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Is meconium from healthy newborns actually sterile?

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Abstract

In a previous study, bacteria were able to be isolated from umbilical cord blood of healthy neonates and from murine amniotic fluid obtained by caesarean section. This suggested that term fetuses are not completely sterile and that a prenatal mother-to-child efflux of commensal bacteria may exist. Therefore, the presence of such bacteria in meconium of 21 healthy neonates was investigated. The identified isolates belonged predominantly to the genuses *Enterococcus* and *Staphylococcus*. Later, a group of pregnant mice were orally inoculated with a genetically labelled *E. fecium* strain previously isolated from breast milk of a healthy woman. The labelled strain could be isolated and PCR-detected from meconium of the inoculated animals obtained by caesarean section one day before the predicted date of labor. In contrast, it could not be detected in samples obtained from a non-inoculated control group.

Keywords: Meconium; Gut; Fetus, *Enterococcus*; *Staphylococcus*

1. Introduction

Since Tissier's time [37], the idea that term fetuses are sterile, and that initial bacterial colonization of the newborn gut occurs only when the baby initiates transit through the labor channel via contamination by maternal vaginal and fecal bacteria, has been widely accepted [21]. In this context, meconium, amniotic fluid and chorioamnion tissue have been considered sterile under normal conditions; therefore, the presence of bacteria in such environments is only investigated when there are symptoms of infection or circumstances that may facilitate it, such as premature rupture of membranes or cervical dilatation. However, clinical practice supports the potential presence of bacteria in fetal meconium, since meconium-stained amniotic fluid is considered a marker of microbial invasion of the amniotic cavity in women with preterm labor and intact membranes [9,27,32]. Obviously, identification of any microorganism at this location is a potential concern under such circumstances.

In contrast, the presence of bacteria in such locations during healthy pregnancies has not been assessed despite the fact that bacteria can be isolated and/or PCR-detected in umbilical cord blood, amniotic fluid and fetal membranes without any clinical or histological evidence of infection or inflammation in the mother-infant pair [3,13,14,35]. Since amniotic fluid surrounds and is continuously swallowed by fetuses, the first objective of this study was to elucidate whether meconium obtained from healthy hosts is actually sterile or if, on the contrary, it contains bacteria. Recently, it has been shown that the maternal digestive tract may be the origin of bacteria found in amniotic fluid [7,14,16]; therefore, the second objective of this study was to elucidate whether oral administration of a bacterial strain to pregnant mice may lead to its presence in fetal meconium.

2. Materials and methods

2.1. Source and isolation of bacterial isolates

First meconium was collected from 21 term newborns in the Servicio de Obstetricia, Hospital Universitario Doce de

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Octubre, Madrid (Spain). The newborns were selected as donors according to the following criteria: (a) meconium was spontaneously evacuated within the first 2 h of life and before they were breastfed; (b) babies were born to healthy mothers after normal pregnancy; and (c) mothers did not receive probiotic supplementation during pregnancy. Informed consent was obtained from their mothers. Ethical clearance for the study was obtained from the Ethical Committee in Human Clinical Research (Hospital Clínico, Madrid, Spain).

Approximately half of the samples (samples 1–11; $n = 11$) were immediately processed after collection, while the remaining ones (samples 12–21; $n = 10$) were stored at 4–8 °C for 4 days. In order to avoid potential bacterial contamination derived from contact between meconium and perianal skin/nappies, one of the poles of each meconium sample was removed using a laser scalpel. Then, an internal meconium portion was underwent microbiological analysis. Proper peptone water dilutions of the meconium samples were plated in triplicate onto brain heart infusion (BHI, Oxoid, Basingstoke, UK), violet red bile agar (VRBA; Difco, Detroit, MI), and Columbia nadilixic acid agar (CNA, BioMerieux, Marcy l'Etoile, France) agar plates, which were aerobically incubated at 37 °C for 24 h. In parallel, the same samples were also cultured on Wilkins-Chalgren (WCh, Oxoid) and de Mann, Rogosa and Sharpe (MRS, Oxoid) agar plates, which were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in an anaerobic work station (MINI-MACS, DW Scientific, Shipley, UK) at 37 °C for 48 h. Comparison of meconium bacterial counts obtained in the different culture media was done using Student's *t*-tests. An exploratory multivariate statistical method for identifying similar characteristics in an observation group (hierarchical cluster analysis) was performed using Euclidean distances and the single linkage (nearest neighbor) method. Statgraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD) was used for statistical analysis and generation of a dendrogram.

Between 5 and 10 isolates from each culture medium in which growth was observed (~35 isolates per sample) were randomly selected, grown in BHI or WCh broth depending on the original culture media and conditions and stored at −80 °C in the presence of glycerol (30%, v/v). The isolates were examined by phase-contrast microscopy to determine cell morphology and Gram-staining reaction. Subsequently, a percentage of the isolates representative of all the colony and cell morphologies observed was submitted to 16S rDNA sequencing. Briefly, a single colony growing on solid media was removed with a sterile plastic tip and resuspended in 100 µl of sterile deionized water in a microcentrifuge tube. Then 100 µl of chloroform/isoamyl alcohol (24:1) was added to the suspensions, and after vortexing for 5 s the mixture was centrifuged at 16,000 × *g* for 5 min at 4 °C [33]. Then 5–10 µl of the upper aqueous phase was used as a source of DNA template for PCR amplifications that were carried out in DNA thermal cyclers (Techne, Cambridge, UK). PCR amplifications were performed using primers plb16 (5′-AGA GTTTGATCCTGGCTCAG-3′) and mlb16 (5′-GGCTGCTGG CACGTAGTTAG-3′), and the following program: [(96 °C for 4 min) × 1 cycle] + [(96 °C for 30 s; 48 °C for 30 s; 72 °C for 45 s) × 30 cycles] + [(72 °C for 4 min) × 1 cycle]. The primers, based on conserved regions of the 16S rRNA gene [18], were used to direct PCR amplification of an approximately 500 bp portion of such a gene. Five µL of the PCR mixtures were analyzed on a 1.2% (w/v) agarose (Sigma, St. Louis, USA) gel with ethidium bromide staining. A 100 bp ladder (Invitrogen, Carlsbad, USA) was used as a molecular weight standard. The amplicons were purified using the Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced at the Genomics Unit of the Universidad Complutense de Madrid, Spain. The resulting sequences were used to search sequences deposited in the EMBL database using the BLAST algorithm, and the identity of the isolates was determined on the basis of the highest scores (>98%).

2.2. *Oral administration of a labelled* Enterococcus fecium *strain to pregnant mice*

E. fecium HA1, a breast milk isolate [22], was selected to investigate the potential mother-to-child transfer of commensal bacteria through the placental barrier in a murine model. Since labelled *E. fecium* cells were required, a genetic label previously developed by our group was used [11]. Briefly, a 189 bp PCR fragment comprising the junction between the 35S rRNA promoter of the cauliflower mosaic virus (CaMV) and the EPSPS gene of *Agrobacterium tumefaciens* was amplified from transgenic soya kindly provided by Biotools (B&M Labs, Madrid, Spain) using primers EPSP1 (5′-CGCGGATCCTGATGTGATATCTCCACTGACG-3′) and EPSP2 (5′-CGCGGATCCTGTATCCCTTGAGCCATGTTGT-3′). EPSP1 was based on primer P35S-F2 [38], while EPSP2 was designed from the artificial sequence present in Roundup Ready soya (EMBL accession number: AX033493). Both primers were designed with BamHI sites in their 5′-tails (underlined sequences) to facilitate cloning of the fragment in pTG262, a lactococcal-*E. coli* shuttle vector that confers resistance to chloramphenicol (Cm) [8]. The PCR mixture (25 µl) consisted of 75 mM Tris–HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 200 mM dNTPs, 50 pmol of each primer, 0.7 U Taq DNA polymerase (Biotools), and 2.5 µl of transgenic soya genomic DNA as template. DNA was amplified in a Techne thermal cycler as follows: 94 °C for 10 min, 25 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s; and then a final extension at 72 °C for 3 min. Once obtained, the PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), digested with BamHI, and ligated into pTG262. Subsequently, the resulting plasmid was introduced into *E. fecium* HA1 cells following the procedure described by Bhowmik and Steele [4], and strain *E. fecium* JLM3 was obtained. The stability of the recombinant plasmid in the new strain was confirmed by PCR after eight successive subcultures in MRS (Oxoid) broth.

Just after mating, four 10-week-old pregnant BALB/c mice (group A) were orally inoculated with 8 $\log_{10}$ colony-forming units (CFUs) of genetically labelled *E. fecium* JLM3 previously suspended in 200 µL of milk. Subsequently, they

received the same dose daily until labor. Another group (group B), quantitatively and qualitatively identical to group A, received non-inoculated milk and served as a control. All pregnant mice were housed individually. One day before the predicted labor date (day −1), pregnant mice were submitted to caesarean section to aseptically collect meconium samples from term fetuses (8 samples from each group), which were cultured on MRS agar plates. The plates were incubated for 24 h at 37 °C. Among colonies that grew on MRS plates, 20 colonies from each group were randomly selected and subcultured on MRS plates supplemented with Cm (7.5 μg/mL). Finally, to detect the genetically labelled strain, PCR analyses were performed using the Cm-resistant colonies as templates. When required, results are expressed below as means ± standard deviations.

The experimental design was approved by the Ethical Committee for Animal Experimental Research (Universidad Complutense de Madrid, Spain).

3. Results

3.1. Bacterial diversity in meconium

In this study, samples of meconium were collected from 21 healthy neonates born by either vaginal delivery or caesarean section. In general, inoculation of suitable dilutions of the meconium samples led to bacterial growth in the culture media tested (Table 1). However, there were some exceptions, since no colonies could be isolated on VRBA plates from 11 samples, and the same happened with three samples for CNA and one sample for MRS plates. For the other media and/or the rest of the samples, the mean count values varied, depending on the sample: from 2.04 to 10.41 log CFU/g in BHI; from 3.84 to 10.48 log CFU/g in VRBA; from 1.88 to 10.39 log CFU/g in CNA; from 1.84 to 10.66 log CFU/g in WCh; and from 1.82 to 10.69 log CFU/g in MRS plates (Table 1).

Although all meconium samples were obtained within the first 2 h of life, samples 1–11 (group A; $n = 11$) were immediately processed, while samples 12–21 (group B; $n = 10$) were stored at 4–8 °C for 4 days. The differences in the bacterial counts between the two groups were highly significant ($P \leq 0.001$, Student's t-test) (Table 1) and revealed that prolonged storage of the samples at 4–8 °C may lead to overestimation of the bacterial concentration in meconium. Subsequently, the bacterial counts obtained in the five culture media tested were used to investigate the relatedness of the samples by cluster analysis. The dendrogram (Fig. 1) showed that the samples could be clustered into 2 well defined groups. The first cluster ($n = 10$) included all samples from group A (with the exception of sample 8) which were characterized by bacterial counts $\leq 6 \log_{10}$ CFU/g (Table 1). This cluster contained most of the samples from which bacterial growth could not be detected on VRBA, CNA and/or MRS agar plates (Table 1). The second cluster ($n = 11$) included all samples from group A and sample 8 (Fig. 1). Such samples were associated with bacterial counts $>6 \log_{10}$ CFU/g (Table 1) Therefore, multivariate analysis also demonstrated that clustering for bacterial counts was strongly associated with the sample processing time and/or storage temperature.

Table 1
Bacterial mean counts ($\log_{10}$ CFU/g ± SD) in meconium samples

Group	Sample	BHI	VRBA	CNA	WCh	MRS
A	1	3.73 ± 1.35	ND	4.39 ± 0.30	3.60 ± 1.35	2.82 ± 0.00
	2	6.17 ± 0.14	3.84 ± 0.00	6.32 ± 0.06	5.19 ± 0.18	5.57 ± 0.10
	3	6.00 ± 0.07	5.69 ± 0.00	ND	5.87 ± 0.14	6.00 ± 0.12
	4	6.00 ± 0.14	ND	ND	6.90 ± 0.24	ND
	5	4.11 ± 0.39	ND	2.72 ± 0.23	4.27 ± 0.33	4.20 ± 0.17
	7	2.04 ± 0.29	ND	1.88 ± 0.31	1.84 ± 0.01	1.82 ± 0.22
	7	3.84 ± 0.21	ND	ND	3.93 ± 0.337	3.91 ± 0.45
	8	8.85 ± 0.06	ND	9.11 ± 0.10	8.79 ± 0.19	8.79 ± 0.07
	9	4.00 ± 0.04	ND	2.08 ± 0.29	7.01 ± 0.16	3.69 ± 0.00
	10	5.22 ± 0.14	ND	5.22 ± 0.11	5.29 ± 0.09	4.52 ± 0.30
	11	5.42 ± 0.77	ND	5.46 ± 0.07	4.71 ± 0.08	4.19 ± 0.28
	Mean	5.03 ± 1.79	4.76 ± 1.31	4.65 ± 2.44	5.22 ± 1.90	4.55 ± 1.91
B	12	10.05 ± 0.29	9.12 ± 0.03	10.06 ± 0.09	10.55 ± 0.02	10.69 ± 0.05
	13	9.55 ± 0.04	7.70 ± 0.32	9.62 ± 0.03	10.27 ± 0.10	10.29 ± 0.07
	14	10.41 ± 0.03	10.48 ± 0.00	9.17 ± 0.16	10.47 ± 0.09	10.41 ± 0.13
	15	9.87 ± 0.14	9.46 ± 0.05	9.94 ± 0.02	9.89 ± 0.07	9.84 ± 0.00
	16	10.32 ± 0.04	10.15 ± 0.03	9.75 ± 0.21	10.28 ± 0.06	9.65 ± 0.03
	17	8.69 ± 0.06	8.55 ± 0.24	6.28 ± 0.15	8.79 ± 0.02	6.08 ± 0.12
	18	9.01 ± 0.88	7.71 ± 0.43	7.67 ± 0.29	8.01 ± 0.05	7.44 ± 0.22
	19	9.86 ± 0.01	ND	10.06 ± 0.15	10.66 ± 0.03	10.62 ± 0.23
	20	10.33 ± 0.09	ND	10.39 ± 0.06	10.54 ± 0.1	10.43 ± 0.04
	21	10.08 ± 0.07	10.18 ± 0.00	6.72 ± 0.08	9.46 ± 0.02	7.05 ± 0.11
	Mean	9.82 ± 0.58*	9.17 ± 1.10*	8.97 ± 1.50*	9.89 ± 0.88*	9.25 ± 1.71*

ND: not detected. *Statistically significant difference with respect to group A ($P < 0.001$).

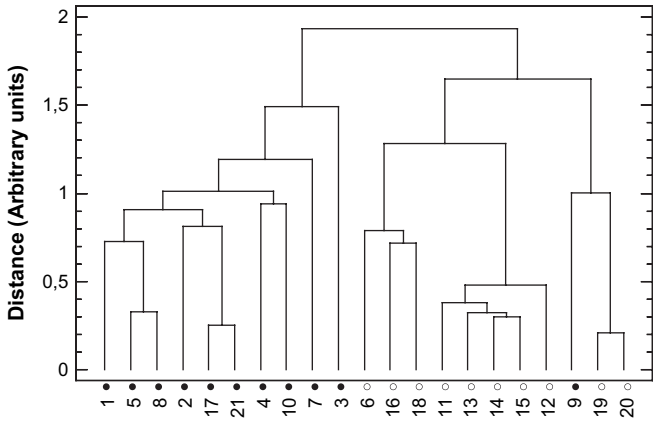

Fig. 1. Dendrogram obtained by hierarchical cluster analysis of bacterial counts achieved from meconium samples. (●): group A samples. (○): group B samples.

Identification of isolates from the different growth media revealed that enterococci were present in 17 (80%) of the 21 samples, with *E. fecalis* being the predominant species, since it was present in the 17 samples (Table 2). Staphylococci were the second bacterial group in quantitative terms, and they were detected in 11 (52%) of the samples; *Staphylococcus epidermidis*, which could be isolated from 10 samples, was the predominant staphylococcal species. *Escherichia coli* and *Enterobacter* spp. were detected in 6 and 5 samples, respectively, and formed the third predominant group (Table 2). The rest of the bacterial species identified in this study (*Streptococcus mitis*, *Streptococcus oralis*, *Bifidobacterium bifidum*, *Leuconostoc mesenteroides*, *Rothia mucilaginosa*, *Klebsiella* spp.) could only be isolated from one of the samples (Table 2). The number of different bacterial species detected in a single meconium sample varied between 5 and 1.

3.2. *Oral administration of a labelled* E. fecium *strain to pregnant mice led to its presence in meconium*

Following oral administration of *E. fecium* JLM3 to pregnant mice, MRS counts (day −1) in the samples of meconium obtained from mouse term fetuses ($n = 8$) were $2.6 \pm 0.8 \log_{10}$ CFU/ml. It is interesting to remark that bacteria were also isolated from meconium of group B animals, although at a lower level ($1.7 \pm 0.9 \log_{10}$ CFU/ml); however, the labelled strain could be PCR-detected in samples obtained only from group A mice (Fig. 2). A total of 20 colonies grown on MRS plates from group A samples were subcultured on MRS-Cm plates and 15 showed resistance to chloramphenicol. PCR results showed that, among the Cm-resistant colonies, 12 contained the genetic label specific to the transformed *E. fecium* JLM3 cells (Fig. 1). PCR sequencing revealed that the PCR product corresponded exactly to the genetic label. In parallel, 20 colonies isolated on MRS plates from group B samples were subcultured on MRS-Cm plates and only 5 showed resistance to chloramphenicol. As expected, none of these 5 Cm-resistant colonies harbored the genetic label (Fig. 2).

Table 2
Bacterial species identified in meconium samples

Genus	Species	Sample (infant)																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Staphylococcus	*S. epidermidis*	−	+	−	−	+	−	+	+	+	+	+	−	−	+	+	−	+	−	−	−	−
	S. caprae	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
	S. aureus	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Enterococcus	*E. fecalis*	+	+	−	+	+	+	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+
	E. fecium	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Streptococcus	*St. mitis*	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
	St. oralis	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Leuconostoc	*L. mesenteroides*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Bifidobacterium	*B. bifidum*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Rothia	*R. mucilaginosa*	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Enterobacter	*spp*	−	−	−	−	−	+	−	−	+	−	+	−	−	−	+	+	−	−	−	−	−
Escherichia	*E. coli*	−	−	+	−	−	−	−	−	+	−	−	+	+	−	+	−	−	+	−	−	−
Klebsiella	*spp*	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Parabacteroides	*P. distasonis*	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Bacteroides	*B. dorei*	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−

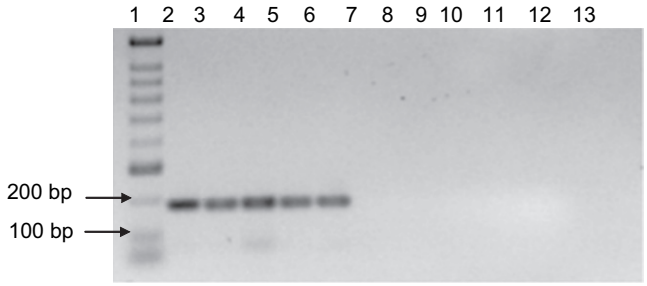

Fig. 2. PCR detection of *E. fecium* JLM3 among colonies isolated from meconium. Lane 1, 100 bp ladder (Bioline, London, UK); lane 2, PCR positive control (genomic DNA obtained from transgenic soy); lanes 3–7, colonies obtained from group A mice that grew on MRS-Cm agar plates; lanes 8–12, colonies obtained from group B mice; lane 13, PCR-negative control (*E. fecium* HA1).

4. Discussion

In this study, bacteria could be isolated from meconium obtained from healthy neonates, which suggests that, contrary to what has been hypothesized up to the present, this biological material may not be sterile. The number of bacterial species detected in the samples varied between 1 and 5, and *E. fecalis*, *S. epidermidis* and *E. coli* were the predominant species. Different studies have shown that high concentrations of coagulase-negative enterococci, staphylococci, and enterobacteria colonize the gut of vaginally and caesarean-delivered term and preterm neonates even from the first day of life [1,2,5,20,34]. Such bacterial groups may play important biological roles in the neonatal gut. *S. epidermidis* is the predominant bacterial species in breast milk of healthy women, and administration of milk to preterms is associated with a decrease in infection rates. Since *S. epidermidis* is one of the main causes of neonatal infection, the staphylococcal strains provided first by meconium and later by breast milk may successfully compete with potentially pathogenic strains found in the hospital environment. *E. coli* is also among the first colonizers of the infant gut [10] and mother-to-infant transmission of fecal isolates of *Enterobacteriaceae* has been described previously, although the route of transmission is unknown [36]. The species *E. coli* contains non-pathogenic and pathogenic bacteria, and commensal strains generally represent normal inhabitants of the human gut. In fact, *E. coli* strain Nissle 1917 (O6:K5:H1) is employed in an infant probiotic preparation and different studies have shown that its oral application to full-term and premature infants reduces the number and incidence of infections, stimulates specific humoral and cellular responses and induces non-specific natural immunity [6,19]. It is possible that other bacteria, such as lactobacilli or bifidobacteria, the isolation of which is difficult in standard culture media and/or conditions may be present in meconium of a higher number of neonates.

The presence of such bacteria in meconium may explain the origin of these first gut colonizers. Such bacteria could initiate gut colonization as an adaptation to fetal gut for life outside the mother, since gastrointestinal bacteria are considered the earliest and strongest stimulus for development of gut-associated lymphoid tissue [15]. Later, the role of meconium as a source of commensal bacteria to the infant gut is continued by breast milk, since it also contains such microorganisms [12,22,24,30]. In contrast, some studies have questioned the importance of transit through the vagina, which would have, if any, a minor role in bacterial colonization of the newborn [22,25,26].

In relation to mice assays, MRS counts (day −1) in meconium samples from group A fetuses were approximately 1 $\log_{10}$ CFU/ml higher than those obtained from group B fetuses. Such a difference may be due to the high enterococcal doses received by group A pregnant mice. The fact that oral administration of an enterococcal strain to pregnant mice led to their presence in meconium is not surprising, since it has been reported that bacteria from the digestive tract can reach amniotic fluid through the blood stream [16]. Another study which focused on the influence of the composition of oral microbiota of pregnant women in the pregnancy outcome showed that some bacteria, such as *Actinomyces naselundii*, were associated with lower birth weight and earlier delivery, while others, such as lactobacilli, were linked to a higher birth weight and delivery date. The results of such a study suggested that oral bacteria can enter the uterine environment through the bloodstream and may influence the delivery process [7]. This would explain why the bacterial species isolated from meconium are usually associated with the mouth and/or gut microbiota of the mother. Interestingly, the presence of streptococci and staphylococci in chorioamnion samples of healthy mothers who underwent caesarean section has been described previously [3]; in addition, bifidobacteria have been recently isolated from human meconium [17]. These bacteria could have a transient spread from the digestive tract to extra-digestive locations. It has been demonstrated that dendritic cells can penetrate the gut epithelium to directly take up bacteria from the gut lumen [29]. Once associated with dendritic cells, live bacteria can spread to other locations through the blood stream, since there is a circulation of cells of the immune system within the mucosal-associated lymphoid tissue (MALT) system. Antigen-stimulated cells move from the intestinal mucosa to colonize distant mucosal surfaces, such as those of the genitourinary tract [31]. In addition, during late pregnancy and lactation, there is selective colonization of the mammary gland by cells of the immune system (the so-called entero-mammary pathway) and this mechanism has been proposed to explain the presence of maternal gut bacteria in breast milk [23,28]. This process is responsible for the abundance of such cells in human milk. It is likely that the same mechanism may be responsible for the presence of gut bacteria in meconium.

Most of the bacterial species isolated in this study are included among opportunistic pathogens in neonates suffering from underlying conditions. This negative role probably reflects easy access of such bacteria to predisposed infants since, in fact, they may be part of their endogenous microbiota even at a fetal stage.

Overall, our results suggest that meconium of term infants is not a sterile environment and therefore gut colonization may start before birth. In addition, the bacterial composition of the maternal gut could affect (both qualitatively and quantitatively)

the bacterial content of infant meconium. Work is in progress to assess the bacterial diversity of this biological material using molecular microbiology techniques.

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