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ORIGINAL ARTICLE

Presence of two *Lactobacillus* and *Bifidobacterium* probiotic strains in the neonatal ileum

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The overall purpose of this study was to examine the lactobacilli and bifidobacteria microbiota in the human ileum at a very early stage of life. Ileostomy effluents from two infants, taken at different time points, were plated on *Lactobacillus* selective agar and *cys*-MRS containing mupirocin to select for bifidobacteria. In one case, a stool sample following ileostomy reversal was subsequently analyzed microbiologically. Pulse-field gel electrophoresis and 16S rRNA sequencing was used to investigate the cultivable population of bifidobacteria and lactobacilli and denaturing gradient gel electrophoresis (DGGE) to examine the non-cultivable population. The probiotic strain, *Lactobacillus paracasei* NFBC 338, was recovered at both time points from one of the infants and dominated in the small intestine for a period of over 3 weeks. Moreover, the probiotic strain, *B. animalis subsp. lactis* Bb12, was obtained from the other infant. This study shows the presence of two known probiotic strains in the upper intestinal tract at an early stage of human life and thus provides some evidence for their ability to colonize the infant small intestine.

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Introduction

The gastrointestinal tract of the fetus is initially sterile, but microbes from the mother and the surrounding environment colonize the gut of the term infant following delivery until a dense and complex microbiota develops. In full-term vaginally delivered infants, colonization starts immediately after delivery and microorganisms such as enterobacteria and streptococci appear in feces. In addition, the composition of the gut microbiota is influenced by the diet of the infant. Breastfeeding positively influences gut colonization with bifidobacteria, whereas formula-fed infants have been reported to have a more diverse microbiota, including bifidobacteria, bacteroides, clostridia and streptococci (Mitsuoka, 1996; Favier *et al.*, 2002). Furthermore, the gut flora of preterm infants differs from term infants for several reasons—immature gut motility, delayed onset of enteral nutrition, the extensive use of broad-spectrum antibiotics, barrier nursing and hygiene practices, and the relatively aseptic neonatal intensive care environment. These factors can lead to colonization by facultatively anaerobic bacteria, whereas colonization by bifidobacteria is often delayed (Stark and Lee, 1982a, b; Gewolb *et al.*, 1999).

The influence of intestinal bacteria on human health can be considered harmful, beneficial or neutral. *Bifidobacterium* spp and *Lactobacillus* species are among those microorganisms believed to be beneficial and can contribute to digestion, immune stimulation and inhibition of pathogens (Fuller, 1989). Members of the genus *Bifidobacterium* are present in large numbers in newborn infants and are considered to be of importance in early infancy (Tannock, 1994; Chierici *et al.*, 2003). The most common *Bifidobacterium* species in infants have been reported to be *Bifidobacterium infantis*, *Bifidobacterium breve* and *Bifidobacterium longum* (Matsuki *et al.*, 1999; Malinen *et al.*, 2002). As infants mature, *B. infantis* and *B. breve* are replaced by *Bifidobacterium adolescentis* and *B. longum* (Matsuki *et al.*, 1999). *Lactobacillus* colonization is

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less well understood and studies report low colonization rates in Western countries (Stark and Lee, 1982a, b; Matsumiya *et al.*, 2002). *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacilli casei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri* and *Lactobacillus brevis* have been the most commonly isolated species from the human intestine (Mitsuoka, 1992). The *Lactobacillus* species that are most commonly found in infant feces are *L. acidophilus*, *L. salivarius* and *L. fermentum* (Cooperstock and Zedd, 1983; Benno and Mitsuoka, 1986). Studies in germ-free animal models have suggested that colonization of the gastrointestinal tract plays a crucial role in the development of the gut immune system by mechanisms related to the maturation of the intestinal epithelium and immune compartment cells (Umesaki and Setoyama, 2000). In particular, *Lactobacillus* and *Bifidobacterium* strains modify *in vitro* the functional pattern of monocytes and lymphocytes towards increased production of IL-12 and IFN-γ, the so-called type 1 cytokines (Th1 cytokines). Th1 cytokines function to help defend against a viral or bacterial attack in the blood and tissues because Th1 cytokines instruct the immune system to produce cells and antibodies that are especially effective against such infections.

Bifidobacterium and *Lactobacillus* are frequently used as probiotics for human consumption (Fuller, 1989). The alleged benefits of probiotic consumption include improved gastrointestinal health, antitumor, anti-infective, immunomodulatory and motility effects (Mitsuoka, 1992; Sanders, 1994; Lee and Salminen, 1995; Aimutis, 1999). Adhesion and colonization (albeit transient) of probiotic bacteria in the gastrointestinal tract of the host is believed to be one of the most essential features required for delivering their health benefits (Bernet *et al.*, 1994). Preterm infants represent a special situation for probiotics as such subjects have immature organs, often receive intensive care, experience delayed feeding and frequently treated are with broad-spectrum antibiotics shortly after birth (Zhang *et al.*, 2005).

Molecular biological methods are increasingly being applied to study the gastrointestinal tract ecology (Tannock, 2001). Denaturing gradient gel electrophoresis (DGGE) analysis is one of the most suitable and widely used methods for studying complex bacterial communities in various environments (Muyzer and Smalla, 1998). Total bacterial DNA from the habitat of interest is extracted, and subsequent processing is used to generate a 'genetic fingerprint' of the community (Walter *et al.*, 2001). Using DGGE, it is possible to identify constituents that represent only 1% of the total population in the human GI tract (Muyzer *et al.*, 1993). More recently metagenomic approaches have been used to explore the complex microbial community within the GIT (Tringe *et al.*, 2005).

It has been established that microbiota patterns differ between stomach, upper small bowel, lower small bowel and rectum in healthy adults (Flourie *et al.*, 1986; Evans *et al.*, 1988; Marteau *et al.*, 1994; Evans, 1998). However, it is not known if surgical interruption of the developing intestinal ecosystem in the infant with an ileostomy results in modification of expected microbiota or adaptation of the neoterminal ileum to yield a microbiota pattern similar to that of the infant colon. The aim of this study was to examine the lactobacilli and bifidobacteria microbiota in the human ileum at a very early stage of life.

Materials and methods

Subjects

'Infant D' was a preterm infant who had complex medical complications of prematurity. Medical therapy for a patent ductus arteriosis was followed by spontaneous intestinal perforation. This required laparotomy, perforation repair and creation of an ileal stoma. 'Infant K' was a term infant who developed early intestinal obstruction that required surgical management and formation of an ileal stoma in the first week of life. Further investigation revealed that the infant had cystic fibrosis. The ileostomy was reversed and intestinal continuity restored at 39 weeks of age. Four ileostomy samples were taken from the two infants, from infant D at 50 and 74 days old and from infant K at 24 and 31 weeks old. A fecal sample was also taken from infant K at age 44 weeks following ileostomy reversal. The diet of infant K did not change between the pre- and postoperative periods. Both infant D and infant K were vaginally delivered and were both breast- and formula-fed. None of the infants received probiotics in their diet. We have no information regarding whether or not the mother received probiotics. Infant D was treated with antibiotics (metronidazole) for the first 3 weeks of life and between days 50 and 60. Fully informed consent was obtained from all parents.

Intestinal content sampling

The swab samples taken did not allow accurate enumeration of bacteria (quantitative assessment) but did allow the isolation of strains from the samples (qualitative assessment). Samples were stored at 5°C and delivered to the laboratory for processing within 5 h of sampling or stored at −20°C for DNA extraction. The swabs were vortex mixed and serially diluted in maximum recovery diluent (MRD, Oxoid Ltd, Hampshire, UK).

Growth conditions and selection of colonies

Serial dilutions of the samples were pour-plated onto *Lactobacillus* selective agar (LBS) (Becton Dickinson Co., Cockeysville, MD, USA) and modified de Man, Rogosa and Sharpe (MRS) agar (mMRS) supplemented with 0.05% (w/v) L-cysteine hydrochloride (98% pure; Sigma Chemical Co., St Louis, MO, USA). To preselect for bifidobacteria,

100 μg of Mupirocin (Oxoid) per ml was added to the mMRS medium as antimicrobial susceptibility disks by using the disk diffusion method (Rada, 1997). Agar plates were incubated anaerobically (anaerobic jars with Anaerocult A gas packs; Merck, Darmstadt, Germany) at 37°C for 72 h. Twenty colonies were randomly selected from each sample, which would represent the predominant strains comprising the population in the sample. These colonies were subcultured in mMRS broth for 24 to 48 h.

Pulse-field gel electrophoresis
High-molecular-weight DNA was isolated from stationary-phase cultures using previously described procedures (Simpson *et al.*, 2003). The restriction enzymes *Apa*I, *Xba*I or *Spe*I (New England Biolabs, Beverly, MA, USA) were used for genomic digests, which were resolved with a contour-clamped homogeneous electric field CHEF-DR III pulsed-field system (Bio-Rad Laboratories, Richmond, CA, USA) at 6 V cm^{-1} for 18 h with a 1- to 15-s linear ramp pulse time and with 0.5 × Tris base-borate-EDTA running buffer maintained at 14°C. Gels were stained in distilled water containing 0.5 μg of ethidium bromide per ml for 30 min and then destained for 60 min. Pulsed-field gel electrophoresis (PFGE) gels were visualized by ultraviolet (UV) transillumination, and the sizes of the PFGE fragments were estimated by comparison with a low-range PFGE marker ranging from 2.03 to 194 kb (no. N0350S; New England Biolabs).

16S rRNA sequencing
Two 16S ribosomal RNA (rRNA) primers—CO1 for the 5′ end (5′-AGTTTGATCCTGGCTCAG-3′) and CO2 for the 3′ end (5′-TACCTTGTTACGACT-3′)—were used to generate an approximate 1.5-kb 16S rRNA product under PCR conditions described previously (Simpson *et al.*, 2003). This product was partially sequenced by using the primer CO1 by LARK technologies (Essex, UK). Comparison of the 16S rRNA sequences obtained by using the BLAST program allowed the assignment of a strain to a particular species. In general, when 16S rRNA similarity values exceed 98%, the strains are considered to belong to the same species (Stackebrandt and Goebel, 1994).

Plasmid profiling
Plasmid DNA was isolated by the method of Anderson and McKay (1983), with one minor adjustment at the lysis step. Briefly, the culture was inoculated (2% v/v) and grown for approximately 4 h. The cells were harvested (6000 *g* for 20 min) and resuspended in lysis solution ((sucrose (6.7% w/v), Tris (50 mM) and EDTA (1 mM) at pH 8)), supplemented with lysozyme (Sigma Chemical, Poole, UK) and Mutanolysin (Sigma) (20 mg ml^{-1} each), and incubated for 30 min at 37°C. All subsequent steps in the plasmid isolation procedure and vertical gel electrophoresis are identical to those described by Anderson and McKay (1983). Plasmids isolated from *Lactococcus lactis* DRC3 were used as standard molecular weight sizes as described previously (Desmond *et al.*, 2005).

*PCR-DGGE analysis (*Bifidobacterium*)*
Bacterial DNA was extracted from swabs using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany) by following the manufacturer's instructions (lysis temperature, 95°C). To investigate the bifidobacterial population in the samples, the PCR was performed as a nested approach. The first PCR applied primers Im26-f (5′-GATTCTGGCTCAGGAT GAACG-3′) and Im3-r (5′-CGGGTGCTICCCCACTTT CATG-3′) described by Kaufmann *et al.* (1997), amplified a 1417-bp fragment of the bifidobacterial 16S rRNA gene. PCR volumes of 50 μl contained 5 μl of 10 × PCR buffer, 5 μl of $MgCl_2$ (2.5 mM), 8 μl of deoxynucleoside triphosphates (dNTPs) (0.2 mM each), 1 μl of each primer (5 pmol), 0.5 μl of Taq polymerase (5 U μl^{-1}), 28.5 μl of sterile Milli-Q water and 1 μl of DNA solution. The following PCR program was used: initial denaturation at 94°C for 5 min; three cycles of denaturation at 94°C for 45 s, annealing at 57°C for 2 min and extension at 72°C for 1 min; 30 cycles of denaturation at 94°C for 20 s, annealing at 57°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min, followed by cooling to 4°C. The presence of PCR products was verified on a 1.5% agarose gel. To eliminate the remaining oligonucleotides and original template DNA, purification of the amplicons was performed by use of the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A second PCR was performed using the amplicons of the first PCR as template DNA. The primer set used (F357-GC and 518R) (5′GC-clamp-GCCTACGGAGGCAGCAG-3′ and 5′-ATTACCGCGG CTGCTGG-3′, respectively) amplifies the V3 region of the bacterial 16S rRNA gene (Muyzer *et al.*, 1993). The forward primer contained a GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGG-3′) to facilitate separation of the amplicons in a DGGE gel. PCR volumes of 50 μl contained 5 μl of 10 × PCR buffer, 2 μl of $MgCl_2$ (1 mM), 8 μl of dNTPs (0.2 mM each), 2 μl of each primer (5 μM), 0.5 μl of *Taq* polymerase (5 U μl^{-1}), 29.5 μl of sterile Milli-Q water and 1 μl of 10-fold diluted DNA solution (obtained from the first PCR). The following PCR program was used: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 7 min, followed by cooling to 4°C. PCR products were analyzed on DGGE gels on the basis of the protocol of Temmermann *et al.* (2003). A Dcode universal mutation detection system (Bio-Rad, Hercules, CA, USA) was used, utilizing 16-cm by 16-cm by 1-mm gels. Eight

percent polyacrylamide gels were prepared and run with 1 × Tris-acetate-EDTA (TAE) buffer diluted from 50 × TAE buffer (2 M Tris base, 1 M glacial acetic acid and 50 mM EDTA). The denaturing gradient was formed with two 8% acrylamide (acrylamide-bis/acrylamide 40%) stock solutions (Severn Biotech Ltd, Kidderminster, UK). A 100% denaturing solution contained 40% (v/v) formamide and 7.0 M urea. The gels were poured from the top by using a gradient maker (CBS Scientific Linear Gradient Maker, Bio-Rad) and a pump (Bio-Rad), and gradients of 50–70% were used for the separation of the generated amplicons. Before polymerization of the denaturing gel (24-ml gradient volume), a 6-ml stacking gel without denaturing chemicals was added and the appropriate comb was subsequently inserted. PCR amplicons were separated by electrophoresis at a constant voltage of 60 V in a 0.5 × TAE buffer at a constant temperature of 60°C for 16 h. Gels were stained in ethidium bromide for 30 min, allowing digital capture of the DGGE band profiles under UV light.

*PCR-DGGE analysis (*Lactobacillus*)*
Amplification was performed using a DNA Thermal Cycler (Eppendorf Mastercycler gradient 5331, Hamburg, Germany) and the specific primers Lac1 (5′-AGCAGTAGGGAATCTTCCA-3′) and Lac2GC (5′GC-Clamp-ATTYCACCGCTACACATG-3′) (Walter *et al*., 2001). PCR volumes of 50 µl contained 5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (1.5 mM), 0.5 µl of dNTPs (0.2 mM each), 0.25 µl of each primer (5 pmol), 0.25 µl of *Taq* polymerase (5 U µl^{-1}), 41.25 µl of sterile Milli-Q water and 1 µl DNA solution. The following PCR program was used: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 1 min, extension at 68°C for 1 min and final extension at 68°C for 7 min, followed by cooling to 4°C. The presence of PCR products was verified on a 1.5% agarose gel. To eliminate the remaining oligonucleotides and original template DNA, purification of the amplicons was performed by use of the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DGGE analysis of PCR amplicons generated with primers Lac1 and Lac2 was performed essentially as described for bifidobacteria but with the following modifications; the gels contained a 30–50% gradient of urea and formamide increasing in the direction of electrophoresis.

Results

In this study, two infants with ileostomies were examined with regard to their lactobacilli and bifidobacteria populations. Ileostomy samples were taken at different time points, and in one case a per-anal stool sample was taken following ileostomy reversal. This allowed us to examine the colonization of *Bifidobacterium* and *Lactobacillus* in the distal small intestine at an early stage of life. Hence, the underlying purpose of the study was to gain a better understanding of the developing intestinal ecosystem in the neonate, which may hold the key to prevention of several important diseases. Culture-based isolations were further compared with culture-independent results using DGGE.

Isolation and genetic fingerprinting using PFGE
Infant D. For each ileostomy sample, 20 isolates were selected at random from lactobacilli selective plates. These isolates represented the predominant strains comprising the populations in the samples. To genetically fingerprint the isolates, genomic digestion with the restriction enzyme *Apa*I and PFGE was used. The isolates from the first time point (50 days old) isolated on LBS produced one macro restriction pattern (Figure 1a). The same occurred with the isolates from the second time point (74 days old), which also produced one macro restriction pattern (Figure 1b). When the macro restriction patterns for the two time points were compared, these were identical, indicating that the same strain survived in the microbial niche over the 3-week period. This strain was identified as *Lactobacillus paracasei* according to 16S rRNA sequencing. Interestingly, this strain exhibited the same PFGE pattern as the probiotic strain *Lactobacillus paracasei* NFBC 338 (Figure 1c) (Gardiner *et al*., 1998, 2000; Desmond *et al*., 2005). This was further confirmed by plasmid profiling (Figure 1d). On the basis of selection on MRS containing cysteine hydrochloride (0.5 g l^{-1}) and mupirocin (50 mg l^{-1}), bifidobacteria appeared to be absent.

Infant K. No growth was obtained from the cecal samples following culturing on bifidobacteria-specific media, but when the ileostomy had been reversed it proved to consist of two different strains, which according to 16S rRNA sequencing were most similar to *Bifidobacterium animalis subsp. lactis* and *Bifidobacterium scardovi* (Figure 2a). *B. animalis subsp. lactis* represented 95% of isolates, while the remaining 5% was identified as *B. scardovi* (Figure 2a). The *Bifidobacterium animalis subsp. lactis* strain isolated exhibited the same PFGE pattern as the probiotic strain *Bifidobacterium animalis subsp. lactis* Bb12, following restriction with both *Xba*I and *Spe*I (Figure 2b). When the samples were cultured on *Lactobacillus* selective media, lactobacilli were not obtained in any of the time points instead, there were three different strains of *Enterococcus faecium* isolated on the *lactobacilli* selective media from the third time point, which proved that the *lactobacilli* selective media was not selective entirely for lactobacilli. Interestingly, these macrorestriction patterns of the *Enterococcus faecium* isolates are very similar,

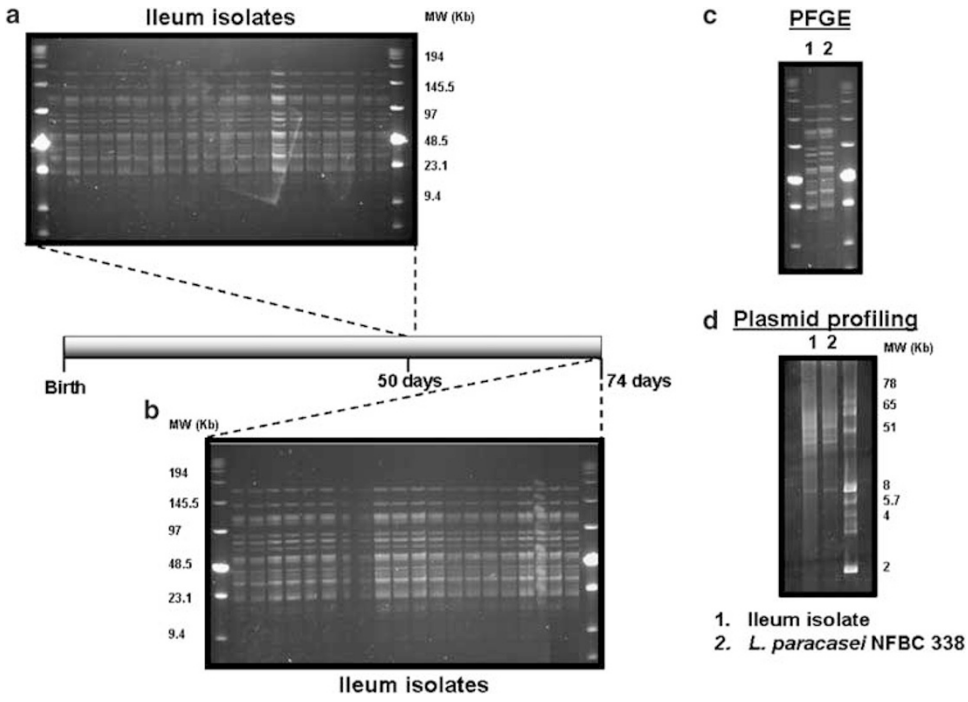

Figure 1 *L. paracasei* NFBC 338 is present in the neonatal ileum of infant D. (**a**) PFGE macro restriction pattern following genomic digests with *Apa*I of isolates from the first time point (50 days old) and (**b**) PFGE macro restriction pattern following genomic digests with *Apa*I of isolates from the second time point (74 days old). (**c**) Comparison with the macro restriction pattern of the probiotic strain *L. paracasei* NFBC 338 revealed that the ileum isolates exhibited the same PFGE pattern as *L. paracasei* NFBC 338. This was further confirmed by plasmid profiling (**d**). Molecular weight (MW) markers are shown in outside lanes of the gels. PFGE, pulsed-field gel electrophoresis.

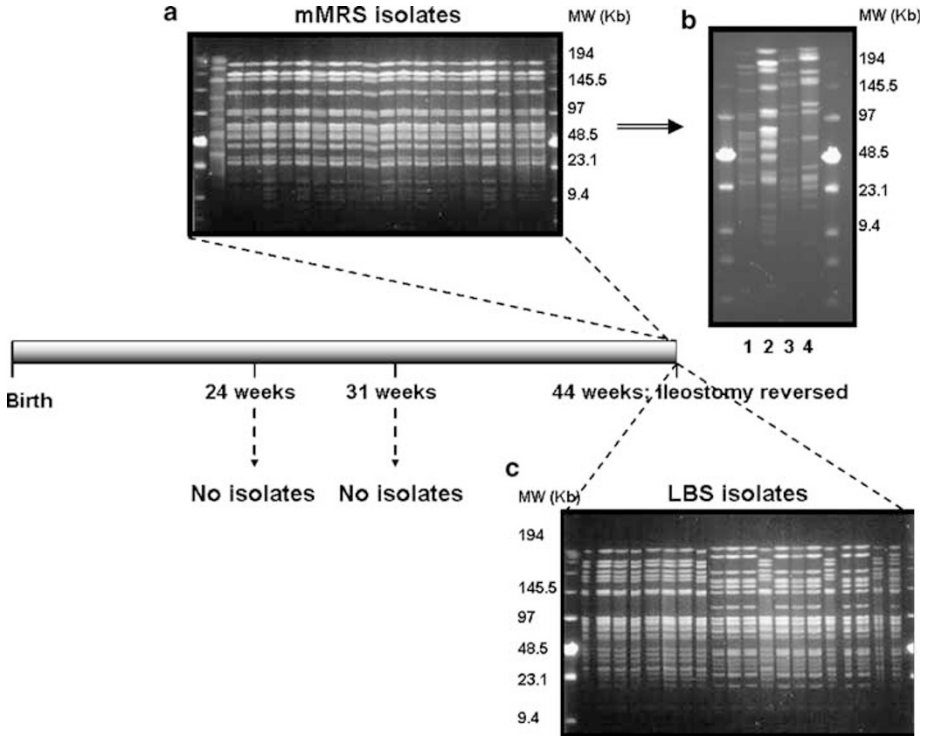

Figure 2 (**a**) PFGE macro restriction pattern following genomic digests with *Xba*I of mMRS-isolates from the third time point, 44 weeks when the ileostomy had been reversed, infant K. (**b**) PFGE macro restriction pattern following genomic digests with *Xba*I on *B. animalis subsp. lactis* strain from infant K (lane 1) and probiotic strain *B. animalis subsp. lactis* Bb12 (lane 2), which proved indistinguishable from PFGE patterns. *Spe*I restriction to differentiate the strains, *B. animalis subsp. lactis* from infant K (lane 3) and *B. animalis subsp. lactis* Bb12 (lane 4), proving that differentiation was impossible. (**c**) PFGE macro restriction pattern following genomic digests with *Apa*I of LBS isolates from the third time point (44 weeks), infant K. Molecular weight (MW) markers are shown in outside lanes. PFGE, pulsed-field gel electrophoresis.

suggesting a strong genetic relatedness between the strains (Figure 2c).

Detection of lactobacilli and bifidobacteria in ileostomy samples (infant D) using DGGE

The primer set Lac1 and Lac2GC resulted in an amplicon for both cecal samples, and DGGE analysis of the PCR-amplified 16S rRNA fragments revealed an identical profile for both the samples, which further matched the migration profile of *L. paracasei*.

Bifidobacterial genus-specific primers were used to amplify a fragment of the 16S rRNA gene. No PCR products were obtained from the cecal samples when using the genus-specific primers Im-3 and Im-26, indicating that either the numbers were below the detection level or that *Bifidobacterium* species were completely absent in these samples.

Comparison of results of DGGE analyses with those of bacteriological culture (infant D)

To obtain an insight into the composition of the dominant *Lactobacillus* species at different time points, 20 colonies were randomly selected from lactobacilli selective plates and subcultured from each time point. These revealed that it was one dominant strain that was the same in both time points and, according to 16S rRNA sequencing, belonged to the species *L. paracasei*. This cultured species could also be detected by PCR-DGGE where the DGGE profile of these samples matched the profile of *L. paracasei* (Table 1).

Detection of lactobacilli and bifidobacteria in the ileostomy and per-anal stool samples (infant K) using DGGE

No PCR products were obtained from the samples when using the primers Lac1 and Lac2GC, indicating that either the numbers were below the detection level or that the *Lactobacillus*-like species were absent.

The *Bifidobacterium*-specific primers Im-3 and Im-26 resulted in an amplicon for all samples in this case. These amplicons served as template DNA for the V3 primer combination V3R and V3F during a second PCR step. The mobility of the PCR products obtained by these primers in DGGE was compared to the PCR pattern of bifidobacteria reference strains. Because of the high G + C content of bifidobacteria, the conventional 35–70% denaturing gradient was replaced with a 50–70% denaturing gradient. DGGE of rRNA gene amplicons revealed that there were three amplicons associated with each sampling time, which were identified as *Bifidobacterium bifidum*, *B. longum*, *Bifidobacterium scardovi* and *Bifidobacterium animalis*, when compared to the migration distances of the reference strains (Figure 3). The reference strains *B. scardovi* and *B. animalis* showed the same migration distance in the gel. As these species were cultured from the samples, they should also be identified by DGGE. These four strains associated with the samples also proved to be the same at all three sampling times.

Comparison of results of DGGE analyses with those of bacteriological culture (infant K)

Although a DGGE profile could be obtained for the cecal samples (Figure 3), bacteria were not cultured on the bifidobacteria selective media from these samples, which indicates that either the strains are non-cultivable by the method used in this study or non-viable. A DGGE profile was also obtained from the per-anal fecal sample, which revealed that this sample contained four different strains, *B. bifidum*, *B. longum*, *B. animalis* and *B. scardovi*, when compared to the migration distances of the reference strains. In the latter case, two of these strains, *B. animalis subsp. lactis* and *B. scardovi*, were also

Table 1 Species detected by PCR-DGGE and by culture from infant D and K

Infant	*Sample*	*Bacterial species*	*Detected by*	
			PCR-DGGE	*Culture (no of colonies, out of 20)*
D	Cecal (50 days)	*L. paracasei*	+	+ (20)
	Cecal (74 days)	*L. paracasei*	+	+ (20)
K	Cecal (24 weeks)	*B. animalis subsp. lactis*	+	—
		B. scardovi	+	—
		B. longum	+	—
		B. bifidum	+	—
	Cecal (31 weeks)	*B. animalis subsp. lactis*	+	—
		B. scardovi	+	—
		B. longum	+	—
		B. bifidum	+	—
	Fecal (44 weeks)	*B. animalis subsp. lactis*	+	+ (19)
		B. scardovi	+	+ (1)
		B. longum	+	—
		B. bifidum	+	—

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

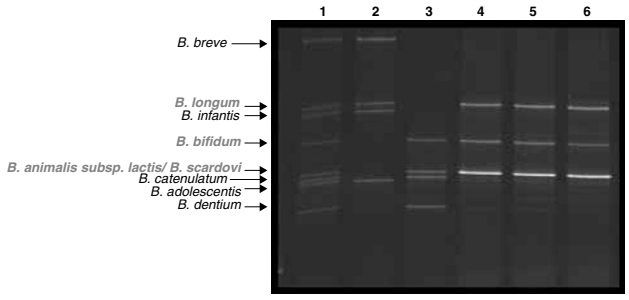

Figure 3 DGGE of bifidobacterial PCR-products (V3-region) from infant K: first time point; cecal sample (lane 4), second time point; cecal sample (lane 5) and third time point; fecal sample (lane 6). The mobility of the PCR products obtained in DGGE was compared to the PCR pattern of reference strains obtained with the same primer set (lanes 1, 2 and 3). DGGE, denaturing gradient gel electrophoresis.

obtained by culturing. Table 1 summarizes the comparisons of culture and PCR DGGE results. All of the cultured species could be detected by PCR-DGGE.

Discussion

A better understanding of the developing neonatal intestinal ecosystem may hold the key to prevention or modification of several important diseases. Adhesion and colonization of probiotic bacteria, such as lactobacilli and bifidobacteria in the gastrointestinal tract of the host, is believed to be one of the essential features required for delivering their health benefits (Bernet *et al*., 1994). Establishment and maintenance of a 'normal' intestinal microflora may help abrogate gastrointestinal and other inflammatory conditions (Kalliomaki *et al*., 2001). This study was designed to examine the lactobacilli and bifidobacteria microbiota in the human ileum at a very early stage of life.

Bifidobacterium species are common members in the infant gut, comprising 91% of the total cultivable microflora in breast-fed babies and up to 75% in formula-fed infants (Bennet and Nord, 1987). In this respect it is interesting that Bifidobacteria were not isolated from ileostomy effluents at any time point in the study. They were obtained only by culturing when the ileostomy had been reversed in 'infant K' and consisted of two different strains, *Bifidobacterium animalis subsp. lactis* and *Bifidobacterium scardovi*, according to PFGE and 16S rRNA sequencing. These species are not among the most common *Bifidobacterium* reported in infants however (Matsuki *et al*., 1999). Interestingly, the *B. animalis subsp. lactis* strain isolated is a well-known commercial probiotic strain, *B. animalis subsp. lactis* Bb12, based on possessing identical genomic fingerprint patterns. *B. animalis subsp. lactis* Bb12 has been reported to increase antimicrobial protection in the gastrointestinal tract, to combat infectious and other diseases and so could be an important health adjunct for infants (Juntunen *et al*., 2001; Prioult *et al*., 2003). DGGE profiles of this infant showed four different strains of bifidobacteria that were the same at three time points. Apart from *B. animalis* and *B. scardovi*, *B. longum* and *B. bifidum* were also detected. These two species were not obtained by culturing in any of the time points, indicating that either the strains are non-cultivable with the method used or non-viable. Furthermore, according to DGGE, the colonization pattern did not change following ileostomy reversal.

Persistent *Bifidobacterium* appeared to be absent in 'infant D' according to both culturing and amplification with *Bifidobacterium*-specific primers, which could be due to the premature birth and antibiotic treatment of this infant. A single strain of *Lactobacillus* dominated in the small intestine for a period of over 3 weeks according to both PFGE and DGGE in this infant. This strain was identified as the probiotic strain, *L. paracasei*, NFBC 338 when compared to the genotype of different *L. paracasei* strains. This strain has been developed specifically for probiotic cheese applications (Gardiner *et al*., 1998), and clinical trials have showed that it increases the overall *Lactobacillus* populations in the adult human gut (unpublished). Repeated isolation of a single strain from the same individual suggests that the strain colonizes and replicates in the gut. Colonization by *Lactobacillus* strains, however, does not appear to be a common trait in early infancy. A study of Japanese infants revealed that only 2/86 infants harbored the same strain of lactobacilli at 5 days and 1 month of age (Matsumiya *et al*., 2002). In the study by Ahrne *et al*. (2005), persistent colonization by a single strain over at least 3 weeks was indicated in 17% of the infants in the first 6 months of life. 'Infant K' did not yield *Lactobacillus* at any time point by either culturing or DGGE. Heilig *et al*. (2002) studied the development of the *Lactobacillus*-like community in infants by PCR on fecal DNA from a baby from delivery (day 1) to 5 months later (day 147) at regular intervals. No PCR products were obtained up to day 55, indicating that the template was absent or that the amount of template was too low to be detected.

We are not aware of any previously published studies of the composition of the human neonatal ileal microbiota, owing to sampling difficulties. Indeed, it is worthwhile to emphasize that such cases are extremely rare but provide an almost unique opportunity to examine the human ileum at a very early stage of life. Few attempts have been made to isolate potential adherent probiotic bacteria directly from the human intestinal mucosa (the environment into which they are subsequently reintroduced and required to function). This study indicates the presence of two different probiotic strains (*L. paracasei* NFBC 338 and *B. animalis subsp. lactis* Bb12) in the upper intestinal tract at an early stage of human life,

which provides evidence for their ability to colonize the human small intestine.

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