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The microbiome: an emerging key player in aging and longevity

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Abstract

Revolutionary advancements of high-throughput sequencing and metagenomic tools have provided new insights to microbiome function, including a bidirectional relationship between the microbiome and host aging. The intestinal tract is the largest surface in the human body that directly interacts with foreign antigens – it is covered with extremely complex and diverse community of microorganisms, known as the gut microbiome. In a healthy gut, microbial communities maintain a homeostatic metabolism and reside within the host in a state of immune tolerance. Abnormal shifts in the gut microbiome, however, have been implicated in the pathogenesis of age-related chronic diseases, including obesity, cardiovascular diseases and neurodegenerative diseases. The gut microbiome is emerging as a key factor in the aging process. In this review, we describe studies of humans and model organisms that suggest a direct causal role of the gut microbiome on host aging. Additionally, we also discuss sex-dimorphism in the gut microbiome and its possible roles in age-related sex-dimorphic phenotypes. We also provide an overview of widely used microbiome analysis methods and tools which could be used to explore the impact of microbiome remodeling on aging.

1. Introduction

Over a century ago, Elie Metchnikoff proposed that age-related dysfunction could result from increased colon permeability-driven chronic system inflammation [1]. Recent advances in DNA sequencing technologies have allowed investigation of the composition and functional dynamics of complex microbial communities with great resolution and without the need for cultivation [2]. During the past two decades, microbiome research thrived to establish a causal relationship between the microbiome and host aging (Reviewed in [3–6]).

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Conflict of interest

The authors declare that they have no conflict of interest to disclose.

The microbiota consists of all the microbes (*i.e.* bacteria, archea, viruses, protozoa and fungi) and a distinct profile of microbiota is found on all host surfaces that are in direct contact with the outside environment (*e.g.* gut, skin, mouth, vagina, *etc.*) [7–15]. The microbiota performs essential functions that contribute to the physiology of the host through a symbiotic relationship [16, 17]. Consequently, perturbations of microbiomes have been proposed to exert negative effects on the host organism. The gut microbiome remains the most extensively studied microbiome and will be the main focus of our review.

Intestinal mucosa is the largest surface of the body that directly interacts with environmental antigens. Thus, the intestinal mucosal immune system monitors the gut environment through a variety of pattern-recognition receptors and is in active communication with the systemic immune system via the local mesenteric lymph nodes [18, 19]. The adult human gut microbiome, composed of approximately 10^{13} to 10^{14} micro-organisms, plays various essential roles in the host including degradation of food, lipid storage and metabolism, vitamin synthesis, suppression of harmful microbial species and maintenance of intestinal barrier integrity [20–25]. Dysbiosis of the gut microbiome is associated with defects in gut barrier integrity and enhanced pro-inflammatory cytokines [26, 27]. Thus, aberrant alterations of the gut microbiome have been attributed to pathogenesis of various metabolic diseases including adiposity, insulin resistance, atherosclerosis and cardiovascular disease, as well as multiple sclerosis, depression and anxiety [28–32].

In this review, we describe changes to the gut microbiome throughout lifespan of the host and key findings that implicate a central role of the gut microbiome in host aging. In addition, we discuss microbiome-relevant biological factors (*e.g.* sex) that may contribute to aging. Finally, we provide an overview of microbiome collection considerations, data analysis pipelines and potential confounding factors, that must be considered when analyzing and interpreting microbiome data.

2. The microbiome in response to aging and pro-longevity interventions

a. The aging gut microbiome: the human side

The microbiota co-evolves with its host and thus the composition of the microbial community within the intestinal tract fluctuates throughout lifespan, in response to genetic and environmental stimuli [3, 33–35]. Based on recent studies that reported the presence of bacteria in the placenta, amniotic cavity and umbilical cord, microbial colonization may initiate as early as *in-utero* [36–38]. During infancy, the gut microbiome undergoes significant fluctuations, which is namely driven by factors including delivery method, feeding, antibiotic exposure, maternal diet and environmental factors [39–41]. Colonization of microbial species in the gastrointestinal tract during early stages of life is reported to affect later health of the host organism [42]. Nonetheless, the microbiome composition reaches a stable structure after the first three years, its profile resembling that of an “adult-like” microbiome [43–45]. After stable recolonization of the microbiome, diet becomes a major force shaping the microbiome composition of the host throughout early adulthood [46, 47].

In general, healthy adults are reported to present with high levels of bacteria from the *Bacteroidetes* and *Firmicutes* phyla, and relatively lower proportions of the *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* phyla [8, 48–50]. *Bacteroidetes* found in the gut mainly functions in polysaccharide metabolism and calorie absorption, whereas *Firmicutes* are important for production of Short-Chain Fatty Acids [SCFAs] [51–55]. The *Firmicutes*/*Bacteroidetes* [F/B] ratio is reported to increase from birth to adulthood and studies show that high F/B ratios are associated to a dysbiotic microbiome [34, 56]. Studies have shown that the F/B ratio can be used as an important indicator of gut microbiome state and thus host health [57–60].

Clinical studies have reported significant differences in microbial composition between young and elderly human subjects [6, 61]. A key transition from healthy adult to elderly microbiota is characterized by a decrease in microbial diversity. Reduced microbiota diversity in aged individuals have been suggested to result in the expansion of distinct groups of bacteria which has been implicated on the development of age-associated type 1 diabetes mellitus, rheumatoid arthritis and colitis [62–64]. However, whether reduced microbiota diversity directly impacts host aging, or is a mere bystander, remains poorly understood.

Generally, in aged individuals, a decrease in *Bifidobacterium* and *Lactobacillus,* and increase in *Enterobacteriaceae* are observed [39, 65, 66]. Such changes in the microbiome structure are believed to result from changed lifestyle, dietary pattern, reduced mobility, weakened immune strength, reduced intestinal functionality, changes in gut morphology, use of medication, recurrent infections and more [27, 34, 35, 39, 61, 65, 67]. However, it is important to note that these generalizations do not apply to certain aged groups from different geological locations or genetic backgrounds [67, 68]. Interestingly, in centenarians and supercentenarians, health-associated bacteria genera, including *Bifidobacteria* and *Christensenella*, are especially abundant [69, 70]. Although these observations are correlative, studies in model organisms support pro-longevity and pro-health effects of these microbes [71–73]. For example, supplementation of *Bifidobacteria* to *C. elegans* resulted in reduced accumulation of lipofuscin, a marker of aging, improved locomotor function and increased longevity [72]. Additionally, transplantation of *Christensenella* to germ-free mice has been shown to amend obese-associated microbiome and reduce weight gain [73].

b. The aging microbiome in model organisms

Baseline microbial composition of the gut microbiota varies across species and taxa [26, 74–76]. However, similar to what has been observed in humans, extensive remodeling of the gut microbiome during aging has also been observed in a number of model organisms, spanning *Drosophila melanogaster*, the African turquoise killifish *Nothobranchius furzeri* and mice [26, 74–77].

In *D. melanogaster*, the aging gut microbiome is characterized by an expansion of *Gammaproteobacteria* [74, 75], and microbiota transplantation from aged donors to young flies leads to reduced longevity. Metagenomics analysis also showed that age-related changes in *Drosophila* microbial species were somewhat similar to that observed in human inflammatory disorder patients and aged human gastrointestinal tract [75]. For example,

increased levels of *Enterobacteriaceae*, the most abundant family of *Gammaproteobacteria*, were also observed in aged humans and mouse model of colitis [78, 79]. In a study of the aging microbiome in the African turquoise killifish, the microbiota of young individuals was found to be more enriched in species from the *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* phyla, whereas the aged microbiota was enriched for species from the *Proteobacteria* phylum [76]. Interestingly, similar to observations in humans, the aging killifish microbiome was also characterized by decreased diversity of the gut microbial community [76]. Additionally, transplantation of microbiome from young to middle-aged killifish improved locomotion and longevity of recipient subjects [76]. The mouse aging microbiome showed a number of shifts in relative abundance of bacteria phyla, including increased presence of *Clostridium* and decreased levels of *Lactobacillaceae* as observed with human aging [80]. Increased abundance of *Clostridium* was also observed in the aging gut microbiome of rats, although (contrary to humans) rats seemed to acquire increased microbial diversity throughout life [81]. Interestingly, the relative proportion of *Firmicutes* and *Bacteroidetes* is also altered with aging in the gut microbiome of aging mice [26]. A direct role of the microbiome in promoting overall health in mammals is also suggested by the fact that fecal microbiota transplants from wild-type mice can significantly improve the health and lifespan of progeroid mice [82]. Together, these studies provide a strong rationale for microbiome-based interventions against age-related decline and pathologies.

c. Effects of pro-longevity interventions on the aging microbiome

Modulation of the microbiota is emerging as a potential mechanism underlying pro-health and longevity effects of various interventions (Table 1). Interestingly, a number of pro-longevity interventions seem to have rejuvenating effects on the microbiome. A recurrent effect is the expansion of bacteria from the *Lactobacillae* taxa, which occurs in the context of independent interventions [83, 84]. Interestingly, a study reported that weight loss in the context of calorie restriction in mice seems to require an intact microbiome [83]. Thus, it will be important to determine whether microbial community remodeling in the context of pro-longevity interventions is a mere bystander, or an actual mediator of pro-health effects.

3. A bidirectional relationship between the gut microbiome and aging?

During the past two decades, studies have provided evidence that age-associated shifts in the gut microbiome contributes to increased predisposition of aged individuals to certain diseases, including cardiovascular diseases, cancer, obesity, cancers, diabetes and neurodegenerative diseases [3, 4, 85–87]. Aging is a complicated process that affects physiological, metabolic and immunological functions of the organism and thus is accompanied by inflammation and metabolic dysfunctions [88]. The overall age-related increase in chronic inflammation and deterioration of systemic immune system led to coining the term “inflamm-aging” [89]. A direct causal role of the gut microbiome on host aging has been suggested by a number of studies using various experimental models [76, 90]. In this section, we discuss studies that suggest the existence of a bidirectional relationship between the gut microbiome and host aging.

a. Interaction between the host immune system and the gut microbiome

Through millions of years of evolution, the host and its surrounding microbial environment have co-evolved into a complex organism [17, 18, 91]. Microbes beneficial to the host are able to reside within the host in a state of immune tolerance, whereas those that exert a pathogenic effect activates robust immune responses of the host [17]. The symbiotic co-existence between the host and microbiota is feasible due to the anatomical separation of microbial species from the host by a physical barrier. The intestinal barrier is responsible for adjusting metabolic homeostasis and systemic antimicrobial responses by detecting microbial-cell components and metabolites through its extensive repertoire of innate immune receptors [92–96]. For example, activation of pattern-recognition receptors (*e.g.* Toll-like receptors) by the gut microbe or its products induces the production of antimicrobial peptides and mucus [92]. Perturbations of such receptors have been reported to result in intestinal inflammation and susceptibility to enteric infections [97].

Relevant to aging, decline of the immune system in the aged intestinal epithelium have been suggested to contribute to age-onset dysbiosis [98, 99]. An important characteristic of age-onset dysbiosis is reduced microbiota diversity, which is suggested to lead to an expansion of distinct groups of bacteria [39, 100, 101]. Concurrently, bacteria that is reported to be involved in maintenance of immune tolerance in the gut, such as *Bifidobacteria* and *Lactobacilli*, are found in reduced level in aged groups, whereas those that are found in increased levels, such as *Enterobacteriaceae* and *Clostridium*, are involved in infection and intestinal inflammation stimulation [27, 66, 102, 103]. Together, these studies suggest that the host immune system shapes not only the host's immune response to microbiome changes, but also the structure of the microbiome itself [104].

Cumulative evidence has implicated a close functional relationship between the immune system of the host and the microbiome, to an extent that the gut microbiome is important for proper development and expansion of intestinal mucosal and systemic immune system [105, 106]. Supporting the notion that the microbiome can directly shape the immune states of the host, the transcriptomic profile of African turquoise killifish guts derived from animals that received young or old gut microbiota transplants showed clear differences, especially in expression of immune-related genes [76]. Interestingly, studies using germ-free mice models also suggest a bidirectional relationship between the host immune system and the gut microbiome. Germ-free mice showed significant alterations in innate immune system composition compared to classical Specific-Pathogen Free [SPF] mice, including deficiencies in macrophage, monocyte and neutrophil populations [107]. Such alterations of the immune system in germ-free mice were partially rescued when mice were treated with specific bacteria and/or bacterial components (*i.e.* bacterial polysaccharide), demonstrating a direct role of the gut microbiome on immune system establishment of the host [108]. Interestingly, experiments using germ-free mice also revealed that, in addition to regulating the abundance of immune cells, the microbiome may also regulate bactericide properties of macrophages [26].

b. Increased intestinal barrier permeability with age

Increased permeability of the intestinal barrier with age has been described across animal species, including worms, flies, mice and rats [26, 74, 75, 109–111]. Age-related deterioration of intestinal barrier function has been proposed to result in leakage of gut microbes into the systemic circulation, and ultimately lead to increased antigenic load and systemic immune activation [112, 113] (Figure 1). For example, age-associated remodeling of the gut microbiome in mice was shown to result in increased production of pro-inflammatory cytokines and intestinal barrier failure [26]. Consistently, the blood of aged mice contained increased levels of muramyl-dipeptide, a component of Gram-positive and Gram-negative bacteria cell wall [26]. Additionally, in a clinical study on aged type 2 diabetes patients, live gut bacteria were found to translocate into the blood stream, suggesting perturbations of the intestinal barrier integrity [114]. In *Drosophila*, the age-related increase in *Gammaproteobacteria* was suggested to lead to increased intestinal permeability, inflammation and mortality [74, 75]. The study showed that regardless of chronological age, intestinal dysbiosis serves as an indicator of age-onset mortality in flies [75].

A number of molecular mechanisms has been suggested to underlie intestinal barrier permeability with age. Mouse studies suggest that host cytokine signaling may play a key role in barrier function breakdown [26]. Indeed, TNF-α signaling was found to play a role in age-related intestinal barrier breakdown, as (i) *Tnfa* knock-out mice did not accumulate bacteria byproducts in their blood with aging, and (ii) anti- TNF-α therapy led to significant remodeling of the gut microbiota [26]. Mechanistically, age-associated epithelial tight-junctions permeability and declined function of Paneth cells of the intestinal mucosa have been speculated to result in intestinal barrier permeability [115, 116]. However, further research will be needed to fully understand the mechanisms of age-associated increase in gut permeability.

c. Changes in production of microbiome-derived metabolites with age

The gut microbiome plays various essential roles in the host including degradation of food, lipid storage and metabolism, vitamin synthesis, suppression of harmful microbial species and maintenance of intestinal barrier integrity [25]. Microbiome-derived SCFAs, including butyrate, propionate, acetate and valerate, are important energy source for the epithelium and ultimately affects hypoxia-inducible factor-mediated fortification of the epithelial barrier [117]. Interestingly, a decline in SCFA levels, including that of butyrate, were observed in aged humans, whereas centenarians presented with a rearrangement in the population of specific butyrate-producing bacteria [70, 118]. Additionally, the blood and intestine of germ-free mice presented with significantly lower levels of SCFAs compared to conventionally raised mice, supporting a role of the microbiota in regulating host SCFA levels [119–121]. For example, studies have shown that administration of butyrate restores the observed abnormal absorptive colonic motor activity and blood-brain barrier permeability in germ-free mice [122, 123]. Microbiota-derived metabolites has also been reported to play a role in intestinal epithelial stem cell proliferation [4]. For example, butyrate and nicotinic acid, both by-products of the gut microbiota, are involved in suppression and promotion of stem cell proliferation in the colon, respectively [124]. In addition, microbiota-derived

neurostimulators, including serotonin, glutamate, gamma-aminobutyric acid, have been reported to regulate proliferation of intestinal epithelial stem cells through the enteric nervous system [125]. Collectively, functional products of the microbiota have been implicated in the regulation of intestinal barrier integrity and function.

Other microbiota-derived metabolites have been shown to directly affect numerous systems of the host, although their functions in relation to host aging is in need of further investigation [126]. For example, Trimethylamine N-Oxide [TMAO], a byproduct of microbial metabolism, is associated with cardiometabolic diseases, such as atherosclerosis and type 2 diabetes [127, 128]. Interestingly, microbial metabolites may also affect the host through epigenetic alterations: indeed, microbiota-derived butyrate can affect the immune response of colonic macrophages through the inhibition of histone deacetylases [129, 130]. The microbiome has also been shown to contribute to various neurological conditions through the so-called “microbiota-gut-brain axis” [131]. It is noteworthy that the microbiome can influence behavioral aspects of the host – the level of SCFAs is reported to affect feeding behavior of the host and thus energy homeostasis [132]. Given that diet is a key factor in remodeling of the microbiome structure, an integrative assessment of various physiological conditions of the host is required to precisely understand the functions and effects of the microbiome.

4. Sex-dimorphism in the gut microbiome, and possible impact on aging

Although aging is a conserved process across species and biological sex, accumulating evidence has shown that many age-related phenotypes are sex-dimorphic, and may thus modify aspects of aging between animals of opposite sex [133]. Concurrently, disparities between the sexes are observed in manifestation of certain age-associated diseases, including obesity, multiple sclerosis and Alzheimer’s disease [134, 135]. However, due to experimental pragmatism, still few studies systematically evaluate how sex interacts with aging phenotypes, including age-related microbial dysbiosis. Fundamentally, key phenotypic sex differences are driven by genetic and/or hormonal mechanisms of the host [136]. Intriguingly, recent studies suggest that there may be substantial involvement of the gut microbiome in tuning sex-dimorphic phenotypes.

a. Sex-dimorphism in the gut microbiome

Mouse model studies have shown that the composition of the microbiome starts to diverge between male and female individuals after the onset of puberty [137, 138]. As described above, the gut microbial composition of healthy human adults is reported to consist of high levels of *Bacteroidetes* and *Firmicutes* [8, 48, 49]. Interestingly, studies have shown females present with higher F/B ratio compared to that of males [16, 139]. Additionally, *Proteobacteria*, *Veillonella* and *Blautia* are found in higher levels in females compared to males, but in much lower proportions [16, 140]. Although increased F/B ratio is associated with gut dysbiosis, a systematic analysis of such disparities between the two sexes and understanding of its physiological implications are still lacking [34, 56].

Interesting sexually dimorphic phenotypes have been described in studies using germ-free mice models. For example, Non-Obese Diabetes [NOD] model female mice are more prone

to spontaneously develop type 1 diabetes compared to NOD model male mice [137]. However, such difference between sexes disappeared when mice were raised in germ-free conditions [141]. In support of this finding, microbiota transplantation of conventionally raised NOD male mice microbiota to germ-free NOD female mice reduced the rate of type 1 diabetes incidence in the recipient mice [137].

b. Sex-dimorphism in host-microbiome communication?

In addition to observed sex differences in microbial communities' composition, emerging evidence is suggesting that the microbiome may potentiate the expression of sex-dimorphic phenotypes in the hosts. For instance, a recent study utilizing germ-free mice suggested that the microbiome is required to establish sex-dimorphic gene expression patterns in the liver [142]. Another study, also comparing germ-free to SPF mice, found that presence of microbes was required for sex-dimorphic regulation of lipid metabolism in the small intestine of mice [143]. Consistent with sex-dimorphic modulation of the immune system by the microbiome, the transcriptional response of adult microglia – the resident macrophages of the brain – to chronic (i.e. germ-free *vs.* SPF husbandry) or acute (*i.e.* antibiotic treatment) microbiota depletion was found to be sex-dimorphic [144]. Intriguingly, a recent study showed that microbiota depletion through antibiotic treatment rescued a number of brain phenotypes only in males in a mouse model of Alzheimer's disease [145]. Reestablishment of the microbiota reversed the rescue, supporting a direct implication of the microbiota in this phenomenon [145]. Thus, host responses to commensal microbes can be sex-dimorphic, revealing that the microbiome interacts with the biological sex of the host. However, how these sex-dimorphic interactions are modulated during aging remains largely unknown. Future studies investigating the impact of the microbiome on the aging process should systematically include sex as a variable to address this complex question.

c. Interactions between the microbiome and sex-steroid metabolism

The gut microbiome has been proposed to drive estrogen metabolism and regulate the proportions of recirculated and excreted estrogens and estrogen metabolites in the host organism [146–148]. The term "estrobolome" has been coined to define "the gene repertoire of the microbiota of the gut capable of metabolizing estrogens" [149, 150]. Indeed, the human gut microbiome is able to hydrolyze estrogen sulfate and glucuronide conjugates [151]. Thus, through manipulation of the gut microbiome, circulating estrogen levels can be shifted in a dosage-dependent manner [148]. Consistently, in a recent study, germ-free female mice presented with significantly lowered levels of 17-β estradiol, the major form of estrogens in females, compared to conventionally raised mice [142]. In the same study, transcriptome analysis of sexual development marker genes and histological studies of follicle development in germ-free female mice indicated that sexual maturation is perturbed in microbiota-depleted mice [142].

Interestingly, estrogens have been shown to impact gut microbiome structure and contribute in gut homeostasis maintenance [152]. In a metabolic syndrome study, the microbiome structure of males and ovariectomized [OVX] females were observed to share similar profiles [153]. When the two test groups were supplemented with 17-β estradiol, both males and OVX females showed alteration of the gut microbiome and suppression of Western diet-

induced obesity phenotypes. Collectively, these findings demonstrate a close bidirectional relationship between the gut microbiome and female sex hormones in affecting host health.

5. Microbiome data analysis: experimental and analytical “omics” pipelines

Traditionally, research on microbial interactions was focused on single pathogenic organisms through culture-based methods that capture only a small proportion of the bacterial microbiota [154]. However, recent findings suggest that disease pathogenesis is dependent not only on single pathogens, but also on global changes in the host microbiome [155, 156]. Advancements in next-generation sequencing techniques have enabled culture-independent analyses to capture the global changes in the microbiome. In addition, the advent of various model organisms and experimental tools, including germ-free rodent models and microbiota transplantation methods, have helped characterize microbial communities as key factors in not only dietary metabolism and host nutrition, but also in the pathogenesis of a number of chronic age-associated disease, including diabetes, cardiovascular diseases and neurodegenerative disorders [157–161].

As the field expands, microbiome analysis methods and standards are rapidly advancing to allow accurate characterization and interpretation of the microbiome data. This section will provide a primer on microbial sample collection guidelines and widely used microbiome data analysis methods: marker gene, metagenomics and metatranscriptomics analysis (Table 2).

a. Guidelines for microbiome sample collection

For human studies, oral, skin and vaginal samples are generally collected by a physician during a clinic visit – microbial samples can be collected by swabbing the appropriate area using a sterile soft cotton tip or nylon swab [162]. For model organisms, the same type of swab can be used for sample collection. Samples should be immediately flash-frozen and stored at −80°C until further processing [162]. Among various microbial samples, fecal sample collection for gut microbiome analysis presents with the most challenges because on demand collection of fecal samples is difficult. For human fecal samples, various transportation kits, including the Fisherbrand™ Commode Specimen Collection System (Fisher Scientific), OMNIgene Gut kit (DNA Genotek) and Cary Blair Transport Medium (Remel), have been developed in order to preserve microbial composition during shipping from site of sample collection to laboratories for further analysis [163–165].

For mouse studies, gut microbiome samples can be collected by picking freshly defecated fecal pellets or extracting fecal pellets from the distal colon after euthanasia. When collecting from the distal colon, fecal pellets need to be homogenized in order to ensure even distribution of microbial species of the colon. Immediate freezing of fecal samples is crucial – storage of microbiome samples at room or higher temperature for extended times results in expansion of specific microbes, such as aerobic microbes but not anaerobes, introducing bias to the data [166]. Studies have shown that microbiome samples are stable for 2 years after

being frozen at −80°C [162, 167]. Additionally, it is important to avoid multiple freeze-thaw cycles as it has been shown to affect microbial sample stability [168].

Due to the nature of the microbiome, microbiome data can be significantly affected by external factors, such as lifestyle, diet, medication and physiology. For example, for mice, housing conditions (single- or group-housed), time of cage/bedding change and fasting prior to sample collection can have significant effects on the microbial composition [169]. Additionally, technical variability is a critical issue in microbiome data analysis. Indeed, technical aspects, from DNA extraction to the choice of sequencing platform, have been found/ to substantially affect data reproducibility [170, 171]. Studies have also reported that the choice of DNA extraction kits, contaminants from carriers and storage methods may contribute to data variability [172–175]. Thus, standards and controls must be carefully chosen, and complete metadata should be provided along with the raw microbiome sequencing data in order to promote reproducibility and translatability of microbiome research.

b. Microbiome data analysis pipelines: Marker gene, metagenomics and metatranscriptomics analysis

Development of various microbiome-related experimental protocols and analytical tools have provided great opportunities in age-related microbiome research. In this section, we provide a general overview of widely used microbiome data analysis pipelines: marker gene, metagenomics and metatranscriptomics analysis (Table 2). Additionally, we discuss age-related microbiome studies that utilize each analytical method.

Marker gene analysis: high-level, low-resolution overview of microbial composition—Marker genes are conserved genes that contain a highly variable region, flanked by highly conserved regions that serve as primer binding sites, that can be used for detailed identification of microbial species: 16S rDNA PCR amplification is commonly used for bacteria and archaea, and ITS (internal transcribed spacer) for fungi. Marker gene analysis is well-tested, fast and cost-effective. Consequently, a significant proportion of microbiome research, not limited to age-related studies, is based on marker gene analysis data [176]. Additionally, its quantification generally correlates well with genomic content of microbial species [177–179]. However, it is important to note that marker gene data is susceptible to biases rising from variable region selection, amplicon size and number of PCR cycles [180, 181]. Thus, choice of amplicon primer will have significant effect on the resolution of data [182], and it is highly recommended to review primers used in the Earth Microbiome Project [183]. Marker gene analysis has been extensively reviewed elsewhere [184]. PhyloChip is another 16S rRNA gene-based method for tracking microbial communities – this microarray-based technique analyzes all nine variable regions of the 16S rRNA gene [185]. In terms of aging research, PhyloChip was used in a study to analyze age-associated changes in the microbial composition in hoatzin, a South American strict folivorous bird [186].

Typically, similar sequences detected from the marker gene analyses are clustered together into Operational Taxonomic Units [OTUs]. This process, called OTU picking, consolidates

similar sequences into single features and thus, merges sequence variants and may lose subtle - but real - biological sequence variants. Recent studies have started to prefer the oligotyping method to capture position-specific information from marker gene sequencing data [187]. In this method, exact sequence variants are used to distinguish between closely related, but distinct, taxa. Widely used algorithms such as Deblur and DADA2 implements this method to allow detection of subtle variations between sequences and thus enable greater sensitivity in microbiome analysis from the marker gene method [188, 189]. Marker gene data can also be used to infer putative biological functions of the identified microbial community through predictive functional profiling [179, 190, 191]. This analysis method links the feature-abundance data from the marker gene analysis with available microbial genomes to predict the metagenome content and biological functions. A variety of open source microbial genome references are available, including Silva, Greengenes, and iMGMC [192–194]. It is important to note that different microbial genome references are reported to show varying degrees of sensitivity towards different microbial composition arising from specific host organism and/or sampling site [194, 195]. Thus, choice of genome reference can have substantial differences on the final result (Figure 2).

Metagenomics: high-resolution with genome-level information—Metagenomics is used to sequence all the microbial genomes within a given sample. This technique captures all the DNA molecules present in the sample, spanning not only bacteria, but viral and eukaryotic DNA (including that of the host). Compared to marker gene analysis, metagenomics data yields more detailed genomic information and taxonomic resolution. Additionally, this technique allows detection of microbial species to strain level and enables *de novo* metagenome assembly using short DNA sequence reads, if desired [196, 197]. In addition, metagenomic studies directly enable the detection of the actual gene products present in the sample, thus giving a true window into the biological functions that the microbiome may perform [198, 199]. However, it is substantially more expensive than marker gene analysis, and thus more challenging to scale up to bigger comparative studies. For a thorough review of metagenomics analysis, refer to [200, 201].

Relevant to aging, metagenomic profiling of gut microbiome from young and elderly individuals and centenarians revealed distinct characteristics of microbiome structure and function of the different age groups, identifying 116 microbial genes that significantly correlated with aging [202]. For example, the study showed that a key feature of the gut microbiome profile of centenarians is the overall increase in *Proteobacteria* and a re-arrangement in *Firmicutes* compared to young adults [202]. *Proteobacteria* has been reported to contribute to systemic inflammation [203, 204]. In support of these findings, high levels of plasma interleukin-6 and interleukin-8 were detected in centenarians, although a possible pro-longevity effect of the abundant *Proteobacteria* in centenarians needs further investigation [70]. Additionally, a study by Pasolli E. *et al.* conducted a large-scale metagenomic analysis of human microbiome data from 9,316 metagenomes spanning 46 datasets from various populations, body sites, including oral cavity, gut, skin and vagina, and host ages, spanning ages of less than 1 to over 65 [205]. Utilization of such resources will provide new insights and comprehensive understanding of the functional relationship between the microbiome and host aging.

Metatranscriptomics: characterization of microbial gene expression— Metatranscriptomics uses RNA-sequencing technique to profile transcription of microbiomes present in a sample. Metatranscriptomics has been argued to best represent functionality of live microbiome and thus can provide unique insights of the sample [206]. When preparing metatranscriptomics libraries, a number of considerations are required due to host RNA contamination, such as from abundant host rRNA, and preservation of RNA quality. To note, metatranscriptomics may miss the activity of rare species in the samples, due to relatively lower gene expression. For a more thorough review, refer to [207, 208].

To our knowledge, there are only a limited number of metatranscriptomics studies that have been performed in the context of aging. In 2018, a large-scale investigation on 372 human male fecal metatranscriptomics was published [209]. The study involved male subjects from varying age groups, from 18 to 81 of age. Interestingly, the study revealed noticeable differences between metatranscriptomic and metagenomic data [209]. Such finding suggests the importance of multi-omics in microbiome research to accurately characterize taxonomic profiles and interpret physiological effects.

c. Multi-omics and multi-site analysis of the microbiome

Characterization and analysis of the microbiome have revealed immense taxonomic and genomic diversity of the microbiome and implicate more important functions of the microbiome to be revealed in future studies. However, due to the complex functional interaction between the host and the microbiome, establishment of a causal function of the microbiome should be done with caution [210]. A multi-omics approach involving analyses of transcriptomes, proteomes, metabolomes, and immunomes along with microbiome data analysis pipelines discussed above will provide unique insights in characterizing and understanding the roles of the microbiome. Additionally, a comprehensive analysis of different microbiomes, such as skin, oral and vaginal, will be fundamental in elucidating causal functions of the microbiome on host health and longevity.

The human skin is reported to possess a distinctive microbial composition and is estimated to inhabit approximately one billion bacteria per square centimeter of skin [12, 211]. Similar to other microbiomes, the skin microbiome has been shown to undergo various changes in composition throughout lifespan of the host [212]. For example, in a study of cheek microbiomes, specific genera of the *Bacteroidetes* and *Firmicutes* phyla were found on the young and specific genera of the *Actinobacteria* phyla were found only in the older age group [212]. Interestingly, Shibagaki, N. *et al.* suggested that the age-associated changes in the skin microbiome is largely influenced by the oral bacteria [213]. More specifically, microbial species that were found in greater abundance in the older age group, and thus contributed to differentiate the skin microbiomes of the different age groups, were identified as bacteria frequently found in the oral cavity, including *Streptococcus*, *Rothia* and *Veillonella* [213]. The oral microbiome has been shown to affect the whole body of the host and has been associated with a number of systemic diseases [214]. As an initiation point of digestion, the oral microbiome has been shown to impact the gut microbial composition [215]. Interestingly, the gut microbiome has been shown to affect appetite and feeding behavior of the host through the release of SCFAs [132]. Consequently, in term, the gut

microbiome can affect microbial composition of the oral cavity. Together, these studies indicate a functional network among the different microbiomes on a single host and emphasize the importance of systematic investigation in microbiome research.

6. Summary and Perspective

Recent availability of methodological and analytical tools has prompted researchers around the world to investigate the functions of the microbiome and their effects on the well-being of its host. For example, a recently published study developed a human gut microbiome aging clock based on a gut metagenomics data-trained deep learning model [216]. The model was shown to achieve the mean absolute error of 5.91 years, demonstrating that generalizable indicators of age can be derived from microbiome data [216]. With increasing understanding of the importance of the gut microbiome in host longevity, it is anticipated that we will be able to identify and predict risk factors of age-onset gut dysbiosis in the near future.

In this review, we described general changes in the microbiome with age and key findings that implicate a bidirectional relationship between the host and the microbiome. We also discussed sexual dimorphism and various confounding technical factors that must be considered when analyzing and interpreting microbiome data. Over a century ago, Elie Metchnikoff hypothesized that frailty might be delayed by manipulating the gut microbiome with host-friendly bacteria found in yogurt [217]. Intriguingly, a study of yogurt consumption in Japanese individuals observed significant disparities between the two sexes in terms of induced changes to the gut microbiome, emphasizing widespread sex-dimorphism in host physiology [218]. Although it is certain that rigorous and reproducible characterization of microbiome data will offer great opportunities to develop new diagnostic biomarker in aging, key confounding (or biologically-relevant) factors, including sex-dimorphism, will need to be carefully considered. Finally, a comprehensive analysis of different microbiomes (*e.g.* oral, skin) and how they interact with each other, will be fundamental in elucidating causal functions of the microbiome on host health and longevity.

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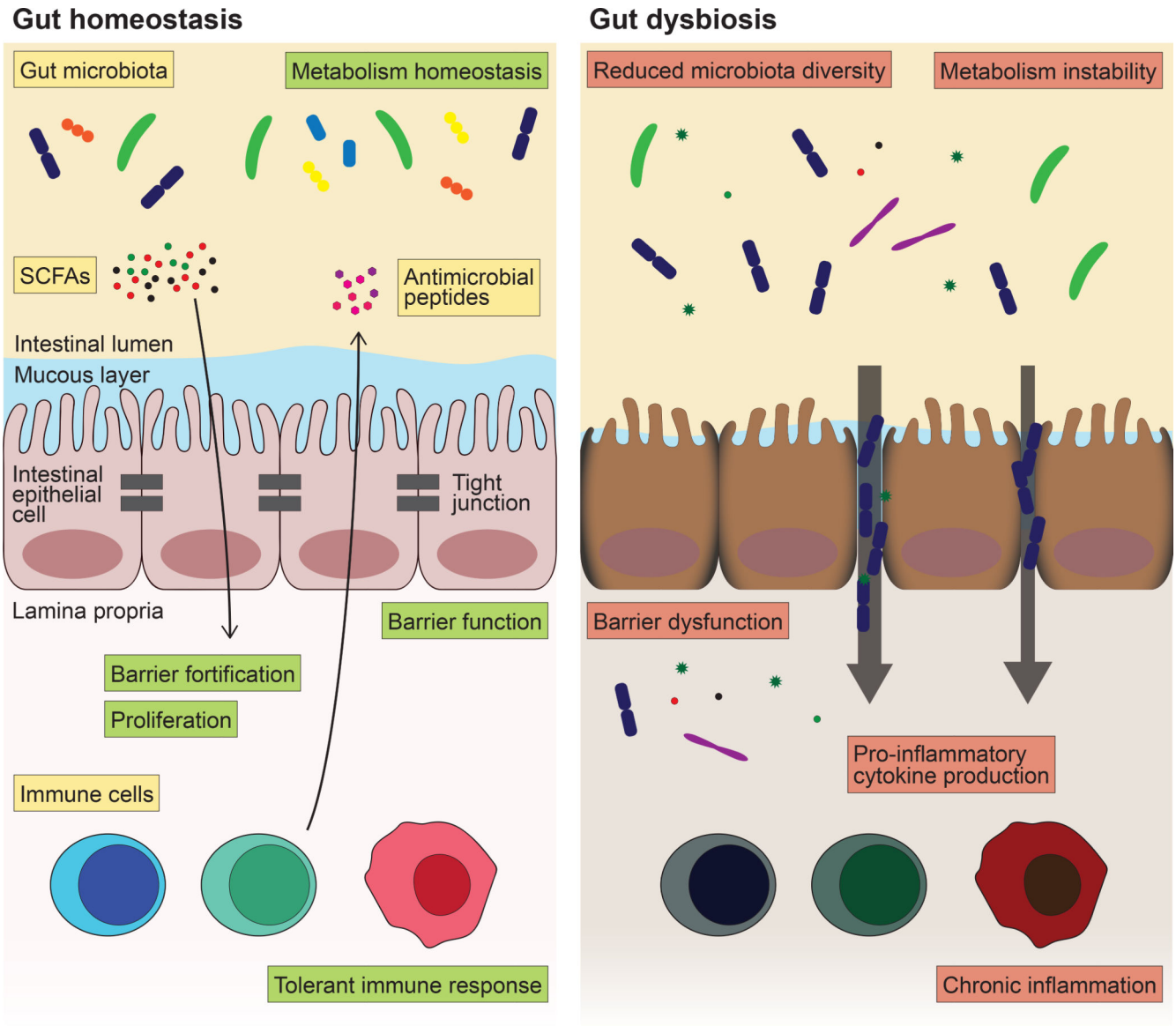
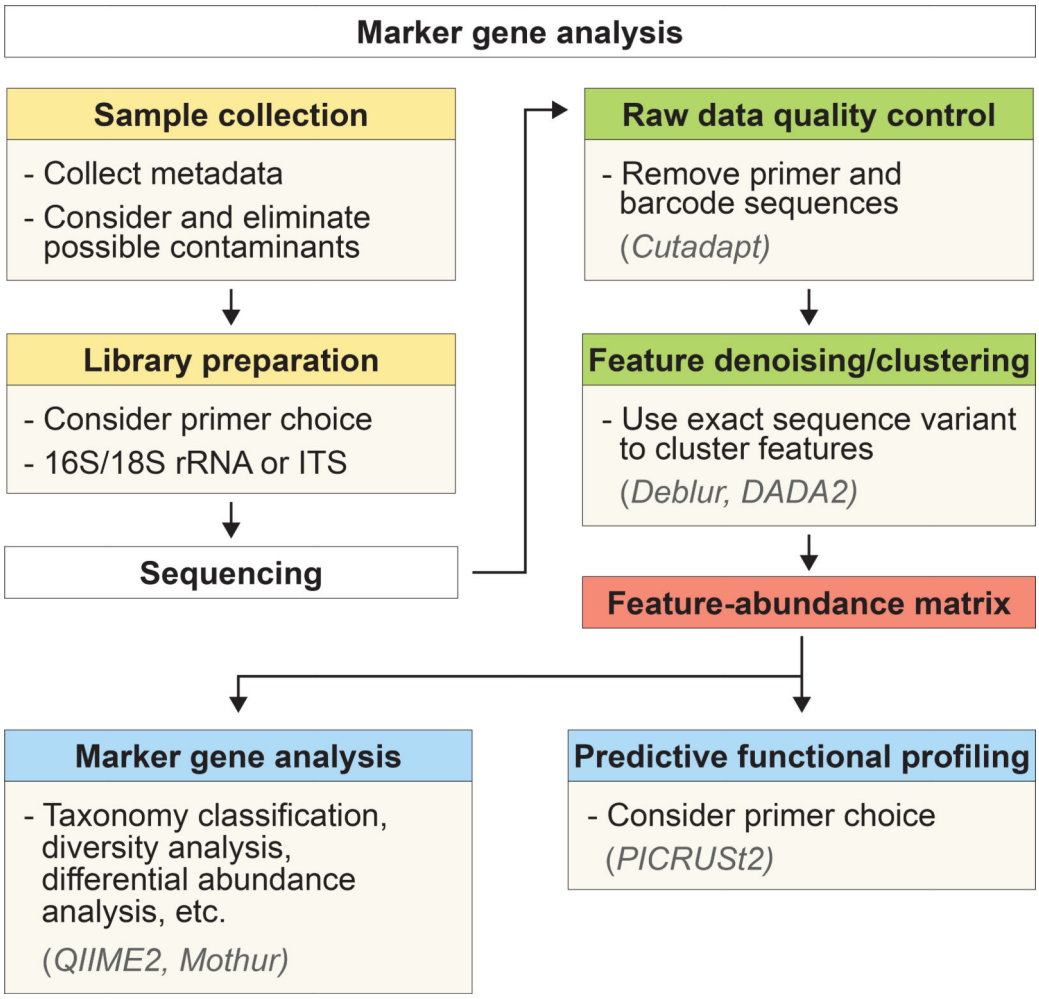


[Figure 1]. The bidirectional relationship between the gut microbiome and aging

(Left panel) In a healthy gut, balanced microbial composition and intestinal barrier integrity maintains gut homeostasis and contains the microbiota in the intestinal lumen. Microbiota-derived metabolites, including SCFAs, participate in a feedback mechanism with the host immune system to fortify the barrier function, produce mucus and promote intestinal stem cell proliferation. An efficient immune system tolerates the host immune responses to avoid excessive activation. (Right panel) In gut dysbiosis (such as with aging), declined intestinal barrier integrity results in translocation of microbes and microbial particles through the intestinal epithelial cell lining. Reduced microbiota diversity leads to overgrowth of distinct microbes and metabolism instability. Aberrant levels of microbiota-derived metabolites instigate abnormal immune responses resulting in chronic inflammation. SCFA: Short-chain fatty acid.



[Figure 2]. General workflow for marker gene analysis.

For general marker gene analysis, after sample and metadata collection, choice of primer set should be carefully considered for library preparation. Quality control of sequenced raw data, such as primer and barcode sequence removal, is strongly recommended for accurate data analysis. Features (exact sequence variants) are denoised/clustered to produce a feature-abundance matrix. Feature-abundance matrix can be used for high-level analysis, including taxonomy classification, diversity and differential abundance analysis and predictive functional profiling.

[Table 1]

Impact of pro-longevity interventions on the aging microbiome.

Pro-longevity intervention	Organism	Site	Effect on microbiome	Microbiome profiling	Reference
Calorie restriction	*M. musculus*	Gut	- Gut microbiota required for CR-induced weight loss - Significant increases in *Lactobacillus*, *Bifidobacterium* - Decreased B/F ratio	V4 16S V3-V4 16S	[83, 84, 219]
	R. norvegicus	Gut	- Increase of *Lactobacillus* - Increased B/F ratio - Changes in microbial SCFA production (Increased propionogenesis, decreased butyrogenesis and acetogenesis)	V4 16S Full length 16S Metaproteomics	[220, 221]
Dwarfism (*Ames*)	*M. musculus*	Gut	- Increased B/F ratio	V4 16S	[219]
Metformin	*C. elegans*	Gut *(food source)*	- Changes the *E. coli* metabolism of folate and methionine - *E. coli* required for longevity extension	N/A	[222]
	H. sapiens	Gut	- Increased *E. coli* abundance - Increased production of SCFAs	V4 16S Metagenomics	[223–225]
	M. musculus	Gut	- Increased B/F ratio- Increased abundance of *Lactobacillus*	V4 16S	[226]
Rapamycin	*M. musculus*	Oral	- Rejuvenation of the oral microbiome	V4 16S	[13]
	M. musculus	Gut	- Increased prevalence of segmented filamentous bacteria - Remodeling of specific OTUs - No change in B/F ratio - Renders microbiome more similar to that of HFD-treated mice	V4 16S Full length 16S (*Sequencing and PhyloChip*)	[227–229]
Resveratrol	*M. musculus*	Gut	- No change in B/F ratio - Reverses HFD-induced changes in bacterial abundances	Full length 16S	[229]
Mediterranean diet	*H. sapiens*	Gut	- Increased taxa associated to lower frailty in aged humans - Predicted increase in microbial SCFA production	V3-V4 16S	[230]
Acarbose	*M. musculus*	Gut	- Increased abundance in *Muribaculaceae* - Increase in microbial SCFAs, including propionate	V4 16S	[231]

HFD: High-fat diet

B/F: Bacteroidetes/ Firmicutes

[Table 2]

Pros and cons of marker gene analysis, metagenomics and metatranscriptomics.

Method	Cost	Analysis	Use in aging research	Pros	Cons
Marker gene analysis	Low	Simple	+++++	- General overview of microbial communities - Large public datasets available	- Cannot discriminate among live, dead or active features - Low resolution (genus level) - Primer choice significantly affect data
Metagenomics	High	Complex	++	- High resolution (species and strain level) - Direct measure of functional microbial genes - Assemble novel microbial genomes	- Cannot discriminate among live, dead or active features - Prone to host contamination-derived errors
Meta-transcriptomics	Medium	Complex	+	- Measure actively transcribing microbial genes - Directly observe microbial activity changes	- Prone to host mRNA and rRNA contamination - May fail to detect rare species or low expressed microbial genes