

KEY DETERMINANTS OF THE INTESTINAL MICROBIOTA IN EARLY CHILDHOOD

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ABSTRACT

The intestinal microbiota develops rapidly after birth, determined by the maternal microbiota, mode of delivery, early infant feeding patterns and the subsequent long-term diet. While short-term dietary changes are associated with rapid effects on the intestinal microbiota, changes are transient and modest in magnitude. A healthy intestinal microbiota with high diversity can support a balanced immune homeostasis, allowing appropriate tolerance responses while simultaneously protecting against harmful exposures. Disruption of the intestinal microbiota in early life leads to a degraded stable microbiota that has been associated with increased risk of disease, particularly chronic non-communicable diseases that have increased in prevalence alongside progressive industrialisation. Strategies that support the establishment of a healthy microbiota in the first years of life and beyond may offer an approach to the prevention of chronic non-communicable diseases.

ACQUISITION OF THE INITIAL INTESTINAL MICROBIOTA IN EARLY INFANCY

The first encounter with microbes commences during gestation, however, the major acquisition of gut microbiota occurs during and after birth. The infant intestinal microbiota composition is initially strongly influenced by the mother's microbiota and mode of delivery (Caesarean section vs vaginal).¹ The microbiota of the mother is determined by genetic and environmental factors, particularly her long-term diet and diet during pregnancy. Moreover, the maternal microbiota can change considerably from the first to the third trimester.² The maternal microbiota composition determines the quality and quantity of microbiota that colonise the newborn gut during the birthing process. These co-called 'pioneer bacteria' influence the subsequent succession of microbiota through modulation of epithelial cell gene expression in the intestines and the intestinal microenvironment.¹

The composition of the succession microbiota in early infancy (first six months of life) is further modified by early feeding patterns (breast milk or formula) and the infant's home environment. Breastfeeding may assist in the development of a healthy gut microbiota by providing bifidobacteria and lactic-acid bacteria, which reinforce colonisation, and by supplying human milk oligosaccharides that promote a healthy microbiota composition. Breastfeeding also facilitates the exchange of microbes between mother and infant. Notably, the

breast milk microbiota is different in mothers who delivered their newborn by Caesarean section compared to mothers who experienced a vaginal delivery.³ Microbes are also acquired via skin contact and exposure to the microbiota of the immediate environment. Molecular analysis of bacterial communities in healthy babies during the first ten months of life demonstrated a progression from a simple profile in the first days of life to a more complex diverse profile with members of the genera *Bifidobacterium*, *Ruminococcus*, *Enterococcus*, *Clostridium* and *Enterobacter* identified by six months of age. The healthy intestinal microbiota around this time is characterised by a large gram-positive bacterial population, which contains significant numbers of bifidobacteria, mainly *B longum*, *B breve* and *B infantis*. The intestinal microbiota in formula- and breastfed infants differs with respect to bifidobacterial numbers and species composition. In breastfed infants, bifidobacteria constitute 60–90% of the total faecal microbiota with the most common bifidobacterial species being *B longum*, *B breve* and *B infantis*.¹ In formula-fed infants the microbiota is more complex. These differences largely disappear when infants are weaned from breast milk onto solid foods.

CHANGES IN THE MICROBIOTA ASSOCIATED WITH WEANING ONTO SOLID FOODS

Weaning is associated with the acquisition of an even more diverse microbiota. A longitudinal study involving 330 healthy Danish infants examined faecal microbial composition at 9, 18 and 36 months of age.⁴ Significant

changes in the gut microbiota were observed between 9 and 18 months, with additional less dramatic changes noted between 18 and 36 months. The composition of the microbiota was most influenced by the cessation of breastfeeding and weaning onto solid foods. This resulted in replacement of *Lactobacilli*, *Bifidobacteria* and *Enterobacteriaceae* with a dominance of *Clostridium* spp and *Bacteroides* spp. Adult-like enterotypes were established between 18 and 36 months, with 30% of infants showing a shift in enterotype between 18 and 36 months. These findings suggest that the gut microbiota undergoes significant changes in the first three years of life, is most influenced by weaning onto solids, and that adult-like faecal microbial patterns are established between 18 and 36 months of age. After the age of around three years, the gut microbial composition stabilises and becomes similar to that of adults.

These age-dependent changes in microbial composition appear to be consistent across different populations and lifestyles. Yatsunenکو et al examined maturation of gut microbial communities in healthy infants and children living in geographically and culturally distinct settings.⁵ Included were 531 individuals from 151 families in three geographic locations:

- Guahibo Amerindians residing in two villages – Platanillal and Coromoto – in Venezuela, whose diets comprised predominantly cassava;
- four rural communities in Malawi whose diets were dominated by maize, and in
- urban United States communities in the greater metropolitan areas of St Louis, Philadelphia and Boulder with Westernised diets.

The average UniFrac distances between each child and all unrelated adults from the same country reduced substantially over the first three years of life for all three populations, despite their different geographic origins. Similarly, microbial diversity increased rapidly reaching adult levels by around three years. Therefore, in these populations with diverse cultures and lifestyles, the gut bacterial community evolved along a similar period of time to assume an adult-like configuration by ~3 years of age. Nevertheless, the subsequent faecal microbiota patterns that become established beyond the first years were different in the three populations. The faecal microbiota of US adults was much less diverse than that of the other two populations; and these differences were also evident in children older than three years of age but not in younger subjects. This suggests that the effects of diet and lifestyle become apparent from around three years of age.

THE EFFECT OF LIFESTYLE ON MICROBIOTA COMPOSITION

In the study by Yatsunenکو et al, unsupervised clustering using principal coordinates analysis (PCoA) of UniFrac distance matrices showed that the faecal microbiota of US

populations clustered separately from non-US populations and, within the non-US populations, Malawians were separated from Amerindians. Similarly, Obregon-Tito et al compared the faecal microbial patterns in traditional societies (Matses and Tunapuco) with US urban populations.⁶ Examination of the microbial community structure (beta diversity) among these three populations using PCoA transformation of weighted UniFrac distances showed a clear separation of the traditional and industrialised populations and, among the traditional populations, the Matses formed a separate cluster. The gut microbiomes of traditional societies were composed of larger numbers of phylogenetically diverse taxa, whereas the gut microbiomes of industrialised societies were composed of fewer closely related taxa.⁶ Furthermore, by combining data from several studies, Obregon-Tito et al showed that the microbiomes of individual populations were clustered by lifestyle. For example, the Matses and Hazda hunter–gatherer communities from two separate continents (Africa, South America) had a greater affinity to each other than to other traditional or urban populations; rural agriculturalists from Africa (Tunapaco and Malawi) and Venezuela (Amerindians) had a greater affinity to each other than to other traditional or urban populations; and urban industrial populations from North America and Italy had greatest affinity with each other.⁶ Overall, between ~64% and 85% of individuals had faecal microbial profiles consistent with their subsistence strategy.⁶

The faecal microbiota associated with either traditional or urban–industrial societies could be distinguished by differential expression of three phyla. The microbiomes of traditional populations were enriched for Proteobacteria and Spirochaetes, whereas those of urban–industrial populations were enriched for Actinobacteria. Deeper resolution of the Bacteroidetes phyla at the genus level revealed that traditional lifestyles were associated with higher levels of Prevotella, whereas urban–industrial lifestyles were associated with higher levels of Bacteroides.^{5–7} Interestingly, Spirochaetes have been found in high abundance only among extant human populations with non-Western lifestyles, such as a traditional community in Burkina Faso and a hunter–gatherer community in Tanzania. Spirochaetes may therefore represent a part of the human–ancestral gut microbiome that has been lost through adoption of industrial agriculture and/or other changes associated with the urban–industrial lifestyle. The shift from a high- to low-fibre diet might be an important factor as Spirochaetes are carbohydrate metabolisers that would be selectively supported by a high-fibre diet.

THE STABLE INTESTINAL MICROBIAL COMPOSITION IS DETERMINED BY THE LONG-TERM DIET

The gut microbial composition is sensitive to changes in diet and the stable microbial composition that is established beyond the first years of life is primarily determined by

the long-term diet. Wu et al⁸ examined the effects of the long- and short-term diet on faecal microbial composition in 98 healthy volunteers. Diet information was collected using two questionnaires that queried their recent diet ('Recall') and their habitual long-term diet (food-frequency questionnaire or 'FFQ'). Faecal-microbial enterotype composition was strongly associated with the long-term diet but not the short-term diet. The *Bacteroides* enterotype was associated with meat consumption, representing the typical Western diet with high intake of animal protein and saturated fats. The *Prevotella* enterotype was associated with intake of carbohydrates, reflecting the carbohydrate-based diet typical of farming societies. Self-reported vegetarians had enrichment in the *Prevotella* enterotype (27% *Prevotella* vs 10% *Bacteroides*). In contrast, short-term changes in diet can induce transient and modest shifts in microbial communities, but fail to support long-lasting changes once the modified diet is stopped. The effects of short-term dietary change were examined in a controlled feeding study.⁸ Ten individuals were randomised to receive either a high-fat/low-fibre or a low-fat/high-fibre diet for ten days in hospital. Stool samples were analysed by 454 pyrosequencing and shotgun metagenomics. Changes in microbiome composition were detectable within 24 hours of initiating the controlled feeding, indicating that dietary modification can induce rapid changes in the gut microbiota. However, all subjects started in the *Bacteroides* enterotype (high protein/fat) and none switched stably to the *Prevotella* (carbohydrate) enterotype. The controlled diets were also associated with functional changes (eg in the bacterial secretion system, protein export and lipoic-acid metabolism). These findings indicate that short-term dietary modification has rapid effects on microbiota composition and function, but is not sufficient to switch individuals between enterotype clusters associated with protein/fat and carbohydrate diets and the magnitude of detectable changes is modest.

CUMULATIVE EFFECTS OF DIET ON THE INTESTINAL MICROBIOTA OVER GENERATIONS

One possible explanation for the greater microbial diversity seen in hunter-gatherers and agrarians is the large quantity of dietary fibre they consume relative to Western populations. Dietary fibre that is accessible to microbiota in the distal gut serves as the primary source of carbon and energy for the distal-gut microbiota. A recent study examined the cumulative impact on the intestinal microbiota of a diet low in 'microbiota accessible carbohydrates' (MAC) over several generations.⁹ Germ-free mice were colonised with faecal microbiota from a healthy anonymous donor (an American male living in the San Francisco Bay Area, California, age 36, omnivorous diet) to generate 'humanised mice'. These humanised mice were then fed a diet rich in fibre derived from a variety of plants (high-MAC diet) for six weeks and then randomly divided into two groups. The first group was switched to a low-MAC diet for seven weeks, after which they were

returned to the high-MAC diet for six weeks. The second group (control) was maintained on the high-MAC diet throughout. Subsequent generations of low-MAC mice were produced by mating parent mice during the low-MAC diet phase and weaning the offspring onto a low-MAC diet. Second generation offspring were then mated while they were on the low-MAC diet to produce the third generation of offspring that were in turn weaned onto a low-MAC diet. This sequence was repeated to generate fourth generation low-MAC mice. The faecal microbiota in each generation of mice was examined while they were on a low-MAC diet and again when they were placed on a high-MAC diet (after weaning of their offspring). Consumption of a low-MAC diet for one generation resulted in a loss of taxa that was largely reversible by return to a high-MAC diet. However, after several generations of a continuing low-MAC diet, there was a progressive loss of diversity, which was not recoverable after the reintroduction of dietary MACs. Fourth-generation mice raised on a low-MAC diet had extensive loss of taxa (72% or 150 of 208 high-confidence OTU) and the majority of taxa (141 out of 208) remained undetectable after return to a high-MAC diet. Most of the lost taxa (112 out of the 141) were from the *Bacteroidales* order with an additional loss of 26 taxa from the *Clostridiales*, making the *Clostridiales* the most numerous high-confidence taxon present in the fourth generation. Restoration of the microbiota to its original state required the administration of missing taxa in combination with dietary MAC consumption.⁹ These findings suggest that taxa driven to low abundance when dietary MACs are scarce are inefficiently transferred to the next generation, and are at increased risk of becoming extinct within an isolated population if there is continuing scarcity of dietary MAC. Furthermore, restoration of a disrupted-gut microbiota (dysbiosis) might require a combination of dietary MACs together with replacement of taxa that may no longer be present in the Western microbiota as a result of many generations on a low-MAC diet that is typical of urban-industrial societies.

THE IMPORTANCE OF INTESTINAL MICROBIOTA IN HEALTH AND DISEASE

Studies in mice have shown that the intestinal microbiota plays a critical role in supporting normal immune development and healthy immune homeostasis. Mice raised under germ-free conditions lacking an intestinal microbiota have:

- abnormal development of the gut-associated lymphoid tissues with underdeveloped Peyer's patches and absent germinal centres;
- fewer IgA plasma cells and CD4+ T-cells in the lamina propria;
- fewer intra-epithelial lymphocytes with reduced cytolytic activity, and
- reduced numbers of T-regulatory cells with impaired suppressor function in mesenteric lymph nodes and Peyer's patches.^{10–14}

These developmental abnormalities result in failure of oral-tolerance induction, overexpression of Th2 responses and exaggerated anaphylaxis.^{12,15–17} Remarkably, both the developmental and functional abnormalities observed in germ-free mice can be corrected by reconstituting the microbiota with single or multiple bacteria species, provided that this occurs in the early postnatal period.^{15,17,18}

A role for the intestinal microbiota in human health and disease has also been demonstrated. Alterations in gut microbiota composition have been linked to later development of a range of chronic disorders, in particular the non-communicable diseases associated with a Westernised lifestyle.^{19,20} For example, children who develop eczema have altered microbiota composition preceding the onset of allergic disease, with lower counts of bifidobacteria and enterococci at one month, higher counts of *C difficile* and *E Coli* at three weeks and three months, and increased colonisation with *S aureus* at six months.^{21–23} Furthermore, within the Bifidobacteria genus, reduced colonisation with *B breve* and increased colonisation with *B catenulatum* in the first three months of life were associated with increased risk of developing eczema at 12 months of age.²⁴ Reduced intestinal microbial diversity was also associated with increased risk of later eczema.^{25–27} Hence, the establishment of a healthy microbiota during infancy is important as this provides the foundation for a healthy gut microbiota later in life which in turn can support healthy immune regulation and a reduced risk of disease.

DECLARATION OF CONFLICT OF INTEREST

The author declares no conflict of interest.

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DEFINITIONS:

Pyrosequencing: Conventional DNA sequencing relies on the dideoxy chain termination technique first described by Sanger and colleagues more than two decades ago (Sanger sequencing). Recently, pyrosequencing has emerged as a new sequencing methodology. Advantages include accuracy, flexibility, parallel processing; it also dispenses with the need for labeled primers, labeled nucleotides, and gel-electrophoresis, thus is readily automated.

Metagenomic sequencing (shotgun sequencing): Sequencing of the 16S rRNA locus is the most widely used method for characterizing the microbiota. The 16S rRNA locus is amplified by PCR and sequenced then bioinformatically characterized to determine which microbes are present in the sample and at what relative abundance. However, 16S rRNA sequencing only provides insight into the taxonomic composition of the microbial community - it does not resolve the biological functions associated with these taxa; and furthermore, it can only analyse taxa for which taxonomically informative genetic markers are known - novel microbes are difficult to identify. Shotgun metagenomic sequencing is an alternative approach that extracts and sequences all genes from all organisms present in a given sample. This provides information on both *the compositional and functional properties of the microbiota*. Shotgun metagenomic sequencing also provides a means to study unculturable microorganisms that are otherwise difficult or impossible to analyze.

Operational taxa units (OTU): Refer to clusters of organisms, grouped by DNA sequence similarity of a specific marker gene, typically the 16S rRNA sequences. Operational taxonomic units are defined based on the similarity threshold set by the researcher (usually 97% similarity). This commonly-used method may not necessarily reflect similarity based upon true microbial species phylogeny or ecology.

Alpha diversity: Refers to the number (*richness*) and distribution (*evenness*) of taxa within a single population. The greater the number of taxa and the more evenly they are represented, the higher the alpha diversity.

Beta diversity describes how many taxa are shared between multiple populations, or in other words, how similar the taxa are between populations.

Enterotypes: The term “enterotypes” has been used to classify gut microbiome samples that cluster together in ordination analyses. Three clusters of microorganisms (‘enterotypes’) were first identified when faecal metagenomes of individuals from four countries were examined. The enterotypes were not influenced by

age, gender, body weight, or nation, but appeared to be determined by the long-term diet. The existence of enterotypes was suggested to indicate that intestinal microbiota variation is stratified rather than continuous, and that there are a limited number of well-balanced host-microbial symbiotic states that become established in response to different conditions such as diet. Although three distinct enterotypes were initially described, later studies reduced this to two clusters, one dominated by *Bacteroides* or Clostridiales species found more commonly in Western (American and Western European) subjects and the other dominated by *Prevotella* more often associated with non-Western subjects. More recently, the existence of “enterotypes” has been questioned because additional microbial taxa that can define these communities have not been identified and studies have also failed to confirm the distinct clusters originally proposed in the “enterotypes” hypothesis.

Principal coordinates analysis (PCoA): A means of visualizing the level of similarity or dissimilarity of individual cases in a dataset. Rather than using raw data, PCoA takes a (dis)similarity matrix as input. With PCoA you can visualize individual and/or group differences in a data plot. Cases that lie close together are more similar, cases that lie further apart are less similar.

Unifrac: A method for comparing biological communities. UniFrac measures the phylogenetic distance between sets of taxa; it provides information on the relative relatedness of community members based on phylogenetic distances between observed organisms. Both weighted (quantitative) and unweighted (qualitative) variants of UniFrac are widely used in microbial ecology, where the former accounts for abundance of observed organisms, while the latter only considers their presence or absence. UniFrac can be used to determine whether communities are significantly different, to compare communities using clustering and ordination techniques, and to measure the relative contributions of different factors, such as chemistry and geography.

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