

Anaerobes in the microbiome

Establishment and development of the intestinal microbiota of preterm infants in a Lebanese tertiary hospital



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ABSTRACT

The establishment and development of the intestinal microbiota is known to be associated with profound short- and long-term effects on the health of full-term infants (FTI), but studies are just starting for preterm infants (PTI). The data also mostly come from western countries and little information is available for the Middle East. Here, we determined the composition and dynamics of the intestinal microbiota during the first month of life for PTI ($n = 66$) and FTI ($n = 17$) in Lebanon. Fecal samples were collected weekly and analyzed by quantitative PCR (q-PCR) and temporal temperature gradient gel electrophoresis (TTGE). We observed differences in the establishment and composition of the intestinal microbiota between the two groups. q-PCR showed that PTI were more highly colonized by *Staphylococcus* than FTI in the first three weeks of life; whereas FTI were more highly colonized by *Clostridium* clusters I and XI. At one month of life, PTI were mainly colonized by facultative anaerobes and a few strict anaerobes, such as *Clostridium* cluster I and *Bifidobacterium*. The type of feeding and antibiotic treatments significantly affected intestinal colonization. TTGE revealed low species diversity in both groups and high inter-individual variability in PTI. Our findings show that PTI had altered intestinal colonization with a higher occurrence of potential pathogens (*Enterobacter*, *Clostridium* sp) than FTI. This suggests the need for intervention strategies for PTI to modulate their intestinal microbiota and promote their health.

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1. Introduction

In the 21st century, a century after Ilya Metchnikoff introduced the theory that lactic acid bacteria are beneficial to human health, the interest in the intestinal microbiota is reaching its culmination [1]. The intestinal microbiota influences the normal development of the intestinal mucosal barrier and has short- and long-term effects on the health of children and adults [2,3]. It has been implicated in many disorders including obesity, asthma, diabetes, heart disease, metabolic syndrome, inflammatory bowel disease [2–4], and cancer [5].

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Intestinal colonization begins during delivery and continues thereafter by microorganisms recovered from the mother and the environment [6,7]. Recent data suggest earlier colonization *in utero* [8]. Full-term, vaginally delivered infants are initially colonized by facultative anaerobes favoring the later proliferation of strict anaerobes, including *Clostridium*, *Bacteroides*, and *Bifidobacterium* [6,7]. By 10 days of life, most healthy full-term neonates are colonized by a heterogeneous bacterial microbiota, with bifidobacterial species dominant in breast-fed infants and a more diversified population in formula-fed infants.

Several factors may affect this colonization process, including the: type of feeding, mode of delivery, antibiotic exposure, and gestational age (GA) [7]. The type of feeding was the first factor known to highly influence bacterial establishment, with breast-feeding enhancing gut colonization by bifidobacteria and

lactobacilli, in contrast to formula feeding [9]. Delivery through cesarean section results in lower biodiversity, a delay in colonization by beneficial bacteria (bifidobacteria and lactobacilli), and lower colonization by *Bacteroides* during the first weeks of life [10,11]. Antibiotics may also reduce the diversity and delay colonization of intestinal microbiota [12]. Gestational age (GA) plays a significant factor in the establishment of the gut microbiota, as severely premature infants are colonized differently than late-preterm infants, with a high inter-variability [13]. Data carried out in very low birth-weight neonates reported aberrant profiles with a major delay in colonization by anaerobes, such as *Bifidobacterium* and *Bacteroides*, whereas *Clostridium* predominated and staphylococci and *Enterobacteriaceae* other than *Escherichia coli*, such as *Enterobacter cloacae*, reached high levels [18]. Other factors can also influence the colonization process, such as environmental exposures, medical practices, and geographic differences [14].

Alteration of the intestinal microbiota in preterm infants (PTI) has been associated with the development of short-term diseases, such as sepsis or necrotizing enterocolitis (NEC) [13,15]. NEC is the most common gastrointestinal emergency in PTI and is a devastating condition with a high morbidity and mortality. An aberrant microbiota in PTI appears to be one of the key factors leading to NEC development [16–18], although its pathogenesis remains unclear and no specific microorganism has been shown to be consistently involved.

Early microbial intestinal dysbiosis in PTI has also been associated with numerous long-term health consequences including type-1 diabetes, Crohn disease, allergic diseases, such as asthma and atopic dermatitis, obesity, and autism spectrum disorders [7,15].

Most studies on microbial colonization have been performed in industrialized countries and on western children, although differences in microbial establishment have been observed among infants from different environments. Differences have been observed in children across Europe [14] or between those raised in rural and urban environments [19], likely due to differences in neonatal care. The aim of this study was to analyze and quantify the establishment and development of the intestinal microbiota in Lebanese PTI using culture-independent techniques (i.e. real time PCR (qPCR) and TTGE), in the absence of relevant data on the country and Middle East region. A group of full-term infants (FTI) was included as there is no literature on bacterial establishment in Lebanese FTI.

2. Materials and methods

2.1. Preterm and full-term infants

PTI were recruited among patients admitted to the neonatal intensive care unit (NICU) at Hôtel-Dieu de France Hospital (Beirut, Lebanon) from January 2013 to December 2014. Enrolled PTI were born at a gestational GA of <37 weeks and had no congenital malformations (e.g. gastrochisis, atresias) and/or metabolic diseases. NEC cases were also excluded from this study. Signed and written consent was obtained from parents before inclusion of PTI to this protocol, approved by the ethics committee of Saint Joseph University of Lebanon (CEHDF 426). Clinical data for all PTI were registered on a special form which included: gender, GA, delivery mode, birth weight, length, head circumference, APGAR score, *antepartum* and *intrapartum* antibiotics and steroids, maternal infections and diseases, neonatal infections, type of feeding, days of life at the start of enteral feeding, antibiotic courses <72 h, intubation, transfusions, and parenteral feedings.

FTI born at a GA >37 weeks and with no congenital malformations or metabolic diseases participated in this study as a comparative group. Clinical data recorded included gender, GA, delivery mode, birth weight, type of feeding, and days of life at the start of enteral feeding.

2.2. Fecal sampling

Fecal samples were collected weekly for PTI during their stay at the NICU. Diapers were stocked at 4°C and collected by the laboratory within 4 h. After homogenization with a sterile loop, approximately 1 g of each stool was placed in a sterile cryogenic tube for subsequent molecular analysis. All samples were immediately frozen at -80°C until analysis. For FTI, stool samples were collected at the first and between the second and third weeks of life and then treated as the PTI samples.

2.3. Microbiota analysis

2.3.1. DNA extraction from fecal samples

Total DNA was extracted from fecal samples using the bead-beating method previously described and adapted from Magne et al. [20]. Approximately 125 mg of each sample was suspended in 125 µL 4 M guanidinium isothiocyanate – 0.1 M Tris (pH 7.5) and 500 µL 5% N-lauryl sarcosine - 0.1 M phosphate buffer (pH 8). After 15 min of incubation at 70°C, 750 µL 0.1 mm glass beads (Biospec, Bartlesville, USA) were added and the samples homogenized in a mini-bead beater 16 (Biospec) twice for 2 min. After centrifugation, the pellet was washed three times with TENP [50 mM Tris-Cl (pH 8), 20 mM EDTA (pH 8), 100 mM NaCl, and 1% of polyvinylpolypyrrolidone] to ensure removal of all polyphenol to avoid inhibiting the subsequent qPCR reactions. All obtained supernatants were pooled and extracted with an equal volume of equilibrated phenol (Sigma, Saint Louis, USA) and purified twice with chloroform – isoamyl alcohol 24:1 (Sigma). Nucleic acids were precipitated by the addition of one volume of 100% isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.4) and incubated at -20°C for 30 min. The precipitated DNA was centrifuged for 10 min at 20,000× g at 4°C. DNA pellets were washed with 70% ethanol, allowed to air dry, and finally re-suspended in DNA-free water. The extracted DNA was frozen at -0°C until analysis.

2.3.2. Quantitative analysis of intestinal microbiota by qPCR

Quantification of the major bacterial genera or groups was carried out by qPCR using the primers shown in the supplementary files (Table S1). All reactions were performed using Hard-Shell® Low profiles 96-plates sealed with Microseal® B adhesive seals (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR® Green Supermix (Bio-Rad) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The total bacterial copy number was determined using primers Eub339 and Eub 788. The reaction mixture contained 2 µL template fecal DNA (diluted 1:10 to 1:100 in pure water), 0.16 µM–0.32 µM of each primer, and 2X SYBR® Green supermix in a reaction volume of 25 µL PCR.

Thermal cycling consisted of an initial cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 1 min at the appropriate primer-pair Tm and 2 min at 68 °C. The fluorescent product was detected at the last step of each cycle. Standard curves were obtained from serial dilutions of a known concentration of plasmid DNA containing a 16S rRNA gene insert from each species or group. The plasmid concentration was determined by spectrophotometry and the quantity of target gene in a sample was determined using these standard curves. Samples were analyzed in duplicate in at least two independent PCR runs. The bacterial concentration in each sample was calculated by comparing the Ct values obtained from standard curves. Results were converted to log₁₀ CFU/g of feces after taking into account the number of 16S rRNA operons (Ribosome Database Project) in each genus or family. The detection limit depended on the bacterial groups and ranged between 10⁴ and 10⁶ CFU/g.

2.3.3. Temperature temporal gel electrophoresis analysis

Primers Eub339F and Eub788R were used to amplify the V3 to V4 region of the bacterial 16S rRNA genes, as previously described [20]. Samples were amplified in an eppendorf master-cycler (Eppendorf AG, New-York, USA) using the following program: 94°C for 10 min; 30 cycles of 96°C for 15 s, 55°C for 1 min, 72°C for 4 min; and finally 72°C for 15 min. A Dcode™ universal mutation detection system (Bio-rad) was used for sequence specific separation of the PCR products. Electrophoresis was performed as previously described [20]. The ladder consisted of the following organisms in order of migration: *Bacteroides* sp., *Prevotella* sp., *Enterococcus faecium*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Bifidobacterium longum*.

After electrophoresis, the gels were analyzed using Diversity Database Software 2.1 (Bio-rad). The number of bands was used as an indicator of biodiversity. Bands of interest were excised from TTGE gels, washed, and incubated overnight at 4°C in sterile water to allow for DNA dispersion. Re-amplification by PCR was performed on an aliquot using the same primers and conditions as above (supplementary files S1). Amplicons were then sequenced and compared with those present in the National Center for Biotechnology Information.

2.4. Statistical analysis

The results were analyzed using SPSS software 21.0 (SPSS Inc, Chicago, IL). The normality of distribution of the continuous variables was evaluated using the Kolmogorov-Smirnov test. Because variables were not normally distributed, non-parametric tests were used. Mann-Whitney tests were used to compare bacterial levels between the PTI and FTI groups. Kruskal-Wallis tests were used to compare bacterial levels between the different time points in PTI and FTI. The association between bacterial colonization and perinatal factors (GA, delivery mode, type of feeding, and antibiotic treatment) were studied using the Chi square test or Fisher's exact test, as appropriate. All associates were considered to be significant when p values were less than 0.05.

For microbiota, an arbitrary value of 2 log₁₀ colony-forming units per gram of fecal content (log₁₀ CFU) was used when species or targeted taxonomic groups were not detected.

3. Results

3.1. Characteristics of the infants

Of the 81 eligible consecutive PTI studied between October 2012 and November 2014, 15 were excluded; nine because of NEC, four due to metabolic and congenital diseases, and two due to early death. The remaining 66 PTI included eight pairs of twins and one set of triplets. In parallel, 17 healthy FTI born at a GA of 37 or more weeks were included in this study.

Characteristics of the PTI and FTI are reported in Table 1. Breast-fed infants were either exclusively fed breast-milk or had a mixed diet, whereas formula-fed infants were exclusively fed infant formula. PTI were mainly born by cesarean delivery, breast-fed, and exposed to antibiotics. FTI were also mainly born by cesarean delivery, breast-fed, but less exposed to antibiotics (23.5% vs. 75.7% for PTI, p = 0.0005). Forty-six PTI were exposed to antibiotics with 28 receiving short-length treatment of less than 48 h with ampicillin and cefotaxime, and 18 receiving long-term treatment of three days or more (median seven days, range 3–19 days). Thirteen PTI were treated with antibiotics for three days to two weeks with various combinations of amikacin (9 PTI), vancomycin (6 PTI), imipenem (6 PTI), ceftazidime (6 PTI), clarithromycin (4 PTI) and/or gentamicin (1 PTI). Five PTI were given

a prolonged antibiotic treatment beyond two weeks, with a combination of the following antibiotics: ceftazidime, amikacin, vancomycin, imipenem and clarithromycin.

We analyzed a total of 127 samples, for an average of 2.6 samples per PTI. We defined the time window of the samples as follows: three to nine days of life (dol) as week 1; 10 to 16 dol as week 2; 17 to 23 dol as week 3; 24 to 30 dol as week 4. We sampled 48 PTI at week 1; 52 between weeks 2 & 3, and 27 at week 4. We collected two samples for FTI, one at week 1 and another at weeks 2 or 3. All fecal samples were studied using qPCR and TTGE.

3.2. Establishment of the intestinal microbiota

The analyses of bacterial colonization using qPCR, in both PTI and FTI, are summarized in Fig. 1.

The most frequent and abundant genus of the aerobic microbiota found in PTI after one week of life (Fig. 1A) was *Staphylococcus* (median 6.8 log₁₀ CFU/g of feces; 83.3% were colonized). The median levels of the genera *Enterococcus* and *Enterobacteriaceae* were lower (5.6 log₁₀ and 5.0 log₁₀, respectively) with a high percentage of non-colonized infants (33.3% and 43.8%, respectively). Colonization by lactobacilli was low (35.4%) with a low median (2 log₁₀). The most frequent genus of the anaerobic microbiota in PTI after one week of life was *Bifidobacterium* with 64.5% infants colonized at a median of 5.6 log₁₀. By contrast, there was a low level of colonization by the *Bacteroides/Prevotella* group, *Clostridium* cluster I, the *Clostridium leptum* group, the *Clostridium coccoides* group, and *Clostridium* cluster XI. Among these groups, the highest percentage of colonized infants was 18.8% for *C. leptum*. However, colonization by anaerobes reached high levels for two of these infants (up to 10 log₁₀).

For the PTI cohort, the levels of *Staphylococcus* and *Enterococcus* varied little during the second to fourth weeks of life (Fig. 1B and C). In contrast, colonization by *Enterobacteriaceae* increased (median 8.8 log₁₀ at weeks 2 and 3, and 9.1 log₁₀ at week 4). Lactobacilli remained at low levels (median 3.4 log₁₀). For anaerobic microbiota, there was a slight change in colonization by *Bifidobacterium* (median 6.3 log₁₀ at week 4) and nearly no variation for the other anaerobic taxonomic groups, except *Clostridium* cluster I, for which the levels increased from 16.1% at week 1–30.8% at weeks 2 and 3, and 41% at week 4.

For the FTI cohort (Fig. 1D and E), the percentage of colonization by *Staphylococcus* was low in the first week (23.5% colonized) but increased at weeks 2 and 3 (45% colonized infants) whereas the levels of colonization remained low (median 2.6 log₁₀ at week 1, 3.6 log₁₀ at week 2). There was substantial colonization by *Enterococcus* and *Enterobacteriaceae* in the first week (59% and 82.3%, respectively) which increased during weeks 2 and 3 (81.8%–100%, respectively). Colonization levels by lactobacilli were low in the first week (median 2 log₁₀) and increased in the second and third weeks (median 5.9 log₁₀), despite a slight increase in colonization percentage (less than 18% colonized at weeks 2–3). Anaerobic colonization for FTI was low during the first week and increased only slightly during the second and third weeks. Only *Clostridium* cluster I increased largely both in the level and percentage of colonization (median 5 log₁₀ and 54.5% colonized). The percentages of colonization for PTI and FTI are shown in supplementary file S2.

3.3. Impact of delivery mode, gestational age, type of feeding, and antibiotic treatment on the gut bacterial establishment in PTI

3.3.1. Delivery mode

The mode of delivery only modestly influenced the gut composition of preterm newborns (supplementary file S3). PTI

Table 1

Clinical characteristics of infants included in this study.

Characteristics	Preterm infants n = 66	Full-term infants n = 17
Mean gestational age, weeks (STD)	32.8 (± 2.1)	38.5 (± 0.9)
Mean birth weight, g (STD)	1913 (± 564)	3221 (± 540)
Number of stools/infant (STD)	2.6 (± 1.2)	2
Delivery: cesarean/vaginal ^a ($p = 0.038$)	57/9	11/6
Male/Female	38/28	7/10
Number of twins/triplets	14/3	0/0
Feeding: Breast milk/formula ^b	52/14	14/3
Antibiotic treatment: yes/no ^a ($p = 0.0005$)	46/20	4/13
Length of antibiotic treatment		
Less than 48 h/More than 7 days ^b	28/18	4/0

^a Significant difference between preterm infants and full-term infants.^b No significant difference observed between the two groups.

delivered by cesarean section were more highly colonized by *Staphylococcus* than vaginally delivered PTI (87.1% vs. 62.5% at week 1) but less colonized by *Enterococcus* (65% vs. 75% at week 1). In addition, vaginally delivered PTI were also more highly colonized, but not significantly, by *Lactobacillus*, *Bifidobacterium*, and *C. leptum* groups than those delivered by cesarean. Despite slightly higher colonization of vaginally delivered PTI by the *Bacteroides/Prevotella* group at week 1, the difference was no longer observable by weeks 2 and 3.

3.3.2. Gestational age

GA had a small impact on the intestinal microbiota of PTI (data not shown). We observed no significant differences in colonization between PTI born before a GA of 33 weeks (extremely preterm and very preterm) and PTI born between 33 and 37 weeks (moderate to late preterm).

3.3.3. Type of feeding

The type of feeding affected the composition of the intestinal microbiota (Fig. 2). We observed no significant differences between breast-fed and formula-fed infants after one week of life, but there was a significant difference after two and three weeks of life with greater colonization by the *C. leptum* group in breast-fed infants ($p = 0.05$). Breast-feeding also favored colonization by *Staphylococcus*, *Enterococcus*, *Lactobacillus*, and *Bifidobacterium* with an increase in colonization rates from the second week of life through the fourth week (Fig. 2).

3.3.4. Antibiotic treatment

PTI were divided into three groups depending on the use and length of antibiotic treatment starting from birth: PTI without perinatal antibiotics, PTI with short antibiotic treatment (less than two days), PTI with longer antibiotic treatment (more than three days) (Fig. 3).

3.3.4.1. Week 1. Colonization by aerobes was not affected (*Staphylococcus*, *Enterococcus*, and *Enterobacteriaceae*) or slightly reduced (*Lactobacillus*) by antibiotics both in the short-term and long-term treated groups at week 1. Antibiotic treatment had a non-significant impact on anaerobe colonization, reducing colonization by *Bifidobacterium* from 86.7% in the non-treated group to 50% in the short-term treated group and 60% in the long-term treated group. Colonization by *Bacteroides/Prevotella* group was also lower in the long-term treated group (6.7%) relative to the non-treated group (20%), whereas it was only slightly lower (16.7%) in the short-term treated group. There was also a slight decrease in colonization by *Clostridium* cluster I from 20% to 16.7% in the short-term treated group and 13.3% in the long-term treated group. Only colonization by *C. leptum* was significantly affected with the long-

term treated group being less colonized than the non-treated group ($p = 0.044$).

3.3.4.2. Weeks 2 and 3. We observed no significant differences between treated and non-treated PTI at weeks 2 and 3 concerning colonization by the *C. leptum* group and other genera and taxonomic groups. PTI treated for a short period regained a similar level of colonization by *Bifidobacterium* as non-treated PTI (83.3% vs. 87.5%), whereas it was slightly lower for long-term treated infants (75%). Colonization by *Clostridium* cluster I was higher in PTI following long course treatment (41.6%) than in non-treated PTI (12.5%) or PTI treated for a short period (33.3%).

3.4. Analysis of the bacterial establishment by TTGE

TTGE profiles revealed low species diversity in both PTI and FTI groups. The fecal microbiota in PTI and FTI were very simple at week 1 with between one and 13 major bands (mean value: 4.4) in individual PTI and between 1 and 9 (mean value: 4.8) in FTI. The fecal microbiota were slightly more diversified at weeks 2 and 3 with between two and 13 bands (mean value: 6) in PTI and two and 10 bands (mean value: 6.8) in FTI.

Fig. 4 shows the TTGE profiles of two PTI labeled C45 and C66. Samples collected at different days of life represent the colonization process and the establishment of intestinal microbiota in these two infants. Infant C45 showed the typical colonization process of PTI in our study: initial colonization by *Staphylococcus* and *Bifidobacterium* in the first week, an increase in diversity by weeks 2 and 3, with the presence of dominant bands of members of the *Enterobacteriaceae* family (*Citrobacter freundii*), *Enterococcus*, and *Bifidobacterium*. Infant C66 showed initial colonization by *E. faecium* and *S. epidermidis* and a typical delay in the colonization by *Bifidobacterium* during the first weeks of life due to broad-spectrum antibiotic treatment (imipenem) from day 1 to day 10 of life.

Cluster analysis of TTGE profiles of PTI and FTI at week 1 and weeks 2–3 (referred to as week 2.5 on the cluster analysis shown in Fig. 5) was performed using the UPGMA method based on the Dice similarity coefficient using Diversity Database software (Bio-Rad). Comparison of stool samples of PTI reveal no particular clusterization, showing that the samples were diverse at week 1 and weeks 2–3. However, microbiota of FTI at week 1 and weeks 2–3 formed mainly two clusters (Fig. 5), depending on the sampling time, with very few exceptions. Each cluster presents many common species, the first one (cluster 1) consists mainly of FTI infants at weeks 2–3 and the second (cluster 2) mainly of FTI infants at week 1. PTI with profiles found near cluster 2 were mostly born at a GA of more than 34 weeks and were vaginally delivered. Likewise, cluster analysis of week 2–3 samples of PTI and FTI show that the microbiota profiles of some FTI were very similar to those of PTI. Close examination of FTI TTGE profiles revealed that they are less colonized by

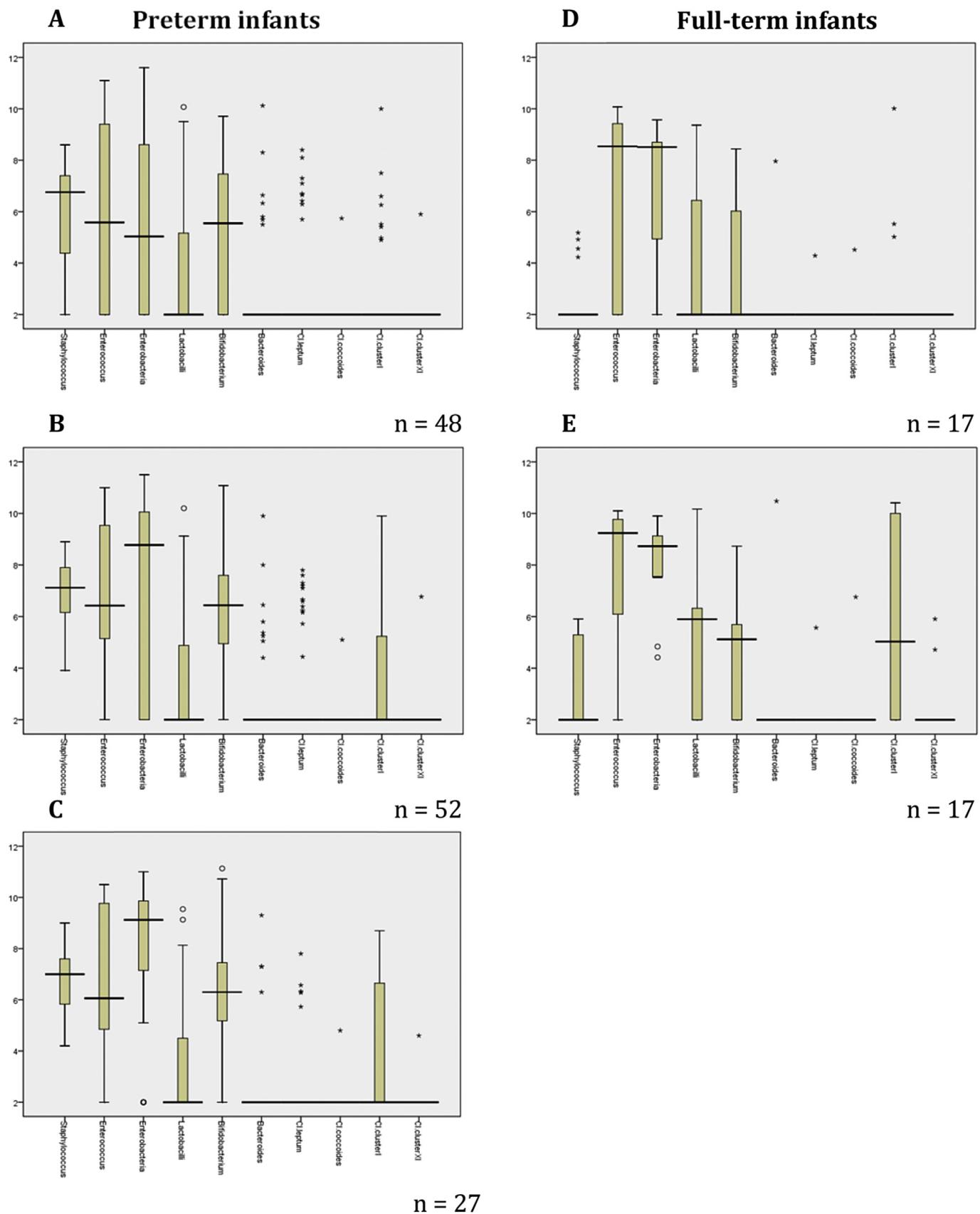


Fig. 1. Bacterial colonization in preterm infants. **1A** at week 1 ($n = 48$), **1B** at weeks 2–3 ($n = 52$), **1C** at week 4 ($n = 27$), and in full-term infants **1D** at week 1 ($n = 17$) and **1E** at weeks 2–3 ($n = 17$).

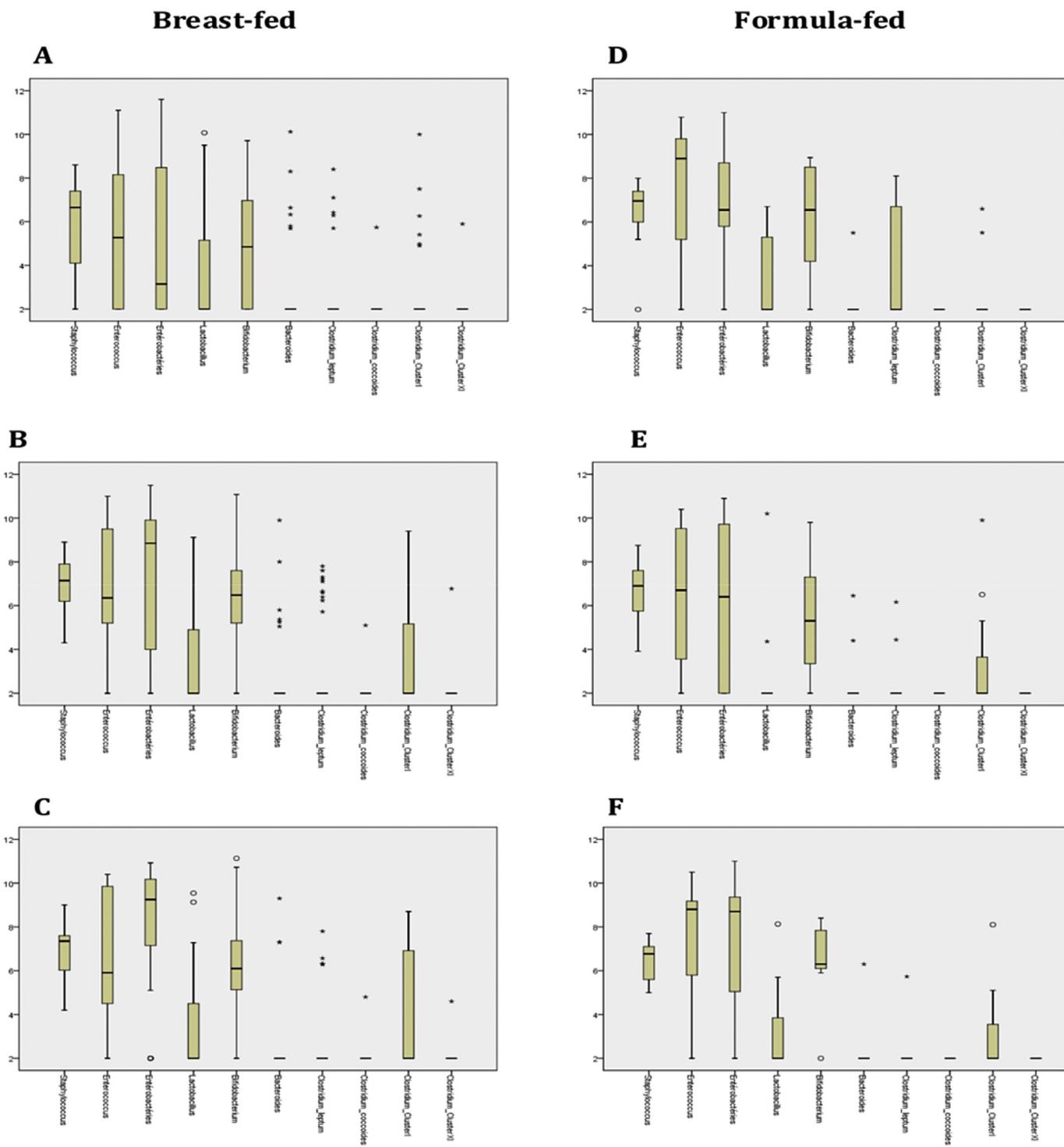


Fig. 2. Bacterial colonization in breast-fed infants: **2A** at week 1 ($n = 38$), **2B** at weeks 2–3 ($n = 41$), **2C** at week 4 ($n = 20$); and in formula-fed infants: **2D** at week 1 ($n = 10$), **2E** at weeks 2–3 ($n = 11$), **2F** at week 4 ($n = 9$).

Bifidobacterium spp than PTI as revealed by sequenced bands on the TTGE profiles. In addition, PTI twins C63–C64 revealed almost identical TTGE profiles (at ~ 0.80 cut-off point) whereas all other twins and triplets C58 – C59 – C60 had different TTGE profiles (with less than 0.50 cut-off point), suggesting high inter-individual variability.

Cluster analysis of all PTI samples reveals that samples of more than 12 infants from weeks 2–3 and week 4 tended to cluster

together, suggesting that the intestinal microbiota does not change between these two sampling windows (data not shown).

4. Discussion

Several studies have been conducted throughout the world on the establishment and development of the intestinal microbiota of FTI living in industrialized countries [6,7], but there have been

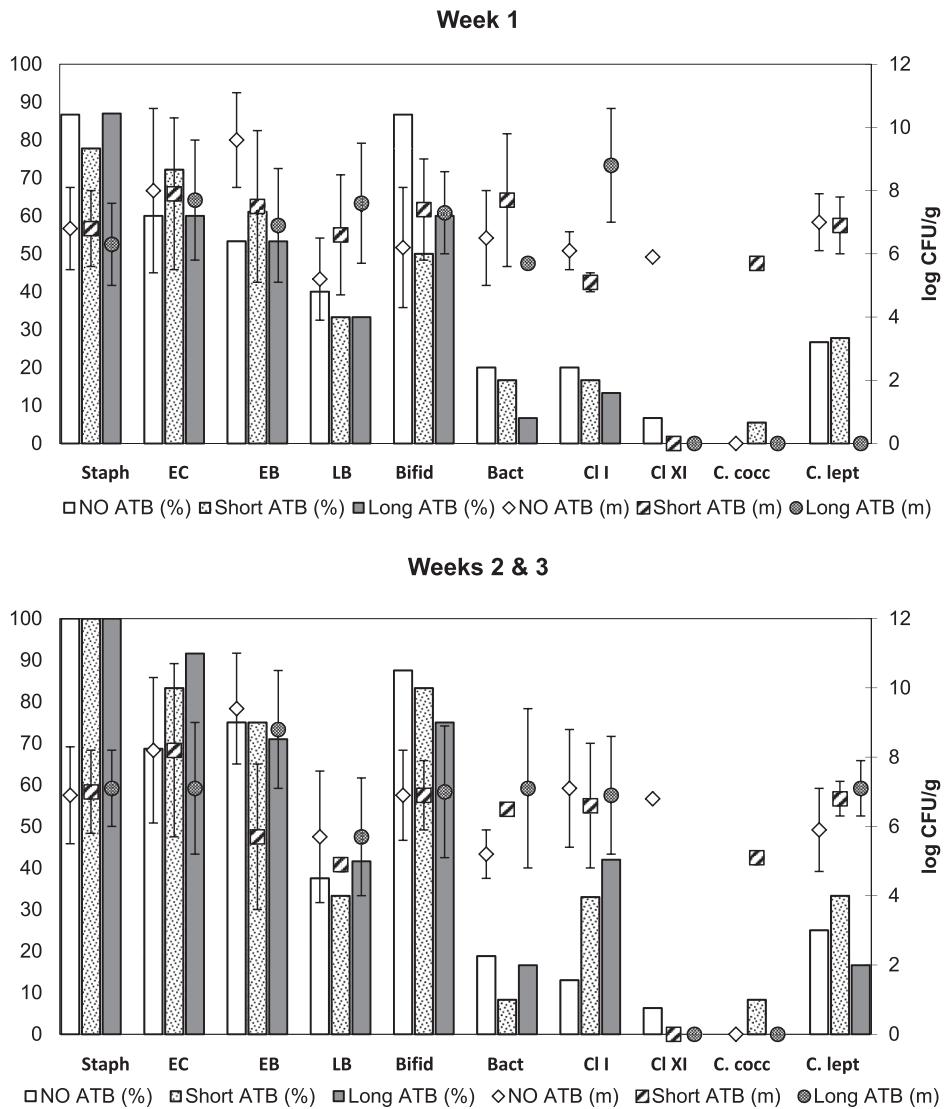


Fig. 3. Histograms showing intestinal microbiota analyses for PTI receiving no antibiotic treatment (white bars), a short course of antibiotics (white with dots), or a long course of antibiotics (grey bars) at week 1 and weeks 2 & 3. Bars show the percentage of colonization and geometric forms (rhombus, squares and circles) showing the level of colonization by each genus or group in colonized infants. Staph = *Staphylococcus*; EC = *Enterococcus*; EB = *Enterobacteriaceae*; LB = *Lactobacillus*; Bifid = *Bifidobacterium*; Bact = *Bacteroides*; CI I = *Clostridium* cluster I; CI XI = *Clostridium* cluster XI; C. cocc = *Clostridium coccoides* group; C. lept = *Clostridium leptum* group.

fewer surveys of PTI and no literature is available on the Lebanese population. To our knowledge, this is the first study on the intestinal microbiota of Lebanese PTI and the first analysis of the effect of perinatal factors on intestinal colonization in Lebanon and the middle-east region, where medical practices may vary from those of western countries.

Here, our results show an altered pattern of intestinal bacterial colonization in PTI relative to that of FTI, with higher levels of facultative anaerobes and delayed colonization by anaerobes during the first weeks of life (except for *Bifidobacterium* spp.). This bacterial establishment pattern is in agreement with studies from other countries [20–25].

After one week of life, the gut microbiota in PTI was dominated by staphylococci, whereas colonization by enterococci and *Enterobacteriaceae* was delayed and colonization by anaerobes was low except for *Bifidobacterium* spp. By the first month of life, PTI were mostly colonized by facultative anaerobes, predominantly

Enterobacteriaceae as shown by others [22,26]. This dysbiosis may be due to the fact that PTI were rapidly separated from their mothers after birth, placed in NICU incubators, and subjected to invasive procedures that may favor nosocomial infections, resulting in greater exposure to environmental bacteria than to bacteria originating from the mother.

We observed in PTI a high frequency of colonization by *Bifidobacterium* (80.7%) by the third week of life, in contrast to previous studies [20–22,27]. However, these studies included infants born at a low or very low GA. The median GA in our population (32.8 weeks) corresponds to the threshold observed in Butel's study for colonization by *Bifidobacterium* [28]. In our work, only six of the 66 preterm infants were born below 30 weeks of gestation, of whom only 50% were colonized by *Bifidobacterium* by one month of life. This high frequency of *Bifidobacterium* colonization may also be related to the high frequency of breastfeeding (78.8% in PTI). Breast milk in our hospital is not pasteurized and may harbor viable

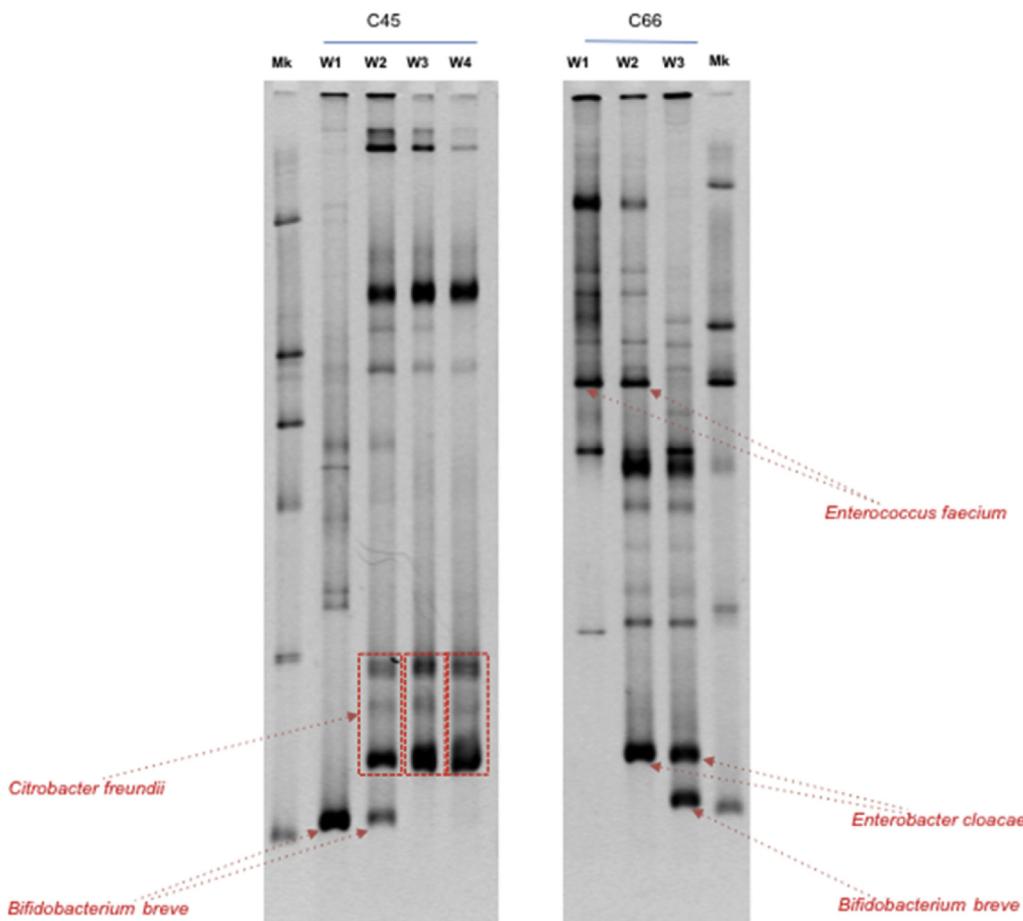


Fig. 4. TTGE profiles of two PTI (cases 45 and 66) showing the colonization process at different weeks of life. The extreme left and extreme right lanes are markers of TTGE profiles of the following species (from top to bottom): *Bacteroides* sp., *Prevotella* sp., *Enterococcus faecium*, *Staphylococcus epidermidis*, *Escherichia coli* and *Bifidobacterium longum*.

bacteria of maternal origin [29,30]. We recently analyzed 20 breast-milk samples, of which all were colonized by staphylococci and 30% by *Bifidobacterium* (Itani et al., unpublished data). These bacteria may participate in bacterial establishment in neonates, as shown in several studies [31]. Furthermore, all studied PTI received prebiotic oligosaccharide supplements (galactooligosaccharide) known to increase *Bifidobacterium* counts [32].

Colonization by *Bacteroides* was also higher than that reported in the literature, but to a lesser extent than that by *Bifidobacterium*, likely due to the higher median GA of our PTI. Colonization by *Clostridium* cluster I, as observed in Ferraris's study, increased throughout the follow-up period, likely due to gut colonization from the environment by this anaerobic spore-forming genus [33].

One of the limitations of our study is the fact that all infants came from the same center and were not followed after discharge from the NICU. A recent study showed differences in intestinal microbiota between two centers and over-time [34]. Another limitation corresponds to the low number of infants vaginally delivered, as PTI were mostly born by cesarean delivery. The influence of cesarean delivery on PTI has been little, as previously reported by several studies where no significant influence of this factor on microbiota composition and establishment was found [22,26,28,35–37]. We observed a non-significant trend toward delayed colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides/Prevotella*. At one month of life, all cesarean delivered PTI were more highly colonized by *Clostridium* cluster I, a genus involved in NEC. A recent study specifically designed to analyze the

influence of the mode of birth reported that vaginally delivered PTI had a higher frequency of *Bacteroides* colonization than those delivered by cesarean [38]. Faster development of the gut microbiota in cesarean delivered PTI was found in one study, but this may be due to confounding factors [35].

Other factors such as the type of feeding and antibiotic treatment had a larger effect on intestinal colonization. At three weeks of life, breastfed PTI were more highly colonized by the *C. leptum* group, which colonizes the gut of PTI and adults. One representative of this group, *Faecalibacterium prausnitzii*, is reported to play an important anti-inflammatory role in protecting against bowel disease and is deficient in patients suffering from Crohn's disease [39]. This may explain why formula-fed PTI are more prone to develop intestinal inflammatory disease and infection. Despite the fact that breast-fed PTI were more highly colonized by *Bifidobacterium* and *Lactobacillus* during the first week of life, the difference was not significant later. This may also be due to the small number of formula-fed PTI who were mostly breastfed or fed a mixed diet. Antibiotic administration during the first hours of life reduced the level of intestinal colonization by the *Clostridium leptum* group and *Bifidobacterium*, likely due to their susceptibility to both amoxicillin and cefotaxime [40], the antibiotics most commonly administrated to PTI. In contrast, the levels of *Clostridium* cluster I colonization were higher. In a study including very low birth weight infants, Arboleya et al. reported that perinatal antibiotics, including *intrapartum* prophylaxis, strongly affected the gut microbiota with an increase in *Enterobacteriaceae* [41]. These antibiotic-mediated

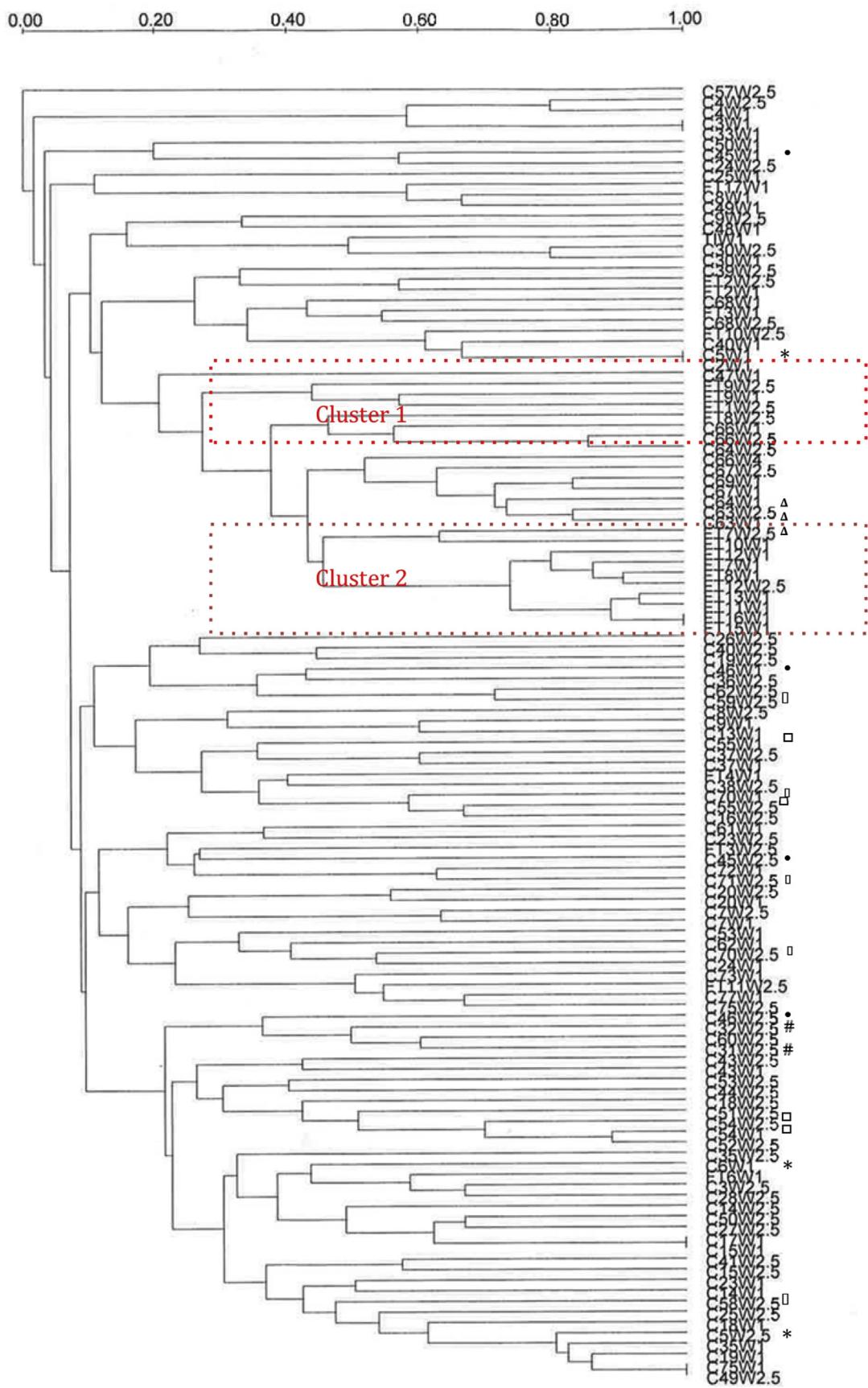


Fig. 5. Dendrogram of TTGE profiles of PTI and FTI at week 1 and weeks 2–3. Samples from twins were marked by symbols next to the abbreviation: C5–C6 represented by *, C31–C32 represented by #, C45–C46 represented by ●, C54–C55 represented by □, C63–C64 represented by △, C70–C71 represented by +, triplets C58–C59–C60 represented by ★.

alterations have been reported to increase the risk of late-onset sepsis and NEC [13].

TTGE analysis in our study revealed a high degree of inter-individual variability as previously described by others [20,35,42]. Samples from FTI tended to cluster into two main groups, which show a different profile of colonization despite the presence of very common species, including *Klebsiella pneumoniae* and *E. coli*, whereas PTI were more highly colonized by *E. cloacae*. Vaginally delivered PTI born at a GA of more than 34 weeks clustered close to FTI. This may be due to several factors which may act synergistically to favor bacterial colonization of the gut: (i) a higher degree of intestinal maturation, (ii) a closer relationship with the mother and her microbiota, (iii) fewer perinatal factors that can reduce bacterial colonization (vaginal delivery rather than cesarean delivery, less antibiotic courses ...). This more favorable bacterial equilibrium can decrease the risk of disease.

In summary, this study identified qualitative and quantitative differences in the establishment and development of the intestinal microbiota in PTI relative to that of FTI. It showed a progressive development of the diversity of the intestinal microbiota in the first month of life. Most PTI had staphylococci in their stools at week 1 and *Enterobacteriaceae* beyond week 2. Many PTI were colonized by *Bifidobacterium* spp. probably due to daily prebiotic supplementation of Lebanese PTI and a higher GA of PTI than those in other studies that focused on extremely preterm infants. Moreover, this study highlights the effects of antibiotic administration on the establishment of the intestinal microbiota, especially on the *C. leptum* group. Our data support the need for a multi-site study that includes a larger number of vaginally-delivered and formula-fed infants to better understand the association between intestinal microbes and intestinal diseases of preterm infants, such as NEC.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anaerobe.2016.11.001>.

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