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Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres

Matteo Fallani,[1]† Sergio Amarri,[2,3] Agneta Uusijarvi,[4] Rüdiger Adam,[5] Sheila Khanna,[6] Marga Aguilera,[1,7] Angel Gil,[7] Jose M. Vieites,[7] Elisabeth Norin,[8] David Young,[9] Jane A. Scott,[10] Joël Doré,[1] Christine A. Edwards[6] and the INFABIO team

Correspondence
Christine A. Edwards
Christine.edwards@glasgow.ac.uk

[1]Institut National de la Recherche Agronomique, Unité d'Écologie et de Physiologie du Système Digestif, Jouy en Josas, France

[2]Department of Paediatrics, Azienda Ospedaliera Santa Maria Nuova, Reggio Emilia, Italy

[3]Department of Paediatrics, University of Modena and Reggio Emilia, Modena, Italy

[4]Department of Clinical Science and Education, Sodersjukhuset, Sachs' Children's Hospital, Karolinska Institutet, Stockholm, Sweden

[5]Department of Paediatric and Adolescent Medicine, University Medical Centre, Mannheim, Germany

[6]Human Nutrition Section, Division of Developmental Medicine, University of Glasgow, Yorkhill Hospitals, Glasgow, UK

[7]Department of Biochemistry and Molecular Biology, University of Granada, Spain

[8]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

[9]Department of Statistics and Modelling Science, University of Strathclyde, Glasgow, UK

[10]Nutrition and Dietetics, School of Medicine, Flinders University, Adelaide, Australia

Although it is well established that early infant feeding has a major influence on the establishment of the gut microbiota, very little is understood about how the introduction of first solid food influences the colonization process. This study aimed to determine the impact of weaning on the faecal microbiota composition of infants from five European countries (Sweden, Scotland, Germany, Italy and Spain) which have different lifestyle characteristics and infant feeding practices. Faecal samples were collected from 605 infants approximately 4 weeks after the introduction of first solid foods and the results were compared with the same infants before weaning (6 weeks of age) to investigate the association with determining factors such as geographical origin, mode of delivery, previous feeding method and age of weaning. Samples were analysed by fluorescence *in situ* hybridization and flow cytometry using a panel of 10 rRNA targeted group- and species-specific oligonucleotide probes. The genus *Bifidobacterium* (36.5 % average proportion of total detectable bacteria), *Clostridium coccoides* group (14 %) and *Bacteroides* (13.6 %) were predominant after weaning. Similar to pre-weaning, northern European countries were associated with a higher proportion of bifidobacteria in the infant gut microbiota while higher levels of *Bacteroides* and lactobacilli characterized southern European countries. As before weaning, the initial feeding method influenced the *Clostridium leptum* group and *Clostridium difficile* + *Clostridium perfringens* species, and bifidobacteria still dominated the faeces of initially breast-fed infants. Formula-fed babies presented significantly higher proportions of *Bacteroides* and the *C. coccoides* group. The mode of birth influenced changes in the

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†**Present address:** Hospitex Diagnostics srl, via P. Lucchese 145, 50019 Sesto Fiorentino, Firenze, Italy.

Abbreviation: FISH, fluorescence *in situ* hybridization.

proportions of bacteroides and atopobium. Although there were significant differences in the mean weaning age between countries, this was not related to the populations of bifidobacteria or bacteroides. Thus, although the faecal microbiota of infants after first complementary foods was different to that before weaning commenced, many of the initial influences on microbiota composition were still evident.

INTRODUCTION

Early events in the bacterial colonization of the human gut may have long-term consequences for the development of chronic diseases, including allergies. We have previously confirmed, in a cohort of 606 European infants, that the initial colonization process is heavily influenced by infant diet (breast milk versus infant formula), mode of birth and perinatal antibiotics, but that country of birth is also a major factor (Fallani *et al.*, 2010). This paper describes the next critical stage in the development of the gut microbiota which occurs during the process of weaning (complementary feeding). The weaning period starts with the introduction of solid (non-milk) foods when infants are exposed for the first time to many different non-digestible carbohydrates. A significant proportion of dietary starch also escapes digestion because of immature pancreatic exocrine function in young infants (Zoppi *et al.*, 1972; Lebenthal & Lee 1980; Parrett *et al.*, 2000). These non-digested carbohydrates enter the colon and influence the microbiota by providing new substrates that may promote the survival and dominance of species not supported by the limited number of non-digestible carbohydrates in human milk and infant formula. As weaning progresses, pancreatic function, small intestinal absorption and colonic fermentation capacity also mature (Parrett & Edwards, 1997) changing the characteristics of the material reaching the colon and also conditions in the fermentation chamber. This is reflected in the microbiota composition and profiles of short chain fatty acids (Midtvedt & Midtvedt, 1992). There are, however, very little data on changes in the composition of the intestinal microbiota during early weaning (Satokari *et al.*, 2002; Wang *et al.*, 2004; Ahrné *et al.*, 2005) and how the influence of pre-weaning feeding methods and perinatal events impact on the microbiota after the introduction of complementary foods.

Therefore, the aim of this work was to characterize the faecal microbiota of 605 infants from five European countries with different lifestyle characteristics during early weaning and to determine which pre-weaning influences were still active. As 531 infants provided samples both pre- and post-weaning, the study also was able to monitor individual changes in the microbiota and determine how much these were influenced by the pre-weaning determinants.

METHODS

Socio-demographic study. Four weeks after the introduction of first solid foods (referred to hereafter as post-weaning), mothers were requested to collect a faecal sample from their infant and complete a questionnaire about diet and environmental exposures. Within the European project INFABIO (http://www.gla.ac.uk/departments/infabio/), post-weaning faecal samples were available from 605 infants recruited in five different centres, from northern to southern Europe: 109 from Stockholm (Sweden), 149 from Glasgow (UK), 113 from Düsseldorf (Germany), 121 from Reggio Emilia (Italy) and 113 from Granada (Spain). The majority of these infants (531 of 605) had been similarly studied at 6 weeks old (baseline measurement) and could therefore provide paired data (Fallani *et al.*, 2010). Of the total 605 infants, 59.1 % had been fully breast-fed, 26.8 % fully formula-fed and 14.1 % had been fed a mixture of formula and breast milk at baseline. The characteristics of the infants from each country that were used in the analysis in this paper are summarized in Table 1. Ethical permission was obtained from local ethics committees at each centre and parents gave written informed consent.

Faecal sample collection, cell fixation and fluorescence *in situ* hybridization (FISH). Each faecal specimen was placed in a sterile plastic box and maintained under anaerobic conditions at 4 °C using an Anaerocult A (Merck) for a maximum of 4 h before processing for cell fixation, as described previously (Rochet *et al.*, 2001). Sample fixation kits were provided at each collection site. Faeces were homogenized by mechanical mixing for 3 min and aliquots of 1 g (wet weight) were added to 9 ml anaerobic PBS. The suspension was mixed to complete homogeneity in a 50 ml stoppered sterile glass jar with a magnetic bar and aliquots of 0.2 ml suspension were added to 0.6 ml 4 % PFA in PBS. After overnight incubation at 4 °C, fixed suspensions were stored at –70 °C, and shipped on dry ice for analysis at a single location.

The FISH method was carried out as described previously (Rigottier-Gois *et al.*, 2003a, b; Fallani *et al.*, 2010) using a panel of 10 group- and species-specific probes covalently linked with indodicarbocyanine (Cy5) at their 5′ end to assess the microbiota composition (Fallani *et al.*, 2006). Enumeration of the different bacterial groups or species was performed by FISH-FC by combining one specific probe labelled with Cy5 together with EUB 338 FITC probe in the same tube.

Statistical analysis. Data are expressed as raw data means ± SD of the proportions of cells that hybridized with each of the 10 oligonuclotide probes relative to the total bacteria. A value of zero was used where a microbial group was undetected below the threshold of sensitivity of 0.4 % (Fallani *et al.*, 2006). Initially, a general linear model was used to assess the relationship between the factors – centre, delivery method, feeding method before weaning, infant and mother antibiotics, and outcome variables/bacterial groups – while correcting for feeding method or delivery method. Comparisons between the main effects were done using the Bonferroni correction factor to compensate for multiple testing with a significance level of 5 % to describe the associations. Variables that were significant at the 5 % significance level in the univariate analyses were then included in a multivariate analysis to identify the independent factors associated with the outcome variable of interest. Multivariate models included interaction terms for country and delivery method, country and feeding method at baseline, country and mother antibiotics, and country and antibiotic exposure of

Table 1. Number and proportion of mothers and infants used in the analysis that belong to each parameter group studied

Not all mothers answered all questions.

Parameter group	All centres	Glasgow	Granada	Reggio Emilia	Dusseldorf	Stockholm
Caesarean section	205 (34.5 %)	43 (30.7 %)	18 (15.9 %)	58 (47.9 %)	46 (41.4 %)	40 (36.7 %)
Mother had antibiotics	175 (29.5 %)	54 (38.3 %)	11 (9.8 %)	44 (36.4 %)	32 (28.8 %)	34 (31.5 %)
Infant had antibiotics	37 (6.53 %)	7 (5.3 %)	6 (5.5 %)	16 (13.4 %)	7 (6.9 %)	1 (0.9 %)
Fully breast-fed at 6 weeks	312 (54.2 %)	58 (43.6 %)	51 (45.1 %)	61 (50.4 %)	58 (56.9 %)	84 (78.5 %)
Formula-fed at 6 weeks	163 (28.3 %)	66 (49.6 %)	41 (36.3 %)	24 (19.8 %)	30 (29.4 %)	2 (1.9 %)
Mixed-fed at 6 weeks	101 (17.5 %)	9 (6.8 %)	21 (18.6 %)	36 (29.8 %)	14 (13.7 %)	21 (19.6 %)

infants, which were tested by using PASW (version 18). Non-significant interactions were not included in the final models.

Similar models were used to assess changes in bacterial populations between pre- and post-weaning periods. The Bonferroni correction factor was used to compensate for multiple testing. Comparisons of the overall changes in bacteria before and after weaning, without consideration of influencing factors, were performed using paired *t*-tests with Minitab (version 14) and a significance level of 5 %.

RESULTS

Microbiota of post-weaning infants (n=605)

Nearly all post-weaning samples (98 %) could be analysed. The remaining samples had a yield of RNA that was too low. The predominant group detected in the post-weaning infants considered together was *Bifidobacterium* with a mean proportion of 36.5 % (±25.8), then *C. coccoides* (14.0±15.0 %) and *Bacteroides* (13.6±15.8 %). Of the sum of bacterial cells detected, 74.3±18.9 % were accounted for with the panel of 10 non-overlapping phylogenetic probes.

In the multivariate analysis, there were very few instances of interactions between factors tested, e.g. centre of origin and delivery method, and these have been taken into account where appropriate.

Impact of centre of origin. As in our previous pre-weaning study, there was a marked difference in the proportions of different bacterial groups detected in infants from the different countries (Table 2), especially for *Bacteroides*, bifidobacteria, *Atopobium* and the *Clostridium leptum* group, while less difference was observed for enterobacteria, *Lactobacillus* and *Streptococcus* groups, and sum of the proportions. The country of birth did not significantly affect the *C. coccoides* group, and *C. difficile* and *C. perfringens* species. Samples from infants in Granada contained significantly greater proportions of the *Lactobacillus* group and significantly lower proportions of bifidobacteria compared with samples from all others centres (P<0.003) after correction for the feeding method. Granada samples also contained greater proportions of *Bacteroides* and *C. leptum* compared with all other centres (P<0.001) except Düsseldorf, greater proportions of enterobacteria compared with all centres (P<0.031) except Reggio Emilia, and greater proportions of members of the *Streptococcus* group compared with Düsseldorf (P=0.05). In contrast, samples from Stockholm and Glasgow contained significantly higher proportions of bifidobacteria compared with those from all other centres (P<0.01). Düsseldorf samples had significantly greater proportions of *C. leptum* compared with all centres (P<0.048) except Granada, and significantly greater proportions of *Bacteroides* compared with Stockholm (P=0.002) and Reggio Emilia (P<0.001). Reggio Emilia samples contained a lower sum of the proportions compared with Glasgow and Stockholm (P<0.001), while samples from Stockholm also had a significantly greater sum of proportions compared with those from Düsseldorf (P=0.025).

Impact of pre-weaning feeding method. The pre-weaning feeding method (breast-fed, formula-fed or mixed-fed) influenced the relative proportions of *Bacteroides*, members of the genus *Bifidobacterium*, *C. coccoides*, *C. leptum* groups, and *C. difficile* + *C. perfringens* after correction for centre effect. Infants breast-fed at 6 weeks but sampled post-weaning presented significantly greater proportions of bifidobacteria (40.7 % vs 29.2 %, P<0.001), and significantly lower proportions of *Bacteroides* (12.1 % vs 17.1 %, P=0.004) and *C. coccoides* (11.1 % vs 15.9 %, P=0.004) than formula-fed infants. Infants who had been breast-fed at 6 weeks (before weaning) also presented significantly lower proportions of *C. coccoides* post-weaning compared with those who had been mixed-fed (11.1 % vs 18.0 %, P<0.001).

Impact of the delivery method. Compared with infants born by Caesarean section, vaginal delivery infants (67 % of the babies) had higher mean proportions of *Bacteroides* (16.1 % vs 10.1 %, P<0.001) after correction for feeding method.

Effect of antibiotics. The post-weaning microbiota of newborns that had received antibiotics perinatally (only 6.5 % of 605 infants investigated) and infants whose mothers received antibiotics were no different in composition from those not exposed to antibiotics.

Impact of weaning age. There was a significant difference in the age of first introduction of solid foods between countries (P<0.0001), with infants in Reggio Emilia weaned earliest (16.9±3.6 weeks) and those in Düsseldorf the latest (22.4±4.6 weeks; Table 1). However, this did not influence

Table 2. Proportions of bacterial groups, genus and species detected in 605 infants' faecal samples coming from five different European countries, assessed by FISH combined with flow cytometry with a panel of 10 oligonucleotide probes

Values (raw data, mean ± SD) indicate the percentage of cells hybridizing with the corresponding probe versus Eub338 probe. For the sum we included the proportion of cells hybridising with the combined probe which measured *C. perfringens* and *C. difficile* together (done for all 605 samples) rather than the data where the two separate Cdif198 and Cperf191 probes measured the individual species (done for only 26 samples). Target groups for each probe included (Fallani *et al.*, 2006): Bif164, *Bifidobacterium* genus; Erec482, species of the genera *Clostridium*, *Eubacterium*, *Ruminococcus* and *Butyribivrio*; Clep866, members of the genera *Clostridium*, *Eubacterium*, *Ruminococcus* and *Anaerofilum* plus *Faecalibacterium prausnitzii* cluster; Ato291, *Atopobium* cluster including members of the genus *Coriobacterium*; Bac303, members of the genera *Prevotella* and *Bacteroides*; Enter1432, Enteric group including *E. coli*; Lab158, members of the genera *Lactobacillus*, *Enterococcus*, *Weissella* and *Pediococcus*; Strc493, *Streptococcus* group including members of the genus *Lactococcus*; Cdif198, *Clostridium difficile* sp.; Cperf191, *Clostridium perfringens* sp.

City of origin (country) (*n*)	Probe											
	Bif164	Erec482	Clep866	Ato291	Bac303	Enter1432	Lab158	Strc493	Cdif198*	Cperf191*	Cperf191 + Cdif198	Sum
Stockholm (SW) (109)	55.7 ± 27.7	9.7 ± 16.0	0.5 ± 2.1	1.3 ± 3.9	8.7 ± 12.5	2.4 ± 5.5	0.2 ± 0.5	1.6 ± 4.4	0.5 ± 0.9	2.7 ± 1.9	0.8 ± 1.6	80.9 ± 17.9
Glasgow (UK) (149)	41.6 ± 24.3	15.0 ± 15.3	0.7 ± 2.5	2.8 ± 6.2	12.8 ± 13.6	2.0 ± 5.7	0.3 ± 0.8	1.0 ± 1.6	0.3 ± 0.8	3.4 ± 3.1	0.8 ± 1.5	77.0 ± 16.8
Düsseldorf (GE) (113)	29.9 ± 20.4	15.4 ± 14.2	2.6 ± 5.2	1.4 ± 4.0	17.4 ± 17.2	3.0 ± 4.9	0.6 ± 1.6	0.9 ± 1.8	0.3 ± 0.5	1.8 ± 1.3	0.9 ± 1.9	72.0 ± 19.7
Reggio Emilia (IT) (121)	37.2 ± 21.1	12.9 ± 12.9	0.6 ± 2.0	1.3 ± 3.8	8.8 ± 11.0	4.2 ± 5.6	0.7 ± 0.9	1.4 ± 2.8	0.7 ± 0.8	1.4 ± 1.3	0.6 ± 0.9	67.7 ± 18.1
Granada (ES) (113)	17.4 ± 19.2	16.4 ± 15.6	4.2 ± 9.4	2.3 ± 6.0	20.5 ± 20.2	6.0 ± 13.0	3.6 ± 11.6	2.3 ± 6.5	1.3 ± 0.9	2.3 ± 1.2	0.9 ± 1.6	73.7 ± 19.9
Total (605)	36.5 ± 17.8	14.0 ± 15.0	1.6 ± 5.2	1.9 ± 5.0	13.6 ± 15.8	3.4 ± 7.6	1.0 ± 5.2	1.4 ± 3.8	0.5 ± 0.8	2.4 ± 2.0	0.8 ± 1.5	74.3 ± 18.9

*Cdif198 and Cperf191 were used separately only on 26 samples chosen among the samples with the highest proportions detected with the combined probe, which did not differentiate between the species, in the different centres (nine in Düsseldorf, six in Glasgow, three in Granada, three in Reggio Emilia and five in Stockholm).

the populations of bifidobacteria or *Bacteroides* after correction for pre-weaning feeding method.

Changes in microbiota in infants who provided samples both before and after weaning (n=531)

The composition of the faecal microbiota of 531 infants who gave samples before weaning (at 6 weeks of age) and 4 weeks after the introduction of first solid foods (Fig. 1) showed that weaning was accompanied by a significant decrease in proportions of bifidobacteria (from 40.9 to 37.9 %, P=0.042), enterobacteria (from 7.3 to 3.1 %, P<0.001) and *C. difficile*+*C. perfringens* species (from 3.2 to 0.8 %, P<0.001), and a significant increase in proportions of the *C. coccoides* (from 5.4 to 14 %, P<0.001) and *C. leptum* (from 0.4 to 1.6 %, P<0.001) groups.

Impact of centre of origin. Changes in bacterial populations before and after weaning changed dependent on the centre of origin after correction for feeding method. This was particularly true for *C. coccoides* and *C. leptum* groups and for enterobacteria, and to a lesser extent for the *Atopobium* cluster and *Streptococcus* group. The country of birth did not impact on the change in the other bacterial groups. In Granada, the increase in the proportions of *C. coccoides* and *C. leptum* groups (from 1.6 to 16.4 % and from 1 to 4.2 %, respectively, P<0.01) and the decrease in proportions of enterobacteria (from 19.3 to 6 %) after weaning were significantly more pronounced compared with the changes in any other centre (P<0.001). Samples from Granada also had significantly different changes in the proportion of the *Atopobium* cluster (from 0.7 to 2.3 %) before and after weaning compared with samples from Stockholm (from 2.4 to 1.3 %, P=0.02) and Düsseldorf (from 2.2 to 1.4 %, P=0.012), and of the *Streptococcus* group (from 1.3 to 2.3 %) compared with Reggio Emilia (from 2.4 to 1.4 %, P=0.002) and Düsseldorf (from 1.3 to 0.9 %, P=0.04). Finally, samples from Reggio Emilia had significantly different changes in the proportion of enterobacteria (from 8.3 to 4.2 %) before and after weaning compared with samples from Stockholm (from 1.7 to 2.4 %, P<0.05) and Glasgow (from 3.5 to 2 %, P<0.05). The centre of origin also affected the change in sum of proportions (P<0.048).

Impact of the pre-weaning feeding method. Changes in bacterial populations during weaning were affected by the pre-weaning feeding method, after correction for country effect; there was a significant difference (P<0.05) in proportional changes only for the *C. leptum* group and for *C. difficile*+*C. perfringens* species after correction for centre. Infants who were formula-fed before weaning had a significantly greater increase in the proportion of *C. leptum* (from 0.5 to 2.3 %) after weaning compared with previously breast-fed babies (from 0.4 to 1.2 %, P=0.028), and a decrease in the proportion of *C. difficile*+*C. perfringens* species (from 3.5 to 0.7 %) compared with mixed-fed babies (from 1.5 to 1.0 %, P=0.012).

Impact of the delivery method. The proportion of *Bacteroides* before and after weaning (16.1 %) did not change in babies born by vaginal delivery, while *Bacteroides* increased after weaning (from 6.9 to 10.1) in infants born by Caesarean section; this represents a significant difference between the two delivery methods (P=0.044). Infants born by vaginal delivery had a decrease in the corresponding proportions of *Atopobium* cluster before and after weaning while babies born by Caesarean section had an increase (2.9 to 2.2 % versus 0.8 to 1.5 %, P=0.044) after correction for feeding method. The delivery method also affected the total sum of detected bacterial groups with a more pronounced increase for infants born by Caesarean section (67.6 to 73.4 % versus 75.5 to 75.9 %, P=0.024) after correction for centre of origin.

Effect of antibiotic treatment. The evolution of proportions of enterobacteria before and after weaning

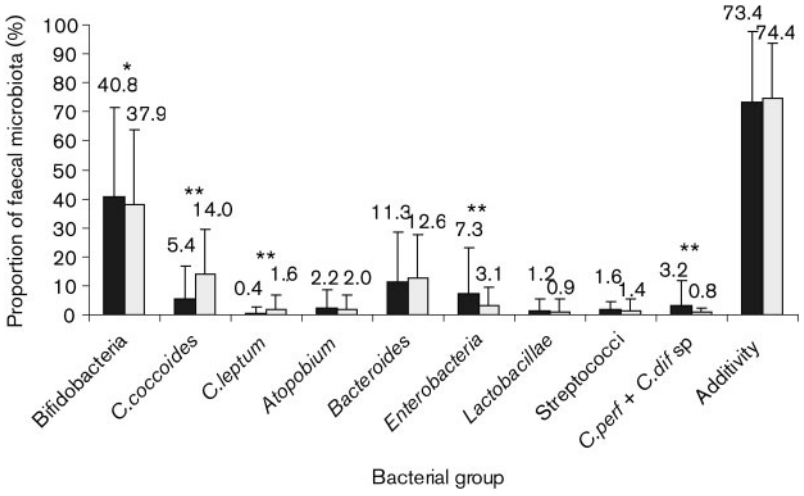

Fig. 1. Composition of the faecal microbiota of 531 infants before weaning (6 weeks of age) (black bars) and 4 weeks after the introduction of first solid foods (grey bars). Values are mean (±SD) proportions of the bacterial groups quantified by FISH-flow cytometry. Asterisks indicate significant differences between the two periods (*P<0.05; **P<0.001).

was markedly affected by perinatal antibiotics ($P=0.003$) after correction for centre, but the greater decrease in treated infants (16.6 to 3.6 %) than in untreated ones (from 6.8 to 3.3 %) was associated with higher pre-weaning proportions of enterobacteria. Proportions of bifidobacteria increased in newborns whose mothers received antibiotics perinatally, while they decreased in infants born from mothers without treatment (36.9 to 38.9 % versus 39.9 to 33.6 %, respectively, $P=0.033$)

DISCUSSION

We have previously reported the factors which affect the infant microbiota at 6 weeks of age (Fallani *et al.*, 2010). This study assessed the factors affecting the faecal microbiota composition of European infants, during early weaning, when more diverse sources of non-digestible carbohydrates would be expected to reach the colon, by analysing 605 faecal samples obtained 4 weeks after the introduction of first solid foods. We also compared the microbiota of infants with paired data at 6 weeks old and at 4 weeks post-weaning ($n=531$). At 4 weeks post-weaning, *Bifidobacteria* still dominated the intestinal microbiota of infants, but their proportions had significantly decreased and the microbiota had diversified. Facultative anaerobes significantly decreased, while proportions of strictly anaerobic clostridia increased. Proportions of *Bacteroides*, however, did not change and still represented one of the most predominant groups in the infant gut microbiota after weaning commenced. This agrees with most previous studies. Martin *et al.* (2000) reported a more diverse microbiota with higher proportions of *Bacteroides* and clostridia (*C. coccoides* and *C. leptum*) after the introduction of solid foods. Wang *et al.* (2004) showed that *Enterobacteriaceae* decreased while clostridia increased after weaning in two Swedish infants. Using real-time PCR and Northern hybridization analyses of 40 infants (0–24 months old), Hopkins *et al.* (2005) reported that *Bacteroides* and *Desulfovibrio* numbers increased and *Enterococcus faecalis* decreased in the 7–12 and 13–24 months age groups, and the *C. coccoides* group and *Faecalibacterium prausnitzii* subgroup increased after 6 months. Doré *et al.* (1998) reported that after weaning was complete, the *Bacteroides-Porphyromonas-Prevotella* group rapidly increased to comprise 30 % of 16S rRNA, while *Bifidobacterium* rRNA was undetectable. In the present study, *C. difficile* and *C. perfringens* also significantly decreased after weaning in accordance with previous observations (Stark & Lee, 1982; Benno *et al.*, 1984; Fallani *et al.*, 2006).

The present study investigated the impact of geographical origin on the development of the gut microbiota of babies born in different European countries before and after weaning. The few previous studies reported here considered the microbiota composition for only one or two countries and the majority investigated a small number of bacterial groups (George *et al.*, 1996; Guérin-Danan *et al.*, 1997; Sepp *et al.*, 1997). We observed that the strong association with geographical origin seen at 6 weeks of age (a gradient of high numbers of bifidobacteria in northern Europe to higher *Bacteroides* and lactobacilli in southern Europe) (Fallani *et al.*, 2010) still persisted 4 weeks after first introduction of solid foods, particularly for *Bacteroides*, bifidobacteria and the *C. leptum* group, despite a high variability in the data (Table 2). It was not possible to consider the impact of each parameter in each country separately as the numbers in each centre were limited. One month after weaning started, the faecal microbiota was still quite different from that of adults (Lay *et al.*, 2005), most evidently for the Swedish infants where bifidobacteria represented >50 % of total bacteria and the *C. leptum* group was virtually undetected. This was also true for Spanish infants, although their post-weaning microbiota appeared closest to that of adults (lowest bifidobacteria and highest *Bacteroides*, *C. coccoides* and *C. leptum* proportions). Thus, early diversification of the faecal microbiota promotes the earlier acquisition of an 'adult-like' microbiota.

The influence of feeding method at 6 weeks persisted after weaning started. Pre-weaning breastfeeding was still associated with higher proportions of bifidobacteria, while formula feeding was characterized by higher proportions of *Bacteroides* and *C. coccoides* in accordance with previous studies (Stark & Lee, 1982; Hopkins *et al.*, 2005). We also observed that during weaning, breastfeeding induced a slower increase of *C. leptum* and a faster reduction of *C. difficile* and *C. perfringens* species compared with formula or mixed feeding. Satokari *et al.* (2002) reported similar changes in the microbiota during weaning between breast-fed and formula-fed infants. In a group of children at 6 months, where half had started to have the breast milk supplemented with solid food, George *et al.* (1996) observed that the number of enterococci and *Bacteroides* increased, whereas enterobacteria and bifidobacteria remained constant.

The influence of mode of birth also persisted after weaning. The delay in faecal colonization and the low number of *Bacteroides* in the Caesarean group (Fallani *et al.*, 2010) was still present after weaning. Vaginal delivery was associated with higher proportions of *Bacteroides* post-weaning. Infants born by Caesarean section had a higher increase in the proportion of *Bacteroides*, *Atopobium* and total additivity. Grönlund *et al.* (1999) studied 30 infants delivered by Caesarean section whose mothers had received antibiotics and 34 vaginally delivered infants, and observed a delay in faecal colonization and low *Bacteroides* in the Caesarean group during the first 6 months of life, even after weaning. *Bacteroides* also showed a higher increase across the weaning period (2–6 months) in the Caesarean group.

At 6 weeks there was a marked effect of antibiotic treatment in infants or their mother perinatally (Fallani *et al.*, 2010). This effect disappeared after weaning started,

suggesting that the effect of early antibiotic administration will fade rather than persist upon weaning even after the treatment has stopped, as formerly reported (Mackie *et al.*, 1999).

In conclusion, in this large-scale longitudinal study, important modifications occurred in the infant gut microbiota after weaning which still remained 'infant-like' 1 month after the introduction of the first solid food. The effects of country of birth, feeding method and mode of delivery seen at 6 weeks persisted after weaning, whereas the impact of antibiotic treatment disappeared. One of the most striking observations was that postnatal conditions and especially early colonization affect the kinetics of acquisition of a mature microbiota. The longer term impact on the development of the microbiota, gut physiology and maturation of the immune system needs to be explored.

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