved and sonicated faeces) to *B. longum* concentration spiked in faeces.

15

### 16 **Quantitative multiplex fluorescent real-time PCR**

17 A quantitative multiplex qPCR system was developed to simultaneously detect the  
18 bifidobacterial *xfp* gene and the conserved region of the 16S rRNA gene belonging to the  
19 domain Bacteria. Therefore, 1  $\mu l$  of total DNA (concentration ranging from 1 to 10  $ng \mu l^{-1}$ )  
20 extracted from 200 mg faecal sample was analyzed in 25  $\mu l$  amplification reactions consisting  
21 of 1  $\times$  qPCR MasterMix (Eurogentec, Seraing, Belgium), 2% Tween 20, 0.9  $\mu mol l^{-1}$  *xfp*-fw  
22 and *xfp*-rv primers, 0.06  $\mu mol l^{-1}$  Eub340 F and Eub781 R primers, 0.225  $\mu mol l^{-1}$  *xfp*-T TM-  
23 probe and 0.2  $\mu mol l^{-1}$  Eub515r TMP (Table 1). To overcome bias caused by inhibitory  
24 compounds in faecal samples, DNA samples were at least diluted tenfold before  
25 amplification. The amplification conditions consisted of an initial cycle of  $50^{\circ}C$  for two

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

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

15 For the detection of total bacteria 16S rRNA gene in the qPCR assay, the plasmid pLME21  
16 (Meile *et al.* 1997) containing the 16S rRNA gene from *B. lactis* was used as a quantitative  
17 standard. Therefore, the plasmid pLME21 (4.1 kb) was isolated from *Escherichia coli* JM109  
18 using the FlexiPrep Kit (Amersham Biosciences, Otelfingen, Switzerland) and  
19 photometrically quantified using a Biophotometer (Eppendorf, Basel, Switzerland) as  
20 recommended by the manufacturer. Dilution series were done with 625 ng, 625 pg, 6250 fg,  
21 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  
22 used as template for the qPCR, one ng pLME21 plasmid DNA corresponding to  $2.4 \times 10^8$  16S  
23 rRNA gene copies.

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

1 Leenhouts *et al.* (1989). Amplification of this DNA was performed with primers xfp-fw and  
2 xfp-rv in a Biometra Gradient PCR apparatus (Biometra, Göttingen, Germany). One  
3 microliter DNA was used as template in a 50  $\mu$ l reaction mixture composed of 1  $\times$  PCR  
4 Buffer (Euroclone, Milano, Italia), 0.2 mmol l<sup>-1</sup> each of dATP, dCTP, dGTP, dTTP  
5 (Amersham Biosciences, Otelfingen, Switzerland), 1.5 mmol l<sup>-1</sup> MgCl<sub>2</sub> (Euroclone),  
6 one  $\mu$ mol l<sup>-1</sup> for each primers and 2.5 U TaqPolymerase (Eurogentec, Seraing, Belgium). The  
7 conditions for PCR consisted of an initial cycle of 95°C for three minutes, 40 cycles of 95°C  
8 for 15 sec, 60°C for 30 sec, and 72°C for one minute, and a final polymerization step of seven  
9 minutes at 72°C. The PCR product (235 bp) was then purified using the GFX<sup>TM</sup> PCR DNA  
10 and Gel Band Purification Kit (Amersham Biosciences, Otelfingen, Switzerland) according to  
11 the manufacturer's instructions and photometrically quantified. Different dilutions of purified  
12 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  
13 fg and 0.00024 fg DNA) were used as a template for the standard curve. One nanogram of *xfp*  
14 PCR product corresponded to  $4.2 \times 10^9$  *xfp* copies. The qPCR assays were replicated three  
15 times independently.

16

## 17 **Statistics**

18 A one-way analysis of variance (ANOVA) was performed using SPSS 13.0 for Windows  
19 (SPSS Inc., Chicago, IL, USA) to analyze the effects of the quantification method on  
20 bifidobacteria and total bacterial counts in faecal samples (qPCR, FISH coupled with  
21 microscopy or flow cytometry). Mean bacterial counts ( $n = 3$ ) were compared using the  
22 student test with a probability level of  $P < 0.05$ . The effects of DNA-stains (DAPI and SYBR  
23 Green I) on total bacteria counts were compared using a paired-samples T-Test ( $P < 0.05$ ).  
24 Data from different methods were compared by calculating intra-assay coefficients of  
25 variation ( $CV_{\text{assay}}$ ), defined as standard deviation from triplicates divided by the mean and

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

4

## 5 **Results**

6

### 7 **Specificity, sensitivity and linearity of qPCR assays**

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

19 To maximize sensitivity and reproducibility of the assay, *xfp*-primer and TM-probe  
20 concentrations used in the *xfp* qPCR assay were optimized with pure cultures of *B. longum*. A  
21 concentration of 900 nmol l<sup>-1</sup> for the primers *xfp*-fw and *xfp*-rv and 225 nmol l<sup>-1</sup> for the TM-  
22 probe *xfp*-T gave the lowest  $C_T$  values and highest fluorescence ( $\Delta R_n$ -values). The sensitivity  
23 and linearity of the assay were tested using different concentrations of *xfp* gene DNA  
24 fragment (produced by PCR) and pLME21 plasmid containing the 16S rDNA of *B. lactis*.  
25 Using the *xfp* DNA fragment of *B. lactis* as standard, the qPCR assay detection range was

1 between 0.0625 fg DNA (corresponding to 260 *B. lactis* cells) and 6250 fg DNA  
2 (corresponding to  $2.6 \times 10^7$  *B. lactis* cells). This assay for bifidobacteria was linear for  $C_T$   
3 values from 16 to 32. The assay with the primer Eub340 F and Eub781 R showed a detection  
4 range from 62.5 fg to 625 pg DNA and was linear for  $C_T$  values between 16 and 31,  
5 corresponding to  $1.5 \times 10^4$  and  $1.5 \times 10^8$  copies of the 16S rRNA gene, respectively.  
6 Both standard curves (Fig. 2) used to quantify bifidobacteria and total bacteria showed a high  
7 linear correlation ( $R^2$ ) coefficient of 0.997 and 0.999 for the *xfp* gene and pLME21 plasmid,  
8 respectively. The slopes of the standard lines for *xfp* and total bacteria were very similar, with  
9 -3.627 and -3.637, for calculated amplification efficiencies of 94.3 and 94.2%, respectively.

10

#### 11 ***Bifidobacterium* DNA recovery and qPCR sensitivity in faecal samples**

12 Only small matrix-dependent effects were observed when comparing the mean *xfp* gene copy  
13 number in spiked autoclaved faecal samples and pure cultures of *B. longum*. After  
14 subtracting the background bifidobacterial cell numbers measured in autoclaved faeces ( $2.5$   
15  $\times 10^3$  *xfp* copies  $g^{-1}$ ), the mean recovery rate of bifidobacterial DNA in spiked faecal samples  
16 was  $78.2 \pm 6.6\%$  ( $n=3$ ), regardless of the initial concentration of *B. longum*.

17 The sensitivity of the qPCR method for *Bifidobacterium* spp. was assessed by analyzing serial  
18 10-fold dilutions of *B. longum* spiked in autoclaved faeces. The detection limits for the  
19 bifidobacteria and 16S rRNA gene (total bacteria) assay were  $2.5 \times 10^3$  and  $2.5 \times 10^5$  bacteria  
20 per g of faeces, respectively.

21

#### 22 **Enumeration of bifidobacteria and total bacteria using FISH coupled with microscopy**

23 Significantly ( $P < 0.05$ ) higher total bacterial concentrations were obtained with DAPI  
24 staining (MS-DAPI) ranging from 10.8 to 11.7  $\log_{10}$  cell numbers  $g^{-1}$ , compared with  
25 EUB338 hybridization (MS-EUB338) ranging from 10.6 to 11.3  $\log_{10}$  cell numbers  $g^{-1}$  in all

1 age groups (Table 2). The mean proportion of bacterial cells hybridized with EUB338 (MS-  
2 EUB338) among DAPI-stained bacteria (MS-DAPI) calculated on the three age groups was  
3 57%. The highest value (84%) was obtained for the BF infant.

4 The bifidobacterial populations (MS-Bif164) recovered in the BF infant faeces ( $10.8 \log_{10}$  cell  
5 numbers  $g^{-1}$ ) were much higher (plus  $1.5 \log_{10}$ ) than in adults (mean of  $9.3 \pm 0.3 \log_{10}$  cell  
6 numbers  $g^{-1}$ ) and children (mean of  $9.2 \pm 0.3 \log_{10}$  cell numbers  $g^{-1}$ ). In contrast the FF infant  
7 harbored a lower concentration of bifidobacteria ( $9.6 \log_{10}$  cell numbers  $g^{-1}$ ). The proportion  
8 of bifidobacteria (MS-Bif164) among total bacteria (MS-EUB338) ranged from 0.9 to 3.1% in  
9 adults; from 1.4 to 6.7% in children; and was 59 % in the BF infant and only 9% in the FF  
10 infant.

11 This microscopic detection method showed low intra-assay coefficients of variation ( $CV_{\text{assay}}$ ,  
12  $n = 3$ ) ranging from 0.2 to 5.6% in MS-DAPI, MS-EUB338 and MS-Bif164 for all age  
13 groups. The highest values were found in children (3.8 and 5.6%) and FF infant (2.9 %) for  
14 bifidobacteria hybridization (MS-Bif164) (Table 2). The  $CV_{\text{total}}$  calculated for each age group  
15 were below 2.2% for total bacteria enumeration with MS-DAPI and MS-EUB338. Higher  
16  $CV_{\text{total}}$  values were obtained with MS-Bif164 (ranging from 3 to 8.1%) due to the high inter-  
17 individual variation of bifidobacteria counts among individuals (especially with infants).

18

### 19 **Enumeration of bifidobacteria and total bacteria using FISH coupled with flow** 20 **cytometry**

21 Typical flow histograms and dot-plots obtained for the BF infant faecal sample stained with  
22 SYBR Green I and hybridized with NONEUB338, EUB338, and Bif164 are shown in Fig. 3.  
23 Compared to the control measured with the NONEUB338 probe for non-specific  
24 hybridization, both probes (EUB338 and Bif164) as well as SYBR Green I gave a strong shift  
25 (more than  $1.2 \log_{10}$  units) in fluorescence intensity, allowing cells to be counted. About 85 to



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

4 Data from flow cytometry quantification are presented in Table 2. The highest total bacteria  
5 concentrations analyzed with FISH coupled with flow cytometry were obtained with SYBR  
6 Green I (FCM-SYBR Green I) in the range 9.6 to 10.9 log<sub>10</sub> cell numbers g<sup>-1</sup>, whereas total  
7 bacteria enumerated with the EUB338 probe (FCM-EUB338) reached 9.2-10.7 log<sub>10</sub> cell  
8 numbers g<sup>-1</sup> and bifidobacteria concentrations (FCM-Bif164) ranged between 7.7 and 10.6  
9 log<sub>10</sub> cell numbers g<sup>-1</sup>. The mean proportion of total bacteria stained with SYBR Green I  
10 (FCM-SYBR Green I) among the ones hybridized with EUB338 (FCM-EUB338) was 58%  
11 for all age groups. Very high and low proportions were observed for BF infant (95%) and FF  
12 infant (10%), respectively.

13 In accordance to MS, the BF infant harbored a very high concentration of bifidobacteria  
14 (10.6 ± 0.2 log<sub>10</sub> cell numbers g<sup>-1</sup>), compared to adults (mean of 9.3 ± 0.4 log<sub>10</sub> cell numbers g<sup>-1</sup>)  
15 and children (mean 9.6 ± 0.2 log<sub>10</sub> cell numbers g<sup>-1</sup>). The bifidobacterial concentration  
16 (FCM-Bif164) obtained for the FF infant was particularly low (7.7 log<sub>10</sub> cell numbers g<sup>-1</sup>, 2  
17 log<sub>10</sub> below the value obtained with microscopic detection). The proportion of bifidobacteria  
18 (FCM-Bif164) among total bacterial populations (FCM-EUB338) represented 1.5 to 35% of  
19 cells in adults, compared to 91% in the BF infant, 47% and 69% in children and only 3% in  
20 the FF infant. The FF infant data showed high intra-assay variations (CV<sub>assay</sub> of 2.7 % for total  
21 bacteria hybridization [FCM-EUB338] and 12.5% for bifidobacteria [FCM-Bif164]). For all  
22 other samples analysed with flow cytometry (FCM), CV<sub>assay</sub> ranged from 0.1 to 6.4%. The  
23 CV<sub>total</sub> in adults were similar for FCM-SYBR Green I (4.5%), FCM-EUB338 (4.1%) and  
24 FCM-Bif164 (4.8%). The CV<sub>total</sub> for children were lower than that of adults for total bacteria  
25 and bifidobacteria.

1

## 2 **Enumeration of bifidobacteria and total bacteria by qPCR**

3 Total faecal bacterial population concentrations measured by the qPCR assay (qPCR Eub-  
4 primers) ranged from  $10^4$  to  $10^9$   $\log_{10}$  cell numbers  $g^{-1}$ , with the exception of one adult (A5)  
5 and the BF infant showing lower total bacterial concentrations ( $9.9 \pm 0.1$   $\log_{10}$  cell numbers  $g^{-1}$ ).  
6 Total bacterial counts estimated with qPCR were lower (minus 0.5 in average) than MS-  
7 EUB338 counts, but higher than FCM-EUB338 values (plus 0.4 in average).

8 As for MS-Bif164, bifidobacteria concentrations measured with qPCR (qPCR xfp-primers)  
9 were similar for adults (mean of  $9.7 \pm 0.4$   $\log_{10}$  cell numbers  $g^{-1}$ ), children (mean of  $9.6 \pm 0.3$   
10  $\log_{10}$  cell numbers  $g^{-1}$ ) and the FF infant ( $9.3 \pm 0.5$   $\log_{10}$  cell numbers  $g^{-1}$ ). The BF infant  
11 harbored a much lower ( $8.6 \pm 0.2$   $\log_{10}$  cell numbers  $g^{-1}$ ) bifidobacteria concentration  
12 estimated by qPCR than with MS-Bif164 and FCM-Bif164. The proportions of bifidobacteria  
13 (qPCR xfp-primers) among total bacterial population (qPCR Eub-primers) were 4% in BF  
14 infant, 8% in the FF infant, 9 and 12% in children, whereas they varied between 8% and 18%  
15 in adults.

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

## 1 **Discussion**

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

7 In this study, a new qPCR method was developed for the detection of bifidobacteria in faecal  
8 samples, and the validity of the assay was assessed by comparison to FISH combined with  
9 microscopy and flow cytometry. Different to published qPCR methods for bifidobacteria  
10 quantification, we used the bifidobacterial *xfp* gene, which encodes the D-xylulose 5-  
11 phosphate / D-fructose 6-phosphate (X5P/F6P) phosphoketolase (Xfp). This gene is common  
12 to all previously described bifidobacteria and to date has only been detected as a single copy  
13 on the bifidobacterial genome. However, it is known that the *xfp* gene is not fully specific to  
14 bifidobacteria, as highly similar sequences have been found with the xylulose 5-phosphate  
15 phosphoketolase gene (*xpkA*) from *Lactobacillus plantarum* (Posthuma *et al.* 2002). The new  
16 xfp-primers and TM-probe were designed to target a partial sequence (positions 2099 to  
17 2595) of the *xfp* gene of *B. lactis* [Acc no. AJ293946], which is highly specific to  
18 bifidobacteria. Alignment of new xfp-primers and TM-probe using the NCBI database  
19 showed high similarities for all bifidobacterial species and two other related species,  
20 *Parascardovia denticolens* and *Scardovia inopinata*, previously named *Bifidobacterium*  
21 *denticolens* and *B. inopinatum*, respectively (Jiang and Dong 2002). A multiplex qPCR assay  
22 was used, combining these newly designed xfp-primers and a previously published sequence  
23 of the 16S rRNA gene to detect total bacteria (Nadkarni *et al.* 2002). To perform two  
24 independent reactions in the same tube, xfp-primer concentrations were adjusted in order to  
25 obtain the most accurate threshold cycles for bifidobacteria detection. Therefore, Eub-primer

1 concentrations for total bacteria detection were minimized to avoid depletion of typical  
2 reactants available for amplifying minority species (Applied Biosystems 1998). The *xfp* qPCR  
3 assay was shown to be sensitive, detecting approximately  $2.5 \times 10^3$  *Bifidobacterium* cells  
4 per g faeces, which is in the low range of detection limits ( $5 \times 10^3$  to  $10^6$  cell numbers g<sup>-1</sup>  
5 faeces) reported in other studies targeting *Bifidobacterium* spp. in faecal samples (Gueimonde  
6 *et al.* 2004 ; Malinen 2003 ; Penders *et al.* 2005).

7 Faecal samples are problematic for PCR analysis since some bacterial cells are difficult to  
8 lyse and multiple substances can inhibit the polymerase enzyme (Matsuki *et al.* 2002).  
9 Recovery of *B. longum* in spiked autoclaved faecal samples was high (78%), indicating that  
10 the assay was not significantly influenced by potentially inhibitory components from stool  
11 samples and could specifically detect bifidobacterial DNA, even at low concentrations in a  
12 complex environment. However, the lysis efficiency of cells growing in MRS-medium may  
13 differ from that of commensal *B. longum* present in stool samples. The recovery rate of *B.*  
14 *longum* with the QIAamp DNA stool mini kit was 78.2% for all concentrations tested, in  
15 agreement with 78.8% reported by Ott *et al.* (2004) for the peqGOLD Stool DNA Kit. It  
16 seems that DNA was not completely destroyed by autoclaving and that low DNA fragments  
17 of the size of the amplicon (235 bp) remained since  $2.5 \times 10^3$  *xfp* copy g<sup>-1</sup> were measured by  
18 qPCR in autoclaved faeces. Both standard curves developed to quantify the total bacterial  
19 population and *Bifidobacterium* spp. had slopes (-3.64 and -3.63, respectively) close to the  
20 optimal theoretical value of -3.32, which confirms a high efficiency for both assays.

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

1 ribosomal RNA (Harmsen and Welling 2002). Moreover dead or metabolically inactive cells  
2 are not detected with EUB338 due to lack of rRNA, accessibility problems and limitations of  
3 the probe and hybridization techniques (Harmsen and Welling 2002; Porter and Pickup  
4 2000). In addition, permeation treatments with lysozyme prior to hybridization with  
5 oligonucleotide probes might result in lysis of Gram-negatives preventing their detection  
6 (Harmsen and Welling 2002). The high detection rate with EUB338 among DAPI-stained  
7 cells in infants can be explained by a dominance of bifidobacteria in faecal microbiota of  
8 breast-fed infants, which are easily permeabilized and whose target rRNA is easily accessible.  
9 The high fluorescence intensity of SYBR Green I used with flow cytometry allowed a good  
10 discrimination between bacterial and non-bacterial material. Flow cytometry with EUB338 or  
11 SYBR Green I gave lower cell counts than microscopy. This can be explained by chains and  
12 cell clusters that produced a single event in flow cytometry analysis, whereas all individual  
13 cells were enumerated by microscopy (Amann *et al.* 1990a). Total cell counts obtained with  
14 qPCR were significantly higher than counts with FCM but lower than for microscopy (DAPI  
15 stained and EUB338 binding). This discrepancy with microscopy was likely due to several  
16 factors. First, the multiplex assay used in this study was optimized for the *xfp* qPCR assay and  
17 not for the total bacteria qPCR assay at the studied concentrations. Another possibility is that  
18 not all bacteria were equally lysed during DNA isolation, or that a differential amplification  
19 of target sequence occurred, caused by differences in G+C composition of 16S rRNA gene.  
20 Genes with high G+C content dissociated less efficiently than genes with low G+C during the  
21 denaturation step in a previous study (von Wintzingerode *et al.* 1997). On the other hand,  
22 qPCR data ranged between the two FISH methods (EUB338), which in agreement with Ott *et*  
23 *al.* (2004) suggests that this method can be directly used for estimating total bacteria number  
24 with no correction of the results for *rrn* operon copy numbers which range from two

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

3 Mean group bifidobacterial concentrations with the three methods were no significantly  
4 different although few differences were observed on individual data, especially for the two  
5 infant faecal samples (BF and FF). Data obtained with Bif164 probe and microscopy showed  
6 good agreement with faecal levels of bifidobacteria reported by other authors using this  
7 method (Franks *et al.* 1998; Gueimonde *et al.* 2004; Harmsen *et al.* 1999; Langendijk *et al.*  
8 1995; Rycroft *et al.* 2001) and were well-correlated with flow cytometry, except for the FF  
9 infant, which showed lower bifidobacterial concentration (< 2 log) with flow cytometry  
10 compared with microscopy. The significant difference between the two hybridization methods  
11 for the FF infant can be explained by difficulties in pelleting during sample preparation,  
12 causing a possible loss of bacteria. The bifidobacterial counts obtained with qPCR were in  
13 good agreement with results obtained in other studies and for most samples were not  
14 significantly different from FCM and microscopy. The higher counts obtained with qPCR for  
15 three subjects can be explained by targeting of extracted DNA, whereas FISH targets rRNA  
16 and its concentration depends on cell activity. In addition, the choice of probe is important.  
17 For hybridization, Bif164 was used, which failed to target all faecal *Bifidobacterium* species  
18 (Arrigoni *et al.* 2002), which is a major disadvantage compared to the *Bifidobacterium* genus  
19 specific hybridization probe Im3 (Kaufmann *et al.* 1997). The low detection of  
20 *Bifidobacterium* spp. and total bacteria in BF infant might be explained by a lower efficiency  
21 in DNA extraction or the presence of a qPCR-inhibiting substance in this sample.

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

1 techniques; and it can be automated. The PCR products can be quantified with better  
2 reproducibility while eliminating the need for post-PCR processing, thus preventing carry-  
3 over contamination (Fukushima *et al.* 2003). This method is therefore highly suitable for high  
4 throughput analyses, although it still remains expensive (Mangin *et al.* 2006). However,  
5 inaccuracies due to DNA isolation from population samples might introduce error and PCR-  
6 based methods are known to amplify DNA sequences from mixed populations especially in  
7 infants with different efficiencies which can lead to biases in bacterial counts (Requena *et al.*  
8 2002). Hybridization techniques also have limits due to differences in target region  
9 accessibility, cell permeability and intracellular ribosome content. Moreover, the  
10 hybridization methods with microscopy and flow cytometry have a low sensitivity (Rinttila *et*  
11 *al.* 2004).

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

25

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3

## 4 **References**

5 Amann, R. I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A.

6 (1990a) Combination of 16S rRNA-targeted oligonucleotide probes with flow  
7 cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**,  
8 1919-1925.

9 Amann, R. I., Krumholz, L. and Stahl, D.A. (1990b) Fluorescent-oligonucleotide probing of  
10 whole cells for determinative, phylogenetic, and environmental studies in  
11 microbiology. *J Bacteriol* **172**, 762-770.

12 Applied Biosystems (1998) User bulletin #5. ABI Prism 7,700 sequence detection system.

13 Arrigoni, E., Jörger, F., Kollöffel, B., Roulet, I., Herensperger, M., Meile L. and Amado, R.  
14 (2002) *In vitro* fermentability of a commercial wheat germ preparation and its impact  
15 on the growth of bifidobacteria. *Food Res Int* **35**, 475-481.

16 Bartosch, S., Fite, A., Macfarlane, G.T. and McMurdo, M.E. (2004) Characterization of  
17 bacterial communities in faeces from healthy elderly volunteers and hospitalized  
18 elderly patients by using real-time PCR and effects of antibiotic treatment on the  
19 faecal microbiota. *Appl Environ Microbiol* **70**, 3575-3581.

20 Cinquin, C., Le Blay, G., Fliss, I. and Lacroix, C. (2006) New three-stage *in vitro* model for  
21 infant colonic fermentation with immobilized faecal microbiota. *FEMS Microbiol*  
22 *Ecol.* **57**, 324-336.

23 Cummings, J. H. and Macfarlane, G.T. (2002) Gastrointestinal effects of prebiotics. *Br J Nutr*  
24 **87** Suppl 2, S145-S151.



1 Delcenserie, V., Bechoux, N., China, B., Daube, G. and Gavini, F. (2005) A PCR method for  
2 detection of bifidobacteria in raw milk and raw milk cheese: comparison with culture-  
3 based methods. *J Microbiol Methods* **61**, 55-67.

4 Franks, A.H., Harmsen, H.J., Raangs, G.C., Jansen, G.J., Schut, F. and Welling, G.W.  
5 (1998) Variations of bacterial populations in human faeces measured by fluorescence  
6 *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes.  
7 *Appl Environ Microbiol* **64**, 3336-3345.

8 Fukushima, H., Tsunomori, Y. and Seki, R. (2003) Duplex real-time SYBR green PCR assays  
9 for detection of 17 species of food- or waterborne pathogens in stools. *J Clin*  
10 *Microbiol* **41**, 5134-5146.

11 Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I.,  
12 Relman, D.A., Fraser-Liggett, C.M. and Nelson, K.E. (2006) Metagenomic analysis of  
13 the human distal gut microbiome. *Science* **312**, 1355-1359.

14 Gueimonde, M., Tolkko, S., Korpimaki, T. and Salminen, S. (2004) New real-time  
15 quantitative PCR procedure for quantification of bifidobacteria in human faecal  
16 samples. *Appl Environ Microbiol* **70**, 4165-4169.

17 Haarman, M. and Knol, J. (2005) Quantitative real-time PCR assays to identify and quantify  
18 faecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. *Appl*  
19 *Environ Microbiol* **71**, 2318-2324.

20 Harmsen, H. J. M., Gibson, G. R., Elfferich, P., Raangs, G. C., Wildeboer-Veloo, A. C. M.,  
21 Argaziz, A., Roberfroid, M. B. and Welling, G. W. (1999) Comparison of viable cell  
22 counts and fluorescence *in situ* hybridization using specific rRNA-based probes for  
23 the quantification of human fecal bacteria. *FEMS Microbiol Lett* **183**, 125-129.

1 Harmsen, H., Elfferich, P., Schut, F. and Welling, G. (1999) A 16S rRNA-targeted probe for  
2 detection of lactobacilli and enterococci in faecal samples by fluorescence *in situ*  
3 hybridization. *Microb Ecol Health Dis* **11**, 3-12.

4 Harmsen, H.J.M., Raangs, G.C., He, T., Degener, J.E. and Welling, G.W. (2002) Extensive  
5 set of 16S rRNA-based probes for detection of bacteria in human faeces. *Appl Environ*  
6 *Microbiol* **68**, 2982-2990.

7 Harmsen, J.M. and Welling, G.W. (2002) Fluorescence *in situ* hybridization as a tool in  
8 intestinal bacteriology. In *Probiotics and prebiotics -Where are we going?* ed  
9 Tannock, G.W. pp. 41-58. Dunedin: Caister Academic Press.

10 Jian, W. and Dong, X. (2002) Transfer of *Bifidobacterium inopinatum* and *Bifidobacterium*  
11 *denticolens* to *Scardovia inopinata* gen. nov., comb. nov., and *Parascardovia*  
12 *denticolens* gen. nov., comb. nov., respectively. *Int J Syst Evol Microbiol* **52**, 809-812.

13 Kaufmann, P., Pfefferkorn, A., Teuber, M. and Meile, L. (1997) Identification and  
14 quantification of *Bifidobacterium* species isolated from food with genus-specific 16S  
15 rRNA-targeted probes by colony hybridization and PCR. *Appl Environ Microbiol* **63**,  
16 1268-1273.

17 Klijn, A., Mercenier, A. and Arigoni, F. (2005) Lessons from the genomes of bifidobacteria.  
18 *FEMS Microbiol Rev* **29**, 491-509.

19 Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H.  
20 and Welling, G.W. (1995) Quantitative fluorescence *in situ* hybridization of  
21 *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its  
22 application in faecal samples. *Appl Environ Microbiol* **61**, 3069-3075.

23 Lebaron, P., Parthuisot, N. and Catala, P. (1998) Comparison of blue nucleic acid dyes for  
24 flow cytometric enumeration of bacteria in aquatic systems. *Appl Environ Microbiol*  
25 **64**, 1725-1730.

- 1 Lee, J.H., Karamychev, V.N., Kozyavkin, S.A., Mills, D., Pavlov, A.R., Pavlova, N.V.,  
2 Polouchine, N.N., Richardson ,P.M., Shakova, V.V., Slesarev, A.I., Weimer, B. and  
3 O'Sullivan, D.J. (2008) Comparative genomic analysis of the gut bacterium  
4 *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture  
5 growth. *BMC Genomics* **9**, 247.
- 6 Leenhouts, K.J., Kok, J. and Venema, G. (1989) Campbell-like integration of heterologous  
7 plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. *Appl Environ*  
8 *Microbiol* **55**, 394-400.
- 9 Malinen, E., Kassinen, A., Rinttila, T. and Palva, A. (2003) Comparison of real-time PCR  
10 with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-  
11 targeted oligonucleotide probes in quantification of selected faecal bacteria.  
12 *Microbiology* **149**, 269-277.
- 13 Mangin, I., Suau, A., Magne, F., Garrido, D., Gotteland, M., Neut, C. and Pochart, P. (2006)  
14 Characterization of human intestinal bifidobacteria using competitive PCR and PCR-  
15 TTGE. *FEMS Microbiol Ecol* **55**, 28-37.
- 16 Masco, L., Vanhoutte, T., Temmerman, R., Swings, J. and Huys, G. (2007) Evaluation of  
17 real-time PCR targeting the 16S rRNA and *recA* genes for the enumeration of  
18 bifidobacteria in probiotic products. *Int J Food Microbiol* **113**, 351-357.
- 19 Mata, L. J., Kronmal, R. A., Garcia, B., Butler, W., Urrutia, J. J. and Murillo, S. (1976)  
20 Breast-feeding, weaning and the diarrhoeal syndrome in a Guatemalan Indian village.  
21 *Ciba Found. Symp* 311-338.
- 22 Matsuki, T., Watanabe, K., Fujimoto, J., Kado, Y., Takada, T., Matsumoto, K. and Tanaka, R.  
23 (2004) Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for  
24 analysis of human intestinal bifidobacteria. *Appl Environ Microbiol* **70**, 167-173.

1 Matsuki, T., Watanabe, K. and Tanaka, R. (2002) Genus- and species-specific PCR primers  
2 for the detection and identification of bifidobacteria. In *Probiotics and prebiotics -*  
3 *Where are we going?* ed. Tannock, G.W. pp.85-105. Dunedin: Caister Academic  
4 Press.

5 Meile, L., Ludwig, W., Rueger, U., Gut, C., Kaufmann, P., Dasen, G., Wenger, S. and Teuber,  
6 M. (1997) *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species  
7 isolated from fermented milk. *Syst Appl Microbiol* **20**, 57-64.

8 Meile, L., Rohr, L.M., Geissmann, T.A., Herensperger, M. and Teuber, M. (2001)  
9 Characterization of the D-xylulose 5-phosphate/D-fructose 6-phosphate  
10 phosphoketolase gene (*xfp*) from *Bifidobacterium lactis*. *J Bacteriol* **183**, 2929-2936.

11 Nadkarni, M.A., Martin, F.E., Jacques, N.A. and Hunter, N. (2002) Determination of bacterial  
12 load by real-time PCR using a broad-range (universal) probe and primers set.  
13 *Microbiology* **148**, 257-266.

14 Orban, J. I. and Patterson, J.A. (2000) Modification of the phosphoketolase assay for rapid  
15 identification of bifidobacteria. *J Microbiol Methods* **40**, 221-224.

16 Ott, S.J., Musfeldt, M., Ullmann, U., Hampe, J. and Schreiber, S. (2004) Quantification of  
17 intestinal bacterial populations by real-time PCR with a universal primer set and minor  
18 groove binder probes, a global approach to the enteric flora. *J Clin Microbiol* **42**,  
19 2566-2572.

20 Penders, J., Vink, C., Driessen, C., London, N., Thijs, C. and Stobberingh, E.E. (2005)  
21 Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in  
22 faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS*  
23 *Microbiol Lett* **243**, 141-147.

- 1 Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F. and Matuchansky, C. (2005)  
2 Review article, bifidobacteria as probiotic agents -- physiological effects and clinical  
3 benefits. *Aliment Pharmacol Ther* **22**, 495-512.
- 4 Porter, J. and Pickup, R.W. (2000) Nucleic acid-based fluorescent probes in microbial  
5 ecology, application of flow cytometry. *J Microbiol Methods* **42**, 75-79.
- 6 Posthuma, C.C., Bader, R., Engelmann, R., Postma, P.W., Hengstenberg, W. and Pouwels,  
7 P.H. (2002) Expression of the xylulose 5-phosphate phosphoketolase gene, *xpkA*, from  
8 *Lactobacillus pentosus* MD363 is induced by sugars that are fermented via the  
9 phosphoketolase pathway and is repressed by glucose mediated by CcpA and the  
10 mannose phosphoenolpyruvate phosphotransferase system. *Appl Environ Microbiol*  
11 **68**, 831-837.
- 12 Requena, T., Burton, J., Matsuki, T., Munro, K., Simon, M.A., Tanaka, R., Watanabe, K. and  
13 Tannock, G.W. (2002) Identification, detection, and enumeration of human  
14 *Bifidobacterium* species by PCR targeting the transaldolase gene. *Appl Environ*  
15 *Microbiol* **68**, 2420-2427.
- 16 Rigottier-Gois, L., Le Bourhis, A.G., Gramet, G., Rochet, V. and Dore, J. (2003) Fluorescent  
17 hybridisation combined with flow cytometry and hybridisation of total RNA to  
18 analyse the composition of microbial communities in human faeces using 16S rRNA  
19 probes. *FEMS Microb Ecol* **43**, 237-245.
- 20 Rinttila, T., Kassinen, A., Malinen, E., Krogius, L. and Palva, A. (2004) Development of an  
21 extensive set of 16S rRNA gene-targeted primers for quantification of pathogenic and  
22 indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* **97**, 1166-  
23 1177.
- 24 Rochet, V.L., Rigottier-Gois, L., Rabot, S. and Dore, J. (2004) Validation of fluorescence *in*  
25 *situ* hybridization combined with flow cytometry for assessing interindividual

1 variation in the composition of human faecal microflora during long-term storage of  
2 samples. *J Microbiol Methods* **59**, 263-270.

3 Rozen, S. and Skaletsky, H.J. (2000) Primer 3 on the www for general users and for biologist  
4 programmers. In *Bioinformatic methods and protocols* vol. 1. ed. Krawetz, S.,  
5 Misener, S. pp. 365-386. Totowa: Humana Press.

6 Rycroft, C.E., Jones, M.R., Gibson, G.R. and Rastall, R.A. (2001) A comparative *in vitro*  
7 evaluation of the fermentation properties of prebiotic oligosaccharides. *J Appl*  
8 *Microbiol* **91**, 878-887.

9 Schell, M.A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M.C.,  
10 Desiere, F., Bork, P., Delley, M., Pridmore, R.D. and Arigoni, F (2002) The genome  
11 sequence of *Bifidobacterium longum* reflects its adaptation to the human  
12 gastrointestinal tract. *Proc Natl Acad Sci USA* **99**, 14422-14427.

13 Schwiertz, A., Le Blay, G. and Blaut, M. (2000) Quantification of different *Eubacterium* spp.  
14 in human faecal samples with species-specific 16S rRNA-targeted oligonucleotide  
15 probes. *Appl Environ Microbiol* **66**, 375-382.

16 Thompson, J.D., Higgins, D.G. and Gibson T.J. (1994) CLUSTAL W: improving the  
17 sensitivity of progressive multiple sequence alignment through sequence weighting,  
18 position-specific gap penalties and weight matrix choice. *Nucl Acids Res* **22**, 4673-  
19 4680.

20 Vaughan, E.E., de Vries, M.C., Zoetendal, E.G., Ben-Amor, K., Akkermans, A.D. and de  
21 Vos, W.M. (2002) The intestinal LABs. *Antonie Van Leeuwenhoek* **82**, 341-352.

22 Vaughan, E.E., Heilig, H.G., Ben-Amor, K. and de Vos, W.M. (2005) Diversity, vitality and  
23 activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular  
24 approaches. *FEMS Microbiol Rev* **29**, 477-490.

- 1 von Wintzingerode, F., Gobel, U.B. and Stackebrandt, E. (1997) Determination of microbial  
2 diversity in environmental samples, pitfalls of PCR-based rRNA analysis. *FEMS*  
3 *Microbiol Rev* **21**, 213-229.
- 4 Wallner, G., Amann, R. and Beisker, W. (1993) Optimizing fluorescence *in situ* hybridization  
5 with ribosomal-RNA-targeted oligonucleotide probes for flow cytometric  
6 identification of microorganisms. *Cytometry* **14**, 136-143.
- 7 Welling, G. W., Elfferich, P., Raangs, G. C., Wildeboer-Veloo, A. C., Jansen, G. J. and  
8 Degener, J. E. (1997) 16S ribosomal RNA-targeted oligonucleotide probes for  
9 monitoring of intestinal tract bacteria. *Scand J Gastroenterol Suppl* **222**, 17-19.
- 10 Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie and H. R. Gaskins (2004) Molecular  
11 ecological analysis of the gastrointestinal microbiota: a review. *J Nutr* **134**, 465-472.

1

2 **List of figures**

3

4 **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.

6

7 **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.

11

12 **Figure 3** Flow cytometry dot-plots and histograms obtained by FISH coupled to flow  
13 cytometry analysis of the BF infant faecal sample. Fixed faecal samples were hybridized in  
14 (a) with NON-EUB338-Cy5 ; (b) with a combination of NON-EUB338-Cy5 and SYBR Green I  
15 ; (c) with a combination of Bif164-Cy5 and SYBR Green I; and (d) with a combination of  
16 EUB338-Cy5 and SYBR Green I. PMT1 histograms show the green fluorescence intensities  
17 conferred by SYBR Green I. The events within the bar correspond to bacterial cells stained  
18 with SYBR Green I. This region was designed according to the background level when NON-  
19 EUB338-Cy5 was used. PMT4 histograms show red fluorescence intensities conferred by  
20 the Cy5 probes. The events within the bar represent the proportion of bacterial cells  
21 hybridized with different group-species probes within the total bacterial cells stained with  
22 SYBR Green I. For proportion of cells, background fluorescence, measured with NON-  
23 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 <i>et al.</i> 1990b)
NONEUB 338	FISH	ACATCCTACGGGAGGC	Non specific binding	(Wallner <i>et al.</i> 1993)
Bif164	FISH	CATCCGGCATTACCACCC	<i>Bifidobacterium</i> spp.	(Langendijk <i>et al.</i> 1995)
Eub340 F	qPCR	TCCTACGGGAGGCAGCAGT	Total bacteria	(Nadkarni <i>et al.</i> 2002)
Eub781 R	qPCR	GGACTACCAGGGTATCTAATCCTGTT	Total bacteria	(Nadkarni <i>et al.</i> 2002)
Eub515r TMP	qPCR	<i>TET</i> <sup>a</sup> -CGTATTACCGCGGCTGCTGGCAC- <i>TAMRA</i> <sup>b</sup>	Total bacteria	(Nadkarni <i>et al.</i> 2002)
xfp-fw	qPCR	ATCTTCGGACCBGAYGAGAC	<i>Bifidobacterium</i> spp.	This study
xfp-rv	qPCR	CGATVACGTGVACGAAGGAC	<i>Bifidobacterium</i> spp.	This study
xfp-T	qPCR	<i>FAM</i> <sup>c</sup> -ACGGCATCTGGAGCTCCTA- <i>TAMRA</i> <sup>b</sup>	<i>Bifidobacterium</i> spp.	This study

<sup>a</sup>TET: reporter stain for 16S system.

<sup>b</sup>TAMRA: quencher.

<sup>c</sup>FAM: reporter stain for *xfp* system.

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

Faeces <sup>a</sup>	Total bacteria (DNA-stains)			Total bacteria (probes / primers)			Bifidobacteria (probes / primers)		
	MS	FCM	qPCR	MS	FCM	qPCR	MS	FCM	qPCR
	DAPI	SYBR Green I		EUB338	EUB338	Eub-primers <sup>c</sup>	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)
<b>Means<sup>b</sup></b>	<b>11•4a (2•1)</b>	<b>10•5b (4•5)</b>	-	<b>11•1a (2•1)</b>	<b>10•2b (4•1)</b>	<b>10•6c (3•1)</b>	<b>9•3a (3•0)</b>	<b>9•3a (4•8)</b>	<b>9•7a (4•6)</b>
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)
<b>Means</b>	<b>11•0a (1•7)</b>	<b>10•1b (1•7)</b>	-	<b>10•7a (1•7)</b>	<b>9•8b (3•4)</b>	<b>10•6a (3•1)</b>	<b>9•2a (3•2)</b>	<b>9•6a (2•2)</b>	<b>9•6a (3•4)</b>
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)

1

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

