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Changes in Neonatal Microbiota Distribution Influenced by the Environment of the Neonatal Intensive Care Unit in the First Month of Life

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Abstract

Commensal bacterial colonization is crucial for human health, and the early neonatal period is important for the establishment of microbial populations. However, studies on the developmental patterns of microbiota in early life, particularly in those exposed to the environment of the neonatal intensive care unit (NICU), are limited. Using a 16S ribosomal RNA polymerase chain reaction assay, this study aimed to evaluate the changes in the levels of representative microbiota in healthy term infants and infants who were admitted to the NICU during the first month of life. Compared with term infants, the NICU group showed lower levels of Bifidobacterium in the early days after birth but achieved the same levels as those of term infants after day 30 of probiotics use. In addition, we found that the presence of *Staphylococcus aureus*, including methicillin-resistant *S. aureus*, from fecal samples was not associated with disturbances in Bifidobacterium during the neonatal period. Clinical factors such as the mode of delivery, antibiotic therapy, and intubation for mechanical ventilation could change the neonatal distribution of microbiota, but the most important factor was insufficient enteral nutrition. These groups, which had experienced poor general conditions and/or underwent surgery early in the neonatal period, showed are markable decrease in Bifidobacterium level at day 30. In conclusion, infants in the NICU developed similar microbiota composition as in the healthy term infants group in 1 month afterbirth; however, insufficient enteral nutrition could lead to disintegration of the microbiota distribution.

Keywords: Antibiotics; Bifidobacterium; Caesarean section; Gastrointestinal tract; Microbiota; Methicillin-resistant *S. aureus*; Neonatal intensive care unit; Preterm infant; Surgery; 16S ribosomal RNA

Introduction

Colonization of the gastrointestinal (GI) tract is crucial for human health. The early neonatal period is particularly important for the establishment of microbial populations. Fetal stools are normally sterile, with some microbiota strains such as *Escherichia coli* and Streptococci being detectable after delivery, and anaerobic genera such as Bacteroides and Clostridium are present in stool samples 4–7 days after birth [1]. Generally, healthy, breast-fed infants predominantly show a great increase in Bifidobacterium levels and a decrease in *E. coli*, Streptococci, Bacteroides and Clostridium at one month after birth [2]. Colonization influences the composition of gut microbiota in early life and may impact the development of certain diseases later in life [3,4]. Traditional plate-counting methods have reported altered microbial colonization patterns in term and preterm infants [5]. However, the techniques used in such studies were not quantitative, and there were limits of detection for some microbiota species. Recently, sequencing of amplified 16S ribosomal RNA (16S rRNA) genes has been performed [6,7]. Some recent reports have discussed the microbiota of preterm infants [8,9]; however, little is known regarding the process of colonization in neonatal GI tracts, especially after long-term exposure to the environment of the neonatal intensive care unit (NICU). Since infants who were hospitalized in the NICU undergo intensive care, including antibiotic therapy, intubation for mechanical ventilation, and surgical procedures [10], we hypothesized that the colonization of their GI tracts may be influenced by such intensive treatment and also by the consequent separation from their mothers. Furthermore, we focused on the patterns of *Staphylococcus aureus* colonization, including methicillin-resistant *S. aureus* (MRSA), which has often been detected in the NICU environment. Using real-time quantitative polymerase chain reaction (qPCR), this study aimed to evaluate the changes in microbiota in healthy term infants and infants who were in the NICU during the first month of life.

Materials and Methods

Patients and samples

This prospective observational case-cohort study was approved by the Institutional Review Board for Human Studies of the University of Kitasato, the Ethical Committee of the Kitasato University Hospital (KUH, Kanagawa, Japan), and Kitasato University Medical Center (KMC, Saitama, Japan). All infants were enrolled after parents provided informed consent. Infants who could be collected their fecal sample at each points and were expected to live beyond the first month of life were eligible. Stool samples were collected from 90 infants born at the KUH and the KMC between June 2013 and May 2014 (Table 1). Fecal samples from 48 infants who were admitted to the KUH NICU were collected two times after birth on days 2.6 ± 0.1 and 31.6 ± 0.4. Forty-two term infants (25 infants born at the KUH and 17 infants born at the KMC) were discharged from each hospital 4–7 days after birth and had a medical examination at one month after birth. Fecal samples were

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Number of all cases	90
Perinatal	
Gestational age (mean weeks ± SE)	36.0 ± 0.5 (25.0-41.1 w)
Birth weight (mean g ± SE)	2348.6 ± 98.7 (542-4138 g)
Male/Female	47/43
PROM (%)	13 (14.4)
Other facilities birth (%)	5 (5.6)
Cesarean section (%)	53 (58.9)
Postnatal	
NICU (%)	48 (53.3)
Antibiotic use (%)	30 (33.3)
Probiotics (*Bifidobacterium breves*) use (%)	23 (25.6)
Brest fed (%)	25 (27.8)
Intubation for respiration management (%)	30 (33.3)
Operation (%)	10 (11.1)
Outcome	
Hospitalization at one month after birth (%)	29 (32.2)
Hospitalization days (mean days ± SE)	40.0 ± 6.2 (5※-393 days)
Death	2 (2.2)
※Virginal birth: 4 ~ 5 days, Cesarean section 7 ~ 8 days	

Table 1: Clinical profile of 90 infants and subject groups.

collected twice shortly following birth (2.0 ± 0.2 days) and again one month after birth (30.2 ± 0.6 days). The samples were put in sterile plastic tubes and stored at −80°C until DNA extraction.

DNA extraction

After fecal samples were melted and weighed, 700 µl buffer ASL (Qiagen, Germany) was added to each 40 mg sample and homogenized by Tissue Lyser II (Qiagen, Germany) for 5 min. DNA was extracted from these homogenized solutions using the QIAamp DNA Stool Mini Kit (Qiagen,Germany). DNA was eluted in a final volume of 200 µL and stored at −20°C until analyzed.

Quantitative analysis by PCR

Quantification of each fecal bacterial population was detected by qPCR using the primers shown in Table 2 [11-14]. All reactions were performed on Multiplate 96 well plates (BIO RAD, Japan)with the Chromo 4 system (BIO RAD, Japan) using the Sso Advanced TM Universal SYBR® GreenSupermix (BIO RAD, Japan). Five microliters of extracted DNA sample (~ 5 ng) and 100 pmol/l of each primer were used in the 25 µl PCR. Thermal cycling consisted of an initial cycle of 95°C 3 min, followed by 45 cycles consisting of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. After amplification, a melting curve analysis was performed from 60°C to 95°C and read every 1°C with a 10 s hold. Standard curves were made with pure cultures of appropriate strains. Samples were analyzed in duplicates in at least two independent PCR runs.

Staphylococcus aureus analysis

For DNA amplification, *mecA* and *nuc* primers for detection were designed, and they are presented in Table 2 [15]. Multiplex PCR was optimized on an Eppendorf thermo-cycler (RocheCo., Germany), in a final volume of 25 µl containing 2.5 µl of 10x PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 mmol of 10 pmol/l deoxynucleotide triphosphate (dNTP) mix, 10 pmol/l of each primer, 0.25 U of Taq polymerase, and 5 µl of template DNA sample. The amplification conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 5 min. The PCR products were loaded onto a 1.5% (w/v) agarose gel with 0.5 µg/ml of ethidium bromide and were detected using gel electrophoresis.

Statistical analysis

Results are presented as the median value and the average value for the indicated number of experiments. Statistical significance was determined using the Mann–Whitney U test for two-group data and the Kruskal–Wallis one-way ANOVA followed by the Dunn's post hoc test formulti-group data using Origin [GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA)]. Statistical significances of *p<0.05, **p<0.01, ***p<0.001 are indicated.

Results

Development and population of microbiota in the neonatal period

The quantification of three microbiota species (Bifidobacterium, Enterococcus, and Enterobacteriaceae) in fecal samples from 90 infants, including 48 in the NICU group and 42 in the non-NICU group, were detected by qPCR at days 0–3 (2.3 ± 0.1 days) and at 1 month (31.0 ± 0.4 days) after birth. Each of three analyzed species is a typical species that forms majority in the intestinal microbiota [1-3]. We found that each three microbiota species increased at one month compared with that of days 0–3 (Figure 1). Especially, the level of Bifidobacterium showed lower than that of Enterococcus and Enterobacteriaceae at day 0-3, but increased remarkably and become the most dominant species after one month. Multiplex analysis (Kruskal–Wallis analysis) reveled that Bifidobacterium levels showed a significant increase compared with Enterococcus and Enterobacteriaceae at day 30 (p<0.0001, U=182.9; data not shown).Compared with NICU with non-NICU group, NICU group had significantly lower levels of Bifidobacterium (p<0.001) and Enterobacteriaceae (p<0.001) at day 0-3 (Table 3). After a month,

Target organism	Strain used standard curves	Primer set	Sequence (5' to 3')	Product size (bp)	temp (°C)	References
Bifidobacterium	*Bifidobacterium longum* (JCM 1217^T=ATCC 15707)	g-Bifid-F	CTCCTGGAAACGGGTGG	550	60	[11]
		g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Enterobacteriaceae	*Escherichia coli* (JCM 1649^T=ATCC 11775)	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	195	58	[12]
		Eco1652R	CTCTACGAGACTCAAGCTTGC			
Enterococci	*Enterococcus feacalis* (JCM 5803^T=ATCC 19433)	Enc-F	CCCTTATTGTTAGTTGCCATCATT	144	58	[13]
		Enc-R	ACTCGTTGTACTTCCCATTGT			
Staphylococcus aureus	*Staphylococcus aureus* (JCM 2151=ATCC 6538P)	nuc-F1	GCGATTGATGGTGATACGGTT	267	55	[14]
		nuc-R2	AGCCAAGCCTTGACGAACTAAAGC			
MRSA		mecA F	TGCTATCCACCCTCAAACAGG	284	50	[15]
		mecA R	AACGTTGTAACCACCCCAAGA			

Table 2: Primers used in this study.

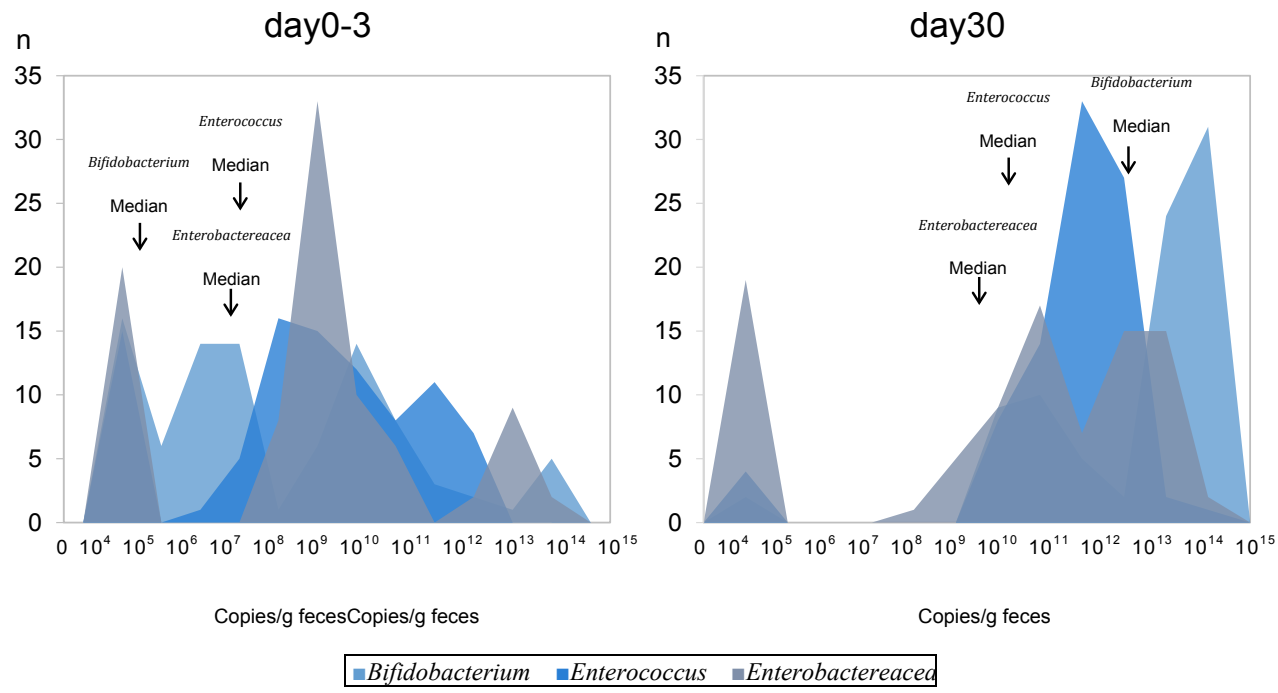

Figure 1: Total population of microbial species at days 0–3 and 30 (90 cases). The median value of three species was denoted as each arrow.

	N	GA (mean weeks ± SE)	BBW (mean g ± SE)
NICU (+)	48	33.5 ± 0.7	1812.0 ± 135.6
NICU (-)	42	38.8 ± 0.2	2961.9 ± 63.6

		Bifidobacterium	Enterococcus	Enterobactereace
Day 0-3	P	<0.0001***	0.2299	<0.0001***
	U	370.0	859.0	393.5
	Median (+)	5.98×10^{4}	1.39×10^{7}	1.445×10^{7}
	Median (-)	6.65×10^{8}	1.002×10^{8}	2.68×10^{8}
Day 30	P	0.7644	0.6741	0.0036**
	U	970.5	955.5	653.0
	Median (+)	5.045×10^{12}	3.245×10^{10}	7.42×10^{8}
	Median (-)	1.115×10^{13}	2.735×10^{10}	5.86×10^{10}
※*p<0.05, **p<0.01, ***p<0.001				

Table 3: Microbiota population of two group at days 0–3 and 30 (NICU (+): 48 infants who admitted in NICU, NICU (-): 42 healthy term infants).

Enterobacteriaceae level of NICU group showed still low, however, the level in Bifidobacterium was almost the same of non-NICU group (p=0.7644).

In the NICU group, infants born before 34 gestational weeks were administered probiotics (*B. breves*) via a GI tube between days 0 and 7. Thus, the influence of probiotic administration was investigated. The probiotics group consisted of 23 infants [Gestation age (GA), 28.7 ± 0.6 weeks; Birth body weight (BBW), 1051.2 ± 110.4 g)], and the non-probiotics group had 67 infants (GA, 38.5 ± 0.2 weeks; BBW, 2794.0 ± 67.0 g). There were statistical differences in Bifidobacterium levels between the probiotics and non-probiotics groups at days 0–3 (p<0.05) but no difference at day 30 (p=0.9766). This result indicated that Bifidobacterium levels of preterm infants (<34 weeks) were low level at day 0-3, but increase to the same level of term infants under the probiotics use at day 30.

S. aureus analysis

S. aureus is one of the most common indigenous bacteria, and it is well known that *S. aureus* species can lead to serious, preterm opportunistic infections and critical conditions for neonates [16,17]. In this study, we also performed *S. aureus* analysis using specific primers as the same qPCR method. In 90 cases, *S. aureus* was detected at least once during the neonatal period in 44infants (44/90; 48.9%), 50.0% (21/42) infants in the non-NICU and 47.9% (23/48) infants in the NICU groups tested positive for *S. aureus*. To analyze the influence of *S. aureus* colonization, we divided 90 infants into *S. aureus*-positive or -negative groups. Compared with the *S. aureus*- negative group, there were no significant difference in three microbiota levels at day 0-3, but Enterococcus level showed lower at day 30 (Table 4). Additionally, we analyzed *mecA* and *nuc* genes from each *S. aureus*-positive fecal sample using gel electrophoresis, following multiplex PCR. Six out of 44 *S. aureus*-positive samples were negative for *mecA* and *nuc* genes; 38 samples were detected as MRSA, and all 23 samples from the *S. aureus*-positive NICU group were positive for the *mecA* and *nuc* genes. To investigate the differences between *mecA*-negative and *S. aureus*-positive groups, we compared *Bifidobacterium* levels from the *mecA*-negative *S. aureus* group (six cases) with the *mecA*-positive *S.*

	N	GA (mean weeks SE)	BBW (mean g ± SE)
S. aureus (+)	44	35.8 ± 0.7	2228.1 ± 143.1
S. aureus (-)	46	36.1 ± 0.7	2463.9 ± 135.6

		Bifidobacterium	Enterococcus	Enterobactereace
Day 0-3	P	0.6273	0.8248	0.9565
	U	951.5	984.0	1005
	Median (+)	3.40×10^5	3.91×10^7	2.64×10^7
	Median (-)	2.975×10^5	7.845×10^7	2.36×10^7
Day 30	P	0.8899	0.0145*	0.4734
	U	994.5	710.5	923.0
	Median (+)	6.625×10^{12}	8.455×10^{10}	4.885×10^9
	Median (-)	6.605×10^{12}	2.18×10^{12}	1.93×10^{12}
※*p<0.05, **p<0.01, ***p<0.001				

Table 4: Influence of *S. aureus* colonization (Infants who was detected *S. aureus* at least one time in neonatal period denoted as (+)).

aureus (15 cases) in the non-NICU group. There were no significant differences for each species (data not shown). Focusing on the NICU group, Bifidobacterium was prevalent in both the *S. aureus*-positive and -negative groups. There were no differences in the increase in Bifidobacterium levels between *S. aureus*-positive and -negative groups during the neonatal period.

Influence of clinical factors

In this study, we divided all infants into some sub-groups and compared with the level of three species (Bifidobacterium, Enterococcus, and Enterobacteriaceae). Each of the sub-groups examined in this study was considered important for understanding neonates' conditions and managing neonates in the NICU in daily clinical practice.

Influence the mode of delivery: Compared 37 VB infants with 53 CS infants at days 0–3 and day 30, CS group showed significant low level in Enterobacteriaceae at day 0-3 (Table 5). Since the rate of infants born *via* CS in the NICU group was high (33/48; 68.8%), we investigated the influence of the mode of delivery inhealthy non-NICU infants. In the non-NICU group, the CS group (20 cases) showed significantly lower levels of Bifidobacterium (p=0.0255) than that of the VB group (24 cases) at days 0–3, but there were no significant differences at day 30 (data not shown).

Influence of antibiotic therapy: Preterm infants have a potential risk for serious infections in the perinatal period, and they often undergo treatment with broad spectrum antibiotics in first few days of life. In this study, 30 antibiotic-treated infants were compared with 60 non-antibiotic-treated infants. The antibiotic-treated infants in Enterobacteriaceae at day 30 showed lower than that of non-antibiotic-treated infants (Table 6A). To avoid the effect of gestational week, we analyzed 28 cases of ≧ 32w cases in NICU group, 10 antibiotics-treated infants who were born after 32 gestational weeks were compared with 18 non-antibiotic-treated infants. Compared the two groups, there were a significant difference in Bifidobacterium levels at day 30 (p<0.05), antibiotics-treated infants showed lower value rather than that of non-antibiotics-treated group (Table 6B).

Influence of intubation: In the NICU, respiratory diseases are one of the most important and serious concerns. Frequently, the requirement for tracheal intubation and mechanical ventilation therapy in the NICU exists because of their respiratory disorders. When 30 intubated infants (GA, 31.6 ± 1.0 weeks; BBW,1570.9 ± 190.9 g) were compared with 60 non-intubated infants (GA, 38.2 ± 0.3 weeks; BBW, 2737.5 ± 73.5 g), intubated infants in Enterobacteriaceae at day 0-2 and 30 and Bifidobacterium at day 0-2 showed lower than that of non-intubated infants, but there were no difference in Bifidobacterium levels at day 30 (Table 7A). Same as antibiotics analysis, 10 intubated infants who were born after 36 gestational weeks (GA,38.6 ± 0.5 weeks; BBW, 2727.2 ± 266.1 g) were compared with 11 non-intubated infants (GA, 38.2 ± 0.4 weeks; BBW, 2471.1 ± 131.7 g) (Table 7B). Compared the two groups, there were tendency of lower Bifidobacterium levels in intubated infants at day 30, but no significance (p=0.1301). Interestingly, we found that *S. aureus*-positive case in intubation group (6/10; 60.0%) were high rate compared with non-intubated group (2/11; 18.2%).

Influence of nutrition: Since there are reports that the composition of intestinal microbiota is strongly influenced by diet [18], we divided

	N	GA (mean weeks ± SE)	BBW (mean g ± SE)
Vaginally-born (VB)	37	38.1 ± 0.5	2729.6 ± 109.5
Cesarean section (CS)	53	34.5 ± 0.7	2082.7 ± 138.6

		Bifidobacterium	Enterococcus	Enterobactereace
Day 0-3	P	0.1281	0.6886	<0.0001***
	U	795.0	931.0	464.0
	Median (VB)	2.93×10^7	9.63×10^7	3.47×10^8
	Median (CS)	1.80×10^5	3.55×10^7	1.86×10^7
Day 30	P	0.7736	0.2382	0.0559
	U	945.0	836.0	748.5
	Median (VB)	7.34×10^{12}	2.00×10^{10}	1.90×10^{11}
	Median (CS)	6.29×10^{12}	4.55×10^{10}	1.12×10^9
※*p<0.05, **p<0.01, ***p<0.001				

Table 5: Influence of the mode of delivery.

	N	GA (mean weeks ± SE)	BBW (mean g ± SE)
Antibiotics (+)	30	31.3 ± 0.9	1520.2 ± 190.3
Antibiotics (-)	60	38.3 ± 0.2	2762.9 ± 66.5

		Bifidobacterium	Enterococcus	Enterobactereacea
Day 0-3	P	0.0074***	0.1721	0.0003***
	U	590.0	740.0	492.0
	Median (+)	1.34×10^5	1.39×10^7	1.094×10^7
	Median (-)	1.215×10^8	9.39×10^7	4.375×10^7
Day 30	P	0.2182	0.3175	0.0006***
	U	755.5	782.5	505.0
	Median (+)	3.455×10^{12}	3.435×10^{10}	2.775×10^7
	Median (-)	1.044×10^{13}	2.735×10^{10}	2.69×10^{10}
※*p<0.05, **p<0.01, ***p<0.001				

Table 6A: Influence of antibiotic treatments.

	N	GA (mean weeks ± SE)	BBW (mean g ± SE)
Antibiotics (+)	10	37.6 ± 0.9	2585.8 ± 320.3
Antibiotics (-)	18	37.2 ± 0.5	2298.4 ± 101.3

		Bifidobacterium	Enterococcus	Enterobactereacea
Day 0-3	P	0.3987	0.7950	0.3044
	U	72.0	84.0	68.0
	Median (+)	1.023×10^5	1.39×10^7	2.395×10^7
	Median (-)	1.815×10^4	3.053×10^7	1.675×10^7
Day 30	P	0.0306*	0.7593	0.7502
	U	45.00	83.0	83.0
	Median (+)	2.683×10^{10}	2.080×10^{10}	2.51×10^9
	Median (-)	8.345×10^{12}	2.815×10^{10}	4.23×10^{10}
※*p<0.05, **p<0.01, ***p<0.001				

Table 6B: Influence of antibiotic treatments ≧ 32 weeks born case in NICU group (28 cases).

	N	GA (mean weeks ± SE)	BBW (mean g ± SE)
Intubation (+)	30	31.6 ± 1.0	1570.9 ± 190.9
Intubation (-)	60	38.2 ± 0.3	2737.5 ± 73.5

		Bifidobacterium	Enterococcus	Enterobactereace
Day 0-3	P	0.0063*	0.2362	<0.0001***
	U	584.5	761.0	422.5
	Median (+)	1.09×10^5	1.39×10^7	5.27×10^6
	Median (-)	1.215×10^8	9.915×10^7	7.015×10^7
Day 30	P	0.2556	0.2647	0.0009***
	U	766.5	769.0	520.0
	Median (+)	3.455×10^{12}	3.435×10^{10}	2.775×10^7
	Median (-)	9.435×10^{12}	2.735×10^{10}	2.69×10^{10}
※*p<0.05, **p<0.01, ***p<0.001				

Table 7A: Influence of intubation for mechanical respiration.

	n	GA (mean weeks ± SE)	BBW (mean g ± SE)
Intubation (+)	10	38.6 ± 0.5	2727.2 ± 266.1
Intubation (-)	11	38.2 ± 0.4	2471.1 ± 131.7

		Bifidobacterium	Enterococcus	Enterobactereace
Day 0-3	P	0.6019	0.7560	0.7439
	U	47.0	50.0	50.0
	Median (+)	7.045×10^4	2.43×10^7	1.695×10^7
	Median (-)	1.47×10^4	7.95×10^6	1.86×10^7
Day 30	P	0.1307	0.7917	0.3399
	u	33.0	51.0	41.0
	Median (+)	3.65×10^9	2.08×10^{10}	3.875×10^9
	Median (-)	6.92×10^{12}	3.09×10^{10}	1.12×10^9
※*p<0.05,**p<0.01, ***p<0.001				

Table 7B: Influence of intubation for mechanical respiration ≧ 36 weeks born case (21 cases).

90 infants into the breast-fed only group (25 infants; GA, 33.1 ± 1.3 weeks; BBW, 1950.6 ± 243.4 g) and the combination-fed (breast-fed and formula-fed combination) group (65 infants; GA, 37.1 ± 0.4 weeks; BBW, 2510.7 ± 94.3 g). The result was that Bifidobacterium was prevalent in both groups at day 30, and there were no significant differences in either group (Table 8A). Additionally, there were nine infants who experienced stopping enteral nutrition and/or insufficient nutrition (<100 ml/kg/day) at the time of day 30. In this group, all nine case underwent surgery at neonatal period, including six cases of congenital heart disease (four cases of patent ductus arterious, one case of transposition of the great arteries, one case of a trioventricular septal defect), one of huge lymphangioma , one of myelomeningocele, and one of anal atresia. In their microbiota profiles, there were no significant difference in three species at day 0-3, however at day 30, Bifidobacterium levels in insufficient enteral nutrition group were significantly decreased than that of other infants ($p<0.05$) (Table 8B). Especially, the microbiota profiles of microbiota distribution at day 30 in one abdominal surgery case demonstrated significantly low levels of Bifidobacterium, a 1010-fold decrease compared with the average levels; alternatively, *S. aureus* and Enterobacteriaceae were prevalent in their intestinal environment.

Discussion

In this study, we demonstrated that the population of gut microbiota in early life could be influenced by clinical factors, and have made some important observations about neonatal microbiota.

First, the NICU group showed lower levels of the representative microbial species in the early birth period. We found that Bifidobacterium, which was the most dominant species in the NICU group, reached approximately the same levels as that in term infants at day 30 after birth. We believe that this is because many of the preterm infants enrolled in this study were generally stable and established their enteral feeding fully by 1 month after birth. Additionally, all infants born before 34 gestational weeks had received probiotics during the first week after birth. However, we did not have data of infants born before 34 gestational weeks who did not receive probiotics in this study; thus, it is impossible to examine the degree of change in Bifidobacterium count due to presence or absence of probiotics. Previous reports suggest that premature birth usually results in delayed and abnormal qualitative patterns of gut colonization (often described as aberrant) in comparison with that in healthy term infants [19,20]. There appeared to be significant differences in the composition of the intestinal microbiota in preterm *versus* term infants; these differences included decreased bacterial diversity and an increase in pathogens potentially related to necrotizing enterocolitis (NEC) [21,22]. Stewart et al. [23] using molecular techniques, suggested that Enterobacter and Staphylococcus species were associated with NEC. Additionally, preterm infants showed retarded Bifidobacterium colonization and a high prevalence of *S. aureus*, Enterobacteriaceae, Enterococcaceae, and their lactic acid bacteria from the genus Lactobacillus and Weissella [24]. There were no infants diagnosed with NEC in this study period; we thought this fact was related with our result that Bifidobacterium level was increased as same level as that of healthy neonates at one month after birth. However, there were possibilities that the low levels of microbiota compared with that in healthy infants in early life could lead to microbiota distribution changes and the development of NEC.

The second point addresses *S. aureus*. Generally, healthy neonates start to be exposed to indigenous bacteria, including *S. aureus*, from

	N	GA (mean weeks ± SE)	BBW (mean g ± SE)
Brest-fed	25	33.1 ± 1.3	1950.6 ± 243.4
Combination	65	37.1 ± 0.4	2501.7 ± 94.3

		Bifidobacterium	Enterococcus	Enterobactereace
Day 0-3	p	0.0330*	0.6537	0.4371
	u	577.0	762.0	726.0
	Median (Brest-fed)	6.31×10^7	9.15×10^7	155×10^7
	Median (Comb)	1.48×10^5	3.55×10^7	276×10^7
Day 30	p	0.7352	0.8738	0.0852
	u	774.5	794.5	622.0
	Median (Brest-fed)	3.66×10^{12}	2.88×10^{10}	1.12×10^8
	Median (Comb)	6.92×10^{12}	3.12×10^{10}	4.95×10^9
※*p<0.05、**p<0.01、***p<0.001				

Table 8A: Influence of nutrition type (Brest-fed: 25 infants who used only Brest-fed milk, Combination: 65 infants who used formula-fed and Brest-fed milk).

	n	GA (mean weeks ± SE)	BBW (mean g ± SE)
Insufficient nutrition (+)	9	33.2 ± 2.1	1986.2 ± 336.3
Insufficient nutrition (-)	81	36.3 ± 0.5	2388.9 ± 102.9

		Bifidobacterium	Enterococcus	Enterobactereace
Day0-3	p	0.1087	0.4487	0.0689
	u	245.0	307.0	230.0
	Median(+)	1.46×10^5	1.64×10^7	2.66×10^6
	Median(-)	5.95×10^5	4.93×10^7	2.76×10^7
Day30	p	0.0116*	0.9703	0.1977
	u	180.5	361.5	268.0
	Median(+)	5.030×10^{10}	3.26×10^{10}	1.12×10^8
	Median(-)	7.690×10^{12}	3.08×10^{10}	4.82×10^9
※*p<0.05、**p<0.01、***p<0.001				

Table 8B: Influence of insufficient enteral nutrition.

their mother's skin and their surrounding environment. In the gut environment, Staphylococci, Clostridia, and Streptococci are considered potential pathogens, in contrast with Bifidobacterium and Lactobacillus species, which are beneficial bacteria required for maintaining homeostasis in GI tracts [1-4,25]. Alternatively, the majority of *S. aureus* that has been detected in the hospital environment has the *mecA* type of methicillin-resistant gene which is usually regarded as the harmful hospital infections. One report suggested that 50%–80% of the *S. aureus* isolates from 12 major hospitals were methicillin resistant [16]. In this study, we investigated the *S. aureus*-positive group and found that there were no significant differences in Bifidobacterium and Enterobacteriaceae levels between *mecA*-positive and -negative groups. In addition, Enterococci levels decreased at day 30 in the positive group, but the overall balance of microbiota was maintained in each group. Based on these results, we speculated that the colonization of *S. aureus* alone would not disturb the increase and prevalence of Bifidobacterium.

The last point addresses how clinical factors affect the microbiota of neonates. Results of each subgroup analysis were limited by the small sample size. Previous reports suggested that the mode of delivery is a key factor in shaping the developing infant microbiota [26]. Vaginally-born (VB) infants are initially colonized by fecal and vaginal bacteria from the mother, whereas infants born *via* cesarean section (CS) are initially exposed to bacteria originating from the hospital environment and health-care workers [27]. Since some of these differences are sustained throughout early childhood, birth via CS has been associated with the development of allergy and asthma as well as type I diabetes, celiac disease and obesity [28]. In this study, the CS group in non-NICU group showed lower Enterobacteriaceae levels than VB infants; this result was consistent with a previous report [26]. Although different results exist in several previous studies, this result concerning the mode of delivery is important, and infants should be followed-up for changes in microbial distribution and clinical conditions after the neonatal period.

Antibiotic therapy is one of the most common treatments for infants in the NICU. It was reported that early antibiotic therapy has the potential to cause harm as well as benefits to the infants by impeding the initial microbial colonization [18,29]. Compatible with these results, we found that there were significant differences in Enterobacteriaceae level in antibiotics-treated infants. Additionally, the antibiotic-treated infants showed lower level in Bifidobacterium at day 30 compared with non-treated infants in ≧ GA 36 weeks group. From this point of view, we should recognize that the use of empiric, broad-spectrum antibiotics in early life could influence the distribution of microbiota, which may present risks for infants' future health. Since the previous reports of neonatal microbiota, including preterm infants, mainly focused on the influence of the mode of delivery, type of nutrition, and antibiotic therapy [9,21,30], we also investigated microbiota in infants who experienced more interventional treatment and were in critical condition in the NICU. In this study, Enterobacteriaceae levels of the intubated group showed lower level at both day 0-3 and 30. Additionally, *S. aureus* positive case of intubated group was higher than that of non-intubated infants in ≧ GA 36 week group. Well known as ventilator-associated pneumonia due to mechanical ventilation treatment presents a high risk of respiratory and oral infectious diseases [31], we should recognize additionally that the risk of changing the infants' intestinal environment exists. Beneficial factors in breast milk are widely recognized, and the beneficial Bifidobacterium is the most prevalent in term, breastfed infants [21,30]. In this study, Bifidobacterium was the most prevalent compared with other species in both breast-fed and combination-fed (breast- and formula-fed) groups at day 30. There were no significant differences in the levels of the three microbial species between either group. These results may reflect that we did not compare the only breastfed with the only formula-fed group because even in extra-low birth weight infants, the combination-fed groups were provided with mother's milk at least once during this period. Furthermore, we investigated about the influence of food intake cessation. In the point of nutritional management in NICU, it is important that adequate nutrition should start immediately after birth and breast-fed enteral nutrition could improve their prognosis and prevent the NEC [32]. However, there exist few infants who could not continue the enteral feeding because of their poor general condition. We found in this study that Bifidobacterium levels in insufficient enteral nutrition group were significantly decreased than that of other infants at day 30. In this group, all case underwent surgery at neonatal period. Surgery may influence the neonatal abdominal environment and many factors thought to be involved in inducing a disorder of microbiota colonization through direct invasion, secondary infections, general anesthesia, impaired gut perfusion and oxygenation, and the cessation of food intake. We estimate that the cessation of enteral nutrition is one of the most involved factors for colonization, although we could not answer these questions completely. Further research is required to clarify these points. Conversely beneficial small bacterial overgrowth following duodenectomy can lead to chronic complication such as D-lactic acidosis and Vitamin B12 deficiency anemia, which was described in our previous study [33,34]. It was reported that the disruption of normal colonization in the neonatal period could continue for few years [35]; hence, we need a long-term follow up for such infants' microbiota profiles. In conclusion, we analyzed the representative microbiota species from the fecal samples of 90neonates using the 16S rRNA PCR assay method. We found that infants in the NICU developed similar microbiota composition as in healthy term infants by 1 month after birth; however insufficient enteral nutrition could lead to disintegration of the microbiota distribution.

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