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ORIGINAL ARTICLE **Clinical Mechanisms in Allergic Disease**

Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants

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Clinical & Experimental Allergy

Summary

Background Culture-dependent methods have shown that meconium, the newborn's first intestinal discharge, is not sterile, but the diversity of bacteria present in this material needs to be further characterized by means of more sensitive molecular techniques.

Objective Our aims were to characterize molecularly the meconium microbiota in term infants, to assess whether it contributes to the future microbiota of the infants' gastrointestinal tract, and to evaluate how it relates to lifestyle variables and atopy-related conditions.

Methods We applied high-throughput pyrosequencing of the 16S rRNA gene to study the meconium microbiota in twenty term newborns from a Spanish birth cohort. For comparison, we characterized the microbiota in fecal samples from seven pregnant women days before delivery and in two series of infant samples spanning the first seven months of life. We also compared our data with vaginal and skin microbiota characterized in independent studies. Different types of meconium microbiota were defined based on taxonomic composition and abundance and their associations with different factors were statistically evaluated.

Results The meconium microbiota differs from those in adult feces, vagina and skin, but resembles that of fecal samples from young infants. Meconium samples clustered into two types with different bacterial diversity, richness and composition. One of the types was less diverse, dominated by enteric bacteria and associated with a history of atopic eczema in the mother ($P = 0.038$), whereas the second type was dominated by lactic acid bacteria and associated with respiratory problems in the infant ($P = 0.040$).

Conclusions & Clinical Relevance Our findings suggest that the meconium microbiota has an intrauterine origin and participates in gut colonization. Although based on a small population sample, our association analyses also suggest that the type of bacteria detected in meconium is influenced by maternal factors and may have consequences for childhood health.

Keywords 16S rRNA, atopy, eczema, gastrointestinal tract, pyrosequencing
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Introduction

Meconium, the newborn's first intestinal discharge, represents material ingested or secreted by the gastrointestinal tract (GIT) during fetal life, including amniotic fluid, epithelial cells and mucus. Amniotic fluid and meconium have been considered sterile under normal conditions, with bacterial colonization of the GIT starting during birth, through cross-contamination with vaginal and fecal bacteria. However, recent culture-dependent analyses have detected microorganisms in both environments even when no membrane rupture

has occurred, and a bacterial efflux from mother to fetus has been experimentally demonstrated in mice [1–3]. These studies have established the presence of live bacteria in meconium. However, given that a large proportion of gastrointestinal bacteria are not culturable, molecular methods are needed to obtain a more thorough characterization of the meconium microbiota. In two pioneering works, this microbiota has been assessed in premature infants by 16S-rRNA-based techniques, and related to factors such as gestational age, intrapartum antibiotic use and late-onset sepsis [4, 5].

The presence of microorganisms in meconium suggests that an internal route may transport maternal bacteria to the fetal GIT. Such a route might proceed through the bloodstream to the placenta, from where bacteria could transfer into fetal circulation or into the amniotic fluid [2, 3, 6]. Bacteria have been recovered from the umbilical cord blood of healthy neonates born by elective Caesarean section (C-section), supporting a mother-to-child efflux of microorganisms through the placenta [2]. Transport of maternal GIT bacteria could be facilitated by dendritic cells, capable of penetrating the epithelium and taking up bacteria from the lumen [7]. Furthermore, in pregnant and lactating women bacterial translocation to mesenteric lymph nodes could be enhanced, as has been demonstrated in mice [8].

The bacteria reaching the fetal GIT could influence the development of the microbiota and the immune system and hence have relevant consequences for health. The GIT microbiota is known to affect immune modulation and induction of immunological tolerance during infancy, and has been related to the relative occurrence of different atopic diseases [9–19]. A protective role against atopy has often been reported for Lactic Acid Bacteria (LAB), mainly bifidobacteria and *Lactobacillus* [9, 12–14, 20–22], although not confirmed by large prospective case–control studies [15, 23]. In contrast, high abundances of *Escherichia coli* and other enterics have been linked to eczema [10, 24, 25] and other allergies [12, 20, 22]. Eczema development has also been strongly associated with reduced GIT microbiota diversity [16,18,19].

Here, we employ high-throughput pyrosequencing to obtain a thorough characterization of the microbiota present in the meconium of term-birth infants. We compare the microbiota detected in meconium to other microbiotas that could contaminate it during birth, and to those found in infant fecal samples spanning the first 7 months of life. Finally, we explore potential relations between the composition of the meconium microbiota and available clinical and lifestyle metadata associated with the meconium samples, including the occurrence of eczema and of asthma-related conditions in both the mothers and the children.

Methods

Study subjects

This study used meconium samples and metadata from 20 children enrolled in the Valencia birth cohort of project INMA (Infancia y Medio Ambiente - Childhood and the Environment, http://www.proyectoinma.org), which investigates the effect of environmental exposures and diet during pregnancy on fetal and child development [26]. Briefly, pregnant women from a well-defined geographic area in Valencia and attending the first prenatal visit at La Fe Hospital during November 2003–June 2005 were recruited before week 13 of gestation and followed up until delivery. Their children were enrolled at birth and have been followed up until 4 years of age (N: 601).

The mothers of the 20 children for whom meconium samples were analysed were all born in Spain, had non-vegetarian diets and, in most cases (18/20), had normal body mass indexes (18.5–24.9) before pregnancy. All had healthy pregnancies with no complications (no fever, urine infection, gestational diabetes, high blood pressure or amniotic fluid losses). The 20 infants were born at term (> 37 weeks of gestation), none of them had a low birth weight (< 2500 g) and only one was small for the gestational age (length below the 10^{th} percentile within the INMA cohort).

In addition to the INMA meconium samples, we collected and analysed fecal samples from seven healthy pregnant women, as well as two series of mother–infant samples. The series included one maternal sample, meconium and four infant samples collected at one week, one month, three months and seven months of age. All women were residents of the city of Valencia and their samples were obtained days before delivery. Infants were vaginally delivered at La Fe Hospital and breastfed throughout the length of the study; solid foods were introduced to their diets between the 3 months and the 7 months sample collections.

Informed consent was obtained from all participants in the study, which was approved by the Ethics Committees of La Fe Hospital and the Center for Public Health Research (CSISP).

Sample collection

Meconium samples were collected at La Fe Hospital. All of the meconium passed was collected into sterile glass flasks with the help of a spatula and immediately stored at −20°C. Fecal samples from mothers and infants were collected in sterile containers with 10 mL phosphate buffered saline (PBS), immediately placed in home freezers and brought to the laboratory within days for storage at −80°C.

DNA isolation, 16S rDNA amplification and pyrosequencing

To account for the possibility of external contamination, we peeled a thin external layer from the frozen meconium samples using a scalpel to obtain internal (MI) and external (ME) portions that were analysed separately. Meconium fractions and fecal samples were resuspended in PBS and centrifuged at 1258 g for 2 min to remove fecal debris. DNA was then extracted using the QIAamp DNA Stool Mini Kit of QIAGEN (DNA isolation for pathogen detection protocol). 16S rDNA PCR amplicons were obtained using barcoded primers (8F, 5′-AGAGTTTGATCMTGGCTCAG-3′, and 357R, 5′-TGCTGCCTCCCGTAGGAGT-3′) attached to the 454 Roche adaptors. This allows for simultaneous pyrosequencing of 20 samples in 1/4th of a Titanium run. Sequences trimmed and filtered by the 454 Sequencing System Software (version 2.6) were further inspected, and any sequences having more than 2% ambiguous callings or a mean per base quality score under 20 (Q20) were removed. Sequences (Table S1) were deposited in the NCBI Short Read Archive (accession number SRP009092.1).

Phylogenetic analysis of 16S rDNA

After elimination of short sequences (< 50 bp), 16S rDNA fragments were taxonomically assigned by comparison against the sequence data sets of the Ribosomal Database Project-II (RDP) [27]. Hierarchical taxa assignment was performed with the RDP Classifier tool, which assigns sequences to the different taxonomical categories within the RDP hierarchy, ranging from phylum to genus, and evaluates the support level for each assignment by means of bootstrap analysis. For each sequence, we recovered and used for further analysis the assignation at the lowest category with a confidence level at or above 80%. The number of taxonomically assigned reads per sample is presented in Supporting Table 1.

Estimation of diversity and comparison of community structure

We calculated taxon richness in the meconium microbiota by means of the Chao1 estimator [28] and employed the Shannon index [29], which correlates positively with both taxon richness and evenness, as a measure of biodiversity. The 'vegan' library [30] of the R statistical package [31] was used for these computations and the resulting values were compared between MI and ME through R implementations of the Mann–Whitney and Wilcoxon tests.

We also employed R to perform comparisons of microbial communities among environments by means of heatmaps and clustering based on taxa composition and abundance (Bray-Curtis distance). In addition, we compared communities by means of Fast UniFrac [32] followed by Principal Component Analysis (PCoA). The UniFrac method takes into account the phylogenetic distance among the 16S rRNA gene sequences present in different communities and can only be applied when compositional data has been obtained by sequencing of the same 16S rRNA region. For input in Fast Unifrac, we combined in a single file for joint processing all of the 16S rRNA sequences from the environments to be compared (MI and ME in Figure S1; MI and feces from this study plus vagina and skin from [33] in Fig. 3). 97%-similarity sequence clusters were then obtained with the cd-hit-est programme [34] and representative sequences from each cluster were aligned using Mothur [35] and the Greengenes Core Set database [36]. The alignment was used for phylogenetic reconstruction in FastTree with Jukes-Cantor + CAT models [37] and the resulting phylogeny served as input for Fast UniFrac together with taxa abundance tables. Finally, the resulting matrixes of Fast UniFrac distances among environments were subjected to PCoA.

To define the different types of meconium microbiota we utilized the sample clustering analysis based on the Bray-Curtis distance. Mann–Whitney tests were then applied to evaluate the differences in Chao1 estimator and Shannon diversity index between the defined microbiota types.

Statistical evaluation of microbiota associations with clinical data and other covariates

At the first and third trimesters of gestation, INMA cohort women answered questionnaires administered by trained interviewers focused on sociodemographic, clinical, nutritional, environmental and lifestyle information. These questionnaires recorded any history of asthma, allergic rhinitis and eczema. Perinatal information was obtained from La Fe Hospital clinical records and information on children's health was obtained in interviews performed at the end of the first and fourth years of age, by INMA paediatricians (first year) or INMA field workers (fourth year). In these interviews, parents reported whether their children had been diagnosed with bronchitis (respiratory problems and fever) or atopic eczema. The diagnosis of atopic eczema in Spain follows the criteria established by Hanifin and Rajka [38–40]. Parents also reported if their children had suffered from mucus congestion in the chest or from wheezing, and whether the wheezing had required the prescription of medication during the fourth year of age.

Using this information, we evaluated any potential associations of the microbiota in meconium with differ-

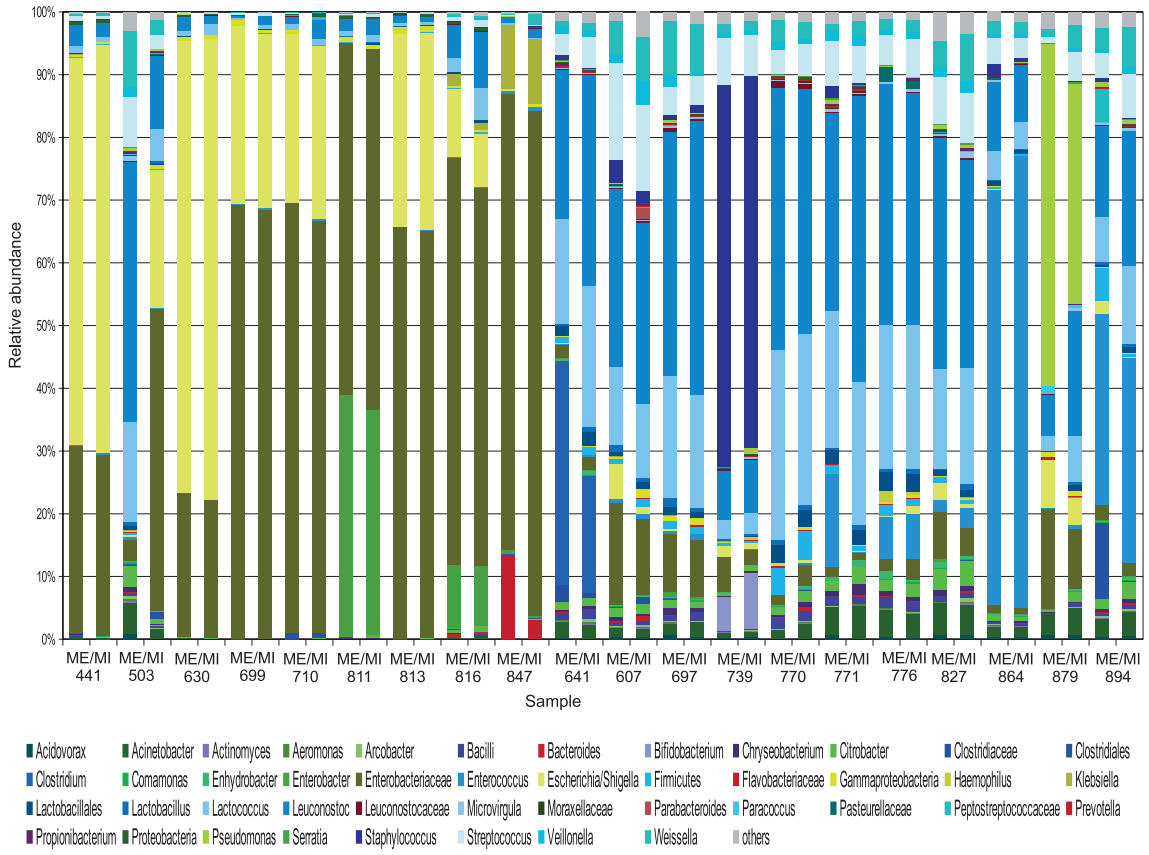

Fig. 1. Relative abundances of the most abundant taxa in the microbial communities of the external (ME) and internal (MI) fractions of the 20 INMA meconium samples analysed in this study. We considered only sequences taxonomically assigned in RDP with a bootstrap value over 80%, stopping sequence assignation at the lowest phylogenetic category identified at or above this support level.

ent atopic diseases in mothers and children, as well as with respiratory problems in children that could be symptomatic of asthma development. In addition, we examined whether other maternal and perinatal factors had any relation to the microbiota. The evaluated maternal factors included age, parity, social class, education level, zone of residence, smoking during early or entire pregnancy and intake of antibiotics, dairy products and organic products during pregnancy. The evaluated perinatal factors included gestational age, gender, whether the amniotic fluid was clear or stained by meconium, the type of initiation of labour and the delivery mode and whether the newborn was breast- or formula fed at birth.

We specifically investigated the potential associations between INMA variables and the following characteristics of the meconium microbiota: 1) type (A vs. B), 2) Shannon diversity index, 3) Chao1 taxon richness estimator and 4) relative abundance of the main bacterial families detected in our analysis. Associations with microbiota types A and B were evaluated by means of bivariate analyses. Simple logistic regression models were built, where the dependent variable was meconium composition separated into the two defined types. The odds ratio (OR) with 95% confidence intervals (95% CI) was used as the measure of association. Distribution differences were also contrasted with the χ^2 test for categorical variables or the Mann–Whitney test for continuous variables (maternal age and gestational age). For evaluation of associations with diversity, richness and relative abundance of bacterial families we employed the Mann–Whitney or the Kruskal–Wallis tests, depending on the possible number of values for the variable. All these analyses were carried out using the statistical packages Stata v.9s (Stata Corporation, College Station, TX, USA) and the statistical package for social sciences v.15.0 (SPSS Inc., Chicago, IL, USA).

Results

Bacterial composition, biodiversity and richness of meconium samples

Figure 1 presents relative taxa abundances in the MI and ME fractions of the INMA samples, showing a highly uniform composition for the two fractions (except for sample 503). A PCoA plot further confirms that the MI and ME fractions of each sample are sepa-

rated by short Unifrac distances (Figure S1). Furthermore, MI and ME are also generally similar in both their diversity and taxon richness. No overall difference in diversity and richness exists between fractions (Mann–Whitney test: $P = 0.304$ for Shannon and $P = 0.267$ for Chao1), although, for the Shannon index, some variability can be detected between the two fractions of a given sample (Wilcoxon test: $P = 0.015$ for Shannon and $P = 0.090$ for Chao1). With basis on the similarities we observe at several levels, we employ only MI fractions in all further analyses.

Figure 1 also reveals that two distinct types of microbiota composition can be detected in meconium samples. Clustering analysis based on the Bray-Curtis distance was applied to the MI fractions to confirm the existence of these two types, that we define as A and B (Fig. 2). Type A presents the family Enterobacteriaceae as the most abundant bacterial taxon (58.69%), mainly represented by the *Escherichia/Shigella* genus (24.68%). In contrast, Firmicutes, and particularly LAB of the families Leuconostocaceae, Enterococcaceae and Streptococcaceae, predominate in type B, with *Leuconostoc* (25.86%), *Enterococcus* (16.79%), *Lactococcus* (14.01%), *Staphylococcus* (6.75%) or *Streptococcus* (6.34%) as the most abundant genera. The phylum Bacteroidetes is present at a frequency > 1% in only three samples, which contain, respectively, 3% *Bacteroides,* 2% *Parabacteroides* and 1.9% *Chryseobacterium* (Figs 1 and 2). In terms of diversity and richness, comparisons of the values of the Shannon index and the Chao1 estimator between the two clusters detected significant differences ($P = 4.721e^{-04}$ and $P = 0.013$, respectively), with lower values overall within the Enterobacteriaceae-dominated group A.

Comparison to other microbiotas from women and infants

To evaluate the potential for external contamination of the meconium samples during or after childbirth, we compared the microbiota detected in MI to those present in different areas of the body of pregnant women. To this aim, we analysed microbial composition in fecal samples from pregnant women days before delivery (this study), vaginal and skin samples from Venezuelan women 1 hour before delivery (from [33]) and samples of three different vaginal regions from US women at different stages of pregnancy (from [41]). The Unifrac-

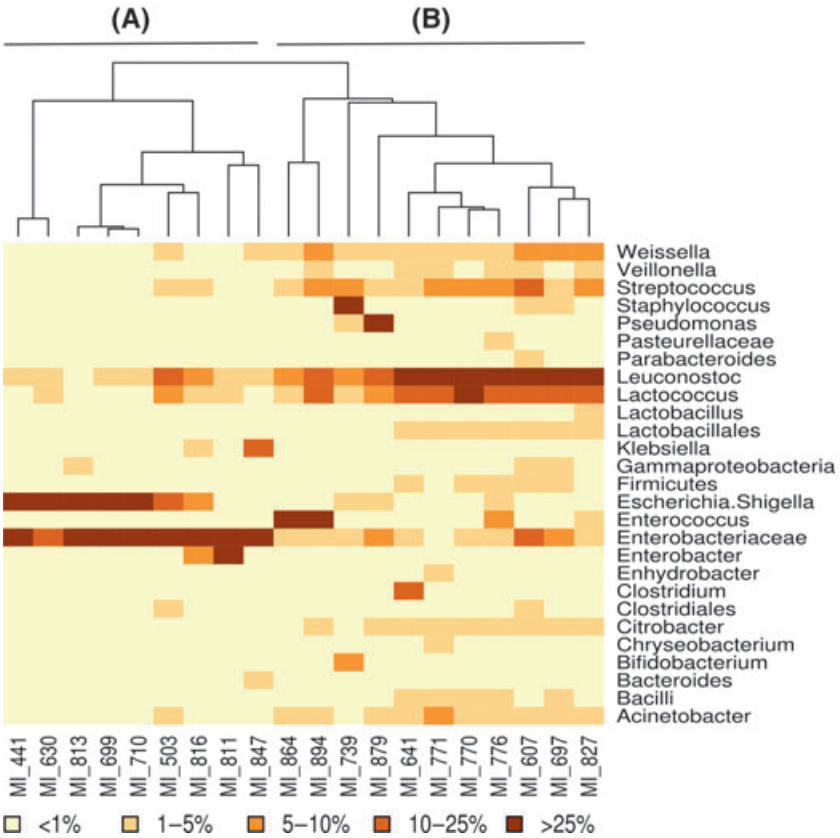

Fig. 2. Heatmap and clustering based on taxon composition and abundance (Bray-Curtis distance) in the microbiota of 20 INMA meconium samples (MI). Colours in the figure depict the percentage range of sequences assigned to main taxa (abundance > 1% in at least one sample). On top, hierarchical clusters A and B.

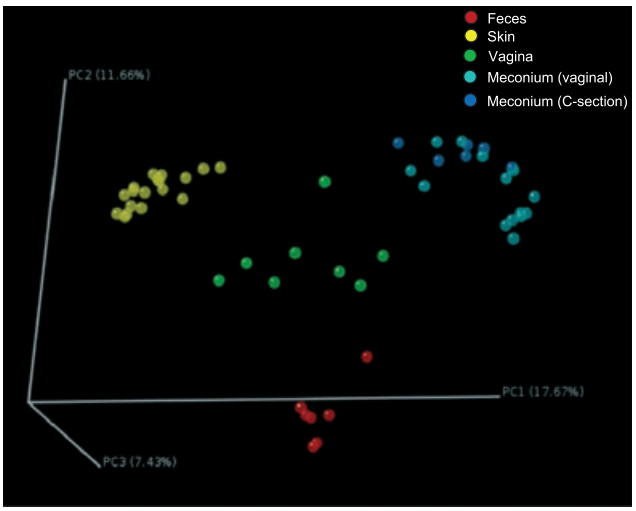

Fig. 3. PCoA of unweighted Fast UniFrac distances among microbial communities in MI fractions from vaginal (cyan) or C-section (blue) deliveries, and in feces (red), skin (yellow) and vagina (green) of pregnant women shortly before delivery. Samples are from this study (meconium and fecal samples) and from [33] (skin and vagina).

based PcoA in Fig. 3 shows that meconium samples, both from vaginal and C-section deliveries, cluster away from the fecal samples and the Venezuelan skin and vaginal samples. US vaginal samples could not be included in the Unifrac analysis because their microbial composition was assessed using a different region of the 16S rRNA gene, so they were compared with MI samples and with Venezuelan vaginal samples by means of heatmaps and clustering based on the Bray-Curtis distance. Figure S2 shows that all US vaginal samples group with the Venezuelan ones, in a cluster separate from MI samples.

We also assessed the similarity between the MI microbiota and those present in the infant GIT at different periods. To this aim, we analysed two series of infant fecal samples spanning the first seven months of life along with the corresponding meconium and maternal samples. Figure 4 shows that meconium samples cluster with other samples from the first time periods based on their microbiota composition, even if the clustering patterns of each series are not identical. For the M02 series, the meconium clusters with the first three infant samples; for the M21 series, the meconium clusters with the one-week and one-month samples, whereas the three months sample already clusters with those of the seven months infant and the mother.

Using the M02 and M21 series, we also assessed whether specific organisms present in meconium could be detected in the corresponding infant samples. On the

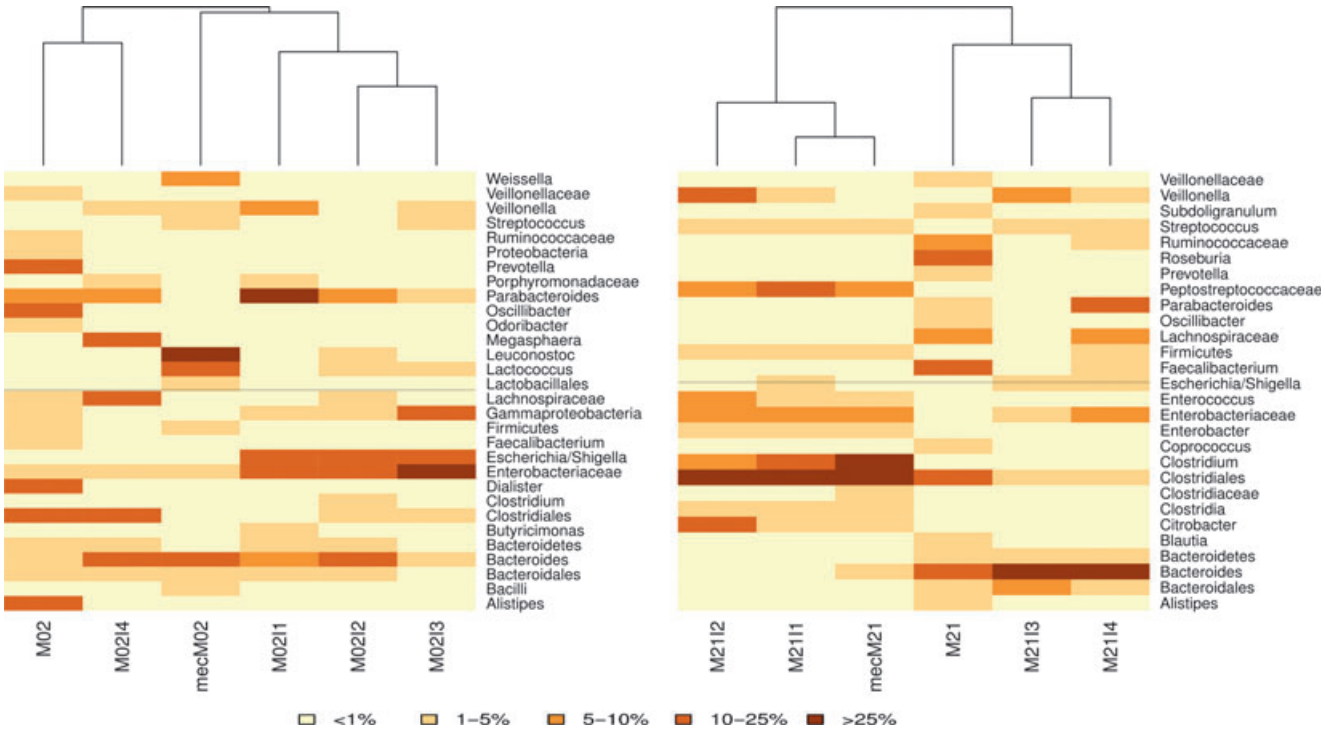

Fig. 4. Heatmaps and clustering based on taxon composition and abundance (Bray-Curtis distance) for two series of mother–infant samples (mothers M02 and M21). mec, meconium; I1, fecal sample from infant at 1 week old; I2, fecal sample from infant at 1 month old; I3, fecal sample from infant at 3 months old; I4, fecal sample from infant at 7 months old. Maternal samples were obtained days before delivery. Colours in the figure depict the percentage range of sequences assigned to main taxa (abundance > 1% in at least one sample).

basis of 100% identity clustering, we detected 21 M02 and 132 M21 meconium sequences in at least one of the infant samples in the corresponding series. These included sequences belonging to *Leuconostoc*, *Lactococcus, Clostridium*, *Enterobacter*, *Citrobacter*, *Bacteroides* and *Parabacteroides*, as well as different unclassified Clostridiales and Enterobacteriaceae. The latest infant samples investigated, from 7 months of age, still contained sequences present in meconium, including sequences assigned to *Leuconostoc*, *Lactococcus*, *Clostridium* and *Enterobacter,* as well as one of the unclassified Clostridiales. A small minority of the meconium sequences present in infants could also be detected in the corresponding maternal samples (2 in M02 and 7 in M21), including sequences belonging to *Leuconostoc*, *Citrobacter*, *Bacteroides, Clostridium* and different unclassified Clostridiales.

Associations of the meconium microbiota with clinical, sociodemographic and lifestyle variables

Table 1 presents the distribution of microbiota types A and B depending on the presence or absence of atopy-related conditions in mothers and children. The strongest association was detected between maternal eczema and a microbiota of type A ($P = 0.038$). All meconium samples from infants whose mothers had a history of eczema had this microbiota, whereas the majority of samples from other infants were of type B. In accordance, Table 2 shows that maternal eczema is also associated with a significantly different distribution of values for Shannon diversity ($P = 0.040$) and Chao1 taxon richness ($P = 0.028$), and with differences close to significance ($P = 0.054$) for the relative abundances of Enterobacteriaceae and Leuconostocaceae. Among

Table 1. Meconium microbiota type and atopy-related problems in mothers and children

		Meconium microbiota type					
		A		B			
		N	%	N	%	*P* (χ^2 test)	OR (95% CI)
Maternal history of atopic disease							
Asthma	No	7	41.2	10	58.8	0.413	0.35 (0.03–4.65)
	Yes	2	66.7	1	33.3		
Rhinitis	No	8	50.0	8	50.0	0.369	3.00 (0.25–35.33)
	Yes	1	25.0	3	75.0		
Eczema	No	6	35.3	11	64.7	**0.038**	Nc
	Yes	3	100.0	0	0.0		
Health problems at first year of life							
Mucus congestion	No	6	66.7	3	33.3	**0.040**	8.00 (1.00–63.96)
	Yes	2	20.0	8	80.0		
Bronchitis	No	8	47.1	9	52.9	0.202	Nc
	Yes	0	0.0	2	100.0		
Wheezing	No	5	38.5	8	61.5	0.636	0.62 (0.09–4.40)
	Yes	3	50.0	3	50.0		
Atopic eczema	No	8	44.4	10	55.6	0.381	Nc
	Yes	0	0.0	1	100.0		
Health problems at fourth year of life							
Mucus congestion	No	5	45.5	6	54.5	0.914	1.11 (0.16–7.50)
	Yes	3	42.9	4	57.1		
Bronchitis	No	8	50.0	8	50.0	0.180	Nc
	Yes	0	0.0	2	100.0		
Wheezing	No	7	50.0	7	50.0	0.375	3.00 (0.25–36.32)
	Yes	1	25.0	3	75.0		
Atopic eczema	No	4	33.3	8	66.7	0.180	0.25 (0.03–2.00)
	Yes	4	66.7	2	33.3		

Meconium Microbiota Type A: Family Enterobacteriaceae as the most abundant bacterial taxon.
Meconium Microbiota Type B: Families Leuconostocaceae, Enterococcaceae and Streptococcaceae as the most abundant bacterial taxa
OR (95% CI): odds ratio (and 95% confidence interval).
Nc: not calculated due to the 2 × 2 table containing a 0
Significant *P*-values (< 0.05) are in bold.

Table 2. Significant associations between INMA variables and meconium microbiota diversity, richness and relative abundance of the most highly represented bacterial families*

		Shannon diversity			Chao1 richness			Enterobacteriaceae abundance			Leuconostocaceae abundance			Enterococcaceae abundance			Streptococcaceae abundance		
		M†	IQR‡	*P*-value§	M	IQR	*P*-value	M	IQR	*P*-value	M	IQR	*P*-value	M	IQR	*P*-value	M	IQR	*P*-value
Maternal eczema	No	2.0	0.9	**0.040**	79.0	47.1	**0.028**	0.111	0.767	*0.054*	0.242	0.319	*0.054*	0.003	0.008	0.146	0.125	0.213	*0.093*
	Yes	0.9	0.2		26.3	20.0		0.952	0.014		0.026	0.018		0.002	0.001		0.014	0.010	
Maternal	Primary	0.9	0.7	**0.032**	34.0	38.0	0.328	0.936	0.469	0.350	0.030	0.232	*0.096*	0.002	0.003	0.231	0.014	0.183	*0.077*
educational	Secondary	1.5	1.0		43.0	64.0		0.733	0.908		0.104	0.260		0.002	0.032		0.074	0.184	
level	University	2.2	0.1		82.0	11.0		0.111	0.085		0.387	0.048		0.009	0.004		0.275	0.051	
Organic products	No	1.5	1.2	**0.028**	43.0	54.0	0.179	0.733	0.886	0.479	0.104	0.389	0.358	0.002	0.008	0.546	0.074	0.227	0.179
during pregnancy	Yes	2.3	0.4		82.0	68.0		0.157	0.122		0.368	0.146		0.006	0.007		0.275	0.166	
Mucus congestion	No	1.0	1.2	0.243	35.0	56.0	0.182	0.951	0.800	**0.001**	0.032	0.227	*0.095*	0.002	0.005	*0.065*	0.018	0.114	*0.065*
at 1st year	Yes	2.0	0.7		81.0	47.0		0.059	0.059		0.327	0.321		0.005	0.074		0.235	0.225	

*Relative abundances are absolute sequence counts assigned to the family normalized by the total number of taxonomically-assigned sequences per sample.
†M = median.
‡IQR = interquartile range.
§Significant *P*-values (< 0.05) are in bold and *P*-values < 0.1 are in italics. The Kruskal-Wallis test was applied for the 'Educational level' variable because three different educational levels were recorded, whereas all other variables were evaluated with the Mann-Whitney test because they took only yes/no values.

children, we detected a skewed distribution of microbiota types depending on the presence or absence of eczema by four years of age, with two thirds of the meconium samples of children who developed this disease having a microbiota of type A, vs. only one third in children who did not. However, this association did not reach statistical significance ($P = 0.180$).

In contrast to eczema, asthma and rhinitis in the mother were not related to microbiota type ($P = 0.413$ and $P = 0.369$). In children, a significant association was detected between the occurrence of mucus congestion during the first year and a microbiota of type B ($P = 0.040$; OR=8.00, 95% CI: 1.00–63.96). The relative abundance of Enterobacteriaceae was also significantly different in 1-year-olds having suffered mucus congestion ($P = 0.001$), but there was no difference in terms of overall meconium microbiota diversity ($P = 0.243$) and richness ($P = 0.182$; Table 2). Moreover, no association between the presence of mucus congestion and microbiota type remained by four years of age ($P = 0.914$). The presence of other respiratory problems that could be symptomatic of asthma development in children (bronchitis, wheezing) was not associated with microbiota type (Table 1), nor was the intake of medication for wheezing by four years of age ($P = 0.671$).

Regarding the influence of perinatal factors, we detected no statistically significant associations with meconium microbiota type (Table 3). In a previous study that analysed microbiota composition in meconium through 16S-rRNA-based techniques, the gestational age of preterm newborns was associated with microbiota diversity [4], but we did not detect any effect of this factor among the infants in our study, who were all born at term ($P = 0.552$). We also investigated the relationship between microbiota type and the mode of childbirth, which is known to be strongly associated with the composition of the microbiota detected on different regions of the body of newborns shortly after birth [33]. The distribution of meconium samples between microbiota types differed depending on delivery mode, as most C-section samples (5/6) belonged to type B, whereas vaginal delivery samples were distributed similarly between the two types. However, this difference was not statistically significant ($P = 0.127$; OR: 5.83, 95% CI: 0.52–64.82).

Analyses of potential associations of the meconium microbiota with sociodemographic factors revealed an interesting trend. Maternal educational level displayed an association with microbiota type that was close to significance ($P = 0.051$), with 100% of university-educated women having type B, and 75% of women with primary school education or with no studies having type A (Table 3). Moreover, a statistically significant association was detected between this sociodemographic

Table 3. Relationship between maternal and perinatal variables and meconium microbiota type

		Meconium microbiota type					
		A		B			
		N	%	N	%	*P*-value	OR (95% CI)
Parity	0	5	35.7	9	64.3	0.202	0.28 (0.04–2.09)
	≥ 1	4	66.7	2	33.3		
Social class*	SC I + II	1	20.0	4	80.0	0.194	0.21 (0.02–2.44)
	SC III–V	8	53.3	7	46.7		
Educational level	Primary or less	3	75.0	1	25.0	*0.051*	Nc
	Secondary	6	54.5	5	45.5		
	University	0	0.0	5	100.0		
	Urban + metropolitan	5	55.6	4	44.4	0.391	2.18 (0.36–13.22)
	Semi-urban + rural	4	36.4	7	63.6		
Antibiotics intake during pregnancy	No	8	50.0	8	50.0	0.369	3.00 (0.25–35.33)
	Yes	1	25.0	3	75.0		
Smoking during early pregnancy	No	6	46.2	7	53.8	0.888	1.42 (0.18–7.28)
	Yes	3	42.9	4	57.1		
Smoking during entire pregnancy	No	7	38.9	11	61.1	*0 099*	Nc
	Yes	2	100.0	0	0.0		
Organic products intake during pregnancy	No	9	52.9	8	47.1	*0.089*	Nc
	Yes	0	0.0	3	100.0		
Dairy products intake during pregnancy†	< 420 g/day	4	40.0	6	60.0	0 653	0.66 (0.11–3.92)
	> 420 g/day	5	50.0	5	50.0		
	Girl	3	50.0	3	50.0	0.769	1.33 (0.16–9.08)
	Boy	6	42.9	8	57.1		
Amniotic fluid	Meconium-stained	3	60.0	2	40.0	0.510	0.50 (0.06–4.00)
	Clear	6	42.9	8	57.1		
Spontaneous initiation of labor	No	1	20.0	4	80.0	0.194	0.22 (0.02–2.45)
	Yes	8	53.3	7	46.7		
Cesarean section	No	7	53.8	6	46.2	0.127	5.83 (0.52–64.82)
	Yes	1	16.7	5	83.3		
Breastfeeding at birth	No	1	25.0	3	75.0	0 435	0 38 (0 03–4 55)
	Yes	7	46.7	8	53.3		
Continuous variables		Meconium Microbota Type					
		A		B			
		Median		Median		*P*-value	OR (95% CI)
Maternal age (in years)		28.0		30.0		0.456	1.07 (0.85–1.34)
Gestational age (in weeks)		40.3		40.4		0.552	1.32 (0.67–2.58)

Meconium Microbiota Type A: Family Enterobacteriaceae as the most abundant bacterial taxon

Meconium Microbiota Type B: Families Leuconostocaceae, Enterococcaceae and Streptococcaceae as the most abundant bacterial taxa

OR (95% CI): odds ratio (and 95% confidence interval)

Nc: not calculated due to the 2 × 2 table containing a 0

P-values < 0.1 are in italics.

*Social class was defined from the maternal or paternal occupation during pregnancy with the highest social class, using a widely used Spanish adaptation of the international ISCO88 coding system [69]

†Intake of dairy products during pregnancy categorized by median; dietary information was collected using a validated semi-quantitative food frequency questionnaire of 101 food items

variable and meconium microbiota diversity ($P = 0.032$; Table 2).

Among lifestyle habits during pregnancy, we identified two factors that were exclusively associated with one type of meconium microbiota. All women who smoked during the entire pregnancy had an A type microbiota ($P = 0.099$), whereas all women who consumed organic foods had a microbiota of type B ($P = 0.089$). Moreover, organic food consumption was significantly associated with the meconium microbiota diversity ($P = 0.028$; Table 2). As organic food consumption was more frequent among highly educated mothers ($P = 0.001$), the trend observed for this dietary variable may be related to the association

between the meconium microbiota and educational level described above.

Discussion

The microbiota of meconium

The lack of significant differences in composition and taxon richness between ME and MI suggests that the majority of bacteria detected in these samples do not come from external sources, as we would then expect a higher number of taxa in external areas. Nevertheless, to maximally avoid external contamination, we carried out all further analyses using only MI fractions.

The overall microbiota composition of MI fractions is clearly distinct from that of the microbiotas that could most easily contaminate the meconium during or shortly after childbirth, such as those present in the vagina, feces or skin of pregnant women (Fig. 3 and Figure S2). Unfortunately, the meconium and pregnant women samples in our comparisons were not from the same individuals. However, the composition of the human microbiome varies systematically across the different habitats of the body, so that, in spite of substantial interpersonal variability, the microbial composition of a given sample is primarily determined by the body habitat from which it originated [42]. Hence, we would expect that the vaginal, fecal and skin microbiotas of INMA pregnant women would be similar enough to those employed in our analyses to behave similarly in respect to meconial samples. This assumption is supported by the fact that vaginal samples from different geographical, ethnic and cultural regions, different intravaginal locations and different gestational periods cluster away from MI samples (Figure S2). Therefore, our results suggest that the microbiota detected in meconium is not gathered perinatally due to contact with maternal habitats, pointing towards a likely accumulation of microbes during intrauterine life. This is in marked contrast to previous analyses demonstrating that the microbiota recovered from other types of newborn samples, including skin, oral mucosa, nasopharyngeal aspirate and rectal swabs, closely resembles vaginal or skin microbiota, depending on the mode of delivery [33].

The identity of the specific microbes detected in MI also supports their intrauterine origin. The main taxa detected were *Escherichia/Shigella* in type A meconia and *Leuconostoc*, *Enterococcus* and *Lactococcus* in type B, a set of taxa almost identical to that identified in meconium by culture-dependent techniques, which includes *Enterococcus*, *Staphylococcus*, *Escherichia* and *Leuconostoc* [3]. These genera do not correspond to the dominant groups in fecal (*Bacteroides, Clostridium*), skin (*Corynebacterium*, *Propionibacterium)* or vaginal (*Lactobacillus*) environments of pregnant women [6, 33], further supporting that contamination from these environments is not the source of the meconial microbiota. Moreover, the taxa detected in type B are strikingly similar to those recently identified by pyrosequencing in colostrum samples [43], suggesting that these bacteria may travel through the maternal circulation and reach both the placenta and the mammary gland.

To assess whether the meconium microbiota resembles that found later on in the GIT, we analysed two series of samples collected from two infants and their mothers, not belonging to the INMA cohort. The meconium microbiota of both infants corresponded to that of INMA samples of type B. When compared with other samples in their series, meconia clustered with other samples from the first stages of life, suggesting that the meconium microbiota is indeed contributing significantly to the colonization of the infant's GIT during this early period (Fig. 4). In particular, the similarity between the MI microbiota and that in the one-week-old infant is particularly striking for series M21, indicating a likely higher contribution of meconium bacteria in this case. Moreover, we detected numerous meconium bacterial sequences in the infant feces from the different time periods sampled, including those taken at 7 months, indicating that some of the organisms that reach the fetal GIT *in utero* can be present in the infant long into the first year. A minority of these sequences were also present in the feces of the mothers, suggesting that they may originate from the maternal GIT.

Relationships with lifestyle and allergy

The diversity and type of bacteria that first reach the fetal GIT could affect the propensity to allergic disease through influences on the development of the fetal immune system. The immunology of the fetal GIT is far from being fully understood, but antigen presenting cells, T cells and dendritic cells are all known to appear in this environment between the 11^{th} and 16^{th} weeks of gestation, and a T cell progenitor population is likely to be present within the fetal lamina propia [44–46]. The co-stimulatory T cell molecule CTLA-4, characteristic of regulatory T cells (Treg), is also detectable in the fetal GIT, indicating that immune homeostasis and tolerance are probably developing. There is also evidence that immunological priming can start prenatally and that the fetal GIT is capable of hosting adaptive immune responses [46, 47]. It is likely therefore that microbes reaching the fetal GIT can be recognized by the local immune system and can modulate the early establishment of immune homeostasis and the induction of tolerance by influencing T cell differentiation.

Development and maturation of the fetal gut immune system occur under close control of the maternal environment and can be affected by a variety of maternal factors, including immune status and exposure to microbes. Maternal allergic sensitization might reduce the expression and function of Toll-Like Receptors (TLR) in fetal immune cells [48–50], therefore reducing the fetal capacity for recognizing microbial antigens and responding through signalling processes that shape T cell differentiation. Accordingly, Treg cell numbers, gene expression and function have been shown to be impaired in the cord blood of atopic mothers [51], whereas levels of IgE are elevated [52]. Remarkably, there is also emerging evidence that pregnancy smoking, like maternal allergy, can affect immune system development by attenuating TLR function in the feto-placental unit, resulting in altered T cell responses and increased cord blood IgE [53–56]. Importantly, the concentration of T cell-associated chemical mediators in cord blood has been shown to affect atopy development in children [57–59], so that maternal effects on the fetal immune system are likely impacting on the child's propensity to atopic disease. In fact, a history of atopic disease in the mother is one of the strongest predictors of the development of childhood allergies [51, 60, 61], and antenatal smoking has been implicated in the increase in the prevalence of eczema up to 3 years of age [62]. In contrast, an increased maternal exposure to microbes during pregnancy, as in mothers who are in contact with farm animals, results in an elevated number and function of cord blood Treg cells and lower IgE, and a decreased risk of atopic disease [63, 64]. Our results suggest that, beyond the influence of maternal immune factors, maternal health and lifestyle could also affect TLR function and immune system development by altering the diversity and type of bacteria that first reach the fetal GIT.

In this respect, it is noteworthy that maternal eczema and pregnancy smoking, two factors known to affect immune system development and risk of eczema in a similar manner, were associated in our analyses with the same type of meconium microbiota (A), and that this microbiota type was also majoritary among children with occurrences of eczema by the fourth year. However, the associations of type A with smoking and childhood eczema did not reach statistical significance and analyses of a larger number of individuals should be undertaken to address this issue. Importantly, infants who develop eczema have also been shown to carry in their GIT a microbiota that shares the main characteristics of the meconium microbiota of the A type, i.e. low diversity and high abundances of *E. coli* and other enterics [10, 16, 18, 19]. Our results then suggest that the association between eczema and a low-diversity GIT microbiota dominated by enterics may extend back to the intrauterine stage and may be initiated by maternal factors.

It is also interesting that we detected opposite trends in the relationships of pregnancy smoking and organic food intake with the meconium microbiota. This difference might be related to the opposite effects of these lifestyle habits on levels of oxidative stress, as cigarette smoke contains high levels of free radicals, whereas organic farming decreases the free radicals and increases the antioxidants present in food [65–67]. The balance between free radicals and antioxidants could affect the development of the fetal immune system, as oxidative stress has been shown to promote Th2 cell differentiation [68, 69]. Our results suggest that oxidative stress may also influence which type of bacteria reach the intrauterine environment, presumably through effects on inflammation and immune function [56]. Organic food consumption could also directly influence the type of bacteria present in the maternal GIT, as organic farming can increase the diversity or alter the composition of bacterial communities in soil [70, 71], crops [72] and animals [73], and therefore can modify exposure to bacteria through the food chain.

Finally, our analyses also revealed that the presence of mucus congestion, in contrast to eczema, was significantly associated with a meconium microbiota of type B when assessed in 1-year-old infants. However, although the presence of mucus congestion and noise in the chest during early childhood is considered an asthma-like respiratory symptom, this condition can also be unrelated to atopy, and derive instead from transient viral and bacterial infections. In fact, the majority of young children with asthma-like symptoms are not diagnosed with the disease by six years of age, when a definite diagnosis is more reliably made [74]. In our analyses, the association between mucus congestion and microbiota type was no longer present at four years. Moreover, the other children respiratory problems analysed that could also be symptomatic of asthma development (wheezing and bronchitis) were not associated with microbiota type at either age (i.e. one or four years), and neither was a history of asthma in the mother. Therefore, we consider that the association of type B with the presence of mucus congestion by one year of age represents only a weak indication that this type of microbiota might be related with the development of asthma.

Conclusions

Overall, the different associations we detect indicate the existence of interactions between maternal and fetal immune systems and meconium microbiota formation that may exert long-term influences on health. Furthermore, maternal diet and behaviour could affect meconium microbiota composition and impact through this

route the development of the immune system and of the future gut microbiota in the infant. However, the size of the population we analysed was small and several of the associations were only weakly supported, so that further studies will be needed to confirm our results. Nevertheless, our findings suggest that further research may enable the use of specific meconium bacteria as biomarkers of increased risk for immune disease or as probiotics for early immune modulation, and support approaches aimed at the favourable priming of the infant gut microbiota and immune system through maternal lifestyle modifications and/or probiotic use during pregnancy.

Finally, we would emphasize that, in addition to the microbiota originally present in the newborn's GIT, many other influences will affect gut colonization and immune modulation. From birth, different microorganisms will be incorporated to the GIT depending on the mode of delivery [33] and the type of feeding [75]. All of these incomes and their synergistic relations may play a role in shaping the infant's gut microbiota and immune system, contributing to the individual's overall health and propensity to allergy and other immune diseases.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Principal Component Analysis of weighted Fast UniFrac distances among microbial communities in external and internal fractions of the 20 INMA meconium samples analyzed in this study. ME external meconium; MI internal meconium.

Figure S2. Heatmaps and clustering based on taxon composition and abundance (Bray-Curtis distance) comparing the microbiota of INMA meconium samples (MI) to that of vaginal samples from pregnant women collected in Venezuela [33] (Vvag), and the U.S. [41] (**A,** USInt −vaginal introitus−; **B,** USPos −posterior fornix−; **C,** USMid −mid vagina−). Colors in the figure depict the percentage range of sequences assigned to main taxa (abundance >1% in at least one sample).

Table S1. Summary of the number and type of samples sequenced and analyzed in this study with corresponding numbers of pyrosequencing reads after quality control.