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Early colonization by *Enterobacteriaceae* in the developing gut microbiota of preterm infants: A culture-based insight

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Abstract: The gut microbiome plays a significant role in human health and disease pathogenesis. In preterm infants, gut dysbiosis often results in early dominance of opportunistic pathogens, particularly members of the *Enterobacteriaceae* family. *Enterobacteriaceae* can serve as potential reservoirs of opportunistic pathogens, antibiotic-resistance determinants and may contribute to major neonatal morbidities, including necrotizing enterocolitis (NEC) and late-onset sepsis (LOS). This study aims to isolate and identify *Enterobacteriaceae*, as well as to explore the diversity of isolated *Klebsiella pneumoniae* from meconium/early stool samples of preterm infants collected from a neonatal intensive care unit (NICU) in Johor Bahru, Malaysia. A combination of culture-based isolation technique, phenotypic and genotypic identification methods was used to identify the culturable *Enterobacteriaceae* in the gut of preterm infants. Three main bacterial families were successfully isolated and identified from the stool samples of preterm infants, which include *Enterobacteriaceae*, 46% (76/166 isolates), *Staphylococcaceae*, 22% (37/166), *Enterococcaceae*, 22% (37/166) and others, 10% (16/166). It was clear that *Enterobacteriaceae* was one of the predominant bacterial groups during the early gut colonization in preterm infants, making up to 46% of all isolates. Within the *Enterobacteriaceae* family, *K. pneumoniae*, 74% (56/76 isolates) dominates, followed by *Escherichia coli*, 15% (11/76), *Klebsiella aerogenes*, 9% (7/76), *Citrobacter europaeus*, 1% (1/76), and *Citrobacter freundii*, 1% (1/76). The high number of *Enterobacteriaceae* isolates, particularly *K. pneumoniae*, followed by *E. coli* are concerning as these bacterial species are often associated with hospital-acquired infections in the NICU. Our findings provide insight into the early gut colonization patterns of culturable gut bacteria in preterm infants. It highlights the importance of continuous microbiological monitoring and infection control measures within the NICU to mitigate potential *Enterobacteriaceae*-associated infections.

Keywords: *Enterobacteriaceae*, Preterm infants, Stool, *Klebsiella pneumoniae*, Gut microbiome

1. Introduction

The gut microbiome is a complex microbial ecosystem expressing 100 times more genes than the human host and plays a significant role in human health and disease pathogenesis [1–3]. The gut microbiota have various functions including, metabolism and energy regulation [2, 4, 5], fermentation of polysaccharides to short-chain fatty acids [2, 4, 5], maturation and regulation of the immune system [6, 7], absorption of minerals and nutrients [8, 9], synthesis of vitamins [10–12], and potential to breakdown of toxic components [13–15]. Hence, they are important contributors to human health. Dysbiosis of the gut microbiota composition is associated with various diseases, including cancers [16–18], cardiovascular diseases [19–21], immunity-related diseases [22–24], neurodevelopmental disorders [23, 25], obesity and diabetes [26–32].

The gut microbiota of an infant is formed in the early life, right after birth (first 1,000 days) [33, 34]. Preterm infants are those born prematurely at < 37 completed weeks of gestation and are often of low birth weight (< 2.5kg), whereas term infants are born at 40 weeks gestational age [35, 36]. Compared to term infants, preterm infants are highly vulnerable and exhibit distinct gut microbiome colonization patterns [37–39]. Interestingly, Khan *et al.* [40] found that the colonization of fecal microbiota in preterm infants is very dynamic across different time points after birth. According to Sim *et al.* [41], at 6 weeks of age, the gut microbiome of term infants shows a significant increase in *Bacteroides*, *Bifidobacterium*, and *Lachnospiraceae*, while preterm infants show a significant increase in *Enterobacteriaceae* and *Enterobacter*. Factors such as the mode of delivery [42], immature gut [39, 43, 44], exposure to antibiotics [39, 44–50], extended hospital stay [39, 51], and limited feeding of breastmilk [39, 47, 52] contribute to a microbiome characterized by low diversity, delayed establishment of beneficial bacteria (*Bifidobacterium*), and early dominance of Proteobacteria, particularly the family *Enterobacteriaceae* [38, 39, 41, 43, 48, 49]. Although factors such as mode of delivery [42, 53–57] and gestational age [44, 51] are commonly associated with gut dysbiosis in infants, these effects may be temporary; for instance, the microbiota of infants born via caesarean section may eventually resemble that of infants born vaginally [53, 57]. Additionally, the effects of gestational age may be influenced by other factors such as gut immaturity, longer hospitalization, and increased antibiotic exposure, which are more pronounced in preterm infants with lower gestational age than in those with higher gestational age [44]. Gut dysbiosis during the early life has been associated with the development of metabolic and immunological diseases, for instance, asthma [58, 59], allergies [60, 61], and type I diabetes [62–65]. In preterm infants, necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) are two lethal complications closely linked with gut dysbiosis [66, 67].

Enterobacteriaceae have a major impact on clinical and public health, such as causing foodborne disease and outbreaks, and healthcare-associated infections [68]. Importantly, it is one of the bacterial family that dominates the gut of preterm infants during their first months of life [69]. Members of the *Enterobacteriaceae*, including *Citrobacter*, *Enterobacter*. *Escherichia*, and *Klebsiella* are among the first colonizers of the preterm infant gut [35, 40, 69]. While some bacteria are commensal, others are opportunistic pathogens capable of translocating across the immature intestinal barrier to organs and tissues. In combination with preterm infants' immature immune system, this consequently increases the risk for systemic infections [70, 71]. Besides that, *Enterobacteriaceae* can serve as potential reservoirs of opportunistic pathogens, antibiotic-resistance determinants, and may contribute to major neonatal morbidities, including NEC and LOS [69, 72–74]. Furthermore, bacteria under the *Enterobacteriaceae* family have high levels of antibiotic-resistant genes and are able to

facilitate the transfer of these antibiotic-resistant genes via mobile genetic elements to other pathogens [75].

With these clinical implications, the aim of this study was to isolate and identify *Enterobacteriaceae* from meconium/early stool samples of preterm infants admitted to the neonatal intensive care unit (NICU) in Johor Bahru, Malaysia. The diversity of *Klebsiella pneumoniae* isolated from the stool samples of preterm infants was further explored. A combination of culture-based isolation techniques, phenotypic and molecular identification methods was used to determine the composition of culturable *Enterobacteriaceae* in the gut of preterm infants.

2. Materials and Methods

2.1. Collection and processing of preterm infants' meconium/early stool sample

A total of 28 meconium/early stool samples were collected from preterm infants (N= 28, gestational age < 33 weeks, birth weight < 1500g) admitted to the NICU of Hospital Sultanah Aminah Johor Bahru, Malaysia (Research no. NMRR-19-3773-52331 (IIR)). This was a non-interventional study conducted with informed consent and no exclusion criteria was applied to the preterm infants. The collection of meconium/early stool samples was conducted during their stay in the NICU according to approved ethics guidelines. The stool samples were placed in sterile containers, transported on dry ice to the laboratory at the Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, and immediately stored at -80 °C until further analysis.

2.2. Isolation and purification of presumptive Enterobacteriaceae isolates

To isolate the *Enterobacteriaceae*, the meconium/early stool samples were diluted (1:10 w/v) in phosphate-buffered saline (dilution factor: 10^{-1}─10^{-5}) and inoculated onto CHROMagar™ Orientation plates based on the existing protocol with slight modification [76]. The plates were incubated overnight under aerobic conditions at 37 °C. The presumptive isolates were selected according to the colony morphology, with representative colonies selected based on the distinct color group presented on CHROMagar™ Orientation plates [77]. The presumptive colonies were purified by streaking onto Tryptic Soya Agar (TSA) (HiMedia, India) plates and incubated overnight under aerobic conditions at 37 °C. The purified colonies were streaked onto sterile TSA slant and stored at 4 °C until further genotypic identification.

2.3. DNA extraction

Genomic DNA extraction of the presumptive *Enterobacteriaceae* clinical isolates were carried out via a commercial DNA extraction kit (GF-1 Bacterial DNA Extraction Kit, Vivantis, Malaysia; Catalog No. BA-100) according to manufacturer's protocol. One loopful of each isolates was picked from the respective TSA slants and inoculated into Tryptone Soya Broth (TSB) (HiMedia, India), and incubated overnight at 37 °C, at 200rpm. Approximately 1-3ml of overnight culture was centrifuged at 6,000g for 2 minutes, and the supernatant was discarded. The pellet was resuspended with 100µl Buffer R1, centrifuged at 10,000g for 3 minutes, and the supernatant was discarded. The pellet was resuspended in 180µl of Buffer R2 and 20µl of Proteinase K, then incubated at 65 °C for 30 minutes in a water bath with occasional mixing every 5 minutes. After incubation, 400µl of Buffer BG was added, and the tube was inverted several times to achieve a homogenous solution. The tubes were subsequently incubated at 65 °C for 10 minutes. Absolute ethanol (200µl) was added with immediate mixing to prevent precipitation of DNA and transferred (maximum volume of 650µl) into a clean column and centrifuged at 10,000g for 1 minute. The flow through was discarded and 650µl of Wash Buffer was added to the column and centrifuged at 10,000g for 1 minute, in which the flow through was discarded. The column was centrifuged again at 10,000g for 1 minute to remove residual ethanol. The column was then placed into a clean microcentrifuge tube and 100µl of sterile water was added, and the tube was left to stand for 2 minutes. The DNA was then eluted via centrifugation at 10,000g for 1 minute. The DNA was then stored for further analysis at -20 °C.

2.4. Genotypic identification of Enterobacteriaceae isolates based on 16S rRNA gene sequencing

The 16S rRNA-based PCR assay was performed to identify the presumptive isolates. Two primers were used for 16S rRNA-based PCR assay: p27F forward primer (5'-AGA GTT TGA TCC TGG CTC AG -3') and p1492R reverse primer (5'-GGT TAC CTT GTT ACG ACT T -3'). The expected amplicon size is 1,500bp. A final volume of 20µl for the reaction mixture was prepared with 1µl of DNA template, 10µl of 2X *Taq PLUS* PCR Smart mix 1 (SolGent™, Korea), 7µl of sterile distilled water and 1µl of each primer. PCR amplification was performed using thermocycler (Kyratec, Super Cycler Thermal Cycler, Australia) with the following conditions: initial denaturation at 95°C for 5 minutes, 35 cycles of 94°C for 50 seconds, 55°C for 1 minute, and 72°C for 1 minute 30 seconds, and a final elongation at 72°C for 8 minutes. The PCR products were then separated in 1.5% agarose gel and visualized under a gel documentation system (ChemiDoc™ XRS, Bio-Rad, USA) before Sanger

sequencing. After that, the retrieved 16S rRNA gene sequences were manually trimmed using BioEdit Sequence Alignment Editor Software [78], and then submitted to BLAST (National Center for Biotechnology Information of the National Library of Medicine) for the identification of bacterial isolates based on the calculation of levels of sequence similarity.

2.5. Phylogenetic analysis of 16S rRNA gene sequences of K. pneumoniae: Evolutionary relationships of taxa

The 16S rRNA gene sequences of the *K. pneumoniae* isolates were utilized to build a phylogenetic tree with reference to the protocol devised by Lee *et al.* [79] and Law *et al.* [80]. The 16S rRNA gene sequences obtained were aligned with representative sequences of closely related strains in the genus *Klebsiella* obtained from GenBank/EMBL/DDBJ databases using CLUSTAL-X software [81]. The alignment was manually verified and adjusted before the reconstruction of phylogenetic tree. Phylogenetic tree was reconstructed with neighbor-joining algorithm using MEGA version 7.0 [82], and the evolutionary distances for this algorithm were computed using Kimura's two-parameter model [83]. The stabilities of the resultant tree topologies were analyzed through bootstrap analysis based on 1000 resampling method of Felsenstein (1985) [84].

3. Results

3.1. Isolation of presumptive Enterobacteriaceae isolates from meconium/early stool of preterm infants based on colony morphology

In the present study, presumptive *Enterobacteriaceae* isolates were isolated from 28 meconium/early stool samples of preterm infants (gestational age < 33 weeks, birth weight < 1500g) obtained from the NICU of Hospital Sultanah Aminah Johor Bahru. The stool samples used in our study were mainly collected from week 1. In cases where the stool sample was insufficient, stool samples collected during week 2 or week 3 were used. A total of 166 isolates were obtained from 23 stool samples based on colony morphology selection on CHROMagar™ Orientation plates. The remaining 5 stool samples yielded no growth. From the 166 isolates obtained, the observed colony colors on CHROMagar™ Orientation plates include metallic blue (55 isolates), turquoise blue (38 isolates), white (56 isolates), dark pink to reddish (7 isolates), metallic blue with red halo (3 isolates), pink (3 isolates), and light grey (4 isolates). The majority of isolates observed were white, followed by metallic blue and turquoise blue. Among the colony colors observed, dark pink to reddish, metallic blue with red halo, pink, and light grey were only observed in a few of the isolates (Table 1).

Table 1: List of stool samples, isolates, and colony morphology description.

Stool sample no.	Total number of isolates obtained*		Colony morphology
1	**3**	3	White, Circular
2	**6**	4	White, Punctiform
		1	White, Circular
		1	Turquoise blue, Circular
3	**8**	8	Metallic blue, Circular
4	**15**	11	Turquoise blue, Circular
		4	White, Punctiform
5	**14**	5	Metallic blue, Circular
		8	White, Circular
		1	White, Irregular
6	**13**	3	Metallic blue, Irregular
		10	Metallic blue, Circular
7	**10**	2	Metallic blue, Circular
		3	White, Circular
		5	Turquoise blue, Circular
8	**3**	3	Metallic blue, Circular
9	**9**	9	Metallic blue, Circular
10	**9**	4	Turquoise blue, Circular
		3	Dark pink to reddish, Irregular
		2	White, Circular
11	**10**	3	Metallic blue, Circular
		3	Turquoise blue, Circular
		3	Pink, Punctiform
		1	Dark pink to reddish, Punctiform
12	**5**	2	Turquoise blue, Circular
		3	Dark pink to reddish, Circular
13	**1**	1	White, Punctiform
14	**3**	3	Metallic blue, Circular
15	**9**	3	Metallic blue, Circular
		6	Turquoise blue, Circular
16	**8**	6	Turquoise blue, Circular
		2	White, Circular
17	**6**	5	White, Circular

		1	White, Punctiform
18	**5**	3	Metallic blue, Circular
		2	Metallic blue with red halo, Irregular
19	**7**	3	White, Circular
		3	Metallic blue, Circular
		1	Metallic blue with red halo, Circular
20	**5**	2	White, Circular
		3	White, Punctiform
21	**12**	8	White, Circular
		4	Light Grey, Circular
22	**4**	4	White, Circular
23	**1**	1	White, Circular

***Total isolates: 166**

3.2. Genotypic identification of isolates based on 16S rRNA gene

Upon submitting the retrieved 16S rRNA gene sequence of the 166 isolates to BLAST (National Center for Biotechnology Information of the National Library of Medicine) that identifies isolates based on the calculation of levels of pairwise sequence similarity, the majority of the identified isolates belonged to the family *Enterobacteriaceae*, 46% (76/166 isolates), followed by *Staphylococcaceae*, 22% (37/166), and *Enterococcaceae*, 22% (37/166). The remaining 16 isolates belonged to others, 10% (16/166). Among the *Enterobacteriaceae* family, 56 out of the 76 isolates were identified as *K. pneumoniae* (91.32%–100% pairwise sequence similarity) while the remaining isolates consisted of 11 *Escherichia coli* (99.58%–100%), 7 *Klebsiella aerogenes* (99.28%–100%), 1 *Citrobacter europaeus* (99.79%), and 1 *Citrobacter freundii* (99.36%). On the other hand, the identities of the 37 *Staphylococcus* spp. were *Staphylococcus epidermidis* (15/166 isolates; 99.5%–100% pairwise sequence similarity), *Staphylococcus haemolyticus* (11/166; 99.86%–100%), *Staphylococcus* spp. (9/166; 99.86%–100%), *Staphylococcus hominis* (1/166; 100%), and *Staphylococcus warneri* (1/166; 99.93%), while the identities for the 37 *Enterococcus* spp. consisted of *Enterococcus faecalis* (21/166; 98.4%–100%), *Enterococcus faecium* (12/166; 99.37%–100%), *Enterococcus avium* (3/166; 98.99%–100%), and *Enterococcus* spp. (1/166; 99.82%). Lastly, the remaining other isolates were identified as *Streptococcus agalactiae* (6/166 isolates; 98.99%–100% pairwise sequence similarity), *Corynebacterium* spp. (5/166; 99.5%–100%), *Burkholderia multivorans* (4/166; 99.72%–100%), and *Niallia* spp. (1/166; 100%) (Table 2).

Table 2: The identity of isolates from preterm stool samples based on 16S rRNA gene sequence similarity.

Stool sample no.	Isolates (MPB)	Color of bacterial colony	Identity (16S rRNA gene sequence similarity %)
1	158	White, Circular	*Staphylococcus* spp. (99.93%)
	160	White, Circular	*Staphylococcus* spp. (99.86%)
	163	White, Circular	*Staphylococcus epidermidis* (99.93%)
2	164	White, Punctiform	*Corynebacterium* spp. (100%)
	166	White, Circular	*Staphylococcus epidermidis* (100%)
	171	White, Punctiform	*Corynebacterium* spp. (99.93%)
	172	White, Punctiform	*Corynebacterium* spp. (99.93%)
	173	White, Punctiform	*Corynebacterium* spp. (99.68%)
	174	Turquoise blue, Circular	*Niallia* spp. (100%)
3	12	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
	13	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
	14	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.84%)
	15	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	16	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.86%)
	17	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	18	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	19	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.84%%)
4	23	Turquoise Blue, Circular	*Enterococcus faecium* (100%)
	24	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	25	White, Punctiform	*Enterococcus avium* (100%)
	26	White, Punctiform	*Enterococcus avium* (98.99%)
	27	White, Punctiform	*Enterococcus faecium* (99.86%)
	28	Turquoise blue, Circular	*Enterococcus faecium* (100%)
	29	Turquoise blue, Circular	*Enterococcus faecium* (99.79%)
	30	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
	31	White, Punctiform	*Enterococcus avium* (99.36%)
	32	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
	33	Turquoise Blue, Circular	*Enterococcus faecium* (100%)
	34	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
	35	Turquoise Blue, Circular	*Enterococcus faecium* (99.86%)
	36	Turquoise blue, Circular	*Enterococcus faecalis* (99.86%)
	37	Turquoise Blue, Circular	*Enterococcus faecium* (99.37%)
5	1	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	2	Metallic Blue, Circular	*Klebsiella pneumoniae* (98.39%)
	3	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.57%)
	4	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.57%)

	5	White, Circular	*Staphylococcus epidermidis* (100%)
	6	White, Circular	*Staphylococcus epidermidis* (99.5%)
	7	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.22%)
	8A	White, Circular	*Klebsiella pneumoniae* (99.79%)
	8B	White, Circular	*Staphylococcus epidermidis* (100%)
	9A(i)	White, Circular	*Klebsiella pneumoniae* (97.99%)
	9A(ii)	White, Circular	*Klebsiella pneumoniae* (100%)
	9B	White, Circular	*Staphylococcus epidermidis* (100%)
	10	White, Circular	*Staphylococcus hominis* (100%)
	11	White, Irregular	*Staphylococcus epidermidis* (100%)
6	95	Metallic Blue, Irregular	*Klebsiella pneumoniae* (100%)
	96	Metallic Blue, Irregular	*Klebsiella pneumoniae* (100%)
	97	Metallic Blue, Irregular	*Klebsiella pneumoniae* (100%)
	98	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	99	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.5%)
	100	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	101	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
	102	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	103	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	104	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	105	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
	106	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.28%)
	107	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
7	138	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	140	White, Circular	*Staphylococcus haemolyticus* (100%)
	141	White, Circular	*Staphylococcus haemolyticus* (100%)
	142	White, Circular	*Staphylococcus epidermidis* (100%)
	143	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	144	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
	145	Turquoise blue, Circular	*Enterococcus faecalis* (99.93%)
	146	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
	148	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
	149	Turquoise blue, Circular	*Enterococcus faecalis* (100%)
8	150	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	151	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	152	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
9	41	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	42	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	43	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	44	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.92%)

	45	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	46	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	48	Metallic Blue, Circular	*Klebsiella pneumoniae* (91.32%)
	49	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	50	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
10	51	Turquoise Blue, Circular	*Klebsiella aerogenes* (100%)
	52	Turquoise Blue, Circular	*Klebsiella aerogenes* (99.54%)
	53	Dark Pink to Reddish, Irregular	*Escherichia coli* (100%)
	54	Dark Pink to Reddish, Irregular	*Escherichia coli* (100%)
	55	Dark Pink to Reddish, Irregular	*Escherichia coli* (99.78%)
	56	White, Circular	*Escherichia coli* (99.58%)
	58	Turquoise Blue, Circular	*Klebsiella aerogenes* (99.28%)
	59	White, Circular	*Staphylococcus haemolyticus* (99.86%)
	60	Turquoise Blue, Circular	*Klebsiella aerogenes* (99.93%)
11	139	Metallic Blue, Circular	*Klebsiella aerogenes* (100%)
	147	Metallic Blue, Circular	*Klebsiella aerogenes* (100%)
	153	Metallic Blue, Circular	*Klebsiella aerogenes* (100%)
	154	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	155	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	156	Turquoise Blue, Circular	*Enterococcus faecalis* (99.93%)
	157A(i)	Pink, Punctiform	*Escherichia coli* (100%)
	157A(ii)	Pink, Punctiform	*Escherichia coli* (100%)
	159A(i)	Pink, Punctiform	*Escherichia coli* (100%)
	161	Dark Pink to Reddish, Punctiform	*Escherichia coli* (100%)
12	162	Turquoise Blue, Circular	*Enterococcus faecalis* (99.93%)
	167	Dark Pink to Reddish, Circular	*Escherichia coli* (100%)
	168	Dark Pink to Reddish, Circular	*Escherichia coli* (100%)
	169	Dark Pink to Reddish, Circular	*Escherichia coli* (100%)
	170	Turquoise Blue, Circular	*Enterococcus faecalis* (98.4%)
13	181	White, Punctiform	*Corynebacterium* spp. (99.5%)
14	175	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	176	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	177	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
15	108	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	109	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	110	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	111	Turquoise Blue, Circular	*Enterococcus faecium* (100%)
	112	Turquoise Blue, Circular	*Enterococcus faecium* (99.65%)

	113	Turquoise Blue, Circular	*Enterococcus* spp. (99.82%)
	114	Turquoise Blue, Circular	*Enterococcus faecium* (99.93%)
	115	Turquoise Blue, Circular	*Enterococcus faecium* (99.37%)
	116	Turquoise Blue, Circular	*Enterococcus faecium* (99.91%)
16	117	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	118	Turquoise Blue, Circular	*Enterococcus faecalis* (99.09%)
	119	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	120	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	121	Turquoise Blue, Circular	*Enterococcus faecalis* (99.86%)
	122	Turquoise Blue, Circular	*Enterococcus faecalis* (100%)
	125	White, Circular	*Staphylococcus haemolyticus* (99.86%)
	126	White, Circular	*Staphylococcus haemolyticus* (100%)
17	123	White, Circular	*Streptococcus agalactiae* (100%)
	124	White, Circular	*Streptococcus agalactiae* (99.93%)
	127	White, Circular	*Streptococcus agalactiae* (99.93%)
	128	White, Circular	*Streptococcus agalactiae* (99.35%)
	129	White, Circular	*Streptococcus agalactiae* (99.93%)
	130	White, Punctiform	*Streptococcus agalactiae* (98.99%)
18	83	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.93%)
	84	Metallic Blue, Circular	*Klebsiella pneumoniae* (99.86%)
	85	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	86	Metallic Blue with Red Halo, Irregular	*Citrobacter europaeus* (99.79%)
	87	Metallic Blue with Red Halo, Irregular	*Citrobacter freundii* (99.36%)
19	88	White, Circular	*Staphylococcus epidermidis* (100%)
	89	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	90	White, Circular	*Staphylococcus* spp. (100%)
	91	Metallic Blue with Red Halo, Circular	*Klebsiella pneumoniae* (99.93%)
	92	White, Circular	*Staphylococcus haemolyticus* (100%)
	93	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
	94	Metallic Blue, Circular	*Klebsiella pneumoniae* (100%)
20	131	White, Circular	*Staphylococcus* spp. (100%)
	133	White, Punctiform	*Staphylococcus* spp. (99.93%)
	134	White, Punctiform	*Staphylococcus* spp. (100%)
	135	White, Punctiform	*Staphylococcus* spp. (100%)
	136	White, Circular	*Staphylococcus* spp. (100%)
21	63	White, Circular	*Staphylococcus haemolyticus* (99.93%)
	64	White, Circular	*Staphylococcus haemolyticus* (99.93%)

	65	Light Grey, Circular	*Burkholderia multivorans* (99.72%)
	66	Light Grey, Circular	*Burkholderia multivorans* (99.86%)
	67	White, Circular	*Staphylococcus epidermidis* (99.86%)
	68	White, Circular	*Staphylococcus epidermidis* (100%)
	69	White, Circular	*Staphylococcus* spp. (99.93%)
	70	Light Grey, Circular	*Burkholderia multivorans* (100%)
	71	Light Grey, Circular	*Burkholderia multivorans* (99.85%)
	72	White, Circular	*Staphylococcus haemolyticus* (100%)
	73	White, Circular	*Staphylococcus haemolyticus* (99.93%)
	74	White, Circular	*Staphylococcus haemolyticus* (99.93%)
22	75	White, Circular	*Staphylococcus epidermidis* (99.93%)
	76	White, Circular	*Staphylococcus epidermidis* (99.93%)
	77	White, Circular	*Staphylococcus epidermidis* (99.58%)
	78	White, Circular	*Staphylococcus epidermidis* (100%)
23	137	White, Circular	*Staphylococcus warneri* (99.93%)

3.3. Diversity of K. pneumoniae isolates derived from meconium/early stool samples of preterm infants

The 16S rRNA gene sequences of the 56 *K. pneumoniae* isolates were compared with closely related strains retrieved from DDBJ/EMBL/GenBank, and the results displayed percentages of pairwise sequence similarity ranging from 91.32% to 100%. Their 16S rRNA gene sequences were utilized for the reconstruction of a neighbor-joining phylogenetic tree consisting of a total of 86 *Klebsiella* isolates together with their closely related strains to understand their taxonomic relationships (Figure 1).

Neighbor-joining phylogenetic tree constructed based on 16S rRNA gene sequences revealed 20 major clades, designated as Clade A-T; with 15 *Klebsiella* isolates were assigned to Clade A, 3 isolates were assigned to Clade B, 15 isolates assigned to Clade C, 13 isolates were assigned to Clade D, 2 isolates were assigned to Clade E, 2 isolates were assigned to Clade F, 2 isolates were assigned to Clade G, 2 isolates were assigned to Clade H, 6 isolates were assigned to Clade I, 2 isolates were assigned to Clade J, 2 isolates were assigned to Clade K, 2 isolates were assigned to Clade L, 4 isolates were assigned to Clade M, 4 isolates were assigned to Clade N, 2 isolates were assigned to Clade O, 2 isolates were assigned to Clade P, 2 isolates were assigned to Clade Q, 2 isolates were assigned to Clade R, 2 isolates were assigned to Clade S, and 2 isolates were assigned to Clade T (Figure 1).

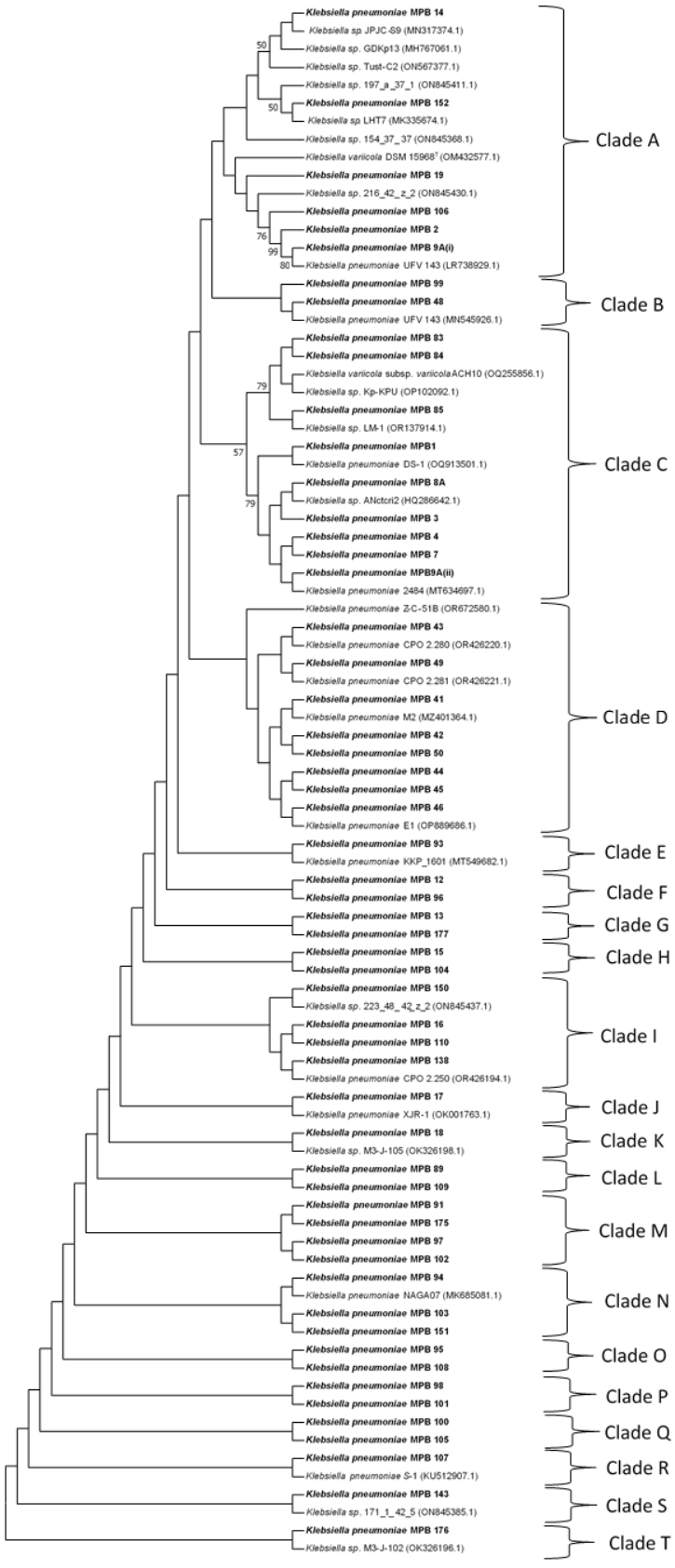

Figure 1. Neighbor-joining phylogenetic tree based on 998 nucleotides of 16S rRNA gene sequence showing the relationship between a total of 56 *K. pneumoniae* isolates and representatives of related taxa. Numbers and nodes indicate percentages (> 50 %) of 1000 bootstrap re-sampling.

Isolates with bootstrap value > 50% were mostly in Clade A and Clade C. Six *K. pneumoniae* isolates were present in Clade A, including MPB 14, MPB 152, MPB 19, MPB 106, MPB 2, and MPB 9A(i), while 9 isolates were present in Clade C, including MPB 83, MPB 84, MPB 85, MPB 1, MPB 8A, MPB 3, MPB 4, MPB 7, and MPB 9A(ii). The isolates assigned to Clade A were derived from 4 different stool samples: MPB 14 and MPB 19 (stool sample 3), MPB 152 (stool sample 8), MPB 106 (stool sample 6), MPB 2 and MPB 9A(i) (stool sample 5). The isolates assigned to Clade C were derived from 2 different stool samples: MPB 83, MPB 84, and MPB 85 (stool sample 18), while MPB 1, MPB 8A, MPB 3, MPB 4, MPB 7, and MPB 9A(ii) (stool sample 5). Interestingly, all 3 *K. pneumoniae* isolates isolated from stool sample 18 were detected in Clade C and all 8 *K. pneumoniae* isolates isolated from stool sample 5 were detected in Clade A and Clade C.

In Clade A, there were a total of 6 closely related strains: *Klebsiella* sp. strain JPJC-S9 was isolated from a domestic wastewater treatment plant in Jaipur, India [85]; *Klebsiella* sp. strain GDKp-13 was isolated from the surfaces of rice seeds in Guangzhou, China [86]; *Klebsiella* sp. strain LHT7 was isolated from rice paddy field mud surrounding the rice root in Vietnam [87]; *Klebsiella* sp. strain 197_a_37_1, *Klebsiella* sp. strain 154_37_37, and *Klebsiella* sp. strain 216_42_z_2 were isolated from environmental samples (wastewater, treated wastewater, river water) in Japan [88]. The constructed phylogenetic tree revealed that *Klebsiella* sp. strain JPJC-S9 clustered within Clade A alongside MPB 14, while *Klebsiella* sp. strain LHT7 grouped with MPB 152.

In Clade C, there were a total of 4 closely related strains: *Klebsiella variicola* subsp. *variicola* strain ACH10 was isolated from blueberry rhizosphere in Spain [89]; *Klebsiella* sp. strain Kp-KPU was isolated from clinical samples [90]; *K. pneumoniae* strain DS-1 was isolated from petrochemical wastewater in Jilin province, China [91]; *K. pneumoniae* strain 2484 was isolated from a cheese stall in a public bazaar in Turkey [92]. *K. pneumoniae* strain DS-1 was clustered with MPB 1, while *K. pneumoniae* strain 2484 was grouped with MPB 9A(ii).

In Clade D, there were a total of 4 closely related strains, which includes *K. pneumoniae* strain Z-C-51B that was isolated from oil palm biomass waste in Indonesia [93]; *K. pneumoniae* strain CPO 2.280 and *K. pneumoniae* strain CPO 2.281, both of which were associated with crown gall disease on raspberry (*Rubus idaeus*) according to Genbank records; *K. pneumoniae* strain M2 was isolated from a patient with urinary tract infection in India, and is associated with a Genbank record citing Desai *et al.* [94] as the reference. *K. pneumoniae* strain M2 formed a monophyletic clade with MPB 41. *K. pneumoniae* strain

CPO 2.280 clustered with MPB 43, while *K. pneumoniae* strain CPO 2.281 grouped with MPB 49.

Clade I has 2 closely related strains, while Clade K, Clade R, Clade S, and Clade T each have 1 closely related strain. *K. pneumoniae* strain CPO 2.250 (Clade I) was associated with crown gall disease on raspberry (*Rubus idaeus*) according to Genbank record. This strain clustered with MPB 138. Both *Klebsiella* sp. strain 223_48_42_z_2 (Clade I) and *Klebsiella* sp. strain 171_1_42_5 (Clade S) were isolated from environmental samples (wastewater, treated wastewater, river water) in Japan [88]. *Klebsiella* sp. strain 223_48_42_z_2 formed a monophyletic clade with MPB 150, while *Klebsiella* sp. strain 171_1_42_5 was claded with MPB 143. Meanwhile, *Klebsiella* sp. strain M3-J-105 and *Klebsiella* sp. strain M3-J-102 were isolated from the human gut microbiome according to Genbank records. The former was clustered with MPB 18 in Clade K, while the latter was grouped with MPB 176 in Clade T. Lastly, *K. pneumoniae* strain S-1 (Clade R) was isolated from agricultural field soil of Banaras Hindu University, India [95]. This strain formed a monophyletic clade with MPB 107.

4. Discussion

The gut microbiome is a complex microbial ecosystem expressing 100 times more genes than the human host and plays a significant role in human health and disease pathogenesis [1]. Interestingly, the gut microbiome has been associated with the role of probiotics for health and the prevention and/or treatment of various diseases in recent years [96–105]. The gut microbiome has various functions, including absorption of minerals and nutrients, synthesis of vitamins, fermentation of fibers to short-chain fatty acids, breakdown of toxic components, and regulation of the immune system [1]. Importantly, the gut microbiome of preterm infants differs from that of term infants [106, 107]. Aujoulat *et al.* [108] demonstrated that *Staphylococcus*, *Enterococcus*, and members of *Enterobacteriaceae* successively dominated the gut microbiota of very preterm infants. In addition, according to findings by Korpela *et al.* [109], with increasing postmenstrual age, the development of microbiota in preterm infants progressed from *Staphylococcus*-*Enterococcus*-dominated composition to *Enterobacter* and lastly towards *Bifidobacterium*-dominated microbiota. Interestingly, the authors found that in preterm infants, *Enterobacteriaceae* peaks on average at 35 weeks postmenstrual age, while term-born infants presented only the declining part of this successional phase and a modest secondary bloom at 45–50 postmenstrual age [109]. This suggests that the gut microbiota of preterm infants develops in a distinct, age-dependent sequence and differs from that of term infants in both composition and developmental trajectory.

Our study successfully isolated 166 bacterial isolates from 28 meconium/early stool samples of preterm infants (gestational age < 33 weeks, birth weight < 1500g) obtained from the NICU of Hospital Sultanah Aminah Johor Bahru (Table 1). The gut microbiome of preterm infants is affected by many factors. Due to the fact that preterm infants have a lower gestational age, are more likely to be delivered via caesarean section, fed with formula milk, exposed to antibiotic treatment, and have long exposure in an ICU environment, together these factors contribute to a bacterial community rich in facultative anaerobes (including *Enterobacteriaceae*) [110]. Among these 166 isolates, 76 gram-negative isolates (46%) belonged to the *Enterobacteriaceae* family. *Enterobacteriaceae* have a major impact on clinical and public health, such as causing foodborne disease and outbreaks, and healthcare-associated infections [68]. Interestingly, most genera within the *Enterobacteriaceae* family have a G+C content of 49 to 59 mol% [68]. Several significant pathogens of animals and humans belonging to *Enterobacteriaceae* include species of *Cronobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Kluyvera*, *Klebsiella*, *Raoultella*, *Shigella*, and *Salmonella* [111].

In this study, the preliminary isolation of presumptive *Enterobacteriaceae* isolates from preterm infants' stool samples was carried out by inoculation of stool samples onto CHROMagar™ Orientation plates, and colonies were selected based on colony morphology, with particular attention to colony color . The reason for using CHROMagar™ Orientation as the primary screening medium is that it is a non-selective chromogenic culture medium intended for use in the qualitative direct detection, differentiation, and presumptive identification of pathogens based on colony color [77]. Furthermore, the differences in colony colors produced by microorganisms on the medium allow easier recognition of mixed growth [77]. There were other studies that also utilized CHROMagar™ Orientation for differentiation and presumptive identification of gram-negative bacteria [76, 112]. Based on the colony appearance guide provided by CHROMagar™ Orientation [77], the colony appearance of *Klebsiella*, *Enterobacter*, *Serratia* appear metallic blue; *Enterococcus* appears turquoise blue; *E. coli* appears dark pink to reddish; *Citrobacter* appears metallic blue with red halo; *Streptococcus agalactiae* appears light blue; *Candida albicans* appears colorless; *Pseudomonas aeruginosa* appears translucide, cream to blue; *Proteus* is characterized by a brown halo; *Staphylococcus saprophyticus* appears pink, opaque, small; and *Staphylococcus aureus* appears golden, opaque, small. From the 166 isolates obtained in our study, the observed colony colors include metallic blue, turquoise blue, white, dark pink to reddish, metallic blue with red halo, pink, and light grey. However, among these colors, dark red to reddish, metallic blue with red halo, pink, and light grey were only observed in few of the isolates compared to the other colors mentioned.

After obtaining the pure isolates, the genotypic identification was performed based on the 16S rRNA gene. The 16S rRNA gene is an essential constituent present in all bacteria, and it can be used to distinguish the 3 main kingdoms- *Archaea*, *Bacteria*, and *Eukarya* [113]. The 16S rRNA gene is the gold standard in the identification of taxonomic and phylogenetic relationships among different bacteria [113]. This gene contains highly conserved regions and variable regions with approximately 1600 nucleotides long (base pairs) [113]. Conserved regions are used as tools to study distant phylogenetic relationships, while regions between the conserved regions with higher mutation rates are used to discriminate closely-related bacteria [113]. There are also hypervariable regions, for example gamma region, that diverged over evolutionary time, which can be used for species discrimination [113]. Importantly, horizontal gene transfer events are usually implausible to happen within the highly constrained rRNA genes [113]. The comparison of the 16S rRNA gene sequences allows for the differentiation between organisms at the genus level across all major phyla of bacteria, and also it can classify strains at the species and subspecies level [114].

Based on our results (Table 2), 63 isolates have been identified belonging to the genus *Klebsiella* based on 16S rRNA gene, but only 55 out of 63 isolates (87.30%) were in accordance with the colony color metallic blue on CHROMagar™ Orientation, while the colony colors of the other isolates appeared as white (3 isolates), turquoise blue (4 isolates,), and metallic blue with red halo (1 isolate). There were 11 isolates identified as *E. coli*, but only 7 out of 11 isolates (63.64%) were in accordance with the colony color dark pink to reddish on CHROMagar™, while 3 isolates appeared as pink colonies and 1 isolate appeared as white colonies. For *Enterococcus*, 33 out of the 37 isolates (89.19%) identified were in accordance with the colony color turquoise blue on CHROMagar™ Orientation, while 4 other isolates appeared as white colonies. On the other hand, 6 isolates were identified as *Streptococcus agalactiae*, but all 6 isolates appeared white colonies on CHROMagar™ instead of appearing as light blue colonies (0%). Nevertheless, 2 isolates identified as *C. europaeus* and *C. freundii* (100% as metallic blue with red halo colonies), were in accordance with the color on CHROMagar™ Orientation. These findings demonstrated that phenotypic identification (for example, by colony morphology) is less specific than the 16S rRNA gene sequencing molecular identification method. Therefore, this underscores the limitation of relying on a single bacterial identification method. To ensure reliable identification, genotypic identification, such as 16S rRNA gene sequencing, is essential to accurately characterize bacterial isolates at least to the genus level.

Our study demonstrated that *Enterobacteriaceae* (n= 76; 46%) was the most abundant bacterial family detected and cultured from the meconium/early stool samples of preterm

infants (< 33 weeks of gestational age). This was followed by *Staphylococcaceae* (n= 37; 22%), and *Enterococcaceae* (n= 37; 22%). At the genus level, *Klebsiella* (n= 63), *Staphylococcus* (n= 37), *Enterococcus* (n= 37), and *Escherichia* (n= 11) were the four most prevalent, which accounted for 89.16% of all preterm stool isolates.

Multiple studies have consistently reported that the facultative anaerobes, including members of *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus* are among the dominant bacterial groups in the gut microbiota of preterm infants [40, 49, 115–118]. *Enterobacteriaceae* belongs to the phylum Proteobacteria, whereas *Enterococcus* and *Staphylococcus* are classified under the phylum Firmicutes. Thus, it is in-line with Morrow *et al.* [119] who found that the dominant phyla in preterm infants were Proteobacteria and Firmicutes, with only minor contributions of 1–2% from Bacteroidetes and Actinobacteria. At lower taxonomic such as family and genus, Patel *et al.* [115] found *Enterobacteriaceae*, *Staphylococcaceae*, and *Enterococcaceae* constituted the majority of bacterial families from preterm infants' rectal swabs, while Wandro *et al.* [116] found fecal samples from preterm infants were dominated by facultative anaerobes, including members of *Enterobacteriaceae*, *Staphylococcus*, *Enterococcus*. However, it is worth noting that the cohort in Wandro *et al.* [116] included preterm infants with conditions such as NEC or LOS, and concluded that the preterm infant gut microbial communities were personalized and showed stronger associations with antibiotic exposure than with clinical outcomes such as NEC or LOS. Arboleya *et al.* [49] also found *Enterobacteriaceae* and *Enterococcaceae* were the predominant microbial groups in preterm infants. Similarly, Magne *et al.* [117] found *Enterobacteriaceae* family and the genera *Staphylococcus, Streptococcus, and Enterococcus* to be the most often retrieved bacterial groups from preterm infants. Additionally, Schwiertz *et al.* [118] reported that *K. pneumoniae, E. coli*, and *Enterococcus* sp. were the bacteria most commonly detected in preterm infants.

Citrobacter spp. was detected in our study (n= 2), and has also been reported in preterm infants by Khan *et al.* [40], where it was among the dominant genera detected around 1 day after birth. The authors further stated that over time, the relative abundance of the three major bacterial families (*Exiguobacterium, Acinetobacter*, and *Citrobacter*) declined, while the bacterial groups of *Enterococcus*, *Klebsiella*, and *Escherichia* gradually increased and became the main bacterial groups by 42 days after birth [40]. Taken together, these findings are broadly consistent with our detection of *Enterococcus* (n= 37; 22%) and *Enterobacteriaceae* (n= 76; 46%) members; however, *Staphylococcus* spp. (n= 37; 22%) also represented a major proportion in our study. In another study, Morrow *et al.* [119] found that at the genus level, the most common genera in order of relative abundance were

Enterobacter, *Staphylococcus*, *Escherichia*, *Enterococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, and *Clostridia*. Notably, the first four genera represented over 90% of all microbial reads [119]. In our study, *Klebsiella*, *Staphylococcus*, *Enterococcus*, and *Escherichia* were the top four genera, accounting for 89.16% of all preterm stool isolates. Consistent with Morrow et al [119], there was an overlap in dominant taxa, particularly *Staphylococcus*, *Enterococcus*, and *Escherichia* [119].

NEC and LOS are two common premature birth complications with high morbidity and mortality [32, 120]. Gut microbiota dysbiosis plays a potential role in contributing to both NEC and LOS [67, 120–122]. It has been suggested that microbial translocation from the gut is an important pathway initiating NEC and LOS in very low birth weight infants and preterm infants [120, 123]. That being said, Morrow *et al.* [119] found that in terms of the subsequent risk of NEC, two distinct forms of dysbiosis in preterm infants occurred during the first two weeks of life. The first form occurred during days 4 to 9, where the microbial community prior to the onset of early NEC cases consisted mainly of Firmicutes (≥ 98%), mostly from the Bacilli class, with *Staphylococcus*, and *Enterococcus* as the dominant genera. The second microbial pattern occurred during day 10 to 16, preceding later-onset NEC cases. This was characterized by a gram-negative Proteobacteria profile, particularly the *Enterobacteriaceae* family [119]. Consistent with this, a pooled meta-analysis by Pammi *et al.* [67] found the abundance of Firmicutes and Bacteroidetes decreased while Proteobacteria increased preceding NEC diagnosis in preterm infants. At a finer taxonomic level, specific members of *Enterobacteriaceae* have also been implicated. Torrazza *et al.* [124] detected a novel signature sequence from the first stool samples of preterm infants with NEC in the first week of life. The sequence was distinct from but matched closest to *K. pneumoniae*, which was highly associated with the development of NEC later in life [124]. Besides that, Mai *et al.* [125] detected specific Operational Taxonomic Units (OTUs) that increased in NEC preterm infants during the week prior to NEC diagnosis. Notably, most of these OTUs grouped to the *Enterobacteriaceae* (*K. pneumoniae*, and *E. coli*) [125]. Additionally, Mshvildadze *et al.* [126] detected *Citrobacter* species in 3 out of 4 cases of preterm infants with NEC.

With regards to LOS, several studies have highlighted the role of *Enterobacteriaceae* colonization in preterm infants. Smith *et al.* [73] found that intestinal colonization of *Enterobacteriaceae* was associated with LOS in preterm infants, especially the carbapenem-resistant *E. coli* and *Klebsiella* spp., which can be challenging to treat. Similarly, a prospective study based in a NICU in Shanghai by Liu *et al.* [32] found *Klebsiella* was the only dominant genus detected during LOS progression in preterm infants. In addition, El Manouni El Hassani *et al.* [121] hypothesized that colonization by *E. coli*, and possibly *K.*

pneumoniae, was important in the pathogenesis of gram-negative LOS and can be detected up to few days before the onset of LOS. This finding aligns with a systematic review based on 7 studies comprising 106 sepsis cases in preterm infants, which showed that the majority of studies supported that the potential pathogen responsible for sepsis was detectable in stool samples prior to the onset of LOS, some of which with increased relative abundance of identified pathogens, including *K. pneumoniae*, and *E. coli* [127]. Collectively, these findings suggest that intestinal colonization by *Enterobacteriaceae* may represent a shared microbial signature associated with, and often preceding both NEC and LOS. This highlights the importance of early gut colonization dynamics in preterm infants and supports the concept that dysbiosis involving *Enterobacteriaceae* may contribute to both NEC and LOS.

Established literature reported that an estimated 10% of preterm infants' fecal samples harboured *Klebsiella* spp., and the proportion could vary depending on geographical location and presence of unstable gut microbiome, immaturity of the gastrointestinal tract, and immune system [128, 129]. Importantly, *K. pneumoniae* was more often detected in the stools of preterm infants than term infants within the first few days of life [49]. Based on an analysis of fecal microbiota of 12 preterm infants, Khan *et al.* [40] found that the colonization process is highly dynamic at different time points after birth. The authors found an increase in *Klebsiella* and *E. coli* at around 7 days after birth, and they slowly increased, becoming the main microbiota. At 42 days of life, they became the predominant gut microbiota with proportions of 32.6% and 38.2%, respectively, suggesting that some opportunistic pathogens can gradually grow during fecal microbiota development in preterm infants [40]. This is in agreement with Patel *et al.* [115] who found increased dominance of *Enterobacteriaceae* over time, accounting for 60% of Proteobacteria samples in week 1, 84% in week 2, and by weeks 3 to 5, it reached 88%. It is worth noting that, despite the significant increase in *Enterobacteriaceae*, the overall Proteobacteria only changed from 55% to 59% from weeks 1 and 3 to 5 [115]. Collectively, these studies illustrate a clear trajectory wherein opportunistic *Enterobacteriaceae* expand over time in the developing preterm gut. This pattern provides a plausible context for the high isolation rate of *Enterobacteriaceae* (76 out of 166 isolates) observed in the present study.

The identification of *Klebsiella* spp. as the most frequently isolated gram-negative organism isolated from stool samples of preterm infants in this study is consistent with several NICU-based intestinal colonization studies using either stool cultures or rectal swabs [45, 107, 130, 131]. A Malaysian study reported that *K. pneumoniae* was the most frequently detected gram-negative organism, identified in 23 of 43 (53.5%) preterm infants with at least one positive culture from stool and/or tracheal specimens [130]. A Moroccan study reported

that, out of 641 *Enterobacteriaceae* isolates, *K. pneumoniae* (319/641) was the predominant bacterium, followed by *E. coli* (261/641,) based on rectal swabs of neonates (average gestational age of (35.2 (± 3.2) weeks; mean birth weight of 2612.1 g (±1023.2)) [131]. Besides that, a study based in China by Jia *et al.* [107] found *Klebsiella* was the predominant genus in the gut microbiota of preterm infants based on meconium and stool samples. This finding was consistent with Zou *et al.* [45], who found *Klebsiella* among the predominant genera in the gut microbiota of preterm infants based on stool samples. However, a study from India reported *E. coli* as the major intestinal colonizer in very low birth weight infants based on stool or meconium samples (or rectal swabs in the absence of stool), followed by *K. pneumoniae* and *E. faecalis* [132]. Nonetheless, the frequency of *K. pneumoniae* gut colonization may differ as the gut microbiome composition of preterm infants can be influenced by various factors such as mode of delivery [133], gestational maturity at birth [44], geographical location (including hospitals) [134], type of feeding [135], and differences in antibiotic usage [45, 116].

Among the species within the *Enterobacteriaceae* family, *K. pneumoniae* is a clinically significant opportunistic pathogen that colonizes the gut of preterm infants [130]. Moreover, *K. pneumoniae* is one of the pathogens associated with both nosocomial and community-acquired infections [136, 137]. Based on our findings, the highest number of culturable *Enterobacteriaceae* was *K. pneumoniae*, 74% (56/76 isolates). Preterm infants, particularly those admitted to the NICU, are at a higher risk of infection due to the combination of extended hospitalization, frequent use of antibiotics and invasive devices, and relative immunocompromise from an immature immune system [138, 139]. Consistent with this vulnerability, Ikuta *et al.* [140] reported that *K. pneumoniae* was the pathogen associated with the most neonatal deaths in 2019, estimated at 124, 000 (95% uncertainty intervals of 89, 000–167, 000).

Given the clinical importance of *K. pneumoniae* and its high burden in neonates, we further explored the diversity of *K. pneumoniae* detected in this study. Our phylogenetic analysis revealed a high level of diversity within the species *K. pneumoniae* isolated from stool samples of preterm infants. These isolates are related to various *Klebsiella* spp. and are distributed across a total of 20 clades (Clade A to Clade T) (Figure 1). Notably, all *K. pneumoniae* isolates obtained from stool samples 5 and 18 clustered within either Clade A or Clade C. Phylogenetic reconstruction further identified Clade C as a distinct clade predominantly composed of only *K. pneumoniae* isolates from stool samples 5 and 18. These isolates form a well-supported cluster, suggesting close genetic relatedness among isolates associated with these two preterm infants.

One of the closely related strain worth mentioning in our study is *Klebsiella* sp. strain JPJC-S9, which was isolated from a domestic wastewater treatment plant in Jaipur, India [85]. In a subsequent study, the authors investigated the antibiotic resistance profile of *Klebsiella* sp. strain JPJC-S9 using AST-N280 cards to evaluate if it poses a risk to human health and contributes to the spread of antibiotic resistance [141]. The strain known as *K. pneumoniae* spp. *pneumoniae* in their study was tested against 19 antibiotics, and the susceptibility was based on their minimal inhibitory concentration following Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines. Results showed resistance only to two antibiotics—ampicillin, and amoxicillin-clavulanic acid, while the rest were sensitive [141]. Based on our phylogenetic analysis, *Klebsiella* sp. strain JPJC-S9 clustered within Clade A alongside MPB 14. Thus, MPB 14 may display a comparable susceptibility profile.

In addition, there were five closely related strains from the same study which monitored carbapenem-resistant *Enterobacterals* in the environment for the assessment of the spread in the community [88]. *Klebsiella* sp. strain 154_37_37, *Klebsiella* sp. strain 171_1_42_5, *Klebsiella* sp. strain 197_a_37_1, *Klebsiella* sp. strain 216_42_z_2, and *Klebsiella* sp. strain 223_48_42_z_2 were isolated from environmental samples (wastewater, treated wastewater, river water) in Japan by Urase *et al*. [88]. The study detected *K. pneumoniae* complex carrying IMP (imipenemase)-type carbapenemase and Guiana extended-spectrum (GES)-type carbapenemase. Findings detected genes encoding carbapenemase in *Klebsiella* sp. strain 154_37_37, *Klebsiella* sp. strain 171_1_42_5, *Klebsiella* sp. strain 197_a_37_1, *Klebsiella* sp. strain 216_42_z_2, and *Klebsiella* sp. strain 223_48_42_z_2 were bla_{GES} (carbapenemase-producing (CP)), bla_{GES} (CP), bla_{GES} (CP), bla_{GES} (CP) + $bla_{\text{IMP-6}}$ (IMP-type metallo-β-lactamase), and bla_{NDM} (New Delhi metallo-β-lactamase), respectively [88]. Phylogenetic analysis in our study revealed that MPB 143 and MPB 150 clustered with *Klebsiella* sp. strain 171_1_42_5 (Clade S) and *Klebsiella* sp. strain 223_48_42_z_2 (Clade I), respectively. The former formed a monophyletic clade with MPB 143, while the latter was cladded with MPB 150. However, this grouping was supported by low bootstrap values (< 50%), indicating weak statistical support for the inferred relationship. Nonetheless, MPB 143 may carry bla_{GES} (CP), while MPB 150 may carry bla_{NDM}, although further investigation is required. `

Overall, based on 20 of the 30 closely related strains included in our phylogenetic analysis, we noticed these 20 closely related strains were isolated from diverse sources including human clinical isolates (a patient with urinary tract infection, clinical samples), the human gut microbiome, environmental sources (cheese stall, wastewater, treated wastewater, river water, petrochemical wastewater, domestic wastewater treatment plant, agricultural

field soil), plants (surfaces of rice seeds, rice paddy field mud surrounding rice root, blueberry rhizosphere, raspberry (*Rubus idaeus*) associated with crown gall disease), and oil palm biomass waste. Hence, demonstrating that *K. pneumoniae* is able to colonize diverse niches and that these strains which were detected in the gut of preterm infants may also be derived from the environment or clinical reservoir. If this is so, it further highlights the risks for infections, antibiotic transmission, and the importance of environmental and infection control practices in the neonatal units.

Besides *K. pneumoniae*, a few other species were isolated and identified from the meconium/early stool of preterm infants in our study. It is worth noting that the mode of delivery of preterm infants tends to be via caesarean section, and the infant's microbial community resembles the skin microbiota of their mothers and the environment [42, 54]. *Staphylococcus* is a genus representative of skin bacteria, commonly associated with caesarean delivery [142]. Within the *Staphylococcus* species identified in our study, majority were identified as *S. epidermidis* and *S. haemolyticus*, followed by *S. hominis*, and *S. warneri* all of which generally are residents of the skin, suggesting initial colonization of the skin-associated bacteria [143–146]. In fact, Aujoulat *et al.* [108] found that coagulase-negative staphylococci, particularly *S. epidermidis* was detected in all very preterm infants, and were most represented from the first days of life. *Corynebacterium* spp. were also detected in this study, and the *Corynebacterium* genus is also one of the residents on healthy human skin [147]. However, it is worth noting that *Corynebacterium* is reported as part of the breast milk microbiome as well as a reagent contaminant in low biomass samples [148]. A high number of *E. faecalis* and *E. faecium* were isolated from the preterm stool samples in our study. *E. faecalis* and *E. faecium* are commensal bacteria of the human intestine. Approximately 10^6 to 10^7 *Enterococcus* are present in the human intestine (< 1% found in the ileum, and up to 1% in the colon), most of which are either *E. faecalis* (10^5–10^7 colony-forming units per gram of feces) or *E. faecium* (10^4–10^5 colony-forming units per gram of feces), while *E. avium* is occasionally detected [149]. Furthermore, Aujoulat *et al.* [108] found *Enterococci*, detected in 60% of very preterm infants were early, highly represented, and persistent colonizers of the premature gut. Hence, justifying *Enterococcus* spp. as one of the main bacterial taxa in our study.

This study has several limitations. First, the relatively small sample size of N= 28. This affects the generalizability of these findings. Second, the lack of detailed information on the mode of delivery, antibiotic regimens, and the type of feeding to preterm infants limited our ability to assess whether an association exists between the gut microbiota and these factors. Third, the use of the conventional bacterial culture method may have failed to

isolate some bacterial taxa, due to limitations in growth conditions (for example, *Bifidobacterium*, which grows anaerobically), and low abundance in samples. Nonetheless, larger studies with more diverse cohorts, such as the inclusion of preterm infants from multiple NICUs across different regions in Malaysia, are needed in future studies to increase statistical power and validate our findings, allowing for a better understanding of the gut microbiome of preterm infants.

Future research on antibiotic susceptibility profiling and longitudinal follow-up of the gut microbiome should be incorporated to better understand how fluctuations in *Enterobacteriaceae* populations influence health outcomes and microbiome development in preterm infants. Besides that, future research should also employ culture-independent methods such as whole-genome shotgun metagenomic sequencing to profile the full microbial communities in preterm infants, capturing microbial genes, genome composition, and pathways [1, 150, 151]. Metatranscriptomic sequencing analysis provides a snapshot of the gene expression profile under specific conditions and at a given moment, instead of its potential as inferred from DNA-based shotgun metagenomic analysis [1]. Therefore, metatranscriptomic analysis can be employed to complement shotgun metagenomics by elucidating what genes are actively transcribed from a potential repertoire of annotated genes as revealed by shotgun metagenomic analysis [1].

In light of these limitations, probiotics may play a role in promoting a healthy gut microbiome in preterm infants. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host [3, 152]. Bifidobacterium and lactobacilli, are two commonly studied probiotics because of their generally safe status and technical robustness [153]. Nonetheless, other bacterial genera, including *Enterococcus*, *Streptococcus*, *Bacillus*, and members of the yeast genus *Saccharomyces*, can have probiotic properties [154, 155]. Interestingly, it is worthy to note that in Malaysia, several strains of *Streptomyces* have been isolated and evaluated as a probiotic having antimicrobial, antioxidant, and cytotoxic potentials [156–164]. That being said, a systematic review based on research from the year 2012 to 2022 found that the commonly used probiotics for preterm infants were bacteria from the families *Bifidobacteriaceae* (*Bifidobacterium* spp.) and *Lactobacillaceae* (*Lactobacillus* spp., *Lacticaseibacillus* spp.) [165]. Several possible distribution of mechanisms among probiotics include colonization resistance, acid and short-chain fatty acid production, regulation of intestinal transit, normalization of perturbed microbiota, increased turnover of enterocytes, and competitive exclusion of pathogens [152].

Since there is a delayed establishment of beneficial bacteria and higher levels of opportunistic pathogens in the gut of preterm infants, probiotics could be a potential supplement to mitigate gut dysbiosis [49, 70, 165–169]. This can be observed in the study by Alcon-Giner *et al.* [143], whereby preterm infants fed probiotics supplementation consisting of *Bifidobacterium* and *Lactobacillus* demonstrated a microbiome that was dominated by *Bifidobacterium*, and that their gastrointestinal environment was more closely resembled that of full-term infants. In addition to maintaining a healthy gut microbiome, probiotics contribute to preventing the spread of antibiotic resistance genes. As demonstrated by Guitor *et al.* [75], preterm infants administered with probiotics during their hospitalization had reduced diversity and prevented persistence of antibiotic resistance genes in the gut microbiome. Similarly, Bargheet *et al.* [51] also supported that probiotics helped in microbiota development by promoting microbial community interconnectivity and stability in the first week of life and minimizing resistome development induced by antibiotic use and hospitalization. Hence, this would be highly beneficial for preterm infants as they tend to be hospitalized, experience gut dysbiosis, and are frequently administered with antibiotics.

5. Conclusions

Our findings provided an insight into the culturable *Enterobacteriaceae* in preterm infants. The combined use of cultural (phenotypic) and genotypic methods in our study provided a more reliable approach to characterize the diversity of *Enterobacteriaceae* from preterm stool samples. Overall, our study demonstrated that within the hospital ward, there may be a typical bacterial colonization pattern, characterized by a few main bacterial genera. Our study successfully isolated and identified 3 main bacterial families from 28 meconium/early stool samples of preterm infants from the NICU of Hospital Sultanah Aminah Johor Bahru, Malaysia, which include *Enterobacteriaceae*, 46% (76/166 isolates), *Staphylococcaceae*, 22% (37/166), and *Enterococcaceae*, 22% (37/166). This shows that *Enterobacteriaceae* is one of the predominant bacteria during the early gut colonization in preterm infants, making up to 46% of all isolates. Particularly, within the *Enterobacteriaceae* family, *K. pneumoniae* (56/76 isolates, 74%) dominates, followed by *E. coli* (11/76, 15%), *K. aerogenes* (7/76, 9%), *C. europaeus* (1/76, 1%), and *C. freundii* (1/76, 1%).

The high number of *Enterobacteriaceae*, particularly *K. pneumoniae*, followed by *E. coli*, is concerning, as these bacterial species are often associated with hospital-acquired infections in the NICU [130, 170–173]. Furthermore, their presence in the meconium/early stool samples suggests that preterm infants are susceptible to early colonization by opportunistic pathogens, possibly driven by antibiotic exposure, type of feeding, hospital environment, and delayed establishment by beneficial bacteria. Importantly, the phylogenetic relationships of

the 56 *K. pneumoniae* isolates and their closely related strains demonstrated that *K. pneumoniae* is able to colonize diverse niches and that these strains, which were detected in the gut of preterm infants may also originate from the environment or clinical reservoirs. It highlights the importance of continuous microbiological monitoring and infection control measures within the NICU to mitigate *Enterobacteriaceae*-associated infections.

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