# The Influence of Timing of Maternal Administration of Antibiotics During Caesarean Section on the Intestinal Microbial Colonization in Infants (MAMI-Trial): A Randomized Controlled Trial

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# The influence of timing of Maternal administration of Antibiotics during caesarean section on the intestinal Microbial colonization in Infants (MAMI-trial): a randomized controlled trial.

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## Abstract

**Background:** To reduce the risk of maternal infections, revised guidelines for caesarean section (CS) now advise maternal antibiotic administration prior to skin incision instead of after cord clamping. Unintentionally, this results in perinatal exposure to antibiotics in all CS born neonates. Aim of this study is to investigate the effect of timing of maternally administered antibiotics during CS on the infant microbiome.

**Methods:** In this randomized controlled trial, women scheduled for an elective CS received antibiotics prior to skin incision (group A: intrauterine antibiotic exposed infants) or after clamping of the umbilical cord (group B: intrauterine unexposed infants). Women delivering vaginally were included as controls (group C). Faecal microbiota was determined from all infants at one, seven and twenty-eight days and three years after birth by means of whole-metagenome shotgun sequencing and 16S rRNA gene sequencing. The trial is registered with https://www.trialregister.nl/, NTR6000.

**Findings:** Differences between intrauterine antibiotic exposed infants (n=20) and non-exposed infants (n=20) born via CS were limited at day one and seven. However, at twenty-eight days, the whole metagenome based microbiome of infants from the former group consisted of a lower abundance of bifidobacteria compared to the latter ($p<0{\cdot}001$). In the first month of life evident differences between CS and vaginally born infants (n=23) were present. At three years of age, no differences in microbiota were observed between the three subgroups.

**Interpretation:** We observed that maternal administration of antibiotics during CS according to the revised guidelines leads to disturbance of gut colonization with bifidobacteria in the infant. This has previously been associated with disturbed priming of the immune system, even when these microbial disturbances are restored later in infancy. Our results therefore challenge

the statement in the current guidelines that prophylactic maternal prescription of antibiotics prior to CS does not influence infant health.

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## INTRODUCTION

Over the last few years, international obstetric guidelines have been revised in order to reduce the incidence of maternal and neonatal infections.[1,2] Implementation of these adjusted guidelines have resulted in an increased use of antibiotics perinatally.[1,2] However, it seems that maternal administration of intrapartum antibiotics has unintended, but profound, effects on the infant gut microbiota colonization in vaginally born infants, while evidence of consequences in caesarean born infants is lacking.[3] The effects of maternal intrapartum antibiotic use are commonly characterized by decreased microbial diversity and decreased abundance of taxa within the phylum Bacteroidetes and the genus *Bifidobacterium*, with a concurrent increase of members of the phylum Proteobacteria.[3,4] Long-term health effects for infants of perinatal exposure to antibiotics are largely unknown. Yet, increasing evidence suggests that antibiotic exposure and the following microbiome aberrations early in life, even when restored later in life, may be associated with an increased risk for impaired imprinting of the immune system, and consequently development of immune-mediated conditions such as asthma, allergies and type 1 diabetes.[5,6]

One of the revised protocols leading to an increased fetal exposure to antibiotics worldwide, is the National Institute for Health and Care Excellence (NICE) (2011) guideline for caesarean section (CS).[1] In this modified guideline, it is advised to administer antibiotics prior to skin incision, instead of after clamping of the umbilical cord. This policy has been shown to reduce the maternal risk on infectious morbidities, especially of endometritis and wound infections.[7] As a consequence, all infants born by CS are currently exposed to broad-spectrum antibiotics via the umbilical cord when adhering to the adjusted guideline. Although no increase in incidence of neonatal sepsis was observed,[7] long-term consequences or effects on the gut microbiota remain unknown. We hypothesized that exposure to antibiotics in children delivered by CS, related to the revised international guidelines, influences the microbial colonization

process and may impact health later in life. In this randomized controlled trial (RCT), we evaluated this effect by comparing the microbiome composition of CS born infants with and without intrauterine antibiotic exposure, according to the revised and previous protocol respectively.

## METHODS

### Study design and setting

This RCT was conducted at the obstetrics and paediatrics department of the Amsterdam UMC, location VUmc, a tertiary referral centre. Participants were recruited between March 2015 and November 2017. The study protocol of this study (NTR6000)[8] was approved by the local medical ethical committee (2014.468). Written informed consent for participation was obtained from all parents. If mothers did not want to participate, they received intrapartum antibiotic prophylaxis (IAP) after clamping of the umbilical cord according to the local hospital guideline.

### Study population

Pregnant women visiting outpatient clinics of the department of obstetrics and gynaecology during the third trimester of an uncomplicated pregnancy and scheduled for a primary CS were eligible to participate. Uncomplicated pregnancy was defined as a normotensive singleton pregnancy, with a normal-weight fetus, delivering at a gestational age $\geq$ 37 weeks. An overview of all maternal and neonatal exclusion criteria is listed in Table 1.

A control group of women visiting the outpatient clinic for a vaginal delivery was included simultaneously during the study period, in order to compare CS with vaginally born infants. The same exclusion criteria were retained for this group as for women delivering via CS. Over time the inclusion rate of the women delivering vaginally was adapted to the primary CS inclusions to facilitate inclusions in the same seasons.

### Randomisation and maskin

Included women scheduled for a CS were randomly allocated to be treated according to the current or the previous NICE guideline on timing of prophylactic antibiotic administration during CS. The women treated according to the current guidelines[1] received 1500 mg

cefuroxime 30 minutes prior to CS (group A). Those women allocated to be treated in accordance with previous NICE guidelines,[9] received 1500 mg cefuroxime after clamping of the umbilical cord (group B). Randomization to subgroups was done by means of www.randomizer.org in permuted blocks of 10. Women delivering vaginally (group C) did not receive antibiotics and were not randomised.

This study was not placebo controlled, since both CS groups received antibiotics; only the timing of prophylactic antibiotic administration was different between groups A and B. The gynaecologist administering the antibiotics during CS was not blinded. However, the investigators analysing the samples and performing the statistics were blinded.

**Sample size calculation**

Since there is limited literature available on the influence of antibiotics during CS on infantile microbiota colonization,[3] a formal power analysis could not be performed for this study. We planned 20 inclusions per arm of investigation to enable detection of differences over time in line with the trial by Nogacka et al.[4]

**Sample and data collection**

Faecal sample collection

The first stool sample (meconium) was collected in a sterile container (Stuhlgefäß 10 mL, Frickenhausen, Germany) by nurse or midwife, and immediately stored at -20°C. When discharged, parents were asked to collect faecal samples (approximately 2 grams) at home from their newly born children in provided containers at seven and twenty-eight days after birth. These samples were stored at home in a regular freezer and subsequently transported in cooled condition to the hospital on the day of the regular postpartum check-up, 6 weeks after the delivery. At arrival in the hospital, the samples were collected by the investigator and stored at

-20°C until further handling. At the age of three years, parents collected a fourth faecal sample at home and stored them in a regular freezer. After collection, the faecal samples were transported in frozen condition to the hospital. At arrival in the hospital the samples were stored at -20°C until further handling.

Umbilical cord blood collection

To determine to what extent neonates were exposed to cefuroxime administered to the mother, umbilical cord blood was collected from infants of group A directly after clamping of the umbilical cord and delivery of the placenta. Blood samples were collected in an Ethylene-Diamine-Tetra-Acetic acid (EDTA) tube and directly transported to the laboratory. Samples were centrifuged and plasma was stored at -80 °C until the concentration of cefuroxime was determined.

Data on health status

Parents of all included infants were instructed to complete a questionnaire (Supplement 1) at the age of three years. The questionnaire was slightly adjusted from a previously used questionnaire[10] and included items on feeding practices, anthropometric measurements, medication and health related problems like allergy, respiratory and gastro-intestinal symptoms.

**Sample handling**

DNA extraction and sequencing methods

DNA from faecal samples of days one, seven and twenty-eight was extracted as described previously.[11] All faecal samples were analysed using 16S rRNA gene sequencing to characterize the taxonomic composition. V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from faecal samples collected during the first month of life. From

faecal samples collected at the age of three, the V4 region of the 16S rRNA gene was amplified. Extracted DNA from samples of days seven and twenty-eight was additionally used for WMS sequencing to further distinguish possible differences in more detail at these time points. These time-points were chosen since the effect of the perinatal antibiotics was expected to be most pronounced with limited influence of confounding environmental factors in these samples. In contrast to meconium, at day seven the amount of human DNA has decreased with a concurrent increase in DNA of the small number of pioneer bacterial species present in the early microbiome[12]. At day twenty-eight, the diversity has increased due to an increased prevalence of *Veillonella*, *Streptococcus*, *Bifidobacterium* and Enterobacteriaceae[13]. Consequently, associations between perinatal factors and taxonomic composition in previous studies were more pronounced after one month compared to early samples from the first week of life[12,13]. DNA from samples collected at the age of three was not sequenced with WMS, since the microbiome has reached a more stable state and differences due to perinatal influences were likely to have disappeared by then.[6] A more detailed description of the performed DNA extraction, 16S rRNA gene sequencing and WMS sequencing is shown in supplementary 8.

Cefuroxime analysis

Cefuroxime plasma concentrations (mg/L) were determined using a validated high performance liquid chromatography – ultraviolet detection analysis at the department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, The Netherlands. Validation was carried out according to EMA guidelines. The lower limit of quantitation was 0.4 mg/L and upper limit of quantitation was 100 mg/L. Variation coefficient was less than 4% over the entire working range.

**Statistical analysis**

Demographic data

Demographic data was given descriptively. Comparison between both CS groups was done using the $\chi^2$ test or Fisher's exact test for dichotomous variables and Student's t-test or Mann-Whitney U for normally and non-normally distributed continuous data. For the health outcome variables at the age of three, comparisons of continuous variables between the three study groups was done using a one-way ANOVA for normal distributed variables and Kruskal Wallis test for non-normal distributed variables. The $\chi^2$ test was used to compare dichotomous outcome variables. Differences were considered significant if the two-sided p value was <0·05.

Microbiota analysis 16S

At each time point the relative abundances of all detected taxa were subjected to a Wilcoxon Rank Sum test to calculate p-values for the difference between the two CS groups and between CS born and vaginally born infants. Within-sample diversity was calculated using the Shannon diversity index on the genus level data for each group at each time point. Between-sample diversity was calculated based on Bray-Curtis distances on the genus level data, and the dissimilarity matrix was then used for the calculation of principal coordinate analysis (PCoA). The PCoA procedure was performed using Canoco 5 software for multivariate data exploration.[14]

Whole metagenome shotgun sequencing

Relative abundances of the detected taxa were subjected to a Wilcoxon Rank Sum test to calculate p-values for the difference between the two CS groups and between the CS born and vaginally born infant groups.. The resulting large sets of p-values were corrected for multiple testing by assessing the positive false discovery rate (pFDR,)[15] hence all reported p-values in the text are adjusted p-values. Furthermore, DESeq2 (v. 1.29.6)[16] was also used for the

differential abundance testing. At each time point the same approach was followed for the functionally annotated data sets.

**Role of funding source:**

This research was partially supported by Nutricia Research (Utrecht, the Netherlands) by financing costs for the microbiota analysis. This source had no role in the design of this study and did not have any role in interpretation of the data or decision to submit results. Whole metagenome sequencing was supported by a Canadian Institutes of Health Research (CIHR)-Canadian Association of Gastroenterology-Crohn's Colitis Canada New Investigator Award (2015–2019), a Canada Research Chair Tier 2 in Translational Microbiomics (2018-2019) and a Canadian Foundation of Innovation John R. Evans Leadership fund (awards #35235 and #36764) for JvL. Researchers from the funding sources declare their independence. All authors, external and internal, had full access to all of the data (including statistical reports and tables) in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The guidelines on good publication practice (GPP2) have been followed.

## RESULTS

### Patient population

During the inclusion period 4.138 women delivered of which 2.933 vaginally and 572 and 633 respectively via a primary and secondary CS. A total of 380 of the 572 women delivering via a primary CS were screened for eligibility to participate. After screening, 265 women were excluded because they did not meet the in- and exclusion criteria and one who was unable to store the samples correctly. A total of 192 women, possibly meeting the inclusion criteria were not screened during the inclusion period and 58 women declined to participate. Women declined participation mainly because they did not want their unborn child to be exposed to antibiotics (n=24). A total of 34 women declined participation without given a reason. A total of 56 women scheduled for a primary CS were found eligible to participate and were included. After randomization a total of 9 were excluded from group A and 7 from group B (Figure 1). Stool samples were collected during the first month of life of the remaining 20 intrauterine antibiotic exposed (group A) and 20 non-exposed neonates (group B).

During the inclusion period 290 women delivering vaginally were screened, of whom 44 gave consent for participation (group C). After inclusion, a total of 21 were excluded in the analysis (Figure 1). After three years, six infants were lost to follow up in group A and five and three in group B and C, respectively. Demographic and clinical characteristics of all included mothers and infants are shown in Table 2. None of the variables differed significantly between the study groups.

### Microbiome analysis

16S rRNA based diversity analysis

The microbiota showed no significant differences in Shannon diversity indices at all four time points between the two CS groups when analysed by 16S rRNA gene sequencing. Figure 2

demonstrates the Shannon diversity at day one, seven and twenty-eight. At day seven mean Shannon diversity was lower in group A (1·03) compared to group B (1·36), however this difference was not significant (p=0·23). Compared to vaginally born infants, both CS groups had a significant lower diversity at day twenty-eight (p<0·001) (Figure 2). This difference disappeared at three years of age.

Beta diversity analysis plots showed no clear differences between both CS groups during the first month of life (Supplement 2. Figure 1a). Principle coordinates of the vaginal group clustered together at day twenty-eight, where both CS groups did not. After three years, no differences between the three groups were found in the principle coordinate analysis (Supplement 2. Figure 1b and 1c).

16S rRNA based microbiome composition: difference between both CS protocols

Absolute abundance of the four most present phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) showed no difference between the two CS groups during the first month of life (Supplement 2. Figure 2). Also at genus level, no statistical significant differences were found between the two CS protocols. At three years of age, no differences were found in taxonomic composition between group A and B. An overview of the phyla and genera compared between the groups based on the 16S sequenced data along with adjusted p-values are demonstrated in Supplement 3.

16S rRNA based microbiome composition: difference between vaginally and CS born infants

Compared to vaginally born infants, the microbiota of CS born infants harboured a decreased abundancy of Firmicutes on day seven ($p_{DESeq2}$=0·002), however on day twenty-eight the Firmicutes abundance was lower in the vaginally born infants ($p_{DESeq2}$<0·001) (Supplement 2.

Figure 2). In CS born infants a decreased abundancy of Bacteroidetes was observed at day twenty-eight at phylum level ($p_{DESeq2}$=0·076).

On genus level no changes in taxa abundances were significantly different on day one. However, on day seven there were four genera that showed a deceased abundancy in CS born infants: *Dialister* ($p_{DESeq2}$<0·001), *Lactobacillus* ($p_{DESeq2}$=0·013), *Prevotella* ($p_{DESeq2}$<0·001), and *Megaspaera* ($p_{DESeq2}$<0·001). At day twenty-eight a total of 55 genera showed significant changes between CS born and vaginally born infants (Supplement 4 and Supplement 5).

Whole metagenome based microbiome composition: difference between both CS protocols

At phylum level, no differences were found between the two CS protocols at day seven. At twenty-eight days after birth, the microbiota of CS born infants from group A, the phylum Chlorobi ($p_{DESeq2}$=0·006) was more abundant compared to group B. The abundancy of this phylum was very low in all groups (Supplement 2. Figure 3). Figure 3 demonstrates the abundance of the four most prevalent phyla at days seven and twenty-eight after birth.

The abundance of members from four genera were significantly different between the two CS groups at day seven. At day twenty-eight, members of twenty-three genera differed between both CS groups using DESeq2 (Supplement 2. Figure 4 and 5). All of these genera were present in very low abundancies, except for the genus *Bifidobacterium*. The microbiota of CS born infants from group A was significantly depleted by this genus compared to the microbiota of CS born infants from group B at day 28 ($p_{DESeq2}$=0·009) (Figure 4 and Supplement 2. Figure 5). In Supplement 6 the p-values for comparisons of the abundancy of all phyla and genera between group A and B are depicted based on the WMS sequencing data. When focussing on species belonging to the genus *Bifidobacterium*, no significant differences were found between both CS groups (Figure 5).

Analyses of subsystems (sets of functional roles that together implement a specific biological process or structural complex)[17] did not reveal any differences between both CS protocols. At day seven and twenty-eight the abundance of respectively seven and two genes was significantly different (Supplement 2. Figure 6 and 7). These genes were all present in very low amounts.

Whole metagenome based microbiome composition: difference between vaginally and CS born infants

At phylum level, members of the phylum Bacteroidetes were significantly more abundant within vaginally delivered infants at day 28 ($p_{DESeq2}$=0·0004) with a concurrent decrease in Proteobacteria ($p_{DESeq2}$=0·002) (Figure 3). At genera level, only the Bacteroides was more abundant within vaginally born infants for both statistical test ($p_{DESeq2}$=0·04) (Figure 4.). Furthermore, the abundancy of numerous genera was significantly different using either one or both of the two statistical tests at day 7 and 28. An overview of the phyla and genera compared between the vaginally born infants and CS born along with adjusted p-values are demonstrated in Supplement 7.

**Cefuroxime cord blood levels**

In 17 of 20 included infants of group A umbilical cord blood was analysed to determine cefuroxime levels. Two samples were excluded since two mothers received prophylactic clindamycin because of a suspected cefuroxime allergy and in one case the blood sample was collected incorrectly. The median cefuroxime level of the analysed samples was 13·7 mg/L (interquartile range 11·2 - 17·8 mg/ L), which is above the minimal inhibitory concentration (MIC) of most bacterial species.[18]

**Questionnaire 3 years after birth**

No differences were observed in the health status at the age of three years between the groups A, B and C. No significant differences were found in the body weight index (BMI), nor in the incidence of atopic disorders as allergies and eczema (Supplement 2. Table 1.).

## DISCUSSION

In this randomized controlled trial, the effect of timing of maternal prophylactic antibiotic administration during CS on the microbiome and health state of infants up to three years of age was evaluated. CS born infants intrauterine exposed to antibiotics demonstrated a significantly lower abundance of bifidobacteria as measured by WMS sequencing at 28 days compared to unexposed CS born infants. In addition, this study confirmed that CS in general, irrespective of timing of the maternal antibiotic administration, severely affected initial microbial colonization compared to those born vaginally. No differences in health status were observed at the age of three years between the different groups.

The rate of infants born by CS has continued to increase worldwide. Currently, reported rates vary from around a quarter to more than half of all infants, depending on a wide range of factors.[19] This study confirms that that these CS born infants have an altered microbiome compared to vaginally born infants and demonstrates that these differences are independently of being exposed to antibiotics via the umbilical cord. In this study, CS born infants depicted a decreased diversity and an increased abundance of Proteobacteria during the first month of life. In vaginally born infants, maternal IAP decreases the diversity and abundance of beneficial bacterial taxa in the infant and might increase the risk for negative long-term health outcomes.[3,4] It is contra-intuitive to assume that maternal IAP has an effect on microbial colonization only in vaginally born children and not in CS born infants. In this study, it was indeed confirmed that intrauterine exposure to antibiotics in CS born infants leads to a 'second hit' on the microbiome, in addition to the generic effect of the route of delivery. The microbiota of intrauterine antibiotic exposed CS born infants, consisted of more Proteobacteria, in particular more of the genus *Klebsiella*, and decreased abundancy of Actinobacteria, mainly bifidobacteria at day twenty-eight compared to non-exposed CS born infants. After adjusting

for multiple testing, the abundancy of bifidobacteria remained statistically significantly different between the two CS groups. The early microbiota plays an important role in the development of the immune system and the risk on diseases such as asthma, eczema, diabetes and obesity later in life.[5,6] In general, bifidobacteria are considered to confer positive health benefits in this process.[20-22] For example, bifidobacteria produce acetate and lactate which act as a barrier against enteropathogenic infections. Bifidobacteria are also known to play an important role in the maturation of B-cells and a delayed in colonization is associated with a decreased number of memory B-cells later in infancy.[23,24] Furthermore, low abundancy of this genus during early infancy, is associated with elevation of inflammation markers such as pro-inflammatory monocytes and MAIT-cells, and with immune dysregulations.[22] These previous findings suggest an ongoing immune activation, instead of a homeostatic balance between tolerance and inflammation. This is characterized by elevated inflammatory markers, activated immune cell populations and a perturbed immune cell network, suggesting a disturbance in the immune imprinting during the first critical months of life in infants with a decreased abundance of bifidobacteria.[22-24] In line with this, an increasing amount of evidence indicates the importance of the role of bifidobacteria and its function in the development of the immune system and the risk on multiple non-communicable diseases later in life.[5,20,21,25]

Revisions in the NICE guidelines for CS[1] have led to unintended intrauterine exposure to antibiotics in CS born infants with at least a transient effect on the microbiome. A reduction of maternal infectious morbidities was the reason for the NICE guideline modification in 2011.[1] Women receiving antibiotics prior to CS were affected in 3·9%, predominantly by endometritis and wound infections, compared to 6·9% of women receiving antibiotics after cord clamp (risk ratio: 0·57 and number needed to treat: 33·3).[7] It was claimed that administration of antibiotics before onset of the CS decreases the incidence of maternal complications without negatively affecting neonatal outcomes, like the incidence of neonatal sepsis and duration of hospital

admission.[7] According to the NICE guideline, physicians should inform parents that no negative effects on the infant have been observed. Importantly, effects on neonatal gut microbial colonization or long-term effects associated with antibiotic exposure were not investigated before implementation of these adjusted guidelines, and thus not taken into account in this recommendation. Notably, during the informed consent conversations with parents for this study, over 50% of parents declined to participate, mainly because they preferred to be treated according to the previous NICE guidelines, preventing unintended exposure of their infants to antibiotics. In fact, the majority of parents considered the uncertain risk of antibiotic exposure more important than the proven protective effects on risk of maternal infection. Until more evidence is obtained on whether the protective effects of IAP for the mother during CS outweigh the potential harmful consequences for the child, this uncertainty should be taken into account in the informed consent process with the parents.

This is the first study evaluating timing of antibiotics during CS in a randomized design using metagenomics. Only one study investigated the effect of timing of antibiotic administration during CS on the infant microbiota using 16S rRNA gene sequencing.[26] In that study, the effect of the protocol adjustment on the infant microbiota was measured after ten days and nine months. No differences were demonstrated at the taxonomic composition at ten days postnatally, but a significantly decreased microbial species richness was found in intrauterine antibiotic exposed infants after nine months. In line with the findings from Kamal et al. (2019), we found no differences in the microbiota between the two protocols after seven days. In contrast, after one month of life, we demonstrated that the abundance of bifidobacteria was significantly decreased. Differences in outcome may result from different time points and different analytical techniques. Early in life, the diversity and amount of bacterial DNA is low, whereas at one month of age the diversity has increased and associations between perinatal factors and taxonomic composition are expected to be more pronounced.[12,13] In our study,

significant differences were only found using WMS sequencing, and not by 16S rRNA sequencing. Both methods are substantially different and can yield quantitatively and qualitatively different results.[27-29] While with rRNA sequencing only a single region of one bacterial gene is being amplified, in WMS sequencing random primers are used to sequence across the entire genome.[27-29] This allows for taxonomic identification of a larger number of species and is thought to be superior in the characterization of the complexity of the microbiome (within the limitations of available annotated genomes), and making it possible to infer microbial function.[27-29] Previous studies showed only a weak correlation between amplicon sequenced data and WMS sequencing data and this may explain why we observed differences only by using WMS. Since both methods have their own advantages and are therefore considered as complementary, it is considered useful to analyse samples parallel with both techniques.[27-29]

Strengths of this study include the randomized controlled study design and application of strict in- and exclusion criteria to limit the risk of bias. Furthermore, the cefuroxime cord blood concentrations in exposed neonates provided valuable information on the degree of antibiotic exposure. Despite the short exposure period of 30 minutes, a median concentration of 13·7 mg/L could be found in the umbilical cord, which is above the MIC of most bacterial species.[18]

Limitations of this study include the relatively small sample size. The study was underpowered to provide good insight in the long-term health outcomes. Secondly, the majority of infants were fully breastfed during the first month of life, potentially leading to a type I error, since these neonates might have been exposed to cefuroxime through lactation, regardless of timing of antibiotics during CS. However, given the short half-life of cefuroxime, the low peak concentrations in breast milk,[30] and particularly since the distribution of breastfed infants was similar in both study arms, observed differences between both study groups were most likely influenced by intrauterine antibiotic exposure and not by exposure via breastmilk.

In conclusion, we observed that the revised guidelines on antibiotics in CS lead to disturbance of early colonization with bifidobacteria. This has previously been associated with disturbed priming of the immune system, even when these microbial disturbances are restored later in infancy. Therefore, our results challenge the statement in the current NICE guidelines that maternal prescription of intrapartum antibiotics prior to CS does not influence infant health. Moreover, this study underlines that CS born infants show an aberrant microbiota, compared to vaginally born children, which is not restricted to perinatal exposure to antibiotics. Because of the ongoing worldwide increase in CS rates, prospective studies including a larger number of inclusions are needed to assess the relationship between observed dysbiosis in early infancy following intrauterine antibiotic exposure and health consequences later in life, in order to improve clinical decision making.

**Research in context**

*Evidence before this study*

Microbial colonisation, especially of bifidobacteria, is essential for the development of the innate immune system and health later in life. Intrapartum maternal use of antibiotics has been shown to effect this colonisation process and long-term health in vaginally delivered infants. Well performed research on the effects of maternal antibiotic use during pregnancy or delivery in caesarean born infants is lacking.

*Added value of this study*

In this study, it is demonstrated that maternal administration of prophylactic antibiotics prior to skin incision in caesarean delivering women, according to the current international guidelines, affects initial infant gut colonization with bifidobacteria.

*Implications of all the available evidence*

Because of the ongoing worldwide increase in caesarean section rates, prospective studies including a larger number of inclusions are needed to assess the relationship between observed dysbiosis in early infancy following intrauterine antibiotic exposure and health consequences later in life, in order to improve clinical decision making.

## DECLARATIONS

### Contributors

TdM and JdV designed the study and had responsibility overall of the study. DB, NS, LV, and TD included participants and collected data and material. JL supervised the performance of whole metagenome sequencing. GR supervised the performance of 16S rRNA gene sequencing analysis and the statistics of the sequenced data. DT performed the cefuroxime analysis. TD and TM led the writing of this editorial, and all other authors contributed equally with comments and feedback. TG is the guarantor for this paper. All authors read and approved the final manuscript. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

### Declaration of interest

All authors have completed the Conflict of Interest Statement from https://www.thelancet.com/for-authors/forms?section=icmje-coi and declare: financial support from Danone Nutricia Research for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work. NKH de Boer has served as a speaker for AbbVie and MSD. He has served as consultant and/or principal investigator for TEVA Pharma BV and Takeda. He has received a (unrestricted) research grant from Dr. Falk, TEVA Pharma BV and Takeda. The other authors have no financial disclosures that would be a potential conflict of interest. All authors declare no conflict of interest.

### Data sharing

The datasets generated and analysed during the current study are not publicly available but are available on reasonable request and after approval by a review panel. The lead author (TdM) affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned (and, if relevant, registered) have been explained.

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## Figures

**Figure 1: Trial profile**

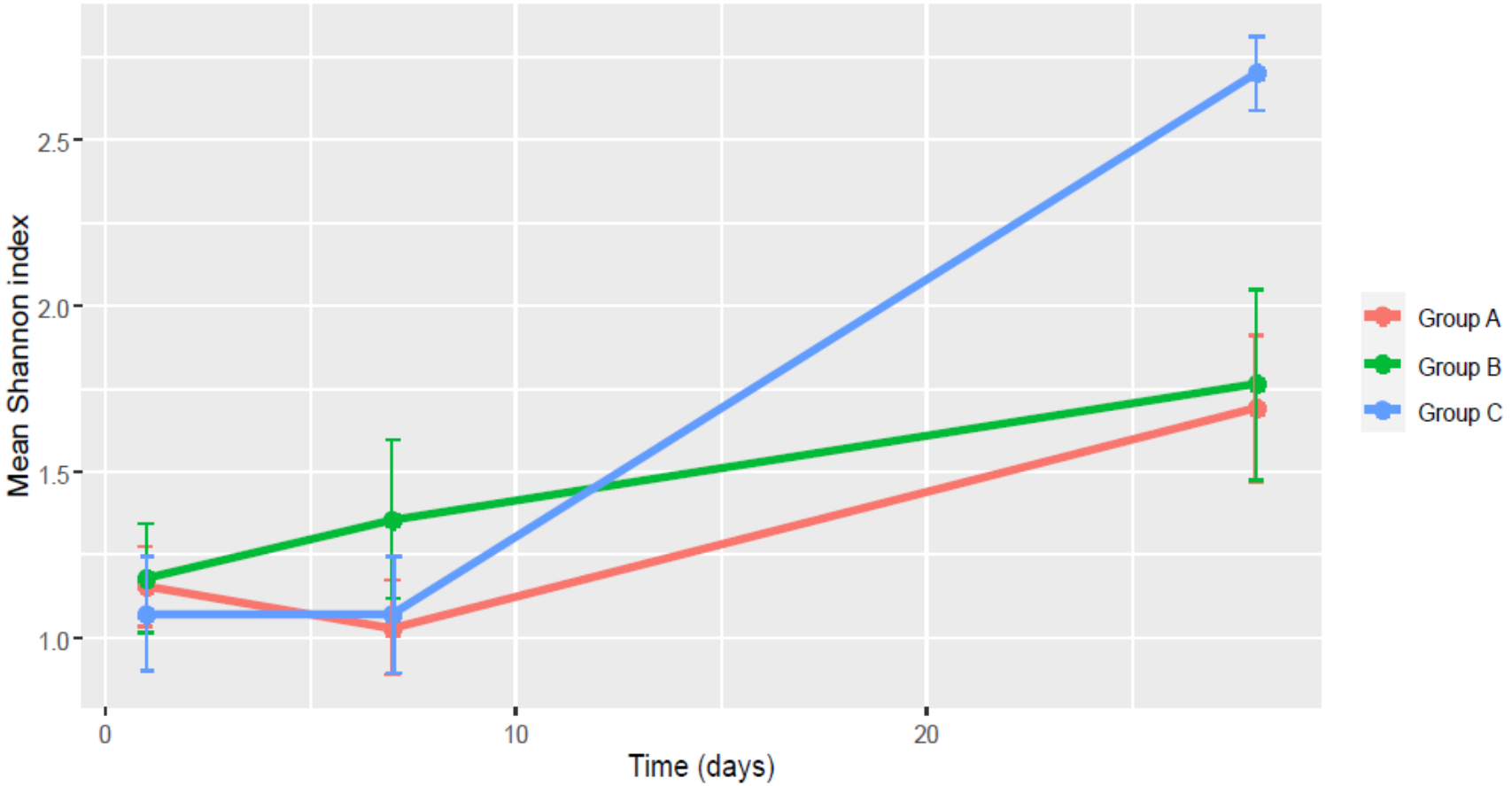


**Figure 2: Mean Shannon diversity indices of the faecal microbiota.** Faecal samples were obtained at 1, 7 and 28 days postpartum from infants of mothers delivering via caesarean section and receiving prophylactic antibiotics before skin incision (group A) or after skin incision (group B). Faecal samples were also collected from a third group of vaginally born infants. Samples were analysed by 16S rRNA gene sequencing. At day 1 and 7 no significant difference was present between infants from all three groups. At day 28 Shannon diversity index of vaginally born infants was significantly higher compared to both CS groups ($p<0{\cdot}001$).

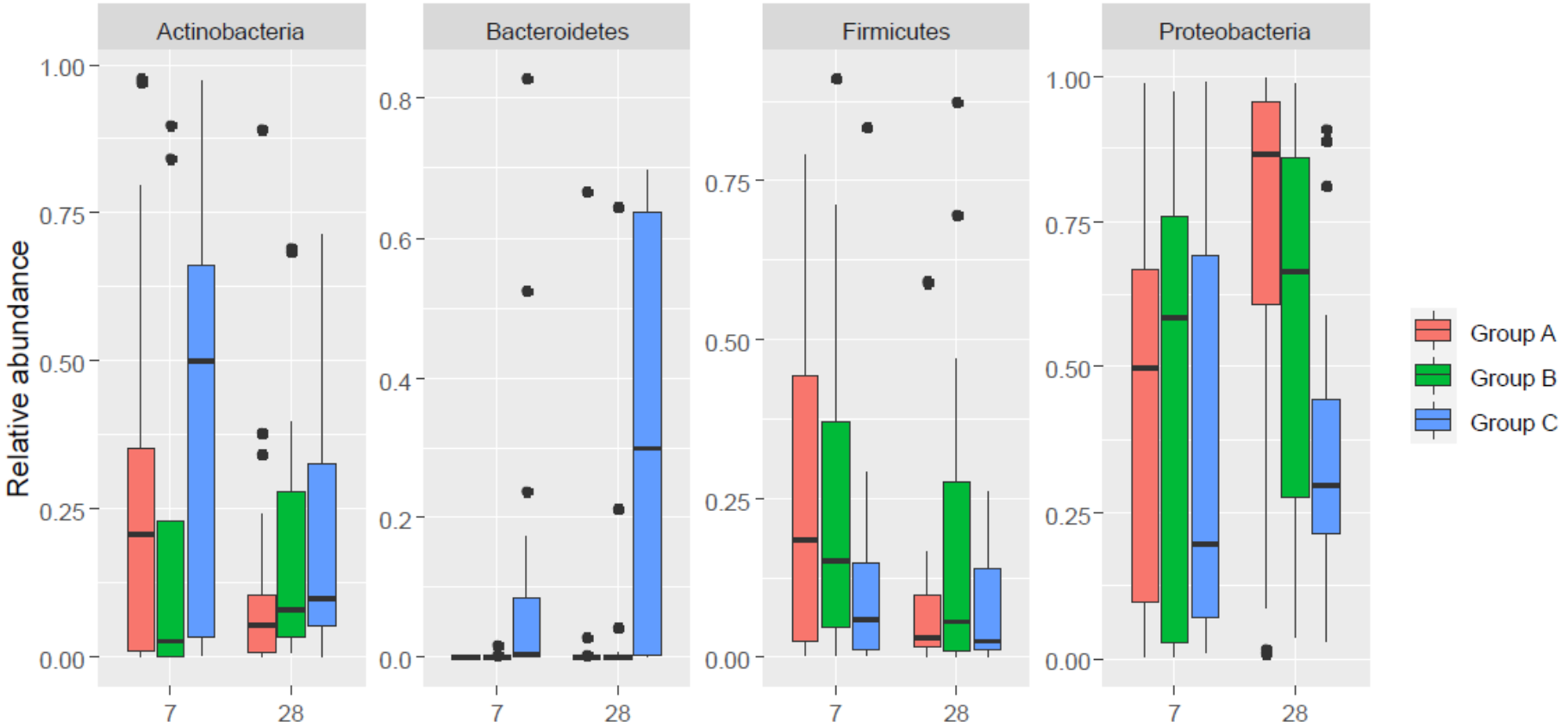


**Figure 3: Absolute abundance of the four most abundant phyla (Actinobacteria, Bacteriodetes, Firmicutes and Proteobacteria) in faecal samples obtained at 7 and 28 days analysed by whole shotgun metagenomics.** No differences were observed between intrauterine antibiotic exposed infants born via caesarean section (Group A) and non-exposed caesarean born infants (Group B). The microbiota of vaginally born infants (Group C) consisted of a higher abundance of Bacteroidetes at day 28 (p=0·0004) and a lower abundance of Proteobacteria (p=0·002)

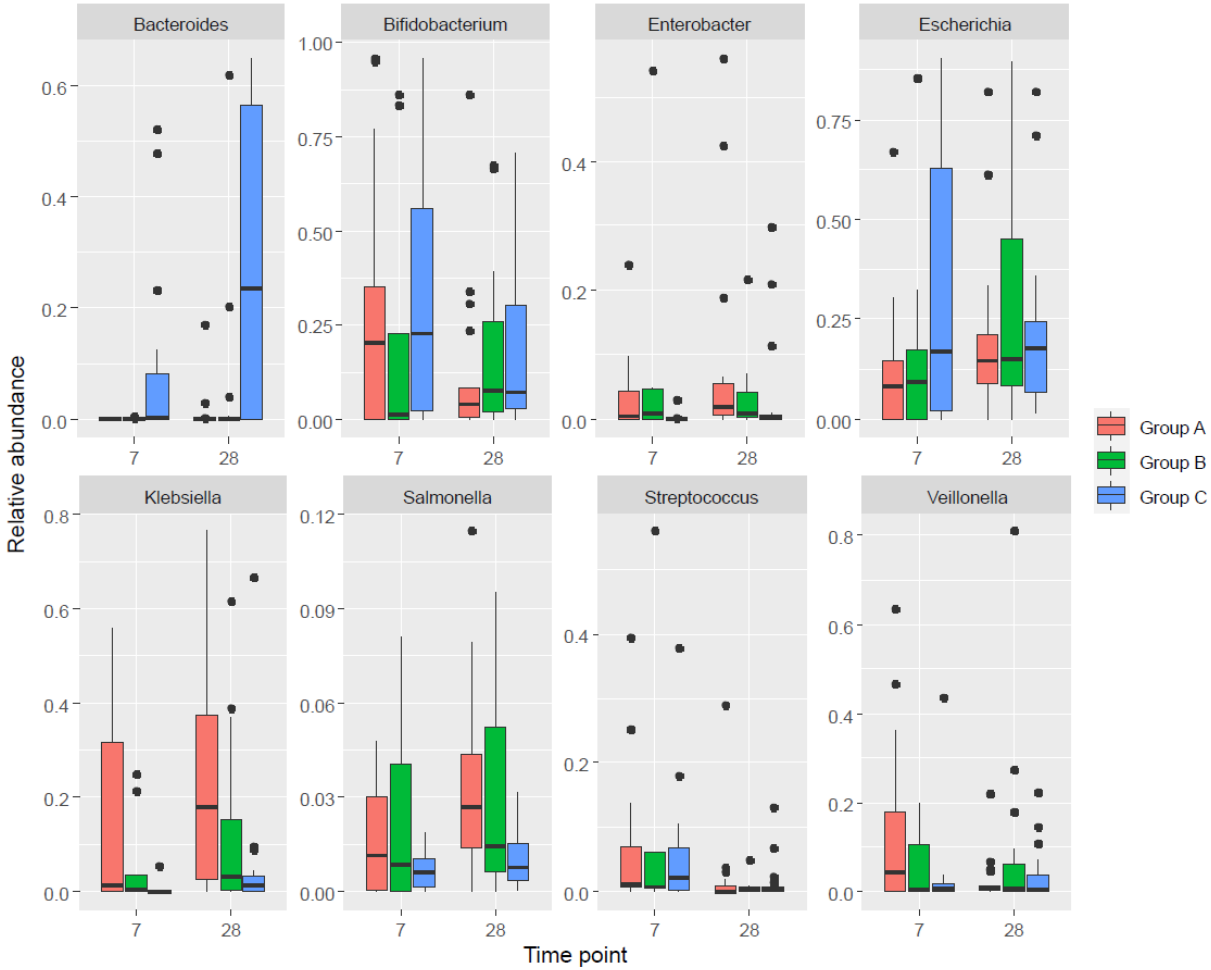


**Figure 4: Absolute abundance of eight genera in faecal samples obtained at day 7 and 28 days analysed by whole shotgun metagenomics.** At day twenty-eight the abundancy of bifidobacteria was significantly lower in intrauterine antibiotic exposed caesarean born infants (group A) compared to caesarean born infants not exposed to intrauterine antibiotics (group B) (p=0·009). Numerous genera of the microbiota of vaginally born infants (group C) differed significantly at day 7 and 28 compared to the microbiota of the caesarean born infants (Supplement 4).

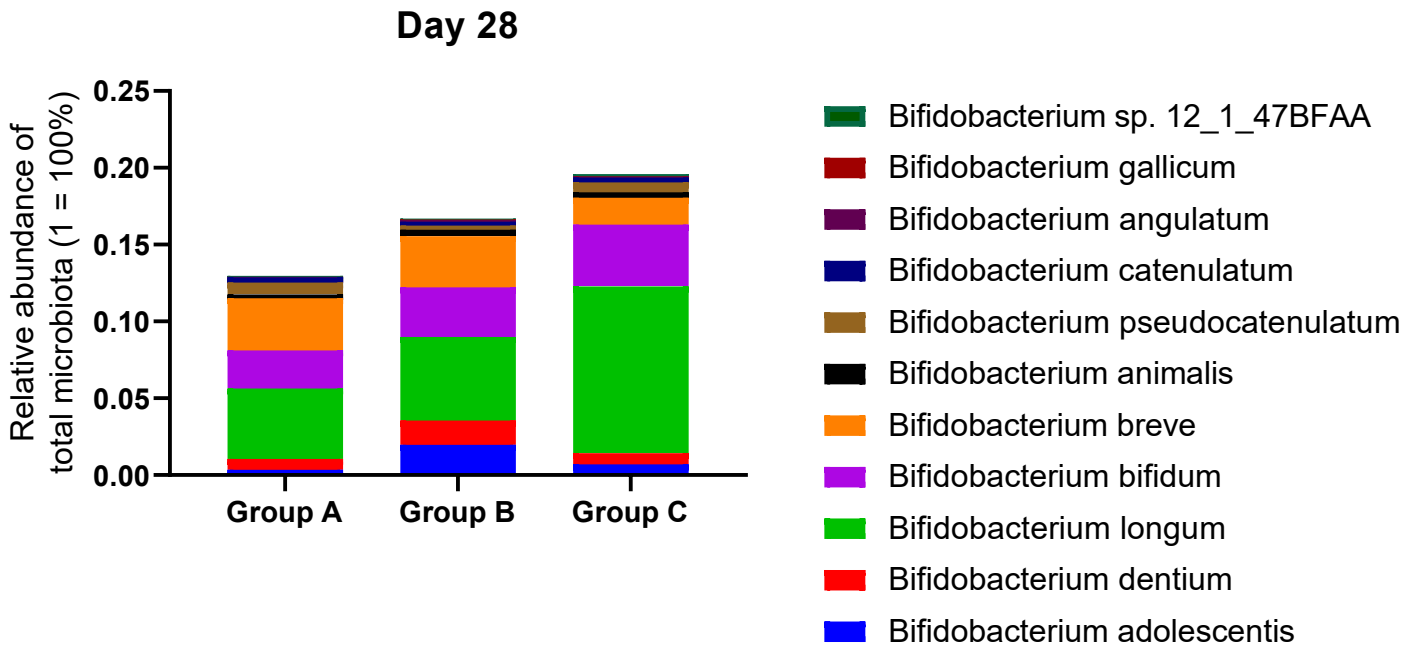


**Figure 5. Relative abundance of 11 *Bifidobacterium* species with a relative abundance of >0·1% in intrauterine antibiotic exposed caesarean born infants (group A), non-exposed caesarean born infants (group B) and vaginally born infants (group C) at day 28.** Despite the lower abundancy of bifidobacteria in group A compared to groups B and C, no significant differences were present in species belonging to the genus *Bifidobacterium* at day 28.

## Tables

**Table 1. Maternal and neonatal exclusion criteria**

| |
|---|
| **Maternal exclusion criteria** |
| Delivery < 37 weeks gestation |
| Aged ≤ 17 years |
| Body mass index (BMI) ≥ 25* |
| Antibiotic use during pregnancy |
| Antibiotic use during first month postpartum |
| Immunosuppressive usage within 3 months prior to delivery |
| Inflammatory bowel disease |
| Coeliac disease |
| Rupture of membranes before cesarean section (group A and B) |
| Prolonged rupture of membranes for >18 hours (group C) |
| Diabetes Mellitus type I/II |
| Gestational diabetes requiring insulin |
| History of major gastro-intestinal surgery |
| Alcohol or tobacco use in second and third trimester |
| Drug use during pregnancy |
| **Neonatal exclusion criteria** |
| Congenital gastro-intestinal anomalies |
| Gastro-intestinal surgery during first month of life |
| Antibiotic or immunosuppressive medication use during first month of life |

* Was adjusted to BMI ≥ 30 at November 2015. Abbreviations: BMI = body mass index

**Table 2. Mother and infant baseline characteristics.** Women delivering via caesarean section received antibiotics prior to skin incision (group A) or after clamping of the umbilical cord (group B). Vaginally delivering women (group C) were included as a controls and were not exposed to antibiotics.

| **Characteristics** | **Group A** (n=20) | **Group B** (n=20) | **Group C** (n=23) | **P value** |
|---|---|---|---|---|
| **Maternal age at birth** (median [IQR]), years | 36·6 (5·9) | 36·0 (6·7) | 32·3 (5·1) | 0·550 |
| **BMI** (median [IQR]), kg/m² | 22·8 (2·7) | 23·8 (3·7) | 21·9 (2·5) | 0·594 |
| **Gravida** (median [IQR]) | 3 (1·8) | 3 (1·8) | 2 (2·0) | 0·620 |
| **Para** (median [IQR]) | 1 (0) | 1 (1·8) | 1 (1·0) | 0·779 |
| **Maternal diet at birth** (n[%]) | | | | |
| Vegetarian | 1 (5) | 1 (5) | 3 (13) | 0·970 |
| Non-vegetarian | 18 (90) | 19 (95) | 20 (87) | |
| Missing | 1 (5·0) | 0 (0) | 0 (0) | |
| **First or repeat caesarean section** (n[%]) | | | | |
| First | 5 (25) | 9 (45) | NA | 0·185 |
| Repeat | 15 (75) | 11 (55) | NA | |
| **Gestational age** (median [IQR]), weeks + days [days] | 39+0 (3·8) | 39+0 (2·8) | 39+6 (12·0) | 0·383 |
| **Birth weight** (mean[SD]), gram | 3518 (380) | 3442 (593) | 3385 (484) | 0·634 |
| **Sex** (n[%]) | | | | |
| Female | 12 (60) | 7 (35) | 14 (61) | 0·113 |
| Male | 8 (40) | 13 (65) | 9 (39) | |
| **P-value birthweight** (n[%]) | | | | |
| *p <10* | 0 (0) | 3 (15) | 0 (0) | 0·341 |
| *p 10-p50* | 8 (40) | 6 (30) | 11 (48) | |
| *p 51-p89* | 9 (45) | 8 (40) | 10 (44) | |
| *p >90* | 3 (15) | 3 (15) | 2 (9) | |
| **Apgar score** (median [IQR]) | | | | |
| *1 minute* | 9 (0) | 9 (0) | 9 (1) | 0·947 |
| *5 minutes* | 10 (0) | 10 (0) | 10 (1) | 0·862 |
| **Meconium stained amniotic fluid** (n[%]) | 0 (0) | 1 (5) | 3 (13) | 0·311 |
| **Feeding type*** (n[%]) | | | | |
| *Breastfed* | 10 (50) | 10 (50) | 15 (65) | 0·403 |
| *Formula fed* | 6 (30) | 3 (15) | 4 (17) | |
| *Combination* | 4 (20) | 7 (35) | 4 (17) | |

Data are mean (SD), median (IQR) or n (%). BMI=body mass index; IQR=interquartile range; SD = standard deviation. *Breastfed: ≥ 80% breastmilk; Formula fed: ≥ 80% formula feeding; Combination: 21-79% breastmilk and 21-89% formula feeding.

**Table 1. Maternal and neonatal exclusion criteria**

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Data are mean (SD), median (IQR) or n (%). BMI=body mass index; IQR=interquartile range; SD = standard deviation. *Breastfed: ≥ 80% breastmilk; Formula fed: ≥ 80% formula feeding; Combination: 21-79% breastmilk and 21-89% formula feeding.

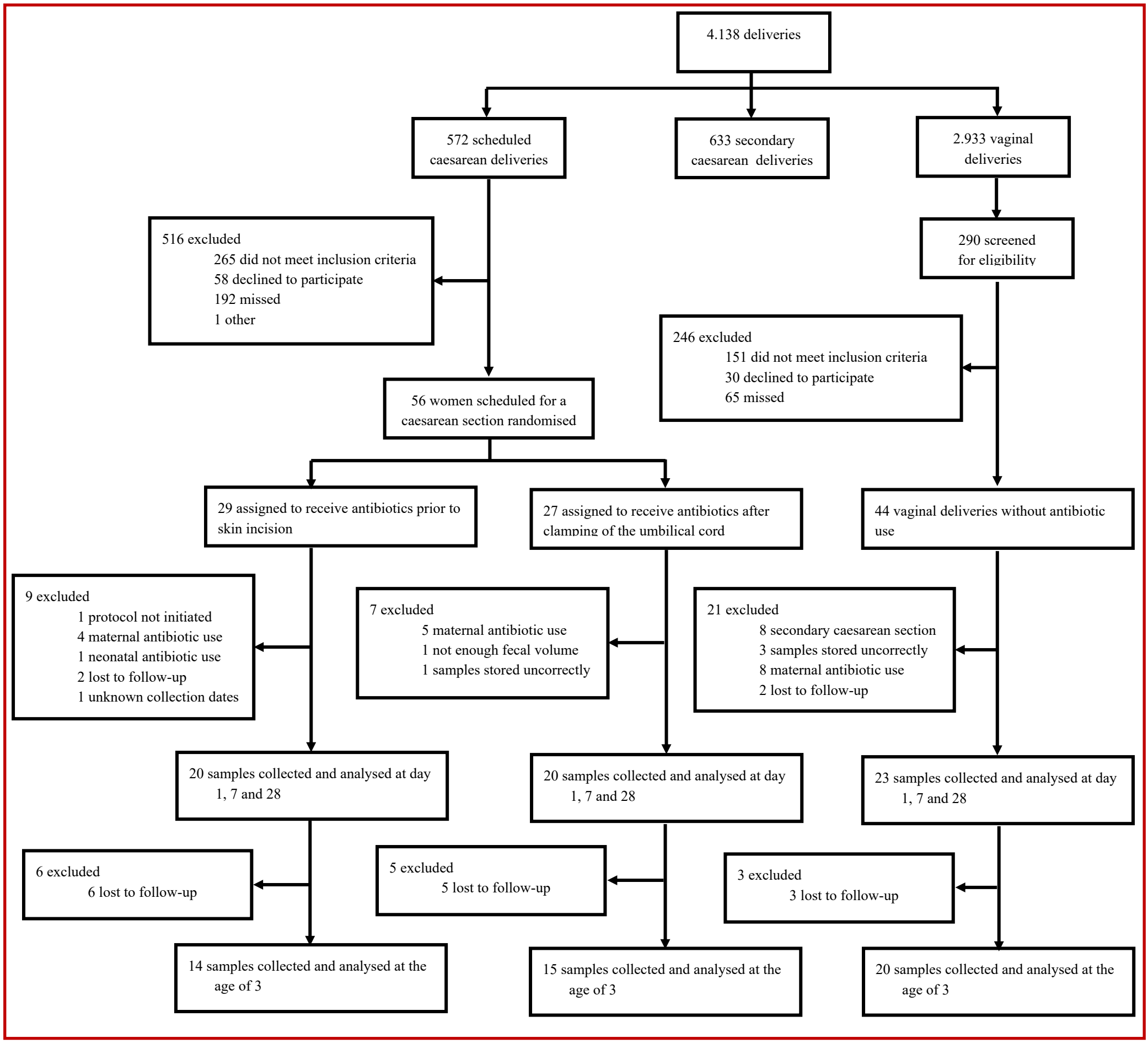
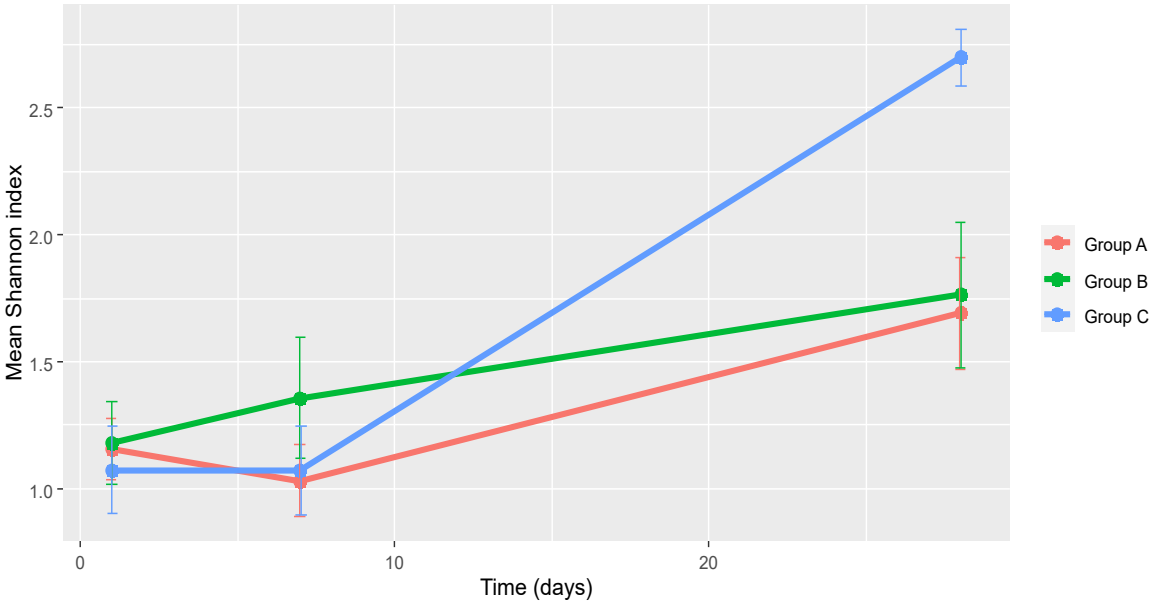
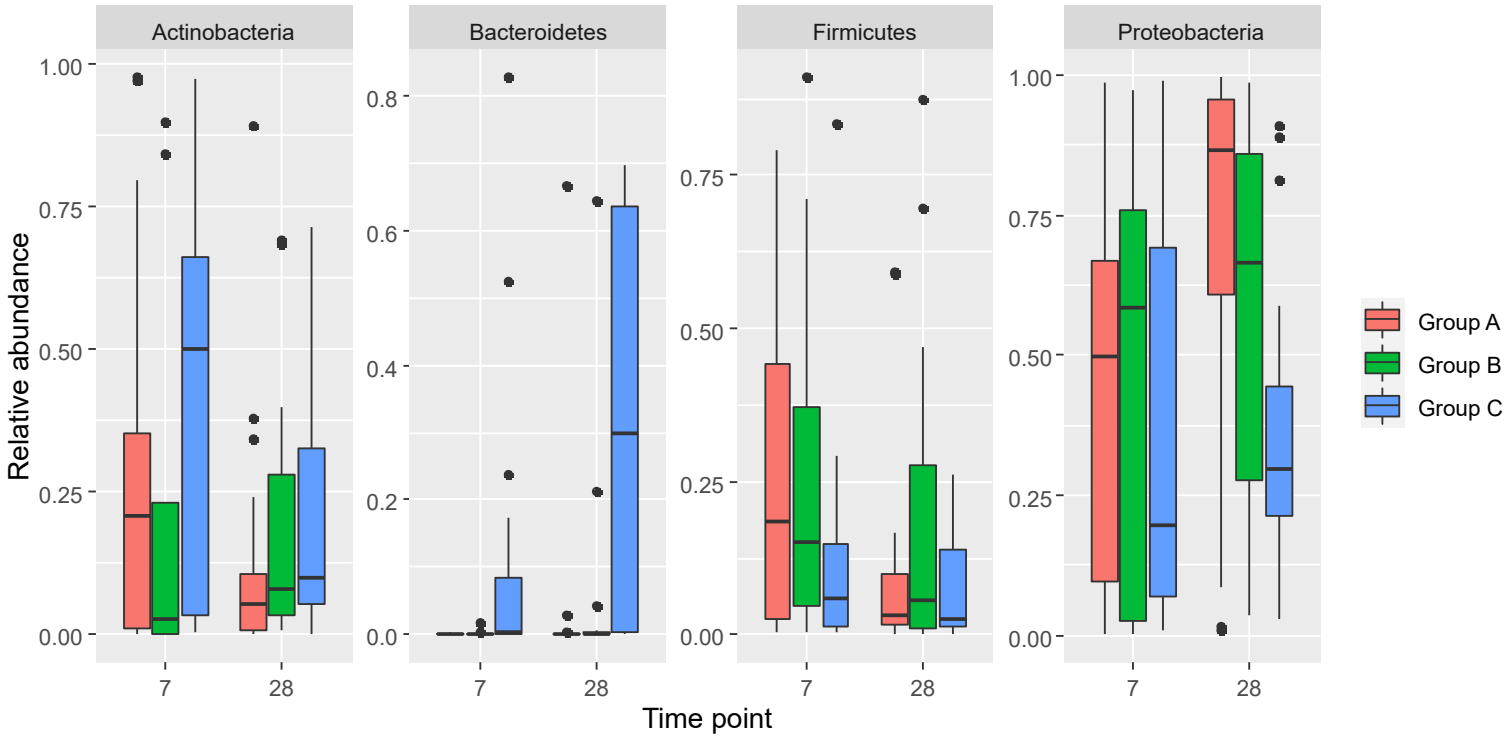
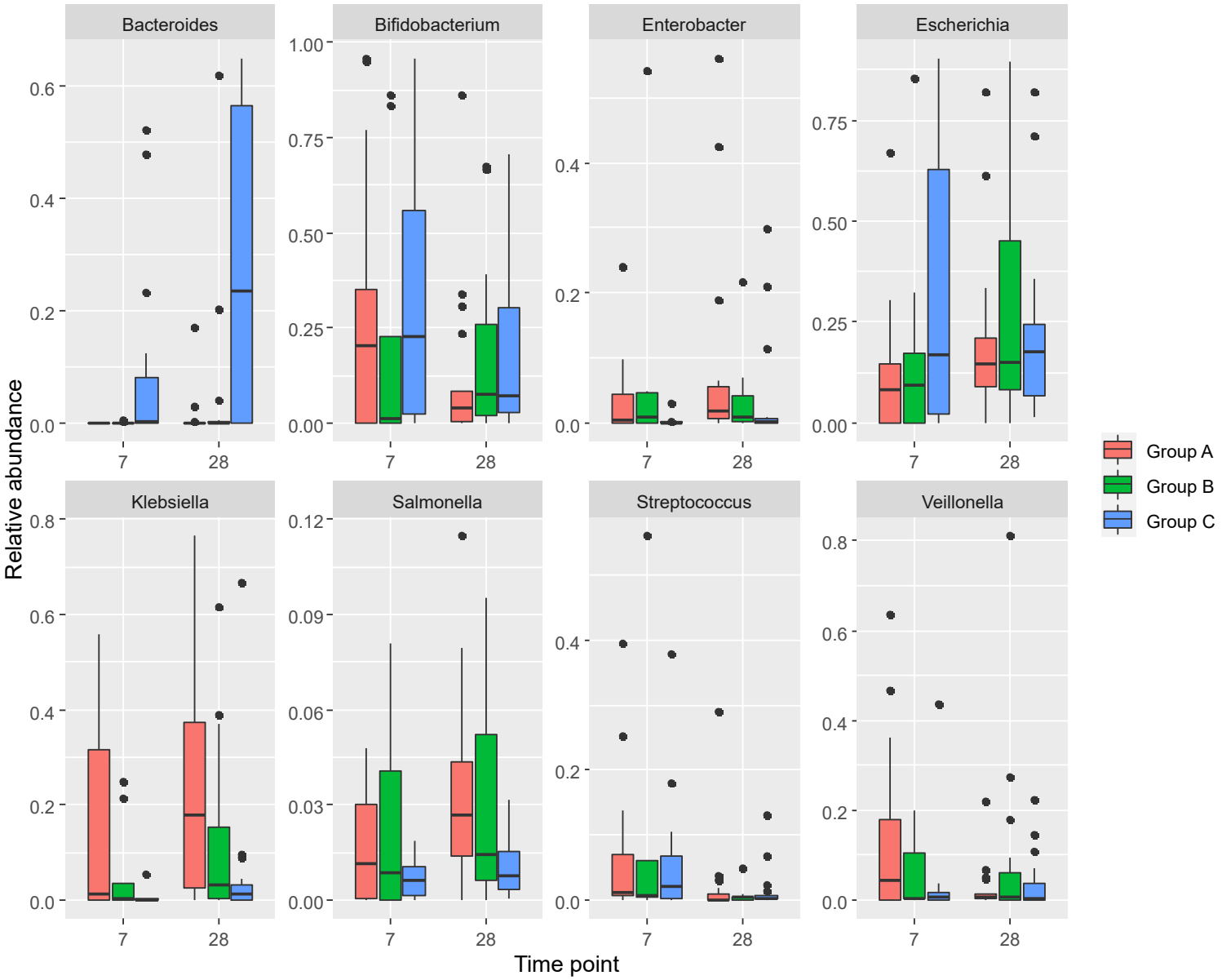
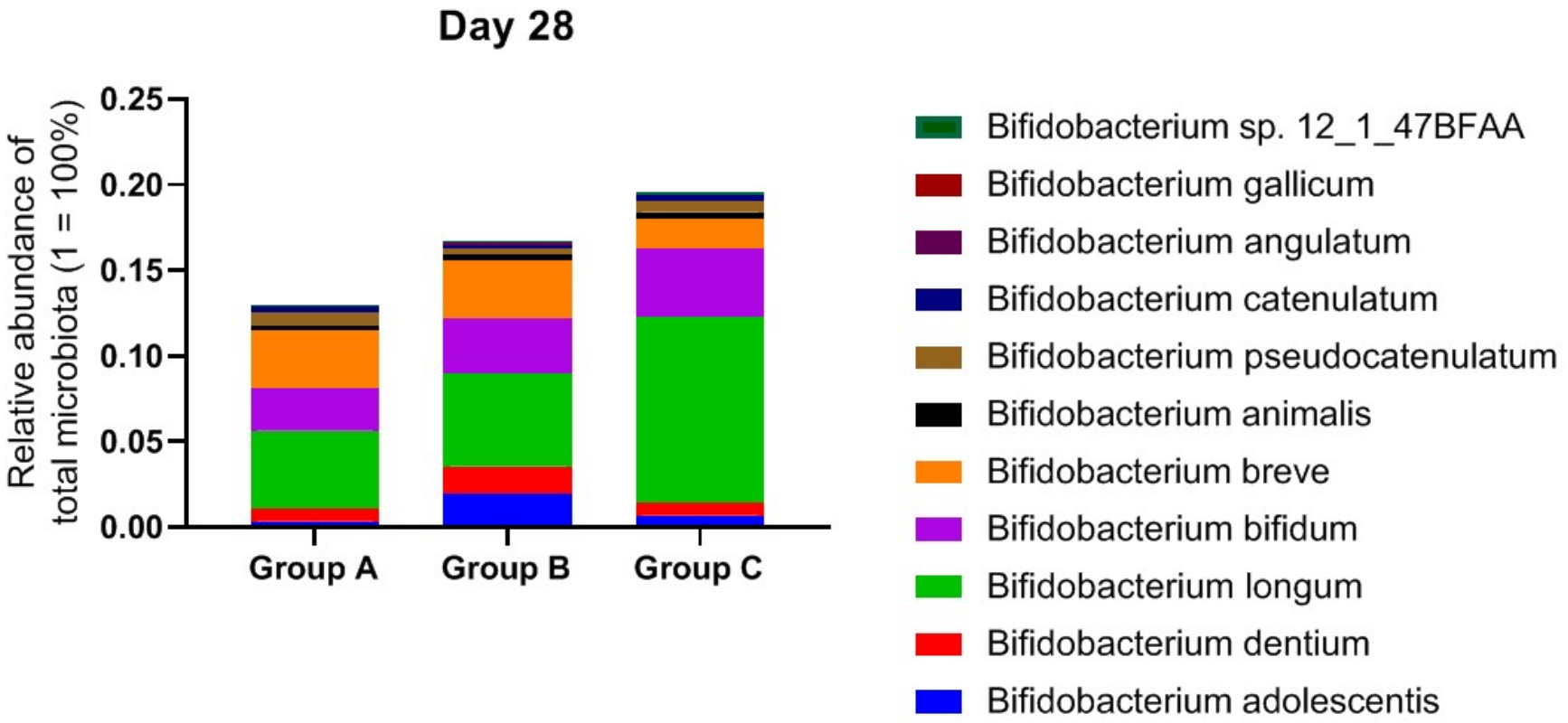



**Figure 1: Trial profile**








Day 28
Relative abundance of total microbiota (1 = 100%)
0.25
0.20
0.15
0.10
0.05
0.00
Group A
Group B
Group C
Bifidobacterium sp. 12_1_47BFAA
Bifidobacterium gallicum
Bifidobacterium angulatum
Bifidobacterium catenulatum
Bifidobacterium pseudocatenulatum
Bifidobacterium animalis
Bifidobacterium breve
Bifidobacterium bifidum
Bifidobacterium longum
Bifidobacterium dentium
Bifidobacterium adolescentis