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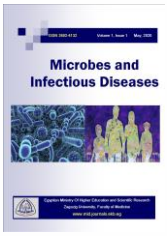

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Original article

Characteristics of intestinal microbiome in very low birth weight preterm neonatal intensive care unit patients

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ABSTRACT

Background: Trillions of microbial cells in the human body are thought to be vital to human survival. Microbial cell populations are at maximum density within the intestinal compartment, referred to as the gut microbiota. **Aim:** To describe the neonatal microbiota in hospitalized preterm patients and to detect the relation between necrotizing enterocolitis (NEC), sepsis and preterm gut microbiota. **Patients and methods**: This was a prospective study on preterm very low birth weight neonates. Rectal swabs were collected at postnatal weeks one, two and three. The Illumina MiSeq system was employed to sequence the 16S rRNA gene. Results were compared to that of routine blood culture and were correlated with the use of antimicrobials. **Results:** Rectal swabs of the first week had predominantly no organisms (89.7%). *Klebsiella* genus was the most predominant bacteria in all rectal swabs (5.8%, 22.0%, 22.0% of the 1st, 2nd and in 3rd swabs results respectively). In spite of the non-significant results on the level of number of operational taxonomic unit (OTU) and chao indices, the association of microbiota change with NEC and sepsis could not be excluded. The significant organisms at phylum level in NEC and sepsis groups was Proteobacteria. There was no significant correlation between change of antibiotic therapy and results of rectal swabs at the 1st, 2nd and 3rd weeks. **Conclusion:** The gut of preterm neonates born by CS who received antibiotics was mostly sterile at first week. Subsequent colonization by pathogenic organisms such as *Klebsiella* and *Enterobacter* occurred after the first week.

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Introduction

The establishment of gut microbiota commences with birth, and possibly during fetal development. The bacteria from the mother's vaginal, intestinal, and skin flora are critical sources of initial bacterial exposure. The gut's microbial population experiences significant growth and changes primarily in the initial years of life, with notable alterations in gut microbiota composition continuing up to the age of 2 to 3 years [1]. However, the diversity akin to that of an adult's gut microbiota may not be achieved until the age of 5 [2].

In the infancy phase, the microbiota showcases limited diversity, predominantly occupied by the Actinobacteria and Proteobacteria phyla [3]. As the infant grows, especially within the first year, there's an increase in microbial diversity,

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leading to the establishment of a more adult-like microbiota profile, distinguished by unique temporal patterns for each infant [4]. By the age of approximately 2.5 years, the infant's microbiota composition, diversity, and functional attributes start to mirror those found in adults. While adult gut microbiota composition is comparatively stable, it remains susceptible to modifications triggered by various life events [5,6].

Numerous critical and interlinked factors influence the evolution of the human gastrointestinal (GI) microbial composition during this transition period. These factors encompass age [7], diet [8], genetic makeup of the host [9], antibiotic usage [10], birth method, feeding type, and the infant's birth setting [11].

Perinatal and nosocomial infections represent a significant risk for newborns, particularly those in neonatal intensive care units (NICUs), affecting not just survival rates but also the neurodevelopmental trajectories of these infants [12,13]. Therefore, preventing and managing microbial infections is crucial in neonatal care. Preterm infants are at a heightened risk of such infections due to their exposure to invasive medical procedures, including the use of central catheters for nutrition and mechanical ventilation for breathing support [14]. Moreover, it has been hypothesized that late onset sepsis (LOS) in the neonates is preceded by alteration in the intestinal microbiome. Earlier gestational age associated with immature barriers such as the intestinal mucosa are additional factors for the sepsis risk. Thus, the colonizing bacteria are known to modulate the immune response and the metabolic functions [15].

A disruption in the normal development and balance of gut microbiota, known as gut dysbiosis, is linked to the onset of various intestinal conditions, such as necrotizing enterocolitis (NEC). This condition is marked by a scarcity of beneficial commensal microbes, reduced bacterial diversity, and the predominance of pathogenic bacteria, which provoke an intense inflammatory reaction [16].

Aim of the work

To describe the neonatal microbiota and its development in hospitalized preterm patients and to detect the relation between necrotizing enterocolitis (NEC), sepsis and preterm gut microbiota.

Patients and methods

This prospective study was conducted in the NICU of Cairo University Children's Hospital, spanning 20 months from November 2020 to June 2022. Laboratory analyses were performed in the Clinical Pathology Sequencing Lab.

Informative consent was taken from the legal guardian of preterm infants and the study was approved by the ethical committee of Faculty of Medicine, Cairo University.

Inclusion criteria were preterm neonates (≤ 34 weeks gestation), very low birth weight (≤1.500 gm), delivered by caesarean section, and admitted at the NICU. Patients with major congenital malformations, congenital gastrointestinal anomalies, chorioamnionitis or premature rupture of membranes were excluded from the study.

The minimum sample size calculated was 68 patients with a confidence level of 90%, and a margin of error of 10%. Sample size was calculated according to Wayne Daniel [17].

Clinical data for all enrolled infants included gestational age assessment using New Ballard score [18], anthropometric data including weight, and antibiotic intake were recorded. NEC was diagnosed according to the modified Bell staging criteria.

Blood cultures are done in the NICU whenever sepsis is suspected whether clinically or laboratory. In the current study, this was applied for all patients in each of the first 3 weeks of life.

Rectal swabs for 16S Metagenomics were collected at postnatal week one, week two and week three. Samples were stored at − 80 °C until processing. DNA extracted from stool was used to sequence the 16S rRNA gene according to Illumina 16S Metagenomics library preparation [19], and run on Illumina MiSeq (Illumina, Inc. San Diego, California). Bioinformatic analysis was done using EZ Biocloud online analysis program. This program performs a comprehensive bioinformatics analysis using Chunlab's expertly curated prokaryotic reference database. It provides bacterial taxonomic profiles at different levels based on the high taxonomic accuracy and coverage of the database. This profile comes with other functionalities, such as alpha-diversity, beta-diversity and biomarker discovery (LEfSe). EZ Biocloud generates taxonomic profiles with an accurate relative abundance by using optional normalization methods. It supports read count normalization and gene copy number normalization for 16S-based MTP results [20].

Statistical methods:

The statistical evaluation was conducted utilizing IBM SPSS® Statistics version 21, provided by IBM® Corporation, located in Armonk, NY, USA. The representation of numerical data was in the form of median and interquartile ranges, while qualitative data was depicted through frequency and percentage. The normality of numerical data was assessed using both the Kolmogorov-Smirnov and Shapiro-Wilk tests. To examine the correlation among variables, the Mann-Whitney test was applied for quantitative data, and the Fisher's Exact test was used for qualitative data. A threshold of p-value less than 0.05 was established to denote statistical significance.

Results

Sixty-eight preterm very low birth weight neonates delivered by Caesarean section were recruited for this study. Their gestational ages ranged from 27-34 weeks with a median of 31 and their birth weights ranged from 0.870- 1.4 kg with a median of 1.3 kg. According to the local antibiotic policy of the NICU, 1st line antibiotics included Ampicillin/sulbactam plus Aminoglycoside (Unasyn, Gentamicin) and 2nd line antibiotics included Vancomycin/linezolid plus Meropenem/ imipenem which were given whenever sepsis was suspected either clinically or laboratory. For some cases, targeted protocol was started according to the results of microbiological culture and sensitivity. Most of the cases in this study received 1st and 2nd line treatment. There wasn't any significant correlation between different antibiotic protocols and rectal swab results.

Rectal swabs were collected at 1st week, 2nd week and 3rd week for evaluation of the gut microbiome by 16S metagenomics protocol from Illumina using next generation sequencing. According to workflow of this protocol, 16S rRNA is done by conventional PCR prior to sequencing. Out of the 204 collected samples, 53 showed positive 16SrRNA by PCR. The characteristics of the different datasets and results of the 16S rRNA PCR and the bacterial 16S metagenomics are listed in **Table 1**. For the 3 weeks, the microbiota was dominated by Proteobacteria and some Firmicutes, with some inter-individual variations. Among Proteobacteria, the most abundant family was *Enterobacteriaceae*, at the genus level mostly to be *Klebsiella* (19.1 % ,19.6%, and 17.8 at 1st, 2nd and 3rd weeks respectively), *Enterobacter* (8.3%, 10.3%, and 11.5% at 1st, 2nd and 3rd weeks respectively) and *E.coli* (4.6%, 6.6% in 2nd and 3rd weeks respectively). The most represented families of Firmicutes were *Paenibacillus* (4.5%) and *Enterococcacae* (4.3%) in week 2, and *Enterococcacae* (3.2%) in week 3.

We evaluated the differences in alpha diversity between the 3 weeks samples. The median Chao 1 index of week 3 (75.1) was higher than that of week 2 (73.5) and week 1 (56.4), while the median Shannon index of week 2 (1) was higher than that of week 3 (0.9) and week 1 (0.7). By comparing alpha diversity values in the 3 different groups, there was no statistical significance between any of the weeks as shown in **Figure 1**.

As regards beta diversity based on principle coordinates analysis, Bray-Curtis on genus level between the 3 groups, there was no statistical significance between week 1 and week 2 (P= 0.5), week 1 and week 3 (P= 0.5), or week 2 and week 3 (P= 0.5). Lefse analysis between 1st and 2nd week demonstrated significant predominance of Proteobacteria phylum presented mainly by Gammaproteobacteria class that included predominantly Enterobacterales order in 1st week, while others were predominant in 2nd week as shown in **Figure 2** (e.g. Phylum Bacteroidetes and *Actinobacteria*, *Enterobacter* at genus level).

Lefse analysis between the 1st and 3rd week showed significant predominance of Gammaproteobacteria class in the 1st week mainly Enterobacterales, while the 3rd week had higher predominance of the other microbiota as shown in **Figure 2** (e.g. *Enterococcus*, *Corynbacteria*…).

Lefse analysis between 2st and 3rd week showed predominance in 2nd week (*Bacteroidetes*, *Paenibacillus*) as shown in **Figure 2**.

We detected definite NEC according to Bell staging criteria in 12 neonates out of the 68 patients. **Table 1** describes the difference between confirmed NEC and non-NEC. For the 2 groups, the microbiota was dominated by Proteobacteria and mostly very close distribution of taxa on the genus level, apart from *Acinetobacter* in the NEC group. Number of OTU and Chao diversity index showed no significant difference, however Shannon diversity index showed highly statistically significant different P value 0.001 as shown in **Figure 3**. Lefse analysis of confirmed NEC group showed significant prevalence of the following taxa in the NEC group: *Enterobacteriaceae* genus, *Morganellaceae* family, *Proteus* genus and *Bifidobacterium* as shown in **Figure 4**. Regarding

beta diversity there was no significant difference between the 2 groups.

As regards sepsis, the majority of cases had no sepsis in the 1st week (42 neonates, 61.8 %) and in the 3rd week (32 neonates, 47.1%), while in the 2nd week, 31 neonates (45.6 %) were suspected to have sepsis. The most predominant bacteria isolated from blood culture was *Klebsiella* in 6 neonates from week one (8.8%) and in 7 neonates in week 2 (10.3%), while coagulase-negative *Staphylococcus* bacteria (CONS) was the predominant isolate in 3rd week patients (4 neonates, 5.9%).

The characteristics of the microbiota in sepsis and non -sepsis groups are listed in **Table 1**. For the 2 groups, the microbiota was dominated by Proteobacteria and mostly very close distribution of taxa on the genus level, apart from *Acinetobacter* and *Paenibacillus* in the sepsis group. There was no significant difference between the 2 groups regarding number of OTU, Chao nor Shannon diversity indices. Beta diversity did not show any significance between the 2 groups as well. Regarding Lefse analysis, the sepsis group showed predominance of *Acinetobacter* genus, a member of the *Moraxellaceae* family as shown in **Figure 5**.

Table 1. Characteristics of datasets and results of the next-generation sequencing (NGS) analysis of the intestinal microbiota in the 3 weeks, NEC and Non NEC groups, and sepsis and no sepsis groups.

		Week 1	Week 2	Week 3	NEC	Non NEC	Sepsis	Non sepsis
Number of valid reads: Median, min.-max.		53112, 30161-65346	46852, 2558-83987	55949, 18119-99187	51558.25	52012.32	52737.86	51092.18
Coverage, Median		99.97	95.18	99.96				
Number of positive 16sRNA samples		6 out of the 68	21 out of the 68	26 out of the 68				
Number of OTUs: Median, min.-max.		46.5, 23- 96	57.7, 21- 87	62.7,37 -98	60.75	57.91	57.29	59.75
Percentage of main phyla, (Mean)	Proteobacteria	99.4	88.9	96.2	94.7	93.8	92.9	95.1
	Firmicutes	1.52	23.7	9.7	4.41	5.8	6.45	4.67
Alpha diversity, Median	Chao 1 Index	56.38	73.55	75.07	77.91	71.10	70.73	73.57
	Shannon Index	0.66	1.01	0.98	1.28	0.89	1.04	0.90

OTUs: Operational Taxonomic Units

Figure 1. Boxplot graph describing the median, interquartile range (IQR) and range of Chao1 and Shannon diversity indices, representing the alpha diversity in the 3 weeks. Comparative analysis is done between the parameters of the 3 groups and the P value is calculated.

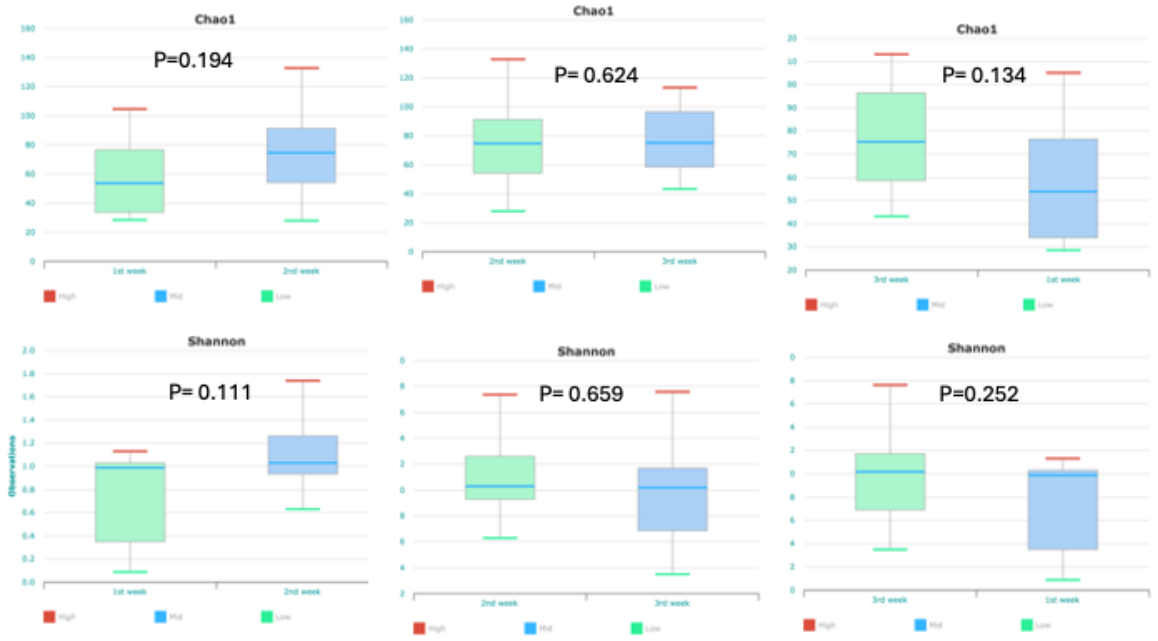


Figure 2.LDA (Linear discriminant analysis) score between the 3 different weeks calculated based upon the Lefse analysis describing the effect in the significantly different taxa in each group.

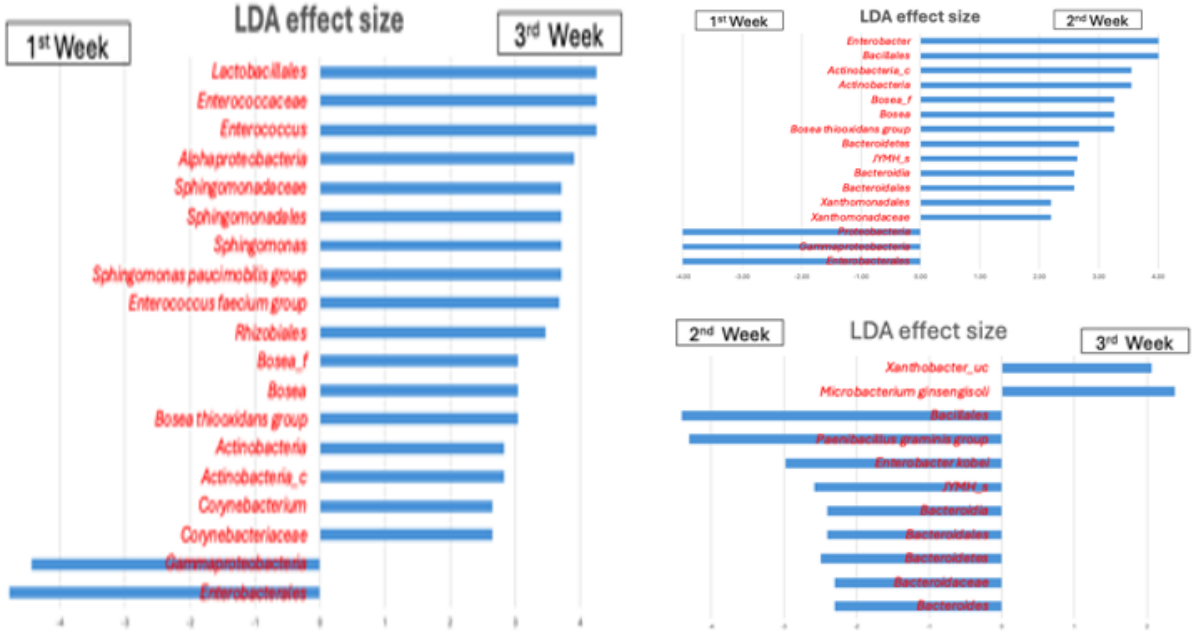


Figure 3. Boxplot describing the median, interquartile range (IQR) and range of the alpha diversity parameters in the NEC and non-NEC groups. Alpha diversity parameters include Chao and Shannon indices. High significance was shown in the Shannon diversity index.

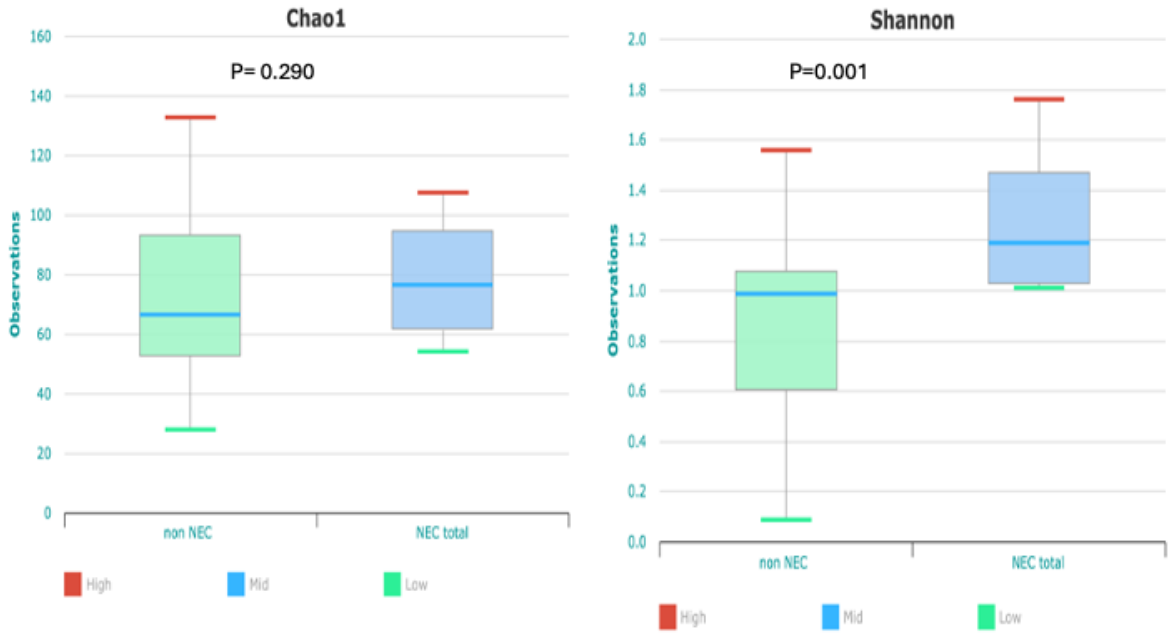


Figure 4.LDA (Linear discriminant analysis) score between NEC and non-NEC group calculated based upon the Lefse analysis describing the effect in the significantly different taxa in each group.

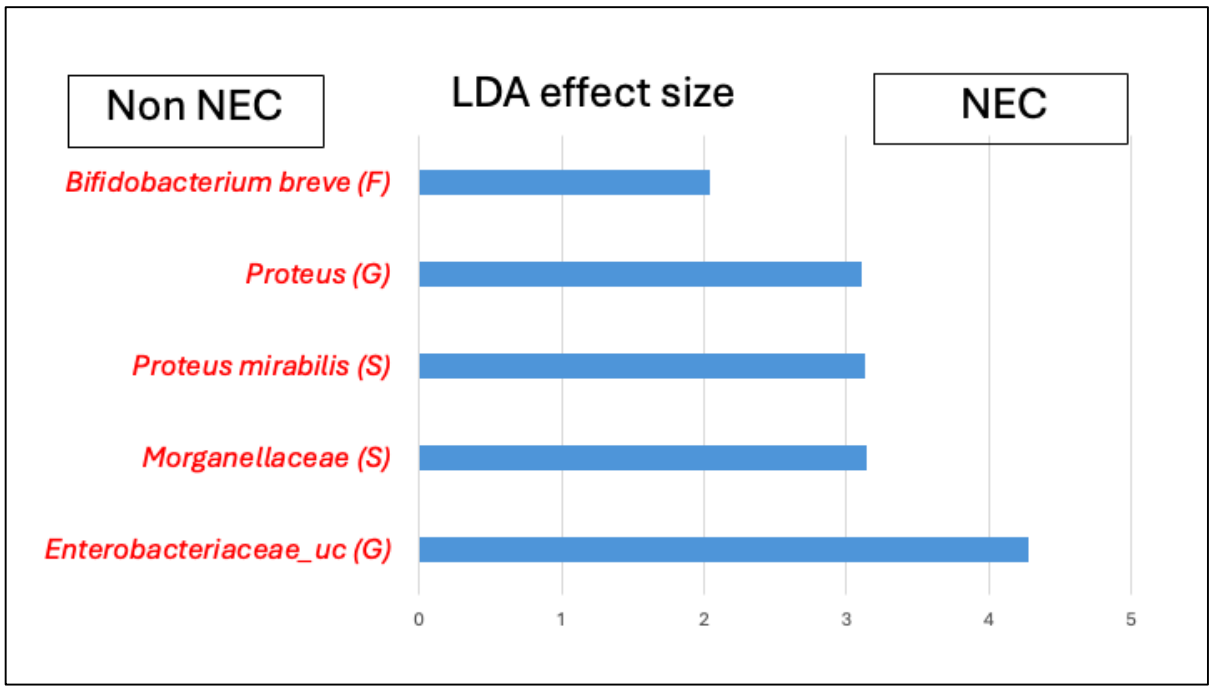
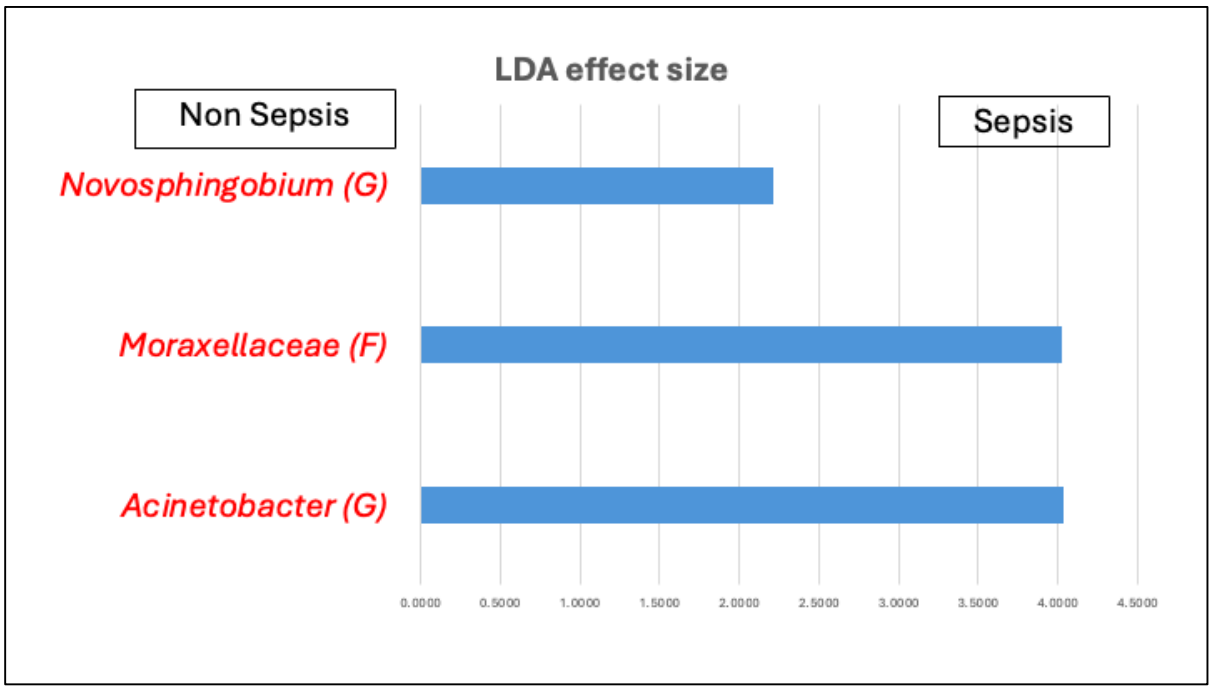


Figure 5.LDA (Linear discriminant analysis) score between sepsis and non-sepsis groups calculated based upon the Lefse analysis describing the effect in the significantly different taxa in each group.



Discussion

The significance of the human gut microbiome for both health and disease is increasingly recognized [21]. Postnatal alterations in the composition of the gut microbiota are linked to a broad spectrum of health issues in infancy and later stages of life, including infantile colic, wheezing, allergies, functional gastrointestinal disorders, obesity, and an overall impact on immune system development [22,23]. The factors contributing to these microbiota disturbances are becoming clear, with notable impacts identified from cesarean section births, formula feeding, and the use of antibiotics on the neonatal gut microbiome [24,25]. Specifically, antibiotic treatments significantly modify the species diversity and overall community structure, or ecology, of the gut microbiome, leading to long-lasting effects in both children and adults [26,27]. These alterations in the microbiome composition due to antibiotics favor the emergence of antimicrobial resistance [28].

In this study, neonatal gut microbiota had the development of mainly *Enterobacteriaceae* 98.2%, 82.9%, 87.7% in 1st, 2nd and 3rd weeks respectively. Firmicutes started to be present by the 2nd week in lower relative abundance 23.7% and 9.7 % in 2nd and 3rd weeks respectively. Among the phylum Firmicutes, *Enterococcaceae* was the most common 3.9%, 3.2 % in 2nd and 3rd weeks respectively. Silvia et al. reported on a cohort comprising 20 healthy Caucasian infants, all full-term, vaginally delivered, and breastfed, born following uncomplicated pregnancies, and compared them with 21 Caucasian preterm infants. The investigation revealed elevated concentrations of certain microbes, such as *Enterobacteriaceae*, *Enterococcaceae*, and the *Lactobacillus* group (which includes *Weissella*), in the preterm neonates. Concurrently, a decrease in anaerobic bacteria levels, notably *Bifidobacterium*, *Bacteroides*, and *Atopobium*, was observed. This pattern suggests a disruption in the normal development of the anaerobic gut microbiota [29]. Our study showed concordant results regarding *Enterobacteraceae* and *Enteococcaceae* as well as the mentioned anaerobic bacteria but unlike Silvia et al., our preterm neonates showed lower abundance of lactic acid bacteria, this might be due to different feeding type.

In this study, the predominating *Enterobacteraceae* family was mainly represented on the genus level by *Klebsiella* (5.8%, 22 % and 22 % in the 1st, 2nd and 3rd weeks respectively) and *Enterobacter* (4.4%, 8.8% and 10.2% in the 1st, 2nd and 3rd weeks respectively). In this regard, *Klebsiella pneumoniae* has been previously reported to be often present on the preterm infant microbiota [30-32].

Sharma et al. enrolled two groups, preterm and full term. The findings indicated that the majority of infants in both the preterm and term groups were colonized by 2-3 bacterial species, with *Klebsiella pneumoniae* and *E. coli* being the most prevalent. The colonization rates of Gram-negative bacteria were elevated in both preterm and term infants, whereas the prevalence of Gram-positive bacteria was notably lower in preterm infants [33]. This study had similar results as ours as there was predominance of some pathogens on the genus level (*Klebsiella, Enterobacter and E. coli*).

Ho et al. enrolled 45 eligible VLBW preterm infants, some of them born vaginally and some CS. Three stool samples were obtained on an average of ten days. Proteobacteria increased in abundance over time, comprising 46% at ≤ 2 weeks, 83.5% in the 3rd and 77% in the 4th week, compared to our study also there was predominance of Proteobacteria; The result of Ho et al. is somehow similar to our result as the number of positive 16S samples predominating by Proteobacteria increased over time (99.4 %, 88.9 % ,96.3% in 1st, 2nd and 3rd weeks respectively) despite that the relative abundance did not show significant increase (99.5 % in 1st week, 89.9 % in 2nd week and 96.3 % in 3rd week) [32].

In our study, Proteobacteria were the predominating taxa in the 3 weeks, Firmicutes were only present in the 2nd and 3rd weeks and had a relative abundance of 23.7% and 9.7% in 2nd and 3rd weeks respectively. Unlike our results, Khan et al. reported predominance of Firmicutes in preterm hospitalized neonates from Chinese hospital, that decreased after 3rd week and was replaced by higher Proteobacteria. This discrepancy can be explained by the vast difference of fecal microbiota in infants from different geographical areas as Khan et al justified [34].

According to the local antibiotic policy of the NICU unit, all included patients in this study received 1st and 2nd line antibiotic regimens. Interestingly, only around 25% of them showed positive 16SrRNA PCR. Gibson et al. encompassed 82 preterm neonates, all under 33 weeks of gestation with very low birth weight (VLBW; less than 1,500 grams). Stool samples were gathered both within 48 hours before starting antibiotic treatment and within 48 hours following the conclusion of the therapy. The study found that total exposure to antibiotics in these preterm infants was significantly linked to a decreased species richness in their gut microbiota ($P < 0.001$) [35]. In our study, we suggest that antibiotic intake did not only result in decreased richness, but furthermore was a reason that most of the collected rectal swabs were negative for the bacterial screening test (151 out of 204 collected swabs).

In the current study, definite NEC was detected in 17.5% of the total patients. Proteobacteria phylum was predominating the 2 groups. On the genus level, *Acinetobacter* was present only in the NEC group. No statistical difference was detected in the different indices except the Shannon diversity index. *Morganellaceae* family, *Enterobacteriaceae* genus, *Proteus* genus and *Bifidobacterium* genus were significantly present in the NEC group by Lefse analysis. In line with our study, Matthew et al. detected predominance of Proteobacteria irrespective of the NEC development, also he detected significant prevalence of *Enterobacteriaceae* family, but we did not agree with him regarding the lower levels of Firmicutes [36]. As per his explanation, elements that may favor the selection of these microorganisms include the routine administration of antibiotics to premature infants at birth, a high frequency of cesarean deliveries, a preference for formula feeding over breastfeeding, and the underdevelopment of the gastrointestinal tract and immune system [36].

In our study, both sepsis and non-sepsis groups were dominated by Proteobacteria; the chao index and the number of OTU were lower in the sepsis group but not statistically significant. Graspeuntner et al. carried out an extensive forward-looking study involving 71 preterm infants who developed late onset sepsis (LOS) and 164 healthy preterm infants. In line with our findings, they found a reduction in microbial diversity in the group affected by LOS [15]. Unlike, Graspeuntner's work, the sepsis group had significant predominance of *Acinetobacter* genus, that could be due to local prevalence / spread of this pathogen in our hospitals [37].

In conclusion, the gut of hospitalized preterm neonates born by CS who received antibiotics was mostly sterile at first week due to antibiotics' suppression that may pave the way for the subsequent pathogenic organisms colonization such as *Klebsiella* and *Enterobacter*. The phylum Proteobacteria predominated in both NEC and non-

NEC groups with no significant differences detected between both groups NEC group showed significantly lower Shannon diversity index. The sepsis group had significantly higher predominance of *Acinetobacter* genus, a prevalent pathogen in the Egyptian hospitals. This study shed some light on the microbiota of a vulnerable group of hospitalized patients. However, it had limitations such as the routine use of antibiotics to neonates in our unit and the excessive manipulations and complications to the VLBW neonates that increased the risk of sepsis and the continuation of antibiotics.

Declaration of interest and funding information

All authors report no conflicts of interest. all authors have approved the final article.

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Authors' contribution

AE and ZE contributed to the conception and design of the study, DH, EM and MS have done the acquisition of data, MS and DH did the analysis and interpretation of data, all authors have contributed to the drafting of the article, AE, ZE and MS revised it critically for important intellectual content.

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