

# Impact of $\beta$ -lactam antibiotics and the delivery method on Bifidobacteria and Bacteroides populations in early infancy: A Japanese cohort study

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## Research

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## Abstract

# Background

Intestinal flora changes dynamically in early infancy. Association between colonisation of *Bifidobacterium* and *Bacteroides*, the dominant bacterial genera in the intestinal flora 6 months after birth, and development of intestinal immunity has been suggested. Antibiotic administration to the mother is an important factor in this colonisation, but few studies have considered antibiotics taken immediately before delivery, including before Caesarean section. The association of the gut flora with the delivery method is also uncertain. Here, we performed a cohort study to determine the influence of antibiotics taken immediately before delivery on the intestinal flora in early infancy, with a focus on *Bifidobacterium* and *Bacteroides*, in 130 healthy Japanese infants.

## Results

Faecal samples were collected from infants at 1, 3, and 6 months after birth (383 samples) in a hospital in Japan. The samples were analysed using a next-generation sequencer. Two  $\beta$ -lactam antibiotics were administered to mothers immediately before delivery: Cefazolin was used before Caesarean sections and ampicillin was used for cases with premature rupture of the membranes and in Group B Streptococcus positive cases. At all ages, *Bifidobacterium* and *Bacteroides* were dominant, and the mean combined occupancy was 60–70%. *Bifidobacterium* was the most dominant at all ages but its abundance was significantly lower in infants who were exposed to antibiotics at delivery, and 1 and 3 months after birth, whereas no difference was found between the delivery methods. *Bacteroides* were the second or third dominant bacteria at all ages but their abundance was significantly lower after Caesarean section compared with that after vaginal delivery, whereas there was no difference due to antibiotic exposure. These effects were confirmed in a linear mixed-effect model. In addition, occupancy by *Bifidobacterium* at 1 and 3 months and by *Bacteroides* at 3 months differed between infants with and without siblings.

## Conclusions

Exposure to antibiotics at delivery has an important effect on *Bifidobacterium*, the dominant bacterial genus of the intestinal flora, at an early age in Japanese infants, whereas *Bacteroides* is more associated with the delivery method. The influence of siblings is also significant. The clinical significance of these results in later life requires further exploration.

## Background

The human body is inhabited by 100–1000 trillion bacteria in the oral cavity, skin, and intestine, influencing the biological health of the host. [1] [2] There are large differences in the composition of human intestinal flora depending on race, country, and lifestyle. [3][4][5] Although *Bifidobacteria* population in intestinal flora in infancy is known to affect Japanese children in the subsequent school period, studies on the intestinal flora of Japanese infants are very few. [6][7][5][8][9][10]

Many studies have indicated an association between intestinal flora and disease. [11, 12][13] Colonisation by intestinal bacteria in early infancy is known to have a major effect on intestinal immunity [14][15][16] and changes in the intestinal flora. Especially the dominant bacteria, *Bifidobacteria* and *Bacteroides* [17][18][19][20][21][22][23] resulting from the start of food ingestion at 6 months after birth, have been linked to subsequent development of diseases, such as allergies. [24][25][26] Various factors are known to influence the bacterial colonisation, including maternal exposure to antibiotics administered as intrapartum antimicrobial prophylaxis (IAP) in Group B Streptococcus (GBS)-positive mothers [27][28][29] and those administered in the late perinatal period. [30] These studies, however, have reported differences in the administration and screening period, thereby resulting in the effects being inconclusive. The influence of the delivery method on the *Bifidobacteria* and *Bacteroides* populations in infants has also been extensively studied. [31][32][16][33] It is a widely accepted hypothesis that since Caesarean section does not require the infant to pass through the birth canal, the infant does not come in contact with the maternal bacterial flora and this influences the microbiome of the infant. [34][35] However, reports of the actual influence of Caesarean section on intestinal flora in an infant differ among studies, and it is, therefore, uncertain. Moreover, the prophylactic administration of antibiotics immediately before surgery, while the foetus and umbilical cord are connected in Caesarean section, has not been considered in these studies. [36][37] The nutrition method, [35][38] presence or absence of siblings, [39][19][40] and gestational age [33][41][42] may also influence the intestinal flora.

We previously examined the influence of all antibiotics given immediately before delivery on the *Bifidobacteria* colonisation in the intestine in a pilot study of one-month-old healthy Japanese infants. [43] This study showed that the use of antibiotics in the mother at delivery has a strong influence on the *Bifidobacteria* population and that this influence may even be more substantial than that of Caesarean section.

Unlike previous studies of IAP, this pilot study was unique in a sense that the antibiotics were used for GBS and IAP in cases of premature rupture of the membranes (PROM) and routinely before Caesarean section; i.e., antibiotics were used in the mother immediately before delivery, while the umbilical cord was still intact. Interestingly, the results revealed a significantly higher bifidobacterial occupancy in infants with siblings. In contrast, *Bacteroides* occupancy was more substantially influenced by delivery mode compared to antibiotic exposure at delivery. Since the pilot study<sup>43</sup> had a cross-sectional design and was limited to 33 one-month-old infants, a continuous study comprising of a larger group of infants was needed to confirm the results.

Based on the above background, the present study defined antibiotic exposure of infants through all antibiotics administered to their mothers immediately before delivery as antimicrobial exposure at delivery (AED). Based on the hypothesis that AED may have a strong influence on the intestinal flora of healthy

Japanese children in early infancy, and especially on the dominant bacterial genera, *Bifidobacteria* and *Bacteroides*, samples were collected from infants until 6 months after birth, and the influence of AED and other factors in the infant population were investigated.

## Methods

### Subjects

This prospective cohort study was performed as a part of a study investigating the association of allergic diseases with time-course changes observed in the microbiome in infants. The subjects were 142 infants and their mothers, who gave consent to registration before delivery or 2 weeks after delivery at Iwate Prefectural Iwai Hospital between February 2018 and March 2019. This hospital is in the Tohoku (Northeast) region of Japan and handles about 800 deliveries each year as a core perinatal medical care centre in the southern Iwate prefecture.

Inclusion criteria were as follows: Infants born by full-term natural delivery or Caesarean section, whose mothers had not been exposed to antibiotics for one month before delivery except for the antimicrobial use just before the delivery. Faecal samples were collected at 1, 3, and 6 months after birth by their parents.

Exclusion criteria were as follows: Premature babies born before 37 weeks of gestation; the samples were collected at 6 months after birth from infants, whose mothers had received antibiotics, the samples collected were handled as dropouts. If the sample collection was missed, the infant was considered as a dropout at that age and after that. If a sample was inappropriate for MiSeq analysis and could not be analysed, data for this infant was excluded from the analysis only at this time-point, while the analytical results for the infant at all other ages were used.

During IAP at delivery, a single dose of cefazolin (CEZ) (1 g) was given systemically to mothers for all Caesarean sections just before the surgery. Ampicillin (ABPC) (2 g) was given at least 4 h before delivery, followed by intermittent administration every 6 h until delivery in GBS-positive and PROM cases. Antibiotics were administered to all subjects at the dose and time defined in the clinical protocol determined by the hospital board.

Containers for collection of faecal samples at 1 month after birth were handed to mothers during hospitalisation for delivery or at the health examination of infants 2 weeks after delivery. The container for the collection of faecal samples at 3 months was sent by mail to the address of each registered infant from Core Technology Laboratories, Asahi Group Holdings, and the container for 6 months was sent by mail from the Department of Microbiome Research, Juntendo University. Each faecal sample collected by the infants' parents was transferred to a test tube (Techno Suruga Laboratory, Shizuoka, Japan) containing 0.001% bromothymol and 100 mM Tris-HCl (pH 9), 40 mM EDTA, 4 M guanidine thiocyanate, and was mixed well as described in a previous study. [44] Mixed faecal samples were delivered to a laboratory of Asahi Group Holdings (Sagamihara, Kanagawa, Japan) and were stored at  $-80^{\circ}\text{C}$  until processing for DNA extraction.

### DNA extraction

The processed samples were subjected to DNA extraction, as described previously. [43] Briefly, the samples (2 mL) were transferred to plastic tubes, centrifuged at  $14,000 \times g$  for 3 min, washed in 1.0 mL of phosphate-buffered saline, and centrifuged at  $14,000 \times g$ . Pellets were re-suspended in 500  $\mu\text{L}$  of extraction buffer (166 mM Tris/HCl, 66 mM EDTA, 8.3% sodium dodecyl sulphate, pH 9.0) and 500  $\mu\text{L}$  of TE buffer-saturated phenol. Next, 300 mg of zirconium beads (0.1 mm diameter) was added to the suspension, and the mixture was vortexed vigorously for 60 s  $\times$  3 times using a Multi-Beads Shocker (Yasui Kikai Corp., Osaka, Japan). After centrifugation at  $14,000 \times g$  for 5 min, 400  $\mu\text{L}$  of the supernatant was purified using a Maxwell Instrument (Promega KK, Tokyo, Japan).

### Sequencing and data processing

16S rRNA gene sequencing was performed using a MiSeq V3 kit as per the manufacturer's protocol (Illumina, CA, USA). Briefly, the V3-V4 region of the bacterial 16S rDNA was amplified by PCR with forward and reverse primers (5'-TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGA GGC WGC AG-3' and 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3'), using the TaKaRa Ex Taq HS Kit (TaKaRa Bio, Shiga, Japan) to amplify 5 ng of DNA from a faecal sample. After the PCR products were purified with Agencourt AMPure XP (Beckman Coulter, CA, USA), the products were amplified using a Nextera XT Index Kit v2 (Illumina, CA, USA). After the second round of PCR, the products were again purified using Agencourt AMPure XP. The library was quantified, normalised, and pooled in equimolar amounts. Sequencing was conducted using a paired-end  $2 \times 300\text{-bp}$  cycle run on an Illumina MiSeq system with a MiSeq Reagent Kit v.3 (600 cycles).

### 16S rRNA-based taxonomic and diversity analysis

QIIME2 (Quantitative Insights into Microbial Ecology, <http://qiime2.org/>) v.2019.4.0. was used for the analysis of the sequences [45]. The quality of the sequences was checked and filtered using QIIME2 plugin DADA2 [46], and chimeric sequences were removed. The primers were trimmed, and the remaining forward and reverse sequences were truncated to a final length of 280 bp. As the reference sequence, "gg-13-8-99-nb-classifier.qza" from the greengenes database was used, and operational taxonomic unit were identified at the genus level. A phylogenetic tree was then created using FastTree [47], and the beta diversity distance was estimated. Beta diversity was visualised by principal coordinate analysis.

### Data collection

The following data were collected from medical records at Iwate Prefectural Iwai Hospital: Delivery method, gender, body weight at birth, perinatal history, records of hospital visits and treatments received by the infant up to 6 months after birth including the use of antibiotics after birth. Additional information related to age (days) at sample collection, feeding method (exclusive breastfeed or added top feed), and siblings were obtained from a questionnaire completed by the mothers. The following data for mothers were also collected from medical records and a questionnaire: Age, delivery method, history of

allergies (food allergy, bronchial asthma, atopic dermatitis, allergic rhinitis), abnormal findings at delivery (including PROM and GBS-positive status), antimicrobial use during late pregnancy, and systemic antibiotics (including types) given at delivery.

## Statistical analysis

The significance of the difference between the two groups was analysed using a non-parametric ANOSIM (analysis of similarities) test based on unweighted UniFrac distances within QIIME2 (<https://qiime2.org/>). Acquired 16S rRNA gene data for bacteria were analysed using SAS® 9.4 (SAS Institute Inc., Cary, NC, USA). Background factors of the mother and child were compared using the Mann-Whitney U-test for continuous variables and the Pearson's chi-square test for categorical variables. The influence of each background factor on occupancies by higher-rank dominant bacterial genera in intestinal flora at each age was examined by logistic regression analysis. The dependence of occupancy of each bacterial genus on factors, for which a significant association was found in diversity analysis and logistic regression analysis, was examined by Mann-Whitney U-test for between-group comparison and by Kruskal-Wallis test with a Bonferroni correction for multiple group comparison. Continuous comparative changes of dominant bacterial genera due to different factors were analysed using a linear mixed-effect model (random intercept and first-order autoregression model). The significance level was set at  $p < 0.05$  in all analyses.

## Results

### Subject data

A total of 142 mother and infant pairs were registered in the study, and 424 samples were obtained. Among these, 130, 127, and 126 samples collected at 1, 3, and 6 months, respectively, adhered to the inclusion criteria of the study. Exclusion of subjects occurred due to the following reasons: Three premature babies dropped out by 1 month of age, 1 infant received antibiotic administration for fever in the neonatal period, and there were eight cases with non-analysable samples at 1 month. While dropouts from 1 to 3 months were due to antibiotic administration in eight cases, no samples were received in one case, and a non-analysable sample was received at 3 months in one case, dropouts from 3 to 6 months were due to antibiotic administration in one case and a non-analysable sample at 6 months in one case.

The background information of the mothers and infants at 1 month, which were influencing factors, are shown in Table 1. Since the dropout cases resulted in no major change in the mean value of the background factors, the data at 3 and 6 months are shown in Supplementary Table S1. Antibiotics were used immediately before delivery in about 55% of cases at each age. Caesarean section, GBS-positive, and PROM cases accounted for about 20%, 15%, and 15% of all cases at each age, respectively. The PROM cases included one emergency and 6 GBS-positive Caesarean sections. These seven cases were included in the Caesarean section group because CEZ was administered as an antibiotic. In 5 PROM cases, delivery rapidly progressed, and no antibiotic was used. Of the infants born by Caesarean section, the Apgar score at birth was low in two cases, oxygen was administered after birth for mild neonatal respiratory disorder in one case, phototherapy was performed for neonatal jaundice in one case, the mothers had diabetes in two cases, the mothers had hyperthyroidism in two cases, and one infant was admitted for respiratory syncytial virus (RSV) infection during the observation period and discharged after symptomatic treatment. All these infants were confirmed to be healthy via a health examination at each age by physicians of the paediatrics and neonatology department.

The top 20 bacterial genera constituting intestinal flora at each age are shown in Fig. 1. At 1 month, based on the occupancy, *Bifidobacteria* population was found to be overwhelmingly dominant ( $49.7\% \pm 34.1\%$ ), while the *Bacteroides* population was found to be the third most dominant ( $7.7\% \pm 12.6\%$ ), but the occupancy was almost equivalent to the second most dominant bacteria, *Streptococcus* ( $7.8\% \pm 12.6\%$ ). *Bifidobacteria* population was also the most dominant at 3 months ( $61.7\% \pm 28.0\%$ ), while the *Bacteroides* population was the second most dominant ( $6.5\% \pm 10.9\%$ ). The *Bifidobacteria* population continued to be the most dominant at 6 months ( $66.2\% \pm 21.6\%$ ), followed by the *Bacteroides* population ( $5.7\% \pm 9.2\%$ ).

Table 1  
Background factors of 1-month-old infants and their mothers.

Background Factors	Data
Number of infants	130
Number of girls	70 (54.0%)
Gestational age at birth <sup>a</sup>	275.1 ± 9.3
Birth weight <sup>a</sup>	3038.9 ± 339.7
Maternal antimicrobial use at delivery	74(56.9%)
Caesarean section	31 (23.8%)
Premature rupture of membrane	22 (16.9%)
Group B Streptococcus positive status	33 (25.4%)
Infants with older siblings	67 (51.5%)
Exclusive Breast feeding	80 (61.5%)
Age of Mothers <sup>a</sup>	31.6 ± 5.1
Maternal history of allergy	55 (42.3%)
Neonatal respiratory disorder	2 (1.5%)
Neonatal jaundice	4 (3.1%)
RSV infection	3 (2.3%)
Maternal history of smoking	9 (6.9%)
Maternal history of Hyperthyroidism	2 (1.5%)
Maternal history of Diabetes Mellitus	2 (1.5%)
<sup>a</sup> Gestational age at birth, birth weight, and maternal age are shown as the mean ± standard deviation. Other factors are given as the number of subjects and a percentage.	

RSV: respiratory syncytial virus

## Analysis at one month

The effects of background factors on the five most dominant bacterial genera of the intestinal flora of one-month-old infants are shown in Table 2. *Bifidobacteria* occupancy was significantly dependent on the exposure to AED (non-AED: Odds Ratio ((OR)), 0.11; 95% Confidence Interval ((CI)), 0.03–0.39) and the existence of siblings (no sibling: OR, 3.03; 95% CI, 1.09–8.4), whereas that of *Bacteroides* significantly depended on the delivery method (vaginal delivery: OR, 0.03; 95% CI, 0.003–0.23), but not on the exposure to AED or the existence of siblings. The fourth dominant genera, *Clostridium* (6.2% ± 15.5%), showed significant effects of the exposure to AED (non-AED: OR, 4.98; 95% CI, 1.69–14.7), delivery method (vaginal delivery: OR, 4.94; 95% CI, 1.1–22.2), the existence of siblings (no sibling: OR, 0.22; 95% CI, 0.07–0.66), and feeding methods (exclusive breastfeeding: OR, 5.88; 95% CI, 2.24–15.4).

Table 2

Influence of background factors of the mother and child at 1 month on occupancies of the five most dominant bacterial genera in the intestinal flora of the infant

Variances	<i>Bifidobacterium</i>			<i>Streptococcus</i>			<i>Bacteroides</i>			<i>Clostridium</i>			<i>Escherichia</i>				
	Odds Ratio	95% CI		Odds Ratio	95% CI		Odds Ratio	95% CI		Odds Ratio	95% CI		Odds Ratio	95% CI			
Gestational days	0.97	0.91	1.04	1.03	0.97	1.09	0.95	0.89	1.02	1.02	0.95	1.10	0.99	0.95	1.0		
Birth weight	1	0.998	1.00	1	0.99	1.00	1	0.99	1.00	1	0.99	1.00	0.99	0.99	1		
Age of mothers	1.00	0.91	1.10	1.02	0.94	1.10	0.93	0.85	1.011	0.94	0.85	1.03	1.05	0.98	1.1		
Male	1.47	0.57	3.75	1.20	0.57	2.53	1.35	0.56	3.23	0.85	0.33	2.18	1.45	0.67	3.1		
Vaginal delivery	1.84	0.49	6.92	1.06	0.32	3.54	0.026	0.003	0.23	**	4.94	1.10	22.23	*	0.48	0.14	1.6
Infants without siblings	3.03	1.09	8.43	*	1.19	0.56	2.57	0.96	0.40	2.33	0.22	0.072	0.66	**	0.84	0.38	1.8
Non-AED	0.11	0.03	0.39	***	1.22	0.53	2.82	0.65	0.27	1.56	4.98	1.69	14.69	**	0.75	0.32	1.7
Mothers without allergy	1.33	0.51	3.50		1.27	0.60	2.72	1.14	0.46	2.78	0.85	0.33	2.23		1.62	0.73	3.5
Exclusively breast-fed	1.06	0.41	2.73		2.06	0.96	4.41	1.61	0.66	3.94	5.88	2.24	15.43	***	0.97	0.44	2.1

The bacterial genera are shown from left to right in the order of higher occupancy (mean). The occupancy was classified into two groups based on the median for each genus, and the odds ratio and 95% confidence interval were calculated by logistic regression analysis. The significance level was set at 5%. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Analysis at three months

The effects of background factors on the five most dominant bacterial genera and other high-ranking genera in the intestinal flora of 3-month-old infants are shown in Supplementary Table S2. *Bifidobacteria* occupancy was significantly dependent on the exposure to AED (non-AED: OR, 0.3; 95% CI, 0.09–0.9) and the existence of siblings (no sibling: OR, 3.73; 95% CI, 1.1–12.6), similar to that seen at 1 month ( $p < 0.05$ ), whereas that of *Bacteroides* was significantly dependent on the delivery method (vaginal delivery: OR, 0.14; 95% CI, 0.03–0.63), also similar to that at 1 month ( $p < 0.05$ ). The fifth dominant genus, *Ruminococcus* (2.9%  $\pm$  8.5%), showed a significant dependence on feeding methods (exclusive breastfeeding: OR, 2.5; 95% CI, 1.13–5.62).

## Analysis at six months

The effects of background factors on the five most dominant bacterial genera in the intestinal flora in 6-month-old infants and on other high-ranking genera are shown in Supplementary Table S2. *Bifidobacteria* occupancy showed no significant dependence on any factor, and *Bacteroides* only showed a significant dependence on exclusive breastfeeding ( $p < 0.05$ ). There were significant effects of the delivery method (vaginal delivery: OR, 0.26; 95% CI, 0.07–0.91) and the existence of siblings (no sibling: OR, 0.26; 95% CI, 0.07–0.91) on the third most dominant genus, *Streptococcus* (4.8%  $\pm$  6.9%). The mothers' allergy history (with history of allergy: OR, 2.67; 95% CI, 1.20–5.94) significantly affected the fourth most dominant genus, *Enterobacteriaceae*; other, and feeding methods (exclusive breastfeeding: OR, 0.29; 95% CI, 0.12–0.66) significantly affected the fifth most dominant genus, *Ruminococcus*.

## Effects of AED

Infants at each age were divided based on the AED status and delivery method, and background factors of the mother and child were compared. The results are shown in Supplementary Table S3. In infants born via Caesarean section, the existence of siblings and age of the mother were significantly higher due to the influence of the previous Caesarean section, and the gestational age and birth weight were significantly lower because the date of Caesarean delivery was decided beforehand unless performed as an emergency. This tendency also existed between the two types of delivery methods in the AED group, but a sub-group analysis of the vaginal delivery group, excluding the influence of Caesarean section, showed no difference in background factors between the AED and non-AED groups. The effects of background factors that influenced the dominant bacterial genera at each age (AED, delivery method, siblings) and the occupancies by *Bifidobacteria* and *Bacteroides* were then analysed.

AED had a significant effect on the diversity of overall intestinal flora at 1 and 3 months (Fig. 2a). *Bifidobacteria* occupancy was significantly lower in AED cases than in non-AED cases in 1-month-old infants, regardless of the use of ABPC or CEZ ( $p < 0.001$ ). In contrast, in 3-month-old infants, occupancy was not affected by AED. *Bacteroides* population was markedly lower in the CEZ group at both 1 and 3 months compared with that of the non-AED group ( $p < 0.001$ ) and this tendency was also noted in the ABPC group. A significant difference was also found between AED and the non-AED groups ( $p < 0.05$ ) (Fig. 2b).

In a sub-group analysis of AED in vaginal delivery cases without CEZ administration (i.e., excluding infants born by Caesarean section), there was a significant difference in diversity ( $p = 0.03$ ) (Fig. 3a). *Bifidobacteria* occupancy in 1-month-old infants was significantly lower in the AED group (all were included in the ABPC group) ( $p < 0.001$ ), and a significant difference was also noted at 3 months ( $p < 0.05$ ). In contrast, occupancy of *Bacteroides* did not differ between these

two groups (Fig. 3b). In the AED group, the exposure to antibiotics did not affect the *Bifidobacteria* and *Bacteroides* populations in the PROM and GBS-positive groups (Supplementary Figure S1).

## Effects of the delivery method

The delivery method significantly influenced the diversity of the intestinal flora at 1 month (Fig. 4a). The occupancy of *Bifidobacteria* did not differ with age, whereas that of *Bacteroides* was significantly lower in 1- and 3-month-old infants born via Caesarean section ( $p < 0.001$ ) (Fig. 4b). A comparison of delivery methods within the AED group gave similar findings (Fig. 5a,b).

## Effects of siblings

The presence of siblings significantly changed the diversity of the intestinal flora at 1 and 3 months (Fig. 6a). *Bifidobacteria* occupancy was significantly higher in 1-month-old ( $p = 0.001$ ) and 3-month-old ( $p < 0.001$ ) infants with siblings. Occupancy of *Bacteroides* did not differ at 1 month but was significantly lower in infants with siblings at 3 months ( $p < 0.05$ ). At 6 months, there was no significant difference in occupancy for either genus (Fig. 6b). Sub-group analysis within the AED group also showed a significant change in the diversity of the intestinal flora at 1 and 3 months (Fig. 5a), and bifidobacterial occupancy was significantly higher in 1- and 3-month-old infants with siblings ( $p < 0.01$  and  $p < 0.001$ , respectively) (Fig. 5b).

Time-course changes in *Bifidobacteria* and *Bacteroides* (linear mixed-effect model)

Comparison of bifidobacterial occupancy in AED and non-AED infants using a linear mixed-effects model (Fig. 7a) showed a significant difference over 6 months after birth, with lower occupancy in the AED group, but the difference in occupancy decreased with time. There was, however, no difference seen due to the delivery method. Occupancy appeared to be higher in infants with siblings, but there was no significant difference by the 6th month. Occupancy of *Bacteroides* did not differ significantly between the AED and non-AED groups (Fig. 7b), but the occupancy was lower in the Caesarean section group and infants with siblings.

## Discussion

The results of this study indicated that the diversity of intestinal flora was influenced by AED, delivery method, and siblings, with significant effects on *Bifidobacteria* and *Bacteroides* populations, which remained dominant at a combined occupancy of 60–70% in the bacterial flora of infants aged up to 6 months. These findings confirm the results of an earlier pilot study [43] in 1-month-old infants.

AED to  $\beta$ -lactamase antibiotics has a major influence on *Bifidobacteria* population in early infants regardless of the use of ABPC or CEZ with the influence being especially marked in 1-month-old infants. The influence of ABPC persisted until 3 months, but then gradually weakened and mostly disappeared by the 6th month. These results also comply with those of the pilot study [43]. Several previous studies have suggested a minor effect of IAP on the *Bifidobacteria* occupancy. However, these studies were limited to use of antibiotics for specific reasons, such as for GBS-positive mothers [48][49] or in the late stage of delivery [30], and the study design and screening timeline of the intestinal flora were inconsistent. A study on the antibiotic administration, including Caesarean section, has been reported [49]. However, to the best of our knowledge, the present study is the first of its kind to evaluate the effects of antibiotic administration to mothers immediately before delivery, including the cases of Caesarean section. The study comprised of a statistically appropriate number of samples, thereby allowing sub-group analyses and the effects were evaluated until 6 months after birth using a 16S rRNA-targeting next-generation sequencer.

The delivery method (vaginal vs Caesarean section) did not affect the *Bifidobacteria* occupancy in 1-month-old infants, but there was a significant difference in the CEZ and non-AED groups. The delivery method did not show any difference in the effect on the *Bifidobacteria* population between the ABPC and CEZ groups. Thus, *Bifidobacteria* occupancy was significantly influenced in the Caesarean section and CEZ groups compared to non-AED infants, but this effect did not change by AED in the vaginal delivery group (i.e., the ABPC group). These results suggest that  $\beta$ -lactam antibiotics may directly influence bifidobacterial occupancy. Influence of delivery method on *Bifidobacteria* population from immediately after birth to early infancy was shown previously, [39, 50–53] but the influence of antibiotics administered before Caesarean section [36, 37, 54] was not considered in these studies, and this effect may have been simultaneously observed. The present study is not capable of judging whether the observed effect is due to the infant not passing through the birth canal in Caesarean section or CEZ. Further studies with the use of the same antibiotics for vaginal delivery and Caesarean section are therefore warranted.

*Bifidobacteria* colonisation was also influenced by the presence or absence of siblings. *Bifidobacteria* occupancy in AED infants was significantly higher in those with siblings. The results suggested that at least until 3 months after birth, the presence of an elder sibling promoted the colonisation of *Bifidobacteria*, even in infants exposed to antibiotics at delivery, i.e., there may be mutual interference of the microbiome between siblings. This may explain the maintenance of high *Bifidobacteria* occupancy in infants, even in the AED group. In a previous pilot study, [43] the presence of siblings was suggested to influence IAP, and the present study confirmed this effect. Previous studies on the effects of siblings have reported that *Bifidobacteria* colonisation occurs more easily in infants with siblings, [19, 39, 40]. However, this effect has been scarcely studied as compared to studies on factors such as AED and the delivery method, particularly in Japanese infants. Thus, the present study is significant in showing the effect of siblings in a large cohort study. This effect was confirmed by a sub-group analysis within the AED group to allow the interpretation in the context of AED. The association of this effect with the intestinal flora of siblings requires a continuous study, including siblings living together.

The influence of Caesarean section was more substantial than that of AED in *Bacteroides*. This effect was firmly maintained at 3 months and persisted at least until 6 months after birth. The same tendency was observed in the sub-analysis of the AED group. This confirmed that birth via Caesarean section is an important factor in the occupancy of *Bacteroides*, compared to AED. The persistence of the influence of Caesarean section on *Bacteroides* until the weaning period and thereafter has been pointed out, [34, 39, 53, 55, 56] similar to our results. However, as described above, all Caesarean section cases received

preoperative CEZ. Thus, although the *Bacteroides* population was not influenced by ABPC, it may have been markedly influenced by CEZ. This possibility is outstanding and was not examined in previous studies.

*Bacteroides* population was not affected by siblings at 1 month, but a significant effect was seen at 3 months, with no influence at 6 months. In contrast to *Bifidobacteria*, siblings negatively influenced the occupancy of *Bacteroides*. The influence of siblings on *Bacteroides* population has been shown before, [39] with the occupancy by *Bacteroides* at 18 months after birth being higher in infants with siblings, in contrast to the findings of this study until 6 months. [57] Similar to *Bifidobacteria*, the effect on *Bacteroides* until 3 months may reflect mutual interference among siblings.

Many studies have examined the clinical significance of colonisation with *Bifidobacteria* and *Bacteroides* in intestinal flora in early infancy. [17–22] The present study evaluated the influence of AED on early infants in terms of the effects on their subsequent health. The samples from this study were also used to assess the relation of allergies with changes in the intestinal flora. This study was performed as a part of a cohort study evaluating the clinical significance of changes in the intestinal flora during early infancy in healthy Japanese infants and their effect on the intestinal flora later in life. Data at 1, 3, and 6 months after birth were used in this study, but data related to longer-term time-course changes in the same individuals are needed for a complete and comprehensive investigation. The composition of intestinal flora shows substantial similarities at different time-points in the same individual, [58] but external factors influencing the intestinal flora increase with growth, such as the increase in baby food intake, interaction with siblings and other infants in group nursing, and further use of antibiotics for various diseases. However, this study is significant as a cohort study with a statistically significant large number of samples performed on intestinal flora in infants in the first 6 months of life, which is a crucial period for the development of the immune system since this is a time at which external factors have the least influence in the entire lifetime [24–26].

The present study is not without limitations despite the undeniable significance. This study was conducted in a single hospital, which does not allow for extrapolation of the obtained results to the population from other regions within and without Japan, with differences in ethnicity or geography. Although the protocol for the use of antimicrobial agents before the delivery was similar to that in previous studies on IAP [27] [29], multi-centre studies are needed for validation of such extrapolation. Also, while this study focused on several factors that could have a significant impact on the gut microbiota of infants, it did not account for some factors such as feeding methods (only exclusively breast-fed infants included), which could affect colonisation characteristics of some bacteria in the intestinal microbiota of infants. Inclusion of another group of infants that were milk-fed for the most part along with top feed is imperative to understand the factors influencing gut microbiota in infants.

## Conclusions

This prospective cohort study confirmed the findings from a previous pilot study indicating that  $\beta$ -lactam antibiotics administered to the mother immediately before delivery have a significant influence on the intestinal flora of healthy Japanese infants at 1 and 3 months after birth. This effect is more important than the effect of the delivery method for *Bifidobacterium*, the dominant bacterial genus. The evaluation of the influence of AED requires the inclusion of all antibiotics used immediately before delivery, including before the Caesarean section. The presence of siblings also affects *Bifidobacteria* colonisation, and this effect persists until 3 months and increases with time. In contrast, for *Bacteroides*, the influence of the delivery method is greater than that of AED. However, it is unclear whether this is an influence of delivery via Caesarean section or CEZ administration. These results provide a new perspective on essential factors influencing the intestinal flora in early infancy, which is vital for the development of intestinal immunity. The clinical significance of the results in the later life of the infants requires further long-term studies.

## Declarations

### *Funding*

None.

### *Consent for publications*

All mothers agreed to publication of the data and written informed consent was obtained for this purpose.

### *Ethics approval and consent to participate*

All experimental procedures used in the study complied with the ethical standards of national guidelines of the Japanese government on human experimentation and the Helsinki Declaration of 1975, as revised in 2008. The study was approved by the institutional review boards of Iwate Prefectural Iwai Hospital (No. 1234) and Juntendo University (No. 2017127). Written informed consent was obtained from all mothers.

### *Data availability*

Data are deposited in Figshare. DOI: 10.6084/m9.figshare.12000255

Sequence data reported in this article are deposited in DDBJ (DNA Data Bank of Japan) Sequence Read Archive. Accession number: DRA010467

### *Competing interests*

The authors declare no competing interests.

### *Author contributions*

Imoto, Aoyagi, Morita, and Amanuma designed the study.

Watanabe, Hashiguchi, and Maruyama evaluated, corrected, and reviewed the study.

Imoto, Aoyagi, Kano, Amanuma, and Maruyama implemented the study (explanation to parents, distributing sample containers, and collecting questionnaires).

Aoyagi, Kano, and Morita performed storage, processing, and analysis using MiSeq of samples. Imoto, Aoyagi, Kano, and Nojiri performed data management and statistical analysis.

Imoto drafted the manuscript.

Watanabe, Hashiguchi, Amanuma, and Maruyama corrected and reviewed the manuscript.

All authors read and approved the content of the manuscript, including the accuracy of the data, ethical legitimacy, and validity of the results.

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## Figures

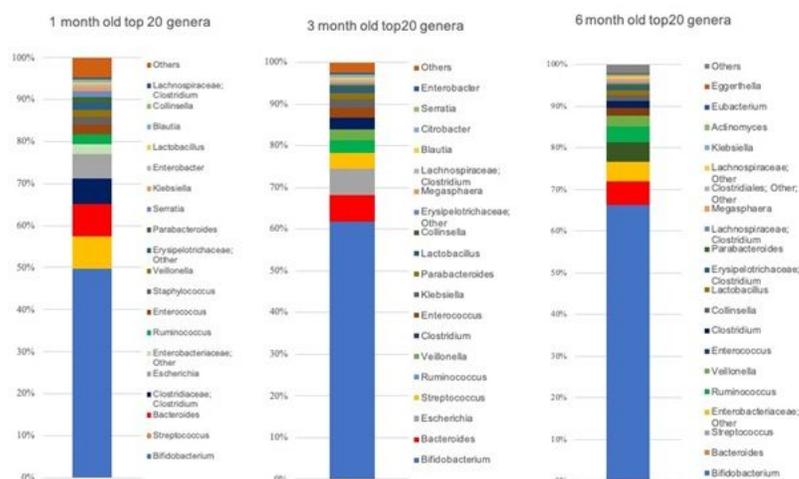
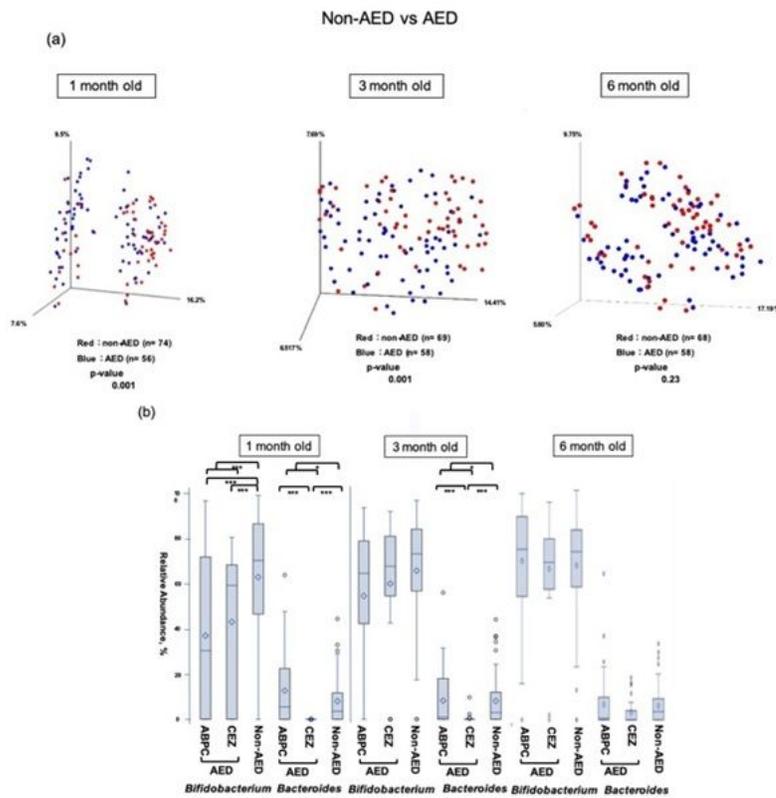


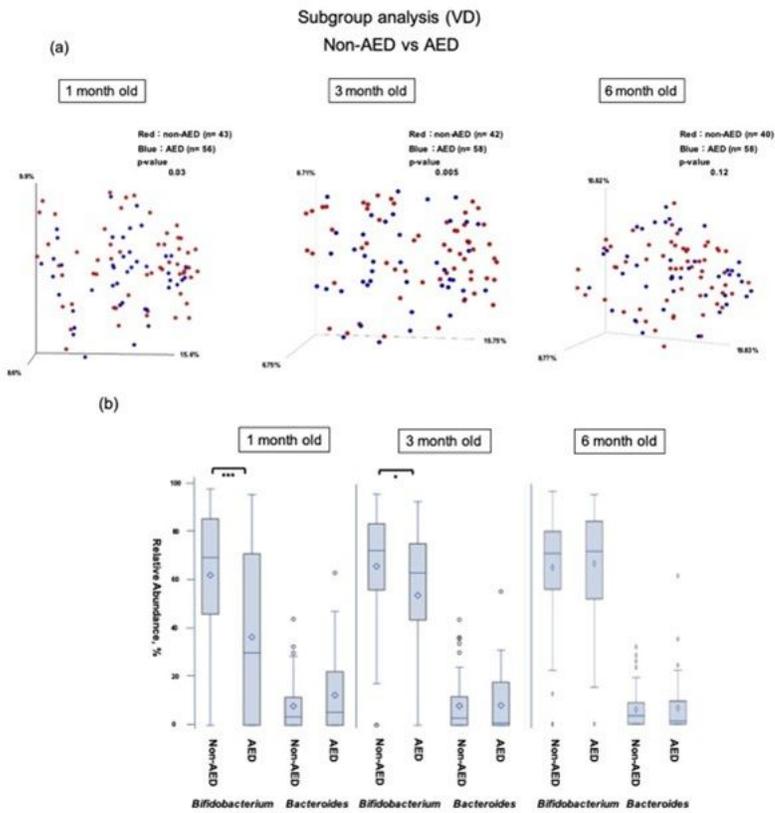
Figure 1

Mean occupancies of the top 20 bacterial genera constituting intestinal flora at 1, 3, and 6 months after birth. At each age, the occupancies of the top 20 genera (others are indicated as 'Others') are shown as 100% stacked columns. The genera are shown in the order of higher occupancy from the bottom to the top.



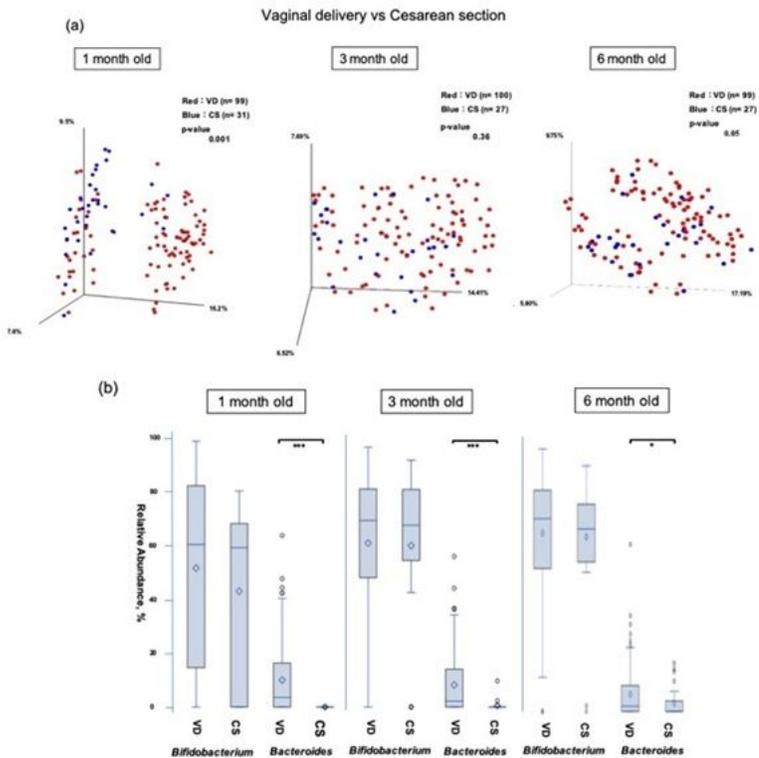
**Figure 2**

(a) Comparison of  $\alpha$  diversity of intestinal flora between infants at each age with (AED) and without (non-AED) antibiotic exposure at delivery. (b) Bifidobacteria and Bacteroides occupancies in infants in the AED and non-AED groups, shown as box-whisker plots at each age. The AED group was also divided into ampicillin-treated (ABPC) and cefazolin-treated (CEZ) groups. Comparison of occupancy among these groups and the non-AED group was performed by Bonferroni multiple comparison test. Comparison between the AED and non-AED groups was performed by Mann-Whitney U-test. The ABPC, CEZ, and non-AED groups included 43, 31, and 56 infants at 1 month (130 in total); 42, 27, and 58 infants at 3 months (127 in total); and 40, 28, and 58 infants at 6 months (126 in total). The significance level was set at 5%. \* $p < 0.05$ , \*\*\* $p < 0.001$



**Figure 3**

(a) Comparison of  $\beta$  diversity of intestinal flora between infants at each age with (AED) and without (non-AED) antibiotic exposure at vaginal delivery. (b) Comparison of Bifidobacteria and Bacteroides occupancies in infants in the AED and non-AED groups with vaginal delivery, shown as box-whisker plots at each age. The AED and non-AED groups included 43 and 56 infants at 1 month (99 in total), 42 and 58 infants at 3 months (100 in total), and 40 and 58 infants at 6 months (98 in total). The significance level was set at 5%. \* $p < 0.05$ , \*\*\* $p < 0.001$  by Mann-Whitney U-test.



**Figure 4**

(a) Comparison of  $\beta$  diversity of intestinal flora at each age between vaginal delivery (VD) and Caesarean section (CS) (b) Bifidobacteria and Bacteroides occupancies between delivery methods, shown as box-whisker plots at each age. The VD and CS groups included 99 and 31 infants at 1 month (130 in total), 100 and 27 infants at 3 months (127 in total), and 99 and 27 infants at 6 months (126 in total). The significance level was set at 5%. \* $p < 0.05$ , \*\*\* $p < 0.001$  by Mann-Whitney U-test.

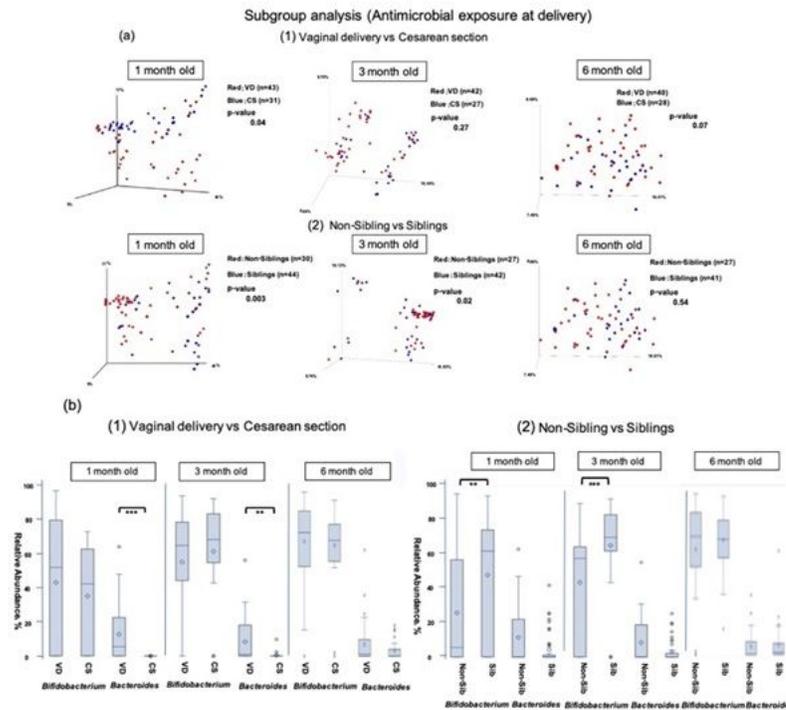


Figure 5

(a) Comparison of  $\beta$  diversity of intestinal flora at each age between delivery methods (1) and with and without siblings (2) in the AED group. VD: vaginal delivery, CS: Caesarean section, Non-Siblings: infants without a sibling, Siblings: infants with older siblings. (b) Bifidobacteria and Bacteroides occupancies in the AED group compared between delivery methods (1) and presence of absence of siblings (2), shown as box-whisker plots at each age. (1) The vaginal delivery (VD) and Caesarean section (CS) groups included 43 and 31 infants at 1 month (74 in total); 42 and 27 infants at 3 months (69 in total), and 42 and 27 infants at 6 months (68 in total). (2) The Sib and non-Sib groups included 44 and 30 infants at 1 month (74 in total), 42 and 27 infants at 3 months (69 in total), and 41 and 27 infants at 6 months (68 in total). The significance level was set at 5%. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Mann-Whitney U-test.

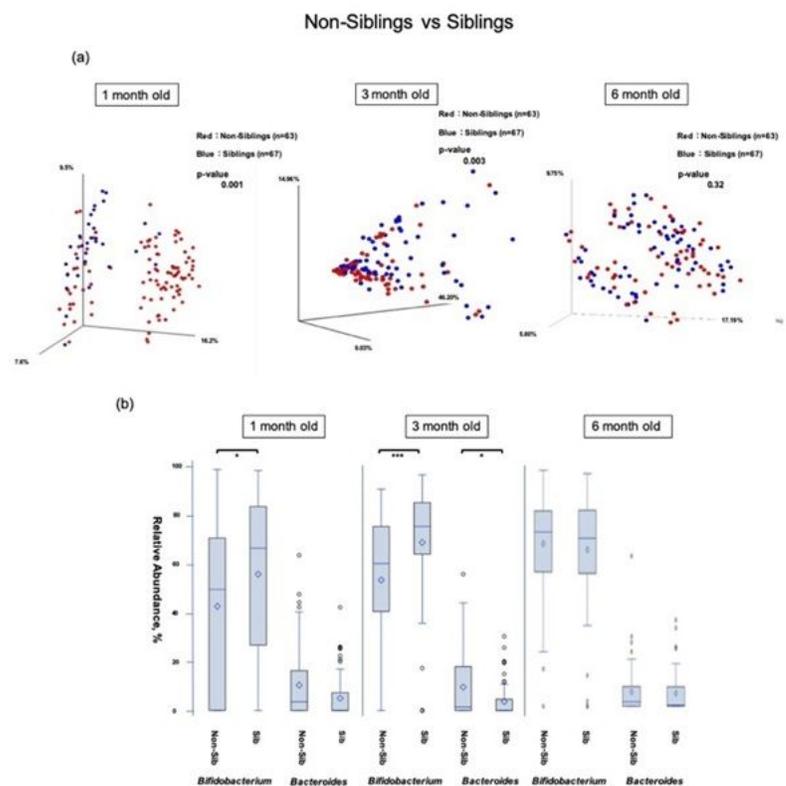


Figure 6

(a) Comparison of  $\beta$  diversity of intestinal flora between infants with and without siblings. (b) Bifidobacteria and Bacteroides occupancies between infants with siblings (Sib) and without siblings (Non-Sib), shown as box-whisker plots at each age. The Sib and non-Sib groups included 67 and 63 infants at 1 month (130 in total), 61 and 66 infants at 3 months (127 in total), and 66 and 60 infants at 6 months (126 in total). The significance level was set at 5%. \* $p < 0.05$ , \*\*\* $p < 0.001$  by Mann-Whitney U-test.

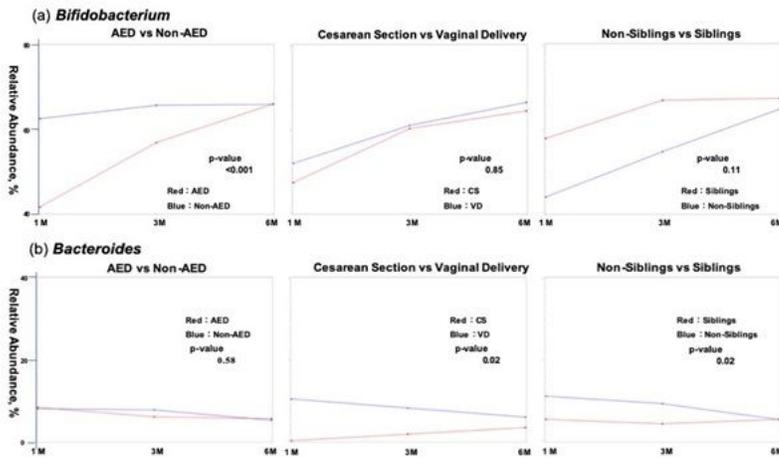


Figure 7

Time-course changes in bifidobacterial (a) and Bacteroides (b) occupancies (from 1 to 6 months after birth) based on AED or non-AED, delivery method, and the presence or absence of siblings, using a linear mixed-effect model. The analysis set at 1 month included 130 infants. Dropouts at 3 and 6 months were handled as missing values.

## Supplementary Files

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