

Soggetto Realizzatore del Programma di Ricerca e Innovazione dal titolo "Ageing Well in an ageing society" - Age-It" (Partenariato Esteso - PE0000015) -
CUP B83C22004880006
MISSIONE 4 COMPONENTE 2, "Dalla ricerca all'impresa" INVESTIMENTO 1.3, NextGenerationUE

BANDO N. 400.2 IRGB PNRR BANDO N. 400.2 IRGB PNRR

Selezione per titoli e colloquio ai sensi dell'art. 8 del *"Disciplinare concernente le assunzioni di personale con contratto di lavoro a tempo determinato"*, per l'assunzione, ai sensi dell'art. 83 del CCNL del Comparto "Istruzione e Ricerca" 2016-2018, sottoscritto in data 19 aprile 2018, di una unità di personale con profilo professionale di Ricercatore III livello, presso l'Istituto di Ricerca Genetica e Biomedica (IRGB) - sede Monserrato (CUP B83C22004880006).

BUSTA N. 1

Il candidato illustri le esperienze più rilevanti descritte nel curriculum con riferimento alle competenze concorsuali.

Descrivere quali sono i passaggi preliminari per il filtraggio delle reads di sequenziamento di ampliconi 16S r-RNA.

Descrivere quali possono essere gli approcci pratici utilizzabili per la modulazione del microbiota intestinale.

Conoscenza della lingua inglese: lettura e traduzione (paragrafo A) dell'articolo "Intrahost evolution of the gut microbiota" tratto dalla rivista "nature reviews microbiology" (<https://doi.org/10.1038/s41579-023-00890-6>).

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BUSTA N. 2

Il candidato illustri le esperienze più rilevanti descritte nel curriculum con riferimento alle competenze concorsuali.

Descrivere quali sono i passaggi preliminari per il filtraggio delle reads ottenute dal sequenziamento full-shotgun metagenomics.

Descrivere il ruolo del microbiota nelle patologie infiammatorie intestinali.

Conoscenza della lingua inglese: lettura e traduzione (paragrafo B) dell'articolo "Intrahost evolution of the gut microbiota" tratto dalla rivista "nature reviews microbiology" (<https://doi.org/10.1038/s41579-023-00890-6>).

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BUSTA N. 3

Il candidato illustri le esperienze più rilevanti descritte nel curriculum con riferimento alle competenze concorsuali.

Descrivere una pipeline di analisi per dati di sequenziamento di ampliconi 16S r-RNA.

Descrivere qual è l'interesse e il possibile impatto clinico dell'individuazione di nuovi biomarcatori microbici per patologie infiammatorie intestinali.

Conoscenza della lingua inglese: lettura e traduzione (paragrafo C) dell'articolo "Intrahost evolution of the gut microbiota" tratto dalla rivista "nature reviews microbiology" (<https://doi.org/10.1038/s41579-023-00890-6>).

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BUSTA N. 4

Il candidato illustri le esperienze più rilevanti descritte nel curriculum con riferimento alle competenze concorsuali.

Descrivere una pipeline di analisi per dati di sequenziamento di full-shotgun metagenomics.

Descrivere i possibili fattori che hanno un impatto sulla composizione del microbiota intestinale durante la vita.

Conoscenza della lingua inglese: lettura e traduzione (paragrafo D) dell'articolo "Intrahost evolution of the gut microbiota" tratto dalla rivista "nature reviews microbiology" (<https://doi.org/10.1038/s41579-023-00890-6>).

Intrahost evolution of the gut microbiota

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Abstract

A massive number of microorganisms, belonging to different species, continuously divide inside the guts of animals and humans. The large size of these communities and their rapid division times imply that we should be able to watch microbial evolution in the gut in real time, in a similar manner to what has been done in vitro. Here, we review recent findings on how natural selection shapes intrahost evolution (also known as within-host evolution), with a focus on the intestines of mice and humans. The microbiota of a healthy host is not as static as initially thought from the information measured at only one genomic marker. Rather, the genomes of each gut-colonizing species can be highly dynamic, and such dynamism seems to be related to the microbiota species diversity. Genetic and bioinformatic tools, and analysis of time series data, allow quantification of the selection strength on emerging mutations and horizontal transfer events in gut ecosystems. The drivers and functional consequences of gut evolution can now begin to be grasped. The rules of this intrahost microbiota evolution, and how they depend on the biology of each species, need to be understood for more effective development of microbiota therapies to help maintain or restore host health.

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Introduction

Every human is colonized by microbial communities, the composition of which varies across different body sites¹. The gut of mammals contains an enormous density of microorganisms that belong to hundreds of different species, known as the gut microbiota. Its taxonomic diversity is commonly profiled by sequencing the 16S ribosomal RNA gene of the gut-colonizing bacteria (Fig. 1a), which can vary substantially across hosts and their health status^{2,3}. Therefore, it is critical to understand how a diverse gut microbial ecosystem can be assembled and maintained.

Two broad ecological interactions have been hypothesized to underlie the stability and resilience of gut microbiota: competition and cooperation. These interactions have been inferred from the dynamics of 16S ribosomal RNA profiles⁴, which can vary substantially after a perturbation (Fig. 1b). Nevertheless, establishment of causal effects in these complex ecosystems requires subsequent empirical studies^{5,6}. Theoretical modelling and empirical data suggest that competition, albeit of weak intensity, is a dominant interaction in the gut microbiota^{7,8}. That does not mean that cooperation is irrelevant in the gut, as examples of strong cooperation between microbiota members have been discovered^{9,10}. The gut ecosystem harbours a high richness of microbial genes, which in numerous instances complement the genetic make-up of their hosts. Many genes are critical for the microorganisms to compete for resources and to be maintained in the gut¹¹ and to prevent replacement by other microorganisms from the external environment^{12–14}. Other genes are key for the ability of the host to harvest energy from the food it ingests¹⁵ and even to modulate the host immune system, a process that starts at birth¹⁶ and that can have consequences for the host later in life¹⁷.

By contrast, the host immune system is also capable of modulating the species diversity of its gut microorganisms¹⁸. Hosts with compromised immune systems, or with polymorphisms in immune genes, have a distinct microbiota composition when compared with healthy hosts^{19–21}. IgA, the most abundant immunoglobulin in the gut,

is thought to have an important role not only in the maintenance of a diverse gut microbiota composition but also in microbial gut biogeography²². In addition, maternal IgA is also important in protecting infants from necrotizing enterocolitis²³, a disease leading to high mortality.

Colonization of the human gut by different species, from the mother and the external environment, occurs at birth, and a complex crosstalk between microbial cells and the immune system starts to happen. High abundances of bacteria and smaller numbers of fungi and archaea start to divide in the human gut²⁴. The large sizes of each of the populations in the infant gut and the novel environment they encounter are likely to lead to the occurrence of intrahost evolution throughout life^{25,26}. High mutational inputs, complex selective pressures, such as competition for fluctuating resources²⁷, intermicrobial interactions⁴, host–microbial interactions²⁸ and short generation times²⁹ lead to the expectation of a gut ecosystem in which rapid evolution should occur (Fig. 1c). In this Review, we explore the mechanisms likely to be responsible for such evolution, as well as recent work showing evidence for how evolutionarily dynamic the mammalian gut microbiota seems to be.

Mechanisms of evolution

Evolution is the change in allele frequencies in a population. The main mechanisms responsible for evolution in any population are migration, mutation, genetic drift, natural selection and recombination. When considering intrahost evolution in the gut, the strength of each of these mechanisms is likely to vary along the lifetime of the host. Migration and drift are, in principle, dominant mechanisms driving microbiota evolution at birth. The gut of a neonate is close to sterile, and a certain amount of random chance occurs in the strains that colonize the gut at this life stage. Bottlenecks associated with colonization events by external strains into an adult host are also contributors to the uniqueness of microbiota of each host when profiled at the strain level.

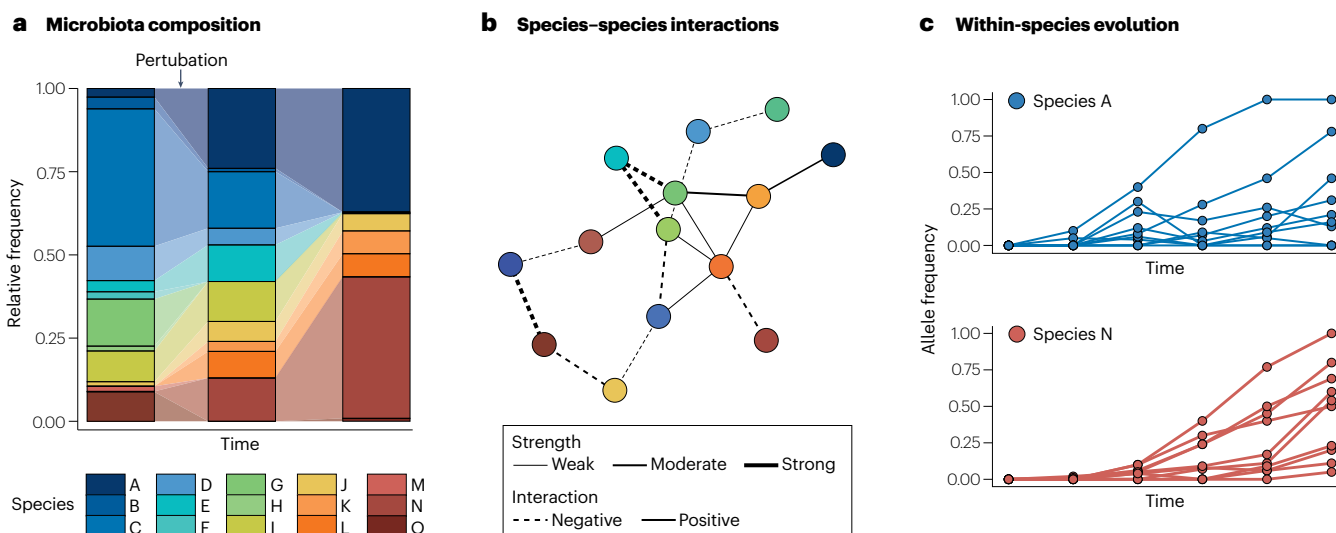


Fig. 1 | Ecological interactions, microbiota composition and intrahost evolution in the gut. **a**, The species composition of the gut microbiota can be profiled by measuring the abundance of 16S ribosomal RNA in faecal samples. **b**, Ecological interactions, such as cooperation and competition, have been estimated by analysing the dynamics of species composition in the gut after

a perturbation of the microbiota (for example, after antibiotic treatment)⁴. **c**, Underneath the composition of each species lies a substantial amount of strain variation, and intrahost evolution of gut microbiota species can also occur, here exemplified for two species in which new beneficial mutations emerge and rise in frequency throughout time.

Mutation is the ultimate source of new alleles, and the gut microbiota will experience mutations recurrently throughout the life of the host. With trillions of microorganisms growing in the adult gut and an estimated genomic mutation rate for DNA-based microorganisms of 10^{-3} per division³⁰, the microbiome can collectively experience many billions of mutations every day. The rate of spontaneous mutations and the distribution of their fitness effects are critical to understanding how natural selection will shape intrahost microbiota evolution. These parameters can be measured by performing mutation accumulation experiments (Fig. 2a). First, these experiments have shown that most mutations will be deleterious, thus present at very low frequencies and not easy to detect in metagenomic surveys. Nevertheless, enrichment strategies for culturable species can help capture and characterize such weak deleterious mutations. Second, many mutations will be neutral or close to neutral, for example, those that do not lead to amino acid changes (synonymous mutations) and thus more likely to be detected. Finally, a small fraction of mutations may confer a fitness benefit and thus increase the chances of their carriers to rise to high frequency. Such mutations will shape the functional diversity of the microbiota either for short or for long periods of the lifetime of the host (Fig. 2b).

Population geneticists build simple analytically tractable null models that are useful to detect when natural selection drives evolutionary change³¹. An important example is the neutral model of Kimura, which assumes that most variation observed within and across species can be explained by an equilibrium between a variation-generating mechanism – mutation – and a variation erosion mechanism – genetic drift. Motivated by the observation that most genetic differences between individuals, of the same or different species, do not cause amino acid alterations, the theory of Kimura gave rise to invaluable tests. One such test is pN/pS: the ratio of observed non-synonymous to synonymous polymorphisms, compared with that expected if one assumes uniform mutation rates across the gene. pN/pS should be equal to 1 under neutral evolution. Despite the limitations of the neutral model to understand prokaryote diversity (reviewed elsewhere³²), pN/pS is widely used in the genomic analysis including those of gut microbiomes³³. The values of this ratio vary across gut-colonizing species, but on average pN/pS is well below 1, compatible with a dominant role of purifying selection, which continuously removes deleterious mutations, shaping within-host and between-host variation in the gut.

One important caveat when interpreting microbiota intraspecific diversity regards the role of genetic linkage. Unlike sexual species, most bacterial species reproduce asexually. This implies that the fate of a mutation with a phenotypic consequence can influence the variation that exists in the rest of the genome. The tight linkage, characteristic of bacterial reproduction, can have important consequences for the rate of adaptive evolution and levels of variation in bacterial genomes³⁴. With a high input of beneficial mutations in large populations, a phenomenon of Darwinian selection known as clonal interference occurs^{35–37}. This mode of selection is typically observed during laboratory evolution both in vitro and in vivo (discussed subsequently). Unlike the simple scenario in which a rare beneficial mutation, which manages to escape stochastic loss when it emerges, rapidly sweeps to fixation before any other beneficial mutation undergoes the same process, under clonal interference multiple genetically distinct clones emerge and compete for fixation. This interference owing to linkage comes at a price: mildly deleterious mutations reduce the rate of fixation of mutations with small beneficial effects³⁸; mutations of weak benefit get outcompeted by mutations of stronger benefit, even if the

latter are rarer³⁶; and weak deleterious mutations can be driven to fixation via hitchhiking on mutations with strong fitness benefits. There is, however, a mechanism that can break this interference: recombination. Bacterial populations have specific ways to engage in genetic exchanges hypothesized to be important drivers of bacterial evolution³⁹, including in the gut^{40,41}. Such horizontal gene transfers (HGTs) involve three main different mechanisms (Box 1), which can cause the transfer of new genes between species^{42–44} (for example, transfer of antibiotic resistance gene); the transfer of genes between strains of the same species^{45,46} (Fig. 2c) and the transfer of DNA sequences within species⁴⁷. Our understanding of rates and fitness effects of HGT within single hosts is still in its infancy, and the relative role of mutations and HGT mechanisms during intrahost evolution is an important open question.

Strain-specific evolution

Adaptation of a strain to simple laboratory environments

Experimental evolution (EE) is commonly used to characterize the tempo and mode of microbial evolution in well-controlled environments under specific selective pressures. Some of these pressures are of great medical importance, for example, increasing concentrations of an antibiotic^{48,49} or the presence of immune cells that can kill bacteria⁵⁰. EE is a powerful method to understand patterns of evolution and adaptation that may take place in natural populations, in which the causes of natural selection are likely multiple and difficult to grasp. A typical experiment involves the propagation of several replicates of a single strain for hundreds to thousands of generations in the same environment. Replication is a key feature underlying the power of EE as it allows to distinguish adaptive changes from neutral or deleterious ones. This is because the probability of observing parallel evolution (that is, the same mutation rapidly spreading in independently evolving populations) is extremely small for non-adaptive mutations.

A key experiment performed in chemostats showed how an initially monomorphic population growing in a single carbon source (glucose) diversifies into multiple phenotypic clusters within only 26 days⁵¹. This may seem counterintuitive, but growth on a single sugar gives rise to waste products that may alter the environment in sometimes important ways. Indeed, genome-scale metabolic models identified thousands of interactions between two strains that could lead to coexistence on a single supplied carbon source: for example, *Escherichia coli* growing on glucose can produce more than 50 alternative nutrients, each potentially selecting for mutants that can consume them⁵². In the case of *E. coli* evolving in chemostats supplied with glucose, a typical newcomer by-product metabolite is acetate, whose presence can lead to the spread of new acetate-consumer specialized mutants. These can be maintained in the populations owing to cross-feeding interactions for many generations, establishing that even in a very simple environment diversifying selection can be pervasive.

Chemostats have been used to follow evolution over relatively short periods and even to challenge some dogmas about how natural selection is expected to work⁵³. However, propagation of populations in batch culture is a methodology more commonly embraced. The longest experiment to date in which adaptation of a strain to a single carbon source is continuously being followed is Lenski's long-term evolution experiment, known as LTEE⁵⁴. The simplicity of conditions and the clever design enabled the elucidation of important principles regarding bacterial adaptation to novel environments (Box 2). A considerable degree of parallel evolution is often seen in Lenski's long-term evolution experiment and other EE studies involving more complex selective

pressures. In populations evolving at high temperatures⁵⁵ or under antibiotics⁴⁸, fixation of mutations in the same genes is observed across independently evolved clones. Such EE experiments identify new resistance mechanisms and show how clonal interference influences

their spread⁵⁶. Similarly, in EE under immune selective pressures, for example, phagocytosis and killing by macrophages, bacteria evolve by accumulating mutations that confer similar adaptive phenotypes, some of which could lead to increased virulence⁵⁰.

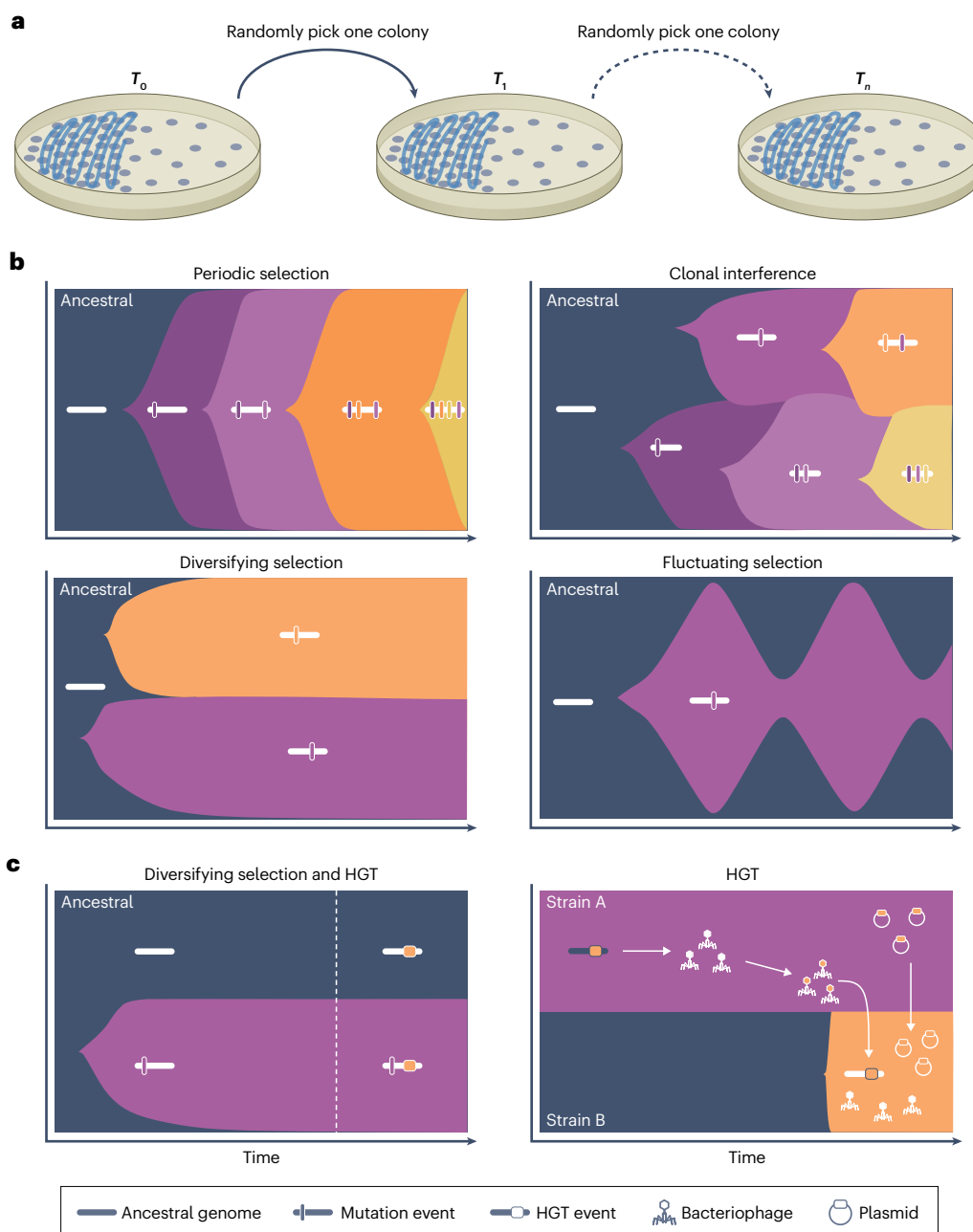


Fig. 2 | Mechanisms of evolution. a, Mutation accumulation experimental design to measure rates and fitness effects of mutations that spontaneously occur and fix in the absence of natural selection, owing to the strong bottlenecks applied at each passage¹³⁷. **b**, Muller plots, in which each distinct colour corresponds to the emergence of a beneficial mutation that changes in frequency with time, exemplifying the dynamics of evolution under different modes of selection: periodic selection, in which beneficial mutations sequentially fix; clonal interference, in which clones with distinct beneficial mutations compete for fixation; diversifying

selection, in which emerging mutations provide an advantage when rare but a disadvantage when common, thus leading to stable polymorphism maintained by negative-frequency-dependent selection; and fluctuating selection, in which the fitness effect of a new mutation changes along time. **c**, Dynamics of evolution in the presence of recombination and horizontal gene transfer (HGT): left, exchange of an allele between two closely related lineages that are maintained by negative-frequency-dependent selection; right, HGT via bacteriophages, in which a new gene from a donor strain A is transferred to strain B; and HGT via a conjugative plasmid.

Intrahost evolution of focal strains in the animal gut

The dynamics of evolution of bacteria within hosts is less understood than in vitro or during pathogenic infections⁵⁷. Nevertheless, some studies have followed the evolution of lineages inside hosts using experimental designs akin to those in vitro. Distinct animal models have been studied, and each brings specific advantages (Box 3). For many models, it is possible to obtain germ-free (GF) and genetically modified animals. These systems can provide important information regarding the eco-evolutionary dynamics of focal strains and the effect of the host and the gut microbiota for such dynamics. Despite the ability to be genetically manipulated, animal models such as nematodes (for example, *Caenorhabditis elegans*) and flies (for example, *Drosophila melanogaster*) lack adaptive immunity and harbour a small and low diversity gut microbiota, in contrast to vertebrate models such as fish (for example, *Danio rerio* and *Nothobranchius furzeri*) and mice⁵⁸. The population size of gut bacteria of individual hosts also varies with the animal model, with smaller population sizes in *C. elegans*⁵⁹ (approximately 10⁴ per worm) and *D. melanogaster*⁶⁰ (10⁴–10⁵ per fly) when compared with honeybees⁶¹ (10⁷–10⁹ per bee) or mice⁶² (10¹¹–10¹² per gram of faeces). Thus, the speed of evolution and the role of genetic drift versus natural selection will be different across these animal models. In GF mice, a focal species can reach enormous population sizes and evolution can be rapidly detected. In a typical colonizer of the mammalian gut, *E. coli*, clones with increased mutation rates (mutators) emerged after 3 weeks of colonization in GF mice⁶³. Consistent with in vitro findings, the study demonstrated that *E. coli* mutators have a short-term advantage over non-mutator strains because they can generate adaptive mutations faster (Fig. 3a). However, evolution in the mice resulted in a trade-off for the mutator strains, as the beneficial mutations accumulated in vivo reduced fitness in secondary environments⁶³. These experiments demonstrate that evolution in the gut of healthy hosts can result in the spread of mutator clones just as it happens in chronic infections, an iconic example being *Pseudomonas aeruginosa* evolution in individuals with cystic fibrosis⁶⁴. Such mutators can be maintained in the lungs of the patient for years, and mutators of *Staphylococcus aureus* and *Haemophilus influenzae* are also observed in individuals with cystic fibrosis, with important consequences for therapeutic interventions for this and other diseases⁶⁵. The spread of *E. coli* clones with varying mutation rates has also been reported in the gut of mice with complex microbiota. In this case, a combination of strong negative-frequency-dependent and weak purifying selection allowed the long-term polymorphism of mutators and non-mutators in the gut⁶⁶.

Beyond mutator emergence, studies using EE in mice have shed light on the tempo and mode of adaptation to the gut. In GF mice, *E. coli* rapidly diversifies by acquiring mutations targeting global regulators of motility, metabolism and the cell membrane^{67–69}. The evolved changes provide support to the hypothesis that resource competition is a strong selective pressure inside the gut⁷⁰. Consistent with this hypothesis, the sole presence of another species altered the evolutionary path taken by *E. coli*, from amino acid metabolism to anaerobic respiration⁶⁸. This path was more similar to the path observed in mice with a richer microbiota⁷¹, in which faster adaptation rates and a high degree of parallel evolution for mutations targeting sugar metabolism were the drivers of *E. coli* adaptation^{72,73}. The spread of mutations with similar fitness benefits, for example, targeting the galactitol metabolism, makes clonal interference and soft sweeps (in which a number of genetic changes targeting the same phenotype repeatedly emerge without reaching fixation in a population) important modes of adaptation when a new *E. coli* strain

Box 1

Mechanisms of horizontal gene transfer

The process of horizontal gene transfer (HGT) is characterized by the transfer of genetic material from one cell to the other. The uptake of foreign genetic material can have a huge impact on the adaptive evolution of microorganisms (reviewed elsewhere¹³⁸). Several mechanisms of HGT can occur in the gut.

Transformation

It occurs when there is an uptake of foreign DNA from the environment. In the gut environment, there is constant growth and death of microorganisms, suggesting that DNA could be readily available for uptake from microorganisms with the ability to perform transformation.

Transduction

The transfer of DNA via bacteriophages. There are various forms of transduction, including generalized, specialized and lateral. Generalized transduction occurs when there is the packaging of any host DNA that is then horizontally transferred¹³⁹. In specialized transduction, aberrant excision events join bacteriophage DNA with adjacent donor host DNA¹⁴⁰, which restricts specialized transduction to a limited set of genes. Lateral transduction takes place when the DNA packaging initiates in bacteriophages with delayed excision, such as those that are still attached to the microbial genome, allowing for the transfer of large DNA regions¹⁴¹. Transduction is likely frequent in the gut environment.

Conjugation

It takes place when DNA is transferred through cell-to-cell mechanisms such as those involving a conjugative pilus¹⁴². Rapid acquisition of antibiotic resistance is an important consequence of conjugation. The role of conjugation during intrahost evolution needs further studies both in health and in disease¹⁴³.

Membrane vesicles, nanotubes and bacteriophage-like gene transfer agents

These non-canonical mechanisms have been described to promote HGT. Membrane vesicles are lipid bilayer enclosed particles involved in the transfer of antibiotic-resistant genes¹⁴⁴. Nanotubes are membranous structures that allow for HGT through cell-to-cell contact¹⁴⁵. Bacteriophage-like gene transfer agents are derived from bacteriophage DNA. Genes encoding bacteriophage-derived holins and endolysins can disrupt the cells, releasing particles that contain DNA¹⁴⁶. The role of these processes in the gut is unknown.

colonizes antibiotic-treated mice⁷². In conditions that allow for a richer microbiota, the same strain evolves either by diversifying selection, generating distinct ecotypes, or by selective sweeps and clonal interference, with continuous fixation of beneficial mutations⁴⁵. The first mode of selection is marked by metabolic mutations and the second

Box 2

Evolutionary lessons from Lenski's long-term evolution experiment

In vitro studies have been instrumental to reveal the evolutionary mechanisms and key rules driving microbial adaptation to new environments. A pioneering study, known as Lenski's long-term evolution experiment, started more than 30 years ago, following the evolution of *Escherichia coli* growing on a single sugar. This apparently simple but extremely important evolution experiment gave rise to an astonishing amount of data that provided crucial information on the tempo and mode of adaptation and the evolutionary paths taken by bacteria. It revealed that the rate of phenotypic evolution is non-constant: it is high in the initial stages and lower in the latter stages. This is what one intuitively expects to occur in a constant environment and is predicted by one of the simplest mathematical models of adaptation^{147,148}. In addition, it described that the rate of molecular evolution does not mirror that of phenotypic evolution: mutation accumulation tends to be much more linear in time than fitness increases¹⁴⁹, a pattern that could be caused by global diminishing returns epistasis¹⁵⁰, a genetic phenomenon in which additional mutations have increasingly smaller effects on the phenotype of an organism as the number of mutations increases. It also showed that the effect of beneficial mutations that spread to high frequency or fixation tends to become weaker as the population is composed of fitter clones. This is expected from different theoretical models of adaptation¹⁴⁷. In Lenski's long-term evolution experiment, mutators emerged in about half of the evolved populations. This shows that the rate of molecular evolution can be highly heterogeneous, even in very simple contexts. Finally, the emergence of complex interactions between clonal lineages has been observed, some of which were caused by diversifying selection¹⁵¹. This is consistent with the predicted emergence of cross-feeding in limited environments⁵². These important evolutionary principles are beginning to be grasped in more complex environments such as the mammalian gut.

by the domestication of bacteriophages that get incorporated into its genome^{45,46}. In the richer microbiota setting, coexistence of *E. coli* strains led to bacteriophage-driven HGT events, from a resident strain to the new colonizer, conferring metabolic advantages⁴⁶. These findings exemplify the important role that HGT can have during intrahost evolution when strains from the same species coexist in the gut. However, there is currently a lack of research on how the interplay between different mechanisms of HGT affects the rate at which mutations accumulate in bacterial chromosomes.

The speed of evolution may differ throughout the lifetime of a host, as strong fluctuations in the gut environment are expected at different ages. Rapid evolution is seen during mother-to-offspring microbiota transmission, in which multiple mutations causing constitutive expression of the *lac* operon are selected during the breastfeeding period⁷⁴.

During host ageing, the increased levels of gut inflammation can also drive rapid evolution of gut commensals. Indeed, a shift in the fitness landscape of *E. coli* was detected in ageing mice, characterized by an increased emergence of mutations targeting stress-response genes⁷⁵ and increased transposition rates⁷⁶. An adaptive target found in ageing mice was a regulator of iron homeostasis, *iscR*, which was shown to undergo fluctuating selection in the mouse gut⁷⁷. Interestingly, the strength of fluctuating selection was modulated by the immune system, antibiotic treatment and microbiota composition⁷⁷. Fluctuating selection was also observed in *Bacteroides thetaiotaomicron*-colonized mice fed with alternating diets. A Western-style diet selects for mutations that target mucin-derived glycan degradation, and periodic changes in diet lead to fluctuating selection on the mutations increasing genetic diversity⁷⁸. These studies suggest that intrahost adaptation is shaped by host age, the immune system and diet.

Intrahost evolution of focal species in the human gut

In principle, the patterns of evolution observed in animal models should also occur in human microbiomes (Fig. 3b). When the evolution of an *E. coli* strain colonizing the gut of a healthy human was followed, no signs of selection were found over 1 year⁷⁹. By contrast, remarkable evidence for the adaptation within the human gut was found in several more prevalent species⁸⁰. Typically, humans are colonized by a single strain of *Bacteroides fragilis*¹³, so resolving the emergence of de novo mutations in this species is much simpler than in species for which host colonization involves multiple strains⁸¹. Through sequencing hundreds of cultured isolates and metagenomic sequencing of faecal samples covering more than 1 year, *B. fragilis* was found to acquire de novo mutations and diversify into coexisting lineages inside a human⁸⁰. Parallel evolution also occurred: 16 genes of *B. fragilis* were repeatedly mutated across the 12 individuals studied, showing that a considerable amount of adaptive evolution naturally occurs in this abundant species of the human microbiota. Furthermore, the genes identified are involved in polysaccharide utilization (*susC*) and cell envelope biosynthesis, and some have unknown functions⁸⁰. This finding illustrates the selective pressures *B. fragilis* populations experience in the gut and identifies genes for which in vitro work is needed to discover their function. Interestingly, in 1 out of the 12 people followed, the pattern of *B. fragilis* polymorphisms was consistent with the emergence of a mutator strain. Its signature could be detected because the types of change observed were mostly GC-to-TA transversions, a completely different mutation spectrum than that seen in the strains of the other individuals⁸⁰.

The combination of isolate and metagenome sequencing also allowed the identification of a few cross-species HGT events, probably mediated by bacteriophages and integrative conjugative elements. It was, however, difficult to determine whether such events are neutral or bring selective advantages to the recipient cells. In two people, dense temporal sampling data allowed a closer look at the dynamics of the adaptive mutations and to discover some new ones, through the rapid increase in the frequency of these new alleles⁸⁰. Rates of increase in frequency translating into selection coefficients of ~2% per day were estimated; yet, the adaptive mutations did not sweep to fixation. Instead, diversity was maintained (in *B. fragilis* and in other species⁴⁷), with at least two lineages staying polymorphic for more than 500 days in one person⁸⁰. Such coexistence could also have been shaped by co-evolution between the strains driven by bacteriophage-dependent killing, as in some strains a prophage could be identified and in others not⁸⁰. The selection coefficients on de novo mutations measured in

B. fragilis are similar to those measured in *E. coli* evolving in the mouse gut⁸², and bacteriophage-mediated selection shaping between-strain diversity has also been observed when a new strain of *E. coli* invades the mouse gut⁴⁶. Analysis of the gut virome of a human has also found rapid evolution in bacteriophages, with a higher substitution rate observed for virulent than temperate bacteriophages⁸³.

Overall, an increasing number of studies in which evolutionary change can be detected in the human gut is revealing similar evolutionary patterns across different species of this complex ecosystem^{47,84,85}.

Evolution of communities

Adaptation of microbial communities in vitro

Most natural habitats are occupied by multiple species, but our understanding of the drivers of their eco-evolutionary dynamics is still in its infancy. Assuming that in a stable ecosystem species do not interact or interact weakly⁷, one expects that adaptation to a new selective pressure (for example, an antibiotic) would be similar when a species is in a community or alone. But if interactions are common, such that one species can severely constrain⁸⁶ or facilitate⁸⁷ the evolution of another, then it is very difficult to make predictions about the tempo and mode of adaptation in the whole ecosystem. EE approaches indicate that composition can affect the adaptation of communities in complex ways. A pioneering study showed that interactions between five strains from different species that naturally co-occur can drive the evolution of new resource preferences, which do not evolve when each species is propagated alone⁸⁸. Moreover, it also showed that evolution could lead to reduced growth rates in some of the strains and that evolution in the communities could change the type of interactions between its species, with an increase in positive interactions between isolates evolved in communities when compared with the ancestral or monoculture isolates⁸⁸. The latter result has important consequences for how we understand species–species interaction networks in gut microbiotas. If intrahost evolution of commensals is pervasive, then the network of ecological interactions will be dynamic.

Intrahost evolution of microbial communities in the animal gut

Animal models have been used to study how microbial communities assemble and evolve. Experiments in *C. elegans* revealed mechanisms of interaction between symbionts and pathogens capable of altering host–microorganism co-evolution⁸⁹. They also allowed the identification of host genes that modulate microbiota composition, capable of altering the pattern of evolutionary change⁹⁰. Experiments in GF mice, in which well-defined species assemble into an ecologically stable gut community, have shown continuous evolutionary change through the emergence of new strains by de novo mutation within each species⁹¹. In a remarkable tour de force, the authors colonized mice with 12 species that commonly inhabit the mouse intestine and followed their evolution for 6 years (Fig. 4). The species consortium (Oligo-MM)⁹² is ecologically stable across mouse generations and contains important properties of a natural mouse microbiota, such as colonization resistance towards pathogens⁹². Owing to the sterility of the habitat and the food, no external input of microbial diversity occurs. Furthermore, with the genome sequences of each of the initially colonizing strains known, this sort of in vivo system can reach levels of control very close to those in vitro EE. Experimental systems such as this can thus allow for calculating rates of evolution by de novo mutation and identifying signatures of natural selection with precision. Analysis of

pN/pS values allowed the detection of positive selection in three of the species colonizing the mouse colony⁹¹. By following the most abundant species in which pN/pS > 1, and sequencing several isolates throughout time, the emergence and maintenance of five strains, which shared mutations with similar temporal frequency dynamics, could be observed. Interestingly, each of the evolved strains of this species showed signs of continuous parallel evolution, and a set of mutations that had spread to detectable frequencies was responsive to selection through changes in the diet of the mice⁹¹. This Oligo-MM colonization model and others^{93–95} offer an excellent system to enquire about rates and fitness effects of interspecies HGT events, an issue that is currently not well understood.

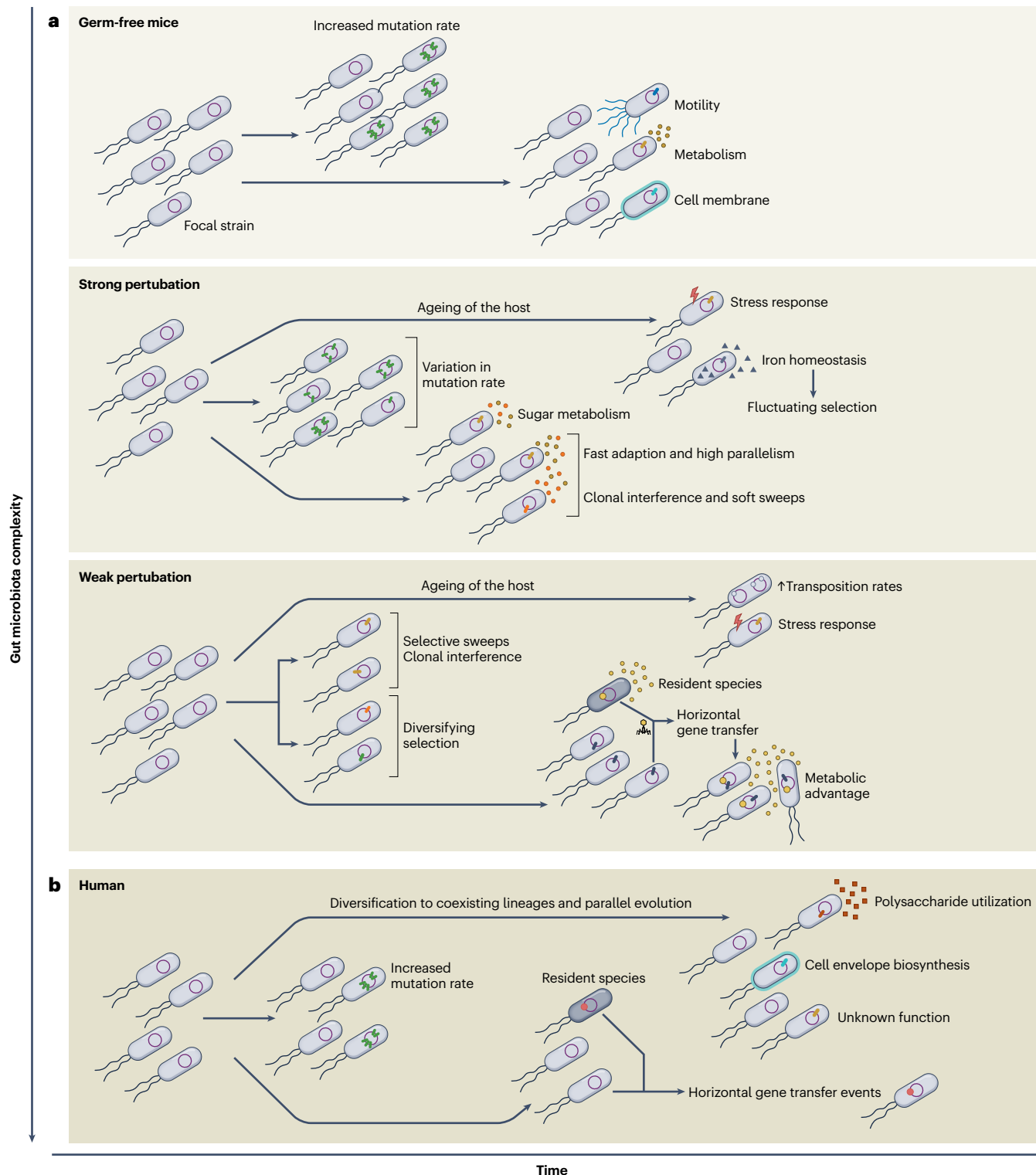
Intrahost evolution of microbial communities in the human gut

Longitudinal data of human metagenomes have also been leveraged to find evolutionary changes across the most abundant species of the human gut (Fig. 4). Using data from the Human Microbiome

Box 3

In vivo models to understand intrahost evolution and quantify the predictability of evolution

Animal models have enabled the elucidation of the dynamics of microbial evolution within hosts with great detail. Laboratory mice have been a common model to study intrahost evolution. The high percentage of genes shared with humans, together with the possibility of genetic manipulation of their genes and the control of dietary conditions, provides a controlled system in which evolutionary principles can be tested. Contributing to this system, synthetic microbial communities, which resemble the composition of the human gut microbiota, have been used to colonize germ-free mice^{92–95}. Other animal models such as zebrafish (*Danio rerio*) larvae, *Drosophila melanogaster* and *Caenorhabditis elegans* have also been used to uncover the details of intrahost evolution using experimental evolution designs. Using zebrafish larvae, two studies examined the intrahost evolution of two distinct bacteria, *Shewanella oneidensis*¹⁵² and *Aeromonas veronii*¹⁵³. The authors observed rapid evolution for both species, leading to increased fitness and high levels of parallelism for enhanced motility^{152,153}. An interesting study in *D. melanogaster* revealed that intrahost evolution of its symbiont *Lactobacillus plantarum* is strongly driven by the host diet, instead of the host itself¹⁵⁴. Furthermore, an adaptive mutation in *ackA* acquired by *L. plantarum* was shown to provide a growth benefit to the host¹⁵⁴. In *C. elegans*, a recent study showed that the intrahost evolution of the pathogen *Staphylococcus aureus* was shaped by host genotype¹⁵⁵. In addition, increased virulence acquired through host metal ion binding was a common trait of the evolved bacteria¹⁵⁵. Overall, the results obtained with these models are consistent with the results obtained in mice and highlight their importance as complementary systems to study intrahost evolution in the gut.



Project, a study revealed that intrahost evolution can occur in several abundant strains of the human gut over short timescales⁸¹. Intra-host evolution for these strains was characterized by gene losses and gains, together with nucleotide variants, that sweep to high frequency in a matter of months. By contrast, over longer timescales,

distantly related strains often replace the resident strains⁸¹. In infants, a tenfold increase in strain turnover, and an increase in the rate of evolution when compared with healthy adults, was found⁹⁶. A dominance of gene-loss events was identified during mother–infant transmission, whereas gene gain became more frequent as time progressed,

Fig. 3 | Strain-specific evolution. **a**, Summary of the evolutionary dynamics of intrahost evolution of *Escherichia coli* and the selection pressures that shape *E. coli* evolution under distinct gut microbiota complexities in mice. In the absence of other members of the gut microbiota (top), intrahost evolution of *E. coli* can lead to an increased mutation rate and mutations that target motility, the cell membrane and metabolism of *E. coli*^{67–69}. In the strong perturbation models of colonization (middle), intrahost evolution of *E. coli* can lead to variation in the mutation rate and alterations in sugar metabolism^{72,73}. In this context, intrahost evolution is described by fast adaptation and high parallelism, together with clonal interference and soft sweeps⁷². Ageing of the host can change the selective pressures acting on *E. coli*, and it was shown that in this context intrahost evolution of *E. coli* alters genes involved in stress response and iron homeostasis⁷⁵, the latter shown to be under fluctuating selection⁷⁷. Finally, after a weak perturbation

(bottom), intrahost evolution of *E. coli* revealed two modes of selection: one characterized by selective sweeps and clonal interference and the other by diversifying selection⁴⁵. In this context, a resident strain of *E. coli* can still be present in the mouse gut microbiota, leading to horizontal gene transfer events driven by bacteriophages that confer a metabolic advantage to *E. coli*⁴⁶. Furthermore, intrahost evolution of *E. coli* in an ageing host revealed increased transposition rates and mutations that target the *E. coli* stress response⁷⁶. **b**, Summary of the evolutionary dynamics of intrahost evolution of *Bacteroides fragilis* and the selection pressures that shape *B. fragilis* evolution in humans. Intrahost evolution of *B. fragilis* in humans can lead to an increased mutation rate, and diversification to coexisting lineages and parallel evolution targeting polysaccharide utilization and cell envelope biosynthesis were also shown to occur⁸⁰. Furthermore, the presence of resident species can lead to horizontal gene transfer events⁸⁰.

reflecting rapid changes in eco-evolutionary dynamics in the early days of life⁹⁶.

A promising method for tracking evolution without the need for culturing gut bacteria strains has been recently developed. It is based on high-throughput chromosomal conformation capture, together with an algorithm to construct metagenomic assembled genomes and detect HGT events⁹⁷. When applying this method to faecal samples of two humans, it was found that some strains could persist in a host for at least 10 years, and the accessory genome is highly dynamic. These results are consistent with other findings of pervasive evolutionary change of gut microbiomes within human hosts^{80,81,98}. Interestingly, changes in human lifestyle have also been found to affect the ecology and evolution of the human microbiota⁴². Not only do HGT events appear frequently within individuals but they are also found across a broad set of bacteria species, with abundant bacteria more likely to engage in HGT⁴². This is consistent with previous studies on Bacteroidales, which are among the most prevalent members of the human gut microbiota, that described extensive HGT events within the human gut^{43,44}. In addition, an increased frequency of HGT events and higher rates of plasmid and transposon exchanges were observed for industrialized populations, in contrast to an increased exchange of carbohydrate-active enzyme genes in non-industrialized populations⁴².

We are just starting to understand the dynamics of intrahost evolution in human guts and the mechanisms that contribute to it^{25,26}. The increasing interest in this field and the continual development of methods and analysis raise hopes for a deeper comprehension of the tempo and mode for this process within a host and the commonalities it may have across hosts with different lifestyles and health status.

Implications of intrahost evolution for disease and microbiota modulation

Evolution of traits

Virulence and resistance are key pathogenic traits in the context of infectious diseases. Intrahost evolution may be an important process underlying the transition from commensalism to pathogenicity and the emergence of medically relevant traits⁹⁹. This can occur through the accumulation of pathoadaptive mutations¹⁰⁰ and/or new genes through HGT (for example, antibiotic resistance determinants¹⁰¹). If a mutant lineage emerges in the gut and can invade and propagate beyond that niche, an arms race between the bacterial cells and the host may ensue. For mice and humans, whose immune systems are equipped with a Darwinian process of generating antibody diversity, that arms race can be co-evolutionary. The bacteria, which are now seen as pathogens, change their phenotypes (for example, by expressing some virulence determinants they may carry) but also their genomes (by acquiring

mutations that increase their growth rate or escape immune pressures in the new environment). The B cell repertoire of the host adaptive immune system changes in composition, and a repertoire change may select for new antigenic variants, a process with consequences for vaccine design (for example, vaccines against *Streptococcus pneumoniae*, a human commensal clinically relevant in children, where it rapidly evolves during nasopharyngeal colonization¹⁰²).

EE in mice showed how rapid evolution in the gut may lead to microbiota-driven diseases. An *Enterococcus gallinarum* strain evolving in GF mice diversified into lineages adapted either to the lumen or to the mucosa¹⁰³. The mucosal-adapted bacteria, which were able to cross the gut barrier and colonize the liver, were more resistant to cellular and chemical immune defences, that is, more resistant to macrophage killing, antimicrobial peptides and lysozyme¹⁰³. Underlying these traits were mutations in regulatory genes causing changes in the expression of approximately 1,000 genes. Furthermore, the liver-colonizing strain caused increased gut and liver inflammation¹⁰³. Although the evolution of niche diversification was found to generally occur in *E. gallinarum*, the evolution of its pathogenic traits was microbiota-dependent, as in healthy mice with a complex microbiota liver translocation did not occur¹⁰³. Studies such as this open new doors to understanding and treating pathologies caused by opportunistic pathogens. They help to estimate the probability of a strain evolving translocation and reveal which gut adaptive traits may constitute pre-adaptions to invade other organs. Predicting which genes will underlie the evolution of an epidemic pathogen is however much more difficult, as such clones should carry evolutionary changes that lead to global adaptation¹⁰⁴.

A species long known for intrahost evolution in the human gut is *E. coli*¹⁰⁵. Its genomic diversity, at the level of single nucleotide polymorphisms and accessory genes, holds the mystery of why it can act as a probiotic, capable of offering colonization resistance¹⁰⁶, or a pathogen, causing recurrent urinary tract infections. Isolation of strains from individuals living in the same house showed that a relatively low number of mutations, many of which alter major regulators (such as *lrhA*), can give rise to strains capable of causing different symptoms in distinct people¹⁰⁷. Besides pathoadaptive mutation accumulation, mobile genetic elements (MGEs), which are pervasive in this species, can also contribute to its adaptation to different human body sites. Although still poorly understood, a habitat-specific signature of MGEs was found when *E. coli* lineages colonizing the gut, the urinary tract or both habitats were compared¹⁰⁸.

Interactions within the microbiota can influence the evolution of antibiotic resistance of a gut commensal in complex ways. Competition may constrain the evolution of resistance, as in the absence of antibiotics resistant determinants can incur fitness costs^{109,110}.

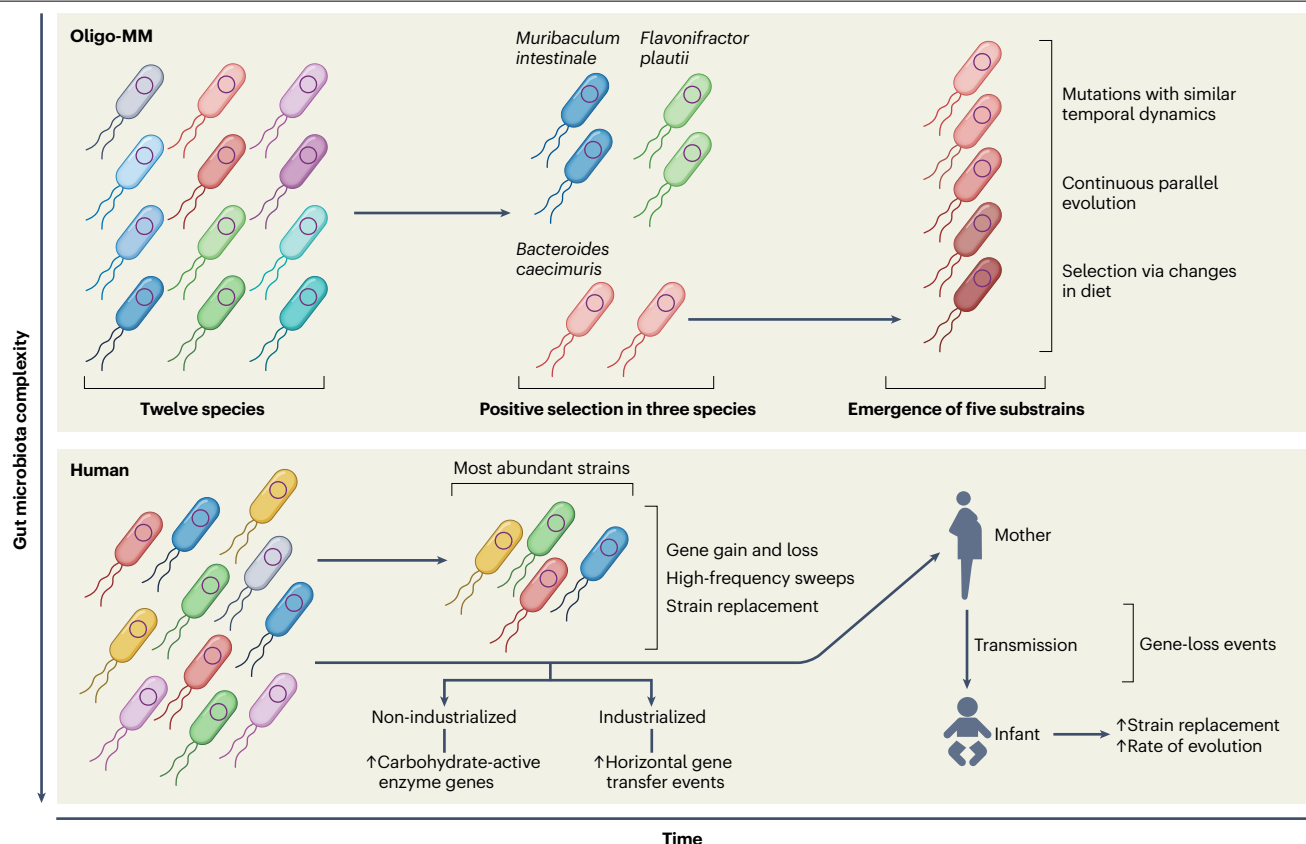


Fig. 4 | Evolution of communities. Summary of the evolutionary dynamics of intrahost evolution of gut microbiota communities and the selection pressures that shape their evolution. Using the mouse model and a synthetic community of twelve different bacterial species (top), intrahost evolution of the community revealed signatures of positive selection for three species⁹¹. Furthermore, for one of the species, the emergence of five substrains was described and characterized, revealing mutations with similar temporal dynamics, continuous parallel evolution and selection signatures driven by changes in diet⁹¹. In humans (bottom), following the most abundant strains during intrahost evolution

revealed gene loss and gains, high-frequency sweeps and strain replacement⁴³. Ecology and lifestyle of humans were also shown to alter intrahost evolution of the gut community, with an increase in carbohydrate-active enzyme genes targeted in non-industrialized humans, in contrast to an increased rate of horizontal gene transfer events in industrialized humans⁴². Furthermore, gene-loss events were described for intrahost evolution of the gut community during mother-to-infant transmission, and intrahost evolution of gut communities in infants revealed an increased number of strain replacement events and rate of evolution when compared with mother-to-infant transmission⁹⁶.

But the presence of many strains can also accelerate resistance evolution through an increased chance of HGT. Studies in mice have shown that the composition of the microbiota affects the cost of resistance^{111,112}, and a study ex vivo found that gut communities could suppress the evolution of β -lactam resistance in *E. coli*¹¹³, even in conditions in which resistant genes were present in the community. Exchange of multidrug resistance genes via conjugation could, however, be captured between strains coexisting in the gut of an infant, even in the absence of antibiotic selection¹¹⁴. Remarkably, the plasmid transferred in the gut of an infant carried a fitness benefit when tested in the mouse gut, whereas it showed reduced fitness in vitro. In addition, MGEs carrying antibiotic resistance genes can be mobilized between commensal and pathogenic strains¹¹⁵. These findings underscore the need for further in vivo studies measuring the fitness effects of plasmids and other MGEs across strains¹¹⁶ as well as new methods to quantify their rates of transfer in the gut¹¹⁷. Better knowledge of the fitness effects of mutations and/or MGEs that confer resistance in natural ecosystems, as well as interactions between

different resistance mechanisms¹¹⁸, will be critical to make predictions about intrahost resistance evolution.

Antibiotics are essential to treat infections, but they can also alter the ecology and evolution of gut bacteria. Even a short course of an antibiotic can lead to a great reduction of microbiota diversity, which may or may not be able to recover to its original state^{119–121}. In healthy humans, antibiotic-induced perturbation has been shown to lead to increases in resistance gene burden in the gut for some antibiotics^{117,122}. Beyond the selective pressure they exert, antibiotics can also increase the genomic mutation rate of bacteria¹²³. Consistent with this, the overall rate for genetic sweeps of individual species was higher during antibiotic perturbation than in healthy hosts⁴⁷. However, the extent to which antibiotic treatment can accelerate microbiome evolution is still not well understood.

Intrahost evolution and microbiota modulation

Probiotics have been used to aid in restoring gut dysbiosis and curb the negative side effects of antibiotics. An in vitro study using a probiotic

Bacillus subtilis revealed that it undergoes rapid evolution in a laboratory environment, with loss of traits that may be important for its function as a probiotic in the mammalian gut¹²⁴. If and how probiotic bacteria are prone to intrahost evolution has received increased attention. Intrahost evolution was found in probiotics such as *Lactiplantibacillus plantarum* and *E. coli* Nissle. In one study, mice, zebrafish and humans were colonized by *L. plantarum*¹²⁵ and, after 1 month, mutations that alter carbohydrate utilization and acid tolerance emerged independent of the host¹²⁵. Another study described distinct evolutionary paths taken by *L. plantarum* when evolving in vitro or in vivo^{126,127}. Host-specific adaptations occurred in genes involved in amino acid biosynthesis and carbohydrate metabolism, and a loss of plasmids was observed¹²⁷. In experiments with *E. coli* Nissle, mice with different levels of microbiota complexity under different diets were colonized with the probiotic strain¹²⁸. After several weeks of evolution, it was found to accumulate mutations in genes relating to processes such as the stress response, adhesion and carbohydrate utilization¹²⁸. An enrichment for mutations that enhance mucin degradation was also observed in microbiotas with low complexity¹²⁸. Whether these genetic changes have consequences for host health remains an open question. Recent work also suggests that intrahost evolution of probiotic lactobacilli increases the risk of developing probiotic-associated bacteraemia¹²⁹. Furthermore, probiotic intake may affect the evolution of resident gut microorganisms¹³⁰. These studies highlight the importance of understanding the pace and consequences of intrahost evolution of probiotic strains.

Another strategy to restore gut dysbiosis is the use of faecal microbiota transfer (FMT). FMT has been successful to treat recurrent *Clostridioides difficile* infections, with an approximately 90% success rate¹³¹. However, and despite an increased interest for the treatment of other diseases such as inflammatory bowel disease¹³², the efficacy of FMT for such diseases can greatly vary¹³³. Two recent studies used a combination of meta-analysis and metagenomic-based clinical trials^{134,135} to understand which factors from the donor or recipient microbiota could increase the efficacy of FMT and the ecological dynamics after FMT. One study suggested that using both upper and lower gastrointestinal tract routes for the administration of FMT and the intake of antibiotics increased the efficacy of FMT¹³⁴. The other study did not detect any association between efficacy of FMT and recipient strain replacement, the reinstatement of specific functions or colonization by donor strains¹³⁵. Nevertheless, both studies revealed that the ecological dynamics after FMT are greatly driven by the microbiota composition of both donor and recipient, which could lead to strain replacement, inhibition of colonization and coexistence^{134,135}. These pronounced ecological changes should change the way natural selection acts on the donor and recipient species that are maintained after FMT. In accordance with this hypothesis, another study using longitudinal samples from healthy FMT donors revealed the occurrence of strain replacement and intrahost evolution driven by the accumulation of mutations and gene-gain and gene-loss events⁸⁴. The extent that intrahost evolution of bacterial species from the donor and/or the recipient after FMT can have on the success of therapy still requires further studies.

Conclusions and future perspectives

A multitude of elegant in vitro experiments in which evolution is studied in real time have been instrumental to discover fundamental rules about microbial adaptation to new environments. It is now becoming clear that intrahost evolution is pervasive in the gut at relevant timescales.

Thus, animal and human guts are the setting for natural experiments in which rapid evolution and complex adaptive dynamics can be observed. Yet, the rules that govern the evolution in these complex ecosystems are just now starting to be unravelled. Similar and broad evolutionary patterns, such as diversification and long-term maintenance of emerging lineages, are detected across different species colonizing the gut. This raises the question: under what conditions and to what extent can intrahost evolution be predictable?

The increasing amount of metagenomic and isolate sequencing data from the gut microbiome shows that multistrain colonization is common in many species in the human gut^{12,43,47,81,84}. Coexistence of strains of the same species should, in principle, increase the rates of genetic exchange and the potential for co-evolution of strains. Such processes could lead to local adaptations within a host, which are not necessarily beneficial after transmission to new hosts. Furthermore, such intrahost evolution can have potential consequences for the health of individual hosts and for new therapies that capitalize on the ability to modulate the diversity and functional capacity of the gut microbiota.

How variable the rates of evolution are, through mutation accumulation and HGT, in species that colonize the gut is an open question. EE in animal models can be a powerful tool to help solve some of the challenges ahead and to complement studies of the human microbiota. However, translating results from these animal models to humans will require considering their different physiologies. The rate at which bacteria accumulate mutations can vary across species¹³⁶, and differences in their generation times may be one of the factors contributing to such variation. Studies of intrahost evolution of gut microbiota, across the lifetime of different hosts, should help illuminate the factors that can contribute to variation in the evolutionary rates of bacteria.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

The authors declare no competing interests.

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