Research article

Clinical characteristics influence cultivable-bacteria composition in the meconium of Indonesian neonates

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A B S T R A C T

Background: Microbial colonization of a neonate's gastrointestinal tract has significant perinatal and lifetime health consequences. However, information regarding the profile of meconium microbiota in neonates and the influence of clinical parameters are lacking in the Indonesian population. This study aimed to preliminary investigate the profile of cultivable bacterial diversity of meconium isolated from neonates born at Cipto Mangunkusumo Hospital (CMH), Jakarta. The cultivable bacteria were isolated from meconium samples and were then processed for cultivation and molecular identification.

Results: Fourteen neonates were enrolled as described, i.e., seven hyperbilirubinemia (Hyp) and seven non-Hyp with ten neonates delivered by cesarean section (CS) and four others by vaginal route (VR), and with five exclusive breastfeeding (Ebf), four formula milk, and five combinations. Microbiological identification, molecular 16S rDNA PCR-Sanger sequencing, and PCA analysis of cultivable bacteria isolated from meconium showed Firmicutes' predominance (84.41%), with an abundant population of *Staphylococcus,* which consist of *S. hominis,* *S. epidermidis,* and *S. haemolyticus.* The influence of mode of delivery showed a lower diversity than the CS populates the VR, but their composition was similar. Concurrently, between feeding patterns, the genera profile did not show much difference; in the non-Ebf group, the total amount of *Staphylococcus* and *Bacillus* showed a higher amount but a less diverse. Interestingly, the non-Hyp group showed more abundant and diverse *Staphylococcus* than that of the Hyp group. In contrast, neonates diagnosed with NEC and proven sepsis showed the same pattern of *Staphylococcus* domination.

Conclusion: *Staphylococcus* predominated the composition of cultivable bacteria in neonates meconium. Due to the small sample size, only the hyperbilirubinemia parameter significantly influenced the profile, i.e., *Staphylococcus*'s proportion ($p = 0.037$).

1. Introduction

The *in-utero* environment was previously thought to be sterile. It was postulated that the infants' gastrointestinal tract obtained bacterial colonization through cross-contamination from the environment. However, several studies [1, 2, 3, 4] have recently found the microbial existences in the placenta, amniotic fluid, and meconium samples that suggested an *in-utero* bacterial exposure. Studies using meconium samples from healthy neonates suggest that gut microbial colonization might be initiated before birth [5, 6, 7]. As the first intestinal discharge of infants, the meconium is believed to represent the material ingested during their fetal life, such as amniotic fluid, epithelial cells, and mucus, thus representing the *in-utero* environment. Ardissone et al. [8] reported the association of the common bacteria genera found abundance in amniotic fluid and the meconium, thus confirming the representative of the in-utero environment while representing the early colonization of gastrointestinal bacteria in newborns.

Microbiota in meconium has been vastly studied due to its possible impact on infant's health and development and diseases that may be contracted during their early days as well as in the long term [2, 9, 10]. It was reported that the bacteria taxonomic diversity is relatively low at birth but increases over time as the infant is colonized with bacteria acquired from breast milk and the environment [11]. The meconium

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microbiota reported from the United States (i.e., State of Florida) and Australia showed a predominance of Proteobacteria followed by Firmicutes phyla. In contrast, China showed Firmicutes followed by Proteobacteria phyla predominant [8, 9, 12]. Other reports presenting infants' data from China and Brazil showed an abundance of Proteobacteria, while the US, Sweden, and Canada predominates by Actinobacteria. Other studies reported that infants from Bangladeshi and Sweden were abundant in Firmicutes [13]. At the genus level, *Staphylococcus* predominated in the meconium samples, as reported by [5, 14].

Factors such as mode of delivery, gestational age, birth weight, feeding pattern, and antibiotic use were found to be related to the diversity and composition of microbiota in neonates, as reported previously [4, 7, 15]. There is a report showing that, in addition to microbiota-derived from feeding and intubation, the gut microbiome of preterm infants is also configured by the immediate incubator and room environment within neonatal intensive care units rather than by individuals who come into contact with the infants (parents and healthcare providers) [16]. A study on meconium from premature births [8] has shown some evidence of the role of gut microbiota that leads to premature birth. Several clinical outcomes often reported in neonates, such as hyperbilirubinemia, necrotizing enterocolitis (NEC), and neonatal sepsis, have also been linked to differences in the diversity and composition of gut microbiota [9, 10, 17]. Recently, an increasing number of studies [1, 4, 5, 6, 14] have been conducted on the diversity and composition of the gut microbiota, as well as the development of gut microbiota in neonates.

Although studies from many countries on the neonatal gut microbiota have been reported widely, there are considerably fewer studies on the neonatal gut microbiota in Indonesian neonates using meconium samples. Furthermore, by the emergence of fecal microbiota transplant (FMT) for the treatment of severe microbial infections by applying frozen healthy person feces, the study of meconium microbiota has also vigorously risen [18, 19]. Hence, to provide preliminary data of Indonesian neonates' meconium bacteria profile, we investigated by performing bacteria isolation and cultivation of the meconium, as well as the 16S rRNA PCR- sequencing method simultaneously, collected from neonates born in the National Referral Hospital Cipto Mangunkusumo (CMH), Jakarta, Indonesia. The influence of clinical characteristics and outcomes, i.e., mode of delivery, feeding patterns, hyperbilirubinemia, NEC, and proven sepsis, were also studied.

2. Materials and methods

2.1. Participants and sampling

This cross-sectional observational study included 14 inborn neonates born from February to March 2019 at the Cipto Mangunkusumo Hospital, Jakarta (Indonesia) (see Supplement 1). The number of enrolled neonates was limited based on the fact that the cultivation approach needs laborious work and intensive collection process, and by the fact that the predominant genus reported widely is in agreement, i.e., *Staphylococcus*. For inclusion into the study, the neonates had to pass the meconium samples in the first 24–48 h after birth, which was collected from the diapers by CMH trained health professional following the Ethics approval. Meconium samples that were less than 150 mg were excluded. We excluded neonates with signs of hemolytic anemia caused by ABO incompatibility (based on blood grouping, Coomb's test, and fall in Hemoglobin), cephalhematoma, and major congenital malformations assess through clinical and laboratory examinations. Subgroups were then formed for further analysis between variables based on the number of patients with specific variables. Total serum bilirubin was collected using the ADVIA Chemistry Total Bilirubin 2 device within 24 h, and before phototherapy (when required) was initiated. Neonatal hyperbilirubinemia is defined as total serum bilirubin (TSB) level above 5 mg per dL (86 μmol per L). However, in this study, due to the lack of appropriate neonate samples enrolled, we divided the group into greater and equal to 4.75 mg per dL as Hyp, and below 4.75 mg per dL as non-

Hyp. Other relevant clinical data recorded for each neonate, such as mode of delivery, feeding pattern, gestational age, necrotizing enterocolitis (NEC) (based on radiologic and clinical finding), and sepsis (proven through positive blood culture), are detailed in Supplement 4.

The meconium samples were all first feces samples within 24–48 h of birth, collected and placed into sterile tubes by well-trained nurses. All samples were stored temporarily at 4 °C before they were transported on the same day. The samples were transported in a cooler box to the Laboratory of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Universitas Indonesia, and immediately processed for culturing.

2.2. Bacteria isolation and identification

Each sample was weighed and diluted in pre-reduced phosphate-buffered saline with 0.1% cysteine to obtain a 10−1 dilution. An aliquot (0.05 mL) of the diluted sample was then inoculated in triplicate on eosin methylene blue (EMB) agar for the isolation of *Escherichia coli*; deMann, Rogosa, and Sharpe (MRS) agar for the isolation of *Lactobacillus*; raffinose Bifidobacterium agar for the isolation of *Bifidobacterium*; mannitol salt agar (MSA) for the isolation of *Staphylococcus*; and MacConkey (MC) agar for the isolation of *Enterobacteriaceae*. Sheep blood agar (BA), brain heart infusion (BHI) agar, and Brucella blood agar (BRU) were used as general media for the isolation of other bacteria groups. All plates were anaerobically incubated at 37 °C for up to 5 days in an anaerobic jar, except for BA, MSA, and MC plates, which were aerobically incubated at 37 °C for up to 5 days. The isolates obtained were sub-cultured on new agar media for purification and enrichment. Pure single colonies were then enriched in 7-mL BHI broth at 37 °C for 24 h under aerobic and anaerobic conditions, and 600 μL of each broth culture was stored at −80 °C in glycerol (85%, v/v). The isolates were identified visually and microscopically to determine the cell morphology and the Gram-staining reaction. During the screening, selection, and identification, we employed reference strains of bacteria as control.

2.3. DNA extraction

The bacteria isolates were cultured in tryptic soy broth and incubated according to their previous incubation conditions (aerobic or anaerobic) at 37 °C for 24 h. Subsequently, the cultures were centrifuged at 14,000 × g for 2 min to pellet the cells. Genomic DNA was extracted from the pellets using a Presto™ Mini g-DNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. The presence and concentration of bacteria DNA were confirmed by agarose gel electrophoresis (1.0% in 1× tris-acetate-ethylenediaminetetraacetic acid [EDTA] buffer); a UV transilluminator system (Biometra, Germany) was employed to observe and for documentation.

2.4. Polymerase chain reaction and sequencing of 16S rRNA gene

Amplification of the V1–V3 regions of 16S rRNA gene was performed using two universal primers: 27F-modification (5′-AGA GTT TGA TC[A/C] TGG CTC AG-3′) and 534R (5′-ATT ACC GCG GCT GCT GG-3′). The polymerase chain reaction (PCR) amplification was achieved in a total volume of 50 μL comprising 6.5 μL of ddH2O, 25 μL of 2× KOD FX Neo buffer (Toyobo, Japan), 10 μL of 2-mM dNTPs, 0.75 μL each of 20-mM primer 27F, and 534R, 6 μL of bacteria DNA template, and 1 μL of KOD FX Neo polymerase (Toyobo, Japan). The amplicons were sequenced using Sanger sequencing by1[st] Base, Singapore). During PCR-sequencing, we employed g-DNAs of reference bacteria as control.

2.5. DNA sequence analysis and nucleotide sequence accession numbers

The DNA sequences data obtained from DNA sequencing were analyzed by using Basic Local Alignment Search Tool (BLAST™) (http://www.ncbi.nlm.nih.gov/BLAST) in GenBank database to determine

the species identification of bacteria isolates. Approximately 0.4–0.5 kb DNA sequences data of 16S rRNA gene of bacteria isolates obtained in this study were submitted to the GenBank database under accession numbers MN075325–MN075401 by employing a web-based sequence submission tool BankIt™ (https://www.ncbi.nlm.nih.gov/WebSub/).

2.6. Phylogenetic tree analysis

The phylogenetic trees that were constructed from 16S rRNA sequences of all 77 bacteria isolates obtained were distributed into three clinical parameters, i.e., mode of delivery, feeding pattern, and totals serum bilirubin. Most analyses were performed using the MEGA X software [47]. The sequences were aligned using MUSCLE (EMBL-EBI, UK). The evolutionary distances were computed using the maximum composite likelihood method and units of the number of base substitutions per site. The codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated (complete deletion option).

2.7. Statistical analysis

All statistical analyses were performed using R v3.5.2 (R Foundation for Statistical Computing, Austria). Paired-sample T-tests were applied to determine differences between the number of species per genus in each subgroup clinical parameters involved in this study that yielded adequate numbers for statistical analysis, i.e., feeding pattern, mode of delivery, hyperbilirubinemia vs. non-hyperbilirubinemia groups. PCA of the cultivable bacteria (number of species in each genus) was used to group the bacteria profiles of all meconium samples to categorize the samples according to their clinical parameter group. The genus was used as the variable to understand the differences in the clusters between the microbiota profile of the groups. The PCA plot also contained the principal components (PCs), representing the maximum variance of the data.

2.8. Ethics approval and consent to participate

This study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia number KET-146/UN2.F1/ETIK/PPM.00.02/2019, Protocol Number 19-02-0163, on February 18, 2019. The study was performed following the ethical standards of the Declaration of Helsinki (1964). Written informed consent was obtained from the parents before sample collection.

3. Results

3.1. Characteristics of neonates

All neonates born at average gestational age 35 weeks (mean; 33–38 weeks) and average birth weight of 2.219 g (mean; 1.645–3.670 g) represent seven Hyperbilirubinemia (Hyp) and seven non-Hyp. Of these neonates, ten were delivered by cesarean section (CS), and four were born by vaginal route (VR) delivery. Feeding pattern variation comprised of exclusively own mother's breast milk (35.71%; 5 out of 14 subjects), formula milk (28.57%; 4 out of 14 subjects), and a combination of mother's breast milk and formula milk (35.71%; 5 out of 14 subjects). A complete list of the characteristics is shown in Supplement 1.

3.2. Bacteria profile of meconium

Microscopic identification of the 77 pure bacteria isolates from meconium samples revealed 48 isolates (62.34%) as coccus-shaped bacteria with Gram-positive staining, and the remaining 29 isolates (37.66%) were identified as rods or irregular rod-shaped bacteria with either Gram-positive or -negative staining (Supplement 2). Molecular identification with Accession Number (AN) of the isolates (Supplement 2) revealed that the most abundant phylum was Firmicutes (84.41%; 65

out of 77 isolates), of which *Staphylococcus* and *Bacillus* were the most commonly encountered genera (53.24%; 41 out of 77 isolates and 19.48%; 15 out of 77 isolates, respectively), as shown in Figure 1. *S. hominis*, *S. haemolyticus*, and *S. epidermidis* were present in approximately 6–7 out of 14 samples. The remaining species of this phylum were only present in 7%–14% (1–2 out of 14) of the samples. The phylum with the most diverse genera was proteobacteria (11.69%; 6 genera; 9 out of 77 isolates), and the least abundant phylum was actinobacteria (3.90%; 2 genera; 3 out of 77 isolates). Every species belong to those phyla was occurred in a small number of samples (7%; 1 out of 14 samples). All bacteria composition profile is listed in Table 1.

3.3. Mode of delivery influence

In contrast to the microbiota from the cesarean section (CS) neonates, the vaginally-route (VR) bacteria profile in this study was less diverse. Still, both were predominated by Firmicutes, as presented in the reconstruction of phylogenetic trees provided in Supplement 3E and 3F.

Data matrix used to reconstruct the bacteria profile of VR -born neonates consisted of 22 taxa, with 20 of them belonged to Firmicutes (four genera). Two of them belonged to Actinobacteria (one genus). The outgroup from the phylogenetic tree of microbiota from VR -born neonates was *Corynebacterium singular*. *Staphylococcus epidermidis* and *Staphylococcus warneri* seemed to be sister, although their bootstrap value is low (bootstrap value 65).
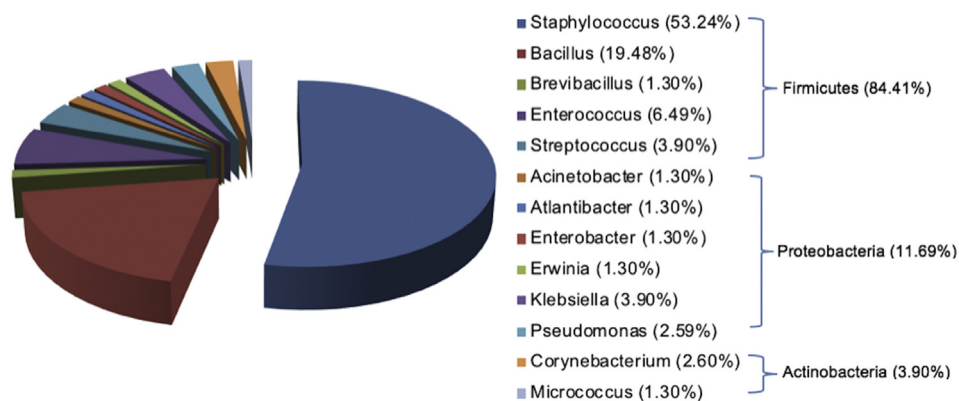
Meanwhile, the data matrix used in the reconstruction of the microbiota of CS-born neonates phylogenetic trees consisted of 55 taxa in which 45 of them belonged to Firmicutes (four genera), one of Actinobacteria (one genus), and nine of Proteobacteria (six genera); this showed that although there were more genera represented for CS-born neonates, Firmicutes composition was more abundant. *Staphylococcus pasteuri* and *Staphylococcus warneri* seemed to be sister with a bootstrap value 98. The same occurred for *Bacillus purnilus* and *Bacillus aerius* with a bootstrap value 97. *Brevibacillus agri*, although they have different genera than *Bacillus simplex*, they appeared to be sister, although with a low bootstrap value 39. The microbiota from phylum Proteobacteria seemed to be the outgroup Proteobacteria from the phylogenetic tree of microbiota cesarean-born neonates.

We also investigated whether there are significant differences in the number of species from the profile of microbiota from both groups (CS-born and VR -born neonates) using PCA and T-test; results showed that some samples possessed similar cultivable-microbiota composition. The score plot containing PC1 (60%) and PC2 (24%) accounted for 84% of the total variance (Figure 3 A), with the T-test value presented in Table 2.

3.4. Feeding pattern influence

The feeding pattern was divided into two groups, i.e., exclusive breastfeeding (exclusively mother's breast milk) and non-exclusive breastfeeding (formula milk; both mother's breast milk and formula). Both exclusive breastfeeding (EBF) and non-exclusive breastfeeding (non-EBF) meconium microbiota profile, which was determined phylogenetically, showed that *Staphylococcus sp.* predominated the population, followed by *Bacillus sp*. However, the phylogenetic tree of EBF showed a more diverse bacteria profile than non-EBF (see Supplement 3G and 3H). EBF neonates harbored a more diverse meconium microbiota composed of more bacteria genera, such as *Acinetobacter* sp., Atlantibacter sp., *Enterobacter* sp., Klebsiella sp., and *Corynebacterium* sp., which were not found in non-EBF microbiota profile.

The data matrix used for the phylogenetic reconstruction of the EBF tree comprised 33 taxa, of which 24 belonged to Firmicutes (representing three genera), seven to Proteobacteria (5 genera), and two to Actinobacteria (one genus), which was used as the outgroup. Species from the same genus, such as *Staphylococcus warneri* and *Staphylococcus pasteuri* appeared to be sister, with a bootstrap value 94. *Pseudomonas stutzeri*

- Staphylococcus (53.24%) ┐
- Bacillus (19.48%)   │
- Brevibacillus (1.30%)   ├ Firmicutes (84.41%)
- Enterococcus (6.49%)   │
- Streptococcus (3.90%)   ┘

- Acinetobacter (1.30%) ┐
- Atlantibacter (1.30%) │
- Enterobacter (1.30%) │
- Erwinia (1.30%)   ├ Proteobacteria (11.69%)
- Klebsiella (3.90%)   │
- Pseudomonas (2.59%)   ┘

- Corynebacterium (2.60%) ┐ Actinobacteria (3.90%)
- Micrococcus (1.30%)   ┘

Figure 1. The profile of cultivable bacteria composition of neonates' meconium. The most abundant genus is *Staphylococcus* from phylum Firmicutes, and the three main phyla identified in this study are Firmicutes, Proteobacteria, and Actinobacteria.

appeared sister to *Acinetobacter baumannii* with a bootstrap value 96, although they come from a different genus.

Data matrix used for the phylogenetic reconstruction of the non-EBF tree comprised 44 taxa, 41 belonging to Firmicutes (representing five genera), two to proteobacteria (two genera), and one to Actinobacteria (one genus). *Bacillus flexus* appeared to be sister to *Bacillus aryabhattai*, with a bootstrap value 98. *Pseudomonas stutzeri* appeared to be sister to *Erwinia billingiae* with a bootstrap value 100, although they come from a different genus.

The total amount of *Staphylococcus* and *Bacillus* was higher in the non-EBF group than EBF group. The significant differences in the number of species from the microbiota profile of both groups were compared using PCA and T-test, which resulted in the differences between the genera being not statistically significant. The score plot containing PC1 (60%) and PC2 (24%) accounted for 84% of the total variance (Figure 3 B), with the T-test value presented in Table 2.

3.5. Hyperbilirubinemia influence

Based on several clinical parameters collected, TSB value showed more variable microbiota than NEC and sepsis. Several demographic parameters that are risk factors for the development of hyperbilirubinemia (Hyp), such as gestational age, birth weight, mode of delivery, antibiotic exposure, and feeding pattern, were observed in our study. We discovered that some risk factors showed a high incidence of Hyp, such as nutritional intake other than breast milk (66.67%; 6 out of 9 subjects), preterm gestational age (53.85%; 7 out of 13 subjects), low birth weight (54.55%; 6 out of 11 subjects), and no antibiotic exposure (54.55%; 6 out of 11 subjects). In contrast, the incidence of Hyp appeared similar (50%; 5 out of 10 subjects; 2 out of 2 subjects) for those born through cesarean and vaginal delivery, as shown in Figure 2.

The meconium microbiotic profile based on the incidence of hyperbilirubinemia as the measured clinical outcome was determined phylogenetically. The phylogenetic tree of microbiota in cesarean-born neonates with hyperbilirubinemia (see Supplement 3A and 3B) showed a more diverse bacteria profile than vaginally delivered neonates. However, the mode of delivery showed an equal risk (50%). The phylogenetic reconstruction was categorized into two groups based on the clinical outcome: hyperbilirubinemia and non-hyperbilirubinemia (see Supplement 3C and 3D). The data matrix used for the phylogenetic reconstruction of the hyperbilirubinemia tree comprised of 27 taxa, of which 20 belonged to Firmicutes (representing four genera), six to Proteobacteria (four genera), and 1 to Actinobacteria (one genus), which was used as the outgroup. Species from the same genus such as *Bacillus lichenformis* and *Bacillus velezensis* appeared to be sister, with a bootstrap value 99. *Enterococcus faecalis* appeared sister to *Streptococcus agalactiae* with a bootstrap value 90, although they come from a different genus. The data matrix used for the phylogenetic reconstruction of the non-

hyperbilirubinemia tree comprised 50 taxa, of which 45 belong to Firmicutes (representing five genera), three to Proteobacteria (two genera), and two to Actinobacteria (one genus), which was used as the outgroup. *Bacillus flexus* appeared sister to *Bacillus aryabhattai*, with a bootstrap value 99. The total amount of *Staphylococcus*, *Bacillus, Enterococcus*, and other genera was mostly higher in the non-hyperbilirubinemia group than the hyperbilirubinemia group. However, the differences between the genera were only statistically significant for *Staphylococcus*. PCA and T-test were applied to the whole dataset of the species to determine if differences existed between the hyperbilirubinemia and non-hyperbilirubinemia groups' bacteria profile. The PCA results showed that although the cultivable meconium bacteria profile was divided into two groups (hyperbilirubinemia and non-hyperbilirubinemia), they demonstrated a predominant bacteria member for PC1 and PC2, i.e., *Staphylococcus* and *Bacillus*, respectively. The score plot containing PC1 (60%) and PC2 (24%) accounted for 84% of the total variance (Figure 3 C), with the T-test value presented in Table 2.

3.6. Other clinical outcomes influence

Necrotizing Enterocolitis (NEC) and sepsis were found in one subject. Neonates born from cesarean delivery were diagnosed with NEC, and neonates born with vaginal delivery were diagnosed with sepsis. Both of them were non-EBF. Due to the lack of subjects, the data for analyzing the correlation between these clinical outcomes and the bacteria profile cannot be determined. Thus, we tried to describe and compare the pattern of both subjects.

Even though Firmicutes were predominant in both meconium microbiota, they have a different species of *Staphylococcus*; this could be observed from the phylogenetic tree of neonates with non-exclusive breastfeeding in Supplement 3H. *Staphylococcus hominis*, *Staphylococcus edaphicus*, *Staphylococcus cohnii* subsp. *urealyticus*, and *Staphylococcus hominis* subsp. *novobiosepticus* were found in a neonate with NEC. There was also *Bacillus simplex* found in a neonate with NEC.

Meanwhile, *Staphylococcus warneri* and *Staphylococcus epidermidis* were found in the neonate with sepsis. *Streptococcus agalactiae* was also found in a neonate with sepsis. However, due to a lack of neonates recruited, we discovered that neonates' data with NEC and or sepsis could not be statistically analyzed further.

4. Discussion

The cultivation method performed in this study showed the advantage of obtaining bacteria species identified as commensal safe-microbes. At the genus level, we found *Staphylococcus* as the most abundant of the dominant phylum Firmicutes of neonates' meconium, which agrees with reports by [5, 13]. The three most significant populations present, i.e., *S. hominis* (12.99%), *S. epidermidis* (11.68%), and *S. haemolyticus* (10.39%),

Table 1. Composition of neonates' meconium cultivable-bacteria diversity.

Genus	Number	Species
Staphylococcus (53.24%) 41 out of 77 isolates	1	*Staphylococcus hominis* (12.99%) 10 out of 77 isolates
	2	*Staphylococcus hominis subsp. Novobiosepticus* (7.79%) 6 out of 77 isolates
	3	*Staphylococcus haemolyticus* (10.39%) 8 out of 77 isolates
	4	*Staphylococcus epidermidis* (11.68%) 9 out of 77 isolates
	5	*Staphylococcus warneri* (3.90%) 3 out of 77 isolates
	6	*Staphylococcus cohnii subsp. Urealyticus* (2.59%) 2 out of 77 isolates
	7	*Staphylococcus caprae* (1.30%) 1 out of 77 isolates
	8	*Staphylococcus edaphicus* (1.30%) 1 out of 77 isolates
	9	*Staphylococcus pasteuri* (1.30%) 1 out of 77 isolates
Bacillus (19.48%) 15 out of 77 isolates	1	*Bacillus velezensis* (2.59%) 2 out of 77 isolates
	2	*Bacillus pumilus* (1.30%) 1 out of 77 isolates
	3	*Bacillus subtilis* (1.30%) 1 out of 77 isolates
	4	*Bacillus flexus* (2.59%) 2 out of 77 isolates
	5	*Bacillus licheniformis* (2.59%) 2 out of 77 isolates
	6	*Bacillus aerius* (1.30%) 1 out of 77 isolates
	7	*Bacillus simplex* (1.30%) 1 out of 77 isolates
	8	*Bacillus aryabhattai* (1.30%) 1 out of 77 isolates
	9	*Bacillus paramycoides* (5.19%) 4 out of 77 isolates
Brevibacillus (1.30%) 1 out of 77 isolates	1	*Brevibacillus agri* (1.30%) 1 out of 77 isolates
Enterococcus (6.49%) 5 out of 77 isolates	1	*Enterococcus hirae* (3.90%) 3 out of 77 isolates
	2	*Enterococcus faecalis* (2.59%) 2 out of 77 isolates
Streptococcus (3.90%) 3 out of 77 isolates	1	*Streptococcus agalacticae* (2.59%) 2 out of 77 isolates
	2	*Streptococcus anginosus* (1.30%) 1 out of 77 isolates
Acinetobacter (1.30%) 1 out of 77 isolates	1	*Acinetobacter baumannii* (1.30%) 1 out of 77 isolates
Atlantibacter (1.30%) 1 out of 77 isolates	1	*Atlantibacter hermannii* (1.30%) 1 out of 77 isolates
Enterobacter (1.30%) 1 out of 77 isolates	1	*Enterobacter hormaechei* (1.30%) 1 out of 77 isolates
Erwinia (1.30%) 1 out of 77 isolates	1	*Erwinia billingiae* (1.30%) 1 out of 77 isolates
Klebsiella (3.90%) 3 out of 77 isolates	1	*Klebsiella pnuemoniae* (3.90%) 3 out of 77 isolates
Pseudomonas (2.59%) 2 out of 77 isolates	1	*Pseudomonas stutzeri* (2.59%) 2 out of 77 isolates
Corynebacterium (2.59%) 2 out of 77 isolates	1	*Corynebacterium singulare* (1.30%) 1 out of 77 isolates
Micrococcus (1.30%) 1 out of 77 isolates	1	*Micrococcus luteus* (1.30%) 1 out of 77 isolates
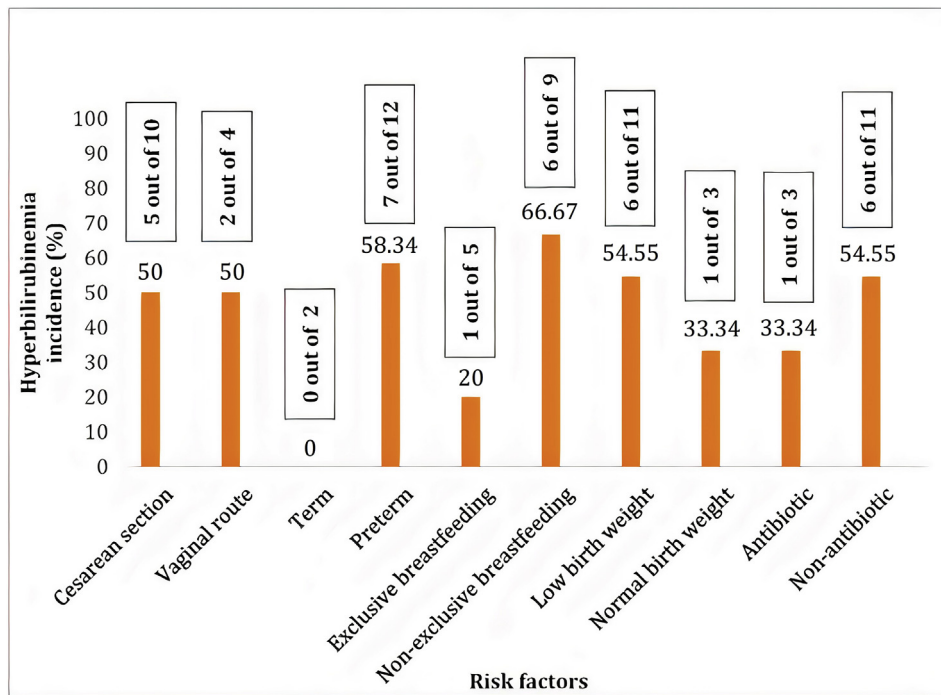
Figure 2. Percentage of the incidence of hyperbilirubinemia as the clinical outcome based on the risk factors such as mode of delivery (cesarean section, CS and vaginal route, VR), gestational age (preterm: less than 37 weeks, and term: 37 weeks and above), feeding patterns (exclusive breastfeeding, Ebf [exclusively mother's breast milk] and non-exclusive breastfeeding, non-Ebf [formula milk; both mother's breast milk and formula]), birth weight (low birth weight [≤2500 g] and normal birth weight [>2500 g]), and antibiotic exposure.

were also consistent with previous studies reported in countries of United States, Australia, China, Bangladesh, and Sweden [8, 9, 12, 13] besides Proteobacteria and Actinobacteria. It was also reported that data from China and Brazil showed Proteobacteria predominant, while from the US, Sweden, and Canada showed mainly Actinobacteria [13].

We found a comparable rate of VR and CS deliveries regarding the role of mode of delivery towards the neonatal intestinal microbiota. CS-born neonates undergo a delayed microbiotic colonization, prolonging the duration of condition with immature microbiota and resulting in lower species diversity. Meanwhile, VR born neonates who had relatively lower microbiota diversity showed rapid colonization to compensate for the diversity deficiency [9, 20]. The high microbiotic diversity in CS-born neonates could be a protective factor against hyperbilirubinemia [9]. Mode of delivery has been extensively regarded as one of the essential aspects of the first gut microbiota colonization from several studies that

discovered a similarity between the microbiota from a neonate's meconium and the mother's microbiota [15, 21]. Dominguez-Bello et al. [6] compared the bacteria found in neonates' meconium with the microbiota from the mothers' vagina and skin, revealing a high similarity between the mothers' vaginal microbiota and their own babies' gut microbiota. Furthermore, the first gut colonizers of neonates born by CS resembled the human skin microbiota, especially bacteria from Staphylococcus spp., which were found to be predominant in this study [6].

Feeding type has been demonstrated to influence microbiota composition. This happens directly by providing the substrates for bacterial proliferation and function and sources of bacterial contamination originating from the nipple and surrounding skin, and milk ducts for breast milk; from the dried powder, the equipment for preparation and the water used for suspension for formula milk [22, 23]. Our study found that in both exclusive breastfeeding and non-exclusive breastfeeding

Table 2. Species count across samples for bacteria composition obtained from neonates' meconium.

Genus	Sample group								
	C-s	V-r	P-value*	Ebf	Non-ebf	P-value*	Hyp	Non-hyp	P-value*
Staphylococcus	26	5	0.06121812	12	19	0.78046714	9	22	0.03720944**
Bacillus	8	3	0.91565562	2	9	0.23203011	4	7	0.47559972
Enterococcus	1	2	0.26497365	0	3	0.08051624	1	2	0.55242108
Brevibacillus	1	0	0.34343640	0	1	0.34659351	0	1	0.35591768
Streptococcus	0	2	0.18169011	1	1	0.70997348	1	1	1.00000000
Acinetobacter	1	0	0.34343640	1	0	0.37390097	0	1	0.35591768
Atlantibacter	1	0	0.34343640	1	0	0.37390097	1	0	0.35591768
Erwinia	1	0	0.34343640	0	1	0.34659351	1	0	0.35591768
Enterobacter	1	0	0.34343640	1	0	0.37390097	1	0	0.35591768
Klebsiella	1	0	0.34343640	1	0	0.37390097	1	0	0.35591768
Pseudomonas	2	0	0.16785066	1	1	0.70997348	0	2	0.17230830
Corynebacterium	0	1	0.39100222	1	0	0.37390097	0	1	0.35591768
Micrococcus	1	0	0.34343640	0	1	0.34659351	1	0	0.35591768

C-s (Cesarean section); V-r (Vaginal route); Ebf (Exclusive breastfeeding); Non-ebf (Non-exclusive breastfeeding); Hyp (hyperbilirubinemia); Non-hyp (non-hyperbilirubinemia).

*t-test; **p < 0,05 indicates significant difference.
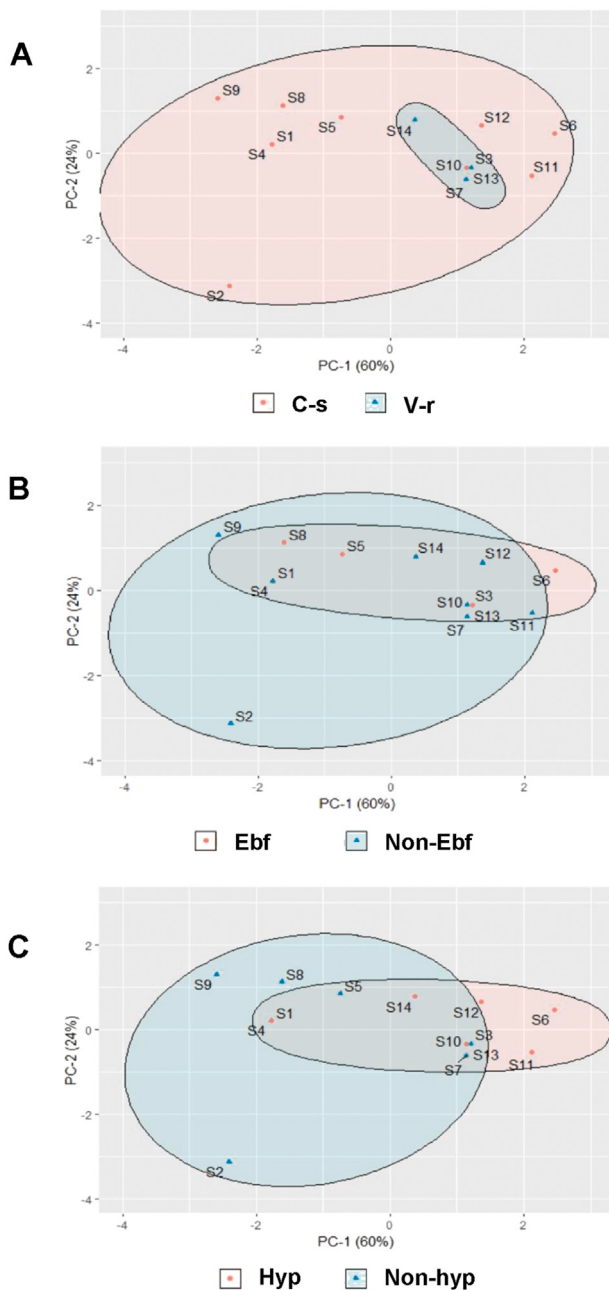
Figure 3. Principle Component Analysis (PCA) of cultivable bacteria composition of neonates' meconium microbiota profile, Cipto Mangunkusumo Hospital (CMH), Jakarta. S1– S14 is the profiles of each subject, which are represented by single point. The analysis was performed at the genus level to understand the clusters between the microbiota profiles of **A**. the mode of delivery, i.e., vaginal route (V-r) and cesarean section (C-s) groups; **B**. the feeding patterns, i.e., exclusive breastfeeding (Ebf) and non-exclusive breastfeeding (Non-ebf) groups; **C**. hyperbilirubinemia (Hyp) and non-hyperbilirubinemia (Non-hyp) groups. The PCs represented the maximum variance of the data. Red circles, **A**. cesarean section; **B**. exclusive breastfeeding; **C**. hyperbilirubinemia; blue triangles, **A**. Vaginal route; **B**. non-exclusive breastfeeding; **C**. non-hyperbilirubinemia.

meconium microbiota, *Staphylococcus sp.,* and *Bacillus sp.* were dominant. However, exclusively breastfed newborns had a more diverse meconium microbiota composed of more bacteria genera such as *Acinetobacter sp., Atlantibacter sp., Enterobacter sp., Klebsiella sp.,* and *Corynebacterium sp.*; this differs from the result of previous studies that showed breastfed newborns had been demonstrated to carry a more stable and uniform population when compared to the formula-fed ones (non-exclusive

breastfeeding) [24]. Moreover, the mode of breastfeeding (direct breastfeeding and indirect breastfeeding) could be assumed as a cause of more diverse meconium microbiota in exclusive breastfeeding (EBF) neonates. *Enterobacteriaceae* and potential pathogens were shown to be enriched with indirect breastfeeding, consistent with culture-dependent studies where pump expression increased the abundance of *Enterobacteriaceae* and other Gram-negative bacteria in milk [25, 26]. However, another study has found no significant differences in gut microbial communities between EBF and non-EBF infants [27]. Our study result could be attributed to the fact that breast milk can alter gut microbiota after delivery to obtain probiotic bacteria that confer health benefits to neonates [4].

Several studies linked the role of gut microbiota to hyperbilirubinemia. Vitek et al. [28] studied the chemistry and metabolism of bilirubin, including the microbiota capable of reducing the unconjugated bilirubin from the catabolism of hemoglobin. Dong et al. [9] reported that breastfeeding could increase the risk of hyperbilirubinemia, and Fuchs et al. [29] reported that antibiotic exposure could lead to hyperbilirubinemia. The use of certain antibiotics (e.g., ceftriaxone) may displace bilirubin from its serum albumin-binding site, resulting in the progression of hyperbilirubinemia [29]. As reported previously, clinical risk factors of older gestational age and greater birth weight show a significant association with a lower incidence of hyperbilirubinemia [30]. Hypothyroid is also one risk factor known to influence hyperbilirubinemia, but from the neonatal screening, none of our samples were found to have abnormal thyroid results [31]. Other possible risk factors for the development of hyperbilirubinemia were not included in the study due to the limited availability of data; initiation of feeding were only ranged as within 24 h, no data on the different hours [32] or the lack of definitive association found from previous studies [32, 33, 34].

In addition, in this study, breastfeeding and antibiotic exposure were also associated with a decreased incidence of hyperbilirubinemia in CMH neonates' population. Breast-feeding is considered a risk factor for hyperbilirubinemia. Thus, it could be proposed that the negative effect is more prominent only when there is a mutation in the UGT1A1 gene [35]. Another study [9] has recently revealed that the microbiota showing *Clostridium perfringens* was more abundant in the hyperbilirubinemia group. The use of antibiotics causes a shift in the gut's microbiome, as reported in a study using an animal model [36]. In this study, we were unable to isolate the anaerobe bacteria *Clostridium perfringens* in any of our samples, which could be probably due to the limitation of growth conditions-handling. Another possibility could be due to the fact that facultative anaerobes are prominent pioneers of intestinal microbiota in the first week of life, i.e., *Staphylococcus*, *Streptococcus*, and *Enterococcus*, and that obligate anaerobes from the Firmicutes, Bacteroidetes, and Actinobacteria phyla take over at the age of 1 month as reported [9, 10, 17] The meconium microbiotic profile of the two different groups (hyperbilirubinemia vs. non-hyperbilirubinemia) did not exhibit many differences, except in the non-hyperbilirubinemia group where *Staphylococcus* is more abundant than in the other group. It appears that the hyperbilirubinemia groups showed a higher data variance than the non-hyperbilirubinemia group toward PC1. Conversely, the non-hyperbilirubinemia group's data variance was higher than the hyperbilirubinemia group's data variance against PC2. The genus *Staphylococcus* contributed most (-0.97408063) to the variance in PC1, whereas *Bacillus* contributed most (-0.92189572) to the variance in PC2.

Gut microbiota is also closely linked with neonatal sepsis and NEC. Microbiota profile of neonates diagnosed with NEC and late-onset sepsis (LOS) were reported to have lower diversity even when compared to preterm infants [37, 38]. Mai et al. [39] reported four phyla (i.e., Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria), which dominated the microbiota of the fecal sample. The proportion of Proteobacteria in those samples showed an increase, with *Firmicutes* found to decrease over the week before the infants would be diagnosed with NEC. *Firmicutes* was dominant in earlier time point of neonates with NEC [39]. Another study [40] found that NEC could be caused by either Firmicutes

dysbiosis or Proteobacteria dysbiosis at different time points where Firmicutes dysbiosis detected in the first 4–9 days and Proteobacteria dysbiosis detected in days 10–16.

In contrast to Mai et al., *Bacillus* and *Solibacillus* (Firmicutes) were over-represented three days before the onset of NEC and LOS, as reported by [38]. This finding supported our data, which found Firmicutes as the most dominant phylum in both groups. LOS patients were found to have a higher abundance of *Klebsiella* in their gut microbiota during LOS progression [38]. The most dominant and infectious bacteria in the hospital environment could cause LOS [38]. However, due to the lack of data and samples for statistical analysis, we cannot analyze the association and causality of the microbiota and both groups.

Interestingly, there is a report of preterm infants with a large cohort study on gestational age at birth. It appears that gestational age influences the pace, but not necessarily the progression of bacterial colonization, and showed a more massive influence compared to other exogenous factors, including antibiotic use, diet, and mode of delivery, as reported by [41]. Commensal microbes also engage in competitive colonization, which provides further protection against pathogen overgrowth. The gut microbiome also biosynthesizes essential vitamins and hormones [42], as well as a range of anti-inflammatory compounds [43, 44, 45, 46]. Hence, to provide more insight into the meconium microbiome of the Indonesian population, the use of more comprehensive specimens is underway in the investigation performing Next Generation Sequencing.

We have several limitations to this study. Firstly is the limited number of samples. We circumvented this issue by employing a PCA analysis instead of a variables analysis. Secondly, the cultivable method that we performed fortunately aided the lack of meconium yielded from the neonates while allowed us to profile the cultivable meconium microbiota. Nevertheless, as a preliminary study, this report serves as an impetus for further investigation, for example, analyzing more samples with a more sophisticated molecular technique (Next-generation sequencing) for a holistic microbiome profile of the meconium.

5. Conclusion

The predomination of Firmicutes' *Staphylococcus* in cultivable bacteria composition of meconium from neonates born at Cipto Mangunkusumo Hospital (CMH), Jakarta, Indonesia, is found similar throughout the clinical parameters used in this study, i.e., mode of delivery, feeding patterns, and hyperbilirubinemia. However, a significantly higher abundance of this genus was exhibited only by the non-hyperbilirubinemia group. Parameters for clinical influence and outcomes such as NEC and sepsis can be optimistically analyzed further to confirm the result by recruiting more neonates in our future study.

Declarations

Author contribution statement

R. Amandito, A. Malik: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

R. Rohsiswatmo: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools, or data; Wrote the paper.

K.J.T. Jonathan, G. Ong, F.A. Prasetyaningsih: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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