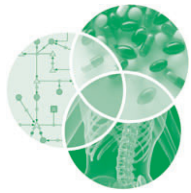
Focus Article



The role of the microbiota in ageing: current state and perspectives

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Since the application of high-throughput technologies to investigate complex microbial communities, alterations in the human gut microbiota have been associated with an increasing number of diseases and conditions. This field of research has developed into an area of intense study which is quite different to the microbial investigations that have preceded it in terms of both the broadness of the area of research and the complexity of the analyses. In this review, we discuss gut microbiota changes observed in ageing in the context of the physiological changes that accompany senescence, examine what correlations can be established or inferred, and we discuss what key questions remain to be answered in the field.

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INTRODUCTION

Investigation of the bacterial component of the human microbiome has gained a lot of interest in the past few years. One of the main reasons for this interest is that the human gut microbiota has been implicated in an increasing number of disease conditions.[1–3] The gut microbiota is highly diverse and stable in adult life, but there are a number of life stages and conditions during which the microbiota composition and function change. The first of these is the establishment phase of the microbiota early in life. Once fully established, the microbiota is relatively stable from childhood through adulthood. In later years, there is a subsequent shift of the microbiota composition that is associated with health deterioration and dietary changes.

It has been suggested that the emerging literature on health-microbiota associations has not appreciated the complexity of interactions that should be accounted for when dealing with large observational datasets.[4] Conversely it must also be acknowledged that the beneficial as well as potentially undesirable effect of the microbiota on dietary, immunological and metabolic conditions can no longer be ignored by individuals studying these conditions from a purely physiological perspective.

Life Without a Microbiota

The high-throughput sequencing revolution in microbial community analysis has shown that the establishment and maintenance of beneficial interactions between the microbiota and host may be a key requirement for some aspects of the health of the host. Germ-free mice can survive without a microbiota, even though they lack these beneficial interactions. However they suffer from behavioral deficits[5] and a number of morphological and immunological issues due to altered metabolism,[6] development, and physiology, including organ development and morphogenesis.[7] This shows that the microbiota is important for normal development. These differences in the phenotype of germ-free mice can be partially reversed through the introduction of a microbiota.[5]

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The microbiota is also important for the maintenance of the immune system[8] and the lack of a microbiota protects mice from some aspects of diet-related obesity.[9]

Microbiota Effects Upon the Immune System

A major question concerning the biological significance of alterations in the microbiota regards cause or effect. In our studies of the microbiota in the elderly we reported changes in the microbiota and the inflammatory status of the individuals.[10] So the question arises, 'Does the immunological state of the individual affect the microbiota, or does the microbiota affect the immunome of the individual'? Current knowledge tells us that both statements are likely to be true. The gut microbiota has a large impact on the immune system due to the considerable number of bacterial antigenic substances that the immune system must deal with on a daily basis. In fact there is no larger immune organ in the body than the gut[11] and gut–microbe interaction is also critical for the establishment of a healthy immune system.[12] Thus it is completely plausible that the gut microbiota has a role in stimulating production of the inflammatory molecules that are a hallmark of persistent inflammation in the elderly, termed inflammaging. Experiments are in progress in our lab in pre-clinical models and in human dietary trials to further investigate cause and effect relationships for the microbiota in age-related health loss.

Certain bacteria can trigger differential immune cell responses. For instance, members of the class Clostridia are important for T-cell differentiation, and have been shown to help mice resist colitis and IgE responses.[13] Other segmented filamentous bacteria (SFB) that appear to be absent in humans induce responses by T-helper cells.[14] The production of butyrate and other short-chain fatty acids (SCFAs) by bacteria maintains the barrier function of the epithelium and mucus layers,[15] which prevents gut bacteria from entering the bloodstream. Even in otherwise healthy individuals, defects in the epithelial barrier function of the gut can lead to significant changes in the phenotype of the individual and the occurrence of comorbidities.[16,17] Indeed a number of the studies examining disorders associated with changes in the microbiota have identified an immunological component that might be linked to impaired barrier function including coeliac disease, inflammatory bowel disease (IBD), and rheumatoid arthritis (reviewed by Vanuytsel et al.[18]).

As well as acting as a barrier separating the microbiota from the *lamina propria*, the epithelium also controls the microbiota through the production of antimicrobials and secretory IgA (sIgA), as well as allowing passage of phagocytes and lymphocytes if the epithelium barrier is breached.[19] The antimicrobial particles are cathelicidin-related peptides, which includes the defensins family.[20] These molecules have been shown to alter the commensal bacterial population in the gut of mice, decreasing the *Firmicutes*:*Bacteroidetes* ratio.[21] Commensal bacteria in the human gut are resistant to the action of sIgA and are found to be coated with immunoglobulins.[22] Indeed the presence of the commensals is important for the normal level of IgA production in the gut.[23] The presence of IgA prevents numerous potential pathogens from colonizing the gut and so maintains the commensal populations in the gut.

What's in It for the Microbiota?

The mammalian body maintains the microbiota using the proverbial 'carrot and stick' approach. The 'stick' is the immune surveillance keeping the microbiota in check as discussed above, but the 'carrot' is the maintenance of an environment conducive to the survival of the specialized human microbiota and the availability of specific gut energy sources.

As well as mucin providing a barrier separating the microbiota and their antigens from the immune cells located in the epithelium, it also serves as a semi-separate ecosystem within the gut, with a different microbiota composition and microbiome gene function. The mucus layer harbors species that inhabit the lumen as well as commensals that can use the mucin as a nutrient source, often relying on cross-feeding to achieve this.[24] This function of the mucin layer alters and maintains the commensal population within the mucin layer and within the luminal area. These factors contribute to allowing the gut commensals to out-compete potential pathogens.

The symbiotic relationship between the host and the microbiota has very clear connotations for the development and maintenance of a healthy immune system, and health in general. So how does the microbiota develop in the infant gut? Why does it go wrong? And what changes in elderly subjects contribute to drive health decline? The following sections answer these key questions.

THE INFANT MICROBIOTA

In Utero Microbiota

Until recently, it was thought that the fetus was sterile *in utero*, and subsequently colonized during the process of birth. In recent years, a number of publications have suggested that this may not be correct. Amniotic fluid was shown to contain microbes,[25,26]

as were the umbilical cord,[27] the placenta,[28] and the meconium.[29] Using oral administration of a genetically labeled strain of *Enterococcus faecium*, Jiménez et al. were able to establish that transmission occurs from the gastrointestinal tract of pregnant mice to the amniotic fluid.[27] Because the fetus swallows amniotic fluid, it was conceivable that transmission would thus occur. Using the same approach, the labeled *E. faecium* strain was detected in the meconium of the pups after aseptic cesarean section.[29] Together these studies suggest that conditions *in utero* are not sterile.

When analyzing microbiota of environments or samples previously presumed to be sterile such as the umbilical cord, the amniotic fluid, and the meconium, a number of concerns are raised. For instance, Jiménez et al.[29] noted that bacterial counts significantly differed between meconium samples that were processed immediately and samples that were processed after 4 days of refrigeration. Many of the immediately processed samples showed no detectable bacterial counts on some culture media. A major concern of using culture-based techniques on low bacterial load samples is that the techniques may not be able to detect cells within the sample. Storage prior to assessment can lead to overestimations of counts. Differential growth rates in such conditions may also result in over-approximations of the proportions of some bacteria within a sample compared with others. For these reasons, immediate extraction of DNA from a sample, and amplification of the 16S rRNA gene for sequencing is preferable in many cases. Using 16S rRNA gene amplicons to differentiate between sterile and low biomass samples brings a new set of problems, as a recent study suggests that contamination can make up a large proportion of the reads produced from sequencing DNA from low biomass samples.[30] As many studies suggested that conditions *in utero* were sterile, the possibility of contamination in these new experiments that indicate an *in utero* microbiota must therefore be considered.

The Type of Birth Affects Gut Microbiota of the Infant

While fetuses may be almost devoid of bacteria *in utero*, the natural birth process ensures that an infant will be exposed to a complex inoculum of microorganisms. Under normal circumstances, this exposure occurs in the mother's birth canal. However, for the significant number of mothers undergoing cesarean section, their infants are not exposed to the vaginal microbiota. Instead they are exposed to the microbiota of the skin. With different body sites harboring different microbial populations,[31] the result of cesarean section delivery is that the microbiota of new-borns, at all body sites tested, resembles the skin microbiota of the mother,[32] whereas naturally-delivered infants have a microbiota more similar to that of the vaginal canal.

In the first 2 years of life, the progression of the gut microbiota toward an adult-like microbiota is quite clear at the phylum level.[33] In the first 3 months, the relative abundance of *Proteobacteria* declines, and abundance of *Actinobacteria* rise. After this, the *Actinobacteria* decrease, and at this point, the relative abundance of phylum *Firmicutes* begins to increase. These trends continue through to age 6 months,[33] with the emergence of a low level of *Verrucomicrobia*. Vaginally-delivered infants have a greater relative abundance of phylum *Bacteroidetes* and a lower abundance of *Firmicutes* than children delivered via cesarean section.[33] Only after the first year do the levels of *Bacteroidetes* begin to rise in cesarean section-delivered children to levels similar to those in vaginally-delivered children. Even at the age of seven, levels of clostridia are still significantly higher in vaginally-delivered children.[34] The genus *Bifidobacterium* is numerically dominant throughout the first year at least, regardless of delivery mode, a finding that recurs throughout the literature.[35,36] High levels of *Bifidobacterium* are driven by breast feeding, highlighted by a much slower colonization in bottle-fed infants.[37] Other differences are caused by breast feeding over formula feeding, including lower clostridia levels, and differential colonization times.[38] The role of diet is therefore an important factor in the development of the gut microbiota. Mothers weaning off breast feeding at different stages, and introducing other foods at different time-points has a large effect on the timescales for microbiota development discussed above, which are estimates with large variations, and which results in conflicting literature on this topic.

Jakobsson et al. also showed that microbiota diversity is relatively stable for the first 3 months after birth,[33] but after this point, it begins to rise significantly. There is some evidence for significantly different levels of microbiota diversity between vaginally-delivered and cesarean section-delivered children,[33,39] and with diversity linked to a number of diseases, this difference raises some concerns. Low levels of diversity are also observed for the microbiota of premature infants.[40,41] However, many preterm children are administered antibiotics or are born by cesarean section, so the effect of premature birth itself is often confounded by these other factors. Further study needs to be performed on larger cohorts of preterm infants who were not administered antibiotics where possible, and directly compared with term infants.

The Impact of the Early Microbiota on the Host

Many studies have investigated how birth delivery mode, breast feeding, and antibiotics can change the infant gut microbiota. But what downstream effects do these differences have? Gut microbiota can change the gene expression patterns of immune-regulatory cells, and prime them to respond in certain ways.[42,43] Therefore it can be extrapolated that differential populations can have different effects on the immune system. Furthermore, alternate expression patterns of the microbes within one of these different populations may also impact on immune cells. It is therefore unsurprising that an altered microbiota early in life correlates with an altered immune profile later on.

Infants delivered by cesarean sections have been reported to have an increased risk of developing food allergy,[44,45] allergic rhinitis,[44] asthma,[44] wheezing,[45] and sensitivity to other allergens.[45] Increases in levels of IgA and IgG in children born by cesarean section have been observed,[42] and may be an underlining trigger for such conditions. However there are many examples for a lack of correlation between cesarean section birth and immunoglobulin levels or indeed the conditions listed above.[46,47] Most of these studies of autoimmune conditions and immune-regulatory markers lack analyses of the underlying microbiota, and focus on large age ranges for children, many of which may be too early to observe the condition in question. Without assessing the microbiota, either the population structure (using 16S rRNA gene amplicon analysis), the gene catalogue (using metagenomic analysis), or the transcriptional profile (using metatranscriptomics), correlations will remain uncertain, and statistical models will not be correctly controlled. For this reason, large, multidisciplinary, longitudinal systems approaches are particularly necessary to identify the impact that the early microbiota can have on the development and life-time disease risk of the human infant.

THE ELDERLY GUT

Senescence-Driven Immunological Changes

Ageing is accompanied by increased risk of infectious diseases, cancers and comorbidities.[48] Ageing as a cellular and organismal process is poorly defined and so is an area of intense research. However one can distinguish a large number of immunological changes in the elderly subject. Senescence can be caused by a number of stressors or mechanisms, but whatever the mechanism that moves a cell toward a senescent state,[49] a consensus of studies have reported the production of pro-inflammatory cytokines and chemokines as a central property of the senescent cell.[50] These inflammatory molecules may maintain their production levels through positive feedback loops. Villeda et al. have demonstrated that exposure to blood from younger animals counteracts ageing at the molecular, structural, functional, and cognitive levels in the aged hippocampus of mice.[51] Therefore, as well as molecules in the blood that promote senescence, there are molecules in blood of young animals that are important for the maintenance of health.

Dietary Changes in the Elderly

One of the recurring themes in the microbiota literature is the modulation of the microbiota by the diet. The long-term diet is one of the driving forces that define the microbiota, affecting its composition and metabolic activities. As the diet of elderly individuals is known to change for a number of reasons, such as chewing and swallowing difficulties, and loss of teeth, smell, and taste (reviewed in Cusack and O'Toole[52]), it is likely that some of the microbiota changes are due to the change in diet as people age. Increased consumption of high sugar and high fat food is a common dietary shift seen with age[10] especially in residential care. It is interesting to hypothesize that this may also be due in part to the effect of a low diversity microbiota on food cravings,[53] but it will also reduce the amount of fiber consumed and so change the activity of the microbiota in the gut.

Subsequent Microbiota Alterations Perpetuate Health Decline

As people approach an advanced age, the microbiota begins to change.[54,55] In the community setting, these changes are small and correlate with age, but when people enter long-term residential care settings, these changes are exaggerated.[10] This is evident in the difference between the microbiota composition of young and elderly individuals living in the community, where we recorded increases in *Escherichia* species, and decreases in *Ruminococcus* and *Blautia* species,[10] along with reduction in abundances of species that produce butyrate.[56] Changes in these species and products have been associated with inflammation,[10,56] reduced available energy within the gut, and subsequent increased transit time through the gut.[57] Energy availability, by way of SCFAs produced by the microbiota, may regulate gut transit. Reduction of the production of these SCFAs in the elderly subject may result in transit impairment, which can impact on the wellbeing and further dietary choices of the individual, thus perpetuating gut and microbiota issues for

the elderly. Any method reducing or preventing these changes could improve the health of the elderly.

CHALLENGES AND PROSPECTS

Multifactorial Studies

Many of the studies discussed here relate the microbiota to health. A number of these have not considered the role of diet and how it affects the microbiota. Others have not accounted for the large number of variables that can significantly impact on findings. For these reasons, there are a number of contradictory findings between studies that can only be resolved by large, multiomics approaches that not only account for diet and microbiota when examining health, but control for other confounding variables correctly. With the ever-improving technologies available, and increasing ability to analyze such extensive datasets, each study should aim to include more detailed data such as metagenomics or metatranscriptomics to obtain a multifacetted view of the processes occurring within the microbiota and how they relate to health parameters.

Intervention Possibilities

As detailed above, birth by cesarean section or preterm birth and treatment with antibiotics have been associated with numerous immunological dysregulatory conditions. As many expecting mothers opt for nonessential cesarean sections, an assessment of the long-term risks to the children of these unnecessary interventions should be carried out to determine if nonessential cesarean sections should be discouraged. In cases where cesarean sections are medically indicated, there is the possibility of deliberately inoculating the infant with the vaginal canal microbiota. These approaches have the potential to reduce the number of occurrences of autoimmune diseases, and therefore should be explored.

Elderly subjects suffer from a large range of conditions, from hypertension to Alzheimer's disease. The current approach is to treat conditions as they arise. Many of these subjects receive multiple medications for these different conditions (polypharmacy). Receiving a cocktail of drugs can reduce the impact of some, and can lead to numerous side effects. Some medications have also been shown to modulate the microbiota[58] while others are activated or inactivated by gut microbes.[59] So any approach that can reduce the need to consume some medications would be advantageous. With this in mind, there are two possible routes to consider. The first is to restore a healthy-type microbiota to the elderly individual, either by encouraging the growth of younger adult-associated bacteria, or by more radical measures such as a fecal microbiota transplantation (FMT; Refs 60, 61). Establishment of artificial consortia as an alternative to FMT requires laboratory culture of fastidious bacteria and is not an easy approach for nonspecialist laboratories, while fecal microbiota transplants are not clinically validated for applications other than *Clostridium difficile*-associated diarrhoea[62] as of yet and still present a number of potential risks. The second approach is to prevent the transition of the microbiota from the stable young adult profile. Encouraging a varied diet that resembles that consumed by younger adults, providing supplementary fibers that allow selective maintenance of associated microbiota, or providing probiotics, are some of the possible interventions to improve elderly health and reduce the burden on healthcare systems.

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