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Research Article

Changes in healthy infant gut microbiota over the past decades

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Short Title: *Bifidobacterium presence over 20 years*

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Abstract

Introduction

Bifidobacteria typify the gut microbiota of healthy, breast-fed infants. Altered gut microbiota composition in early infancy characterized by decreased *Bifidobacterium* abundance has been linked with a heightened risk of non-communicable diseases. Our goal was to assess factors impacting on the gut microbiota composition in infants throughout the allergy and obesity epidemics of the past decades.

Methods

We studied deliveries from a series of clinical studies, grouped by the year of birth into three time periods (1997-2001, 2005-2009, 2015-2022). Altogether 48 full-term breastfed infants' having fecal samples available at the age of 1-3 months were studied for microbiota profiling by 16S rRNA gene amplicon sequencing. Perinatal factors including mode of birth and antibiotic exposure during pregnancy and at birth were taken into account.

Results

The richness and diversity of the infant gut microbiota decreased significantly over the three time periods. Reduced abundance of the phylum Actinobacteriota, and its genus *Bifidobacterium* was detected in children born in 2015-2022 as compared to those born during the time periods 1997-2001 and 2005-2009. The time period of birth was the strongest determinant of the gut microbiota composition, followed by maternal pre-pregnancy body mass index, antibiotic exposure during pregnancy and mode of birth. The relative abundance of members of the genus *Bifidobacterium* was significantly associated with elapsed time (1997-2022) and intrapartum antibiotic exposure.

Conclusions

The depletion of gut microbiota richness and diversity, and the selective reduction of relative abundance of the genus *Bifidobacterium* have occurred parallel to the increase in the prevalence of non-communicable diseases.

Introduction

The prevalence of non-communicable diseases (NCDs) has been steadily increasing over the past decades. Among these, allergy and obesity comprise common chronic conditions in children.[1,2] Particularly the rate of propagation of obesity is causing concern; there are now more obese and overweight people than those who are underweight, while four decades ago the number of the overweight was half of that of underweight.[3] The World Health Organization defined obesity as an epidemic in 1997 and set an international target for halting the rise by 2025. Since the NCDs have their origins in early childhood,[4] and the increase has taken place in a relatively short period of time, the explanation may be found in early life exposures. Microbial contact during the gut colonization process constitutes the most massive external modulator of host physiology.

The disappearing microbiota hypothesis postulating that the modern allergic and metabolic diseases may reflect loss of microbe contact has attracted strong research interest.[5] The elements suggested to explain the phenomenon include frequent antibiotic use, reduced rates of breastfeeding, an increase in caesarean section (CS) deliveries, modern dietary habits, smaller family sizes and improved sanitary and hygiene practices.[5,6] All these determinants impact on the compositional development of the child´s microbiome. The microbiome of the healthy breastfed child is characterized by *Bifidobacterium* predominance, with colonization initiated during the first days of life and with a peak at around 1 month until weaning.[7] Gut microbiota is essential for the healthy development of the immune system,[8,9] and disturbances have been consistently linked with heightened risk of NCDs.[10,11]

We undertook to find direct evidence for the proposed microbiota depletion during the early phase of the gut colonization process in an exploratory study of subjects from the same geographical and cultural area in Southwest Finland over a 25-year time period. We characterized perinatal exposures and circumstances of 48 deliveries from 1997 to 2022, coinciding with the rise of NCDs and obesity epidemic. The bacterial composition of 48 fecal samples from infants aged 1-3 months were analyzed using 16S rRNA gene amplicon sequencing.

Methods

Study participants and design

The study population consisted of pregnant and breastfeeding women and their children from a series of intervention studies conducted between the years 1997-2022 [12–14] at the Turku University Hospital in Turku, Finland.

Mother-infant pairs who were given probiotics in the intervention trials and children exposed to antibiotics during the first weeks of life were excluded from this study.

A total of 48 (**FIG 3.**) infants from whom fecal samples had been obtained at the age of 1-3 months met the criteria. The subjects were classified into three time periods based on the year of birth. The first time period: 1997–2001 with 11 infants, the second time period: 2005–2009 with 18 infants, and the third time period: 2015–2022 with 19 infants. Fecal samples were analyzed by 16S rRNA gene amplicon sequencing to determine the bacterial composition in the gut. Comprehensive clinical data was gathered (**TABLE 1).**

DNA extraction and 16S rRNA amplicon sequencing

Total DNA was extracted from the fecal material (approx. 100 mg) using the automated assisted method based on magnetic beads (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain) following the manufacturer’s instructions with previous treatments to improve the DNA extraction. In brief, samples were treated with lysozyme (20 mg/mL) and mutanolysin (5 U/mL) for 60 min at 37°C and a preliminary step of cell disruption with 3-µm diameter glass beads during 1 min at 6 m/s by a bead beater FastPrep 24-5 G Homogenizer (MP Biomedicals). Purification of the DNA was performed using DNA Purification Kit (Macherey-Nagel, Duren, Germany). DNA concentration was measured using Qubit® 2·0 Fluorometer (Life Technology, Carlsbad, CA, USA) for further analysis.

DNA libraries were constructed with the amplification of the V3-V4 variable region of the 16S rRNA gene as described previously. A multiplexing step was conducted by the NextEra XT Index Kit (FC-131-2001) (Illumina, San Diego, CA, USA) and DNA quality of the library PCR product was measured by a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA) to verify the size; the expected size on a Bioanalyzer trace is ~550 bp. The libraries were sequenced using was a 2 × 300 bp paired-end run on a Illumina platform (FISABIO sequencing service, Valencia, Spain). Controls during DNA extraction and PCR amplification were also included and sequenced.

Computational and statistical analysis

Raw 16S rRNA gene sequencing data were processed using the DADA2 pipeline [15] to perform quality filtering, read trimming and merging, removal of chimeras, and inference of exact amplicon sequence variants (ASVs). Taxonomic assignment was performed using the SILVA v138.1 reference database.[16]

All statistical analyses were performed in R (v4.4.1). [17] Figures were generated using the ggplot2 package. P-values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) method.

To identify key covariates explaining variation in the microbiome, we performed a multivariable PERMANOVA based on Aitchison distances (Euclidean distance of CLR-transformed counts). For this, the ASV table was transformed using centered log-ratio (CLR) transformation, and distance matrices were computed. The PERMANOVA model for the fecal samples included the following variables: Time period, maternal BMI, birth method, antibiotic use during pregnancy, antibiotics at birth, infant sex, infant height and weight at birth, infant head circumference, gestational age, breastfeeding at sample collection, sample time point (1 vs 3 months), and maternal age. Results indicated that time period and antibiotic use during pregnancy were the variables explaining the largest proportion of variance, and all subsequent analyses were adjusted accordingly.

Alpha diversity metrics, including Shannon (diversity) and Chao1 (richness) indices, were calculated using the *phyloseq* package (v1.48.0). [18] Differences in alpha diversity between groups were assessed using ANOVA models adjusted accordingly, followed by pairwise comparisons using the *emmeans* package with FDR correction.

Beta diversity was assessed using Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances. The ordinations were visualized with ellipses representing 95% confidence intervals. Permutational multivariate analysis of variance (PERMANOVA) was conducted using the adonis2 function in the vegan package (v2.6-6.1) [19] to test for compositional differences between groups, with models adjusted for accordingly.

Differential abundance analysis was performed using ANCOM-BC2.[20] Models for the fecal samples were adjusted for antibiotic use during pregnancy, and all included pairwise comparisons between periods. Prior to this analysis, ASVs detected fewer than five times in less than 30% of the samples were removed, and samples with fewer than 1,000 total reads were excluded. Only taxa with FDR-adjusted q-values < 0.005 were considered statistically significant and visualized in a heatmap showing log fold changes. Taxa were labeled at the genus and species level when available, or at the genus and then phylum level otherwise.

For community composition visualization at the phylum and genus levels, ASVs were agglomerated based on taxonomic rank and transformed into relative abundances. Filtering steps described above were applied, and bar plots of the top taxa per time period were generated using *ggplot2.[21]*

Finally, Spearman correlations between variables were computed and visualized using the *corrplot* R package.[22]

Results

Clinical characteristics of the mothers and their children in the perinatal period

The clinical characteristics of the children and their mothers are presented in Table 1. The infants were divided into three groups by the year of birth, time periods 1-3 (1997-2001 n=11, 2005-2009 n=18, 2015-2022 n=19). All the infants were full-term and breastfed. The CS rates were comparable over the time periods. None of the children received antibiotics or probiotics, while antibiotic contact occurred during pregnancy in 13% and intrapartum in 31% of the subjects. The age of the mothers in the present study corresponded to the average age of laboring in the Finnish population, which was 29.8 years in 1997 and 31.6 years in 2022.

The composition of the infant's microbiota over the three time periods (1997-2022)

Statistically significant differences were detected in the infant gut microbiota profiles over the three time periods (permutational multivariate analysis of variance (PERMANOVA) R2 = 1.2496, p = 0.068, Figure 1A).

The gut microbiota profile of infants born in the third time period (2015-2022) was significantly different (PERMANOVA UNIFRAC distance $R^2 = 0.195$, p = 0.001) from that of the previous time periods (Figure 1B). The differences were also reflected in alpha-diversity, as the infants born in the third time period exhibited lower microbial richness, measured by the Chao1 (Figure 1C), compared to the infants from the first time period (p=0.0513) and second time period (p=0.051).

In taxonomic analyses, reduced abundance of the phylum Actinobacteria (Figure 2A), and its genus Bifidobacterium (Figure 2B) was observed from first and second time period (1997-2001 and 2005-2009) to third time period (2015-2022). At species level*, B. breve*, *B. longum* and *B. catenulatum* decreased from 1997-2001 to 2005-2009 (LFC= -4.14, -2.31 and -3.46, respectively) and 2015-2022 (LFC= -2.53, -2.13 and -2.71, respectively) (Figure 2C). In addition, a decrease was detected in the relative abundance of the genus *Enterococcus*, and its species *E. faecalis* from 1997-2001 to 2015-2022 (LFC=-2.01). Also, the relative abundance of genus *Staphylococcus* (LFC=-2.47), as well as *Veillonella* (LFC=-2.43), *Streptococcus* (-1.78) and *Lacticaseibacillus* (LFC=-3.02) decreased from 1997-2001 to 2015-2022.

An increased abundance of phylum *Proteobacteria* (LFC = 3.54) (Figure 2A and 2C), and its genus *Klebsiella* (LFC = 3.61 and 2.39, respectively) (Figure 2C) was observed from the first and second time period (1997-2001 and 2005-2009) to third time period (2015-2022).

The clinical circumstances impacting on the infant's gut microbiota composition

Time period (R2 = 1.2496, p = 0.068) of birth was the most important driving force of the infant's gut microbiota variation as per microbial beta diversity assessment by multivariate analysis PERMANOVA based on the Aitchison distance (Figure 1A), followed by antibiotic exposure during pregnancy (R2 = 1.2989, p = 0.088).

The factors affecting the bifidobacterial microbiota composition

We found a significant interlink between *Bifidobacterium* abundance and specific perinatal factors. Firstly, the time period negatively correlated with the abundance of genus *Bifidobacterium* (R2 = -0.47, p = 5.035e-04), showing a notable decrease across 1997 to 2022. Secondly, the administration of intrapartum antibiotics negatively correlated with *Bifidobacterium* levels (R2 = -0.42, p = 2.647e-03).

Discussion

Our serial cross-sectional study provides compelling evidence of strong modifications of the infant gut microbiota over the past decades. Importantly, these changes have coincided with the allergy and obesity epidemics witnessed worldwide.[23] Although the timing is no indication of causality, our results point to a decline in gut microbiota richness, specifically in *Bifidobacterium*, that parallels with the emergence of these NCDs.

We observed a marked decrease in gut microbiota richness and diversity over the course of our study periods (1997-2022), which was attributable to the reduced abundance of phylum Actinobacteriota and its genus *Bifidobacterium*. Bifidobacteria are the predominant gut microbiota members in breastfed infant.[10] It is possible that Bifidobacteria, due to their dominance early in life, are merely an indicator of the shift in the gut microbiota, rather than the underlying cause. Earlier cross-sectional studies point out that there are factors in the modern industrialized world that alter our gut microbiota and decrease the early Bifidobacteria colonization in infants, compared to more rural areas.[24–27] The present study provides direct evidence for a reducing relative abundance of the *Bifidobacterium* microbiota over time in the same geographical and cultural population in Southwest Finland. The clinical significance of our findings is highlighted by the fact that early life exposures and circumstances which are known to perturb the gut microbiota have been consistently linked with lasting alterations in the immune and metabolic phenotype of the child.[10] In line with this, reduced abundance of Bifidobacteria in early life has been associated with an increased risk of atopic diseases and overweight.[28] It is important to note that, while statistically significant, the observed effect sizes in the present study were relatively low. On the other hand, the magnitude of gut microbiota alterations required to influence later health are currently inadequately understood.

A number of early-life exposures are suggested to modulate infant gut colonization.[12,29] The incidence of CS is rising worldwide,[30] and it has been documented to disturb the transmission of gut microbiota, explicitly affecting Bifidobacteria.[31,32] The lack of vaginal and fecal microbiota contact during birth is compensated by breastfeeding, partly restoring *Bifidobacterium* in the infants' gut.[32] Multiple studies have demonstrated a significant association between CS and increased risk of NCDs, such as allergy and obesity.[33] As antibiotics are routinely administered during CS, further disturbance of the gut microbiota is likely to ensue. More than a third of newborns are exposed to antibiotics during birth,[34] and the exposure has been shown to decrease microbial richness, *Bifidobacterium* abundance, and carry enhanced risk of later allergies and obesity.[35] These studies suggest that there is a shared determinant increasing the risk of NCDs. In the present study, both birth by CS and antibiotic exposure during pregnancy were associated with decreased gut microbiota diversity in breastfed infants, and lower *Bifidobacterium* abundance was particularly seen in infants exposed to intrapartum antibiotics. Notably, we documented gut microbiota alterations immediately after birth, in the absence of the elements proposed in the disappearing microbiota hypothesis. Our result thus suggests that the underlying causes may need to be sought earlier, potentially in the health of the pregnant mother.

We identified high maternal BMI as a factor associated with reduced gut microbiota diversity and richness. Obesity is a common condition among women of reproductive age. Today, more than half of pregnant women are overweight or obese,[36] and the prevalence is estimated to rise. In addition to obesity-related alterations, the gut microbiota composition seems to change during pregnancy.[37] In particular, lower abundance of Bifidobacteria have been linked with gestational weight gain.[38] Our recent study suggests that gut microbiota richness and diversity in pregnant women has diminished over the past 25 years and the gut microbiota composition has shifted to resemble that of overweight individuals.[39]

These observations may have high clinical significance for the offspring since maternal obesity is associated with high birth weight, increased risk of childhood obesity that is likely to persist into adulthood.[40] The mechanisms of such intergenerational transfer of overweight are complex and may involve increased CS rates, reduced breastfeeding in mothers with obesity or increased maternal age. Nonetheless, there are data to suggest that the offspring obesity risk may be causally mediated by altered gut colonization. For instance, gut microbiota modulation during pregnancy by specific probiotics reportedly improves maternal weight and blood glucose balance.[41] In a similar fashion, probiotic intervention in the perinatal period and early infancy may reduce the risk of obesity in school age.[42] These results taken together suggest that the gut microbiota modulates host metabolism through as of now largely unknown mechanisms and could be transmitted intergenerationally. Maternal health could be the key element in shaping the infant's *Bifidobacterium* abundance and the risk of NCDs in the future, thus complementing the microbiota depletion hypothesis.

Our study is not without its limitations. Firstly, the relatively small sample size obtained from mother-infant pairs from a limited geographical area. While we believe that the limited number of samples in our study does not affect the reliability of our positive findings, it may have prevented us from detecting all potential differences. Although the sample size is small, these results provide a novel insight, expand our knowledge and open new research for validating these observations in larger cohorts. On the other hand, limiting our study population to a single hospital reduces the risk of confounding or bias. Secondly, 16S rRNA gene amplicon sequencing restricts taxonomic resolution to the genus level for certain taxa. Thirdly, storage times of the fecal samples in the study vary as per the study design, but a recent study indicates that varying fecal sample storage times at −80 °C have no significant influence on microbiota analysis results.[43] To further reduce bias, the samples were collected using a uniform protocol and the DNA extraction and sequencing were performed at the same time with the same protocol and equipment.

The gut microbiota is a complex organ affecting our health in ways that are yet to be uncovered, but the significance of the gut microbiota to human health and homeostasis is beginning to unfold. Still the key question relates to the myriads of factors responsible for the depletion of our gut microbiota like maternal weight, which may contribute to the NCD epidemic we witness worldwide. Our study supports the disappearing microbiota hypothesis and generates a new hypothesis on an interlink between the *Bifidobacterium* composition and risk of allergic diseases, and obesity. In addition, it is noteworthy that the key elements diminishing the gut microbiota richness and the proportion of Bifidobacteria appear to be modifiable, for instance, through microbiota-targeted interventions, diet and weight management, and antibiotic usage policies.

Word count 2762

Statements

Statement of Ethics

The study was conducted in accordance with the Helsinki Declaration of 1975 and other relevant ethical guidelines and regulations. The Ethics Committee of the wellbeing services county of Southwest Finland has reviewed and accepted the protocol, and the study permission has been received (TO8/025/20). Oral and written informed consent were obtained from the subject's mothers.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

TO, EI conceptualized the study. MBU, MCC managed biosamples and performed microbiota analyses. TO, MBU, MCC, SR, EI contributed to the collection and assembly of data. TO, MBU, MCC, SR, EI contributed to the writing and/or data interpretation. TO, MBU, MCC, SR, EI wrote the manuscript and edited the manuscript. All authors read and approved of the final manuscript.

Data Availability Statement

The 16S rRNA gene amplicon sequence data generated is available through NCBI Sequence Read Archive Database under project accession number BioProject ID PRJNA1246647 is available from National Center for Biotechnology Information (NCBI) repository (https://www-ncbi-nlm-nih-gov.ezproxy.utu.fi/bioproject/PRJNA844901). All supporting data are included in the manuscript.

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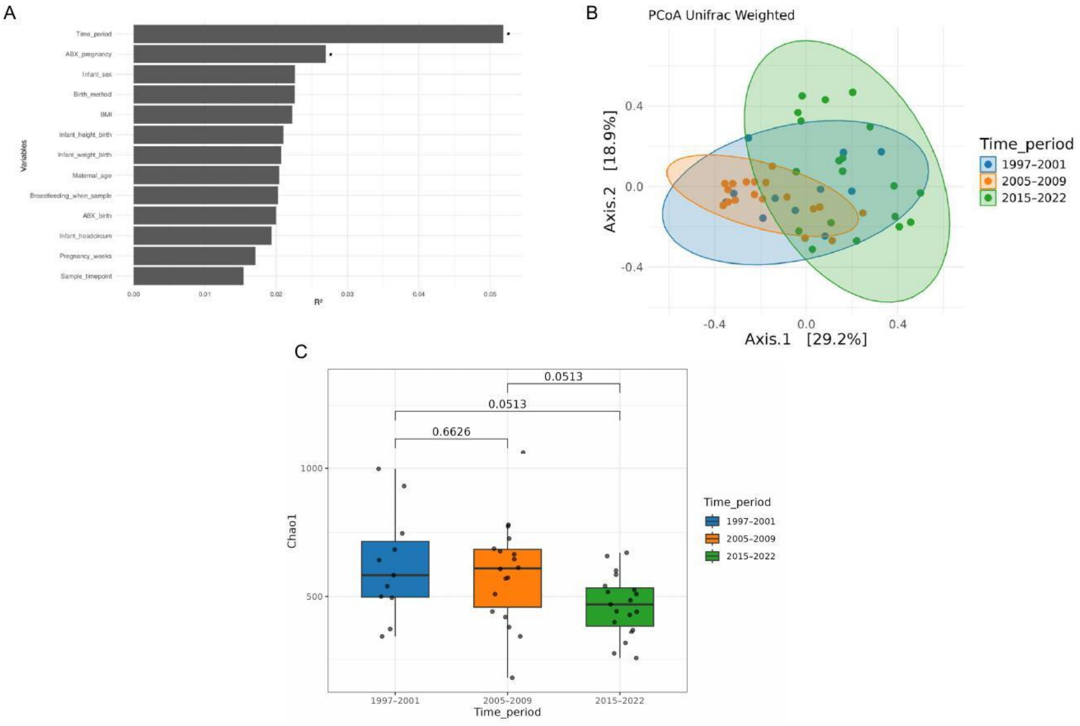
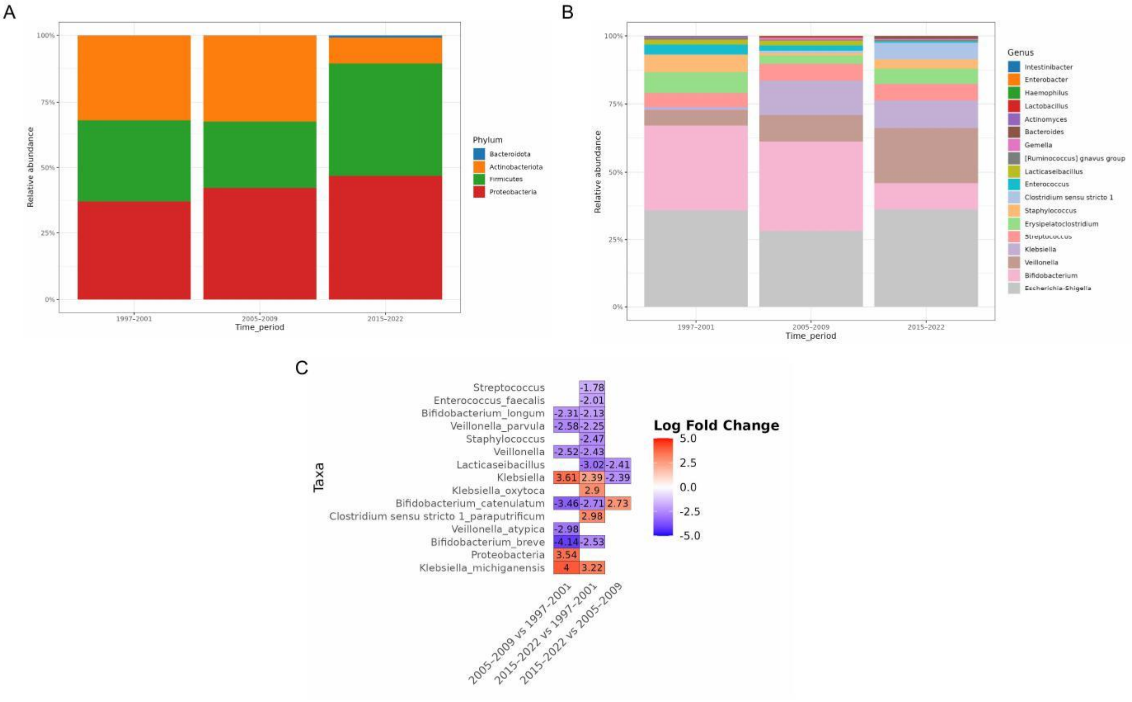
Figure legends

Figure 1. Predictors of infant’s gut microbiota variation and diversity of the infants' gut microbiota between different time periods. Part A: R^2 values from PERMANOVA (adonis2, 999 permutations) on CLR-transformed data. The model included 13 host and environmental variables: birth method (vaginal or CS), infant sex (female or male), mother’s BMI, maternal age, infant height at birth, infant weight at birth, infant head circumference, time period (1997–2001, 2005–2009 and 2015–2022), antibiotics at birth (Yes or No), antibiotics during pregnancy (Yes or No), pregnancy weeks, breastfeeding at the time of sample collection (Yes or No) and sample time point (1 month or 3 months). Significant variables ($p < 0.1$) are marked with dots. Part B: Principal Coordinates Analysis (PCoA) of weighted UniFrac distances. Groups were compared using PERMANOVA (adonis2, 999 permutations), adjusting for Time_period and ABX_pregnancy ($R^2 = 0.195$, $p = 0.001$). Part C: Comparison of alpha diversity across time periods. A two-way ANOVA was performed including Time_period and ABX_pregnancy as fixed effects. Pairwise comparisons between time periods were conducted using estimated marginal means (emmeans function), with p-values adjusted using the Benjamini-Hochberg (BH) method.

Figure 2 Bacterial infant’s gut composition between time periods. Parts A-B: Relative abundance of the top 20 most abundant phyla and genus in the infants' gut microbiota across time periods. Part C: Differentially abundant taxa across time periods identified by ANCOM-BC2. Shown are taxa with FDR-adjusted $q < 0.005$. Models were adjusted for antibiotic use during pregnancy. time periods were conducted using estimated marginal means (emmeans function), with p-values adjusted using the Benjamini-Hochberg (BH) method.

Figure 3 The study population


A
Time_period
ABX_pregnancy
Infant_sex
Birth_method
BMI
Infant_height_birth
Infant_weight_birth
Maternal_age
Breastfeeding_when_sample
ABX_birth
Infant_headcircum
Pregnancy_weeks
Sample_timepoint
Variables
0.00
0.01
0.02
0.03
0.04
0.05
R²
B
PCoA Unifrac Weighted
0.4
0.0
-0.4
Axis.2 [18.9%]
-0.4
0.0
0.4
Axis.1 [29.2%]
Time_period
1997-2001
2005-2009
2015-2022
C
0.0513
0.0513
0.6626
1000
500
Chao1
1997-2001
2005-2009
2015-2022
Time_period
Time_period
1997-2001
2005-2009
2015-2022



Taxa	2005–2009 vs 1997–2001	2015–2022 vs 1997–2001	2015–2022 vs 2005–2009
Streptococcus		-1.78	
Enterococcus_faecalis		-2.01	
Bifidobacterium_longum	-2.31	-2.13	
Veillonella_parvula	-2.58	-2.25	
Staphylococcus		-2.47	
Veillonella	-2.52	-2.43	
Lacticaseibacillus		-3.02	-2.41
Klebsiella	3.61	2.39	-2.39
Klebsiella_oxytoca		2.9	
Bifidobacterium_catenulatum	-3.46	-2.71	2.73
Clostridium sensu stricto 1_paraputrificum		2.98	
Veillonella_atypica	-2.98		
Bifidobacterium_breve	-4.14	-2.53	
Proteobacteria	3.54		
Klebsiella_michiganensis	4	3.22	

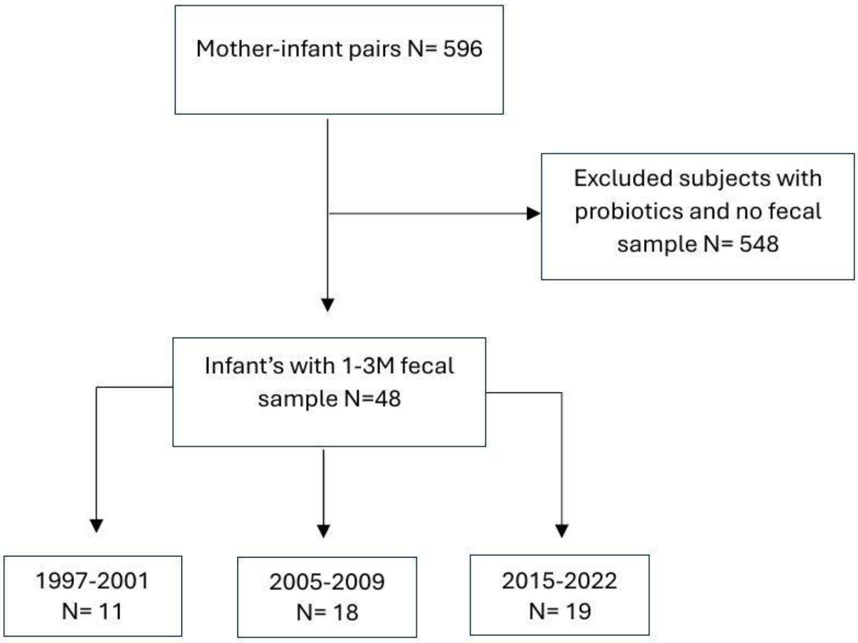

Mother-infant pairs N= 596
Excluded subjects with probiotics and no fecal sample N= 548
Infant's with 1-3M fecal sample N=48
1997-2001 N= 11
2005-2009 N= 18
2015-2022 N= 19

Table 1 Clinical characteristics of the mother-infant pairs

Time period (TP)	1997-2001	2005-2009	2015-2022	1997-2022
Number of mother-infant pairs with faecal sample (GROUP 1)	11	18	19	48
Maternal age (y), mean (range)	31.1 (26.3-35.1)	31.8 (25.9-37.2)	31.4 (26.0-39.8)	31.6 (25.9-39.8)
Pre-pregnancy BMI (kg/m2), mean (range)	21.5 (20.0-24.2)	24.4 (20.3-28.2)	25.1 (20.2-32.2)	24.0 (20.0-32.2)
Weight gain during pregnancy (kg), mean (range)[1]	12.9 (9.40-17.0)	14.8 (9.20-28.5)	13.9 (1.8-22.6)	14.1 (1.8-28.5)
Gestational diabetes, frequency[2]	0	2	1	3
Gestational age (wk), mean (range)	38.9 (36.6-42.3)	39.7 (35.7-42.4)	39.2 (37.6-41.3)	39.3 (35.7-42.4)
Gravidity, mean (range)	2.1 (1.0-5.0)	2.1 (1.0-5.0)	2.3 (1.0-6.0)	2.1 (1.0-6.0)
Parity, mean (range)	0.45 (0.0-2.0)	0.83 (0.0-4.0)	0.6 (0.0-2.0)	0.6 (0.0-4.0)
Mode of Birth, frequency				
Vaginal Birth	5	10	10	25
Caesarean section	6	8	9	23
Antibiotics during pregnancy, frequency[3]	1	2	4	7
Antibiotics during birth, frequency	3	5	6	14
Antibiotics during the first 7 days of life	0	0	0	0
Child sex, frequency				
Male	7	13	8	28
Female	4	5	11	20
Child height at birth (cm), mean (range)	50.6 (49.0-53.5)	50.4 (42.0-54.0)	50.0 (47.0-54.0)	50.3 (42.0-54.0)
Child weight at birth (g), mean (range)	3520 (3060-4100)	3550 (2140-4270)	3420 (2830-4140)	3500 (2140-4270)
Infants *Bifidobacterium* levels (%), mean (range)	23.5 (0.03-59.2)	36.2 (0.13-86.1)	9.2 (0.02-54.0)	22.6 (0.02-86.1)

[1] data missing for 1 mother in time period (TP) 1 and 2 in TP2

[2] data missing for 7 mothers in TP1, 10 in TP2, 17 in TP3

[3] data missing for 1 mother in TP1