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Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR

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Abstract

To determine the influence of either exclusive breast-feeding or formula feeding on both composition and quantity of the gut microbiota in infants, we have developed real-time, quantitative PCR assays for the detection of *Bifidobacterium* spp. and *Clostridium difficile*. Furthermore, we have monitored the prevalence and counts of *Escherichia coli* by applying a previously described real-time PCR assay. We found all 100 infants tested to be colonized by *Bifidobacterium* spp. The bifidobacterial counts were comparable between the 50 breast-fed and 50 formula-fed infants with median values of 10.56 $\log_{10}$ and 10.24 $\log_{10}$ CFU g^{-1} wet weight faeces, respectively. *C. difficile* was detected in 14% of the breast-fed and 30% of the formula-fed infants. In addition, the *C. difficile* counts were significantly lower in breast-fed infants than in the formula-fed group (median values of 3.28 $\log_{10}$ and 7.43 $\log_{10}$ CFU g^{-1}, respectively; $p = 0.03$). The prevalence of *E. coli* in the breast-fed and formula-fed group was 80% and 94%, respectively. Also, the *E. coli* counts in colonized infants was significantly lower in the breast-fed infants than in the formula-fed group (median values of 9.11 $\log_{10}$ and 9.57 $\log_{10}$ CFU g^{-1}, respectively; $p = 0.004$). We conclude that the prevalence and counts of *C. difficile* as well as *E. coli* are significantly lower in the gut microbiota of breast-fed infants than in that of formula-fed infants, whereas the prevalence and counts of *Bifidobacterium* spp. is similar among both groups.

Keywords: *Bifidobacterium*; *Escherichia coli*; *Clostridium difficile*; Real-time PCR; Intestinal microbiota; Breastfeeding; Infant formula

1. Introduction

The normal human microbiota is a complex ecosystem, consisting of at least several hundred different bacterial species. The gut microbiota plays an important role in human health by producing nutrients and by preventing colonization of the gut by potential pathogenic microorganisms [1]. In addition, the intestinal microbiota is involved in maintaining the health of the host, for instance by influencing the immune system. This impact of the gut microbiota on the immune system, such as the development of oral tolerance, was most clearly shown in studies on germfree mice [2]. Also, diseases of the immune system, like allergic diseases and inflammatory bowel diseases

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(Crohn's disease and ulcerative colitis) seem to be associated with gut microbiota composition [3–5].

Major changes in the intestinal microbial composition occur especially early in life. Until birth the gastrointestinal tract is sterile and subsequent development of the intestinal microbiota is influenced by factors like way of delivery (vaginal/caesarean), hygiene and antibiotic use, developmental stage of the gastrointestinal tract (premature/full term) and type of feeding (breast/formula) [6].

For decades, the quantification of bacterial genera and species present in the intestinal microbiota was based on traditional bacteriological culture and biochemical identification techniques. However, these methods are time-consuming and are limited by low sensitivities, the inability to detect nonculturable bacteria as well as unknown species, and the low levels of reproducibility due to the multitude of species to be identified and quantified [7]. Approximately 40–80% of the bacteria visible by direct microscopic examination of diluted faecal samples cannot be cultured [1]. For example, for bifidobacteria, several selective media have been developed. These media, however, do not equally support growth of all *Bifidobacterium* species. In particular the number of *B. adolescentis* are underestimated by selective plating. Moreover, it has been shown that the different media affect the total numbers of bifidobacteria recovered [8].

To study the gut microbiota composition in a culture-independent way, molecular methods have been developed which are based upon the detection of bacterial 16S ribosomal RNA (rRNA) genes [7,9]. These methods include temperature gradient gel electrophoresis [10,11], denaturing gradient gel electrophoresis [12] and fluorescent in situ hybridization [13,14]. A drawback of these techniques is, however, that they are not very suitable for analysis of large numbers of samples. By contrast, real-time PCR is a technique that is highly suitable for high-throughput analyses. Other advantages of real-time PCR are that it can be performed quantitatively, and that it does not require post-PCR sample handling, which prevents potential carry-over contamination and also results in much shorter assay times [15]. Recently, several assays have been published for the detection of bacterial groups and species in faecal samples and gastrointestinal mucosa [7,16–19].

Here, we describe the use of real-time PCR in the quantitative analysis of the presence of *Bifidobacterium* spp., *C. difficile* and *E. coli* in faecal samples of breast- and formula-fed infants.

2. Material and methods

2.1. Study population

Infants were selected from the KOALA-study, an ongoing birth cohort study on the development of allergic diseases in the Netherlands. In this study, faecal samples of over 1100 infants at the age of one month have been collected.

After exclusion of premature infants, infants born by caesarian section and infants who had received antibiotics during their first month of life, 50 exclusively breast-fed and 50 exclusively formula-fed infants were randomly selected. All children were born at home or at the outpatient department of the hospital. Characteristics of the infants participating in this study are shown in Table 1.

2.2. Bacterial strains and culture conditions

The bacterial strains listed below were obtained from the American Type Culture Collection (ATCC; Manassas, USA.), the National Collection of Type Cultures (NCTC; London, UK), Winclove Bio Industries BV (WBI; Amsterdam, the Netherlands) or were laboratory isolates at the University Hospital of Maastricht (azM; Maastricht, the Netherlands). The following reference strains were used in this study to evaluate the sensitivity and specificity of the PCR primer-probe sets: *Bacteroides distansonis* RE01B05-azM, *Bacteroides fragilis* ATCC 25285, *Bacteroides ovatus* ATCC 8483, *Bacteroides thetaiotaomicron* RE01B01-azM, *Bacteroides uniformis* RE01B04-azM, *Bacteroides vulgates* RE01B03-azM, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium bifidum* WBI 4, *Bifidobacterium breve* WBI 9, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium longum* WBI 2, *Clostridium bifermentans* ATCC 19299, *Clostridium difficile* ATCC 9689, *Clostridium histolyticum* ATCC 19401, *Clostridium novyi* ATCC 7658, *Clostridium perfringens* ATCC 13124, *Clostridium septicum* ATCC 6008, *Clostridium sordellii* ATCC 9714, *Clostridium tetani* NCTC 279, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC35218, *Eubacterium* spp. azM, *Lactobacillus casei* WBI 60, *Lactobacillus plantarum* WBI62, *Lactobacillus rhamnosus* WBI 40, *Leuconostoc* spp. azM, *Peptostreptococcus* spp. azM, *Streptococcus viridans* azM.

The numbers of colony forming units (CFU) were determined by plating different dilutions of an overnight culture (37 °C) of the microorganisms on fastidious anaerobic agar plates (LabM LabGo, Lancashire, UK) for the bifidobacteria, *C. difficile* and other (facultative) anaerobic bacteria, and on blood agar plates (Oxoid CM271, Basingstoke, UK) for *E. coli* and other

Table 1
Characteristics of babies participating in this study

	Breast-fed	Formula-fed
Number of babies	50	50
Male/female	22/28	26/24
Mean (SD) birth weight (g)	3572 (448)	3433 (451)
Mean (SD) length at birth (cm)	51 (3)	51 (2)
Mean (SD) duration of pregnancy (weeks)	40 (1)	39 (1)

(facultative) aerobic bacteria using a spiral-plater (Spiral Systems Inc. Salm en Kip, Utrecht, the Netherlands) and incubating the plates anaerobically or aerobically, respectively, for 24–48 h.

2.3. DNA purification from faeces

Faecal samples were 10-fold diluted in peptone–water (Oxoid CM0009) containing 20% v/v glycerol (Merck, Darmstadt, Germany) and stored at −20 °C. For DNA isolation 0.2 ml of the diluted samples was added to a 2-ml-vial containing approximately 300 mg glass beads (diameter 0.1 mm) and 1.4 ml of ASL-buffer from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The bacterial samples were disrupted in a mini-bead beater (Biospec Products, Bartlesville, Okla.) at 5000 rpm for 3 min. Subsequently, the bacterial DNA was isolated from the samples using the QIAamp DNA Stool Mini Kit, according to the instructions of the manufacturer. The DNA was eluted in a final volume of 200 μl.

2.4. DNA purification from bacterial cultures

DNA from the reference strains used in this study was isolated in a similar manner as the DNA from faecal samples. In short, 0.2 ml of a bacterial suspension was added to a 2-ml-vial containing 300 mg glass beads and 1.4 ml ASL-buffer and bead-beated for 3 min., after which the samples were subjected to the DNA Stool Mini Kit.

2.5. Design of primers and probes

The primers and probes for the detection of bifidobacteria and *C. difficile* were based on 16S rRNA gene sequences, retrieved from the National Center for Biotechnology Information databases using the program Entrez (http://www.ncbi.nlm.nih.gov). The bifidobacterial or *C. difficile* sequences were aligned with sequences from closely related species using the program ClustalW from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw.htm). The alignments revealed sequences specific for either bifidobacteria or *C. difficile*. From these sequences, forward primers, reverse primers and TaqMan probes were designed using the Primer Express 2.0 software program (Applied Biosystems [ABI], Foster City, Calif.). Sequences and coordinates of the selected primers and probes are listed in Table 2.

To check for specificity, the selected primers and probes were compared to all available 16S rRNA gene sequences using the BLAST database search program (www.ncbi.nlm.nih.gov/blast). Primers and probes were manufactured by Sigma Genosys.

2.6. TaqMan assay conditions

For the *Bifidobacterium*–TaqMan assay, amplifications were carried out in a total volume of 50 μl, containing 1× TaqMan universal PCR master mix (ABI), 300 nM of both primers, 150 nM of TaqMan probe, and 20 μl of purified target DNA (see above). For the *C. difficile*–TaqMan assay, amplifications were carried out in a total volume of 25 μl, containing 1× TaqMan universal PCR master mix (ABI), 900 nM of both primers, 200 nM of TaqMan probe, and 10 μl of purified target DNA. The conditions of the *E. coli*–TaqMan assay, which was developed by Huijsdens et al. [7], were adjusted so that amplifications were carried out in a total volume of 25 μl, containing 1× TaqMan universal PCR master mix (ABI), 900 nM of both primers, 200 nM of TaqMan probe, and 10 μl of purified target DNA. The amplification (2 min at 50 °C, 10 min at 95 °C, followed by 42 cycles of 15 s at 95 °C and 1 min at 60 °C) and detection were carried out on an ABI Prism 7000 sequence detection system (ABI).

2.7. Sensitivity, specificity and inhibition

To determine the sensitivity of the real-time TaqMan assay, DNA purified from serial 10-fold dilutions of quantified *B. bifidum* and *C. difficile* cultures were subjected to PCR. Bacterial densities of the dilutions were determined by viable counts. Since the assay conditions for the detection of *E. coli* were slightly adjusted, the

Table 2
Primers and probes used in this study

Target organism(s)	Primer/probe	Target[a]	Sequence (5′–3′)	T_m (°C)	References
Bifidobacterium spp.	Forward primer	42–63	GCGTGCTTAACACATGCAAGTC	59	This study
	Reverse primer	167–147	CACCCGTTTCCAGGAGCTATT	59	This study
	Probe	126–103	TCACGCATTACTCACCCGTTCGCC	70	This study
C. difficile	Forward primer	62–100	TTGAGCGATTTACTTCGGTAAAGA	58	This study
	Reverse primer	175–151	TGTACTGGCTCACCTTTGATATTCA	59	This study
	Probe	105–126	CCACGCGTTACTCACCCGTCCG	69	This study
E. coli	Forward primer	395–414	CATGCCGCGTGTATGAAGAA	59	[7]
	Reverse primer	490–470	CGGGTAACGTCAATGAGCAAA	59	[7]
	Probe	437–467	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	68	[7]

[a] Numbering corresponding to *E. coli* 16S rRNA gene [36].

sensitivity of this assay was also determined in a similar way. To check whether the *Bifidobacterium*–TaqMan assay could detect DNA from bifidobacteria other than *B. bifidum*, PCR was performed using $1–1 \times 10^6$ genome copies of *B. adolescentis*, *B. breve*, *B. infantis*, *B. longum* and *B. bifidum*. The specificity of both the *C. difficile* and *Bifidobacterium* assay was further investigated by testing DNA purified from a mixture of bacterial species other than the target species (10^4–10^5 CFU of each of the species per mixture), either in the absence or presence of *B. bifidum* or *C. difficile*. The bacteria that were selected are listed in the "bacterial strains and culture condition" section. In addition, 40 faecal samples that were either positive or negative in the *C. difficile* TaqMan assay were cultured on a selective medium (Oxoid CM0601B).

To analyze the presence of potential PCR inhibitory substances after DNA purification, faecal samples, as well as pure bacterial culture suspensions, were spiked with rat cytomegalovirus (RCMV) particles before DNA extraction. Each purified DNA sample was subjected to a RCMV DNA-specific TaqMan assay, which has previously been described [20].

2.8. Ultrafiltration of the PCR mix

To remove traces of *E. coli* DNA contaminating the universal PCR master mix, an ultrafiltration step as described by Yang et al. was used [21]. In brief, the Amicon Microcon YM-100 centrifugal filter device (Millipore Corporation, Bedford, MA.) was utilized for filtering the PCR master mix prior to addition of template DNA. The PCR mix was spun through the YM-100 device at 100*g* for 30 min.

2.9. Statistical methods

The $\log_{10}$ CFU g^{-1} of *Bifidobacterium* spp., *E. coli* and *C. difficile* were calculated for each stool sample from the threshold cycle (C_t)-values using the constructed standard curves. Since the data did not exhibit a normal distribution we used the Mann–Whitney rank sum test to compare values between the breast- and formula-fed groups.

2.10. Ethical considerations

The KOALA-study was approved by the Ethical Committee of the University Hospital of Maastricht.

3. Results

3.1. Sensitivity of the assays

The sensitivity of the real-time PCR assays was determined using serial 10-fold dilutions of quantified cultures of *B. bifidum*, *C. difficile* and *E. coli*. As shown in Fig. 1(a), the *Bifidobacterium* TaqMan assay could reproducibly detect $\geqslant 10$ CFU of *B. bifidum* per PCR reaction, which corresponds to 5×10^3 CFU/g faeces. From the average C_t values obtained with each dilution in three replicate amplifications, a standard curve was constructed (Fig. 1(b)). This curve shows that the *Bifidobacterium* TaqMan assay was exponential over a broad dynamic range, from 10 to at least 10^6 CFU starting material. In addition, the slope of the standard curve was −3.3, which is similar to the optimal theoretical value.

To investigate whether our assay was capable of detecting *Bifidobacterium* species other than *B. bifidum*, DNA was extracted from cultures of *B. infantis* ATCC 15697, *B. adolescentis* ATCC 15703, *B. breve* WBI 9 and *B. longum* WBI 2. For each of these species, the assay was performed on serial dilutions of purified DNA ranging from 1 to 1×10^6 genome copies. The bifidobacterial species tested were detected with a similar sensitivity as *B. bifidum* (data not shown).

The lower detection limit of the *C. difficile* TaqMan assay was 2 CFU per PCR reaction, corresponding to 2×10^3 CFU/g faeces. In addition, the amplification was exponential over a broad range of input material, from 2 to at least 2×10^5 CFU/PCR.

The *E. coli* TaqMan could detect as little as 4 CFU/PCR reaction, which corresponds to 4×10^3 CFU/g faeces. The assay was exponential from 4 to at least 4×10^5 CFU/PCR. Ultrafiltration of the PCR master mix resulted in complete removal of contaminating traces of *E. coli* DNA, whereas the sensitivity was not influenced.

3.2. Specificity of the assays

To determine the specificity of the assays, purified DNA from a mixture of non-target bacteria was subjected to the *Bifidobacterium* spp. and *C. difficile* assays, either in the presence or absence of DNA from the target bacteria. The TaqMan assays for *Bifidobacterium* spp. and *C. difficile* were highly specific for the target bacteria: no difference in amplification or quantification of *B. bifidum* or *C. difficile* was observed either in the presence or absence of 'nonspecific' bacteria in the mixtures. In addition, amplification was not observed when *B. bifidum* or *C. difficile* were omitted from these mixtures (data not shown). The specificity of the *E. coli* TaqMan assay has previously been demonstrated [7].

Additional validation of both the sensitivity and the specificity of the *C. difficile* assay was done by comparing the results of the assay with the results of culturing on a *C. difficile* selective plate. All 20 samples which were negative in the TaqMan assay were also negative by culture, whereas 19 of the 20 samples which were positive in the TaqMan assay were also culture positive.

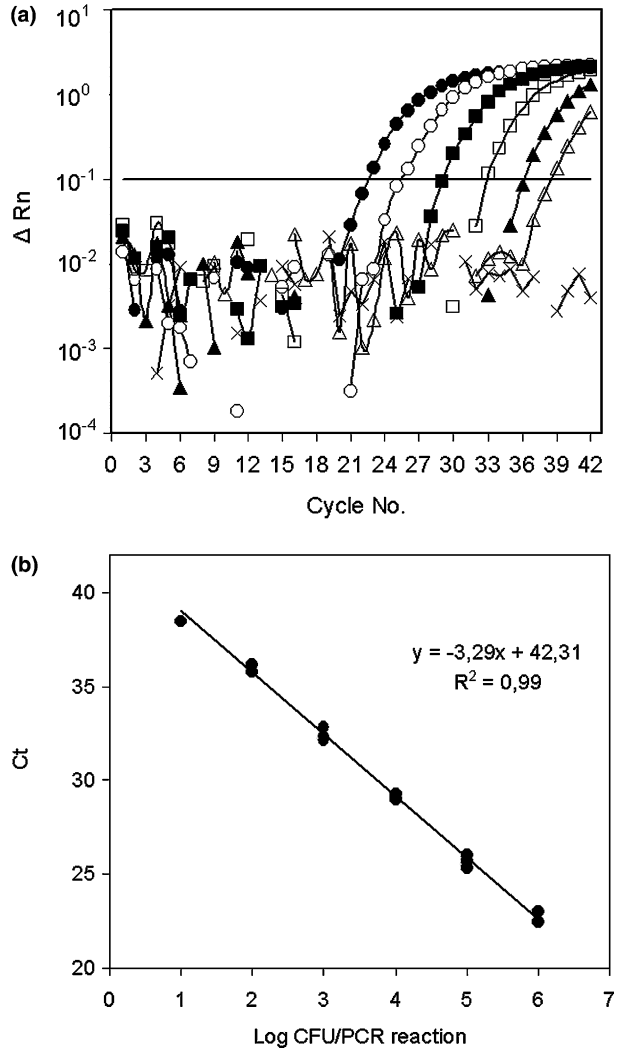

Fig. 1. (a) TaqMan assay of one of the three replicate runs of serial, 10-fold dilutions of *B. bifidum* ranging from 1 to 1×10^6 CFU per PCR reaction. ΔR_n indicates the normalized fluorescent reporter value, subtracted from the background value. No template control (NTC) (-×-); 1×10^6 CFU (-●-); 1×10^5 CFU (-○-); 1×10^4 CFU (-■-); 1×10^3 CFU (-□-); 1×10^2 CFU (-▲-); 1×10^1 CFU (-△-); threshold (–). (b) Standard curve based upon three replicate runs of serial, 10-fold dilutions of *B. bifidum* ranging from 1 to 1×10^6 CFU per PCR reaction.

The *Bifidobacterium* spp. assay was not compared with traditional culturing since there are no reliable selective media for bifidobacteria [8].

3.3. DNA extraction and PCR inhibition control

The DNA extraction and amplification procedures were validated by the addition of a 'spike' consisting of RCMV particles, before DNA isolation. The C_t values of the RCMV-specific TaqMan assay were similar for all stool samples and bacterial cultures as well as the RCMV-positive control (C_t range 32.4–33.7; data not shown).

3.4. Microbial analysis of the faecal samples

The real-time PCR assays were used to analyze faecal samples of 50 exclusively breast-fed and 50 exclusively formula-fed one-month-old infants participating in the KOALA-study. Prevalences of colonization and counts ($\log_{10}$ CFU (g wet weight faeces)$^{-1}$) as determined by real-time PCR are shown in Table 3. All breast-fed as well as formula-fed infants were positive for *Bifidobacterium* spp. Although the number of bifidobacteria in faeces appeared slightly higher in breast-fed infants than in formula-fed infants, this difference was not statistically significant.

The prevalence of colonization by *C. difficile* was more than two times lower in breast-fed infants (7/50) than in formula-fed infants (15/50), and in those infants colonized counts were also significantly lower in breast-fed compared to formula-fed infants (median values 3.28 and 7.43 $\log_{10}$ CFU g^{-1}, respectively; $p = 0.03$).

Ten breast-fed infants were not colonized by *E.* coli, whereas only three formula-fed infants were not colonized by this species. Furthermore, the median value of *E. coli* numbers in colonized breast-fed infants was almost threefold lower compared to the median value of *E. coli* numbers in the colonized formula-fed infants ($p = 0.004$).

4. Discussion

In order to monitor the influence of either exclusive breast feeding or formula feeding on composition as well as quantity of gut bacteria in infants, real-time PCR assays for the detection of *Bifidobacterium* spp. and *C. difficile* were developed. In addition, we also employed a previously described real-time PCR assay for the detection and quantification of *E. coli*. Each of the three

Table 3
Prevalence and counts of *Bifidobacterium* spp., *E. coli*, and *C. difficile* ($\log_{10}$ CFU (g wet weight faeces)$^{-1}$) in faeces determined at one month of age in either breast-fed or formula-fed infants

	Breast-fed ($n = 50$)	Formula-fed ($n = 50$)	P value[b]
Bifidobacterium spp.			
Prevalence	50/50	50/50	
Counts[a]	10.56 (5.52–11.34)	10.24 (6.15–11.37)	0.14
C. difficile			
Prevalence	7/50	15/50	
Counts[a]	3.28 (2.92–6.91)	7.43 (2.71–9.57)	0.03
E. coli			
Prevalence	40/50	47/50	
Counts[a]	9.11 (5.73–10.43)	9.57 (5.14–10.46)	0.004

[a] Median values (ranges) calculated from positive samples only ($\log_{10}$ CFU (g wet weight faeces)$^{-1}$).
[b] P value of Mann–Whitney rank sum test calculated from all samples.

real-time PCR assays was found to be highly specific, since they did not generate positive signals with closely related bacterial species. The assays were also shown to be very sensitive, detecting as little as 10, 2 and 4 CFU of *Bifidobacterium* spp., *C. difficile* and *E. coli*, respectively. Spiking of faecal samples with RCMV resulted in similar values for all faecal samples as well as for the RCMV-positive control. This indicated that the assays are not significantly influenced by potentially inhibitory components from the stool samples.

We found the prevalence and counts of *C. difficile* as well as *E. coli* to be significantly lower in the gut microbiota of breast-fed infants than in that of formula-fed infants. In contrast, the prevalence and counts of *Bifidobacterium* spp. was similar among both groups of infants.

Throughout the last decades numerous studies have been conducted to compare gut microbiota composition between breast- and formula-fed infants, all based upon traditional culture techniques. Although some studies demonstrated significantly higher counts of bifidobacteria in breast-fed infants than in formula-fed infants [22,23], other, more recent studies did not reveal such differences [6,24–26]. Since especially the more recent studies did not show significant differences in bifidobacteria between breast- and formula-fed infants, it has been suggested that this might be the result of modifications of the infant formulas. For instance, the protein and phosphorus content of modern formulas are much lower, giving a lower buffering capacity similar to that of human milk [6]. On the other hand, since most previous studies were performed using traditional culturing methods, discrepant results between these studies may also be due to differences in selectivity and specificity of the media used for counting bifidobacteria [6]. In some studies an extremely low prevalence of bifidobacterial colonization was found in infants aged 4 weeks [27,28], whereas in other studies (almost) all infants were reported to be colonized by bifidobacteria at the same age [25,26,29].

We found *E. coli* to be significantly less prevalent, and in lower numbers, in faecal samples of breast-fed infants than in those of formula-fed infants. This finding is in agreement with data from previous studies in which lower counts of enterobacteria were found in breast-fed infants than in formula-fed infants around the age of one month [24,26,29].

The prevalence of *C. difficile* in our study was also lower in breast-fed infants than in formula-fed infants (14% and 30%, respectively). Furthermore, the breast-fed infants that were colonized by *C. difficile* harbored lower numbers in their faeces than did the formula-fed infants. Other studies on the prevalence of *C. difficile* in infants showed prevalences ranging from 15% to 70% [30–35]. A study among 343 infants born in a Swedish hospital showed carriage rates comparable to our findings: 21% of breast-fed infants and 47% exclusively formula-fed infants carried *C. difficile* at the age of 6 weeks [32].

Several other real-time PCR assays targeting the 16S rRNA gene for the detection of gut bacteria have recently been described. Ott et al. [18] described several TaqMan assays for the detection of gut bacteria using universal primers and specific probes. The sensitivity of the assays was similar to the sensitivity of our assays detecting as little as 10 CFU per reaction, the detection of bifidobacteria was not included in this study.

Matsuki and colleagues [16,17] described several real-time PCR assays for the detection of bifidobacteria not only to the genus-level, but also to the species-level. The detection limit of their assays, however, was only 10^6 CFU bifidobacteria per gram wet weight faeces, whereas our assay could detect as little as 5×10^3 CFU *bifidobacterium* spp. per gram/faeces, which corresponds to 10 CFU per reaction. Guiemondeet al. [19] described an assay for the detection of bifidobacteria in faecal samples with a detection limit of 5×10^4 CFU/g of faeces.

The molecular 16S rDNA-based real-time PCR assays evaluated in this study provide more accurate data on intestinal microbiota composition than traditional culture techniques. Furthermore, these real-time PCR assays do not require anaerobic conditions and they can be applied to high-throughput analyses using both fresh and frozen samples, thereby making this technique very suitable for large-scale epidemiological studies on gut microbiota composition.

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