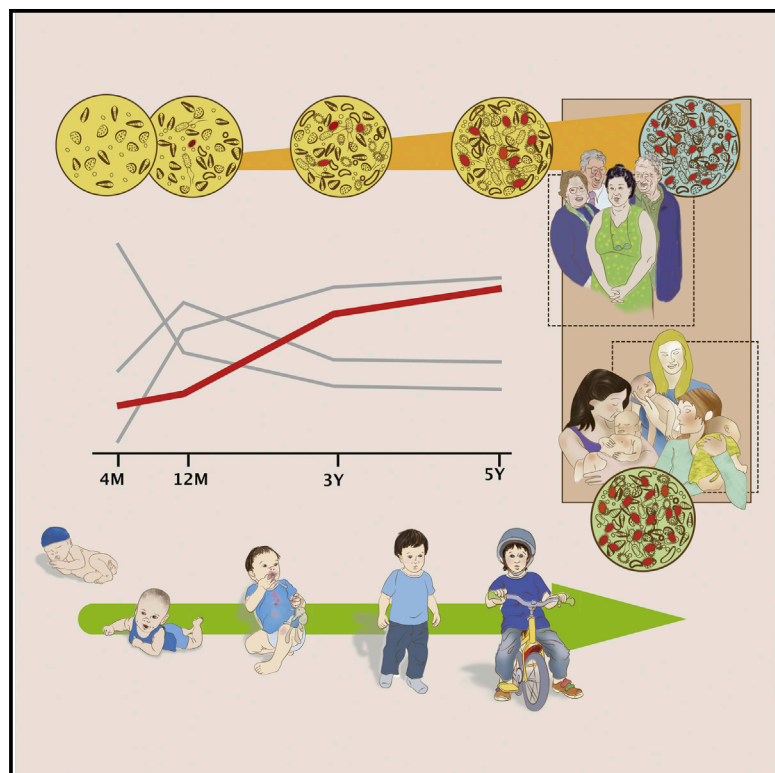# Cell Host & Microbe

Developmental trajectory of the healthy human gut microbiota during the first 5 years of life

Graphical abstract

For a Figure360 author presentation of this figure,
see https://doi.org/10.1016/j.chom.2021.02.021

Highlights

- Children gut microbiota mature along similar trajectories but at different speeds

- Different microbes follow discrete trajectories in the developing gut microbiota

- The effect of c-section on gut microbiota is normalized in 3–5-year-old children

- The gut microbiota has not yet reached adult complexity in 5-years-old children

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In brief

Roswall et al. show in a longitudinal birth cohort with 471 children that the gut microbiota is still developing at 5 years, when microbes associated with high community richness are acquired. The individual dynamics of the gut microbiota may indicate sensitive points for gut microbiota development early in life.

CellPress

Article

Developmental trajectory of the healthy human gut microbiota during the first 5 years of life

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SUMMARY

The gut is inhabited by a densely populated ecosystem, the gut microbiota, that is established at birth. However, the succession by which different bacteria are incorporated into the gut microbiota is still relatively unknown. Here, we analyze the microbiota from 471 Swedish children followed from birth to 5 years of age, collecting samples after 4 and 12 months and at 3 and 5 years of age as well as from their mothers at birth using 16S rRNA gene profiling. We also compare their microbiota to an adult Swedish population. Genera follow 4 different colonization patterns during establishment where *Methanobrevibacter* and Christensenellaceae colonize late and do not reached adult levels at 5 years. These late colonizers correlate with increased alpha diversity in both children and adults. By following the children through age-specific community types, we observe that children have individual dynamics in the gut microbiota development trajectory.

INTRODUCTION

The newborn infant is considered sterile *in utero* during normal pregnancy (de Goffau et al., 2019), but acquires bacteria through transmission from the mother and the environment at delivery (Ferretti et al., 2018). The ecological succession within the gut microbiota is a dynamic process during infancy (Bäckhed et al., 2015; Eggesbø et al., 2011; Palmer et al., 2007), but stabilizes during childhood (Hollister et al., 2015; Stewart et al., 2018; Yatsunenko et al., 2012). The mode of birth, diet (breast or formula feeding), and antibiotic use (Bokulich et al., 2016; Dominguez-Bello et al., 2010; Eggesbø et al., 2011; Koenig et al.,

2011; La Rosa et al., 2014), as well as host factors (Donaldson et al., 2018) are major contributing factors to the initial seeding and development of the gut microbiota and have been associated with health outcomes later in life (Tamburini et al., 2016; Tun et al., 2018). However, it is unclear to what extent these factors contribute to the development of an adult gut microbiota (Derrien et al., 2019). It has been suggested that priority effects, the order by which species appear in the gut microbiota during succession, may have long-lasting effects on the community structure and function (Sprockett et al., 2018).

Breast milk is an important factor for the development and maturation of an infant's microbiota (Bäckhed et al., 2015) and

Table 1. Descriptive data of the study population

	Total cohort		Complete series	
N	**471**		**213**	
Mother's age (years)	31	(28–34)	31	(29–35)
Mother's pre-pregnancy BMI (kg/m2)	23.5	(21,4–26,6)	23.5	(21,5–26,8)
Gestational age (days)	280	(273–286)	281	(273–286)
Birth weight (gram)	3,570	(3,161–3,914)	3,615	(3,135–3,955)
Sampling time mother (days after birth)	2	(0–5)	2	(0–5)
Sampling time infant first week (days after birth)	2	(0–4)	2	(0–4)
Sampling time infant 4 months (days after birth)	122	(119–126)	121	(119–125)
Sampling time infant 12 months (days after birth)	365	(361–371)	365	(360–370)
Sampling time infant 3Y (days after birth)	1,100	(1,093–1,114)	1,099	(1,089–1,107)
Sampling time infant 5Y (days after birth)	1,823	(1,818–1,838)	1,821	(1,816-1,829)
C-section (%)	35.8		35.7	

	Total cohort			Complete series		
	1 week	**4 Months**	**12 Months**	**1 week**	**4 Months**	**12 Months**
Exclusively breast fed (%)	68.3	56		68	59.2	
Mixed fed (breast + formula feeding) (%)	30	23.5		29.9	24.4	
Exclusively formula feeding (%)	1.7	20.5		2.1	16.4	
Any breast feeding (%)	98.3	79.5	9.4	97.9	83.6	7.5

Continuous variables are given as median and interquartile ranges. Distributions in categorical variable are given in percentage.

the developing gut microbiota enters a transitional phase at weaning. Thereafter, the microbiota begins to stabilize (Stewart et al., 2018) and evolve toward an adult-like composition 2–3 years after birth (Bergström et al., 2014; Koenig et al., 2011; Yatsunenko et al., 2012). However, differences in microbiome structure and diversity still exist between children, including preschoolers and school-aged children, and adults (Cheng et al., 2016; Hollister et al., 2015).

Considerable efforts have been focused on studying the infants' gut microbiota (Bäckhed et al., 2015; Bergström et al., 2014; Eggesbø et al., 2011; Ferretti et al., 2018; Palmer et al., 2007; Yatsunenko et al., 2012) and recent efforts have continued with investigations of the microbiota of toddlers and pre-school children (Cheng et al., 2016; Hollister et al., 2015; Zhong et al., 2019). However, the dynamics by which the gut microbiota develops after infancy and weaning toward an adult microbiota is still poorly characterized. Here, we used a longitudinal cohort of 471 Swedish infants (302 born vaginally and 169 born with c-section) to study the gut microbiota dynamics during their first five years of life. Their gut microbiota at 5 years was also compared with that of their mothers and the gut microbiota from a normal adult Swedish population.

RESULTS

Community richness and composition of the gut microbiota in children up to five years

Here, we expand on our previous study (Bäckhed et al., 2015) by increasing the number of children (n = 471) and follow them for the first 5 years of their lives to investigate the establishment

of the gut microbiota. The fecal microbiota was profiled by sequencing the V4 region of the 16S rRNA gene during the first week of life (newborn, NB; n = 246), at 4 months (4M; n = 411), 12 months (12M; n = 397), 3 years (3Y; n = 336), and 5 years (5Y; n = 288) (Table 1). We also analyzed the gut microbiota from 357 of the mothers just after delivery. Because women in the third trimester have an altered gut microbiota (Koren et al., 2012), we compared our results with 101 fecal samples from a normal adult Swedish population (50–64 years of age), the Swedish SciLifeLab SCAPIS Wellness Profiling (S3WP) study (Tebani et al., 2020), which were extracted and sequenced using the same protocol as in the current study as a proxy for the normal adult microbiota. On an average, 56,222 ± 36,381 reads were generated per sample and we identified 1,434 operational taxonomic units (OTUs; with relative abundances higher than 0.2%, Table S1A). The alpha diversity (estimated as richness of the community using phylogenetic diversity [PD]) increased as the children grew older, but at 5 years was still lower than the diversity of the adult microbiota (p = $4.6 \cdot 10^{-5}$; Figure 1A; Table S1B). Mode of birth, sex, antibiotics during the first year of life, or exclusive breastfeeding at 4 months did not significantly affect the increase in alpha diversity over time (Table S1B). We also observed a compositional difference between the mothers' gut microbiota and the S3WP adults (p = $1 \cdot 10^{-4}$, R^2 = 0.026, adonis 9,999 permutations), consistent with that women in the third trimester have an altered gut microbiota (Koren et al., 2012). The mothers had lower alpha diversity and significant differences in 51 of the 96 genera investigated (Table S2). Since the mothers' microbiota did not appear representative for a normal adult
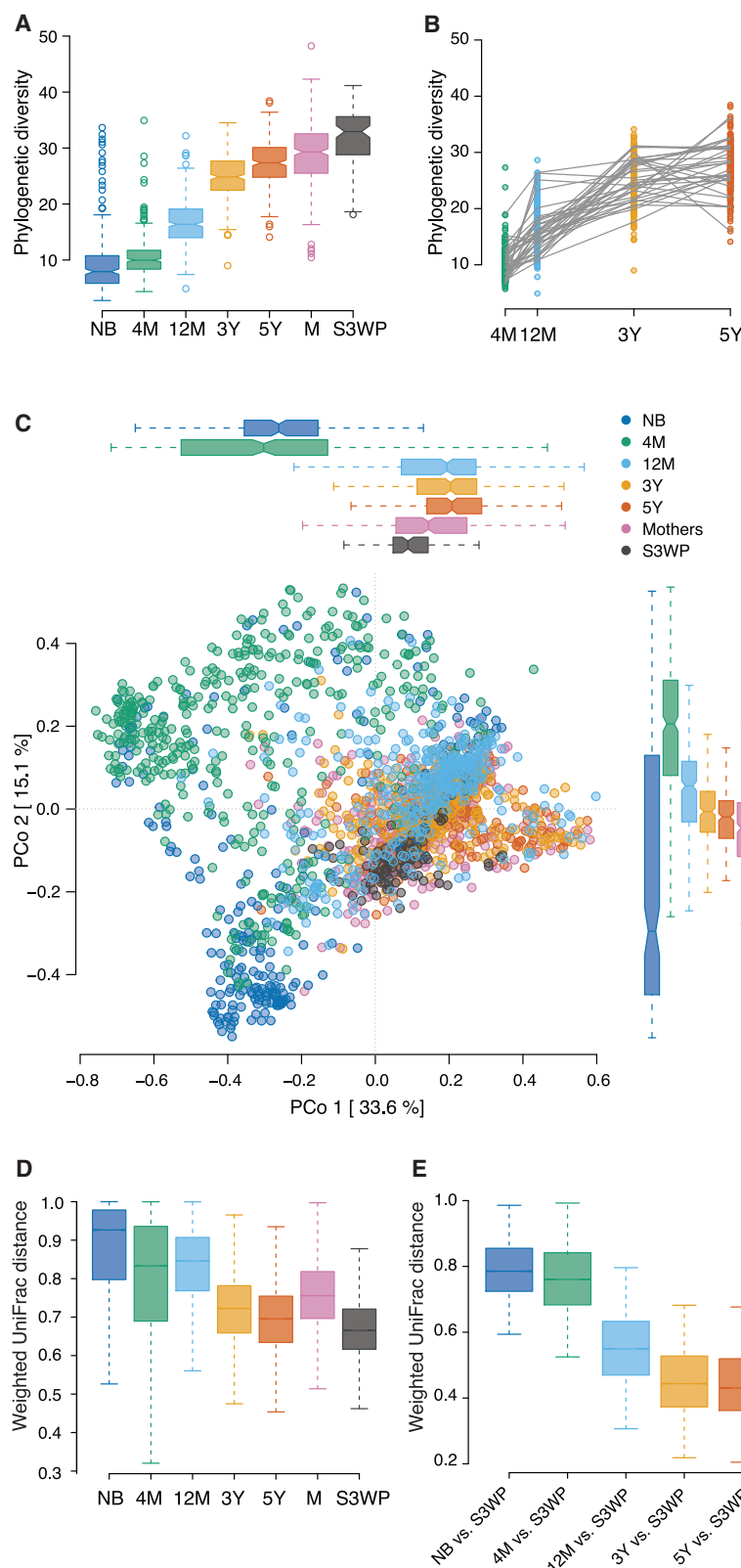
Figure 1. Development of the gut microbiota from newborn up to 5 years compared with adults

(A) Alpha diversity (species diversity) measured as Faith's PD in the total cohort (n = 471) in newborn (NB; n = 246), 4 months (4M; n = 411), 12 months (12M; n = 397), 3 years (3Y; n = 336), 5 years (5Y; n = 288), in mothers (M; n = 357), and in the Swedish SciLifeLab SCAPIS Wellness Profiling (S3WP) adults. All groups are significantly different from each other, statistics in Table S1B.

(B) Longitudinal development of alpha diversity measured as Faith's PD from 4M to 5Y, gray lines of 35 randomly selected children; time is a significant factor in linear mixed effect model, $p < 2.2 \times 10^{-16}$, n = 213.

(C) First and second principal coordinates of dimension reduction for weighted UniFrac measures (values in the brackets indicate the amount of total variability explained by the principal coordinates) in the total cohort. Each point indicates a fecal sample colored by age and adult groups. The boxplots along each axis show the values, grouped by time point and adult group, for the respective coordinates. Notches indicate an approximate 95th confidence interval around medians.

(D) Weighted UniFrac measures within age groups for the children and adult groups in the total cohort, statistics in Table S1C.

(E) Weighted UniFrac measures between age groups and the S3WP adults. UniFrac measures equal to zero indicate identical communities; UniFrac measures equal to 1 indicate completely different communities. Boxes show median and interquartile ranges (IQR); whiskers specify ±1.5*IQR from box's quartile; notches indicate 95% confidence interval.

All statistical comparisons can be found in Table S1.
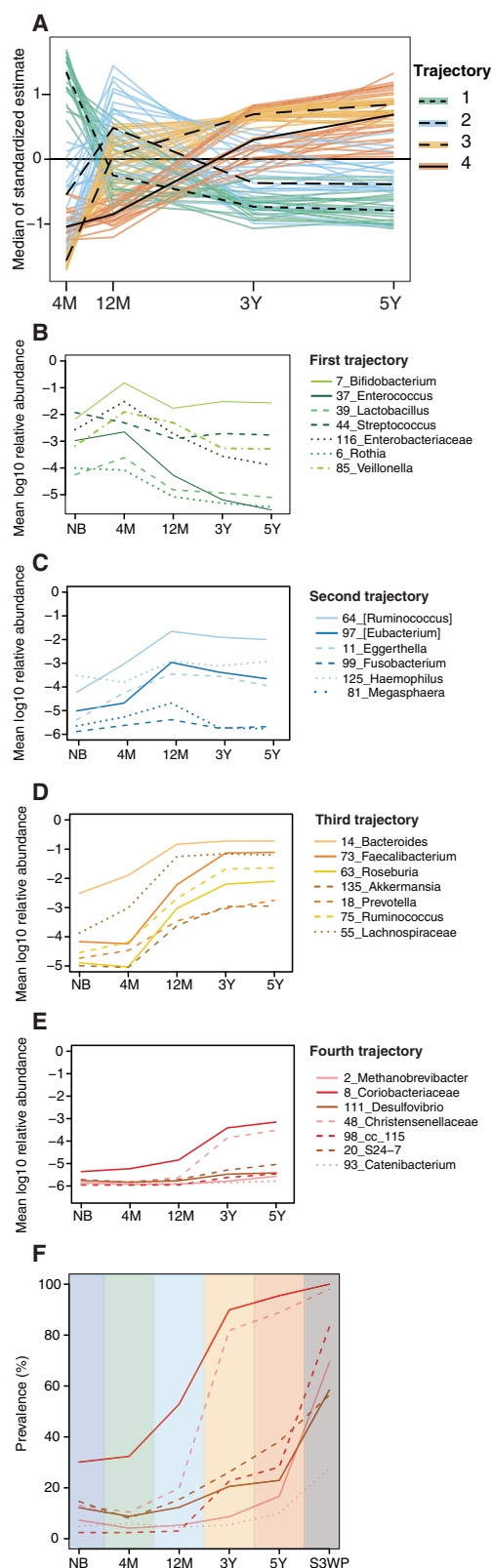
Figure 2. Abundance of microbial genera during the development of the gut microbiota up to 5 years

(A) Estimation of the median abundance of microbial genera using a mixed effect model at 4M, 12M, 3Y, and 5Y in the longitudinal cohort (n = 213). Sets of genera showing the same pattern of variation in abundance are colored with the same color. Relative abundance of representative genera for the four trajectories at each time point in the total cohort (n = 471) and S3WP cohort (n = 101).

(B–E) (B) Examples of representative genera in first, (C) second, (D) third, and (E) fourth trajectories.

(F) Prevalence of genera, from (E), at each time point in the total cohort and S3WP cohort.

Statistics are presented in Table S2.

microbiota we used the S3WP adults for comparisons in further analyses.

In order to better model the gut microbiota development longitudinally within each child, we performed a subanalysis using samples from the 213 children who had complete series from 4 months to 5 years. This analysis revealed that alpha diversity increased significantly with age, gaining 3.9 ± 0.1 units in phylogenetic diversity per month (p < 2.2·10^{-16}) on an average (Figure 1B).

Weighted UniFrac analyses of all samples demonstrated that the overall composition of the gut microbiota changed markedly at the different ages, resembling a more adult microbiota as the children grew older (Figures 1C–1E; Table S1C). However, at 5 years the children gut microbiota composition was still significantly different compared with mothers and adults (adonis, 9,999 permutations, R^2 = 0.03; p = 0.0001 and R^2 = 0.08; p = 0.0001, respectively; Table S1C). The shifts in microbiota composition were largest at younger ages with strongest changes between 4 and 12 months, as age explained 23% of the total variance in compositional variation between 4 and 12 months samples, while age only explained 5% between 12 months and 3 year samples, and 0.9% between 3 and 5 years samples, respectively (boxplots in Figure 1C; Table S1C). Therefore, the microbiota of infants at 4 and 12 months was highly heterogeneous and most different compared with the adults, but the microbiota was more similar to the adults at 3 and 5 years (Figures 1D–1E).

Dynamics and gut microbiota signatures in children from 4 months to 5 years

To further characterize the dynamics in gut microbiota development, we analyzed complete series of samples from 4 months to 5 years in the 213 children by classifying the relative abundance of microbial genera using a time-course analysis (Hejblum et al., 2015). This analysis identified four major trajectories for individual genera in the developing gut microbiota: (1) genera with highest relative abundance at 4 months; (2) genera with peak relative abundance at 12 months; (3) genera whose relative abundance increased rapidly between 4 and 12 months and reached stable levels by 3 years; and (4) genera that increased in relative abundance after 12 months and continued to increase between 3 years and 5 years (Figure 2A; Table S2). Trajectory 1 mainly contained *Bifidobacterium* (the most abundant genus at 4 months) together with lactic acid bacteria (*Enterococcus*, *Streptococcus*, and *Lactobacillus*), gamma-Proteobacteria (Enterobacteriaceae, *Citrobacter*, and *Serratia*) (Figure 2B). In addition, oral bacteria

Rothia and lactate fermenting *Veillonella*, as well as Gram-positive anaerobic cocci (e.g., *Anaerococcus* and *Peptoniphilus*) had a similar colonization pattern. These bacteria are common colonizers of skin and mucosal surfaces of the mouth as well as gastro-intestinal tract (Rajilic-Stojanovic et al., 2020), and are important early colonizers of the mouth (Sulyanto et al., 2019) as well as commensals in the human microbiota (Murphy and Frick, 2013).

Common gut bacteria such as *Eubacterium* and OTUs proposed to belong to *Ruminococcus* ([*Ruminococcus*]) colonized according to the second trajectory (with peak abundance at 12 months). While the prevalence was maintained as the children grew older, their relative abundance decreased. The majority of OTUs belonging to [*Ruminococcus*] could be assigned to the species *Ruminococcus gnavus* (91% of the abundance of [*Ruminococcus*] at 12 months from 16 of a total 30 OTUs). In addition to these gut specific bacteria, we also observed several oral and nasopharyngeal bacteria with higher abundance at this age compared with other time points, such as the genera *Haemophilus*, *Megasphaera*, *Eggerthella*, and *Fusobacterium* (Figure 2C).

We observed highly abundant and prevalent genera such as *Bacteroides*, which had the highest relative abundance at 12M, to colonize after the third trajectory maintaining a high abundance at 3 and 5 years. Several genera that are highly abundant in adults such as *Faecalibacterium*, *Roseburia*, *Akkermansia*, *Prevotella*, and *Ruminococcus* had high prevalence among the children at 1 year of age but increased further in relative abundance as the children grew older (Figure 2D; Table S2).

Genera that are more prevalent and abundant in the adult gut microbiota than in 5-year-old children but display low or non-detected prevalence at 12 months are captured in the fourth trajectory. Among these are some hydrogenotrophic bacteria, such as *Methanobrevibacter*, *Desulfovibrio*, and *Bilophila*, as well as Clostridia within the Christensenellaceae and [Mogibacteriaceae] family, *Adlercreutzia*, and unspecified Coriobacteriaceae. In the fourth trajectory we also observed Tenericutes of the order ML615J-28 and RF39 (Figures 2E and 2F; Table S2). These data suggest that the adequate niche has to be created for these late colonizers to establish.

Age-specific community types

Next, using the total cohort, we tested whether children's gut microbiota at different ages clustered after community types according to age using Dirichlet multinomial mixtures (DMM) (Holmes et al., 2012). We identified 14 community types: 3 were common in samples from newborns (community type NB1,2,3), 4 common at 4 months (community type 4M1,2,3,4), 3 community types were common in samples from 12 months (community type 12M1,2,3), 3 community types were common in samples from 3 year and 5-year-old children (community type Child1,2,3), and 1 community type was common in adult samples (Figure 3A; Table S3A). The clustering in the NB community types was unaffected by mother pre-pregnancy BMI (p = 0.71; Kruskall-Wallis test), pregnancy weight (p = 0.50; Kruskall-Wallis test), antibiotic usage in the mothers (p = 0.15, Fischer's test), or type of feeding (p = 0.05; Fischer's test).

As expected, the prevalent community structures at each age were dominated by genera with the respective trajectory patterns of colonization and establishment. Specifically, bacteria following trajectory 1 (Figure 2B) formed the community types in newborn and 4 months infants, genera following trajectory 2 and 3 formed the community types at 12 months and the community types at 3 and 5 years comprised bacteria in both trajectory 3 and 4 (Figure 3B). The adult community type contained 75% of the S3WP adult samples but also a few samples from 5 years (3.5%) and 3 years (1.1%), but none from younger ages. This observation suggests that a minority of the children had an adult-like microbiota at these ages.

The adult community type was characterized by genera that had higher abundance in adults compared with 5-year-old children (Tables S2 and S3B). As expected and in agreement with data in Figure 1A, the community types with the lowest alpha diversity were dominated by newborns and those with highest alpha diversity were dominated by adults (Figure 3C; Table S3C). These differences in alpha diversity between community types were also observed in samples from the same age groups (Figure S1A). Some adults thus had lower alpha diversity and the same community type as children, suggesting that they had a relatively immature microbiota.

Further analyses revealed that 12M community types segregated by alpha diversity and that the community type 12M3, which had the highest alpha diversity, had higher abundance of *Faecalibacterium* and unclassified Ruminococcaceae. This community type also had lower abundance of *Ruminococcus gnavus*. The community types 12M1 and 12M2 were characterized by lower alpha diversity, and 12M1 had the highest relative abundance of *Bifidobacterium* while 12M2 had the highest relative abundance of *Bacteroides* (Figure 3C; Table S3C). Thirty-nine children at 12 months had a more mature gut microbiota configuration e.g., Child1–3. The microbiota in these children had a higher alpha diversity compared with the rest of the children at 12 months.

The child-community types in 3 and 5-year-old children separated by differences in abundance of *Prevotella*, *Bifidobacterium*, and *Bacteroides*. Community type 'Child3' had higher abundance of *Bacteroides*, *Faecalibacterium*, and *Roseburia*, whereas, 'Child2' had high abundance of *Prevotella*. The 'Child1' community type was dominated by *Bifidobacterium* and was also enriched in bacteria that were late colonizers e.g., trajectory 4 bacteria (Table S3D).

Development of the gut microbiota toward an adult community

To further investigate the growth trajectories, we performed similar analyses on the subcohort with complete sample series (n = 213). The alpha diversity at 12 months positively correlated with alpha diversity at 5 years (Spearman correlation, rho = 0.32, p = 1.5·10^{-6}, Figure S1B). The prevalence of children in 12-M community types decreased over time, whereas the prevalence of children in Child3 community type increased over time (Figure 3A). However, a minority of children had relative immature community types at 5 years of age, i.e., their microbiota had a 12M community type. The gut microbiota of these children was also classified as a 12M community type when the child was 3 years, suggesting that the children had individual dynamics in their gut microbiota development trajectories, but
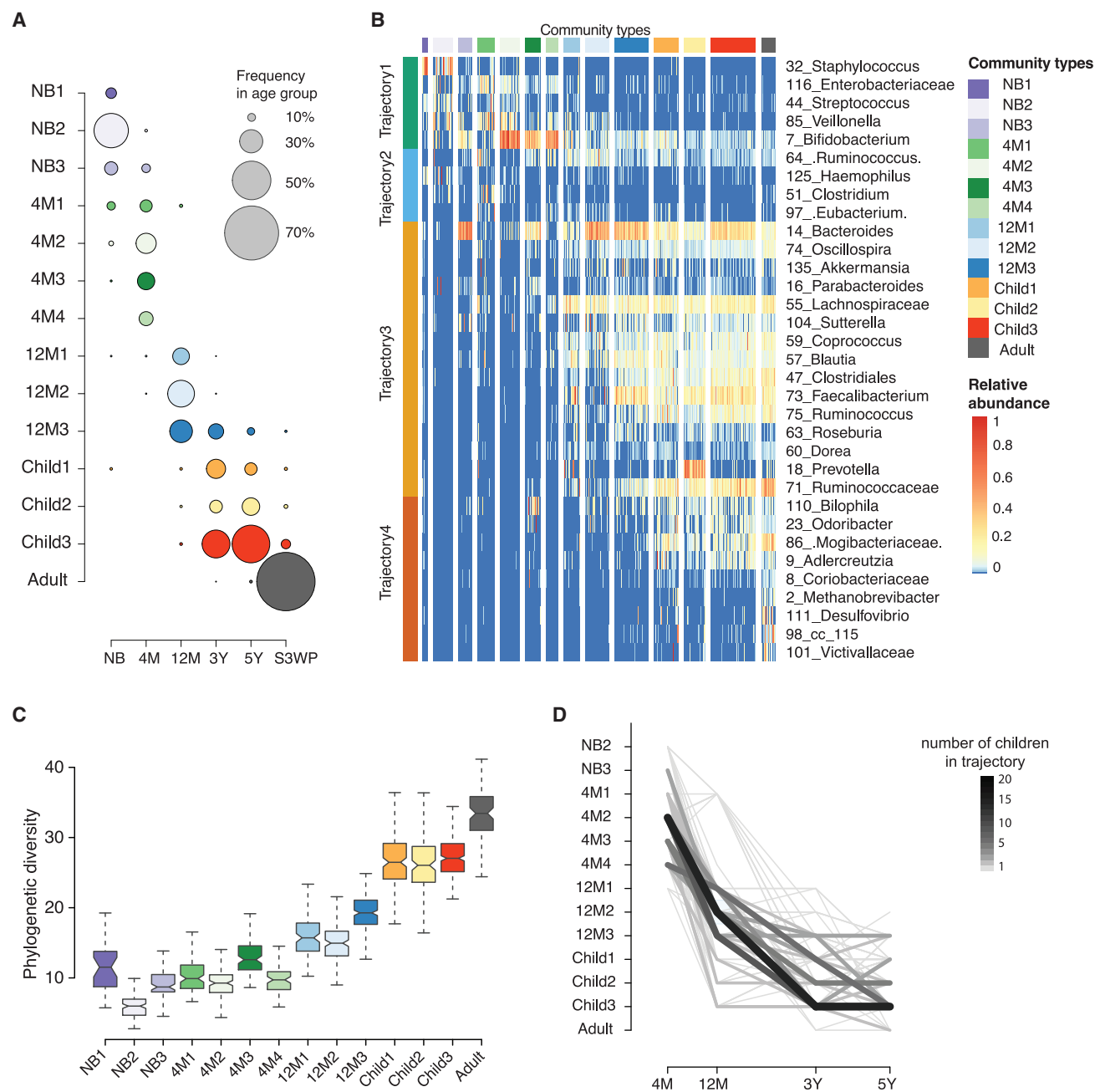
Figure 3. The gut microbiota develop through age-specific community types

(A) Distribution of samples in the identified 14 community types (y axis), clustered using Dirichlet multinomial mixtures at each age time point (x axis) in the total cohort (n = 471). Sizes of circles are scaled by frequency of each community type within at each age.

(B) Heatmap of relative abundance of community types discriminating genera (rows), ordered by the trajectory they belong to. Relative abundance for samples sorted by community types in columns. All statistics are presented in Table S2.

(C) Boxplot of alpha diversity, measured as Faith's PD, distributed between the 14 community types. Boxes show median and interquartile ranges (IQR); whiskers specify ±1.5*IQR from box's quartile; notch indicate 95% confidence interval. Statistics are presented in Table S3A.

(D) Transition between community types in the cohort with complete series of samples (n = 213). Color intensity and line thickness are scaled by number of children in the trajectory.

no child reduced their alpha diversity over time. Children followed individual trajectories from 4 months to 5 years (Figure 3D), but we identified the most common trajectory, followed by 13 children (11 were vaginally born). These children were in

the community type "4M2" at 4 months dominated by *Bifidobacterium*, in the community type "12M2" at 12 months dominated by *Bacteroides* and in the community type 'Child3' at 3 years and 5 years.
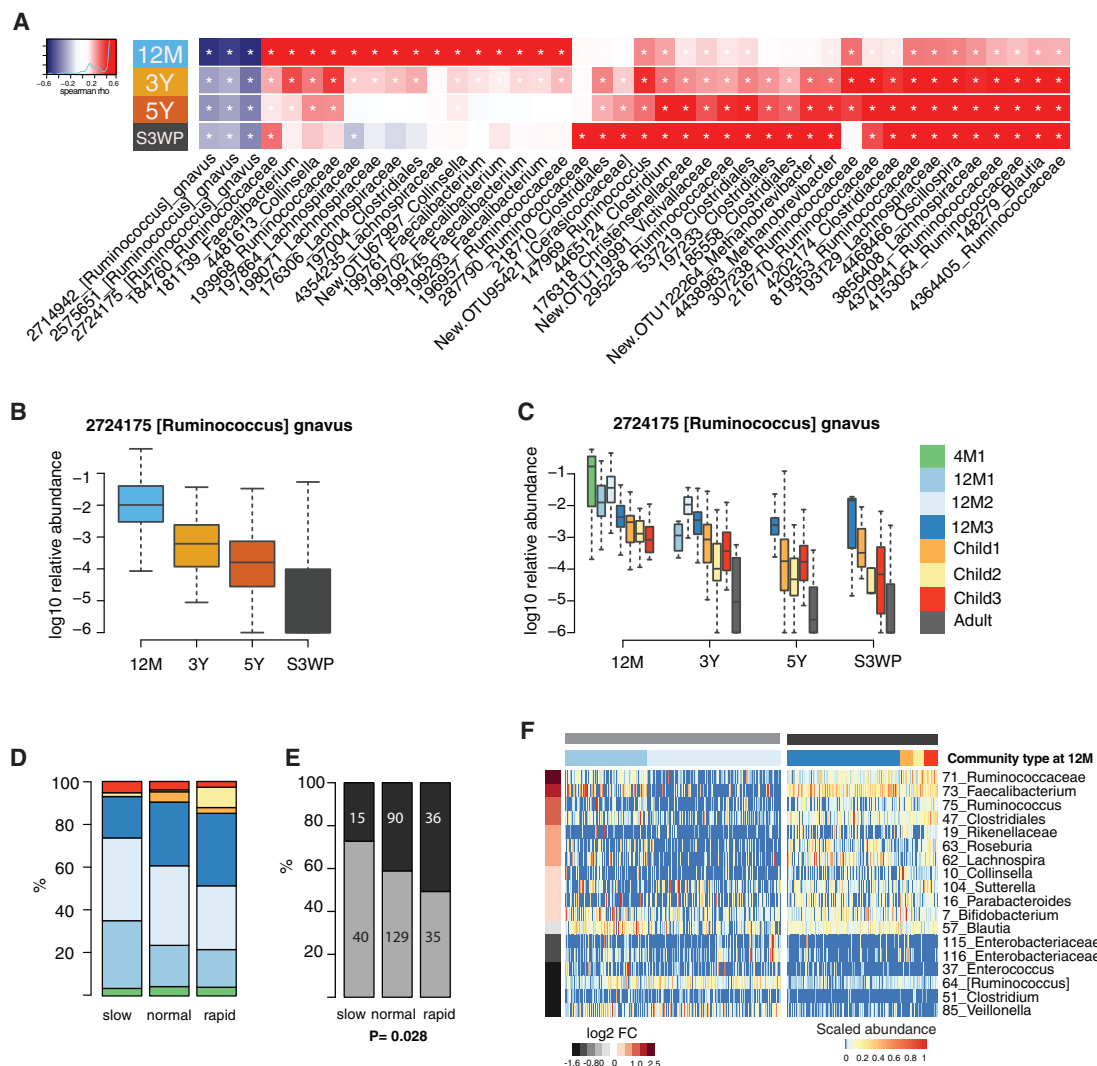
Figure 4. OTUs linked to low community richness is consistent in both children and adults and increased in low diverse community types at all ages

(A) Heatmap of OTUs significantly correlated to community richness within time point (12M; n = 392, 3Y; n = 336, 5Y; n = 288, S3WP; n = 101). Significant OTUs with a Spearman's rho with an absolute value of 0.5 in at least one time point is displayed. *p < 0.05.

(B) Relative abundance of OTU 2724175 at different ages.

(C) Relative abundance of OTU 2724175, belonging to *Ruminococcus gnavus*, distributed between community types at different ages.

(D) Distribution of community types between the three weight-gain development groups at 12 months (12M) (See method for definition).

(E) Distribution of community with lower alpha diversity (low; 12M1+12M2, light gray) and higher alpha diversity (high; 12M3, Child1, Child2, and Child3, dark gray) between the three weight-gain development groups at 12 months (12M).

(F) Heatmap of the L6/genera level that differed significantly in abundance between samples within the high group (community types 12M3, Child1, Child2, and Child3, dark gray) and low group (12M1 and 12M3, light gray) at 12M (n = 376) using Wilcoxon rank sum test after adjusting for false discovery rate. Log₂-fold changes for the comparisons is displayed in the left part of the panel. Boxes in figure show median and interquartile ranges (IQR); whiskers specify ±1.5*IQR from box's quartile.

Alpha diversity-associated bacteria in the developing gut microbiota and adults

Since we observed that the alpha diversity was strongly associated with the development toward a mature microbiota, we next investigated which bacteria correlated with alpha diversity at different ages. Several OTUs linked to increased diversity in the 12M samples belonged to *Faecalibacterium* and several others to unclassified Clostridia with high identity to *Eubacterium*

rectale (Figure 4A; Table S4A). At older ages and in the S3WP adults, high alpha diversity was associated with OTUs annotated as *Methanobrevibacter* and an unclassified Christensenellaceae, as well as unclassified Ruminococcaceae OTUs with poor identity to known species. Interestingly, at all ages OTUs belonging to *Ruminococcus gnavus* (OTU2724175, OTU2575651, and OTU2714942) were negatively associated with alpha diversity and also decreased over time resulting in

significantly lower abundance in adults than in 5-year-old children (Figures 4B, S2A, and S2B), further identifying *Ruminococcus gnavus* as a marker for an immature microbiota in both children and adults.

Association between gut microbiota and growth

Since gut microbiota "immaturity" has been associated with undernourishment in Bangladeshi children (Subramanian et al., 2014), we next investigated how the microbiota composition at 12M might affect weight development during the following 4 years of life in our cohort. Children were distributed after weight development (rapid, slow, and normal) based on individual transitions in standardized weight curves between ages 12M and 5 years (see Star Methods for definitions). Children with slower than expected weight gain (defined as a change > −0.67 standard deviation scores (SDS) according to the growth reference curve between 12 months and 5 years of age) were characterized by lower diversity community types, 12M1 and 12M2, at 12 months compared with the children with normal or faster weight development (Figures 4D and 4E, Fischer's test; p = 0.028, odds ratio 1.99, 95th CI (1.03–4.04)). The microbiota configuration with lower diversity (community types 12M1 and 12M2) had lower relative abundance of unclassified *Ruminococcaceae*, *Faecalibacterium*, and *Roseburia* compared with the more mature microbiota (Figure 4F; Table S4B), suggesting these taxa as markers for a more diverse microbiota and that they are associated with normal weight gain. However, the effect size within a normal cohort between the gut microbiota maturity and growth was much smaller compared with undernourishment (Smith et al., 2013; Subramanian et al., 2014).

Early life-style factors affecting the gut microbiota

The longitudinal design of our study allowed to investigate how factors in early life affected the microbiota at 5 years. In our study, we did not observe any effects of self-reported antibiotic intake during pregnancy (antepartum; n = 68) and during labor (peripartum; n = 50) on community type or overall microbiota composition at any age of the infant (Table S5A).

We and others have previously demonstrated that mode of birth has profound effects on the microbiome of children up to one year of age (Adlerberth et al., 2007; Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Jakobsson et al., 2014). Here, the mode of birth had large effects on the composition and community types of the newborn and infant gut microbiota and the transition between newborn community types and community type at 4 months (Figures 5A, S3A, and S3B). The mode of birth explained 14.4% and 6.2% of the compositional variation at birth and at 4 months. The type of feeding, exclusively formula fed or not (20.4% exclusively formula feeding), explained 1.2% of the variation in the gut microbiota (with a non-significant interaction with mode of birth) at 4 months. As expected, the variance in microbiota composition explained by mode of birth decreased with time to <2% at 3 and 5 years (Table S5A). Importantly, factors such as mother's weight, age, type of feeding, or antibiotic usage did not confound the signal from mode of birth at any age (Table S5A).

We observed the mode of birth-specific transitions between the community types at 4 months and 12 months and higher frequency of c-section born children in the community types with lower alpha diversity (12M1 and 12M2) (p = 0.0005, Fischer's exact test, Figures 5B–5D). However, there were no significant overrepresentations of transition from any specific 4-months community type to the low-diversity community types at 12 months, neither in c-section born (p = 0.65, Fischer's exact test) nor vaginally born children (p = 0.12, Fischer's exact test; Figure 5B).

When longitudinally modeling the alpha diversity increase over time, from 4 months to 5 years using the children with complete sample series, factors such as the mode of birth and antibiotic usage during the first year did not significantly affect the results. However, the microbiota configuration at 4 months and 12 months predicted the increase in alpha diversity (Table S5A).

In cross-sectional comparisons using all samples the alpha diversity was lower at 4 months in c-section born infants but normalized at three years and was even higher at 5 years (Figure 5F; Table S5A). Accordingly, the number of children at 5 years in the "adult" community types were also overrepresented among the c-section born (Fischer's test, p = 0.039, Figure 5F). We also observed 25 genera with significantly different relative abundance between 5-year-olds born with c-section compare to 5-year-olds born vaginally (Table S5B).

DISCUSSION

Here, we characterized the development of the gut microbiota in 471 children in a normal Swedish population from birth to five years of age to extend previous studies, and demonstrated that the gut microbiota acquired an adult-like configuration at 5 years, but still had a lower community richness and missed some critical taxa that are present in the adult microbiota (Koenig et al., 2011; Stewart et al., 2018; Yatsunenko et al., 2012). Our findings highlight the possibility that the microbiota may be particularly sensitive for disturbances during this early establishment that may have profound effects for health later in life (Cho et al., 2012).

We observed age-specific community types at each time point up to 3 years of age, confirming significant development in the gut microbiota during the first years of life (Stewart et al., 2018). Bacteria associated with a more complex microbiota and common in adults, appeared around the time when the children began to eat solid food (Gehrig et al., 2019; Subramanian et al., 2014), which were defined as trajectory 3. Many of these are butyrate producers and likely require the child to eat more complex carbohydrates resulting in increased cross-feeding from other bacteria (Baxter et al., 2019; Flint et al., 2012). The first part of this succession and the increased abundance of genera in trajectory 3, appeared to be required for specialists in trajectory 4 to colonize. Methanogens, such as *Methanobrevibacter*, increase in abundance first after weaning in rats (Maczulak et al., 1989), which is consistent with our findings that these organisms will require a fully reduced environment and complex carbohydrates in the diet for expanding. Furthermore, *Methanobrevibacter* and *Desulfovibrio* are hydrogen consumers and colonize according to trajectory 4, which may enhance the fermentative potential by reducing the amount of hydrogen that inhibits the fermentative process (Gibson et al., 1993).

As previously observed in 3-year-old children (Bergström et al., 2014) and school children (Nakayama et al., 2015; Zhong
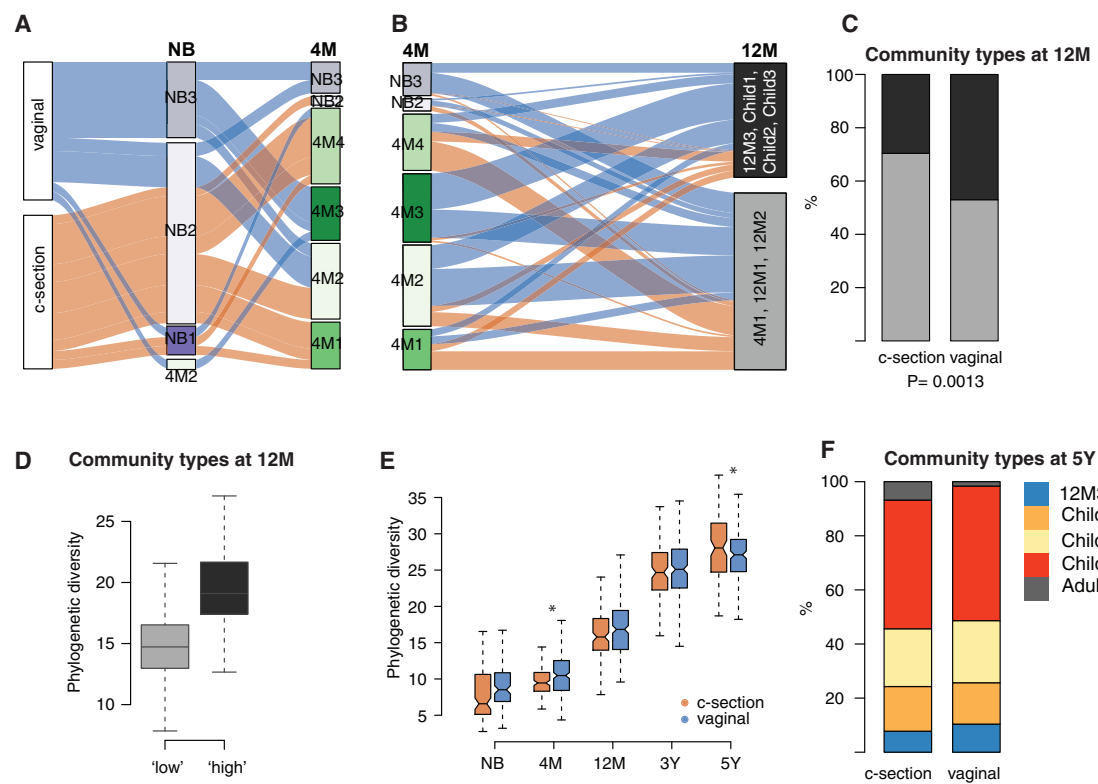
Figure 5. Effect of mode of birth on the gut microbiota up to 5 years

(A) Frequencies of mode of birth in newborn (NB) and 4M community types (n = 227) and transitions between time points. Test of frequencies of transitions between vaginal and c-section born children was performed by Fischer's exact test (p = 0.0005).

(B) Frequencies of mode of birth in NB and 4M and 12M community types and transitions between time points (n = 347). The community types at 12M were grouped based on the alpha diversity within community types (low; 12M1+12M2+4M1, light gray. high; 12M3, Child1, Child2, and Child3, dark gray). Test of frequencies of transitions between vaginal and c-section born children was performed by Fischer's exact test (p = 0.0005).

(C) Frequency of children in 'low' and 'high' community types at 12 months in vaginally and cesarean section born children.

(D) Alpha diversity at 12M time point for children in community types 'low' and community types high. p < 2.2 × 10^{-16} using Wilcoxon rank sum test.

(E) Alpha diversity, measured as PD, at each time point according to mode of birth. * p < 0.05 using Wilcoxon rank sum test within time point.

(F) Frequency of community types in vaginally and cesarean section born children at 5 years.

et al., 2019), we identified community types at 3 years and 5 years driven by differences in *Bacteroides*, *Prevotella*, and *Bifidobacterium*. However, by including adults in our clustering analysis we not only identified a small number of 5-year-old children that clustered with the adults, suggesting a more mature microbiota for their age, but also some adults with less mature microbiota than expected for their age. Different bacterial OTUs correlated with alpha diversity at 12 months, 3 and 5 years, and adults. High alpha diversity was associated with several unspecified Clostridiales, *Methanobrevibacter*, and Christensenellaceae, as well as lower abundance of *R. gnavus* in child and adult microbiota, which is consistent with the decreased abundance of *R. gnavus* over time. Interestingly, both low community richness and high proportions of *R. gnavus* have been repeatedly linked to diseases such as metabolic syndrome (Le Chatelier et al., 2013), obesity (Liu et al., 2017), cardiovascular disease (Jie et al., 2017), and inflammatory bowel disease (Ni et al., 2017). In contrast, increased abundance of *Methanobrevibacter* and *Christensenella* has been linked to metabolic health (Goodrich et al., 2014). Similarly, Archaea, as well as Christensenellaceae, Tenericutes, and *Desulfovibrio* were negatively associated

with BMI and blood triglycerides (Fu et al., 2015). Although our data are insufficient to make claims about future metabolic conditions, experimental studies have demonstrated that, if the microbiota is disrupted by antibiotics before weaning, mice develop obesity later in life (Cho et al., 2012).

The microbiota in undernourished children is immature compared with nourished counterparts (Blanton et al., 2016; Planer et al., 2016; Smith et al., 2013; Subramanian et al., 2014). We did not include children with clinical undernourishment in our study, but could observe that children with lower weight gain than expected between 12 months and 5 years had a more immature gut microbiota at 12 months, although much less pronounced than in children with clinical malnourishment from Malawi and Bangladesh (Smith et al., 2013; Subramanian et al., 2014). However, similar to the malnourished children, the Swedish children with lower weight gain had reduced abundance of *Faecalibacterium* and *Ruminococcus* taxa. Importantly, as our results are based on relative abundances, we cannot exclude that apparently increased or decreased abundances of specific taxa could be due to the variation of total bacterial loads in children fecal samples. To exclude artifacts, proxy

markers of total bacterial counts might need to be assessed, e.g., by quantitative PCR. However, the abundance of *Faecali-bacterium* and *Ruminococcus* species has been correlated also with microbiota gene richness and host metabolic health in shotgun metagenomics studies (Le Chatelier et al., 2013). Thus, it appears as these taxa might have important ecological functions associated with increased microbial diversity, which may promote health.

There is extensive literature on the effects of birth mode on the gut microbial composition during the first year of life (Dominguez-Bello et al., 2010; Dominguez-Bello et al., 2016; Reyman et al., 2019). Furthermore, mode of birth has also been linked with allergy (Feehley et al., 2019) and obesity (Cox et al., 2014; Dogra et al., 2015), potentially by affecting the microbiota at critical windows during the postnatal development of the immune system. In agreement, with the published literature we observed large impact of mode of birth on the gut microbiota early in life, but the impact was small although it still significantly explained compositional variation in the microbiota at 3 and 5 years. C-section was associated with lower alpha diversity at 4 months, which is agreement with previous observations (Bokulich et al., 2016), We also observed an overrepresentation of c-section born children in community types with lower alpha diversity when the children were in the transitional phase, at the chronological age of 12 months. However, these differences normalized as the gut microbiota continued to mature. Future, larger studies are required to identify potential developmental windows when the gut microbiota may be particular sensitive for development of diseases. Furthermore, re-analyses and meta-analyses of previously published datasets in combinations with ours using amplicon sequence variants might facilitate the identification of important taxa independent of reference databases (Callahan et al., 2017).

In summary, here we used a longitudinal birth cohort to describe the development of the human gut microbiota during the first five years of life. We conclude that several bacterial taxa that have been associated with human health are acquired late in childhood and have not reached their adult abundance at five years of age. Furthermore, we observe that the gut microbiota of children in a normal population develop at an individual pace along the microbiota's developmental trajectory, thus highlighting the importance of taking microbiota dynamics into account.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

Conceptualization, J.R., L.O., K.K., J.D., and F.B.; methodology, V.T., P.K.D., formal analysis, L.O. and S.N., investigation, M.K., R.A., M-C.S., and P.K., resources, G.B. and M.U., writing – original draft, J.R., L.O., F.B., writing – review and editing, all authors; supervision, F.B.; funding acquisition, F.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Human faeces	This paper	N/A
Critical Commercial Assays		
NucleoSpin Soil kit	MACHEREY-NAGEL	40780
5PRIME HotMasterMix	Quanta bio	733-2474
NucleoSpin Gel and PCR Clean-up kit	MACHEREY-NAGEL	740609
Quant-iT PicoGreen dsDNA kit	Thermo Fischer	P11496
MiSeq Reagent Kit v2 (500-cycles)	Illumina	MS-102-2003
Deposited Data		
16S rRNA sequencing data	This paper	PRJEB38986
16S rRNA sequencing data	(Tebani et al., 2020)	PRJEB38984
Oligonucleotides		
V4 region 515F and 806R primers. See Table S6	(Kozich et al., 2013), Appendix D	N/A
Software and Algorithms		
R Software	(R Core Team, 2018)	https://www.r-project.org
Qiime (v1.9)	(Caporaso et al., 2010b)	N/A
ChimeraSlayer	(Haas et al., 2011)	N/A
DMM	(Holmes et al., 2012)	N/A
Vegan	(Oksanen et al., 2015)	N/A
TcGSA	(Hejblum et al., 2015)	N/A
Other		
FastPrep-24 Instrument	MP Biomedicals	116004500

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Fredrik Bäckhed (fredrik@wlab.gu.se).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All 16S rRNA-gene sequencing data included in this study have been deposited to the European Nucleotide Archive (ENA). The data for the children and mothers under the study accession PRJEB38986 and the data for the adult cohort (S3WP) under PRJEB38984.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The study was conducted as a sub study in the H2GS project (Gerd et al., 2012) and the study was approved by the ethical review board in Lund (44/2008). Infants and their parents were recruited before delivery when arriving to the delivery ward and informed consent from the parents was collected. The infants and their parents were invited to further clinical visits including height and weight measurements by trained research nurses in a standardized manner (Roswall et al., 2016). Feces and questionnaires were collected when children were newborn (first samples after birth; median 3 days, IQR: 2-6 days) and after 4, 12, 36 and 60 months. Planned c-sections were recruited separately due to organizational issues to increase the n in this group.

We calculated standard deviation (SD) scores for height and weight independent of sex and age and corrected for gestational age according to Swedish national child growth references (Karlberg et al., 2001; Niklasson et al., 1991; Wikland et al., 2002). To explore relationships between the 12 months gut microbiota and effects on later early childhood growth, changes in SD scores between twelve months and five years of age were calculated for weight and length (scores at five years of age minus scores at 12 months of age). A gain in SD score greater than 0.67 SD scores was classified as rapid weight gain, and a decrease in SD scores for weight by more than 0.67 SD scores indicated slow weight gain (Ong and Loos, 2006). A gain in SD score >0.67 represents growth chart centile crossing representative to the width of each percentile band on standard growth charts (second to ninth percentile, ninth to 25th, 25th to 50th etc.).

In total, 471 infants (246 boys/225 girls) and their mothers were included in this project. For detailed description of the study population, see Table 1. Fecal samples from the first visit of 101 individuals of an adult population, the longitudinal Swedish SCAPIS SciLifeLab Wellness Profiling (S3WP) program, were also included (Tebani et al., 2020). Samples from both cohorts were collected and handled in the same way.

METHOD DETAILS

Sample collection
Fecal samples were collected from mother (M, n=357) and child during first week after delivery (NB, n=246) and at 4 (n=411), 12 (n=397), 36 (n=336) and 60 (n=288) months of age. Samples were frozen at −80°C and stored until further analysis. Feeding and growth data were collected at regular child health clinic visits as part of the larger birth cohort (1 week, 1, 4, 6, 12, 18, 24, 36, 48 and 60 M).

A subset of complete series of samples from 4 months to 5 years, referred to as cohort with complete series of samples, comprised 213 individuals. There were no significant differences in gestational age, birthweight, mothers age, rate of c-section and feeding patterns between the longitudinal cohort and the whole sample subset.

Fecal DNA extraction and sequencing
Total fecal genomic DNA was extracted from 100-150 mg of stool using the NucleoSpin Soil kit (MACHEREY-NAGEL, Germany) (Bäckhed et al., 2015). Manufacturer's instruction was followed with the only modification being that the vortex step was replaced with repeated bead beating at 5.5 m/s for 60 s using the FastPrep-24 Instrument (MP Biomedicals). V4 region of 16S rRNA genes from each sample were amplified with 515F and 806R primers, designed for dual indexing (Kozich et al., 2013, Table S6), in duplicate reactions. PCR amplification was performed in 25 μl volume containing 1x Five Prime Hot Master Mix (5 PRIME GmbH), 200 nM of each primer, 0.4 mg/ml BSA, 5% DMSO and 20 ng of genomic DNA. PCR was carried out by initial denaturation for 3 min at 94°C, followed by 25 cycles (denaturation for 45 seconds at 94°C, annealing for 60 seconds at 52°C and elongation for 90 seconds at 72°C) and a final elongation step for 10 min at 72°C. Duplicates were combined, purified with the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany) and quantified using the Quant-iT PicoGreen dsDNA kit (Thermo Fischer). The amplified V4 region of the 16S rRNA gene was sequenced 250bp paired-end on an Illumina MiSeq instrument (Illumina RTA v1.17.28; MCS v2.5) with the V2 MiSeq SBS kit.

Pre-processing of 16S rRNA
Paired-end reads were merged using PEAR (Zhang et al., 2014). To exclude potential sequencing errors, merged sequences were filtered using FASTX (Hannon, 2010) to remove low-quality reads that had at least one base with a q-score lower than 20. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold using an open-reference OTU picking approach with UCLUST (Edgar, 2010) against the Greengenes reference database (DeSantis et al., 2006) (13_8 release) using software package QIIME (Caporaso et al., 2010b) (version 1.9.1). All sequences that failed to cluster against the Greengenes database were used for picking OTUs *de novo*. Representative sequences for each OTUs were assigned taxonomy using the Ribosomal Database Project Classifier (Wang et al., 2007). Representative OTUs were aligned using PyNAST (Caporaso et al., 2010a) and used to build a phylogenetic tree with FastTree (Price et al., 2010). To remove PCR artifacts, chimeric sequences were identified with ChimeraSlayer (Haas et al., 2011); sequences that could not be aligned with PyNAST, singletons and low abundant OTUs (relative abundance < 0.002%) were considered as potential errors, and were therefore excluded from downstream analyses. We obtained a mean ± SD of 56,222± 36,381 sequences/sample (range 6,137–397,922). In total, 1435 OTUs were included in the analyses. Genus level analysis was made on abundance on OTUs collapsed to the same genus (L6-level).

To correct for differences in sequencing depth, samples were subsampled to the same number of reads. Alpha and beta diversity analysis as well as clustering of genera into community types were performed on rarified samples to 6130 reads. In analysis of relative abundance on genera/L6-level and OTU-level counts was scaled counts to the total sum of counts. Values given as relative abundance sums up to 1. Selected OTUs were blasted against NCBI 16S ribosomal RNA sequencing (Bacteria and Archaea) database to obtain a more specific annotation.

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QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using the R environment and language (R Core Team, 2018). Tests for how different factors contributed to compositional variation were performed using adonis from the vegan package (Oksanen et al., 2015). To compensate for the unbalanced groups, sensitivity analysis was made in balanced groups (Tables S1 and S5). Differences in abundance were based on counts normalized to the total sum in each sample. Only genera (L6-level) with an abundance of 0.1% in at least 1% of the 2136 samples were used in abundance testing. For testing of OTUs, only OTUs with an abundance of 0.1% in at least 10% of samples within time points (5% in NB samples) were used in abundance testing.

Differences between timepoints were tested using Wilcoxon signed-rank test in set of complete samples for each individual comparison. Differences between groups were tested using Wilcoxon rank-sum test. Controlling for false discovery rate was done by estimating q-values of significant p values (Storey, 2003). Changes over time was tested in the longitudinal cohort using Linear-mixed-effect model with individual as random variable using the lmer-function in the *lme4*-package (Bates et al., 2015). Factors effect (time, mode of birth) on longitudinal development were evaluated by comparing models with and without factor and without fit with REML.

Genera trajectories in the longitudinal cohort were calculated on $\log_{10}$ transformed L6 relative abundance using mixed effect model with the TcGSA.LR function in the *TcGSA* package (Hejblum et al., 2015), using "cubic" function for time modelling. Clustering of genera into trajectory was made using default settings.

Gut microbiota community types were determined by clustering rarefied counts using Dirichlet multinomial mixtures model (Holmes et al., 2012). The number of community types were chosen by selecting the number of components that gave the minimal Laplace approximation to the negative log model evidence. Samples were assigned to community type by their maximum posterior probability. Differences between community types were tested with Kruskal-Wallis test followed by Dunn's test of multiple comparison with correction for multiple testing using Holm (Holm, 1979).

Correlations to alpha diversity were made on relative abundances of OTUs using Spearman's correlation and controlling for false discovery rate by estimating q-values of significant p values (Storey, 2003).

Test of distributions between community types in categorical factors was made using Fischer's exact test. Test for community types effect on continuous variables distribution was done using linear models.

The alluvial diagrams illustrating proportions of individuals within the age specific community types and their trajectory over community types over time was illustrated using the *alluvial* package (Bojanowski and Edwards, 2016).