Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life

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Bifidobacteria in the infant faecal microbiota have been the focus of much interest, especially during the exclusive milk-feeding period and in relation to the fortification of infant formulae to better mimic breast milk. However, longitudinal studies examining the diversity and dynamics of the *Bifidobacterium* population of infants are lacking, particularly in relation to the effects of weaning. Using a polyphasic strategy, the *Bifidobacterium* populations of breast- and formula-fed infants were examined during the first 18 months of life. *Bifidobacterium*-specific denaturing gradient gel electrophoresis demonstrated that breast-fed infants harboured greater diversity than formula-fed infants and the diversity of the infants' *Bifidobacterium* populations increased with weaning. Twenty-seven distinctive banding profiles were observed from ~1100 infant isolates using ribosomal intergenic spacer analysis, 14 biotypes of which were confirmed to be members of the genus *Bifidobacterium*. Two profiles (H, *Bifidobacterium longum* subsp. *infantis*; and I, *Bifidobacterium bifidum*) were common culturable biotypes, seen in 9/10 infants, while profile E (*Bifidobacterium breve*) was common among breast-fed infants. Overall, inter- and intra-individual differences were observed in the *Bifidobacterium* populations of infants between 1 and 18 months of age, although weaning was associated with increased diversity of the infant *Bifidobacterium* populations. Breast-fed infants generally harboured a more complex *Bifidobacterium* microbiota than formula-fed infants.

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INTRODUCTION

In the early 1900s, Tissier suggested that the better health status of breast-fed infants was associated with higher numbers of bifidobacteria in their faeces compared with their formula-fed counterparts (Dai & Walker, 1999). It is now thought that the high levels of bifidobacteria found in the faeces of breast-fed infants may partially explain why they are, in general, less susceptible to gastrointestinal (GI) diseases in the first year of life compared with formula-fed

infants (Liepke *et al.*, 2002). In addition, breast-fed infants have significantly lower rates of respiratory and urinary infections and food allergies compared with formula-fed infants (López-Alarcón *et al.*, 1997; Wilson *et al.*, 1998; Harmsen *et al.*, 2000b; Wold & Adlerberth, 2000; Hoppu *et al.*, 2001; Howie, 2002). Numerous health benefits have been associated with members of the genus *Bifidobacterium*, including modulation of the immune system (Kirjavainen *et al.*, 2002), vitamin production (Noda *et al.*, 1994), relief of atopic dermatitis symptoms in infants (Isolauri *et al.*, 2000) and reduction of diarrhoea, rotavirus infections and lactose intolerance in children and adults (Saavedra *et al.*, 1994; Jiang *et al.*, 1996).

On average, the *Bifidobacterium* population of a 2-week-old infant contributes 40–60% of the total faecal microbiota; however, bifidobacteria can be non-detectable in some formula-fed infants and constitute 90% of total bacteria in exclusively breast-fed newborns (Harmsen *et al.*, 1999, 2000a). Sakata *et al.* (2005) identified *Bifidobacterium longum* subsp. *longum* as the species most commonly found in 1-month-old infants, irrespective of feeding regime, followed by (in order of predominance) *B. longum*

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; GI, gastrointestinal; ISR, intergenic spacer region; M, monthly; PW, pre-weaning; Rf, retardation factor; RISA, ribosomal intergenic spacer analysis; W, weaning.

The GenBank/EMBL/DDBJ accession numbers for the sequences discussed in this paper are listed in Table 2.

Two supplementary figures, showing *Bifidobacterium* populations present in faecal samples of infants BF4 and BF1 as assessed by FISH analysis, are available with the online version of this paper.

subsp. *infantis*, *B. breve*, *B. catenulatum*, *B. adolescentis* and *B. bifidum*. Other studies have shown that *B. breve* is the member of the genus *Bifidobacterium* found most frequently in both breast- and formula-fed infants (Mackie *et al.*, 1999; Matsuki *et al.*, 1999; Fanaro *et al.*, 2003). The discrepancies between studies may be due to different methods having been used, different milk formulae and geographically distinct infant groups. While the predominance of bifidobacteria can vary according to the infant's environment and type of feeding, levels of these bacteria gradually decrease with age (Mitsuoka, 1996). This observation may be explained by the change of diet, withdrawal of breast or formula milk, physiological changes in the growing infant and maturation of the GI tract (Mitsuoka, 1996; Hopkins *et al.*, 2005). It is generally accepted that *B. adolescentis*, *B. catenulatum* and *B. longum* subsp. *longum* are characteristic of adult *Bifidobacterium* populations, whereas *B. breve*, *B. bifidum* and *B. longum* subsp. *infantis* are more common among infants (Mitsou *et al.*, 2008). However, the transition of the infant *Bifidobacterium* population (in terms of dynamics and diversity) to the adult *Bifidobacterium* population in an individual has not yet been described (Matsuki *et al.*, 2004; Dinoto *et al.*, 2006). In addition, the effect of weaning on the diversity of *Bifidobacterium* populations has not yet been examined fully (McCartney *et al.*, 1996; Klijn *et al.*, 2005; Klaassens *et al.*, 2009).

Investigation into the diversity and dynamics of the predominant *Bifidobacterium* population of human adults demonstrated that genetic fingerprinting methods allowed good discrimination of different biotypes below species level (McCartney *et al.*, 1996). One subject harboured a simple and relatively stable faecal *Bifidobacterium* population across the 12-month study, while the second subject harboured a much more complex and dynamic faecal *Bifidobacterium* population. No common biotypes were identified among the predominant cultivable bifidobacteria of the two donors. The follow-up study from the same research group confirmed the finding that each subject ($n=10$) harboured a unique predominant *Bifidobacterium* population (Kimura *et al.*, 1997). More recently, a number of different fingerprinting/profiling methods have been developed, some of which are rather less labour intensive than the ribotyping and pulsed-field gel electrophoresis protocols used by Tannock and colleagues (McCartney *et al.*, 1996; Kimura *et al.*, 1997). One such method is ribosomal intergenic spacer analysis (RISA), which involves restriction fragment length analysis of the ribosomal intergenic spacer region (ISR) of bacterial genomes (Kabadjova *et al.*, 2002). The use of ISR sequences for differentiation between isolates is known to be more useful than 16S rRNA gene sequence analysis. For example, 16S rRNA gene sequences of species of the genus *Bifidobacterium* can have $\geq 99\%$ similarity (i.e. *B. longum* subsp. *infantis* and *B. longum* subsp. *longum*), whereas the ISR gene sequences have greater sequence divergence in closely related species (Ventura *et al.*, 2001, 2006).

Recently, Turrone *et al.* (2009a) examined the diversity of the *Bifidobacterium* population in colonic mucosal and faecal samples of humans (infants, adolescents and adults) based on ISR sequences of presumptive isolates of the genus *Bifidobacterium*. Inter-individual variation was observed, together with differences based on ecological site the isolates were cultured from (mucosal versus faecal samples), although some caution is warranted for the latter, as stool samples were from infants (no details of age and/or diet were provided) and mucosal samples were from adolescent or adult subjects. Interestingly, Turrone *et al.* (2009a) referred to 'a set of 32 strains which appeared to be commonly distributed among different individuals'. The majority of these (21) were only isolated from mucosal samples and often then only from two different subjects (14), although *B. breve* 246B was isolated from mucosa of eight different subjects. Another study from the same group employed microbiomic analysis (i.e. *Bifidobacterium*-specific 16S rRNA gene libraries) to examine the *Bifidobacterium* population in colonic mucosal samples from five healthy adults (Turrone *et al.*, 2009b). As well as showing inter-individual variation in the *Bifidobacterium* populations, novel *Bifidobacterium* phylotypes were identified, which may reflect the niche investigated and/or the methodology used, although non-specific amplification was also evident. However, it is clear that the diversity of the human gastrointestinal bifidobacterium population has not yet been fully elucidated.

We investigated the development of faecal *Bifidobacterium* populations in relation to diet and age as part of a longitudinal study of the infant faecal microbiota (Roger & McCartney, 2010). The aim of this work was to monitor the infant faecal microbiota using a polyphasic approach that allowed examination of *Bifidobacterium* populations and detection of common or divergent colonization patterns in infants throughout the first 18 months of life.

METHODS

***Bifidobacterium*-specific denaturing gradient gel electrophoresis (DGGE).** DNA extracted during the longitudinal study by Roger & McCartney (2010) was used in PCRs with the *Bifidobacterium*-specific primers Bif164-f and Bif662-GC-r (Satokari *et al.*, 2001). PCR mixtures comprised 10 μ l 5 $\times$ MgCl₂-free buffer (Promega), 5 μ l dNTP mix (containing 12.5 mM of each dNTP; Promega), 1 μ l each of primer Bif164 and primer Bif662-GC-r (both 20 pmol), 6 μ l MgCl₂ (25 mM; Promega), 24 μ l sterile H₂O, 1 μ l GoTaq (1.25 U μ l⁻¹; Promega) and 2 μ l template DNA. PCR was carried out as described by Satokari *et al.* (2001). DGGE was carried out as described by Roger & McCartney (2010), but with minor modifications: the V20-HCDC DGGE system (BDH) was used with gels comprising 45–55% gradients and run at 85 V.

Ladder for *Bifidobacterium*-specific DGGE. Type strains of *Bifidobacterium* species identified as members of the human gut microbiota, namely *B. adolescentis* DSM 20083^T, *B. bifidum* DSM 20456^T, *B. angulatum* DSM 20098^T, *B. breve* DSM 20213^T, *B. catenulatum* DSM 20103^T, *B. gallicum* DSM 20093^T, *B. longum* subsp. *infantis* DSM 20088^T, *B. longum* subsp. *longum* DSM 20219^T and *B.*

pseudolongum subsp. *pseudolongum* DSM 20099^T were used to develop the *Bifidobacterium*-specific DGGE ladder. Type strains were grown on pre-reduced Beerens agar (Beerens, 1991) for 48 h in an anaerobic cabinet (10:10:80, H₂/CO₂/N₂; MACS 1000 work station, Don Whitley Scientific). A single colony of each was inoculated into pre-reduced supplemented brain heart infusion broth [per litre: 37 g brain heart infusion broth; 5 g yeast extract; 4 ml resazurin solution; 0.5 g L-cysteine; vitamin K₁ (1 µg ml⁻¹, final concentration); 10 ml haemin solution (50 mg ml⁻¹)]. After 48 h, the broth cultures were centrifuged at 13 792 g at 4 °C for 5 min. The supernatants were discarded and DNA was extracted from cell pellets by using the QIAmp DNA Stool Mini kit (Qiagen) according to the manufacturer's instructions. PCR was performed using Bif164-f/Bif662-GC-r as described above. The type strains migrated to five different positions in the *Bifidobacterium*-specific DGGE. The bands representing *B. angulatum* DSM 20098^T and *B. catenulatum* DSM 20103^T migrated to the same position. Similarly, the bands representing *B. longum* subsp. *infantis* DSM 20088^T and *B. longum* subsp. *longum* DSM 20219^T migrated to the same position. Bands representing *B. breve* DSM 20213^T, *B. gallicum* DSM 20093^T and *B. pseudolongum* subsp. *pseudolongum* DSM 20099^T migrated to approximately the same position. Five type strains were thus selected for the *Bifidobacterium*-specific DGGE ladder (namely, *B. adolescentis* DSM 20083^T, *B. bifidum* DSM 20456^T, *B. catenulatum* DSM 20103^T, *B. longum* subsp. *longum* DSM 20219^T and *B. pseudolongum* subsp. *pseudolongum* DSM 20099^T).

Analysis of DGGE gels. Gel images were imported into TotalLab TL120DM v2006f. Lanes were created manually and background noise was subtracted by using the 'rolling ball' algorithm with a radius of 50 pixels. Automatic band detection was performed with a minimum slope of 100 and a noise reduction of 5. Peaks smaller than 2 % of the highest peak in a lane were discarded. The edge detection method was fixed to a width of 15 for bands in all gels. Detected bands were compared to those on the original, preserved DGGE gels and corrected manually. Horizontal retardation factor (Rf) lines, corresponding to the seven bands in the *Bifidobacterium* ladder, were used to normalize gels. The same values for all seven Rf lines were used in all gels to allow comparisons once transferred into the database. Matching analysis was then performed (using a vector of 0.003), assessed by eye and corrected manually.

Matching calculations were performed based on the Jaccard similarity coefficient (which matches by the presence/absence of bands), allowing dendrograms to be generated. Pearson correlation coefficient is considered more suitable for highly complex DGGE profiles; however, this algorithm takes into account the whole profile of a lane, including the peak height of each band (van Verseveld & Röling, 2004). In the case of this study, the Pearson correlation coefficient was not a suitable option as a certain degree of variation (in terms of gradient reproducibility) occurred for every gel developed and the amount of template DNA used in each PCR was not always the same.

Cultivation work. This was done on alternate faecal samples from five breast-fed (BF1–BF5) and five formula-fed (FF1–FF5) infants. For details of the sampling schedule, refer to the paper by Roger & McCartney (2010), in this issue. Due to the nature of the collection of samples from some infants (where samples were not always available on particular sample weeks), this did not correlate with alternate pre-weaning (PW), weaning (W) or monthly (M) samples (e.g. PW1, PW3, PW5...).

An aliquot (500 µl) of a 1 in 10 (w/v) faecal homogenate was added to 4.5 ml pre-reduced peptone water (Oxoid) and used to produce a dilution series (10⁻¹–10⁻⁸ in pre-reduced peptone water). An aliquot (40 µl) of each dilution was plated in duplicate onto pre-reduced Beerens agar and incubated in an anaerobic cabinet at 37 °C for 3–5 days. Colonies were counted from the dilution with 30–300 discrete

colonies, and the c.f.u. (g wet weight faeces)⁻¹ was determined. Fifteen colonies were randomly selected and streaked to purity on Beerens agar. Pure cultures were stored on Microbank cryogenic beads (ProLab Diagnostics) at -70 °C.

RISA

DNA extraction from pure cultures. Ten isolates from each sample for which cultivation work was done were resuscitated on Beerens agar. DNA was extracted from the isolates by using the ChargeSwitch gDNA Mini Bacteria kit following the manufacturer's guidelines.

PCR for RISA. Universal primer 785 (McClellan *et al.*, 1996), which targets a conserved region of the 16S rRNA gene, and primer pR (Leblond-Bourget *et al.*, 1996), which targets a conserved region of the 23S rRNA gene, were used to amplify the ISRs (~1500 bp) of isolates. PCR mixtures comprised 10 µl 5 × MgCl₂-free buffer, 5 µl dNTP mix, 1 µl each of primer 785 and primer pR (both 20 pmol), 6 µl MgCl₂, 25 µl sterile H₂O, 1 µl GoTaq and 1 µl template. PCR cycles were 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min, with a final elongation at 72 °C for 10 min. Products were examined by using agarose gel electrophoresis and stored at -20 °C until required.

Enzymic digestion of amplicons. To select the best array of restriction enzymes for DNA digestion, sequences from GenBank for the type strains of different species of the genus *Bifidobacterium* were digested *in silico* using different restriction enzymes (<http://rebase.neb.com/rebase/rebase.html>). The following type strains were used: *B. breve* ATCC 15700^T (GenBank accession nos M58731 and M84776), *B. adolescentis* ATCC 15703^T (M58729), *B. longum* subsp. *longum* ATCC 15707^T (M58739 and M84781), *B. bifidum* ATCC 29521^T (M38018 and M84777), *B. longum* subsp. *infantis* ATCC 15697^T (X70947 and M84783), *B. catenulatum* ATCC 27539^T (M58732 and M84784), *B. angulatum* ATCC 27535^T (M84775) and *B. pseudocatenulatum* ATCC 27919^T (M84785). Seven enzymes (*AluI*, *TaqI*, *HpaII*, *NdeII*, *BamHI*, *Sau96I* and *BstNI*) were identified as giving good differentiation of *Bifidobacterium* species on the basis of the *in silico* analysis. *B. adolescentis* DSM 20083^T, *B. angulatum* DSM 20098^T, *B. breve* DSM 20213^T, *B. catenulatum* DSM 20103^T, *B. gallicum* DSM 20093^T, *B. longum* subsp. *infantis* DSM 20088^T, *B. longum* subsp. *longum* DSM 20219^T and *B. pseudolongum* subsp. *pseudolongum* DSM 20099^T were grown on pre-reduced Beerens agar and DNA was extracted from the strains using the ChargeSwitch gDNA Mini Bacteria kit, as described above. PCR was done using primers pR/785, and products were digested with each of the seven restriction enzymes. Each restriction mixture contained 10 U restriction enzyme, 2 µl buffer, 0.2 µl BSA, 10 µl PCR product and sterile distilled H₂O to give a final volume of 20 µl. Digestions were performed for 1.5 h at 37 or 65 °C, according to the manufacturer's recommendations. Restriction products were separated by electrophoresis using 2.5 % agarose gels in 1 × TAE buffer for 3 h, which were stained by using ethidium bromide (0.5 ng ml⁻¹) for 30 min. After destaining (5 min), the bands were visualized under UV light (Genesnap, Syngene). *HpaII* was selected for the digestion of samples as it gave the greatest differentiation (data not shown).

Analysis of RISA gels. Each profile observed was associated with a single letter and a summary table (Table 1) was organized to illustrate all profiles observed per infant and per sample. Analysis was based both on analysis with TotalLab TL120 v2006f software and visual inspection. The table was then used to detect any common profiles and to observe isolate trends and similarities among infants.

Sequencing and identification of representative RISA biotypes. PCR amplification of the 16S rRNA gene of selected isolates was done using the universal primers pH* (5'-AAGGAGGTGATCCAGCCGCA-3')

and arit (5'-CTGGCTCAGGATGAACGCTG-3'). Almost-complete (>1350 nt) 16S rRNA gene sequences were obtained via the University of Reading's Biocentre. The 16S rRNA gene sequences were subjected to WU-Blast2 searches via the EMBL website (<http://www.ebi.ac.uk/>) to establish the identity of the strains. Isolate identities were confirmed by comparison with type-strain sequences within an in-house database. Sequences of the faecal isolates were proofread manually against type-strain sequences, and submitted to GenBank/EMBL/DDJB for assignment of accession numbers. All sequences were edited via 4Peaks version 1.7.2 (by A. Griekspoor and Tom Groothuis, <http://www.mekentosj.com>).

RESULTS AND DISCUSSION

Bifidobacterium-specific DGGE

In order to monitor the composition of *Bifidobacterium* populations of infants over time, PCR-DGGE was done with the *Bifidobacterium*-specific primers described by Satokari *et al.* (2001). However, *Bifidobacterium* amplicons were not always obtained with these primers (and generally correlated with lower Bif164 counts), although universal DGGE profiles were obtained for all DNA samples (Roger & McCartney, 2010). PCR amplification problems were most frequently observed with PW samples from formula-fed infants. In general, this was consistent with lower Bif164 counts (Roger & McCartney, 2010) and therefore most likely reflects lower copy numbers of *Bifidobacterium* 16S rRNA genes in these samples. However, a more rigorous DNA extraction method may have enabled detection of the *Bifidobacterium* population even in those samples with lower levels. Salonen *et al.* (2010) compared four different faecal DNA extraction methods and demonstrated marked differences in the yields obtained, as well as the relative proportions of certain Gram-positive phyla in community profiling analysis.

A marker was created to help with the analysis of DGGE profiles and to allow presumptive identification of bands in the infant profiles (Fig. 1). DGGE analysis of the type strain profiles demonstrated a single band for all *Bifidobacterium* species, except for *B. adolescentis*, which displayed three bands (Fig. 1). This was consistent with the findings of Satokari *et al.* (2001). *Bifidobacterium*-specific DGGE did not differentiate all the type strains. *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* displayed the same DGGE profiles, as did *B. angulatum* and *B. catenulatum*. *B. breve* and *B. gallicum* produced DGGE profiles with a very faint, fuzzy band (Fig. 1, lanes 29 and 30); this phenomenon was also observed by Satokari *et al.* (2001).

TotalLab TL120DM v2006f software was employed to analyse the DGGE profiles for each sample obtained from the breast- and formula-fed infants. The resulting dendrogram(s) did not demonstrate any specific clustering in relation to diet or age (data not shown). Consequently, the data were reanalysed in relation to presence/absence of each observed band. In order to achieve this, a composite profile was generated and the DGGE data were transformed into figures illustrating the presence/absence of bands (Figs 2 and 3).

DGGE for breast-fed infants. Two samples from the breast-fed group did not produce an amplicon using the *Bifidobacterium*-specific DGGE primers. These were PW6 from BF4, which corresponded to the course of antibiotics received by the infant and a dramatic drop in Bif164 counts (Supplementary Fig. S1, available with the online version of this paper) and sample M2 from BF6.

During the PW phase, BF2 and BF6 each demonstrated a single DGGE profile comprising two bands (Fig. 2a). On the whole, the PW phase *bifidobacterium*-specific DGGE profiles of breast-fed infants displayed between one and

Fig. 1. Composite *Bifidobacterium*-specific DGGE representing the different profiles observed in samples from breast- and formula-fed infants. Lanes: 1, five-strain marker; 2, BF1 month 10; 3, BF2 month 8; 4, BF2 month 18; 5, BF3 month 12; 6, BF3 month 15; 7, BF4 month 15; 8, five-strain marker; 9, BF5 month 15; 10, BF6 month 15; 11, BF7 month 9; 12, BF7 month 10; 13, FF1 month 12; 14, FF2 month 10; 15, five-strain marker; 16, FF3 month 10; 17, FF6 month 10; 18, FF7 month 15; 19, FF4 week 36; 20, BF5 week 26; 21, five-strain marker; 22, *B. bifidum*; 23, *B. adolescentis*; 24, *B. angulatum*; 25, *B. catenulatum*; 26, *B. longum* subsp. *longum*; 27, *B. longum* subsp. *infantis*; 28, *B. pseudolongum* subsp. *pseudolongum*; 29, *B. breve*; 30, *B. gallicum*; 31, five-strain marker. White boxes indicate bands taken as being representative of the respective type strains.

Fig. 2. Representation of *Bifidobacterium*-specific DGGE profiles for breast-fed infants during the PW (a), W (b) and M (c) phases of feeding. The presence of a band at a particular sampling time is indicated by grey shading. Samples are presented in chronological order (left to right). Black arrows, administration of antibiotics; grey arrows, withdrawal of breast milk.

four bands (with a mean number of bands per profile of 1.8). Five of the seven breast-fed infants displayed a band consistent with that of the *B. bifidum* band in the marker at some point during the PW phase (BF2 and BF6 did not). Three breast-fed infants showed bands consistent with the *B. longum* band in the majority of their PW samples (BF2, BF5 and BF7). The profiles from BF1, BF3 and BF4 showed

a band consistent with the *B. breve* band in the marker during the PW phase. No bands corresponding to the *B. adolescentis* and *B. pseudolongum* subsp. *pseudolongum*/*B. gallicum* bands were seen in the PW profiles of breast-fed infants. It appeared that band 9 was a common band amongst the breast-fed infants (i.e. seen in all infants at some time during each dietary phase).

Fig. 3. Representation of *Bifidobacterium*-specific DGGE profiles for formula-fed infants during the PW (a), W (b) and M (c) phases of feeding. The presence of a band at a particular sampling time is indicated by grey shading. Samples are presented in chronological order (left to right).

The profiles obtained during the W phase showed similar diversity to those observed during the PW phase (Fig. 2b), although the profiles of BF5, BF6 and BF7 had an additional band compared with the PW phase and BF7 no longer had a band consistent with *B. bifidum*. Furthermore, the profiles of the twins (BF3 and BF4) and BF5, BF6 and BF7 generally contained more bands in the W phase than seen in the PW phase. This was

reflected in the fact that the mean number of bands per profile was 3.0 during the W phase. Overall the profiles of each infant appeared more stable during the W phase. A band consistent with *B. bifidum* was present in the profiles of four infants (BF7 being the infant who displayed this band during PW but not W). Bands consistent with *B. longum* and *B. breve* were present in the profiles of the same infants who displayed these bands

during PW. Band 9 was again a common band to the profiles of breast-fed infants.

The profiles obtained during the M phase contained between three and seven bands (Fig. 2c), and showed greater diversity than seen for the PW and W phases (with a novel band seen for six of the seven infants; i.e. a band not previously displayed in the profiles). However, the mean number of bands per profile was consistent with the W phase (i.e. 3.0). Band 9 was again common to the *Bifidobacterium*-specific DGGE profiles of breast-fed infants (only absent from the profiles of four of the 43 M phase samples). BF2 was the only infant that did not display a band corresponding with the *B. bifidum* band, but a band consistent with *B. breve* was seen, although only one sample (M6) from BF6 displayed a band consistent with *B. bifidum*. Bands consistent with *B. longum* and *B. breve* were present in the profiles of the same infants who displayed these bands during PW. Bands consistent with *B. adolescentis* were evident in the first three M phase samples of BF4.

There was no obvious effect of withdrawal of breast-milk or administration of antibiotics on the *Bifidobacterium*-specific DGGE profiles of individuals. BF3 and BF4 (twins) displayed a simpler bifidobacterium-specific DGGE profile after breast-feeding ceased, but only one further sample was available, so no conclusions can be drawn from this observation.

DGGE for formula-fed infants. As mentioned above, *Bifidobacterium*-specific DGGE primers did not amplify a product from some samples. Overall, the PW phase *Bifidobacterium*-specific DGGE profiles of formula-fed infants appeared to be relatively simple, with between one and four bands present (Fig. 3a). The mean number of bands per profile was 1.9. Bands consistent with *B. bifidum* and *B. breve* were present in two infants (FF4 and FF5, and FF1 and FF7, respectively). Bands consistent with *B. adolescentis* were seen in all PW phase profiles of FF7. Band 9 was present in the PW phase profiles of four infants (FF2, FF3, FF5 and FF6). Overall, the *Bifidobacterium*-specific DGGE profiles of formula-fed infants were relatively stable during the PW phase.

The *Bifidobacterium*-specific DGGE profiles of formula-fed infants remained relatively simple during the W phase, although alterations in banding profiles were seen for some individuals (Fig. 3b). Between one and five bands were detected and the profiles from three infants (FF2, FF3 and FF4) displayed a single band throughout the W phase. A mean number of 1.4 bands were seen per profile for the W phase samples of formula-fed infants. This was notably less than that seen for breast-fed infants at the same stage. Similar to PW phase profiles, bands consistent with *B. adolescentis* were only seen for FF7, together with a band consistent with *B. breve* and band 8 (on some occasions). A band consistent with *B. breve* was also present in FF5 and FF6, neither of whom displayed this band in their PW profiles. FF5 also contained a band consistent with *B.*

longum in sample W4. A band corresponding with *B. bifidum* was seen in three infants (FF4–FF6); FF4 and FF5 also displayed this band in their PW profiles. Band 9 formed the backbone of the *Bifidobacterium*-specific DGGE profiles of FF2, FF3, FF5 and FF6.

The M phase *Bifidobacterium*-specific DGGE profiles of formula-fed infants remained simple (Fig. 3c), with between one and five bands per profile (mean 1.9 bands per profile). No *Bifidobacterium*-specific PCR products were obtained for FF4 from M phase samples. FF3 maintained a *Bifidobacterium*-specific DGGE profile comprising only band 9 (consistent with the PW and W phase profiles). Indeed, FF2 and FF3 maintained the same profile as seen at the end of the W phase. Band 9 was common among the formula-fed infants, with only FF1 not showing it in M phase DGGE profiles. Bands consistent with *B. adolescentis* were still present in FF7's profiles. The band consistent with *B. bifidum* remained in the profiles of FF5 and FF6, while a band corresponding with *B. breve* was present in FF6 and FF7.

Comparison of DGGE profiles between feeding groups.

Overall, the *Bifidobacterium* profiles were relatively simple (comprising between 1 and 6 bands), in accordance with data described by Klaassens *et al.* (2009), which showed that the *Bifidobacterium* populations of two breast-fed and three formula-fed infants were relatively simple and stable between 6 and 11 months of age. However, Klaassens and colleagues observed less stability in the *Bifidobacterium* profiles of formula-fed infants, most likely reflecting the dietary intervention (prebiotic-supplemented formula). The main difference observed in the current study between the profiles from the breast- and formula-fed infants was the mean number of bands per profile for each dietary phase. Overall, the mean number of bands per breast-fed infant profile was greater than that for the formula-fed profiles (in all dietary phases). In both feeding groups, the mean number of bands per profile increased across the three dietary phases. The *Bifidobacterium*-specific DGGE profiles of breast-fed infants varied more during the study than those of formula-fed infants (which were relatively stable over time).

Bands consistent with *B. adolescentis* were observed in the profiles of FF7 across all dietary phases and in some M phase profiles of BF4. Bands corresponding to *B. bifidum* and *B. breve* were observed in profiles of both feeding groups across the study, while bands corresponding to *B. longum* were observed in profiles of breast-fed infants (throughout the study), but only in the profile of FF5's sample W4. Interestingly, band 9 was seen in both feeding groups and was present throughout the study.

Overall, the diversity of the *Bifidobacterium* populations harboured by breast-fed infants was greater than that of formula-fed infants from 4 weeks of age and increased with weaning. The formula-fed group showed relatively simple and stable *Bifidobacterium*-specific DGGE profiles.

Comparison of DGGE and FISH data

BF1 experienced a drop in Bif164 counts at sample W3 (week 29, Supplementary Fig. S2, available with the online version of this paper) that corresponded with the disappearance of *B. bifidum* from the *Bifidobacterium*-specific DGGE profiles (Fig. 2b). Bif164 counts were relatively stable for the twins (BF3 and BF4) until the start of the M phase, with the exception of BF4's PW6 sample (this correlated with antibiotic administration); interestingly, no amplicon was obtained for *Bifidobacterium*-specific PCR-DGGE for this sample. *Bifidobacterium*-specific DGGE showed intra-individual variability in the twins' PW and M phase profiles. In addition, *Bifidobacterium*-specific PCR was generally unsuccessful for PW samples from FF2, which corresponded with the lowest Bif164 counts of all infants seen during PW. Interestingly, this also correlated with a lack of isolation of any *Bifidobacterium* strains (discussed below) from the FF2 PW samples for which cultivation work was performed.

Taken together, these results indicated that data generated from the FISH and *Bifidobacterium*-specific DGGE assays generally complemented each other. Furthermore, they confirmed that the dynamics of the bacterial population was not always discernible via FISH counts. Interestingly, BF7 showed the greatest diversity in the *Bifidobacterium*-specific DGGE profiles, despite generally having the lowest Bif164 counts of the infants included in the breast-fed group.

Investigation into the predominant culturable biotypes

Fifteen isolates were randomly selected from Beerens plates, when available, from the dilution that resulted in discrete colonies. The objective was to genetically fingerprint 10 randomly selected isolates; 15 were subcultured to allow for loss of strains, due to either unsuccessful subculturing (no growth) or inability to resuscitate after cryogenic storage. In total, isolation work was performed for 123 samples from the 10 infants. On nine occasions, no bacterial colonies were obtained from the dilution series [twice from breast-fed infants' M phase samples (BF1 and BF5), three times from PW samples of FF2, once from a W phase sample of FF2 and three times from formula-fed infants' M phase samples (FF3 and FF4)]. In total, genetic fingerprinting was completed for approximately 1100 isolates.

Initial work comprised screening different restriction enzymes using the type strains of nine *Bifidobacterium* species to determine the relative discriminative power of each enzyme using the amplified 16S–23S rRNA ISR (data not shown). *Hpa*II digests demonstrated the greatest differentiation; however, *B. angulatum* and *B. catenulatum*, and *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* could not be differentiated (Fig. 4a).

Twenty-seven distinctive banding profiles were identified from the infant isolates (Fig. 4b). In general, the infants appeared to harbour relatively simple predominant *Bifidobacterium* populations (with between one and three distinct

Fig. 4. Genetic fingerprinting of bacterial pure cultures using RISA. (a) Banding profiles of type strains of members of the genus *Bifidobacterium* obtained by *Hpa*II digestion of amplified 16S–23S ISR. Lanes: 1, *B. adolescentis*; 2, *B. angulatum*; 3, *B. bifidum*; 4, *B. breve*; 5, *B. catenulatum*; 6, *B. gallicum*; 7, *B. longum* subsp. *infantis*; 8, *B. longum* subsp. *longum*; 9, *B. pseudolongum* subsp. *pseudolongum*; 10, 100 bp ladder. (b) Representative banding profiles obtained by *Hpa*II digestion of amplified 16S–23S ISR of bacteria isolated from infants. Lanes: 1, 100 bp ladder; 2–15, A–N, respectively; 16, 100 bp ladder; 17–28, O–Z, respectively; 29, AA; 30, 100 bp ladder.

biotypes seen per sample) (Table 1). BF3 and BF4 (the twins) displayed the most complex and dynamic *Bifidobacterium* microbiota of all infants, with more than three biotypes seen on a number of occasions. A mean of 6.7 distinctive biotypes were found per infant during the study: 7.2 per breast-fed infant and 6.2 per formula-fed infant.

A representative isolate was selected for each of the 27 profiles and almost-complete 16S rRNA gene sequences were obtained to allow identification of isolates (Table 2). Fourteen of the 27 biotypes were confirmed to be members of the genus *Bifidobacterium*, namely profiles A–K, V, Z and AA. The other profiles included *Carnobacterium divergens* (profile O), *Clostridium baratii* (W), *Clostridium clostridioforme* (X), *Clostridium innocuum* (Y), *Collinsella aerofaciens* (T), *Dorea longicatena* (S), *Enterococcus faecalis* (Q and R), *Lactobacillus casei* (M), *Lactobacillus delbrueckii* (P),

Lactobacillus plantarum (U) and *Staphylococcus pasteurii* (L and N). Of these 13 biotypes, only profiles L and M were isolated from more than one sample (Table 1).

Profiles H (*B. longum* subsp. *infantis*) and I (*B. bifidum*) were common biotypes, seen in nine of 10 infants (only FF3 did not display profile H and FF2 did not display profile I). Profile E (*B. breve*) was common among the breast-fed infants, although FF1 also displayed this biotype. Profiles A, B, C, E, F, H, I and M (of which profile M was not a *Bifidobacterium* species) were observed during all three dietary phases (PW, W and M). Sixteen profiles (of which profiles D, G, V, Z and AA were *Bifidobacterium* species) were only observed on one occasion, i.e. from one sample of one infant, although profiles D (*B. adolescentis*; BF5, M6) and R (*E. faecalis*; FF2, PW1) were shown for all isolates fingerprinted from that sample.

Table 1. Details of the different RISA biotypes identified from bacteria isolated from faecal samples of breast- and formula-fed infants

Letters designate distinctive biotypes based on banding patterns obtained from *HpaII* digestion of amplified 16S–23S rRNA ISRs of faecal isolates selected randomly from Beerens agar plates; superscript numbers represent the number of isolates that displayed each banding profile (maximum of 10 per sample). NC, No colonies were obtained on Beerens plates; NS, no sample collected; –, sample was not processed for cultivation. END, study end.

Sample	BF1	BF2	BF3*	BF4*	BF5	FF1	FF2	FF3	FF4	FF5
PW1	E ¹⁰	H ¹⁰	J ⁶ /A ³ /L ¹	A ⁵ /H ⁵	H ⁹	E ⁷ /A ¹ /Q ¹	R ¹⁰	H ⁹ /I ¹	I ² /K ¹	H ⁷ /C ¹ /I ¹
PW2	–	–	–	–	–	–	–	H ¹⁰	I ⁸ /W ¹	H ⁷ /C ³
PW3	E ¹⁰	H ⁹ /L ¹	A ⁴ /H ⁴ /E ²	C ⁴ /H ⁴ /A ¹ /E ¹	E ⁹ /F ¹	E ⁷ /A ²	NC	–	–	I ⁹
PW4	–	–	–	–	–	–	–	NS	I ⁹ /K ¹	–
PW5	E ⁹ /I ¹	H ¹⁰	E ⁶ /C ² /H ¹ /M ¹	E ⁸ /C ²	E ⁸ /F ¹	E ⁹ /H ¹	NC	NS	I ⁹	H ⁵ /I ⁴ /B ¹
PW6	–	–	–	–	–	–	–	NS	–	–
PW7	E ⁷ /H ³	H ¹⁰	E ¹⁰	E ¹⁰	E ⁶ /H ⁴	NS	NC	NS	M ⁸ /C ¹ /G ¹	NS
PW8	–	NS	–	–	NS	NS	–	NS	NS	NS
PW9	E ⁹ /I ¹	NS	E ⁹ /I ¹	E ⁷ /I ² /H ¹	NS	NS	NS	NS	NS	NS
W1	H ⁷ /I ² /E ¹	–	E ⁸ /H ²	E ⁸ /I ²	–	H ⁷ /A ¹ /E ¹	NC	H ¹⁰	–	–
W2	–	H ¹⁰	–	–	E ¹⁰	–	–	–	–	H ⁶ /B ² /I ²
W3	H ⁵ /E ⁴ /J ¹	–	E ⁷ /F ²	H ⁵ /E ³	–	H ⁷ /E ²	H ¹⁰	H ¹⁰	I ¹⁰	H ⁹ /B ¹
W4	–	H ⁸ /E ²	–	–	E ⁷ /F ³	–	–	H ¹⁰	–	–
W5	H ⁹ /E ¹	–	E ⁴ /N ² /C ¹ /H ¹ /I ¹ /M ¹	E ⁵ /C ² /I ² /H ¹	–	H ⁹ /A ¹	H ¹⁰	–	I ⁶	–
W6	–	E ⁴ /H ² /I ²	–	–	E ⁴ /AA ⁴ /J ²	–	–	H ¹⁰	I ⁸ /X ¹	H ¹⁰
W7	H ⁹ /E ¹	–	H ⁵ /M ³ /C ¹ /I ¹	C ⁴ /I ⁴ /A ¹ /M ¹	–	H ⁷ /E ² /I ¹	F ¹⁰	–	–	–
W8	–	H ⁶ /E ³	–	–	E ⁸ /B ¹ /F ¹	–	–	H ⁷	–	H ¹⁰
W9	–	–	H ⁵ /I ⁴ /M ¹	A ⁵ /E ⁵	–	H ¹⁰	F ⁹ /U ¹	–	–	–
W10	–	H ⁷ /E ³	–	–	E ⁷ /F ² /I ¹	–	–	H ¹⁰	I ¹⁰	H ¹⁰
M1	–	–	–	–	–	–	H ⁵ /C ³	–	NC	–
M2	H ⁴ /K ⁴ /I ¹	–	–	–	–	H ⁸ /I ²	–	NC	–	H ⁹
M3	K ¹⁰	H ⁶ /E ⁴	C ⁴ /H ⁴ /E ²	E ⁷ /C ¹	NC	H ⁶ /A ³ /I ¹	–	–	–	–
M4	NC	–	H ⁵ /I ⁴ /A ¹	E ⁸ /P ²	–	END	H ⁶ /C ⁴	–	NC	–
M5	END	–	C ⁴ /H ³ /O ² /M ¹	H ⁶ /C ² /E ¹ /I ¹	–	–	C ⁸ /H ²	H ⁹ /V ¹	END	H ⁹ /Z ¹
M6	–	E ⁴ /H ⁴	END	END	D ²	–	S ⁴ /F ³ /B ¹ /C ¹ /T ¹	H ⁷	–	H ⁷ /I ² /Y ¹
M7	–	H ⁸ /E ²	–	–	H ⁶ /I ⁴	–	END	H ⁹	–	H ⁸ /I ²
M8	–	E ¹⁰	–	–	H ⁶ /I ³ /B ¹	–	–	END	–	END

*Identical twins.

Table 2. Details of bacteria profiled using RISA

Profile	Strain				Closest phylogenetic relative		
	Name	Source infant	16S rRNA gene sequence (nt)	GenBank accession no.	Name (GenBank accession no.)	Sequence similarity (%) (no. of nt compared, mismatched nt, unmatched nt)*	Origin†
A	LCR1	BF3	1462	HQ259718	<i>B. dentium</i> ATCC 27534 ^T (D86183)	99.5 (1463, 6, 1)	Dental caries
B	LCR2	BF5	1461	HQ259729	<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697 ^T (D86184)	99.9 (1461, 1, 0)	Intestine of infant
C	LCR3	FF2	1419	HQ259738	<i>B. pseudocatenulatum</i> JCM 1200 ^T (D86187)	100 (1419, 0, 0)	Infant faeces
D	LCR4	BF5	1460	HQ259739	<i>B. adolescentis</i> YIT 4011 ^T (AB437354)	98.3 (1463, 20, 3)	Intestine of adult
E	LCR5	BF1	1393	HQ259740	<i>B. breve</i> ATCC 15700 ^T (AB006658)	99.9 (1393, 1, 0)	Intestine of infant
F	LCR6	BF5	1460	HQ259741	<i>B. longum</i> subsp. <i>longum</i> ATCC 15707 ^T (M58739)	99.8 (1447, 2, 1)	Intestine of adult
G	LCR7	FF4	1459	HQ259742	<i>B. bifidum</i> KCTC 3202 ^T (U25951)	100 (1459, 0, 0)	Stool of breast-fed infant
H	LCR8	BF2	1362	HQ259743	<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697 ^T (D86184)	99.8 (1362, 6, 0)	Intestine of infant
I	LCR9	FF4	1469	HQ259744	<i>B. bifidum</i> KCTC 3202 ^T (U25951)	100 (1469, 0, 0)	Stool of breast-fed infant
J	LCR10	BF5	1406	HQ259719	<i>B. breve</i> ATCC 15700 ^T (AB006658)	99.9 (1406, 1, 0)	Intestine of infant
K	LCR11	BF1	1451	HQ259720	<i>B. bifidum</i> KCTC 3202 ^T (U25951)	100 (1451, 0, 0)	Stool of breast-fed infant
L	LCR12	BF2	1481	HQ259721	<i>Staphylococcus pasteuri</i> ATCC 51129 ^T (AB009944)	99.5 (1475, 8, 0)	Human vomit
M	LCR13	BF3	1498	HQ259722	<i>Lactobacillus casei</i> subsp. <i>casei</i> ATCC 393 ^T (D16551)	99.1 (1499, 11, 2)	Cheese
N	LCR14	BF3	1484	HQ259723	<i>S. pasteuri</i> ATCC 51129 ^T (AB009944)	99.8 (1484, 4, 2)	Human vomit
O	LCR15	BF3	1479	HQ259724	<i>Carnobacterium divergens</i> DSM 20623 ^T (M58816)	99.9 (1472, 1, 1)	Vacuum-packed minced beef
P	LCR16	BF4	1489	HQ259725	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> DSM 20074 ^T (M58814)	99.6 (1480, 6, 0)	Sour grain mash
Q	LCR17	FF1	1486	HQ259726	<i>E. faecalis</i> JCM 5803 ^T (Y18293)	100 (1448, 0, 0)	Faeces
R	LCR18	FF2	1379	HQ259727	<i>E. faecalis</i> JCM 5803 ^T (Y18293)	100 (1379, 0, 0)	Faeces
S	LCR19	FF2	1445	HQ259728	<i>Dorea longicatena</i> 111-35 ^T (AJ132842)	99.7 (1347, 2, 3)	Human faeces
T	LCR20	FF2	1433	HQ259730	<i>Collinsella aerofaciens</i> ATCC 25986 ^T (AB011816)	98.4 (1433, 18, 5)	Human faeces
U	LCR21	FF2	1498	HQ259731	<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> JCM 1149 ^T (D79210)	99.9 (1498, 3, 0)	Pickled cabbage
V	LCR22	FF3	1466	HQ259732	<i>B. animalis</i> subsp. <i>lactis</i> DSM 10140 ^T (AB050136)	100 (1464, 0, 0)	Rat faeces
W	LCR23	FF4	1443	HQ259733	<i>Clostridium baratii</i> ATCC 27638 ^T (X68174)	99.9 (1443, 2, 0)	Canine renal epithelioma
X	LCR24	FF4	1335	HQ259735	<i>Clostridium clostridioforme</i> ATCC 25537 ^T (M59089)	93.8 (1335, 79, 3)	Calf rumen
Y	LCR25	FF5	1476	HQ259734	<i>Clostridium innocuum</i> ATCC 14501 ^T (M23732)	98.6 (1476, 19, 0)	Appendicular abscess
Z	LCR26	FF5	1471	HQ259736	<i>B. animalis</i> subsp. <i>lactis</i> DSM 10140 ^T (AB050136)	100 (1471, 0, 0)	Rat faeces
AA	LCR27	BF5	1444	HQ259737	<i>B. longum</i> subsp. <i>longum</i> ATCC 15707 ^T (M58739)	99.9 (1444, 2, 1)	Intestine of adult

*Initial strain identification was based upon WU-BLAST2 searches (<http://www.ebi.ac.uk/Tools/blast2/nucleotide.html>) followed by comparison with an in-house database of type-strain sequences. Strains were aligned with sequences of type strains of species with validly published names and sequence similarity was calculated based upon comparisons of sequences of the same length.

†Information retrieved from www.dsmz.de.

Six of the seven biotypes seen in BF4 were also observed for BF3 (twin). However, a further five biotypes were found from BF4 (two of which were harboured by BF5). Of the biotypes isolated from multiple infants, only profile J (*B. breve*) was isolated from one feeding group and not the other.

In general, RISA and *Bifidobacterium*-specific DGGE results were consistent, even though the two methods employed here target different regions of the rRNA genes. For example, a large number of bands was seen in the *Bifidobacterium*-specific DGGE profiles for BF3, and 11 distinct biotypes were identified by RISA. RISA is based on typing of bacteria isolated on a so-called selective medium and not all isolates we obtained were bifidobacteria (1045 of 1089 isolates were from the genus *Bifidobacterium*), although the predominant biotype isolated from each sample was generally bifidobacteria. This is consistent with the findings of Turrone *et al.* (2009a), who identified that only 704 of the 900 isolates they cultured by using a 'selective' *Bifidobacterium* medium were actually *Bifidobacterium*. While our findings suggest Beerens agar may be more 'selective' than mupirocin-based selective medium, Turrone *et al.* (2009a) examined mucosal and faecal samples from different age groups (infants, adolescents and adults). Unlike Turrone and colleagues, very few *B. adolescentis* and *B. animalis* subsp. *lactis* biotypes were cultured in this study, and a much greater proportion of isolates from the genus *Bifidobacterium* were *B. breve* [27.8 %, compared with 2 % of infant *Bifidobacterium* isolates observed by Turrone *et al.* (2009a), although >90 % of these were from breast-fed infants].

RISA profile E (*B. breve*) was observed in all breast-fed infants and one formula-fed infant (FF1). The *Bifidobacterium*-specific DGGE profiles were consistent with this finding, in that the same infants displayed a band consistent with that of *B. breve* in their *Bifidobacterium*-specific DGGE profiles. However, RISA contained the *B. breve*-like profile in W phase samples of FF1, yet no *B. breve*-like band was seen in the corresponding *Bifidobacterium*-specific DGGE profile for these samples. This indicated that the use of more than one molecular method is necessary during comparison studies. The most commonly observed *Bifidobacterium* species in this study were consistent with those seen previously, namely *B. breve*, *B. bifidum* and *B. longum* subsp. *infantis* (Mackie *et al.*, 1999; Matsuki *et al.*, 1999; Fanaro *et al.*, 2003; Sakata *et al.*, 2005; Mitsou *et al.*, 2008; Klaassens *et al.*, 2009). However, *B. breve* did not appear to be a common member of the formula-fed infants' faecal *Bifidobacterium* population; this was evident in DGGE profiles of only two of the seven formula-fed infants in this study (and the predominant biotype isolated during PW phase for FF1; isolation work was not performed for FF7).

The current data demonstrated that, in general, the *Bifidobacterium* component (relative abundance and diversity) of the infant faecal microbiota has been under-

represented in recent metagenomic studies investigating the human microbiome (Palmer *et al.*, 2007; Koenig *et al.*, 2010; Vaishampayan *et al.*, 2010). Indeed, Koenig and colleagues' comparison of compositional and functional data from a 2.5-year case study of the faecal microbiome of a breast-fed infant highlighted inconsistencies between the 16S rRNA gene data and those generated from shotgun sequencing with subsequent taxonomic assignment of genes, suggesting '16S rRNA gene primer bias' was the most likely explanation for the discrepancy (Koenig *et al.*, 2010). This could also explain the relative lack of abundance of bifidobacteria (and/or *Actinobacteria*) in metagenomic studies of the infant gut microbiota, such as was seen during the longitudinal study carried out by Palmer *et al.* (2007). However, the data from our study (herein and in Roger & McCartney, 2010) generally corroborate the global observations from the longitudinal infant studies performed by Palmer *et al.* (2007) and Koenig *et al.* (2010), i.e. the infant gut microbiota develops in a step-wise fashion with increased diversity over time and that life events (including diet) affect the bacterial succession of the infant microbiome (with initial weaning being a transitional period). In addition, longitudinal investigation of the infant *Bifidobacterium* population concurs with previous microbiomic studies in relation to both inter-individual variations and distinction between the microbiota of breast- and formula-fed infants.

Interestingly, taxonomic assignment of the whole gene sets from shotgun libraries of human faecal microbiome analyses of 13 Japanese individuals (including four unweaned infants and two weaned children) demonstrated a *Bifidobacterium* component for all but one microbiome (that of a 7-month-old unweaned infant; F1 U) (Kurokawa *et al.*, 2007). Although the overall proportion of protein-coding genes assigned taxonomic characterization was low (generally <50 %), the relative proportions of *Bifidobacterium* across the age groups was consistent with the consensus view of the human *Bifidobacterium* population associated with life stage, with *Bifidobacterium* being the main constituent of the unweaned infants' microbiome, but a less predominant constituent of the microbiome of older infants and adults. Kurokawa *et al.* (2007) also mapped their shotgun reads to the available *Bifidobacterium* genome sequences in an effort to examine the *Bifidobacterium* component of the human gut microbiome. Ten of the reads from F1-U (total reads=82 523) mapped to *Bifidobacterium* genome sequences. However, over 50 % of the reads from two of the other unweaned infants mapped with *Bifidobacterium* genome sequences, with >90 % of these mapped to a single *Bifidobacterium* species: *B. breve* or *B. longum* (which correlates with *Bifidobacterium* species identification of two of the three common biotypes from breast-fed infants in the present study). Fewer reads from weaned infants' microbiomes mapped to *Bifidobacterium* genome sequences (1–3 %) and comprised multiple *Bifidobacterium* species. Vaishampayan *et al.* (2010) employed microbiomic analysis of the

Bifidobacterium population similar to that described by Turrone *et al.* (2009b) and found a single *Bifidobacterium* phylotype (most closely related to an oral *Bifidobacterium* strain and *B. adolescentis*) from the infant faecal sample at 11 months of age (no *Bifidobacterium* sequences were obtained from the sample taken 1 month after delivery). Overall, the *Bifidobacterium* data from the current study are in general agreement with those from these two metagenomic studies, demonstrating that the *Bifidobacterium* populations in the infant gut microbiota are relatively simple and these increase in diversity following the introduction of solid food into the diet.

In summary, *Bifidobacterium*-specific DGGE and RISA of isolates both show that breast-fed infants generally harboured a greater number of bands/biotypes compared with formula-fed infants during the first 18 months of life. Taken together with the quantitative data presented by Roger & McCartney (2010), the data from this longitudinal study demonstrate that breast-fed infants harbour both higher levels/a predominance of *Bifidobacterium* and a more diverse *Bifidobacterium* population than formula-fed infants. Moreover, the diversity of the *Bifidobacterium* population appears to increase with the start of weaning, particularly in the breast-fed group. Inter-individual differences were evident in the *Bifidobacterium* population of infants, although common culturable *Bifidobacterium* biotypes were observed, and the greatest similarity was seen in the *Bifidobacterium* populations of the twins. This is the first longitudinal study, to our knowledge, that monitors the *Bifidobacterium* populations of infants and the impact of weaning on this bacterial group in an effort to determine the transition from the infant *Bifidobacterium* population to the adult *Bifidobacterium* population. Weaning correlated with increased diversity, yet the predominant *Bifidobacterium* species remained those more commonly associated with the infant microbiota than that of adults. As such, further work is necessary to fully elucidate the bacterial succession and development of the adult *Bifidobacterium* community.

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