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### **REVIEW ARTICLE**

# Settlers of our inner surface – factors shaping the gut microbiota from birth to toddlerhood

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**One sentence summary:** This review takes an ecological perspective to guide readers through the most important factors controlling the succession and establishment of gut microbiota in infants after birth.

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### ABSTRACT

During the first 3 years of life, the microbial ecosystem within the human gut undergoes a process that is unlike what happens in this ecosystem at any other time of our life. This period in time is considered a highly important developmental window, where the gut microbiota is much less resilient and much more responsive to external and environmental factors than seen in the adult gut. While advanced bioinformatics and clinical correlation studies have received extensive focus within studies of the human microbiome, basic microbial growth physiology has attracted much less attention, although it plays a pivotal role to understand the developing gut microbiota during early life. In this review, we will thus take a microbial ecology perspective on the analysis of factors that influence the temporal development of the infant gut microbiota. Such factors include sources of microbes that seed the intestinal environment, physico-chemical (abiotic) conditions influencing microbial growth and the availability of nutrients needed by the intestinal microbes.

Keywords: infant gut microbiota; abiotic factors; breastfeeding; nutrients; competition

### **INTRODUCTION**

The gut microbiota in adults comprises a large variety of organisms including bacteria, fungi, archaea, protists and viruses. The bacteria vastly outnumber the other groups, and will have our focus in the present review. As most observations about intestinal microbes are based on fecal samples, we use the words 'gut' or 'intestinal' to refer to observations based on feces.

The development of the gut microbiota in an infant undergoes drastic changes: Shortly after birth, only a limited number of living microbes represented by a few species are present in the gut, while a load of up to  $10^{12}$  bacteria per gram and about 150–200 bacterial species can be found later in infancy (Milani *et al.* 2017; Stewart *et al.* 2018).

Reported correlations of the characteristics of the early life microbiota with specific health or disease features in childhood or adulthood include a multitude of conditions, of which many are related to atopy (Fujimura *et al.* 2016; Johnson and DePaolo 2017). However, for most conditions it remains a challenge to establish causal evidence of a microbial influence on later health (Milani *et al.* 2017). Here, we will focus on the external and environmental factors of the gut that affect microbial exposure and

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growth physiology and thereby shape the microbial ecosystem and the temporal succession of bacterial establishment, which we view as a fundament for understanding the role of gut bacteria in host health.

In order for a given organism to establish in a given ecosystem, first, it needs to get there, second, it needs to find an ecological niche in terms of conditions and nutrients, which allows it to proliferate and third, it needs to be able to be competitive within this niche. Unfolding this perspective, we will discuss the sources of microbial seeding to the infant gut, the intestinal physicochemical (abiotic) conditions governing microbial growth and the role of nutrient availability and bacterial cross-feeding and competition for nutritional and topographical niches (Fig. 1). It is important to note that many of these abiotic factors may in themselves be confounders influencing (or reflecting) human health through mechanisms that are independent of the gut microbes. However, this does not make it less important to be aware of them, since they may often be key to understanding observed associations between infant gut microbiota and risk of disease.

#### Sources of microbial seeding

The early-life gut microbiota consists of microbes that are acquired either vertically or horizontally. Studies with captured wild mice of different origins, inbred and co-housed (same room, not co-caged) for up to 11 generations, suggest that transmission of gut bacteria occurs primarily vertically (across generations within mouse origin) and to lesser extent horizontally (across mouse origin along multiple generations; Moeller et al. 2018). The microbes reported to be transmitted vertically were typically strictly anaerobic clostridia and bacteroidia, whereas horizontally transmitted bacteria were usually facultative anaerobic bacilli (Moeller et al. 2018). Thus, taxonomic and phenotypic characteristics of bacteria may affect their transmission mode and it seems that maternal/parental microbiomes are the most pronounced sources of bacteria transmitted to vaginally born offspring. Indeed, in humans, maternal body sites has been reported to contribute with approximately 50% of the bacterial species found in the infant gut throughout the first 4 months of life (Ferretti et al. 2018). Still, horizontally acquired bacteria e.g. from siblings (Laursen et al. 2015, 2017) or unrelated individuals (Korpela et al. 2018), household pets (Azad et al. 2013; Song et al. 2013; Nermes et al. 2015; Levin et al. 2016; Tun et al. 2017), the infant's (complementary) diet (Laursen et al. 2016) and the environment (Shin et al. 2015; Brooks et al. 2017) contribute to the establishment of the complex infant gut microbiota. Further, transmission of bacteria from the infant's own oral microbiota, especially in the neonatal period, has been found to be a significant source of bacteria to the infant gut (Ferretti et al. 2018; Schmidt et al. 2019).

#### Vertically transmitted bacteria

A number of recent papers have strongly suggested that extensive vertical transmission of microbes to the infant gut occurs (Nayfach et al. 2016; Asnicar et al. 2017; Chu et al. 2017; Duranti et al. 2017; Ferretti et al. 2018; Korpela et al. 2018; Yassour et al. 2018; Maqsood et al. 2019). The sources of microbes for the infant gut include maternal vaginal, oral, gut, skin and breast milk microbial communities and data indicate that these together contribute with the majority of species that are establishing in the infant gut (Chu et al. 2017; Pannaraj et al. 2017; Ferretti et al. 2018; Maqsood et al. 2019). Especially the maternal gut (Ferretti et al. 2018; Maqsood et al. 2019) and breast milk (Pannaraj et al. 2017) are influential sources, whereas seeding from maternal oral, vaginal and skin microbiota seem to be mainly significant in the neonatal phase (Chu et al. 2017; Ferretti et al. 2018). In that regard, the heterogeneity often observed in the neonatal gut microbial communities (Bäckhed et al. 2015; Chu et al. 2017) seem to be linked to an initial individual pattern of similarity to the vaginal, oral or skin microbiota of the mother, probably ascribed to transient colonization by microbes transmitted from these sources (Ferretti et al. 2018).

#### Vaginal microbes

The vaginal microbiota is remarkably stable over the course of pregnancy (DiGiulio et al. 2015), and has been grouped into five distinct community types, either dominated by Lactobacillus crispatus (type I), Lactobacillus gasseri (type II), Lactobacillus iners (III) or Lactobacillus jensenii (type V) or a type IV characterized by high diversity and a mixture of Prevotella, Sneathia, Gardenella, Atopobium and Megasphaera spp. (Ravel et al. 2011; Gajer et al. 2012; DiGiulio et al. 2015). Interestingly, the relative abundance of bacterial species in the vagina prior to birth does not seem to be coupled to the chance of vertical transmission (Rasmussen et al. 2020). Nevertheless, vaginally delivered newborns initially harbor a gut microbiota dominated by Lactobacilli, Prevotella and Sneathia spp., resembling the corresponding mothers vaginal microbiota (Dominguez-Bello et al. 2010), while, C-section delivered newborns are initially colonized by typical (maternal) skin microbes such as Propionibacterium, Staphylococcus and Corynebacterium (Dominguez-Bello et al. 2010). All of the four major vaginal lactobacilli; L. iners, L. crispatus, L. gasseri and L. jensenii but also Gardenella vaginalis and Atopobium vaginae, have been found to be transmitted but colonize only transiently in the infant (Ferretti et al. 2018). Within the first day of life, about 16.3% of the neonatal gut microbiota is composed of maternal vaginal species, but this rapidly declines within the first week (Ferretti et al. 2018), indicating that the vaginal microbes persist only transiently in the infant gut environment. Interestingly, multiple studies have shown a depletion of Bifidobacterium and Bacteroides species in the gut of C-section compared to vaginally born infants, spanning the first year of life (Bäckhed et al. 2015; Stewart et al. 2018). However, these are not common vaginal microbes (Mitchell et al. 2020), but can be restored by oral transplantation with bacteria from the maternal gut (Korpela et al. 2020), which unlike the vaginal microbes are adapted for the intestinal environment. It is therefore likely that the lack of exposure to the maternal rectal environment is the main cause of the observed lower levels of these taxa in infants born by C-section.

#### Skin and oral microbes from the mother

The human skin is predominantly colonized by Staphylococcus, Corynebacterium and Propionibacterium (Byrd, Belkaid and Segre 2018), and the areolar/nipple skin is often additionally colonized by Streptococcus (Drell et al. 2017; Pannaraj et al. 2017; note that Propionibacterium is now designated Cutibacterium (Scholz and Kilian 2016)). However, for clarity, we will comply with the genus names used in the cited literature). Species found to be shared between maternal skin and infant gut in the neonatal period include Streptococcus spp., Staphylococcus epidermidis and Propionibacterium acne (Ferretti et al. 2018). While the breast skin microbiota contributes with 5–6.8% of the bacterial species found in the infant gut during the first days of life, this declines to 3.2% after 1 week, and after 4 months bacteria originating from



Figure 1. Influence of selected factors on the developing microbiota. In the first period after birth, the bacterial load in the intestine is low, meaning that many ecological niches are free for the seeded bacteria to explore. The high inter-individual (beta) diversity at this stage is probably reflecting a high influence from the many different sources of seeding to the gut at this stage. When the complexity of diet increases, so does the complexity (alpha diversity) of the bacterial community, and eventually the impact of original seeding sources is no longer detectable. The increasing hostility of the gut environment, characterized by reduced oxygen availability and reduced pH and later by scarceness bacterial of nutrients subject to competition, selects for a community that is optimized for coping with this, thereby reducing the beta diversity, and increasing the impact of competition. (The figure was created in BioRender).

maternal skin are no longer detectable in the infant gut (Ferretti et al. 2018).

Abundant members of the oral microbiota include species within Streptococcus, Rothia, Gemella, Prevotella, Neisseria, Veillonella, Haemophilus and Granulicatella (Yamashita and Takeshita 2017; Williams et al. 2019). Maternal tongue dorsum bacterial species are also found in infant gut throughout the first 4 months of life, accounting for up to 16% of the infant gut microbiota (Ferretti et al. 2018). Here, Streptococcus parasanguinis, Rothia mucilaginosa, Prevotella melaninogenica, Haemophilus parainfluenzae and Veillonella parvula are some of the most commonly species found in both ecosystems (Ferretti et al. 2018). However, it remains a challenge to distinguish strains originating from the mother's oral environment from the infants own oral bacteria. Thus, the apparent sharing of species between maternal oral and infant gut ecosystems may result from seeding of infant's own oral strains into the gut (Ferretti et al. 2018).

#### Fecal microbes from the mother

The adult gut microbiota is dominated by Clostridiales and Bacteroidales, and lower proportions of genera within the Enterobacteriales and Bifidobacteriales (Arumugam et al. 2011). During pregnancy the microbiota undergoes significant changes including a decrease in alpha diversity and relative increases in *Enterobacteriaceae*, *Streptococcus* and *Bifidobacterium* species (Koren et al. 2012). Interestingly, only a limited group of maternal genera appear to be transmitted to the infant, mostly confined to *Bifidobacterium*, *Bacteroides*, *Escherichia* and *Streptococcus* spp. (Bäckhed et al. 2015; Ferretti et al. 2018; Maqsood et al. 2019). Nevertheless, at the species level, 20–50% of the bacteria present in the infant gut during the first 4 months of life are shared

with the maternal gut microbiota (Ferretti et al. 2018). While sharing of bacteria at species level within mother-infant pairs increase with infant age, sharing at strain level tends to decrease (Nayfach et al. 2016), suggesting that strains acquired from the mother gradually get replaced by horizontally acquired strains of the same species. However, within some species, vertically transmitted strains are more likely to persist and stably colonize the infant gut, than horizontally acquired strains (Nayfach et al. 2016; Ferretti et al. 2018; Korpela et al. 2018). These include strains of Bacteroides (Bacteroides vulgatus, B. dorei, B. uniformis), Parabacteroides, Bifidobacterium (Bifidobacterium longum, B. breve, B. adolescentis, B. bifidum) and Escherichia (Escherichia coli). Noteworthy, these strains are typically highly abundant colonizers of the infant gut (Makino et al. 2011; Milani et al. 2015; Nayfach et al. 2016; Asnicar et al. 2017; Ferretti et al. 2018; Korpela et al. 2018; Yassour et al. 2018). Thus, although only a minor selection of the maternal microbes are transmitted, they constitute a significant amount of the total bacterial population in the infant gut.

#### Microbes from breast milk

Human breast milk contains on average 10<sup>3</sup> (range 10<sup>1</sup>-10<sup>5</sup>) bacteria per mL (Jost et al. 2015). As breastfed infants consume 600-1200 mL milk per day (Larsson et al. 2018), this results in a significant exposure of up to 10<sup>7</sup> bacteria daily. Microbes found in breast milk primarily originate from maternal skin, infant oral cavity and from the environment (Moossavi and Azad 2019; Williams et al. 2019; Kordy et al. 2020). Although still subject to debate, internal routes of transfer including enteromammary (Rodriguez 2014) and oro-mammary (Moossavi and Azad 2019) pathways have been speculated to affect the occurrence of microbes in breastmilk. Prevalent and abundant bacteria found in breast milk include skin-associated bacteria such as Staphylococcus, Propionibacterium and Corynebacterium spp. (Jost et al. 2013; Fitzstevens et al. 2017; Treven et al. 2019), oral cavityassociated Streptococcus, Veillonella, Rothia and Gemella (Jost et al. 2015; Treven et al. 2019; Williams et al. 2019), but also bacteria likely to be of environmental origin, such as Acinetobacter and Pseudomonas spp. (Lundgren et al. 2019; Moossavi et al. 2019). Furthermore gut-associated species such as Lactobacillus, Enterococcus and Bifidobacterium spp. are commonly isolated from breast milk, although in low abundance (Jost et al. 2013; Jost et al. 2015; Kozak et al. 2015). During the first month of life, 28% of the infant gut microbial population, at species level, is shared with the maternal breast milk microbiota (Pannaraj et al. 2017). Species found to be shared within mother-infant dyads include Staphylococcus, Streptococcus, Veillonella, Rothia, Enterococcus, Lactobacillus and Bifidobacterium spp. (Makino et al. 2011; Jost et al. 2013; Kozak et al. 2015; Laursen et al. 2017; Pannaraj et al. 2017), and isolation of identical strains within these genera has been demonstrated (Martin et al. 2003; Martín et al. 2012; Kozak et al. 2015). Transmission of Bifidobacterium spp. via breast milk has additionally convincingly been shown by combining gene marker based amplicon sequencing, metagenomic analysis and strain isolation in mother infant-pairs (Makino et al. 2011; Milani et al. 2015; Duranti et al. 2017). Importantly, B. longum and B. breve strains detected in both maternal breast milk and initial infant feces persistently colonize the infant gut for up to 6 months (Makino et al. 2011; Milani et al. 2015). Transfer of bacteria via breast milk, especially lactic acid bacteria and bifidobacteria, thus constitutes an important mechanism by which infants acquire bacteria that colonize their gastrointestinal tract.

#### Horizontally transmitted bacteria

In addition to microbes acquired from the maternal sources described above, the infant is exposed to a variety of other microbes from the environment including siblings, pets, surfaces in the home and in the hospital and from dietary sources. Exposure to new microbes thus increases with increasing age of the infant, however the influence of such exposure on the composition of the gut microbiota decreases as the child grows older and available ecological niches in the gut get occupied (Fig. 1).

# Microbes from siblings, pets and other environmental sources

The presence of siblings clearly impacts the composition of the infant gut microbiota (Laursen *et al.* 2015; Martin *et al.* 2016; Stewart *et al.* 2018; Galazzo *et al.* 2020), and the strongest influence is observed during mid-late infancy and early toddlerhood, i.e. between 6 and 18 months of age (Stewart *et al.* 2018). Microbial richness in toddlers increases with increasing numbers of older siblings (Laursen *et al.* 2015).

An important species, Faecalibacterium prausnitzii, which is considered a marker of gut health (Miquel et al. 2014), is very prevalent and abundant in the adult gut but virtually nondetectable in breastfed infants before age 4–6 months. The increase in abundance and prevalence of this species seems to be accelerated by the presence of older siblings (Laursen et al. 2017). Also, the Bifidobacterium catenulatum group shows a faster increase in prevalence during the first 6 months of life in infants with older siblings as compared to first-borns (Martin et al. 2016).

Several reports demonstrate that also pets in the household represent a source of microbial seeding to the infant gut (Azad et al. 2013; Song et al. 2013; Nermes et al. 2015; Tun et al. 2017). This builds on correlations between household pets and microbial diversity (Azad et al. 2013), but also on evidence of increases in specific bacterial taxa in children exposed to pets (Nermes et al. 2015; Tun et al. 2017), or of the pet serving as a 'vehicle' for transmission of microbes between humans in the household (Song et al. 2013). An example is the animal-derived Bifidobacterium pseudolongum, which is more prevalent in infants who have been exposed to pets, than in controls (Nermes et al. 2015).

Finally, although many measures are taken to protect newborns from nosocomial infections, the hospital environment encountered by the infant immediately after birth have also been suggested to represent a source of microbial seeding (Shin et al. 2015). However, solid proof of transmission of specific strains to the infant gut requires advanced sequence-based methods in order to exclude that given measures of overlap are indeed reflecting occurrence of the same clones, and do not merely reflect parallel occurrence of different clones of the same species in the gut and surroundings, respectively. Indeed, identical strains assembled from metagenomes of hospitalized preterm infant feces and the corresponding hospital room environment has been demonstrated (Brooks et al. 2017). These include strains of opportunistic pathogenic species such as Klebsiella pneumoniae, Staphylococcus epidermidis, Enterococcus faecalis and Pseudomonas aeruginosa. While these are important colonizers of the hospitalized preterm infant (Gasparrini et al. 2019), they are probably less important sources for the term born neonate with only short hospitalization. In this context, it should be noted that the gut microbiota of preterm infants differs from that of healthy infants in many other ways, which have been the topic of a number of important recent studies (Korpela et al. 2018; Gasparrini et al. 2019; Henderickx et al. 2019, Tauchi et al. 2019), but is not within our scope here.

#### Microbes ingested with diet

A number of formula and dietary products for infants are fortified with lactic acid bacteria (probiotics) intended to beneficially influence gut health (Skórka *et al.* 2017). However, typically these strains do not establish in the infant gut and can only be detected in feces during and shortly after the period of repeated ingestion (Mah *et al.* 2007; Derrien and van Hylckama Vlieg 2015). Once the child becomes habituated to foods that have not been boiled or otherwise sterilized, the microbes residing on such foods will evidently end up in the intestinal tract. It is beyond doubt that introduction of complementary and family food leads to increased diversity of the gut microbiota (Laursen *et al.* 2017), however, as reviewed below, the effect of introducing a more complex selection of microbial nutrients is probably a stronger driver of this development than the seeding of new microbes via food.

#### **ABIOTIC CONDITIONS**

All the above mentioned sources, from which seeding occurs, have in common that in order for the microbes to be transmitted and establish in the gut, they have to be able to survive (i) in the source, (ii) during the transmission and (iii) in the gut environment. This means that although the gut environment is primarily anaerobic, the bacteria that grow there must typically have been able to survive exposure to oxygen during seeding, and are thus either facultative anaerobes (Enterobacteriales and Lactobacillales), anaerobes capable of surviving exposure to oxygen (Bifidobacteriales and Bacteroidales) or obligate but spore-forming anaerobes (some Clostridiales such as Clostridiaceae and Lachnospiraceae). An example of a curious exception to this is the strict anaerobic, non-spore forming Ruminococcaceae species F. prautznitzii, which, as described above, colonizes the infant gut quite late and its establishment in the gut seems to be accelerated by presence of siblings (Laursen et al. 2017). It may be speculated that extensive exposure and close contact between individuals is necessary for the establishment of this organism. Interestingly, the presence of antioxidants such as cysteine and riboflavin in the local environment increases survival of F. prautznitzii during oxygen exposure (Khan et al. 2012; Khan, Van Dijl and Harmsen 2014), suggesting that also the abiotic conditions in the environmental compartments may play a role for transmission.

After seeding (arrival of microorganisms into the gut) has taken place, the successional development of the intestinal microbiota during very early life is closely linked to changing abiotic conditions in this environment. This interaction is bidirectional since abiotic conditions, such as oxygen levels and pH, create distinct niches in which specific bacterial physiology types thrive and vice-versa that specific consortia of bacterial strains may alter these and other abiotic conditions over time. In addition to the abiotic factors, the immune system of the infant, as well as multiple factors present in breastmilk may play a role in shaping the infant microbiome. However, this is not within the scope of the current review.

#### Oxygen

Several different gradients of partial oxygen pressure  $(pO_2)$  are present in the adult gastro-intestinal tract. These include a general longitudinal decrease from the proximal to the distal part (He *et al.* 1999), a very steep radial gradient from the intestinal submucosa to the lumen (Albenberg et al. 2014), which is most pronounced in the distal intestinal tract (Friedman et al. 2018) and lastly a gradient along the crypt–villus axis in the small intestine due to counter-current blood flow (Hallbäck et al. 1978). As a consequence of the radial  $pO_2$  gradient, it was demonstrated in humans that a much lower ratio of obligate anaerobe to oxygen tolerant bacteria exists in rectal mucosal biopsies than in fecal samples (Albenberg et al. 2014).

It is frequently reported that the neonatal intestine is relatively more oxygenated than later in life, thus initially only supporting growth of facultative anaerobic bacterial species, such as members of the Enterobacteriaceae family, and not obligate anaerobes (Chong, Bloomfield and O'Sullivan 2018). In line with this, it has been described that after the first week of life, the number and relative abundance of strictly anaerobic species found in a group of 25 infants increased with time (Ferretti et al. 2018). The general belief is that the facultative anaerobes gradually reduce the oxygen levels and thus create the anaerobic environment of the developed gut (Bäckhed et al. 2015). A recent study however challenged this notion, or at least narrow the timespan in which the intestinal lumen is oxygenated in early life (Bittinger et al. 2020). In a cohort of 88 healthy term infants sampled from 2 min to 176 h after birth it was indeed found that the facultative anaerobe E. coli was the most prevalent bacterium during the first 16 h of life. Several anaerobe species were however also abundant in some individuals, including B. vulgatus, B. dorei and Subdoligranulum spp. in this very early time-period, with quite some inter-individual heterogeneity at the species and strain level. Importantly, a mostly anaerobic environment was considered likely due to observed changes in serine, threonine and succinate levels in meconium samples consistent with anaerobic growth of E. coli. These findings indicate that the neonatal gut may be mostly anaerobic shortly after birth. The question thus remains whether it is possible that the neonatal intestinal lumen becomes anaerobic even in the absence of bacterial oxygen consumption, in spite of influx of oxygen from the endothelial cells.

The contribution of living bacteria versus purely chemical reactions to generate anaerobic conditions in the gut lumen has elegantly been explored recently in a germ-free mouse model (Friedman *et al.* 2018), revealing that the partial pressure of oxygen (pO<sub>2</sub>) in the gut lumen is nearly identical in conventional and germ-free (GF) mice. Large longitudinal variations in  $pO_2$  levels from stomach to cecum were found, however no difference was observed between germ-free and conventional animals. The highest levels of oxygen were recorded in the duodenum, which was attributed to the very large surface area allowing higher rates of diffusion of oxygen into the lumen as well as the low biomass causing limited microbial and chemical reduction of oxygen (Friedman *et al.* 2018).

Chemical consumption of oxygen via lipid oxidation was demonstrated as an effective mechanism to reduce  $pO_2$  levels in cecal content from GF animals albeit at much lower rates than observed using cecal content from conventional animals. In addition, the rate of oxygen consumption in GF cecal content was increased to a level similar to the conventional when spiked with facultative anaerobe *E. coli*, but not with the anaerobic but aerotolerant *Clostridium sordelli* (Tally *et al.* 1975; Friedman *et al.* 2018). These findings support the likely contribution of non-bacterial guided reduction in oxygen in the neonate gut, but also indicate that oxygen removal depends on the type of bacteria. Oppositely, increasing the luminal oxygenation by hyperbaric oxygen therapy in mice led to a decreased prevalence of catalase negative, obligate anaerobic *Anaerostipes* spp. (Albenberg *et al.* 2014).

In conclusion, although the neonatal colonic lumen probably rapidly becomes anaerobic, topographical differences in  $pO_2$  are likely to exist and to affect gut microbiota composition in different compartments of the gut.

#### Intestinal pH and transit time

Intestinal oxygen tension is closely connected with other physico-chemical factors including pH and redox potential. While the impact of pH on the differences between the microbial populations present in different sections of the GI tract is irrefutable, little is known about the impact of change in pH on the infant's intestinal microbial population.

The pH in the stomach of newborns at delivery is neutral (Avery, Randolph and Weaver 1966), but fasting gastric pH in neonates rapidly drops to an average around 4.6 at age 2–6 days, and down to 2.6, which is similar to adults, already at 7-15 days of postnatal age (Sondheimer, Clark and Gervaise 1985). Studies assessing the pH in the gut of neonates are scarce, but one study, dated 1952, suggests that the luminal pH of the small intestine is lower in infants fed with cow's milk than in breastfed infants (Barbero et al. 1952). The same study reveals that the pattern of small intestinal pH observed in breastfed infant aged 2 weeks to 3 months, with duodenal, jejunal and ileal pH around 6.4  $\pm$  0.5, 6.6  $\pm$  0.4 and 6.9  $\pm$  0.5, is almost the same as that of older children and adults. Development of novel methodologies for non-invasive measurements of abiotic factors in the intestine is needed to obtain better knowledge about the processes occurring in the infant gastrointestinal tract. Such knowledge is highly relevant not only to understand the impact on intestinal microbes, but also to assess questions about solubility, absorption and metabolism of drugs and nutrients (Vertzoni et al. 2019). In vitro models of infant colonic microbial activity thus suggests that lactate metabolism is strongly modulated by abiotic factors such as pH and retention time (Pham et al. 2019).

In adults, colonic pH plays a key role in determining the outcome of microbial interspecies competition and response to dietary fibers (Chung et al. 2016) as well as in amino acid metabolism (Smith and Macfarlane 1996). In example, whereas propionate-producing Bacteroides spp. dominate fecal microbial communities cultured at pH > 6.0, butyrate-producing Eubacterium/Roseburia or Faecalibacterium spp. dominate when pH is lowered to 5.5 (Walker et al. 2005; Chung et al. 2016), but the species that are dominating depends on the diet (e.g. type of dietary fiber and peptides). Opposite the small intestinal luminal pH (Friedman et al. 2018), the fecal pH is significantly lower in breastfed compared to formula fed infants (Ogawa et al. 1992; Indrio et al. 2007). The low fecal pH in breastfed infants is maintained by human milk oligosaccharide (HMO) degrading, acetate-producing Bifidobacterium species (Matsuki et al. 2016; Henrick et al. 2018), and this is likely to prevent or reduce colonization of non-acid tolerant bacteria. Formula fed infants are not dominated by Bifidobacterium spp. (Fallani et al. 2010), and have a much more diverse microbiota (Stewart et al. 2018), possibly due to the more permissive pH conditions in the colonic environment. Colonic pH is additionally known to be affected by intestinal transit time (Lewis and Heaton 1997), and recently, we have reported that in adults, transit time has a major impact on microbiome diversity and composition as well as on microbial metabolism (Roager et al. 2016). Although it remains to be investigated, we speculate that this is the case also in the infant gut.

# Effects of antibiotic treatment on abiotic conditions in the infant gut

It is well known from animal studies that oral intake of antibiotics courses acute and profound changes in bacterial community composition, which is determined by the dose and class of antibiotic (Tulstrup *et al.* 2015). Lately, the effects of antibiotics on microbial communities in early life and health risks associated with these changes have been investigated in depth and several comprehensive reviews are available (Cox and Blaser 2015; Schulfer and Blaser 2015). Importantly, antibiotic-induced early life disturbances have been associated with increased risk of developing asthma (Aversa *et al.* 2020) and may also have lasting metabolic consequences (Cox *et al.* 2014). Here, we focus on the putative effects of antibiotic use on the abiotic conditions in the intestine.

In the adult colon, there is a steep gradient from the oxygenated endothelial cells into the lumen (Espey 2013). Colonocytes oxidize microbially-produced butyrate as their preferred carbon source, thereby consuming oxygen and generating ATP (Rivera-Chávez, Lopez and Bäumler 2017). Orally administrated antibiotics are known to cause an increase in intestinal oxygen levels, which may result in an expansion of facultative anaerobic bacteria within the Enterobacteriaceae family (Rivera-Chávez et al. 2016). This antibiotic-induced increase in oxygen is partly explained by lack of butyrate production by Clostridia (Rivera-Chávez, Lopez and Bäumler 2017). Antibiotics-induced blooms in Enterobacteriaceae are however also commonly observed during early infancy (Tanaka et al. 2009; Fouhy et al. 2012), although butyrate producing bacteria are only present at very low levels at this age (Appert et al. 2020). This might be explained by induction of nitrate production in the gut. It has thus been shown in mice that streptomycin treatment induces gut mucosal nos2 expression, which increases luminal concentrations of nitrate that functions as an alternative electron acceptor for E. coli and promotes blooming of this species (Spees et al. 2013).

An increase in the oxygen/nitrate levels in the intestine would also manifest as an increase in redox potential, which defines the overall environmental capacity for reducing chemical reactions and is thus a key metric to describe the state of the microbial community (Reese *et al.* 2018). It has recently been suggested that availability of electron acceptors (redox potential) is an important factor driving the structure of bacterial communities, and *Enterobacteriaceae* levels correlate positively with redox potential (Reese *et al.* 2018). An increase in the redox potential, characterized by a higher overall oxidant to reductant ratio, may thus result in opportunistic pathogens taking advantage of new respiratory pathways and is additionally known to increase oxidative stress due to generation of free radicals and ROS (Lushchak 2014).

In order to understand and predict the effects of antibiotic treatment in the infant gut, it is therefore important not only to take into account the effect of a given antibiotic on the targetand non-target bacteria, but also to consider both the direct effects on host gastrointestinal cells and the consequences of removal of non-target bacteria for the abiotic intestinal environment, which to a large degree shapes the microbiota.

# NUTRIENT AVAILABILITY, COMPETITION AND CROSS FEEDING

The intestinal environment is characterized by a continuous turnover of the bacterial population through proliferation and fecal excretion, combined with a continuous supply of nutrients. With respect to these features, the intestine is thus comparable to a chemostat or continuous flow culture. This was extensively investigated and discussed in a series of papers already in 1983 (Freter *et al.* 1983a,b). An important conclusion derived from this perspective is that the number of bacteria that can stably coexist in a chemostat, is determined by the number of different available nutrients. Additionally, in a flow-culture, the populations of bacteria are controlled by one or a few nutritional substrates which a given strain can utilize most efficiently under the prevailing abiotic conditions (Freter *et al.* 1983a).

Translated to the intestinal microbial ecosystem, this means that an increase in the complexity of bacterial nutrients, which in the gut originate from ingested diet as well as from mucosal turnover, will drive an increase in the diversity of the bacterial community. In line with this, it was recently demonstrated that dietary diversity scores were positively correlated with microbial diversity in infant stool samples (109). Also in a large adult population (N > 1500 samples), it was found that a higher number of different types of vegetables in the diet was associated with a higher alpha diversity of the fecal bacterial population (McDonald et al. 2018). Furthermore, it has been elegantly demonstrated in mice, that the abundance of a given strain can be regulated by changing the concentration of a substrate exclusively accessible by this strain (Shepherd et al. 2018). These relations between dietary complexity, nutrient utilization capacity and shaping of a bacterial community become very clear from studies of the development of the infant gut microbiota, as reviewed in the sections below.

#### Breastfeeding

After lactose and fat, HMOs constitute the third most abundant solid component of human breastmilk (Chen 2015). While lactose and fat can be digested by the infant's digestive enzymes, and thus to a lesser degree reach the intestinal bacterial communities, the HMOs are indigestible to the human host, and thus available to the gut bacteria in very high amounts. A multitude of studies show that the gut of breastfed infants (as opposed to formula-fed infants) is dominated by the specific bacterial species, which are able to digest HMOs (Stewart et al. 2018). These include specific species of Bifidobacterium such as B. bifidum, B. breve, B. pseudocatenulatum, B. kashiwanohense, B. longum ssp. longum and B. longum ssp. infantis (Sakanaka et al. 2019; Laursen et al. 2020). For some of these species, it is not only the production of specific enzymes capable of HMO degradation, which makes them highly competitive on this substrate, but also their expression of membrane transporters with high affinity for the different types of oligosaccharides present in breastmilk (Sakanaka et al. 2019). Thus, while B. bifidum employs extracellular enzymes (fucosidases, sialidases and lacto-N-biosidases) to degrade HMOs and transport only the released di- and monosaccharides into the cytoplasm, B. longum subsp. infantis internalizes the intact HMO structures via highly specific membrane transporters and subsequently degrade the HMOs intracellularly (Sakanaka et al. 2019). In addition to the specialized infant bifidobacteria, also species of Bacteroides have been reported to be able to degrade HMOs by use of pathways developed for mucus-utilization (Marcobal et al. 2011), as discussed below. It was moreover recently demonstrated that Roseburia and Eubacterium species utilize selected HMOs as well as HMO and mucin degradants (Pichler et al. 2020). Combined with the ability of these species to utilize plant derived carbohydrates such as xylans and  $\beta$ -mannans (Mirande et al. 2010; Leth et al. 2018; La Rosa et al. 2019), this provides a plausible explanation for their

appearance in the infant gut during weaning (Fallani et al. 2011). Nevertheless, the fact that HMOs are highly selective and constitute a considerable amount of the bacterial nutrients in breastfed infants is underlined by the observation that cessation of breastfeeding has a larger impact on microbiota development than introduction of solid foods (Laursen et al. 2017; Stewart et al. 2018).

#### Bacterial nutrients in infant and child food

In many countries, iron fortification of infant formula or iron supplementation given as droplets for infants are recommended in order to prevent iron deficiency. Iron availability may well be a growth-limiting factor for specific bacteria inhabiting the infant intestine (Lönnerdal 2017), and iron supplementation may thus have an impact on the developing microbiota. However, as recently reviewed by others (Finlayson-Trick *et al.* 2020), the currently available reports about effects of iron supplementation on the intestinal microbial composition in infants are not in consensus.

Longitudinal observational studies have identified associations between toddler diet and gut microbiota (Matsuyama et al. 2019). As the child starts to consume more and more different types of food items, the gradual transition to 'adult' food, and an increased intake of protein as well as fibre is associated with a gradual increase of intestinal microbial diversity and of the abundance of Lachnospiraceae (Laursen et al. 2016). Given that the accessibility of amino acids is not a limiting factor for gut bacteria (Nielsen et al. 2018), the bacterial carbohydrate sources (fibres, polysaccharides), are likely to be major drivers not only of diversity, but also of the composition of the bacterial community, since they are selective for the specific bacteria that have the capacity to digest them (Flint et al. 2012). Bacterial phyla that are highly specialized in carbohydrate utilization, as revealed by the percentage of genes on their genome dedicated to this task, are the Bacteriodetes and the Actinobacteria (Sela and Mills 2010). Noteworthy, of these two phyla, the Actinobacteria have dedicated a larger part of their genome also to transport of oligo- and polysaccharides into the cytoplasm. The genera Bifidobacterium and Bacteroides within these phyla, along with the Roseburia-Eubacterium group, all contain species that can use both HMOs and complex plant polysaccharides as carbohydrate sources, and consistently these taxa are dominating in the transition phase between breastfeeding and family diet (Stewart et al. 2018). Multiple reports from in vitro studies (Holck et al. 2011), animal studies (Shepherd et al. 2018) and human trials (Hansen et al. 2018) reveal that the specific features of the dietary polysaccharides (nature of the monomers, binding structures, branching) are selectively promoting specific bacterial species and subspecies. The composition of dietary polysaccharides are thus likely to shape the development of the child's microbiota during transition from breastfeeding and baby food to other types of solid complementary feeding.

#### Host mucins as bacterial nutrients

The complexity of the nutritional landscape encountered by gut bacteria is increased by the fact that the gut contains several sub-systems, offering different types of niches as the encountered conditions are different for bacteria residing in the mucus layer, in the lumen, in the small intestine and in the large intestine (Pereira and Berry 2017). Although interconnected, these spatial niches govern competitive advantages in distinct directions (Nevola, Laux and Cohen 1987; Poulsen *et al.* 1995). In humans, as faecal samples are often the object of study, such niche-specific competitive interactions are typically not possible to measure, and knowledge about their impact in development of the bacterial community is scarce.

The intestinal mucus is composed of a firmly adherent layer, close to the epithelial cells, which typically does not contain many bacteria, and a loosely adherent layer, which is extensively colonized by a variety of microbes (Atuma et al. 2001). The microbiota of the mucus layer in adults is known to differ from that found in fecal samples (Zoetendal et al. 2002). In 2004, a new species, Akkermansia muciniphila, was described, which specializes in utilizing intestinal mucin as the only carbon- and nitrogen source and grew rather poorly on alternative sugars (Derrien et al. 2004). A. muciniphila has since then been extensively studied, and it is found primarily in the human colon, but also in breastmilk (Geerlings et al. 2018). It is found only in low levels in the infant intestine, but during the first years of life, its abundance reaches that found in adults (Collado et al. 2007), suggesting that the development of the intestinal environment supports the colonization of this bacterium (Geerlings et al. 2018).

Not only Akkermansia, but also gut bacteria belonging to the genera Bacteroides (e.g. Bacteroides thetaiotaomicron and Bacteroides fragilis), Ruminococcus (e.g. Ruminococcus gnavus) and Bifidobacterium (e.g. B. bifidum) are able to degrade glycans from intestinal mucins, although the latter only with moderate efficiency (Tailford et al. 2015). Unlike A. muciniphila, these species abundantly colonize the infant gut early and are capable of utilization of HMOs from breast milk and dietary complex polysaccharides as well as mucins, and thus contain the metabolic flexibility to also become very robust and consistent members of the adult microbiota. Particularly, B. thetaiotaomicron is well studied with respect to its ability to switch between the complex carbohydrate sources offered by diet and host mucins, respectively (Sonnenburg et al. 2005), and is a highly abundant and prevalent member of the adult gut microbiota.

#### **Bacterial cross feeding**

Trophic interactions occur between members of the infant gut microbiota. A well-studied example is interspecies crossfeeding within the genus *Bifidobacterium*, in which different strains can display an 'altruistic' as well as a 'selfish' type of HMO metabolism as described in the following.

By employing extracellular fucosidases and sialidases when grown on HMOs or mucins, B. bifidum can liberate fucose and sialic acid, which B. breve can internalize and metabolize (Egan et al. 2014a,b; Centanni et al. 2019). A similar growth enhancing effect of B. bifidum on B. longum subs. longum has been observed when co-cultured in presence of HMOs (Gotoh et al. 2018). Indeed, in vitro experiments have demonstrated an increased abundance of other Bifidobacterium species when human fecal cultures incubated with HMOs are spiked with B. bifidum, underlining the 'altruistic' activity of this species (Gotoh et al. 2018).

Also some isolates of B. kashiwanohense express fucosidases that liberates fucose in the culture medium when grown on the HMOS 2'FL (2'fucosyllactose) or 3'FL(3'fucosyllactose) (Bunesova, Lacroix and Schwab 2016; James et al. 2019), and may cross feed to fucose-utilizers such as B. breve (Schwab et al. 2017).

Infant isolates of B. pseudocatenulatum grow on HMOs (Matsuki et al. 2016), and HMO-degrading B. pseudocatenulatum strains have been shown to support growth of a non-HMO utilizing B. longum subsp. longum strain isolated from the same infant (Lawson et al. 2020). The opposite may also occur, since when strains isolated from another infant were observed, a HMOdegrading B. longum subsp. longum strain supported growth of non-HMO utilizing B. pseudocatenulatum strains. Fucose, galactose, acetate and N-acetylglucosamine were identified as key by-products of bifidobacterial degradation/metabolism of HMOs that mediate interspecies cross-feeding (Lawson et al. 2020). Thus, cross feeding between Bifidobacterium species is likely to explain the co-occurrence of different Bifidobacterium species commonly observed in the infant gut (Turroni et al. 2012, 2018).

In contrast to the 'altruistic' behavior displayed by *B. bifidum* and other specific bifidobacterial strains, *B. longum* subsp. *infan*tis strains often display a 'selfish' HMO metabolism, internalising the intact HMO structures prior to degradation, and thereby leaving no HMO remnants for others to consume (Sakanaka *et al.* 2019; Lawson *et al.* 2020). Consequently, the microbiota if breastfed infants colonized with efficient HMO-utilizing strains of *B. longum* subsp. *Infan*tis is often completely dominated by this strain, (Laursen *et al.* 2020).

Growing on HMO or mucins in the infant intestine, Bifidobacterium species can also cross feed with strains belonging to other genera, due to their release of simple sugar constituents such as lactose, galactose and N-acetyl glucosamine, and products of bifidobacterial metabolism such as acetate, lactate and 1,2-propanediol, which support growth e.g. of Eubacterium hallii (Schwab et al. 2017; Bunesova, Lacroix and Schwab 2018).

This results in the formation of butyrate and propionate, which increase in abundance during complementary feeding (Differding *et al.* 2020).

Indeed, plant derived substrates such as inulin/oligofructose and arabinoxylan oligosaccharides introduced with complementary feeding have been identified as bifidogenic, but also promote the growth of various butyrate producing bacteria including Eubacterium rectale, Roseburia spp. and F. prausnitzii because Bifidobacterium-produced acetate is utilized by the butyrate producers (Falony et al. 2006; Rivière et al. 2015; Moens, Weckx and De Vuyst 2016).

It has been demonstrated that cross-feeding on arabinoxylan oligosaccharides is mutually beneficial between *B. longum* subsp. longum and *E. rectale*. *B. longum* uses the arabinose part to produce acetate and xylo-oligosaccharides, and from these xylooligosaccharides *E. rectale* releases xylose monomers, which are substrates for *B. longum* (Rivière *et al.* 2015). Thus, it is likely that trophic mutualism between bifidobacteria and butyrate producing taxa exists in the infant gut during complementary feeding.

It has been speculated that lactate cross-feeding and conversion into short chain fatty acids is a key to ecosystem stability in the adult human gut (Wang et al. 2020). Lactate is however also a common metabolic end-product of many early life gut colonizers. Lactate-producing bacteria such as *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Bacteroides* and *Bifdobacterium* may support the growth of lactate consuming bacteria including Veillonella and *E. hallii* (Pham et al. 2016). In example, *B. bifdum* produces lactate, which is consumed by co-cultivated *E. hallii* (Bunesova, *Lacroix* and Schwab 2018). Additionally, a correlation is reported between the lactate consuming *Cutibacterium avidum* (formerly *Propionibacterium*), and the lactate producers *Bifdobacterium* and *Streptococcus* in infant feces (Rocha Martin et al. 2018).

In summary, consumption of degradation products (diet or mucin derived mono- and di-saccharides) and/or metabolites (such as lactate, acetate and 1,2-propanediol) from lactic acid bacteria and bifidobacteria is thus likely to allow propionate and butyrate producers to establish in the infant gut and promote maturation into a diverse and stable adult-like gut microbial community.

#### **ECOLOGICAL ASPECTS**

It is well established, that the developing infant microbiota undergoes an increase in within-individual diversity (alpha diversity), and a decrease in between-individual diversity (beta diversity; Arrieta *et al.* 2014; Bergström *et al.* 2014). In the gut of a newborn infant, where microorganisms are absent or very scarce, the seeding of new microorganisms from the environment will have a major influence on microbiota composition, whereas the impact of competition will increase as the community increases in density and diversity (Fig. 1).

An new ecological aspect originates from a recent study describing the healthy or impaired development of the infant gut microbiota based on a co-varying set of key microbial taxa, a so-called ecogroup (Raman *et al.* 2019). This approach takes longitudinal microbial interactions or networks into account in the description of developing microbial communities. An ecogroup of 15 bacterial taxa was found to explain the majority of variation in the gut microbiome and consistently co-varied during the first 5 years of life in cohorts of Bangladeshi, Peruvian or Indian children, respectively. Importantly, ecogroup taxa configurations were found to be altered in malnourished infants. The study highlights the utility of co-variation networks applied to longitudinal data in order to increase our understanding of the ecological patterns of the developing gut microbiota.

In order to understand the putative impact of order of exposure to microorganisms originating from the sources of seeding discussed above, it is useful to consider the fundamental mechanisms governing competition for ecological niches. It has been suggested that predictions about impact of order of seeding on community assembly (in any ecosystem) can be based on considering the following three features of the species' ecological niches: Overlap, impact and requirement (Vannette and Fukami 2014; Sprockett et al. 2018). A so-called 'priority effect' designates the observation that a given species gains an advantage or a disadvantage by arriving to an ecosystem earlier than others. If two species are competing for the same niche (overlap), the priority effect will be strong. This is speculated to be the case e.g. when specialized HMO consumers compete for this nutritional niche. It has been elegantly demonstrated in a neonatal mouse model, that for the vast majority of intestinal bacteria, early arrival represents an advantage (Martínez et al. 2018). However, it was also observed that in some cases, establishment in the neonatal mouse gut was facilitated by later arrival, suggesting that the preexisting microcoorganisms may impact on the ecological niched either to facilitate or to prevent the establishment of given newcomers, as when facultative organisms consume the intestinal oxygen and thereby facilitates establishment of anaerobic species, but prevent proliferation of aerobes seeded into the gut. Finally, species that are more sensitive to specific requirements, i.e. whose growth rate is sensitive to abiotic and nutritional environmental changes, are predicted to experience stronger priority effects (Vannette and Fukami 2014; Sprockett et al. 2018). This suggests that species such as B. thetaiotaomicron, which have many options for nutritional sources, can robustly colonize the infant intestine irrespective of order of exposure.

Since closely related species might be more likely to compete for overlapping niches (Cavender-Bares *et al.* 2009), it could be speculated that the effect of early arrival would be most pronounced on the competition between bacteria that are phylogenetically closely related, however this was contradicted by experiments in mice (Martínez *et al.* 2018), suggesting that the bacterial functions and features governing competition for niches are not necessarily related to phylogeny.

In addition to investigate the successional development of the neonatal intestinal microbiota based on taxa (Bäckhed et al. 2015; Bittinger et al. 2020) it is also possible to study the succession of bacterial traits (phenotypes), an approach well developed in environmental ecology (Ackerly and Cornwell 2007). Recently, a study of trait-based community assembly of the microbiota in a cohort of 56 infants during the first 3 years of life (Guittar, Shade and Litchman 2019) elucidated some important general features. First, the study demonstrates that the average 16S rRNA gene copy number per bacterial genome decreases as a function of time during the first 2-3 years of life. Since a high number of ribosomal gene copies is affiliated to the ability of rapid initiation of protein synthesis and thus rapid response to conditions allowing growth (Klappenbach, Dunbar and Schmidt 2000), this suggests that the infant gut undergoes a transition from initially fast responding strains to the later predominance of overall less rapidly adapting and slower growing species. Indeed, it has been observed in mice that the in situ ribosome content of E. coli growing in intestinal mucus reflects rapid growth (Poulsen et al. 1995). We thus speculate than in the neonatal intestine, rapid response to changing conditions and capacity for rapid proliferation are selective factors for initial establishment. In support of this, an acute surge in total bacterial density quantified by 16S rRNA gene copies per gram intestinal content has been observed as a consequence of antibiotic treatment of rats, and could partly be explained by a switch to more rapidly adapting bacterial species (Tulstrup et al. 2015).

The observed temporal decrease in rRNA gene copies per genome in the developing gut microbiota was accompanied by a concurrent decrease in predicted motility scores and oxygen tolerance from 3 to 9 months, as well as by increased temperature optimum and increased sporulation capacity in the same period (Guittar, Shade and Litchman 2019), likely reflecting the impact of these traits at different periods of infancy.

#### **CONCLUDING REMARKS**

The first 1000 days after birth of an infant, are often referred to as a window of opportunity for shaping the microbiota, which will characterize the individual throughout life (Rodríguez *et al.* 2015). Since this period is also very important for development of the immune system, where exposure to microbes play a pivotal role (Gensollen *et al.* 2016), the processes governing the microbiota assembly are important to elucidate.

Here, we have taken the perspective of microbial physiology and ecology to describe the development of the microbiota. In the first period after birth, the bacterial load in the intestine is low, meaning that many ecological niches are free for the seeded bacteria to explore. The high inter-individual (beta) diversity at this stage is probably reflecting the many different sources of seeding to the gut at this stage.

However, proliferation of seeded bacteria of a given species requires abiotic conditions that allow for this, which is reflected in the observation that vaginal and skin-derived species remain only transiently in the infant gut, while maternal gut species represent a significant part of the establishing microbiota as reviewed above.

As the bacterial load and diversity increases, competition for nutritional niches originating from diet and mucosa will play a larger and larger role (Fig. 1). As long as the nutritional environment is governed by breastfeeding, bacteria that are efficient in using the HMOs dominate the community. When the complexity of diet increases, so does the complexity (alpha diversity) of the bacterial community, and eventually the impact of original seeding sources is no longer detectable. Additionally, the increasing hostility of the gut environment, characterized by reduced oxygen availability and reduced pH and later by scarceness of bacterial nutrients subject to competition, selects for a community that is optimized for coping with this, thereby reducing the beta diversity.

In order to better understand and predict this development, and eventually to decipher how to govern and optimize it to improve health, we find that it is pivotal to combine longitudinal infant studies with fundamental studies of bacterial growth physiology as well as with ecological models.

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