

Dynamics of Infant Gut Microbiota Are Influenced by Delivery Mode and Gestational Duration and Are Associated with Subsequent Adiposity

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ABSTRACT We found that the relatively simple microbiota of young infants shifts predictably to a more mature anaerobic microbiota during infancy and the dynamics of this shift are influenced by environmental factors. In this longitudinal study of 75 infants, we demonstrate high interindividual variability within the normal range of birth outcomes, especially in the rate of microbiota progression. Most had acquired a microbiota profile high in *Bifidobacterium* and *Collinsella* by 6 months of age, but the time point of this acquisition was later in infants delivered by caesarean section and those born after a shorter duration of gestation. Independently of the delivery mode and gestation duration, infants who acquired a profile high in *Bifidobacterium* and *Collinsella* at a later age had lower adiposity at 18 months of age.

IMPORTANCE This study shows that the acquisition of the early microbiota is strongly influenced by environmental factors such as the delivery mode and duration of gestation, even in healthy neonates. The composition of the early microbiota has been linked with long-lasting effects on health and disease. Here we show that the rate of acquisition of certain microbiota predicts adiposity at 18 months of age and so potentially the risk of later obesity.

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The microbes residing in the human gut have a profound influence on our physiology. Variation in the microbiota of the adult human gut has been linked to many outcomes, including a risk of autoimmune disease (1), response to toxins (2), and behavior (3). In particular, the microbial content of the human gut alters the metabolic state (4, 5) and is associated with the development of obesity (4, 6–8) and insulin resistance (9, 10).

At birth, human infants start accumulating intestinal microbiota until a relatively stable state is reached (11). This process is influenced by environmental factors including the mode of delivery, the mode of feeding, the duration of gestation, and the living environment (for reviews, see references 12 and 13). The rate and trajectory of acquisition of a gut microbiota is hypothesized to have a considerable impact on later health outcomes. In particular, disruption of the early microbiota by antibiotic use (14, 15) and a differential trajectory of microbiota acquisition following cesarean section (16, 17) is associated with increased adiposity later in life. Cox et al. (18) recently found that a subtherapeutic dose of penicillin preweaning is sufficient to temporarily alter the mouse gut microbiota and permanently alter the animal's growth characteristics into adulthood, predisposing it to weight gain and

amplifying the effects of a high-fat diet. Crucially, the transient microbial changes caused by penicillin were causative; penicillin-selected microbes were transferred to germ-free hosts and recapitulated the growth promotion phenotype. The precise kind of bacteria encountered by the infant may be important for health outcomes. For example, increased early microbial contact is thought to protect from autoimmune diseases (19), but early colonization with facultative bacterial respiratory pathogens was associated with an increased risk of asthma development (20).

Here we have studied gut microbiota acquisition in 75 infants participating in the Growing Up in Singapore Toward Healthy Outcomes (GUSTO) birth cohort (21). The microbial content of longitudinal fecal samples during the first 6 months of life was profiled by 16S rRNA sequencing, and we investigated the effect of environmental factors, including the delivery mode and duration of gestation, on the trajectories of microbial development, along with the associative relationships with later adiposity.

RESULTS

The infants in this study represented the normal range of birth weights and durations of gestation (Table 1). The microbiotas

TABLE 1 Characteristics of the subjects included in this study^a

Parameter	No. of infants	Min	Max	Mean	SD	Median
Maternal age (yr)	75	20	40	30.85	5.09	31
Maternal BMI ^a (kg/m ²)	75	17.87	40.09	27.29	5.05	26.68
Maternal subscapular skinfold thickness (mm) at 26 wk	75	10.6	35	21.76	5.98	21
Gestation duration (wk)	75	35.43	41.14	38.82	1.22	39
Infant length (cm) at birth	69	45	52.45	48.87	1.95	49
Infant length (cm) at 12 mo	64	69.2	82.45	75.96	3.21	76.28
Infant subscapular skinfold thickness (mm) at birth	72	2.74	8.3	4.99	1.19	4.9
Infant subscapular skinfold thickness (mm) at 18 mo	51	4.2	14	6.43	1.66	6.2
Birth wt (g)	75	2235	3980	3124.53	393.26	3110
Wt (g) at 6 mo	72	5882.5	9247.5	7681.58	843.61	7650
Wt (g) at 12 mo	64	7195	13,110	9544.10	1123.93	9402.5
% of length at 12 mo vs at birth ^b	59	141.67	170.33	155.41	6.28	154.89
% of subscapular skinfold at 18 mo vs at birth ^c	50	62.65	240	135.64	45.07	122.03
% of wt at 6 mo vs at birth ^d	72	186.55	333.12	248.57	29.78	244.88
% of wt at 12 mo vs at 6 mo ^e	62	110.86	148.73	123.87	7.36	123.30
Difference between length (cm) at 12 mo and at birth ^f	59	21.25	32.55	27.08	2.74	27
Relative % difference in length at 12 mo and at birth ^g	59	41.67	70.33	55.41	6.28	54.89
Difference in wt (g) at 12 mo and at 6 mo ^h	62	720	4055	1833.89	562.98	1718.75
Relative % difference in wt at 12 mo and at 6 mo ⁱ	62	10.86	48.73	23.87	7.36	23.30
Difference in wt (g) at 12 mo and at birth ^j	64	3977.5	9285	6406.53	1038.50	6342.5
% of wt at 12 mo vs at birth ^k	64	212.04	402.06	307.72	42.76	304.65
Relative % difference in wt at 12 mo and at birth ^l	64	112.04	302.06	207.72	42.76	204.65
Difference in wt (g) at 6 mo and at birth ^m	72	2895	6152.5	4563.52	721.50	4496.25
Relative % difference in wt at 6 mo and at birth ⁿ	72	86.55	233.12	148.57	29.78	144.88
Difference in subscapular skinfold thickness (mm) at 18 mo and at birth ^o	50	-3.1	6.9	1.4	2.02	0.98
Relative % difference in subscapular skinfold thickness at 18 mo and at birth ^p	50	-37.35	140	35.64	45.07	22.02
Socioeconomic status	66	-2.56	1.64	-0.35	1.19	-0.12

^a BMI, body mass index.

^b (Infant length at 12 months × 100)/length at birth.

^c (Infant subscapular skinfold thickness at 18 months × 100)/subscapular skinfold thickness at birth.

^d (Infant weight at 6 months × 100)/weight at birth.

^e (Infant weight at 12 mo × 100)/weight at 6 months.

^f Infant length at 12 months — length at birth.

^g [(Infant length at 12 months — length at birth) × 100]/length at birth.

^h Infant weight at 12 months — weight at 6 months.

ⁱ [(Infant weight at 12 months — weight at 6 months) × 100]/weight at 6 months.

^j Infant weight at 12 months — birth weight.

^k (Infant weight at 12 months × 100)/birth weight.

^l [(Infant weight at 12 months — birth weight) × 100]/birth weight.

^m Infant weight at 6 months — birth weight.

ⁿ [(Infant weight at 6 months — birth weight) × 100]/birth weight.

^o Infant subscapular skinfold thickness at 18 months — subscapular skinfold thickness at birth.

^p (Infant subscapular skinfold thickness at 18 months — subscapular skinfold thickness at birth) × 100/subscapular skinfold thickness at birth.

^q Other characteristics recorded were birth order (25 firstborn, 50 other), gender (39 male, 36 female), antibiotic use in labor (19 yes, 55 no, 1 unknown), delivery mode (57 vaginal, 18 caesarean section), ethnicity (19 Indian, 22 Malay, 34 Chinese), infant feeding at 1 week of age (15 exclusively breastfed, 50 partially breastfed, 10 exclusively formula fed), infant feeding at 3 weeks of age (14 exclusively breastfed, 52 partially breastfed, 9 exclusively formula fed), infant feeding at 3 months of age (13 exclusively breastfed, 18 partially breastfed, 43 exclusively formula fed, 1 unknown), and infant feeding at 6 months of age (7 exclusively breastfed, 13 partially breastfed, 51 exclusive formula fed, 4 unknown).

were simple, with three of the four abundant phyla (*Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes*) represented by only one detectable genus and few operational taxonomic units (OTUs); only the phylum *Firmicutes* showed more complexity at the genus level (see Fig. S1 in the supplemental material). There was substantial interindividual variation in the microbiota composition of infant feces (see Fig. S2 in the supplemental material). Day 3 neonate microbiotas were dominated by *Klebsiella* OTU2 and *Escherichia* OTU3, which belong to the family *Enterobacteriaceae* (of the phylum *Proteobacteria*), and these levels decreased over time with a commensurate increase in the levels of members of the genus *Bifidobacterium* (which was the only genus in the phylum *Actinobacteria* detected) represented by one major OTU.

The phylum *Firmicutes* retained similar abundance levels across time but with changing constituent genera (see Fig. S1). The genus *Bacteroides* (the only representative of the phylum *Bacteroidetes* detected) was detected in only a subset of the infants (see Fig. S2).

When all of the samples in the study were clustered on the abundance of all of the variable taxa, three deep-rooted clusters were found (Fig. 1A; for an expanded view with taxon labels, see Fig. S3 in the supplemental material). The relationship between the time point and membership of the clusters was highly significant ($\chi^2 P = 1.4^{-20}$). Most of the day 3 samples were in cluster 2, which was characterized by high levels of *Enterobacteriaceae* family members, especially *Klebsiella* OTU2; in contrast, the majority of the month 6 samples belonged to the biggest cluster, 3, which

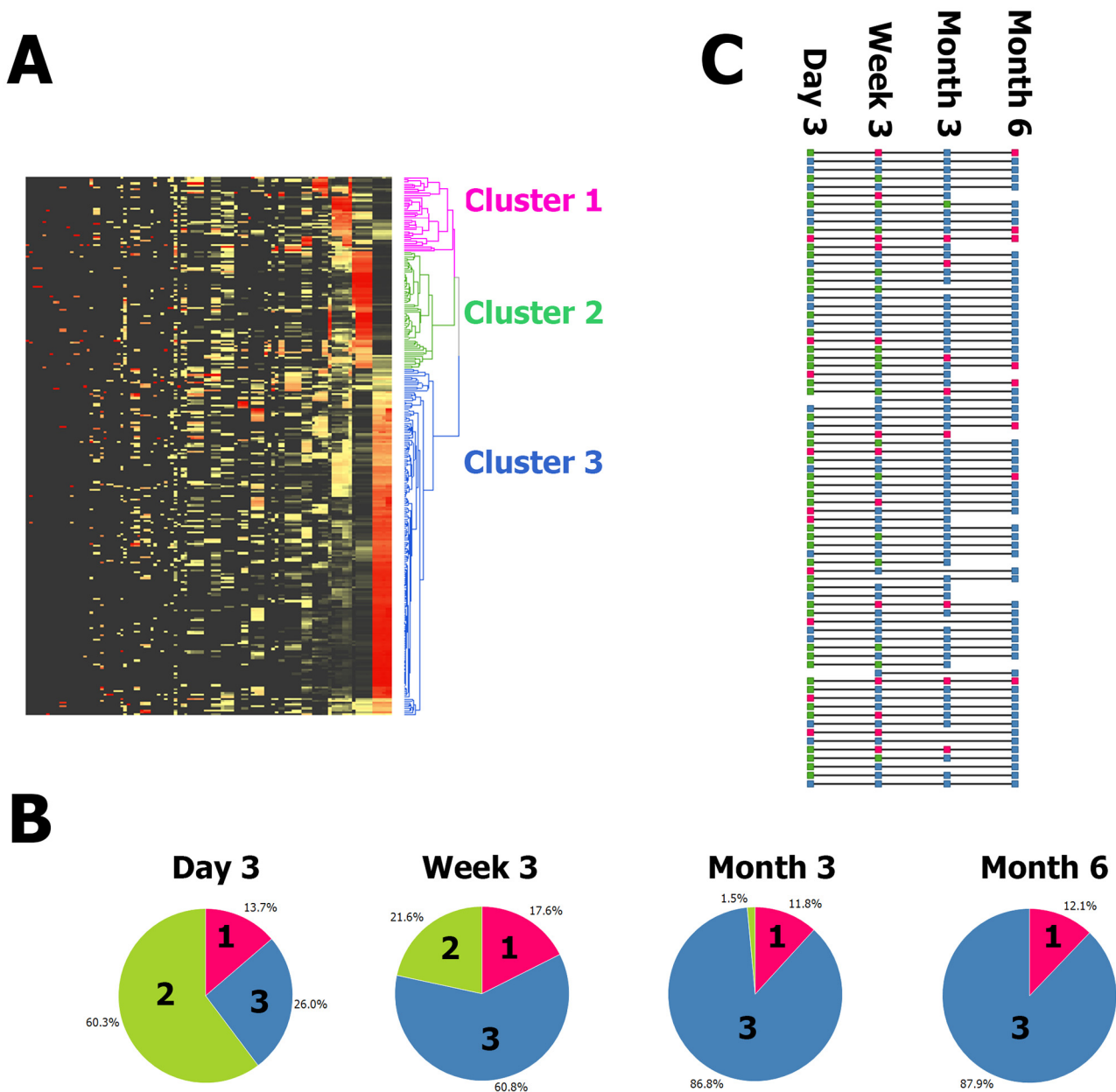


FIG 1 (A) Unsupervised clustering of all samples and all taxa reveals three clusters. *Z* score-normalized data for all detected taxa are displayed in a heat map with black denoting the minimum value, yellow denoting the average value, and red denoting the maximum value. Each row is a sample, and each column is a taxon. Both taxa and samples are clustered by UPGMA with euclidean distance. A sample dendrogram is shown on the right. The sample dendrogram is pruned at distance 238 (chosen by eye) to reveal three deep-rooted clusters. The clusters are pink, green, and blue and numbered 1, 2, and 3 from the top down. (B) The time point was strongly associated with cluster membership ($P = 1.38^{-20}$). Pie charts show the proportions of samples at each time point that were classified in each of the three clusters. Sections are colored as in panel A, i.e., magenta for cluster 1, green for cluster 2, and blue for cluster 3, as well as labeled by cluster number. For example, 60% of the day 3 samples were in cluster 2 and no month 6 samples were in cluster 2; meanwhile, 88% of the month 6 samples were in cluster 3 and 26% of the day 3 samples were in cluster 3. (C) Individuals progress from cluster 2 to cluster 3 with some intermediate cluster 1 and much interindividual rate variation. Each horizontal line represents one subject. Each square is colored to reflect the cluster the subject was classified into at each time point. The squares are colored as in panels A and B, i.e., magenta for cluster 1, green for cluster 2, and blue for cluster 3. A progression from cluster 2 (green) to cluster 3 (blue) is typical, but some subjects start at cluster 3 (blue) and some progress through cluster 1 (magenta).

was characterized by high *Bifidobacterium* OTU1 and *Collinsella* levels. Most subjects progressed from cluster 2 to cluster 3 throughout the 6-month time frame. However, at day 3, 19 of the 73 samples were in cluster 3, demonstrating interindividual variability in progression over time (Fig. 1B). Once a sample from an

individual was classified in cluster 3, later samples from the same individual tended also to be classified within cluster 3. Finally, there was a minority of samples within cluster 1. Cluster 1 was characterized by very high levels of *Firmicutes*, in particular, *Streptococcus*. Although cluster 1 was slightly more frequent in the ear-

lier samples, it appeared across all time points. One subject was stably in cluster 1 across the time points, but others progressed from cluster 2 to cluster 1 to cluster 3 (Fig. 1C).

Next, the individual subjects were classified according to the earliest time point at which a sample was classified in cluster 3. Later samples from the same individual also had to be classified in cluster 3. Infants delivered by caesarean section reached (and remained in) cluster 3 later than infants delivered vaginally. Of the 17 individuals who reached a cluster 3 microbiota at day 3, just 1 (6%) was delivered by caesarean section, while 16 had vaginal deliveries; for those reaching cluster 3 at week 3, the month 3 and 6 comparable figures for the proportions delivered by caesarean section were 25% (6 of 24), 33% (5 of 15), and 40% (2 of 5), respectively (Fig. 2A). This is consistent with univariate analysis showing significantly lower levels ($P = 0.042$) of *Bifidobacterium* OTU1 detected in babies delivered by caesarean section than in babies delivered vaginally at day 3 (Fig. 2B) and significantly higher levels ($P = 5.8E^{-8}$) of *Klebsiella* OTU2 (Fig. 2C).

Babies who reached cluster 3 later tended to be born earlier (Fig. 2D) ($P = 0.016$ by linear regression). This association survived adjustment for the delivery mode (see Table S1 in the supplemental material) ($P = 0.03$). The difference in the mean duration of gestation between infants who reached cluster 3 on day 3 and those who did so by month 6 was only 1 week, suggesting that a shorter gestational duration is associated with a lag in microbial acquisition. This observation is consistent with the within-time point analysis showing that babies born after a shorter duration of gestation tend to have higher *Streptococcus* levels at week 3 (Fig. 2E). There was also an association between reaching cluster 3 later and lower subscapular skinfold thickness at 18 months (Fig. 2F) ($P = 0.01$ by linear regression); the association survived adjustment for gestational duration and delivery mode (see Table S1) ($P = 0.032$). The median subscapular skinfold thickness at 18 months in the infants who reached cluster 3 at month 6 was less than 1 standard deviation below the median defined by the WHO (data for both genders combined) (22). Meanwhile, the median subscapular skinfold thickness at 18 months of infants who reached cluster 3 at day 3 and week 3 was similar to the WHO median (22). Interestingly, *Streptococcus* levels at month 6 were associated with the change in subscapular skinfold thickness between 0 and 18 months (Fig. 2G, $P = 0.018$ by linear regression); again, the association survived adjustment for gestational duration and delivery mode (see Table S1) ($P = 0.02$).

DISCUSSION

The infant microbiotas described in our study are simple, with three of the four dominant phyla (*Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*) represented by just one genus and one or two OTUs each. This is largely consistent with other studies (11, 23). The progression of the microbiota over time is also consistent with other findings (11). La Rosa et al. (24) found that the progression of the gut microbiota in the first 40 days of life in premature infants housed in intensive care units from *Bacilli* to *Gammaproteobacteria* to *Clostridia* was remarkably consistent despite relatively restricted exposure to sources of bacteria. Most of our day 3 term infant gut microbiotas were dominated by *Gammaproteobacteria* (in particular, members of the family *Enterobacteriaceae*), similar to the day 9 to 17 microbiotas of premature infants. We did not detect the predominant *Bacilli* phase noted in premature infants, although we did observe that high levels of the *Bacilli* genus

Streptococcus (a marker in our study of clusters 1 and 2) were associated with increased adiposity at 18 months of age. Although *Clostridia* levels were variable in our infants and increased with time (consistent with reference 24), by month 3, most of our infant microbiotas were dominated by *Actinobacteria* (in particular, *Bifidobacterium*).

In this study, the switch between aerobes and anaerobes occurred between birth and month 3 of infancy, in agreement with previous studies (11, 25, 26). However, there was substantial interindividual variation in when the aerobe-anaerobe switch occurred. We observed that gut microbiota of infants in our study could be classified into three distinct clusters (Fig. 1A). Cluster 3 contained ~88% of the month 6 samples and could be said to represent an anaerobic microbiota appropriate for infants in this age range. Twenty-six percent of our infants had reached cluster 3 by day 3. Cluster 3 was characterized by high *Bifidobacterium*, high *Collinsella*, low *Enterobacteriaceae*, and low *Streptococcus* levels. Interestingly, once cluster 3 was reached by an individual at any time point, samples from later time points tended to stay within cluster 3. In contrast, cluster 2 tended to contain earlier samples and most individuals with cluster 2 membership early progressed to cluster 3 later. The high predominance of *Klebsiella* OTU2 and *Streptococcus* OTU4 in cluster 2 was striking. Both OTUs may contain facultative pathogens, and they are likely to provide a substantially different environment in the developing infant gut than bifidobacteria, which are considered the optimal colonizers of infants (27, 28). Cluster 1 membership did not follow such a strong pattern across time points, although it was slightly more frequent at early time points. High *Firmicutes* levels were characteristic of this cluster and have been observed in the early microbiota of premature infants (25).

Cluster 3 was reached relatively later in infants delivered by caesarean section (Fig. 2A). Lower or later colonization by bifidobacteria in babies delivered by cesarean section is consistent with earlier studies (29). By 6 months, no differences in microbiotas were detected between infants born vaginally and those delivered by caesarean section. This agrees with other studies (30) but does not preclude a long-lasting effect on the immune system or intestinal barrier function of differences in the early microbiota driven by the delivery mode (18, 31–34).

Previous studies have found that preterm infants have a microbiota composition very different from that of full-term infants (35–39) and linked this with detrimental outcomes, such as necrotizing enterocolitis. Even though all of our infants were born at term, the duration of gestation at birth was significantly associated with the infant's microbiota profile over time, with those born after a shorter duration of gestation tending to reach cluster 3 later (Fig. 2D, $P = 0.0163$). A relationship between variation in gestation duration within the normal range and the microbiota profile is remarkable; however, we note that Karlsson et al. (40) also found that the abundance of bacterial groups was associated with a birth weight within the normal range. A shorter gestation duration may be associated with subtle gut immaturity, even within the normal range, with negative consequences for the gut microbiota; this is consistent with recent results showing that early term infants (gestational age of 37 to 38 weeks) show suboptimal outcomes (41, 42). In agreement with reference 24, we found that a shorter gestation duration was associated with a lag in microbiota progression.

An important finding of our study was that reaching cluster 3

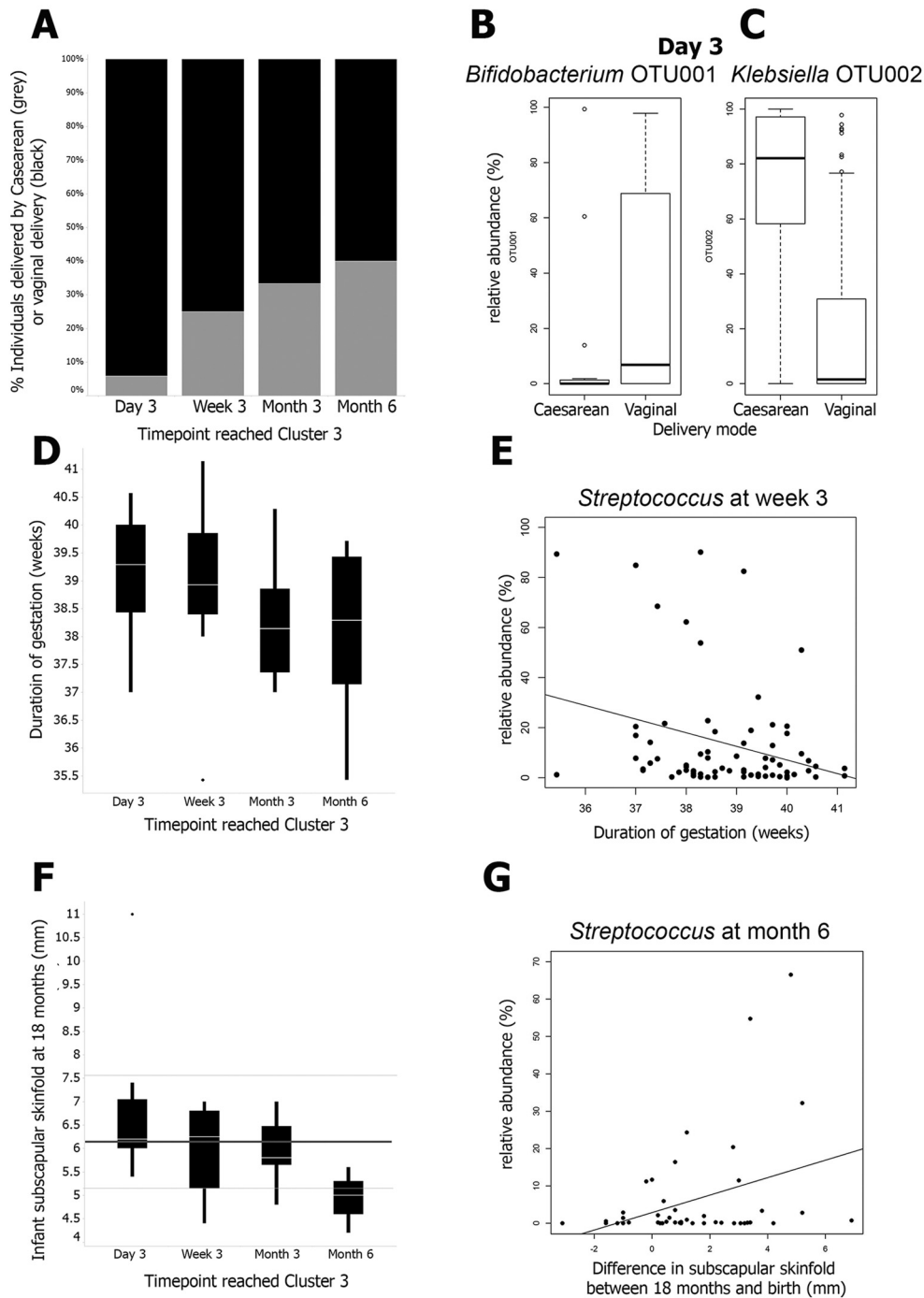


FIG 2 (A) The time point at which a cluster 3 profile is reached is associated with the delivery mode ($P = 0.046$). On the x axis is the time point at which a cluster 3 profile is reached by each individual, and on the y axis are the proportions of individuals who were born by caesarean delivery (grey) and vaginal delivery (black). (B) *Bifidobacterium* OTU001 levels are higher in vaginally delivered infants at day 3 than in infants delivered by caesarean section ($P = 0.042$). The delivery mode is on the x axis, and the relative abundance of *Bifidobacterium* OTU001 at day 3 is on the y axis. (C) *Klebsiella* OTU002 levels are lower in vaginally delivered infants at day 3 than in infants delivered by caesarean section ($P = 5.8E^{-6}$). The delivery mode is on the x axis, and the relative abundance of *Klebsiella* OTU002 at day 3 is on the y axis. (D) The time point at which a cluster 3 profile is reached is associated with gestational age ($P = 0.016$). On the x axis is the time point at which a cluster 3 profile is reached by each individual, and on the y axis is the gestational age in weeks. (E) *Streptococcus* levels are negatively correlated with gestational age at week 3 ($P = 0.011$). The gestational age is on the x axis, and the relative abundance of the *Streptococcus* genus at day 3 is on the y axis. (F) The time point at which a cluster 3 profile is reached is associated with infant skinfold thickness at 18 months ($P = 0.01$). On the x axis is the time point at which a cluster 3 profile is reached by each individual, and on the y axis is infant subscapular skinfold thickness in millimeters at 18 months. For reference, the WHO median subscapular skinfold thickness at 18 months of age of 6.15 mm is denoted by the thick grey line and thin grey lines denote 1 standard deviation from the median (5.15 and 7.5 mm). (G) Month 6 *Streptococcus* levels are positively correlated with the difference in skinfold thickness (millimeters) between 18 and 0 months (i.e., 18-month skinfold thickness minus neonatal skinfold thickness) ($P = 0.018$). The difference in skinfold thickness between 18 and 0 months is on the x axis, and the relative abundance of the *Streptococcus* genus at month 6 is on the y axis.

TABLE 2 Characteristics of infants who reach a month 6-like microbiota earlier or later

Point at which infants reached "month 6-like" microbiota	Tendency of:						Skinfold thickness at 18 mo
	<i>Enterobacteriaceae</i> levels	<i>Streptococcus</i> levels	<i>Bifidobacterium</i> levels	<i>Collinsella</i> levels	Delivery mode	Gestation duration	
Later	Higher	Higher			Caesarian section	Shorter	Thinner
Earlier			Higher	Higher	Vaginal	Longer	Typical

later in infancy was predictive of lower adiposity at 18 months of age (Fig. 2F). This may be due to lasting effects of a differential early microbiota or to causative factors affecting both body composition and the microbiota. Infants who had unusually high *Streptococcus* abundances at month 6 displayed a tendency for a greater increase in adiposity (Fig. 2G); this may be particularly relevant in the light of recent findings linking the extent of adiposity increase in infancy, rather than absolute values of adiposity, to suboptimal health outcomes (43). Adult obesity has been associated with an altered microbiota profile (8). Recent findings obtained with mice show that low-dose penicillin limited to early life acts through an altered microbiota to induce sustained effects on body composition (18). However, the very different microbiotas of mice and human infants make it difficult to apply observations on particular taxa directly to human adiposity.

The infant feeding mode was not significantly associated with the time to cluster 3, although we did find limited support ($P = 0.09$) for the association between breastfeeding status and the abundance of members of the class *Gammaproteobacteria* (at month 6 in our data) shown in reference 24. In addition, multiple other taxa were nominally significantly ($P < 0.05$) associated with the feeding pattern at the univariate level (see Table S2 in the supplemental material). However, the majority of the infants in our study were fed a mixture of breast milk and formula at early time points (49 mixed, 10 exclusively formula fed, and 14 exclusively breastfed at day 3); moreover, the majority ($n = 47$) were exclusively formula fed at 6 months. Our study therefore lacked the discriminatory power necessary to detect effects of infant feeding. Many other groups have noted differences between the microbiotas of breastfed and formula-fed infants (44).

The concept of microbiota maturity was recently introduced by Subramanian et al. (45), who found that an immature microbiota was clearly associated with acute malnutrition. We note that the 24 most age-discriminatory taxa in the analysis of Subramanian et al. included *Bifidobacterium* and *Streptococcus* species, which were important discriminant taxa in our clustering. This similarity was seen despite the radically different circumstances and age ranges of the children included in the study of Subramanian et al. and ours.

Reaching cluster 3 early was associated in our study with factors that are thought to be favorable Table 2 (i.e., longer duration of gestation, vaginal delivery, and adiposity in line with global medians). In contrast, caesarean section delivery, a shorter gestational duration, and relatively low adiposity at 18 months were all associated with a slight microbiota delay Table 2; suggesting that the delay is suboptimal. This is somewhat consistent with the profound gut microbiota immaturity observed by Subramanian et al. in malnourished children.

A limitation of this study was the resolution of our sequence depth. We did not have information on which prokaryotic genes

were expressed, and sequence depth limits the detection of nonabundant taxa (34). A final caveat is that fecal sampling is not a wholly accurate proxy for the gastric and intestinal microbiota. It tends to undersample biodiversity and does not reflect site-specific differences (46). We chose to use the results from sequencing of the V456 region of the rRNA, as these yielded a more complete data set with less missing data; however, V123 was also sequenced and yielded very similar results (see Fig. S4 and Table S3 in the supplemental material).

To our knowledge, this study is the largest of its type in terms of both including 75 individuals and sampling them longitudinally across four time points. The 75 individuals included had normal phenotypes with no extreme values of gestational age or adiposity; the majority received mixed breastfeeding and formula feeding. However, the rate at which a more anaerobic microbiota was obtained correlated with the duration of gestation at birth and the mode of delivery, as well as measures of infant body composition.

Conclusion. An earlier acquisition of an anaerobic microbiome dominated by *Bifidobacterium* and *Collinsella* compared to *Enterobacteriaceae* and *Streptococcus* within the first 6 months of life is associated with a longer gestation duration at birth, vaginal delivery, and typical adiposity at 18 months of age.

MATERIALS AND METHODS

Subjects, sample collection, and DNA extraction. Fecal samples were collected at day 3, week 3, month 3, and month 6 from 75 infants in the GUSTO birth cohort, which includes members of the three main ethnic groups in Singapore, i.e., Chinese, Indian, and Malay; a detailed description of the GUSTO cohort has been published elsewhere (21). This study was approved by both the National Healthcare Group Domain Specific Review Board (reference no. D/09/021) and the Sing Health Centralized Institutional Review Board (reference no. 2009/280/D). All of the participants who provided data gave informed written consent to donate their children's stool samples prior to their participation. Table 1 shows the maternal, delivery, and infant size/growth characteristics of the subjects. Samples were stored under anaerobic conditions until freezing, which took place a maximum of 10 h after sample emission. All samples were stored at -80°C . Total DNA was extracted with the QIAamp DNA stool minikit (Qiagen, Germany) in accordance with the manufacturer's instructions, except for the addition of a series of mechanical disruption steps (11×45 s) with a FastPrep apparatus and Lysing Matrix B tubes (MP Biochemicals, Santa Ana, CA) (50).

DNA sequencing. Two loci covering variable regions V1, V2, and V3 (V123) and V4, V5, and V6 (V456) of the 16S rRNA gene were used to characterize the microbiota by the barcoded-primer approach to multiplex pyrosequencing as described in reference 47 and detailed in the supplemental material. Both sets of primers were selected to ensure the optimal coverage of bacterial phylogenetic diversity, in particular, bifidobacteria. A total of 946,541 good-quality sequencing reads were obtained. The average numbers of reads per sample were 1,494 and 1,667 for the V123 and V456 regions, respectively.

Data processing Data processing was performed according to reference 48, and it is described in detail in the supplemental material. Briefly, denoising was performed with the PyroNoise algorithm, and the sequencing reads were quality checked by using stringent criteria (zero ambiguities, one mismatch permitted in a bar code; reads were trimmed to 250 bp before alignment) and chimera checked. The labels for OTUs were obtained by Ribosomal Database Project (RDP) classification of a representative sequence of each OTU. One dominant OTU belonging to the family *Enterobacteriaceae* was labeled as unclassified at the genus level. A BLAST search performed with a representative sequence of this OTU revealed that it showed 100% identity with several type strains of members of the family *Enterobacteriaceae*. Representative sequence of the corresponding OTU in region V123 yielded a 100% match to *Klebsiella pneumoniae* and more distant matches to other sequences. The analysis was performed primarily with the reads from region V456.

Univariate analysis with respect to phenotypes. Groups were compared by Student's *t* tests assuming homoscedasticity. RDP and OTU data were filtered by setting all singleton read data (i.e., read depth = 1) to zero and removing taxa that had reads detected in only one sample. For taxa at all phylogenetic levels and at each time point, linear regression was run for relative taxon abundance against the continuous phenotypes shown in Table 1; analyses of variance (ANOVAs) were run for relative taxon abundance against the categorical phenotypes. Mode of feeding was divided into the categories exclusively breastfed, mixed, and exclusively formula fed at the four time points (Table 1). False-discovery-rate-corrected *P* values were estimated as described by Benjamini and Hochberg (49) for all phenotypic comparisons conducted at each phylogenetic level.

Detection of clusters. A combined table containing data for subjects at all time points and all of the bacteria at different taxon levels (phylum, class, order, family, genus, and OTU) was produced. The data were normalized by mean value across taxa and samples [$x/(1/n \text{ mean})$]. Hierarchical clustering was performed by the unweighted-pair group method using average linkages (UPGMA) with euclidean distance. The sample dendrogram was pruned at distance 238 (chosen by visual inspection) to reveal three deep-rooted clusters. Cluster 1 (high in *Firmicutes* and its constituent genera *Streptococcus* and *Lactobacillus*) contained 36 samples, cluster 2 (high in members of the family *Enterobacteriaceae*) contained 61 samples, and cluster 3 (high in *Bifidobacterium* and *Collinsella*) contained 181 samples

Assignment of subjects to temporal groups and analysis with respect to phenotypes. Using the cluster definitions above, we next categorized each subject by the time point of first assignment to cluster 3. For example, if subject *x* was in cluster 2 at day3, cluster 2 at week 3, cluster 3 at month 3, and cluster 3 at month 6, the assigned value was 3 months. In this manner, 61 of the 75 subjects were assigned a time point at which they reached (and remained in) cluster 3. Three subjects never reached cluster 3, seven subjects were in cluster 3 during the time course but reverted to another cluster by month 6, and four subjects had missing data penultimate to the time point where cluster 3 was first seen; therefore, a call could not be made. The time point at which the subject reached cluster 3 was then tested against the environmental and phenotypic data by using either a chi-square test or ANOVA, as appropriate.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02419-14/-/DCSupplemental>.

Figure S1, TIF file, 0.7 MB.
 Figure S2, TIF file, 0.3 MB.
 Figure S3, TIF file, 0.5 MB.
 Figure S4, TIF file, 0.7 MB.
 Table S1, XLSX file, 0.01 MB.
 Table S2, XLSX file, 0.01 MB.
 Table S3, XLSX file, 0.01 MB.

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