Supplemental material

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SUPPLEMENTARY FILES

Supplementary Table 1

	AB-	AB^{PEN}	AB^{AMX}	AB^{AMC}
n	126	33	10	13
Gestational age (GA), weeks [IQR]	39.4 [38.5,40.4]	40.5 [39.3,41.1]	40.1 [39.4,40.5]	40.5 [40.2,41.3]
Birth weight, mean grams (SD)	3478 (515)	3733 (540)	3528 (326)	3797 (422)
Birth weight for GA z-score (SD)	0.2 (1.2%)	0.5 (1%)	0.1 (1%)	0.4 (1%)
Sex (Female %)	58 (46%)	16 (49%)	7 (70%)	5 (39%)
Delivery mode (Vaginal %)	83 (66%)	24 (73%)	7 (70%)	10 (77%)
Exclusive breastfeeding at 3m (Yes %)	47 (37%)	12 (36%)	7 (70%)	4 (31%)
Additional AB 1-6m (Yes %)	10 (8.2%)	9 (28%)	1 (10%)	3 (23%)
Additional AB 7-12m (Yes %)	30 (27%)	5 (17%)	0 (0%)	4 (36%)

Supplementary Table S1. Baseline characteristics of the INCA cohort subset included in this study, grouped per antibiotic type. There were no statistical differences between these groups for the listed variables. Birth weight for GA z-score is calculated according to the z-score formula [1] AB: antibiotics, AB-: control infants, AB^{Pen}: antibiotic exposure in first week of life with gentamicin and penicillin, AB^{AMX}: gentamicin and amoxicillin, AB^{AMC}: gentamicin with amoxicillin and clavulanic acid, GA: gestational age, IQR: not normally distributed variables are indicated by their median and inter quartile range [IQR], m: months, SD: standard deviation, There were no statistically significant differences with p-values < 0.05 for AB+ compared to AB- or for AB7 compared to AB2.
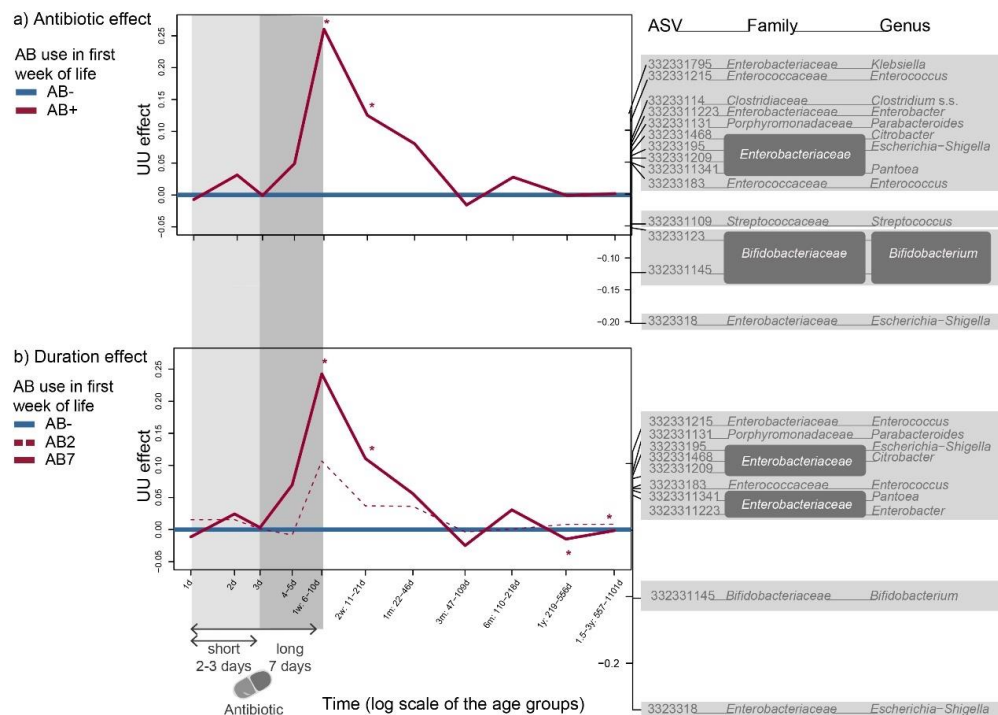
Supplementary Figure 1
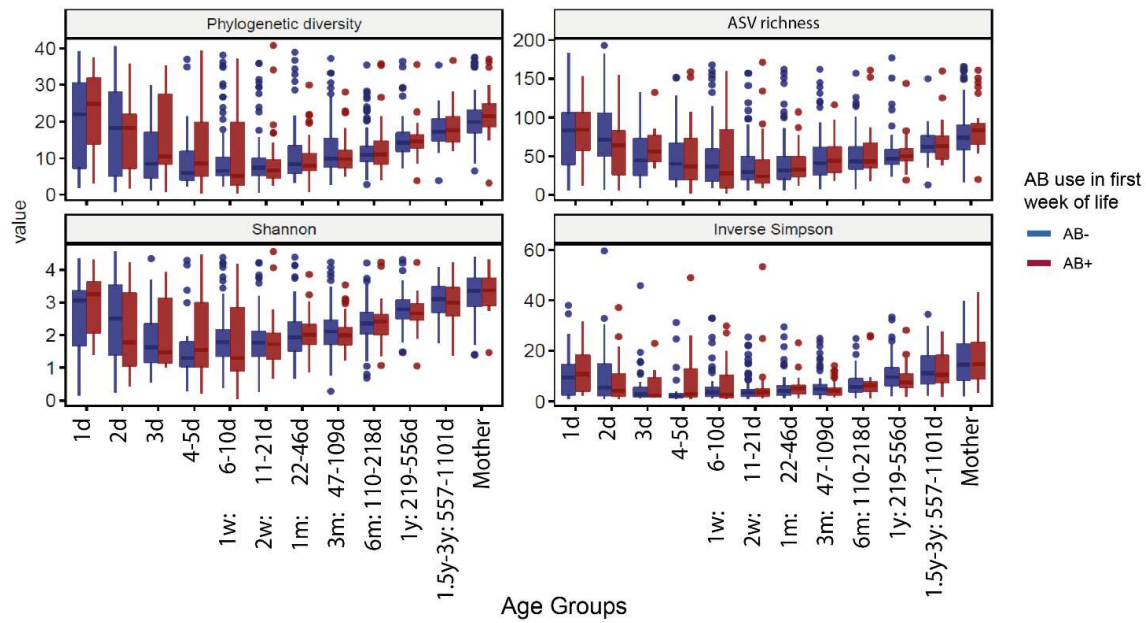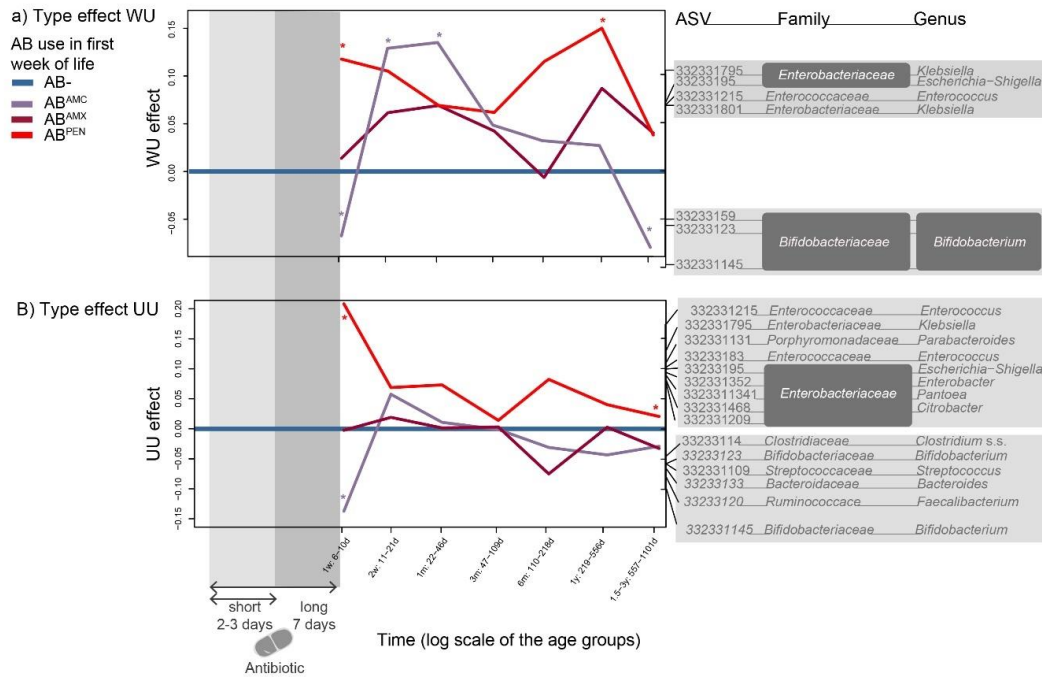

Supplementary Figure 1. Alpha diversity in age categories from birth to 2.5 years of age. Alpha diversity at different age categories of the antibiotic (AB+) exposed and non-AB infants (AB-) did not differ at any of the time points, using one-way Analysis of Covariance (ANCOVA) corrected for additional AB exposure between one and six months of age, ASV: Amplicon sequence variants.

Supplementary Figure 2

Supplementary Figure 2. Unweighted UniFrac (UU) -based Principal Response Curves (PRC) complementary to the PRC in Figure 3. **(a)** The infants who did not receive antibiotics during the first week of life (AB-), were compared as a baseline to the antibiotic exposed infants (AB+). Bacterial genera shown are the main drivers of the differences between AB+ and AB-: taxa on the same side of baseline as the curve are linked to an increased relative abundance at that time point, opposite sides indicate a decrease **(b) AB- was** also compared as a baseline with the different antibiotic durations of 2 to 3 (AB2) or 7 days (AB7) .

Significance was tested at the different time points using an ANOVA like permutation test (* = p-value < 0.05 compared to baseline AB-). Covariates that were controlled for included additional AB exposure between the age of one and six months. AB-: infants who did not receive AB during their first week of life, AB+: infants who received AB during their first week of life also indicated within grey shading, AB2: AB exposure for 2 to 3 days in the first week of life, AB7: AB exposure for 7 days in the first week of life, ASV: Amplicon sequence variants, UU: unweighted UniFrac.

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Supplementary Figure 3

Supplementary Figure 3. Weighted (WU) **(a)** and Unweighted UniFrac (UU) **(b)** -based Principal Response Curves (PRC) comparing the impact of different antibiotic types administered during the first week of life. The infants who did not receive antibiotics during the first week of life (AB-), were compared as a baseline to the different types of antibiotics AB^{PEN}, AB^{AMX} and AB^{AMC}.

Significance was tested at the different time points using an ANOVA like permutation test (* = p-value < 0.05 compared to baseline AB-). AB-: infants who did not receive AB during their first week of life, AB^{PEN}: antibiotic exposure in first week of life with gentamicin and penicillin, AB^{AMX}: gentamicin and amoxicillin, AB^{AMC}: gentamicin with amoxicillin and clavulanic acid. ASV: Amplicon sequence variants, UU: unweighted UniFrac, WU: weighted UniFrac

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Supplementary file 1 – material and methods

Study design summary

In short, 436 infants born at term were recruited from maternity and neonatal wards of four teaching hospitals in the Netherlands. Infants with suspicion of infection, who received a combination of broad-spectrum ABs in their first week of life (AB+), and healthy, unexposed controls (AB-) were included. Blood cultures were taken before AB exposure was started. In case of a negative blood culture, combined with a low clinical suspicion of infection and low C-reactive protein, ABs were discontinued after 2 to 3 days (AB2). Otherwise ABs were continued for 5 to usually 7 days (AB7). Infants without sufficient follow up, less than 7 samples over the first three years of age, were not included for this study.

Detailed material and methods:

Partial 16S rRNA gene amplicons were generated by using two consecutive PCR reactions. First, the V3 region of the 16S rRNA gene was amplified using primers (Probio_Uni/Probio_Rev) with adapters overhanging at the 5' end [2]. Thermal cycling conditions were as follows: 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C; followed by 10 min at 72°C. The PCR amplicons, which were validated and checked on size by electrophoresis on a 2200 Tape Station instrument (Agilent Technologies, USA), were cleaned by magnetic Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH). To enable multiplexing, unique combinations of Tag barcodes (8 base pairs) were attached using a second PCR with the following cycling conditions: 3 min at 95 °C; 8 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; followed by 5 min at 72°C. Each PCR was performed with a Verity Thermocycler (Applied Biosystems, USA). DNA obtained was purified by means of a magnetic purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany). The DNA concentration of the amplified sequence library was determined by a fluorometric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM in 10-µl quantities and combined to prepare the pooled final library. 16S rRNA gene amplicon sequencing was performed using an Illumina MiSeq sequencer and MiSeq Reagent Kit v3 chemicals (Illumina Inc., USA) at GenProbio srl (Parma, Italy) according to manufacturer's instructions.

1.   Hoftiezer, L. *et al.* From population reference to national standard: new and improved birthweight charts. *Am. J. Obstet. Gynecol.* **220**, 383.e1-383.e17 (2019).

2.   Milani, C. *et al.* Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. *PLoS One* **8**, e68739 (2013).