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

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

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

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 other 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 *Butyribiostrum*; 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.

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

*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 changes 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 previously breast-fed babies (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 is depicted in Fig. 1.
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 \((0–24 \text{ months old})\), 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 \((2–6 \text{ months})\) 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.

ACKNOWLEDGEMENTS

This study was carried out with financial support from the commission of the European Communities, RTD programme ‘Quality of life and Management of Living Resources’, QLRT-2001 02606, ‘INFABIO’, coordinated by Professor Christine Edwards (University of Glasgow, UK). It does not necessarily reflect the views of the commission and in no way anticipates the commission’s future policy in this area. Other members of the INFABIO team that were involved in this project are Benoit Thezé (INRA, France), Mohammed Khalid Khan (Glasgow University, UK), Francesca Benatti and Rosa Maria De Mola (both Azienda Ospedaliera Santa Maria Nuova and University of Modena and Reggio Emilia, Italy), Horst Schrotten (University Children’s Hospital, Düsseldorf, Germany), and Oscar Thompson, José Maldonado and Marco Ferrer (all University of Granada, Spain).

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Edited by: H. J. Flint