nature
MILESTONES

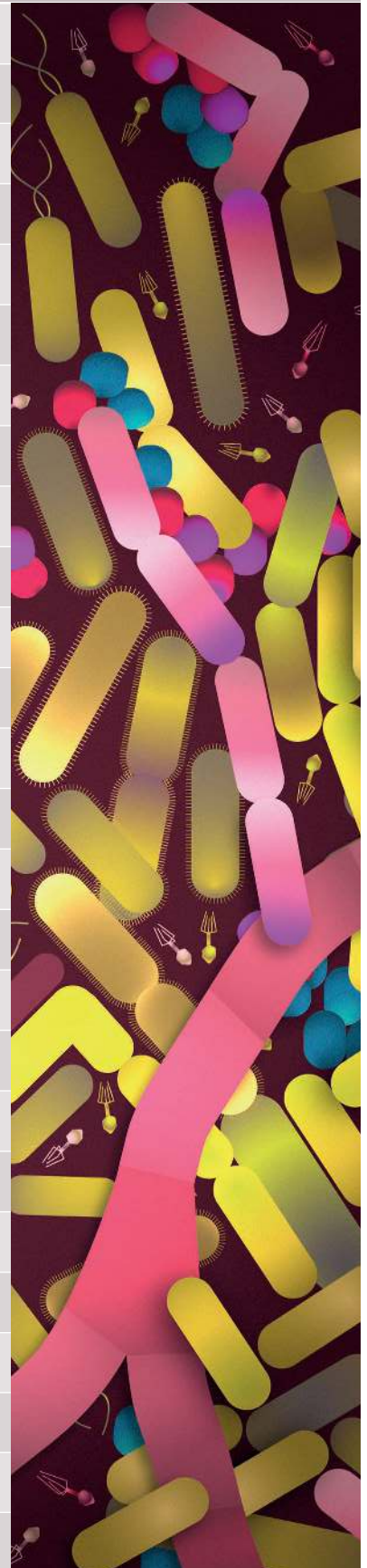# Human microbiota research

MILESTONES IN HUMAN MICROBIOTA RESEARCH

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▣ **FOREWORD**

A field is born

"I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving."
— Antonie van Leeuwenhoek.

Despite being considered by many as a relatively modern field of research, the first descriptions of human-associated microbiota date back to the 1670s–1680s, when Antonie van Leeuwenhoek started using his newly developed, handcrafted microscopes. In a letter written to the Royal Society of London in 1683, he described and illustrated five different kinds of bacteria (although he called them animalcules at the time) present in his own mouth and that of others, and subsequently also compared his own oral and faecal microbiota, determining that there are differences between body sites as well as between health and disease. Some of the first direct observations of bacteria were of human-associated microbiota.

Fast-forward a couple of centuries and, in 1853, Joseph Leidy published a book entitled *A Flora and Fauna within Living Animals*, which some consider to be the origin of microbiota research. Then, the work of Pasteur, Metchnikoff, Koch, Escherich, Kendall and a few others, laid the foundations of how we understand host–microrganism interactions. Pasteur developed the germ theory of disease, but also thought that non-pathogenic microorganisms might have an important role in normal human physiology; Metchnikoff believed that microbiota composition and its interactions with the host was essential for health; and Escherich was convinced that understanding the endogenous flora was essential for understanding the physiology of digestion and the pathology and therapy of intestinal disease. Sound familiar? The themes we explore in these 'Milestones in human microbiota research' largely brought to bear the hypotheses and early work of these microbiology giants, on the shoulders of which the field stands today.

MILESTONES

" the field [...] took off in earnest once methods to culture anaerobic organisms were discovered in the 1940s and 1950s, when members of the microbiota were grown and studied in the laboratory "

In 1890, Koch published his famous postulates, four criteria designed to establish a causative relationship between a microorganism and a disease, and during the first half of the twentieth century, microbiology became more focused on the identification of etiological agents of disease. This was also likely due to the fact that most bacterial pathogens can grow in the presence of oxygen, whereas most members of the gut microbiota cannot and thus could not be studied at the time. Alfred Nissle, a German physician, isolated the *Escherichia coli* Nissle 1917 strain — which remains a commonly used probiotic — in 1917. During World War I, when the first gut eukaryotic microorganisms and bacteriophages were also described, Nissle noticed that one soldier did not succumb to dysentery and thought he might have a protective microorganism in his gut. He isolated the strain and later showed that it antagonized other pathogens, establishing the concept of colonization resistance, whereby human-associated microorganisms prevent the establishment of pathogens in the same niche.

Despite these early insights, the field only took off in earnest once methods to culture anaerobic organisms were discovered in the 1940s and 1950s, when members of the microbiota were grown and studied in the laboratory. This is where we have chosen to start our timeline of milestones, as increasing numbers of researchers became interested in understanding the composition and function of the microbial communities that live on our different surfaces and how they change throughout our lives. The realization that much of the normal physiology of conventional laboratory mice was missing in germ-free mice, and could be reconstituted through colonization with bacteria obtained from faeces, enabled the first in vivo experiments. Comparisons of germ-free and colonised animals in the 1960s led to observations that predicted much of what has since been discovered using methodologies that enable more in-depth analyses. Despite advances in culturing microorganisms, it soon became apparent that there were gross discrepancies between the numbers of existing cells and how many could grow in the lab, what became known as the 'great plate count anomaly'. This key observation motivated the development of sequencing-based approaches to identify unculturable microorganisms, which were pioneered by Woese, Pace, Fox and others to study environmental microorganisms and subsequently adapted to the analysis of human-associated communities, providing an unprecedented view into their composition. A key step in popularising microbiota research, which got it into the mainstream news and made it a household concept, was the finding by the Gordon group, in 2006, that reconstituting mice with the microbial communities associated with a human disease state could transplant the phenotype to the animals. This opened the door to research trying to establish causal relationships between altered microbial communities and disease, which has become a cornerstone of the field.

Although the first use of faecal microbiota transplantation (FMT) in Western medicine was published in 1958 by Ben Eiseman and colleagues, who successfully treated four people suffering from pseudomembranous colitis (before *Clostridioides difficile* was the known cause), FMT was already used in ancient Chinese medicine. Fourth-century Chinese medical literature mentions its use, by Ge Hong among others, to treat food poisoning and severe diarrhoea. In the sixteenth century, Li Shizhen used oral administration of a 'soup' containing fresh, dry or fermented stool to treat abdominal diseases. In seventeenth century Europe, the Italian Fabrizio and the German Paullini documented the use of FMT, and the American microbiologist Stan Falkow candidly recalled his role in preparing first-generation poop pills to reconstitute the gut communities of surgical patients a year before Eiseman and colleagues published their work.

We recognize that an enormous body of work precedes each milestone that we have selected to highlight progress in this field. This foreword aims to pay homage to some of these microbiota pioneers. With this project, divided into 25 milestones, we want to highlight particular areas of research — both established and burgeoning — that have contributed to a better understanding of our microbial selves, as well as methodological advances that have propelled the field forward. We also want to highlight important but lesser known aspects of the field, such as the fact that our microbiota is not just composed of bacteria; that human-associated, health-promoting microbial communities exist on all bodily surfaces, not only our gut; and, importantly, that to have a complete picture of the functional capacity of our microbiota and its roles in human health, we need to look beyond the gut of white, Western populations.

We thank the many researchers from all corners of the field who have advised on the different aspects of this project, as well as those who have participated in the podcasts. It is, of course, impossible to cover everything in a field as broad and diverse as this one, but we hope to have captured the major steps forward. In our attempt to summarise almost 350 years of research, we will have unavoidably missed important contributions and sincerely apologize for any unintended oversights. Although we have focussed these milestones on the study of human-associated microbiota, other vibrant research communities are trying to understand plant- and animal-associated, as well as environmental, microbial communities. We hope that this journey through history will be inspirational and we look forward to the exciting developments that are sure to come, ultimately aiming to harness our understanding of microbial communities to improve not only human health, but that of plants, animals and ecosystems.

Nonia Pariente, *Nature Microbiology*

→ MILESTONE 1

Culturing anaerobes

Understanding the role of our microbiota in health and disease has long been hampered by the strict growth requirements of many of its constituent members. Underpinning modern day investigations into the vast complexity and functions of the human microbiota are fundamental methodologies to culture anaerobic bacteria outside their natural environment.

From the rudimentary oxygen-free culture methods in the era of Pasteur, and subsequent advances in surface culture in the early twentieth century, the mid-1900s saw a substantial expansion and refinement of anaerobic culture techniques, largely due to the pioneering work of Robert. E. Hungate. In a 1944 study of cellulose-degrading microorganisms in the bovine rumen, his revolutionary roll-tube approach enabled the successful culture of *Clostridium cellobioparus* and, in 1950, he published a complete description of his technique. The protocol used rubber-stoppered tubes of boiled culture medium with cellulose agar, through which anoxic gas was bubbled to remove any remaining oxygen. Firstly, passing this gas through a column of hot, reduced copper wire excluded any oxygen from the gas itself, and the subsequent addition of a reducing agent to the medium removed residual traces of oxygen. Rolling tubes under cold water produced a thin layer of solid agarose medium, and for the first time, anaerobiosis was maintained throughout manipulations using a constant flow of anoxic gas. The method, now known as 'the Hungate technique', is still in use to this day.

Several modifications later emerged, such as the VPI (Virginia Polytech Institute) method for larger-scale culture introduced by Moore in 1966, using prereduced medium and prehardened roll tubes. Hungate also made adaptations to culture methanogens, the strictest of anaerobes, reported in 1969. Others, such as Spears and Freter in 1967, similarly recognised the importance of continuously avoiding any exposure to

> " the Hungate technique […] enabled a wealth of anaerobes that had not grown previously in surface cultures to be isolated for further study. "

oxygen, yet the Hungate technique was still more efficient and enabled a wealth of anaerobes that had not grown previously in surface cultures to be isolated for further study.

Alternative approaches used today were also launched in the mid-late 1960s, namely the GasPak and the anaerobic glove-box. The former, a self-contained combustion jar system, quickly made surface culture of anaerobic microorgansims accessible to more laboratories. The glove-box, a sealed chamber with attached gloves, filled with anoxic gases, was also a popular choice, simplifying equipment and procedures for oxygen-free culture.
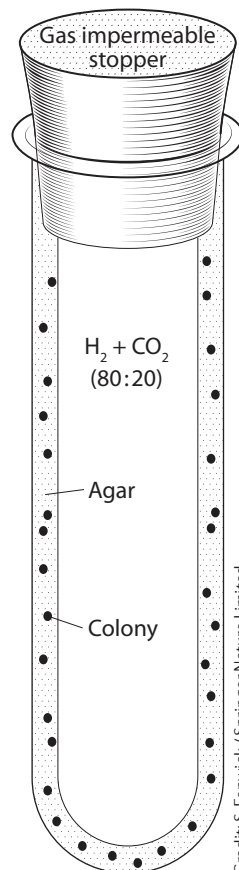
As well as apparatus to create an oxygen-free environment, culturing anaerobes requires appropriate media, which must have a low oxidation-reduction potential, as well as the substrates obtained by microorganisms in their natural habitat. Many researchers working on *Bacteroides* species were instrumental in determining the requirements of specific anaerobic microorganisms, and a recent breakthrough in media composition (the inclusion of antioxidants) has since permitted the aerobic growth of anaerobic bacteria.

Moving into the twenty-first century, the advent of metagenomics

revealed that the majority of environmental microbial biodiversity remained uncultured, inspiring a rebirth of culture techniques. Recent culture-dependent efforts to characterize the human microbiota (see MILESTONE 19) utilised dilution culturing and culminated in the development of culturomics; a high-throughput methodology using hundreds of different culture conditions, prolonged incubations, and matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) spectrometry, combined with 16S ribosomal RNA gene sequencing for the rapid identification of a great number of previously uncultured gut bacteria.

With a large proportion of the human microbiota requiring oxygen-free growth conditions, early breakthroughs in anaerobic culture were crucial in enabling more of our microbiota to be isolated and classified, and for their metabolism, distribution and roles within the microbiota to be studied. Initial methodologies paved the way for higher-throughput technologies that provide vital insights about the functions of the bacteria inhabiting the human body, and their effects on the human host. Now, with our understanding of the importance of the gut microbiota in human health advancing by the day, we are even more indebted to these early researchers and their innovations enabling the culture of anaerobes.

Hannah Clark, *Nature Protocols*

Gas impermeable stopper

$H_2 + CO_2$ (80:20)

Agar

Colony

Credit: S. Fenwick / Springer Nature Limited

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Faecal microbiota transplantation for *Clostridioides difficile* infection

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> ❝ FMT is now an effective treatment option for recurrent *C. difficile* infection and is believed to normalize the microbial diversity and community structure in the colon ❞

In 1958, Eiseman et al. reported the successful treatment of pseudomembranous enterocolitis using a faecal enema. Since then, faecal microbiota transplantation (FMT) has become widely accepted as a successful rescue treatment for recurrent *Clostridioides difficile* infection. FMT is also being investigated for other indications.

Eiseman and colleagues presented the cases of four patients with pseudomembranous enterocolitis. They used enemas with faeces from healthy donors after other therapy options failed. The patients had a rapid recovery from their symptoms.

C. difficile infection can cause debilitating diarrhoeal symptoms when the bacterial spores germinate into vegetative cells that produce enterotoxins, resulting in colonic inflammation and the formation of 'pseudomembranes' consisting of inflammatory cells, dead cells and debris. Over the past couple of decades, *C. difficile* infection has increased in incidence, morbidity and mortality, and has become known as a 'superbug'. Despite antibiotics being the standard treatment for *C. difficile* infection, they also often are the cause of infection owing to their suppressive effects on native gut microbiota and subsequent overgrowth of *C. difficile*. Eiseman and colleagues also noted this link to prior broad-spectrum antibiotic treatment in their patients and speculated that disruption of the 'healthy gut flora' underlies infection.

The intention of performing FMT is restoration of the normal function of the gut microbiota. In Eiseman and colleagues' report, culture of stool samples obtained during infection showed the presence of *Staphylococcus aureus*, which, at the time, was considered to be a possible cause of pseudomembranous enterocolitis. *S. aureus* disappeared from the stool sample cultures after administration of a faecal enema in association with clinical improvement. The authors suggested that normal colonic non-pathogens displaced the colitis-causing pathogen, which, decades later, was demonstrated to be *C. difficile*.

Since Eiseman and co-workers' publication, others have reported success using faecal enemas, *Lactobacillus rhamnosus* GG (a probiotic) and bacteriotherapy (using a mixture of facultatively aerobic and anaerobic bacteria) to treat relapsing *C. difficile* enterocolitis.

FMT is now an effective treatment option for recurrent *C. difficile* infection and is believed to normalize the microbial diversity and community structure in the colon. Currently, a research consortium is recruiting patients to a clinical trial examining whether FMT is safe, and can prevent recurrent *C. difficile*-associated disease. FMT could inhibit *C. difficile* by multiple mechanisms, such as suppression by antimicrobial peptides, inhibition of spore germination and vegetative growth, competition for nutrients, and activation of colonization resistance (MILESTONE 13).

To reduce costs and increase patient and clinician convenience, oral delivery of faecal microbiota has been tested. In 2017, a randomized clinical trial showed that FMT delivery by oral capsule was non-inferior to delivery via colonoscopy, suggesting oral capsules could be an effective treatment approach for recurrent *C. difficile* infection.

FMT has also been investigated for non-*C. difficile* indications, such as ulcerative and drug-induced colitis, and has shown some promise. FMT from lean donors has also been shown to increase insulin sensitivity in men with metabolic syndrome.

Thus, since the pioneering report from Eiseman and colleagues, FMT has become an effective therapy for recurrent *C. difficile* infection and shows promise for treating other diseases.

Louise Stone, Nature Reviews Urology

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Laboratory mice housed under germ-free conditions. Photograph courtesy of Taren M. Thron, California Institute of Technology.

▶ MILESTONE 3

Gut microbiota transfer experiments in germ-free animals

Germ-free animals are raised under sterile conditions to prevent colonization with bacteria and other microorganisms. These animals (mostly mice and rats, but also guinea pigs and chicks) have a life span similar to that of conventional, normally colonized animals, although they seem to have a slower or impaired growth as well as some anatomical and physiological differences (such as an enlarged cecum). By the 1960s, germ-free animals were a well-established tool in nutritional studies aiming to understand the contribution of the intestinal microbiota to host dietary requirements (for example, with regard to the synthesis of vitamins).

In 1965, Schaedler and colleagues introduced a new use for GF animals: the transfer of bacterial cultures to germ-free mice. Such transfer experiments have been essential in studying the effects of the gut microbiota on the host ever since. In their pivotal study, Schaedler and colleagues reported the results of feeding bacterial cultures isolated from the gut of Nelson–Collins–Swiss (NCS) mice (a colony of albino mice that are free of ordinary mouse pathogens as well as intestinal *Escherichia coli* and *Proteus* spp.) to germ-free mice. The germ-free mice were fed food inoculated with individual bacterial

cultures of several anaerobic isolates. After one week, the numbers and localization of these bacterial strains in the gastrointestinal tract were comparable to those observed in the NCS mice and remained stable for several months, confirming the feasibility of microbiota transfer experiments and their usefulness for studying bacterial gut colonization. Importantly, transfer of a *Bacteroides* strain partially reduced the cecum enlargement typical of germ-free mice, and the offspring of germ-free mice that had been colonized with a mixture of strains inherited those strains and subsequently showed normal cecum size and structure. These results directly showed the important and profound effect of the gut microbiota on host development and physiology.

This landmark study paved the way for further research on the effects of the gut microbiota on the host and on the interactions

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In 1965, Schaedler and colleagues introduced a new use for germ-free animals: the transfer of bacterial cultures to germ-free mice […] have been essential to study the effects of the gut microbiota on the host ever since

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between different species of the gut microbiota. For example, one strain of Lactobacillaceae that could 7α-dehydroxylate bile acids in vitro did not have the same catabolic activity when transferred to germ-free rats until additional bacterial strains were introduced. Another study in germ-free rats also showed the metabolic capacity of the gut microbiota, specifically the reduction of bilirubin to urobilins, which had been assumed by some to be produced by the liver. These initial studies laid the ground for detailed work that explored the links between microbial and host metabolism (MILESTONE 12).

In addition to exploring the metabolic capacity of the gut microbiota, GF animals were also essential for elucidating the close links between bacteria and the host that determine tissue homeostasis and immune system development. One striking example is determination of the role of segmented filamentous bacteria (SFB), which had previously been shown to closely interact with the intestinal epithelium. Experiments that led to monoassociation of germ-free mice with SFB showed that these bacteria are key determinants of intestinal lymphocyte numbers and phenotype in mice. Subsequent studies have identified many links between specific microbial taxa and/or molecules and host immune function (MILESTONE 9).

Germ-free animals have been, and still are, indispensable tools for studying functional relationships between the microbiota and the host — although, as always with animal studies, the comparability and applicability of the results to humans need to be verified. Nevertheless, the early studies using these models have inspired several avenues of microbiota research and highlighted the important effects the gut microbiota have on their host.

Lucia Brunello,
Nature Reviews Disease Primers

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bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol. Immunol.* **39**, 555–562 (1995). **FURTHER READING** Johansson, K.R. & Sarles, W.B. Some considerations of the biological importance of intestinal microörganisms. *Bacteriol. Rev.* **13**, 25–45 (1949) | Sommer F. & Bäckhed, F. The gut microbiota — masters of host development and physiology. *Nat. Rev. Microbiol.* **11**, 227–238 (2013).

➡ **MILESTONE 4**

The microbiota influences metabolism of host-directed drugs
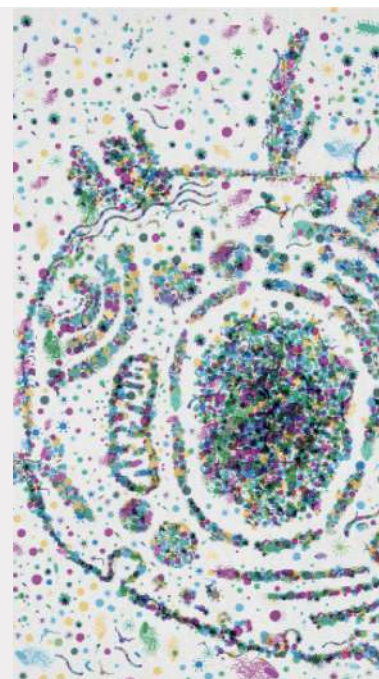
Peppercorn and Goldman demonstrated that the anti-inflammatory drug, salicylazosulfapyridine, could be degraded in conventional rats and when cultured with human gut bacteria, but not in germ-free rats, indicating a role for the gut microbiota in drug transformations. An increasing number of studies have confirmed the role of the microbiota, not limited to the gut, in drug metabolism and highlighted the implications for drug inactivation, efficacy and toxicity.

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➡ **MILESTONE 5**

Microbiota succession in early life

Early life experiences have complex and long-lasting effects that can reach into adulthood — the same can be said of the acquisition and succession of our microbiota during the first years of life. The culmination of years of investigation from many laboratories has led to an in-depth characterization of postnatal microbial acquisition and maturation during the first years of life, and has led to the realisation that this represents a crucial window in our long-term development.

Early studies, dating as far back as 1900, described various aspects of bacterial succession in infants, but in 1981, three studies were reported that set out to quantitatively characterize early acquisition of gut commensals and to study how feeding shapes our initial microbiota. In one study, development of the bacterial community was investigated in infants in Sheffield, England, by culturing specimens taken from the meconium (a baby's first faeces), faeces, mouth and umbilicus in the first six days of life. In another study, the faecal bacterial community was compared between infant cohorts in France that were either bottle-fed or breastfed; and in the third study, faecal bacterial communities from breastfed infants, weaned children and adults born in urban England and rural Nigeria, were compared. These studies provided quantitative measurements of specific bacterial taxa in early life, giving insight into the pioneer species that colonize the infant gut. This paved the way for future high-resolution studies of microbial succession in infants.

With the advent of 'omics' technologies in the following decades, our understanding of when the majority of our microbiota are acquired, and of what species are there, has heightened and the importance of host–microbiota–environment interactions during early life has become realised. The infant gut microbiota undergoes a period of massive change in the first years of life. The initial microbiota adapts over time and is shaped by the availability of different nutrients. As the infant consumes increasingly more complex dietary substrates, there are shifts in composition and an enrichment of bacterial functions related to

> " The infant gut microbiota undergoes a period of massive change in the first years of life "

carbohydrate metabolism and the biosynthesis of amino acids and vitamins. By 2–3 years of age, a stable microbiota develops that resembles that of the adults in the infant's community (see MILESTONE 7).

When colonization first occurs is an open question; however, most scientists think that the foetus develops in a sterile environment and that we acquire the bulk of our initial microbiota during and immediately after birth. Recently, a few studies have found traces of bacterial DNA in the placenta, in the amniotic fluid that surrounds the foetus and in the meconium — suggesting prenatal colonization. However, many scientists think these findings could be the result of contamination and the debate is ongoing. Regardless of possible exposure to microorganisms in utero, the foetus is exposed to microbial molecules that cross the placenta from the mother.

The first major exposure to microorganisms happens during delivery, and is highly dependent on the mode of delivery. The microbiota of neonates that are born vaginally are enriched in bacteria that resemble

Credit: S. Bradbrook / Springer Nature Limited

The environment and people that surround an infant are also a source of microorganisms that can colonize various body sites. Genetically unrelated parents and even pets share a high proportion of their microbiota with infants. Genetics also has a role in determining our microbiota make-up, as evidenced by associations between the heritability of specific taxa and host genes.

The use of antimicrobials, which is essential for preserving life when infants acquire a serious bacterial infection, can impact the ecological succession of the infant microbiota. Antibiotics can impair the diversity and stability of the developing microbiota in infants, with abundances of specific taxa remaining reduced for years after treatment. The impact of antibiotics on the infant microbiota could have long-lasting health implications and their use in early life has been linked to an increased risk of several diseases, including asthma, inflammatory bowel disease and allergies (see MILESTONE 9). More research is required to uncover the underlying mechanisms; however, what is clear is that the microbiota has a vital role in immune, endocrine, metabolic and a variety of other developmental pathways in infants, and without it we would not be here today.

the maternal vaginal microbiota (for example, *Lactobacillus* species), whereas neonates delivered by cae-sarean (C-) section lack these species and are instead enriched in skin commensals such as *Staphylococcus*, *Streptococcus* and *Propionibacterium* species. Over time, these differences gradually reduce between vaginally and C-section-born infants; however, in one study, bacteria associated with C-section remained associated with C-section-delivered infants up to two years of age, showing that birth mode could have long-term impacts on the microbiota.

Postnatal factors further configure the microbiota in early life. Breastmilk contains a complex community of bacteria that may help seed the infant gut microbiota, and in breastfed infants the gut microbiota is dominated by species that metabolise human milk oligosaccharides. Overall, diet has been found to be a major determinant of the infant gut microbiota. Studies of malnourished infants have shown that maturation of the gut microbiota does not occur in a similar manner to healthy infants, even after dietary intervention, and it has been proposed that an 'undernourished' microbiome in infancy can perpetuate growth impairments later in life.

Ashley York,
Nature Reviews Microbiology

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Sequence-based identification of human-associated microbiota

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Over the past twenty years, advances in DNA sequencing technology have deepened our knowledge in every niche of biology, from annotation of the human genome to sequencing the human-associated microbial metagenome, the genetic material of the microorganisms that inhabit almost every surface of the human body.

Study of the human microbiota historically relied on culture-dependent methods to isolate and grow bacterial colonies in a predetermined medium. The inability to cultivate a large portion of microorganisms, however, substantially underestimated the biodiversity of human-associated microbial communities.

An early milestone in this field was the adoption of a technology, previously pioneered by Carl Woese, Norman Pace and others to identify environmental bacteria, that was based on sequencing small subunit ribosomal RNA genes (16S rRNA). Using this approach, Wilson and Blitchington compared the diversity of cultivated and noncultivated bacteria within a human faecal sample in 1996. Since then, sequencing of 16S rRNA genes from complex communities has become a powerful tool for assessing microbial diversity in the human microbiota. In 2005, Eckburg et al. analysed samples not only from faeces, but also from multiple colonic mucosal sites. They sequenced >13,000 16S rRNA genes, which constituted a substantial increase in scope over previous work, and discovered significant inter-subject variability and greater differences between stool and mucosal community composition than previously described.

Sequencing of marker genes (such as 16S rRNA) is traditionally associated with Sanger sequencing, which requires a labour-intensive cloning step and is prohibitively expensive for large-scale microbiome studies. The advent of next generation sequencing (NGS) offered a cost-effective method that eliminated the cloning step by amplifying 16S rRNA genes using primers containing sequencing adapters and barcodes. The massive parallel sequencing throughput offered by NGS has significantly increased the sequence depth of 16S rRNA genes, allowing for taxonomic and phylogenetic analyses of complex microbial communities. Yet, 16S rRNA sequencing cannot always resolve closely related species and may miss the intra-species diversity. To better capture the full picture, shotgun sequencing was developed for direct sequencing of DNA. The advantage is its capability to recover the underrepresented microorganisms that were often masked by high-abundance species. Shotgun sequencing can use short-read (such as Illumina) or long-read (for example, Oxford Nanopore MinION and Pacific Bioscience Sequel) platforms. The short-read approach typically demands extensive computational support for assembling short reads into meaningful sequences. However, an accurate and complete assembly of genome sequences is still hampered by the difficulty in resolving long repetitive regions. Thus, reference-based sequencing methods emerged.

Concerted sequencing efforts have been made to construct microbial reference sequences. In 2007, the National Institutes of Health launched the Human Microbiome Project and, five years later, published the first reference data for microorganisms collected from 242 healthy United States volunteers, covering a number of anatomical sites such as mouth, nose, skin, lower intestine and vagina (MILESTONE 17). The valuable genome references that were generated by this consortium allow reliable identification of individual microbial species, but fall short when new genomes are present in the community.

Long-read sequencing offers an alternative solution for mapping challenging repetitive regions. For example, single molecule, real-time (SMRT) DNA sequencing, in combination with a short-read shotgun DNA library, allows for de novo microbial genome assemblies, although it suffers from high error rates.

Access to genome sequencing has transformed human microbiome research from focusing on identity characterizations, to metagenomics approaches that not only reveal microbial species but also how microbial metabolic activities correlate with human health and disease.

In addition to metagenomics, metatranscriptomic analysis enabled by RNA sequencing offers a way to detect active members present in a microbial community (MILESTONE 14).

The construction of metagenome-assembled genomes, an approach also pioneered in environmental microbiology and recently applied to human-associated communities, will provide a new, unprecedented opportunity for deep characterization of the functional potential of the human microbial ecosystem.

Lei Tang, Nature Methods

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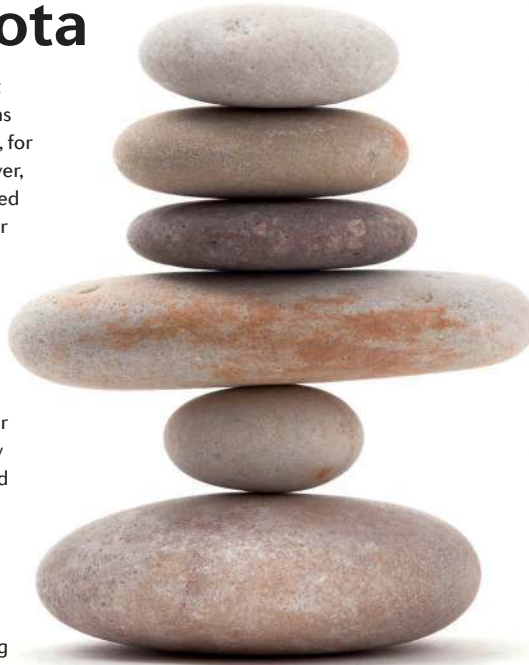
▶ MILESTONE 7

Stability and individuality of adult microbiota

A plethora of studies conducted over the past few decades have revealed strong associations between a disrupted microbiota and diseases, for example, inflammatory bowel disease. However, the key to understanding the role of a disrupted microbiota in human diseases is first to answer the question: what is a 'normal' microbiota? This question still frustrates many microbiologists, even with the advent of high-throughput sequencing and 'omics techniques. Prior to the availability of these technologies, however, several studies were instrumental in answering similar questions to help define 'normal', such as how much microbial variation is there between and within adults, and is the microbiota stable?

In 1998, when conventional microbiological techniques involving plate count analyses had reached an impasse in what they could reveal about human microbial diversity, molecular approaches were instead beginning to be implemented. A study by Willem de Vos and colleagues used polymerase chain reaction amplification of regions of the 16S ribosomal (r)RNA gene, which is often used to infer the genetic relationships between organisms, and then temperature gradient gel electrophoresis (TGGE) to visualize the diversity of the amplified gene. Comparisons of the banding profiles generated by TGGE from 16 adult faecal samples indicated that each individual has their own unique microbial community. Furthermore, by monitoring two individuals over time, the researchers showed that the TGGE profiles were stable over a period of at least six months.

Similar molecular approaches were applied to different sites of the human body, with the goal of improving our understanding of adult human microbial diversity. In 2005, one study moved beyond using faecal microbiota as a surrogate for the entire gut microbiota and sampled multiple colonic mucosal sites from three healthy individuals. Through an analysis of 13,335 16S rRNA gene sequences, this work confirmed marked microbial variation between individuals and showed that the adult gut mucosal microbiota was dominated by Bacteroidetes and Firmicutes, whereas Actinobacteria, Proteobacteria and Verrucomicrobia were relatively minor constituents.

Another study went one step further, in 2009, and examined bacterial diversity of 27 body sites from at least seven individuals and at four different time points. High interpersonal variability was found across all body sites but individuals experienced minimal temporal

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diversity. Other studies dedicated to skin and vaginal microbiomes were also key to our understanding of an individualised microbiota. It was found that comparable skin sites had similar bacterial communities, but that the complexity and temporal stability of the communities were site-dependent, whereas other studies found that the vaginal microbiome differs among individuals and, markedly, change over a short time. Together, these studies revealed that the human microbiota is highly variable both within and between individuals.

One of the goals of many studies characterising the diversity and stability of the human microbiota was to establish whether there was a core microbiota — are there bacterial species that we all share? In 2010, the international MetaHIT (Metagenomes of the Human Intestinal Tract) project published a gene catalogue derived from 576.6 gigabases of metagenomic sequences from the faecal samples of 124 individuals. These genes were found to be largely shared by individuals of the cohort, and 18 species were detected in all individuals. However, a key study that examined the faecal microbiomes of six adult twin pairs and their mothers suggested that there was an identifiable core microbiome at the gene level rather than the microbial species level. In this study, individuals shared >93% of the enzyme-level functional groups, but no bacterial phylotypes were present at >0.5% in all samples.

Many of these concepts were subsequently confirmed in large-population studies published by the Human Microbiome Project (HMP) Consortium. Analysis of samples collected from 242 healthy adults from up to 18 body sites showed that each habitat is characterized by a small number of highly abundant signature taxa, but that the relative abundance of taxa and genes in each habitat varies between individuals.

One drawback to the HMP dataset is the limited temporal scope. Instead, other studies have shed further light on the stability of the adult microbiota. For example, in one study, a human microbiota time series was obtained covering two individuals at four body sites over 396 time-points (daily for up to 15 months). Despite finding stable differences between body sites and individuals, this high-resolution temporal analysis did show pronounced variability in an individual's microbiota across months, weeks and days. In another study, an analysis of the faecal microbiota of 37 individuals found that ~60% of bacterial strains remained stable for up to five years.
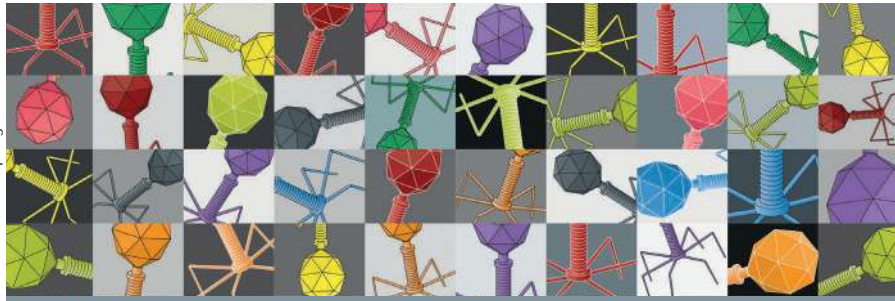
Overall, samples obtained from the same individual are more similar to one another than those from different individuals, suggesting each person has a microbiota that is distinct and stable. Much is still unknown regarding how stable the microbiota is to perturbations, such as those arising from antibiotics, diet and the immune system. However, further studies in-line with those discussed here will no doubt enhance our view of human microbiota dynamics to ultimately understand what is 'normal'.

Iain Dickson,
Nature Reviews Gastroenterology & Hepatology

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> **MILESTONE 8**

Beyond bacteria: studies of other host-associated microorganisms

While bacteria are a major component of the human microbiota, viruses, fungi and archaea are also important members of the community, with potential effects on human health. The development of 16S ribosomal RNA gene sequencing transformed the field of microbiome research by enabling bacterial and archaea phylogenetic analyses without the need for culturing (MILESTONE 6). Fungi can also be identified and classified by sequencing a common nuclear ribosomal internal transcribed spacer region. Viruses, however, are far more challenging to isolate and sequence due to the necessity of a eukaryotic or prokaryotic host and the absence of conserved genes. The modern era of human-associated viral metagenomics actually started in the ocean. In 2001, the research group of Forest Rowher, a marine microbial ecologist at San Diego State University, published a randomized shotgun library sequencing method to analyse genomic DNA from a single bacteriophage. This was rapid, reasonably unbiased and required very little DNA input, a crucial limiting variable for sequencing. The research team further demonstrated the utility of this technique beyond single phage analysis by characterizing the more complex viral make-up of seawater and marine sediment, paving the way for analyses of human viromes and a deeper characterisation of the human microbiome.

By 2003, bacteriophages that infected individual bacterial species had been identified from human faecal waste, but the diversity and relative abundance of different phages remained unknown, as existing approaches were biased towards bacteria already known to be infected by phages. By using the linker-amplified shotgun library approach, Rowher's research group provided the first quantitative description of the composition of the uncultured virome in human faeces collected from a single healthy adult. The majority of phage sequence matches were from temperate phages, which commonly integrate into the host bacterial genome. The faecal virome was also dominated by phages known to infect Gram-positive bacteria, in keeping with prior data on faecal bacterial content.

Viral metagenomics has continued to advance, with higher-throughput techniques enabling more rapid virus discovery and classification in both healthy and diseased tissues, and helping us to understand the role of commensal and pathogenic viruses in the context of the wider microbiome.

In a study published in 2010, by Jeffrey Gordon and Rowher, faecal viromes of healthy adult female twins and their mothers were shown to be individually distinct and stable over the course of a year, in agreement with faecal bacterial data from this same cohort. Other studies have begun to elucidate the effects of disease and diet on the gut virome, finding that phage composition became increasingly similar between individuals on the same diet, and that significant expansion of one phage order is associated with Crohn's disease and ulcerative colitis. Viral microbiome signatures may therefore be associated with environmental influences as well as disease progression, including in tissues beyond the gut.

Shotgun metagenomics has facilitated a more functional and interconnected perspective of the microbiome, with the contributions of fungi and archaea also becoming increasingly understood. The human mycobiome (the fungal community of humans) has also been fruitfully studied. A number of recent studies have highlighted the crucial roles of fungi in both healthy and disease states. For example, enteric commensal bacteria and fungi may have redundant protective and tolerizing functions in the context of regulating the immune response, suggesting that gut homeostasis can be retained through the mycobiome even in the absence of 'good' bacteria. In inflammatory bowel diseases, however, mycobiome dysbiosis contributes to disease progression, with fungi enriched at the expense of bacteria. These data, along with other studies, suggest a complex interplay between fungi and bacteria.

Recently, the human archaeome (the community of human-associated archaea) has attracted interest due to a number of observations, including recognition of the human gut archaeon *Methanosphaera stadtmanae* by the immune system and the discovery of previously undetected human-associated archaea. Methanogenic archaea are amongst the most abundant microorganisms in the human gut and sometimes outnumber even the most abundant bacterial species. In 2004, methanogenic archaea were found to be associated with the onset of periodontal disease, identifying the first link between archaea and human disease. As with bacteria, viruses and fungi, archaea have been isolated from various human anatomical sites, including the gut, skin, vagina and oral cavity. Yet, owing to fundamental differences in biology, they have often remained undetected in microbiome surveys, warranting further investigation of the human archaeome and the role of archaea in human health and disease.

As identification and characterization of the human virome, mycobiome and archaeome in various tissues and disease states continues to improve, it will be increasingly important to delineate function and how these other 'omes' interact as a community to preserve or dysregulate human health.

Saheli Sadanand, Nature Medicine

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Regulation of mucosal immunity by the microbiota

The relationship between the immune system and microorganisms was long viewed as a war rather than a union, as it was mostly studied in the context of host defence against pathogens. Thus, when deficiency in lymphoid organ development and immune cell activity was reported in germ-free animals in the 1960s, this first evidence that microbiota shape immune homeostasis was interpreted as education of the immune system by infections.

This concept laid the foundation for the 'hygiene hypothesis', formalized by David Strachan in 1989 in his influential study reporting lower incidence of hay fever and eczema in children with older siblings. He proposed that infections in early childhood prevent atopy later in life, and that increased allergy prevalence in developed countries may be caused by high standards of personal hygiene. Further developments of the hygiene hypothesis correlated pathogen exposure to decreased allergy risk in humans, and established a role for microbiota in oral tolerance in mice, presumably in shifting an immune set point from T helper 2 (Th2) to Th1 cell responses.
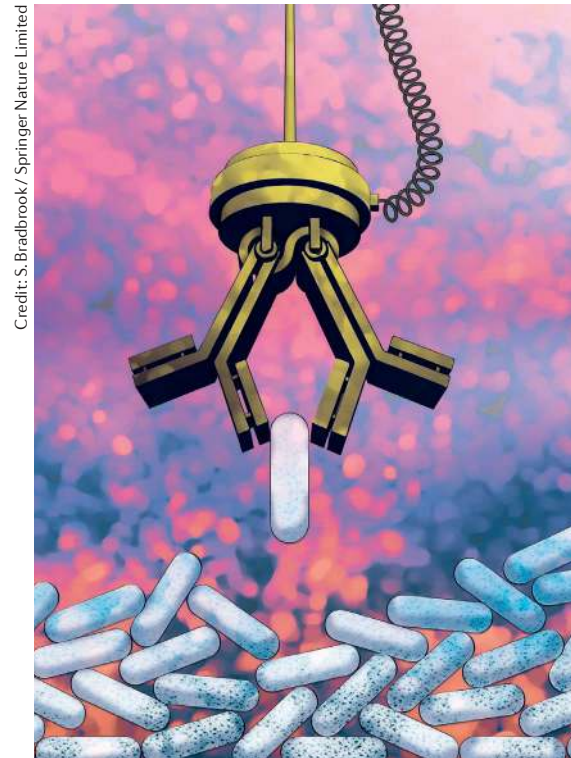
In parallel, fundamental principles of microorganism recognition by the immune system unfolded. In 1989, Charles Janeway proposed that immune responses are initiated by genome-encoded pattern recognition receptors (PRR) on immune cells, which sense conserved microbial molecules. Over the next decade, PRRs specific to various bacterial, fungal and viral components were discovered. However, it became evident that PRRs are not specific to pathogens, posing the question of how commensals, which colonize mucosal surfaces, coexist with the immune system.

The prevailing explanation was that commensals and immune cells are separated by epithelial barriers. This view was challenged in the early 2000s by accumulating examples of immune responses to commensals in healthy mice. Among these, Andrew Macpherson, Rolf Zinkernagel and colleagues found that in mice, commensals are recognized and compartmentalized by gut lumen-secreted IgA. Lora Hooper, Jeffrey Gordon and coworkers reported that a commensal bacterium stimulates antimicrobial peptide production by Paneth cells.

In 2004, Seth Rakoff-Nahoum and Ruslan Medzhitov provided evidence that the immune system senses commensals through PRRs under normal conditions and that this sensing is crucial for tissue repair. This finding opened a new perspective on immune response to microorganisms not as host defence, but as a symbiotic physiological process.

Realization of beneficial roles of immune–microbial interactions prompted a revision of the hygiene hypothesis to postulate that protection from allergic diseases is mediated by early-life exposure to commensals rather than pathogens (MILESTONE 5). The refined hypothesis suggested that the rise of allergies in industrialized societies is caused by loss of commensals. A link between lifestyle, microbiota and allergic diseases has been now confirmed in a large number of human observational studies, and expanded to implicate microbiota in other chronic inflammatory and autoimmune pathologies, as well as metabolic and neurological disease.

Dissecting the underlying cellular and molecular complexity of microbiota–immune interactions has occupied microbiologists and immunologists to this day. Pioneering works by Sarkis Mazmanian, Dennis Kasper and colleagues showed that microbiota-guided maturation of the mouse immune system can be recapitulated by a polysaccharide produced by a symbiotic bacterium, and delineated that its uptake by dendritic cells promotes antigen presentation, inducing expansion and differentiation of CD4+ T cells into Th1 and regulatory T cell lineages. Ivaylo Ivanov,

Kenya Honda, Dan Littman and colleagues uncovered how specific commensal bacteria direct Th17 cell differentiation in the small intestine. In 2013–2014, four groups independently discovered a mechanism of immune tolerance mediated by metabolites of intestinal commensals (MILESTONE 21).

Our intimate companion in sickness and in health, microbiota impacts our physiology to a large extent through interactions with the immune system at mucosal sites. How these interactions, ranging from host defence to active tolerance to symbiosis, integrate into physiological outcomes remains an exciting avenue to explore.

Tanya Bondar,
Nature Communications

Credit: S. Bradbrook / Springer Nature Limited

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MILESTONES

The importance of feeding your microbiota

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Most complex plant polysaccharides are not digested by humans and enter the colon as a potential food source for the microbiota. But, in the late 1970s, the extent to which gut bacteria could metabolize this dietary fibre was largely unknown.

To bridge this gap, Abigail Salyers and colleagues tested the ability of a wide range of anaerobic bacterial species resident in the human colon to ferment plant polysaccharides as well as intestinal mucins (glycosylated proteins that line the gut epithelium). They found that the bacterial strains had a diverse and inducible ability to break down different substrates, with the largest variety of polysaccharides fermented by *Bifidobacterium* and *Bacteroides* species. The researchers proposed that by altering availability of preferred bacterial food sources in the host diet — such as limiting fibre intake — could trigger induction of enzymes capable of degrading the intestinal mucin layer, affecting human health and even colon cancer.

But it was not until 2005 that Jeffrey Gordon's group demonstrated that a change in diet in a mammal could alter the degradative activity of the colonic microbiota in vivo. By colonizing the gut of germ-free mice with *Bacteroides thetaiotaomicron* — which Salyers and colleagues had identified as a human symbiont capable of fermenting a wide range of glycan substrates — the researchers tested, in a physiologically relevant setting, the effects of different diets on expression of bacterial genes.
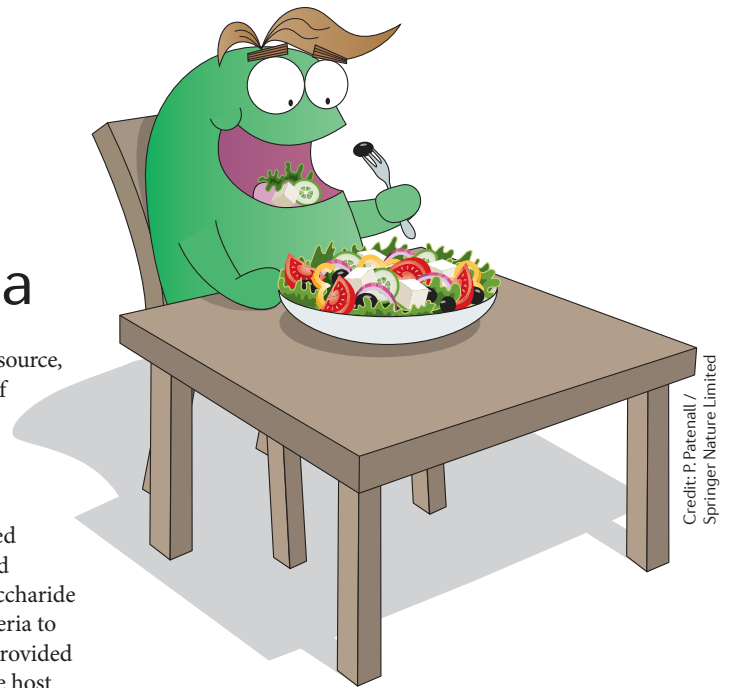
In mice fed a fibre-rich diet, *B. thetaiotaomicron* genes involved in polysaccharide metabolism were significantly upregulated compared to their expression in bacteria grown in a minimal medium. By contrast, in mice fed a diet devoid of complex polysaccharides, the most highly upregulated bacterial genes were those involved in host glycan degradation. The work confirmed in a vertebrate the differential expression of bacterial enzymes dependent on food source, exposing a flexible network of genes for harvesting glycans based on their availability in the host.

Subsequent work on *B. thetaiotaomicron* elucidated many of the gene clusters and pathways involved in polysaccharide metabolism that enable bacteria to use the diversity of glycans provided by a mammalian diet and the host itself. This ability to harvest host glycans, such as during fluctuations of a host's diet, was shown to critically affect survival of *B. thetaiotamicron* in mono-colonized mice fed a fibre-free diet. Such findings, as well as later studies, underscore the important interplay of host diet and glycan metabolism for gut colonization of human commensals and their persistence in populations over time.

We now know that gut microorganisms harbour thousands of genes involved in catabolism of carbohydrates, but how this enzymatic breadth was acquired remains unclear. One potential source of genetic diversity is horizontal transfer of genes from environmental microorganisms to gut bacteria. In a search for enzymes expressed by a marine Bacteroidetes that degrade sulfated polysaccharides found in edible seaweed (such as nori), porphyranases were identified that had a homologue in a gut bacterium, *Bacteroides plebeius*. Additional porphyranases and agarases were subsequently identified in the microbiomes of Japanese but not North American individuals. The findings suggest that transfer of genes from marine bacteria on nori was the likely origin of enzymes in the human gut that diversify the ability of bacteria to harvest energy from food sources, such as algal polysaccharides in seaweed, a major dietary component in Japan.

But do the nutrient foraging properties of gut microbiota have a role in human health? Results in mice suggest they may. A fibre-free diet in mice reduced the thickness of the colonic mucus layer, as predicted, and increased susceptibility to disease caused by a mouse enteric pathogen. In another study, the microbial production of short chain fatty acids from dietary fibre influenced mouse lung disease and immune responses. And microbial metabolism of other components of the human diet, such as L-carnitine in meat, has been linked to atherosclerosis.

Much remains to be understood of the impact of diet and the microbiota on human health and disease. But these studies shed light on the dynamic effect of altering host diet on the makeup and behaviour of microorganisms in the gut and other organs, with potential implications for disease modification and treatment.

Alison Farrell, *Nature Medicine*

> " Such findings […] underscore the important interplay of host diet and glycan metabolism for gut colonization of human commensals "

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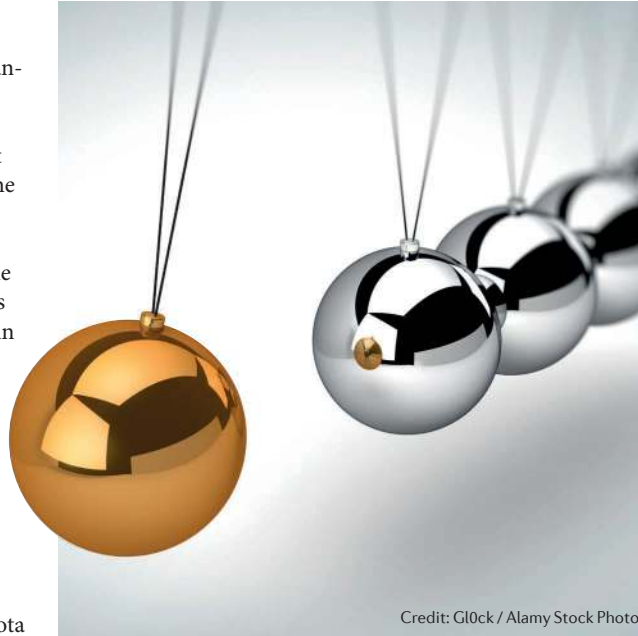
⤷ MILESTONE 11

Transfer of host phenotypes through microbiota transplantation

Chronic inflammatory conditions, such as obesity, diabetes, heart disease, autoimmune disorders and cancer, have long been associated with a Westernized diet. Environmental factors also play a role, with the gut microbiota taking centre stage in the past two decades. Indeed, high-fat diets (HFDs) regulate microbial communities in drastic fashion. The microbiota is now acknowledged as having direct, even causative roles in mediating connections between the environment, food intake and chronic disease.

Early studies using germ-free mice showed that body fat content and insulin resistance are transferable from obese to lean mice through exposure to faecal material. In a pioneering paper, researchers found that the microbiota of obese mice are more efficient at extracting energy from the host diet compared to the microbiota of lean ones and that increased adiposity is transferable. When the microbiota from the cecum of obese mice, which had a higher Firmicutes/Bacteroidetes ratio than lean donors, were transplanted into germ-free recipients, there was a greater increase in body fat than in recipients of microbiota from lean mice. Notably, the structure of the established colonizing community in the recipient was highly similar to that of the original donor, suggesting that the composition of the donor microbiota is critical for development of the obese phenotype.

Subsequent studies using gnobiotic mice (with a defined microbiota) showed that a single endotoxin-producing *Enterobacter* species isolated from an obese human's gut was sufficient to induce obesity, insulin-resistant phenotypes and systemic inflammation in mice fed a HFD. The results, derived from the first gnobiotic mouse obesity model, also represent the first demonstration of a causative role of the microbiota in human obesity.

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These findings have raised the possibility of developing therapies based on modulating the microbiota. One such proof-of-principle study found that small intestinal infusion of the gut microbiota from a lean donor restored insulin sensitivity and increased the microbial diversity (with concomitant short-chain fatty acid metabolism) in obese human subjects with untreated metabolic syndrome. This study also described a role for butyrate from gut microbial metabolism in improving insulin sensitivity, a first step in defining specific species and mechanisms used by gut microorganisms to modulate host physiology. Extending these findings beyond adiposity and insulin resistance, host phenotypes were also found to be transferable in mice by co-housing or through vertical transmission. For instance, a colitis phenotype (resembling human ulcerative colitis) can be induced by genetic mutation or can be transferred, vertically to wildtype progeny or horizontally to wildtype cage-mates, through co-housing — a process that is blocked with antibiotics.

> **"** These findings have raised the possibility of developing therapies based on modulating the microbiota **"**

More recently, the intestinal microbiota has been shown to modulate neurological and psychiatric diseases, as well as cancer, adding to those phenotypes that can be transferred by faecal material or mother-to-child transmission. Intestinal tumours can be induced in mice harbouring an oncogenic mutation in *Kras* with a HFD, independent of obesity, and this effect can be transferred to healthy adult, normal-diet, *Kras* mutant mice via the dysbiotic gut microbial community found in the faecal material of HFD mice. Inflammation-induced colorectal cancer is transferrable to co-housed mice, an effect that is blocked by antibiotic treatment of donor mice. The impact of gut microorganisms on promoting obesity and liver cancer induced by a Westernized diet is trans-generational — it can be seen in the progeny as well as the grandchildren of mother mice consuming the diet, even though the mothers themselves are unaffected by these conditions. Finally, the intestinal microbiota influences brain development and function in mice. For instance, distinct anxiety levels of different mouse strains are linked to distinct microbiota compositions and the phenotypes are transferrable via faecal matter.

There is a large body of anecdotal and direct evidence suggesting that the microbiota has a role in the health of many human functional systems. Being able to transmit phenotypes via whole microbiota, individual microorganisms or, eventually, through individual microorganism-derived metabolites, should prove revolutionary for treatment of disease and maintenance of a healthy human condition.
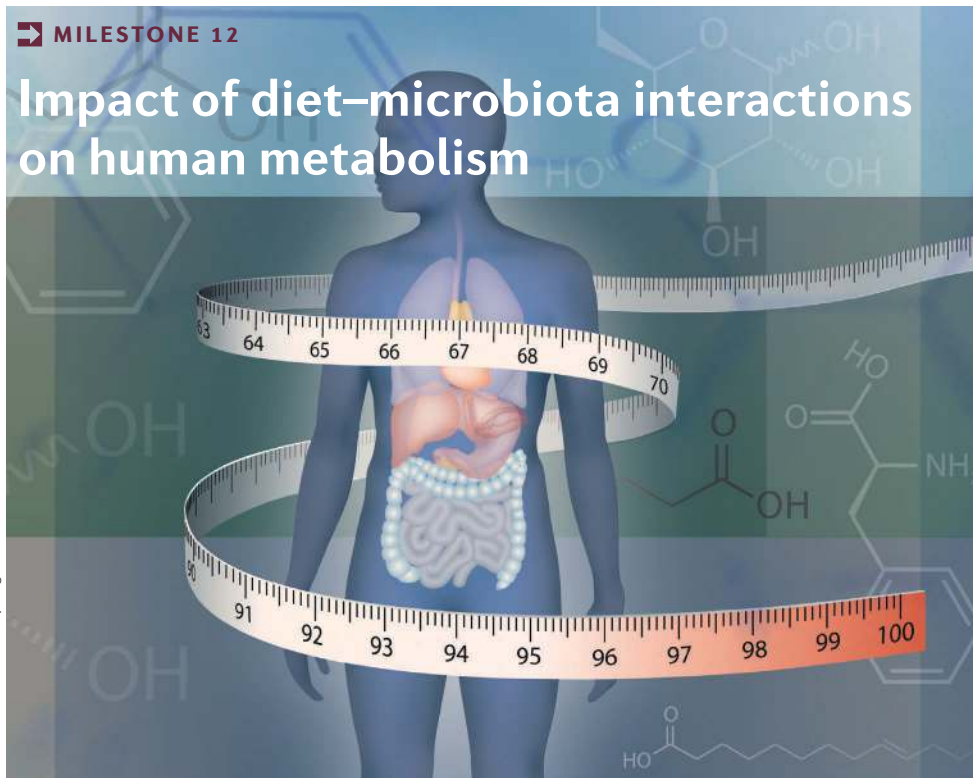
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MILESTONES

Impact of diet–microbiota interactions on human metabolism

Credit: S. Bradbrook / Springer Nature Limited

While studies had made it clear that our gut microbiota could metabolize dietary components (MILESTONE 10), it was yet to be confirmed whether these diet–microbiota interactions had implications for human health.

Jeffrey Gordon's group jump started research into the links between the gut microbiota and obesity with a series of mouse studies. In 2004, this group found that germ-free mice had reduced body fat compared to conventional mice, even though they consumed less food. A year later it was shown that a mouse model of obesity had an altered ratio of the two main phyla present in the gut; the Bacteroidetes and the Firmicutes. A functional analysis of these microbiomes revealed that an obesity-associated microbiota had an increased capacity for energy harvest, and this phenotype could be transferred through faecal microbiota transplant (MILESTONE 11).

Members of the Gordon lab continued this line of research, but this time in humans. In 2006, Ley et al. found that obese individuals had a reduction in the relative abundance of Bacteroidetes compared to lean individuals, and that this could be reversed using diet. This triggered numerous studies of the microbiota in the context of obesity and malnutrition. While many were in agreement and identified an obesity-associated microbiota, others

identified no trend, the opposite trend or found that diet was in fact the main driver, rather than the obese state. Given the conflicting results, three reanalysis papers were published, using publicly available datasets, in an attempt to uncover conserved microbiota signatures of obesity. The overarching results were that phylum-level signatures were not generalizable, especially at the population level, however Shannon diversity and evenness, the number of operational taxonomic units, and obesity status did have significant associations, albeit relatively weak.

However, diet was found to consistently alter the gut microbiota. For

> **These studies highlight the crucial impact that diet can have on the gut microbiota and host metabolism**

example, the high-fibre diets that are typically consumed by rural communities were associated with increased microbial diversity, an enrichment of *Prevotella* species, higher concentrations of health-promoting short-chain fatty acids and a reduction in metabolic disease.

Studies on a range of other metabolic diseases in relation to the microbiota and diet, such as type 2 diabetes and cardiovascular disease, also emerged. A clear link between the ability of the gut microbiota to metabolise dietary phosphatidylcholine into trimethyl-amine-*N*-oxide and the development of cardiovascular disease was confirmed in a series of papers by Stanley Hazen and colleagues.

Given the substantial impact of diet on the microbiota, numerous research groups have attempted to harness this power in order to modulate the gut microbiota to alleviate metabolic disease. In 2015, Zeevi et al. used gut microbiota data, together with blood parameters and metadata, to develop a machine-learning algorithm that could predict an individual's glycemic response to a particular meal, resulting in a personalized diet that could lower post-meal glucose. A more general dietary intervention was recently used for the treatment of type 2 diabetes mellitus. In 2018, Zhao et al. used a high-fibre diet to promote colonization by short-chain fatty acid producers and to improve haemoglobin A1c levels, which was used as a readout of type 2 diabetes status.

These studies highlight the crucial impact that diet can have on the gut microbiota and host metabolism, the resulting implications for human health, and how we can use our knowledge of these interactions to develop nutrition-based treatments.

Emily White, Nature Microbiology

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⇥ **MILESTONE 13**

Mechanisms of colonization resistance

In 1917, as war was tearing its way across Europe, a fascinating scientific observation was being made. The German physician Alfred Nissle had been looking for novel therapies to tackle enteric infections, which in this pre-antibiotic era represented an enormous burden on troops. He noted that one soldier in particular, who had participated in a military campaign in the Balkans, proved stubbornly resistant to dysentery when many of his comrades had been laid low by the disease. Speculating that a component of this soldier's intestinal microbiota might be responsible for this resistance, Nissle acquired stool samples and was able to isolate a strain of bacteria that came to be known as *Escherichia coli* Nissle 1917. Laboratory testing, as well as some self-experimentation on the part of Nissle, showed that this novel strain of *E. coli* was indeed able to antagonise pathogenic bacteria and it soon entered clinical practice. Although his identity is lost to history, the soldier's donation of his

> **"**
> The host's microbiota can manifest colonization resistance through a number of potential mechanisms
> **"**

unique *E. coli* strain is still used to this day as the active component of the probiotic Mutaflor.

In many ways, the findings of Nissle were built on earlier concepts articulated by the 'father' of cellular immunology Élie Metchnikoff, who in a monograph in 1910 had lauded the consumption of soured milk (rich in bacteria) as a means to stave-off infectious disease and enhance human longevity. Indeed, peasants from the Balkans and Caucasus had long-been famous not only for their centenarians but also for millennia-old traditions of yoghurt-making. However, while it seemed that certain strains of bacteria could have beneficial properties on their host, perhaps in part through their direct antagonism of enteric pathogens, the mechanistic basis of these remarkable effects were almost wholly unknown. Arguably, the first in-roads into this question were made in the mid-1920s, in Belgium, by an often-overlooked early pioneer of microbiology — André Gratia.

Along with his colleagues, most notably Sarah Dath, Gratia observed antagonism between co-cultures of different strains of *E. coli*. This effect was attributed to a secreted factor, which came to be known as 'colicin'. This protein now represents the first described member of an unrelated family of narrow-spectrum, bacterially-produced antibiotics known as 'bacteriocins'.

Another important step in understanding the role played by the host's microbiota in resistance to enteropathogenic bacteria was made in the 1950s, by Marjorie Bohnhoff and colleagues at the University of Chicago, and subsequently in the early 1970s by Dirk van Waaij and colleagues in the Netherlands. Secondary infections are a common occurrence following a course of antibiotics, suggesting that some perturbation of the microbiota might be responsible. In order to model this phenomenon, these studies found that mice that had their microbiota heavily-depleted by antibiotics were drastically more susceptible to oral challenge with even mildly pathogenic strains of *Salmonella* or *E. coli*. A 1971 paper by van Waaij and colleagues was especially important for its coining of the term 'colonization resistance' and placing it into a quantitative framework.

The host's microbiota can manifest colonization resistance through a number of potential mechanisms, for example, 'passively' by out-competing bacteria for space and trophic resources, or more actively by the generation of bacteriocidal factors. Three key papers in 2007 illuminated different aspects of colonization resistance. The probiotic strain *Lactobacillus salivarius* UCC118 is known to produce the bacteriocin Apb118. Conor Gahan and colleagues observed that this probiotic strain could protect mice against infection with *Listeria monocytogenes* and this effect was wholly dependent on the production of Apb118. However, *L. salivarius* UCC118 also conferred protection against a strain of *Salmonella* resistant to Apb118, suggesting that colonization resistance by this probiotic is more multi-faceted than

simply the production of a bacteriocin. A second pair of unrelated papers set out to understand how enteropathogens could overcome colonization resistance. In separate mouse studies and using different enteropathogens (*Citrobacter rodentium* or *Salmonella*), it was shown that intestinal inflammation altered the composition of the host's microbiota and made them susceptible to colonization by the invading bacteria. In both cases the bacteria needed to be able to elicit gut inflammation in order to establish themselves — in other words, this appeared to be a case of the enteropathogen co-opting the host's immune response to its advantage.

Colonization resistance has proved to be a useful model for understanding the dynamics of microbial communities in the gut and other barrier surfaces, such as the skin, however in one sense it is strikingly similar to the much earlier ecological concept of 'allelopathy'. Initially outlined in the 1930s to describe interactions between certain plant species, allelopathy was later broadened to describe the suppression of any competitor organism by another through the generation of biologically active factors.

As we teeter towards the dangers a post-antibiotic era, further insights from the study of colonization resistance could offer the hope of novel antimicrobial therapies.

Zoltan Fehervari, *Nature Immunology*

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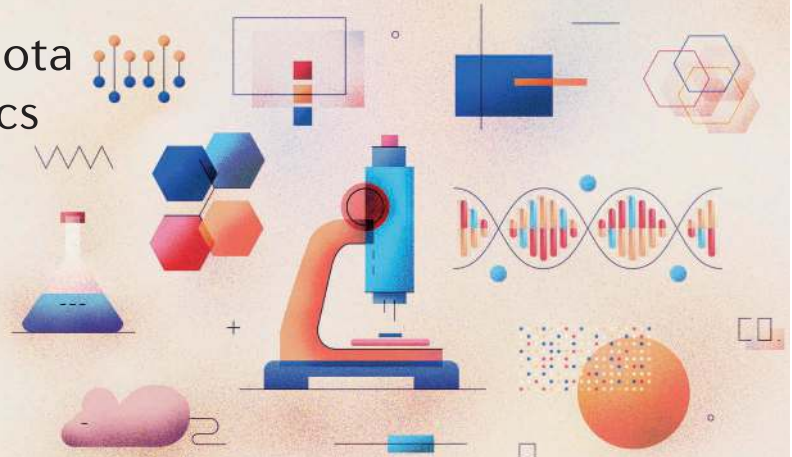
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➡ MILESTONE 14

Functional human microbiota analyses in vivo using 'omics technologies

Eline Klaassens and colleagues applied a metaproteomics approach to uncultured faecal microbiota, providing the first insights beyond taxonomic identification. This was followed by numerous studies using 'omics methods, such as metabolomics and metatranscriptomics, as well as the development of multi-omics pipelines; methods that are still uncovering the functions of the microbiota today.

Credit: Antlii / Alamy Stock Photo

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Molecular cartography of the human skin surface in 3D. *Proc. Natl Acad. Sci. USA* **112**, 2120–2129 (2015) | Heintz-Buschart, A. et al. Integrated multi-omics of the human gut microbiome in a case study of familial type 1 diabetes. *Nat. Microbiol.* **2**, 16180 (2016) | Franzosa, E. A. et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat. Microbiol.* **4**, 293–305 (2019).

⇲ **MILESTONE 15**

Antibiotics alter the gut microbiome and host health

Antibiotics not only act on bacteria that cause infections but also affect the resident microbiota. Although this side effect has long been appreciated, advances in sequencing technologies enabled detailed study of how antibiotics alter the gut microbiome.

Although the composition of the gut microbiota varies between individuals, the community in each individual is relatively stable over time (MILESTONE 7). In 2008, Relman and colleagues studied three healthy individuals and showed that treatment with ciprofloxacin influenced the abundance of about one third of bacterial taxa in faecal samples. These changes decreased the taxonomic richness, diversity and evenness of the community. Although most bacterial groups recovered after treatment, several taxa did not (even after six months) and the level of reconstitution varied between the individuals. A follow-up study showed that a second course of ciprofloxacin had similar effects. There was no correlation between the magnitude of the microbiome shift in the first and second treatments within any individual; each treatment was another 'roll of the dice'.

Owing to the close links between the resident microbiota and the host, such disturbance of the microbiota by antibiotics can be expected to affect host physiology and potentially host health. Indeed, one study found that administration of antibiotics to young mice increased adiposity and levels of metabolic hormones in the blood, and faecal transplantation from antibiotic-treated mice to germ-free mice transferred the metabolic phenotype. Besides effects on metabolism, the gut microbiota also closely interacts with and influences the host immune system, and thus microbiome disturbances have the potential to affect the development of several autoimmune, inflammatory and allergic diseases.

Antibiotic treatment of mice from birth also alters the expression of genes and the number of cells that regulate immune responses. Pulsed

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> **Treatment with ciprofloxacin influenced the abundance of about one third of bacterial taxa [and] decreased the taxonomic richness, diversity and evenness of the community**

antibiotic treatment increased the incidence of type-1 diabetes in susceptible mice, and the relative levels of anti-inflammatory T cells were lower in these mice (prior to the onset of disease) than in untreated mice. Neonatal treatment with antibiotics increased the susceptibility of adult mice to imiquimod-induced psoriasis.

Similar observations have been made for asthma. Children who are exposed to antibiotics in their first year of life had a slightly increased risk of developing asthma, and the risk increased with the number of antibiotic courses. Regression analysis from children with asthma identified that early exposure to antibiotics is a risk factor. The abundance of the genera *Faecalibacterium*, *Lachnospira*, *Veillonella* and *Rothia* (FLVR) were decreased in children at three months of age who had been treated with antibiotics and later developed asthma. In faecal transfer experiments from one of these children, the addition of FLVR decreased disease severity in an

asthma mouse model. The changes in community composition were accompanied by changes in metabolites such as acetate, a short chain fatty acid that is known to influence host metabolism and immune function. In another study, early life exposure to the antibiotic vancomycin indeed increased immunoglobulin E and decreased regulatory T cell levels in mice.

A study looking at antibiotic perturbation of the gut microbiota and the risk of developing inflammatory bowel disease, also showed that transfer of the disturbed gut microbiota from mouse mothers to their newborn pups, promoted and accelerated the development of gut inflammation in the offspring.

Certain interventions could help restore the gut microbial community to its original state if antibiotics are needed. For example, Elinav and colleagues recently found that the administration of certain probiotics hindered, and autologous faecal transplantation helped restore, the microbiome.
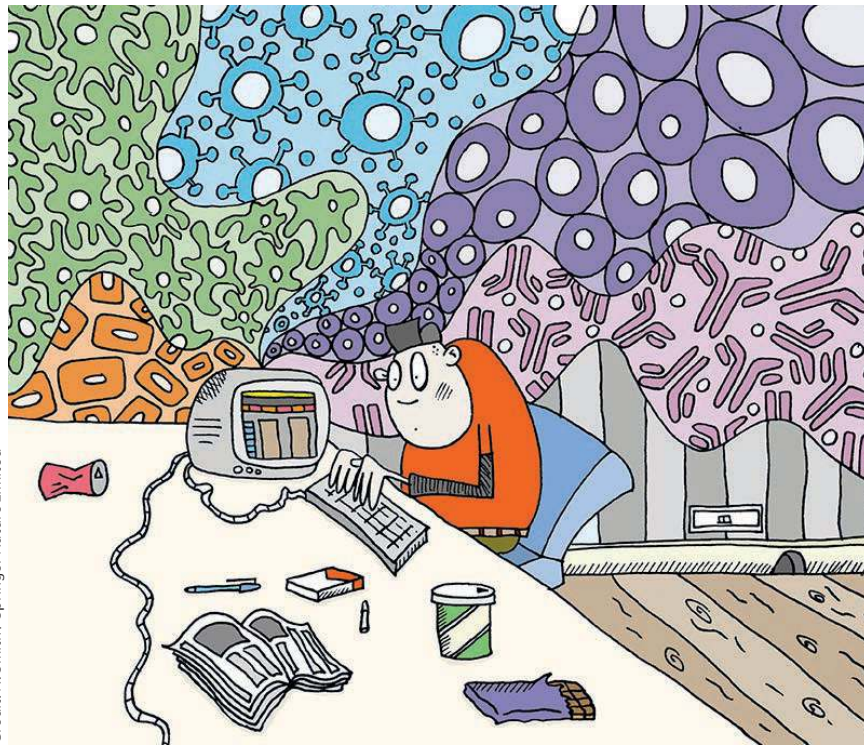
The observation that the gut microbiome can be permanently perturbed even by short-term or low-dose antibiotic treatment, and that this change can have long-term effects on health, cautions against widespread and potentially unnecessary use of antibiotics, particularly in young children and pregnant women, and illustrates that antibiotics should not be considered harmless. However, it also raises hopes for microbiome modulation as a therapeutic treatment for immune conditions.

Megan Cully,
Nature Reviews Drug Discovery

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metabolic alterations affect risk of childhood asthma. *Sci. Transl. Med.* **7**, 307ra152 (2015) | Russell, S. L. et al. Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. *Gut Microbes* **4**, 158–164 (2013) | Livanos, A. E. et al. Antibiotic-mediated gut microbiome perturbation accelerates development of type 1 diabetes in mice. *Nat. Microbiol.* **1**, 16140 (2016) | Schulfer, A.F. et al. Intergenerational transfer of antibiotic-perturbed microbiota enhances colitis in susceptible mice. *Nat. Microbiol.* **3**, 234–242 (2018) | Suez, J. et al. Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell* **174**, 1406–1423 (2018)
FURTHER READING Willing, C. P. et al. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat. Rev. Microbiol.* **9**, 233–243 (2011)

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⬛ **MILESTONE 16**

Bioinformatics tools facilitate the analysis of microbiome sequencing data

The software pipeline QIIME, which stands for 'quantitative insights into microbial ecology', enables the analysis and interpretation of the increasingly large datasets generated by microbiome sequencing.

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➦ **MILESTONE 17**

Microbiome analyses in large human populations

Microbiome composition and function have been implicated in various diseases. However, understanding and exploiting the interactions of the microbiome with the human host is tempered by the huge diversity of the microbiome within and between individuals. Advances in metagenomics and high-throughput sequencing in the early 2000s, inspired projects aimed at capturing microbiome diversity in large human populations. For example, one of the first such projects, Metagenomics of the Human Intestinal Tract, studied faecal samples from 124 individuals, generating 576.7 gigabases of sequence data. The authors noted that this is "almost 200 times more than in all previous studies" and "provides a broad view of the functions important for bacterial life in the gut".

Besides the scientific achievement of this and similar projects, such as the Human Microbiome Project, Belgian Flemish Gut Flora Project, Dutch LifeLines-DEEP study and others, one should note and acknowledge the fruitful collaboration of these large scientific consortia, lately even including citizen scientists in American Gut. Furthermore, dedicated support from several funders has been essential.

As mentioned above, initial studies looked at around 100 individuals; by contrast, today's studies can contain samples from several thousand participants. Larger numbers, as well as refinement and standardisation of protocols and pipelines, and the availability of larger reference data sets — all of which have profited hugely from early microbiome population studies — add to the robustness of results. Sometimes it has been difficult to compare studies and there have been studies with contradictory results. Nevertheless, large population studies have greatly advanced our understanding of what constitutes a 'normal' human microbiome, although this statement should be qualified by the fact that North Americans and Europeans are the best-studied populations. Projects are underway to ameliorate this bias.

In general, the microbiome differs not only between healthy individuals and those with diseases, even between healthy people there is a large diversity — there is no uniform 'healthy' microbiome. Some general measures, such as high taxonomic and functional richness, which usually correlate with a diverse, fibre-rich diet, seem to be beneficial.

Population studies have also identified factors that shape the microbiome, and have helped quantify their impact, including body site, diet, drugs, host genetics and others.

Furthermore, it has been possible to identify disease-specific microbiome signals, for example, for type-1 diabetes mellitus (MILESTONE 9), metabolic syndrome, obesity (MILESTONE 12), inflammatory bowel disease and others. Notably, the second phase of the Human Microbiome Project used an integrative approach that combines several 'omics techniques to study the role of the microbiome in preterm birth, the development of type-2 diabetes mellitus and inflammatory bowel disease over time. Identifying such signals is a first step towards understanding how the microbiome might contribute to disease development, and towards the development of preventative and therapeutic applications.

For example, individual microbiome differences are associated with the response to cancer treatment (MILESTONE 24). This finding and other studies have inspired plans to exploit microbiome differences for individualized therapies and interventions.

In summary, large population studies have greatly advanced our understanding of gut microbiome diversity and have identified numerous potential links to health and disease, inspiring many new research avenues. They have also made essential contributions to establishing methods and standards on which future work can build. Finally, these studies have highlighted the importance of the microbiome, not only for scientists but also for the general public.

Ursula Hofer,
Nature Reviews Microbiology

> " Besides the scientific achievement […], one should note and acknowledge the fruitful collaboration of these large scientific consortia "

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FURTHER READING The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012) | The Human Microbiome Project Consortium. A framework for human microbiome research. *Nature* **486**, 215–221 (2012) | Goodrich, J. K. et al. Human genetics shape the gut microbiome. *Cell* **159**, 789–799 (2014) | Ding, T. & Schloss, P. D. Dynamics and associations of microbial community types across the human body. *Nature* **509**, 357–360 (2014) | Falony, G. et al. Population-level analysis of gut microbiome variation. *Science* **352**, 560–564 (2016) | Zhernakova, A. et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* **352**, 365–369 (2016) | Rothschild, D. et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature* **555**, 210–215 (2018) | McDonald, D. et al. Amercian gut: an open platform for citizen science microbiome research. *mSystems* **3**, e00031-18 (2018) | He, Y. et al. Regional variation limits applications of healthy gut microbiome references and disease models. *Nat. Med.* **24**, 1532–1535 (2018). | Fettweis, J. M. et al. The vaginal microbiome and preterm birth. *Nat. Med.* https://doi.org/10.1038/s41591-019-0450-2 (2019) | Zhou, W. et al. Complex host-microbial dynamics in prediabetes revealed through longitudinal multi-omics profiling. *Nature* **569**, 663–671 (2019) | Lloyd-Price, J. et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* **569**, 655–662 (2019) | Integrative HMP (iHMP) Research Network Consortium. The Integrative Human Microbiome Project. *Nature* **569**, 641–648 (2019)

⇲ MILESTONE 18

The microbiota–gut–brain axis

Credit: K. Lee / Springer Nature Limited

A link between the gut microbiota and the brain has long been surmised, but in recent decades, studies have started to report causal effects of the gut microbiota on our brains and behaviour, and the underlying molecular mechanisms have begun to be elucidated.

Several early studies in animal models provided evidence that stress can perturb the composition of the gut microbiota and that enteric pathogens can affect host behaviour. In 2004, a study showed that germ-free (GF) mice exhibit an upregulated hormonal response to stress induced by physical restraint, implying that the microbiota influences the neuroendocrine hypothalamic–pituitary–adrenal (HPA) axis, the central stress response system. However, the effects of the microbiota — or the absence thereof — on behaviour remained unclear. Seven years later, in 2011, several experimental findings in mice shed light on how a lack of conventional microbiota affects behaviour, gene expression in the brain and the development of the nervous system.

Studies revealed that GF and antibiotic-treated mice displayed reduced anxiety-like behaviour compared with specific pathogen-free (SPF) controls. For example, GF mice were found to spend more time on the open arms of the elevated plus maze (EPM), and in the illuminated compartment of the light–dark box, than their SPF counterparts. The offspring of GF mice that had been conventionalized with SPF microbiota, but not GF mice conventionalized as adults, showed behaviour similar to SPF controls, suggesting that the microbiota may influence the brain during a 'critical period' of development.

Related work showed an effect of differences in gut microbiota on behaviour. Mice treated with a mixture of antimicrobials (ATM) showed more exploratory behaviour, and GF BALB/c mice (which are typically timid) colonized with microbiota from another mouse strain exhibited more exploratory behaviour than those receiving BALB/c microbiota, and vice versa. Furthermore, it was found that treatment of SPF mice with the probiotic *Lactobacillus rhamnosus* (JB-1) reduced anxiety- and depression-like behaviour.

As well as behavioural differences, the brains of animals with altered or absent gut microbiota displayed various molecular differences. These included brain-region-specific changes in levels of brain-derived neurotrophic factor (BDNF; which is known to be modulated in anxiety and depression), differences in the expression of various neurotransmitter receptors and alterations in the turnover of certain neurotransmitters, including serotonin.
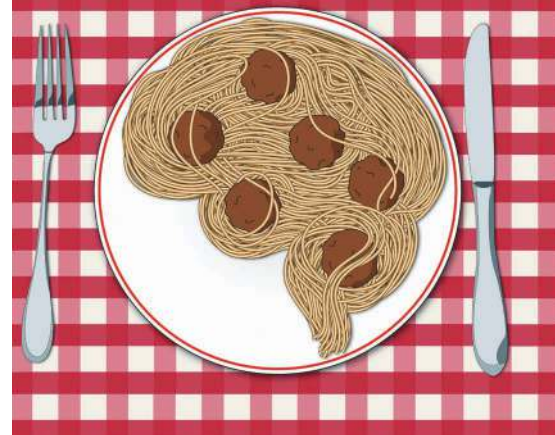
Indeed, much research since has focused on serotonin as a node of gut microbiota–brain interactions. Spore-forming gut bacteria were found to drive the production of serotonin by enterochromaffin cells in the mouse colon, although exactly how this may affect the brain has not been clear. Moreover, male (but not female) GF mice show higher levels of hippocampal serotonin and plasma levels of a serotonin precursor, suggesting that certain influences of the gut microbiota on the brain may be sex-specific.

How the gut microbiota signal to the brain has been the focus of much research. Evidence from models of multiple sclerosis and stroke suggested that changes in the gut microbiota may indirectly influence the central nervous system via effects on immune homeostasis and immune responses. In support of a vagus-nerve mediated route for gut-derived signals, severing the vagus nerve below the diaphragm blocked the anxiolytic and gene expression effects of *L. rhamnosus* (JB-1). By contrast, ablating the vagus nerve or sympathetic nerves did not prevent the effects of ATM on anxiety-like behaviour, and ATM-treated mice showed no overt signs of gut inflammation or alterations in enteric neurotransmitter levels, indicating that some gut–brain communication routes might be independent of the immune and nervous systems.

In fact, later research has started to uncover other means of gut–brain communication — in particular, microorganism-derived products that can directly or indirectly signal to the nervous system. For example, the offspring of immune-challenged mice showed gut dysbiosis, disrupted intestinal integrity and behavioural abnormalities (including anxiety-like behaviour), as well as high serum levels of a microbial metabolite that, when injected into wild-type mice, induced anxiety-like behaviour. Similarly, in a model of Parkinson disease (a neurological disorder associated with α-synuclein aggregation in the brain) the presence of gut microbiota or microbially produced short-chain fatty acids promoted neuroinflammation, motor impairments and α-synuclein pathology.

Nearly all of the work in this field to date has been carried out in animal models, and establishing whether those findings translate to humans will be crucial yet challenging. As an example of such an endeavour, a study investigated the link between faecal microbiota composition and quality of life using data from more than 1,000 people. As well as identifying bacterial genera associated with higher quality of life or depression, they carried out metagenomic analyses that indicated that the potential of microorganisms to synthesize certain neuroactive metabolites may also correlate with mental wellbeing.

Together, the studies described above have laid the foundations for our understanding of the effects of the gut microbiota on the brain and behaviour, and the mechanisms that underlie them, and represent initial efforts to explore the relevance of animal-model findings for humans.

Natasha Bray, Nature Reviews Neuroscience

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→ MILESTONE 19

Modern culturing efforts expand the culturable microbiota

High-throughput anaerobic culturing enabled the recovery of a large part of the diverse human gut microbiota and the creation of individual culture collections.

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→ MILESTONE 20

Global human microbiome

Genetic variation occurs between human populations living in different places, but little was known about variation in microbiomes. To investigate how gut microbiomes differ among human populations, Yatsunenko et al. characterized bacterial species in faecal samples from cohorts living in different regions, including the Amazonas of Venezuela, rural Malawi and US metropolitan areas. The authors found pronounced differences in the composition and functions in the gut microbiomes between these geographically distinct cohorts.

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FURTHER READING Schnorr, S. L. et al. Gut microbiome of the Hadza hunter-gatherers. *Nat. Commun.* **5**, 3654 (2014) | O'Keefe, S. J. D. et al. Fat, fibre and cancer risk in African Americans and rural Africans. *Nat. Commun.* **6**, 6342 (2014) | Obregon-Tito, A. J. et al. Subsistence strategies in traditional societies distinguish gut microbiomes. *Nat. Commun.* **6**, 6505 (2015) | Nishijima, S. et al. The gut microbiome of healthy Japanese and its microbial and functional uniqueness. *DNA Res.* **23**, 125–133 (2016) | Das, B. et al. Analysis of the gut microbiome of rural and urban healthy Indians living in sea level and high-altitude areas. *Sci. Rep.* **8**, 10104 (2018) | Pasolli, E. et al. Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. *Cell* **176**, 649–662 (2019) | Nayfach, S., Shi, Z. J., Seshadri, R., Pollard, K. S. & Kyrpides, N. Novel insights from uncultivated genomes of the global human gut microbiome. *Nature* https://doi.org/10.1038/s41586-019-1058-x (2019).
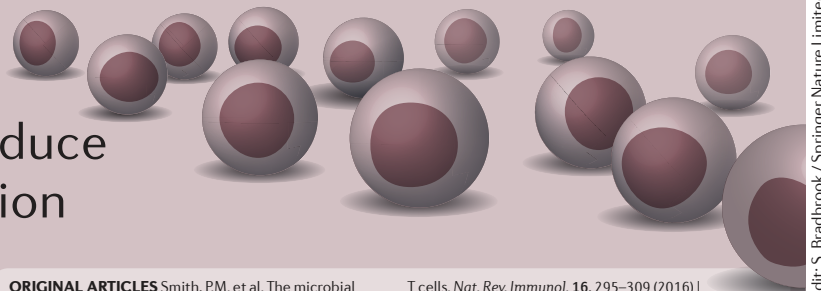
→ MILESTONE 21

Microbially-produced short-chain fatty acids induce regulatory T cell production

Regulatory T cells (Tregs) are crucial in maintenance of immune homeostasis. In 2013, three studies found that microbiota-derived short-chain fatty acids promote the expansion and differentiation of Tregs, revealing a form of chemical-mediated communication between the commensal microbiota and the immune system that affects immune mechanisms.

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↳ MILESTONE 22

Production of antibiotics by the human microbiota

Identification of biosynthetic gene clusters for antibiotics in the genomes of the human microbiota, suggests new sources of antimicrobial drugs whose species-specific production has the potential to modulate the local microbial community structure.

Credit: V. Summersby / Springer Nature Limited

ORIGINAL ARTICLE Donia, M. S. et al. A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. *Cell* **158**, 1402–1414 (2014). **FURTHER READING** Zipperer, A. et al. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* **535**, 511–516 (2016).
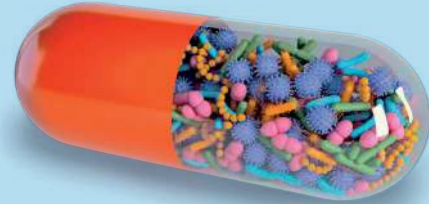
↳ MILESTONE 23

Host-targeted drugs affect microbiota populations

Commonly used medications affect gastrointestinal microbial abundances and bacterial gene expression, which may both positively and negatively contribute to the effects on human health associated with drug treatment.

Credit: Science Photo Library / Alamy Stock Photo

ORIGINAL ARTICLES Tsuda A et al. Influence of proton-pump inhibitors on the luminal microbiota in the gastrointestinal tract. *Clin. Transl. Gastroenterol.* **6**, e89 (2015) | Freedberg, D. E. et al. Proton pump inhibitors alter specific taxa in the human gastrointestinal microbiome: a crossover trial. *Gastroenterology* **149**, 883–885 (2015) | Forslund, K. et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* **528**, 262–266 (2015). **FURTHER READING** Maurice, C. F., Haiser, H. J. & Turnbaugh, P. J. Xenobiotics shape the physiology and gene expression of the active human gut microbiome. *Cell* **152**, 39–50 (2013) | Maier L. et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* **555**, 623–628 (2018) | Zimmermann, M. et al. Separating host and microbiome contributions to drug pharmacokinetics and toxicity. *Science* **363**, eaat9931 (2019).

↳ MILESTONE 24

Human microbiota affects response to cancer therapy

Following earlier studies in mouse models, gut microbiota composition was shown to affect the response of melanoma patients, and those suffering from advanced lung or kidney cancer, to immune checkpoint therapy, as well as tumour control.

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Credit: ImageSource

↳ MILESTONE 25

Metagenome-assembled genomes provide unprecedented characterization of human-associated microbiota

Advances in computational methods, recently pioneered in the environmental microbiology field, enable the reconstruction of bacterial genomes from metagenomic datasets. This approach was used to identify thousands of new uncultured candidate bacterial species from the gut and other body sites, of global populations from rural and urban settings, substantially expanding the known phylogenetic diversity and improving classification of understudied, non-Western populations.

ORIGINAL ARTICLES Pasolli, E. et al. Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. *Cell* **176**, 649–662 (2019) | Almeida, A. et al. A new genomic blueprint of the human gut microbiota. *Nature* https://doi.org/10.1038/s41586-019-0965-1 (2019) | Nayfach, S. et al. New insights from uncultivated genomes of the global human gut microbiome. *Nature* https://doi.org/10.1038/s41586-019-1058-x (2019).

Credit: N. Wallington / Springer Nature Limited