Anaerobe 43 (2017) 4–14

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Anaerobes in the microbiome

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

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ARTICLE INFO

Article history:
Received 31 July 2016
Received in revised form
2 November 2016
Accepted 6 November 2016
Available online 8 November 2016

Handling Editor: Emma Allen-Vercoe

Keywords:
Intestinal microbiota
Preterm infants
Lebanese infants
Bifidobacterium
Real time PCR
TTGE

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.

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].

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

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http://dx.doi.org/10.1016/j.anaerobe.2016.11.001

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 TM 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 $\log_{10}$ 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 10^4 and 10^6 CFU/g.