RESEARCH LETTER

# A novel combined approach based on HTF-Microbi.Array and qPCR for a reliable characterization of the *Bifidobacterium*-dominated gut microbiota of breast-fed infants

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## Abstract

The High Taxonomic Fingerprint (HTF)-Microbi.Array is a fully validated phylogenetic microarray platform for a high taxonomic level characterization of the human gut microbiota. However, suffering from PCR-dependent biases in *Bifidobacterium* quantification, this tool is less appropriate when utilized for the characterization of the *Bifidobacterium*-dominated gut microbiota of breast-fed infants. To overcome this, we implemented a new combined approach based on HTF-Microbi.Array and qPCR for a reliable fingerprint of the infant-type microbiota. This methodology was applied in a preliminary comparative study of the faecal microbiota of eight breast-fed infants, aged 2–6 months, and five young adults. Whereas the adult gut microbiota was largely dominated by *Firmicutes* and *Bacteroidetes*, the infant-type community was mainly dominated by *Bifidobacterium*, with *Enterobacteriaceae* as the second dominant component. In accordance with the most recent literature in the field, the obtained microbiota fingerprints properly depicted the adult- and the infant-type microbiota, demonstrating the reliability of the HTF-Microbi.Array/qPCR combined approach in reflecting the peculiarities of the two intestinal microbial ecosystems.

## Introduction

Selected by human milk oligosaccharides (HMOs; Koropatkin *et al.*, 2012), bifidobacteria traditionally have been considered a dominant component of the gut microbiota of breast-fed infants (Klijn *et al.*, 2005). Although the early culture-independent studies of the infant microbiota provided controversial results (Palmer *et al.*, 2007; Roger *et al.*, 2010), the most recent microbiota surveys, based on next-generation 16S rRNA gene sequencing and shotgun metagenomics, unequivocally demonstrated that *Bifidobacteria* are a predominant constituent of the gut microbiota of breast-fed infants, constituting 50–80% of the total microbiota in the first year of life (Jost *et al.*, 2012; Turroni *et al.*, 2012; Yatsunenko *et al.*, 2012). There is a growing recognition that the gut microbiota of infants is fundamental for the normal development of the immune system (Johnson & Versalovic, 2012; Olszak *et al.*, 2012). Merging the well known immune-modulation properties (Fanning *et al.*, 2012) with the capacity to inhibit enteropathogen growth (Fukuda *et al.*, 2011), *Bifidobacteria* possess the functional requisites to establish an intense – but controlled – immunological cross-talk with the developing gut-associated lymphoid tissue (GALT) of infants, driving the proper maturation of the host immune system (Maynard *et al.*, 2012; Schwartz *et al.*, 2012). Thus, the maintenance of a healthy and *Bifidobacterium*-dominated developmental trajectory of microbiota during infancy is strategic for the immunological well-being, with a strong impact on health throughout life (Hooper & Macpherson, 2010). Extended deviations from this trajectory can compromise the process of immune education, leading to an immune system inclined to inappropriate activation (Ehlers & Kaufmann, 2010; Maynard *et al.*, 2012). This justifies the monitoring and characterization of deviations from the healthy and

*Bifidobacterium*-dominated developmental trajectory of the gut microbiota in infants, and also of the development of chronic inflammatory diseases (Bisgaard *et al.*, 2011).

The HTF-Microbi.Array (Candela *et al.*, 2010) is a fully validated phylogenetic microarray platform which allows the detection and quantification of up to 31 intestinal bacterial groups, covering up to 95% of the human intestinal microbiota (Candela *et al.*, 2012). Although the HTF-Microbi.Array has been revealed as a robust, cheap and fast tool for the high taxonomic level fingerprint of the adult-type human intestinal microbiota (Candela *et al.*, 2010, 2012; Maccaferri *et al.*, 2012), it suffers from the typical PCR-dependent bias in bifidobacterial quantification (Hattori & Taylor, 2009), resulting in a substantial underestimation of members of this group (Candela *et al.*, 2010). This limit makes the HTF-Microbi.Array less appropriate when applied to the characterization of the *Bifidobacterium*-dominated gut microbiota of breast-fed infants, a limit that becomes of particular relevance considering the strategic roles played by this microbial group in infant health. To overcome this limit, we propose a combined approach based on HTF-Microbi.Array and qPCR, allowing a reliable estimation of the *Bifidobacterium*-dominated gut microbiota of breast-fed infants. The new approach was used in a preliminary comparative analysis of the faecal microbiota of eight breast-fed infants aged 2–6 months and five young adults.

## Materials and methods

### Enrolment

Eight breast-fed, genetically unrelated, healthy infants aged 2–6 months were enrolled in this study in the Bologna metropolitan area and surroundings, Italy. The study protocol was approved by the Ethics Committee of S. Orsola-Malpighi University Hospital (Bologna, Italy). Written informed consent from both parents of each infant was obtained. Exclusion criteria were the administration of antibiotics or probiotics or prebiotics within 3 months prior to the sampling. Faeces were collected by parents directly from diapers by means of spoon-equipped sterile tubes, stored at 4 °C, and delivered to the research group within 24 h and immediately processed.

### Faecal slurry preparation and DNA extraction

Fresh faecal samples were diluted 1 : 2 (w/v) in ice-cold Dulbecco's modified Eagle's medium (DMEM) and homogenized in a Stomacher blender (VWR International PBI, Milan, Italy) for 2 min at high speed, until uniform consistency was obtained. Total microbial DNA from faecal slurries was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the modified protocol reported by Candela *et al.* (2010). DNA concentration and quality were evaluated using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

### HTF-Microbi.Array analysis

For this study we employed the ligase detection reaction-universal array (LDR-UA) platform HTF-Microbi.Array (Candela *et al.*, 2010). Based on LDR, this platform relies on the discriminative properties of the DNA ligation enzyme, which catalyzes the ligation of the two adjacent probes specific for each target sequence only in the presence of a perfectly matching template (Castiglioni *et al.*, 2004). A nearly full-length portion of 16S rRNA gene was amplified using universal forward primer 27F and reverse primer 1492R, according to the protocol described by Candela *et al.* (2010). PCR amplifications were performed in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany). PCR products were purified using the High Pure PCR Cleanup Micro kit (Roche, Mannheim, Germany), eluted in 30 μL of sterile water and quantified with NanoDrop ND-1000. Slide chemical treatment, array production, LDR protocol and hybridization conditions were performed as previously reported (Castiglioni *et al.*, 2004; Consolandi *et al.*, 2006). Briefly, LDR reactions were carried out in a final volume of 20 μL containing 500 fmol of each LDR-UA HTF-Microbi.Array probe (Candela *et al.*, 2012), 50 fmol of PCR product and 25 fmol of the synthetic template (5′-AGCCG CGAACACCACGATCGACCGGCGCGCGCAGCTGCAGC TTGCTCATG-3′). LDR products were hybridized on Universal Arrays, setting the probe annealing temperature at 60 °C. All arrays were scanned and processed according to the protocol and parameters already described by Candela *et al.* (2010).

### Evaluation of *Bifidobacterium* relative abundance by quantitative PCR

qPCR assays were performed in a LightCycler instrument (Roche). SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescent signal. Absolute quantification of *Bifidobacterium* population was carried out with the xfp-fw/xfp-rev (Jost *et al.*, 2012) primer set, targeting the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase gene. Total bacteria were quantified using the 8F/338R primer set targeting the 16S rRNA gene (Koenig *et al.*, 2011) (Table 1). For quantification, standard curves were generated with known amounts of genomic DNA from *Bifidobacterium animalis*

**Table 1.** Primer sets used for quantification of *Bifidobacterium* population and total bacteria by qPCR. Primer sequences, amplicon size, annealing and fluorescence acquisition temperature are reported

| Target group | Name | Sequence (5′ to 3′) | Product size (bp) | Annealing temperature (°C) | Fluorescence acquisition temperature (°C) | Reference |
|---|---|---|---|---|---|---|
| *Bifidobacterium* spp. | xfp-fw | ATCTTCGGACCBGAYGAGAC | 235 | 64 | 89 | Jost *et al.* (2012) |
| | xfp-rv | CGATVACGTGVACGAAGGAC | | | | |
| Total bacteria | 8F | AGAGTTTGATCCTGGCTCAG | 350 | 60 | 87 | Koenig *et al.* (2011) |
| | 338R | CTGCTGCCTCCCGTAGGAGT | | | | |

ssp. *lactis* BI07 and *Escherichia coli* K12 for bifidobacteria and total bacteria, respectively. Amplifications were performed in a 20 μL final volume containing 100 ng of faecal DNA, 0.5 μM of each primer and 4 μL of LightCycler-FastStart DNA Master SYBR Green I (Roche). The following PCR cycle was used: (1) initial preincubation at 95 °C for 10 min; (2) 40 cycles of four steps each at the temperature transition rate of 20 °C $s^{-1}$: denaturation at 95 °C for 15 s, annealing at the appropriate temperature (Table 1) for 25 s, extension at 72 °C for 20 s, fluorescence acquisition at the appropriate temperature (Table 1) for 5 s; (3) melting curve analysis. The relative abundance (rel. ab.) of *Bifidobacterium* was calculated as a percentage of the total bacterial content.

### Data analysis

Fluorescence intensities were normalized on the basis of the synthetic ligation control signal as reported by Candela *et al.* (2012). Relative abundance of each bacterial group was obtained by calculating the relative fluorescence contribution of the corresponding HTF-Microbi.Array probe as a percentage of the total fluorescence. Correlation between variables was calculated by Pearson product moment correlation coefficient. Statistical analysis was carried out using SIGMASTAT v. 3.5 (Systat Software Inc, San Jose, CA).

## Results and discussion

The faecal microbiota of eight breast-fed healthy infants aged 2–6 months was characterized by means of the HTF-Microbi.Array platform, as previously described (Candela *et al.*, 2012). Hybridization data depicted an infant-type microbiota largely dominated by *Enterobacteriaceae* (mean rel. ab. 28%) and members of the *Bacteroides/Prevotella* group (mean rel. ab. 23%), with *Clostridium* clusters IX and XIVa and *Enterococcales* as subdominant components (mean rel. ab. 12%, 4% and 7%, respectively). *Bifidobacteria* showed a mean rel. ab. slightly above 1%, appearing to be only a minor component of the ecosystem, confirming the PCR-dependent bias in the detection of *Bifidobacteria* of the HTF-Microbi.Array (Candela *et al.*, 2010). To overcome this bias in bifidobacterial quantification, particularly relevant when studying infant-type microbiota, we implemented a new approach based on the association of the HTF-Microbi.Array with a qPCR protocol designed to specifically quantify the rel. ab. of *Bifidobacterium*. This new combined approach was utilized to re-characterize the faecal microbiota of the eight breast-fed healthy infants enrolled in the study. At first, qPCR was used to quantify both *Bifidobacteria* and total bacteria in each faecal sample, allowing us to obtain a value of rel. ab. of the *Bifidobacterium* population (Table 2). Subsequently, for each faecal sample the non-bifidobacterial fraction was characterized using the HTF-Microbi.Array, with the exclusion of the probes targeting *Bifidobacteria*. For each of the HTF-Microbi.Array probes, the relative contribution of fluorescence intensity was adjusted proportionate to the value corresponding to the rel. ab. of the non-bifidobacterial population determined by qPCR. In Fig. 1a we reported the high taxonomic level fingerprint

**Table 2.** qPCR quantification of the relative abundances of the bifidobacterial and non-bifidobacterial population in the faecal microbiota of infants and adults

| | | Relative abundance (%) | |
|---|---|---|---|
| | Subject | Bifidobacterial population | Non-bifidobacterial population |
| Infants | 3TT | 58.25 | 41.75 |
| | 8AF | 29.52 | 70.48 |
| | 8PS | 49.29 | 50.71 |
| | 4NG | 41.82 | 58.18 |
| | 2SA | 8.79 | 91.21 |
| | 6CF | 16.60 | 83.40 |
| | 603 | 48.67 | 51.33 |
| | 704 | 26.73 | 73.27 |
| Adults | A | 1.87 | 98.13 |
| | C | 2.97 | 97.03 |
| | D | 0.00 | 100.00 |
| | E | 1.69 | 98.31 |
| | F | 0.17 | 99.83 |

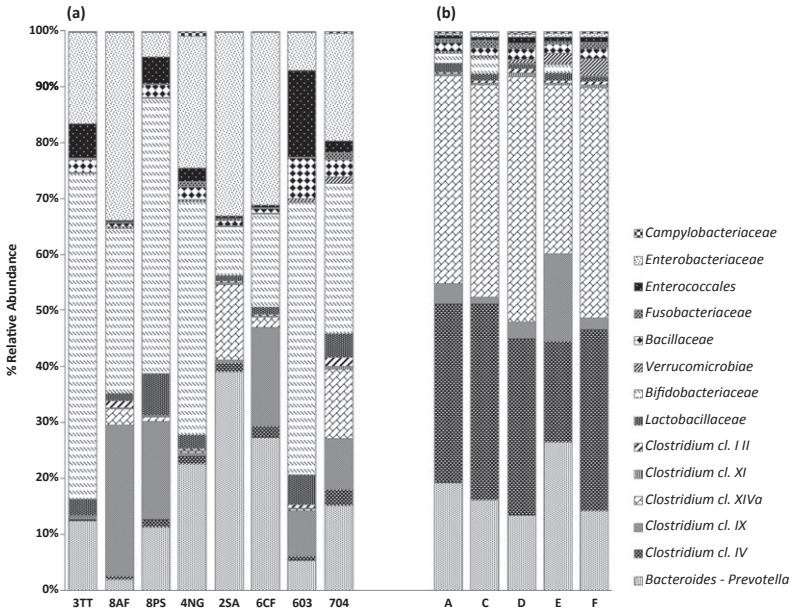


**Fig. 1.** High taxonomic level fingerprint of the faecal microbiota of breast-fed infants (a) and adults (b). Relative abundances of the major gut microbial groups were determined using the HTF-Microbi.Array/qPCR combined methodology. The relative contribution of the bifidobacterial fraction was derived from qPCR data, and the relative contribution of each of the HTF-Microbi.Array probes was adjusted proportionally to the value corresponding to the relative abundance of the non-bifidobacterial population obtained by qPCR.

of the faecal microbiota of the breast-fed infants carried out applying the HTF-Microbi.Array/qPCR combined methodology. The obtained fingerprints reflected well the data reported in the most recent 16S rRNA gene surveys of the infant-type microbiota (Jost *et al.*, 2012; Turroni *et al.*, 2012; Yatsunenko *et al.*, 2012). In fact, all infants showed a *Bifidobacterium*-dominated microbiota with rel. ab. ranging from 9% to 58% (mean 35%). *Enterobacteriaceae* were the second most predominant component of the infant gut microbiota (mean rel. ab. 21%) and, together with *Bifidobacterium,* accounted for the majority of the gut microbial community. *Bacteroides/Prevotella* and *Firmicutes* represented subdominant groups, together accounting for 32% of the total community. *Firmicutes* mainly comprised *Clostridium* cluster IX (mean rel. ab. 11%). *Clostridium* cluster XIVa, *Enterococcales*, *Lactobacillaceae* and *Bacillaceae* were only minor components of the microbiota of breast-fed infants, showing a mean rel. ab. of 4%, 4%, 3% and 2%, respectively. Interestingly, a trend ($P = 0.07$) towards an inverse correlation between *Bifidobacterium* and *Bacteroidetes* in the gut of breast-fed neonates was observed, analogous to that demonstrated by Jost *et al.* (2012). According to the authors, the individual proportion *Bifidobacterium*/*Bacteroidetes* may reflect differences in the composition of the maternal inoculum or may be the result of differences in the nutritional composition of the breast milk.

To validate the new combined HTF-Microbi.Array/qPCR approach, the faecal microbiota of five healthy adults aged between 30 and 40 years was also characterized (Table 2, Fig. 1b). The obtained microbiota profiles well reflected the 'gold standard' of a healthy adult-type intestinal microbiota (Turnbaugh *et al.*, 2007; Costello *et al.*, 2009), largely dominated by *Bacteroides/Prevotella* and *Firmicutes*, which together accounted for 90% of the total community. Differently from what was observed in breast-fed infants, *Firmicutes* were principally represented by *Clostridium* clusters IV and XIVa (mean rel. ab. 30% and 38%, respectively), whereas *Clostridium* cluster IX was subdominant (mean rel. ab. 5%). *Bifidobacteria* were only minor components of the gut microbiota of adults, showing a rel. ab. of between 0.5% and 3% (mean rel. ab. 1.4%). These last data demonstrate the reliability of the combined HTF-Microbi.Array/qPCR methodology, which is capable of properly depicting the adult- and the infant-type microbiota and reflecting the dramatic difference in bifidobacterial abundance between these two ecosystems. Since *Bifidobacteria* are considered a beneficial microbiota component throughout our lives, the right assessment of the bifidobacterial population size in the context of the total gut microbial population is strategic in infants as well as in adults and the elderly.

In conclusion, we have developed a new methodology based on a combined HTF-Microbi.Array/qPCR approach

that overcomes the limits of HTF-Microbi.Array in bifidobacterial quantification without compromising the detection of other bacterial groups. Recently, two alternative approaches based on 16S rRNA gene next-generation sequencing have been specifically designed to optimize the enumeration of *Bifidobacteria* in the gut microbial community (Davis *et al.*, 2011; Sim *et al.*, 2012). While the methodology developed by Sim *et al.* (2012) relies on a 'bifidobacterial optimized' universal primer set and has been applied in breast-fed infants, the approach developed by Davis *et al.* (2011) involves the addition of *Bifidobacterium*-specific primers to the universal primer set and has been used to describe the gut microbial community of adults. Both approaches provided data in general agreement with the one we obtained in our study, where we report a mean rel. ab. of *Bifidobacterium* in about 50% in infants (Sim *et al.*, 2012) and 2% in adults (Davis *et al.*, 2011). Although it has a lower phylogenetic resolution than 16S rRNA gene next-generation sequencing, our HTF-Microbi.Array/qPCR dual approach is a fast and easy-to-use method for a reliable characterization of the *Bifidobacterium*-dominated gut microbiota of breast-fed infants, and may represent a cheap and robust tool, possibly complementary to next-generation sequencing, for the rapid screening of the high taxonomic level profile of intestinal microbiota in infants. Leading to an immune system inclined to inappropriate activation (Maynard *et al.*, 2012; Johnson & Versalovic, 2012), dysbioses of the gut microbiota during our infancy, for instance the protracted reduction of the bifidobacterial fraction (Edwards & Parrett, 2002; Vael & Desager, 2009), have been associated recently with the emergence of systemic disorders later in life, appearing as allergies, obesity and type 2 diabetes (Isolauri, 2012). From this perspective, the combined HTF-Microbi.Array/qPCR approach can be a useful tool for wide epidemiological perspective surveys of the gut microbiota aimed to strengthen associations between the developmental trajectory of the infant microbiota and health later in life.

## References


Bisgaard H, Li N, Bonnelykke K, Chawes BL, Skov T, Paludan-Müller G, Stokholm J, Smith B & Krogfelt KA (2011) Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol* **128**: 646–652.

Candela M, Consolandi C, Severgnini M, Biagi E, Castiglioni B, Vitali B, De Bellis G & Brigidi P (2010) High taxonomic level fingerprint of the human intestinal microbiota by ligase detection reaction–universal array approach. *BMC Microbiol* **19**: 116.

Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, Masetti R, Ricci G, Pession A & Brigidi P (2012) Unbalance of intestinal microbiota in atopic children. *BMC Microbiol* **12**: 95.

Castiglioni B, Rizzi E, Frosini A *et al.* (2004) Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. *Appl Environ Microbiol* **70**: 7161–7172.

Consolandi C, Severgnini M, Castiglioni B, Bordoni R, Frosini A, Battaglia C, Rossi Bernardi L & De Bellis G (2006) A structured chitosan-based platform for biomolecule attachment to solid surfaces: application to DNA microarray preparation. *Bioconjug Chem* **17**: 371–377.

Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI & Knight R (2009) Bacterial community variation in human body habitats across space and time. *Science* **326**: 1694–1697.

Davis LM, Martínez I, Walter J, Goin C & Hutkins RW (2011) Barcoded pyrosequencing reveals that consumption of galactooligosaccharides results in a highly specific bifidogenic response in humans. *PLoS ONE* **6**: e25200.

Edwards CA & Parrett AM (2002) Intestinal flora during the first months of life: new perspectives. *Br J Nutr* **88**: S11–S18.

Ehlers S & Kaufmann SH (2010) Infection, inflammation, and chronic diseases: consequences of a modern lifestyle. *Trends Immunol* **31**: 184–190.

Fanning S, Hall LJ, Cronin M *et al.* (2012) Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *P Natl Acad Sci USA* **109**: 2108–2113.

Fukuda S, Toh H, Hase K *et al.* (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* **469**: 543–547.

Hattori M & Taylor TD (2009) The human intestinal microbiome: a new frontier of human biology. *DNA Res* **16**: 1–12.

Hooper LV & Macpherson AJ (2010) Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* **10**: 159–169.

Isolauri E (2012) Development of healthy gut microbiota early in life. *J Paediatr Child Health* **48**: 1–6.

Johnson CL & Versalovic J (2012) The human microbiome and its potential importance to pediatrics. *Pediatrics* **129**: 950–960.

Jost T, Lacroix C, Braegger CP & Chassard C (2012) New insights in gut microbiota establishment in healthy breast fed neonates. *PLoS ONE* **7**: e44595.

Klijn A, Mercenier A & Arigoni F (2005) Lessons from the genomes of bifidobacteria. *FEMS Microbiol Rev* **29**: 491–509.

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT & Ley RE (2011) Succession of microbial consortia in the developing infant gut microbiome. *P Natl Acad Sci USA* **108**: 4578–4585.

Koropatkin NM, Cameron EA & Martens EC (2012) How glycan metabolism shapes the human gut microbiota. *Nat Rev Microbiol* **10**: 323–335.

Maccaferri S, Candela M, Turroni S, Centanni M, Severgnini M, Consolandi C, Cavina P & Brigidi P (2012) IBS-associated

phylogenetic unbalances of the intestinal microbiota are not reverted by probiotic supplementation. *Gut Microbes* **3**: 406–413.

Maynard CL, Elson CO, Hatton RD & Weaver CT (2012) Reciprocal interactions of the intestinal microbiota and immune system. *Nature* **489**: 231–241.

Olszak T, An D, Zeissig S *et al.* (2012) Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* **336**: 489–493.

Palmer C, Bik EM, DiGiulio DB, Relman DA & Brown PO (2007) Development of the human infant intestinal microbiota. *PLoS Biol* **5**: e177.

Roger LC, Costabile A, Holland DT, Hoyles L & McCartney AL (2010) Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology* **156**: 3329–3341.

Schwartz S, Friedberg I, Ivanov IV, Davidson LA, Goldsby JS, Dahl DB, Herman D, Wang M, Donovan SM & Chapkin RS (2012) A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in immune response. *Genome Biol* **13**: r32.

Sim K, Cox MJ, Wopereis H, Martin R, Knol J, Li MS, Cookson WO, Moffatt MF & Kroll JS (2012) Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing. *PLoS ONE* **7**: e32543.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R & Gordon JI (2007) The human microbiome project. *Nature* **449**: 804–810.

Turroni F, Peano C, Pass DA *et al.* (2012) Diversity of bifidobacteria within the infant gut microbiota. *PLoS ONE* **7**: e36957.

Vael C & Desager K (2009) The importance of the development of the intestinal microbiota in infancy. *Curr Opin Pediatr* **21**: 794–800.

Yatsunenko T, Rey FE, Manary MJ *et al.* (2012) Human gut microbiome viewed across age and geography. *Nature* **486**: 222–227.