

# Dynamic age-associated changes and their driver microbes in healthy gut microbiota of captive crab-eating macaques

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## Research

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# Abstract

**Background:** Previous population studies have indicated age-associated changes in the gut microbiota. However, the actual age effects on microbiota are inevitably confounded by varying environmental factors such as diets and antibiotic use in the populations. Captive crab-eating macaques reared in a well-controlled environment can provide a useful model to recapitulate dynamic age-associated changes in the healthy primate gut microbiota.

**Results:** We show evidence supporting lifelong age-associated changes in the healthy gut microbiota of captive macaques. The Firmicutes to Bacteroidetes ratio and beta diversity but not alpha diversity changed significantly with age. The most significantly age-associated genera were mainly composed of commensals, such as *Faecalibacterium*. Unexpectedly, a subset of the age-associated microbes were suspicious pathogens such as *Helicobacter* and *Campylobacter*, which were enriched in infant macaques, and possibly associated with gut mucosa development. These age-associated microbes were main contributors to the gut microbiota networks. Importantly, topology analysis showed that connectivity of these networks changed with age, and its rapid decrease in elderly macaques might indicate altered microbial interactions associated with host aging. *Prevotella 9*, one of the most abundant age-associated genera, was the driver responsible for the gut microbiota maturation from infants to young adults. In adults, Rikenellaceae RC9 gut group and *Megasphaera* were two key drivers that continuously played an active role in driving microbial community changes of across different stages of adulthood. We also showed evidence of age-associated changes in gut microbial phenotypes and functions, in particular pathways of immunomodulatory metabolite synthesis, and metabolism of lipids and carbohydrates. The driver microbes were key players involved in these functions.

**Conclusions:** Our current study in captive macaques demonstrate evident age-associated changes during the lifelong process of healthy gut microbiota development. The enrichment of suspicious pathogens in healthy infant macaques might indicate the importance of appropriate exposure to these microbes for the developing immune system. The current study provides new insights into the pivotal role of driver microbes and microbial interactions in gut microbiota, and further underlines the importance of network analysis in microbiome studies. Our findings also provide a baseline for better understanding of disease-related changes in the primate gut microbiota.

## Introduction

The human gut microbiota is composed of trillions of microbial cells habitating in the gastrointestinal tract [1]. These microbes altogether encode an extremely large and dynamic genetic diversity, enabling the host to access additional energy and metabolites [2]. The gut microbiota thus plays a substantial role in human physiology and health [3]. In particular, commensal microbes in the gastrointestinal tract interplay with the host immune system, protect the host from pathogens, and modulate the host's physiological functions with commensal-derived metabolites [4–6].

The development of human gut microbiota, with dynamic changes after birth, have been implicated to play an active role concomitantly with the host's development and aging [7]. After first colonization at birth, the postnatal gut microbiota develops rapidly in the first few months of life [8, 9]. By 1 week of age, the infant gut microbiota has already become very similar to that at one-month old [10]. Breastfeeding is one of the key factors that greatly shape the infant gut microbiota, and is linked to the increase of *Bifidobacterium* species [11]. Analysis of fecal bacteria in human populations shows that changes may occur in the gut microbiota as age increases, and could be associated with increased risk of disease, especially age-associated diseases such as type 2 diabetes and hypertension in elderly people [7, 12–14].

Nevertheless, the actual effects of age on human gut microbiota remain to be further elucidated. The human gut microbial community is known to be highly dynamic. The existing population-based studies are inevitably influenced by a number of confounding factors in the populations. The individual human microbiota pattern is vastly variable. And varying environmental factors, such as diets [15] and antibiotic use [16] could dramatically influence the bacterial community [17]. In addition, people of different generations in the same population may have distinct growth experience and life styles due to the rapid urbanization of most human societies, which also shape the human gut microbiota [18]. These confounding factors emphasized the difficulty and importance to study healthy core native gut microbiota. A well-controlled model system that faithfully recapitulates age-associated changes in the gut microbiota is thus needed, and would provide better understanding of the role played by the gut microbiota in the host's healthy development and aging. In addition, humans have a much longer life span and evident difference in the gut microbiota compared to rodents, the lab animals the most widely used in existing gut microbiome studies [19]. In contrast, non-human primates (NHPs) have high similarities to humans in genetics, physiology as well as gut microbial compositions [20]. Moreover, NHPs in captivity have been found to have physiological characteristics and gut microbiota composition similar to those in humans [21]. Captive NHPs are reared with a formula diet and a stable environment, providing a feasible model to study age-associated changes in the gut microbiota of humans and NHPs.

Various microbes in the gut microbiota interact to form a complex biological network. Therefore, not only taxonomic compositions, but also microbial interactions are essential to infer changes in microbial communities. In the current study, we conducted high-throughput sequencing of the 16S rRNA gene to analyze the fecal samples from captive infant, young adult, middle-aged, and elderly crab-eating macaques (*Macaca fascicularis*). Our results revealed compositional, functional and network topology changes of gut microbiota associated with its maturation and development. Moreover, our findings identified core age-associated microbes composed of not only commensals but also suspicious pathogens, emphasizing their importance in the host's development. We also provided novel evidence supporting a substantial role of driver microbes responsible for age-associated changes in the gut microbiota network, which were further linked to altered functions of the microbial community. Such findings, taken together, could provide a baseline for better understanding of gut microbiota changes in diseases.

# Results

## *Age-associated changes of microbiota diversity in healthy captive crab-eating macaques*

The metadata of 16s rRNA gene sequencing of fecal DNA was summarized in **Table S1**. Rarefaction analysis of observed operational taxonomic units (OTUs) indicated that the sequencing efficiently captured the potential total OTUs in the fecal samples (**Fig. S1**). The top five phyla observed in the fecal samples of crab-eating macaques were Firmicutes (44.5%-61.1%), Bacteroidetes (26.4%-39.8%), Epsilonbacteraeota (2.3%-8.0%), Proteobacteria (1.9%-3.8%), and Spirochaetes (1.0%-2.7%) (**Fig. 1a**), with Firmicutes and Bacteroidetes as the two dominant phyla. Furthermore, compared to infants, the Firmicutes to Bacteroidetes (F/B) ratio was found significantly increased in adults (all  $P < 0.05$ ), especially in the middle-aged and elderly. (**Fig. 1b**). The F/B ratio was the lowest in infants (median = 1.09), and increased in young adults (median = 1.28). The highest B/F ratio was observed in the middle-aged (median = 2.74), which slightly decreased in the elderly (median = 2.06) with no significant difference.

Comparison of metrics including the Shannon (**Fig. 1c**) index, Pielou's evenness, observed OTUs, phylogenetic diversity and Simpson index (**Fig. S2**), showed no significant change in alpha diversity among the age groups. In line with alpha diversity, the Venn diagram in **Fig. 1d** showed that 275 (94.18%) genera detected in more than six fecal samples were shared across different ages. As for beta diversity, principle coordination analysis (PCoA) based on the Bray-Curtis distance matrix showed that, the infant samples mainly clustered separately from the adult groups (**Fig. 1e**). The two older adult groups clustered together. The young adult samples fell in-between. Furthermore, permutational multivariate analysis of variance (PERMANOVA) results based on unweighted UniFrac distance indicated significant difference among the four age groups (**Fig. 1f**). The intergroup unweighted UniFrac distance between adults and infants showed a trend similar to the F/B ratio (median = 0.42, 0.47 and 0.46 in young, middle-aged and elderly adults respectively), compared to the intragroup distance in infants (median = 0.38). These results thus pointed to remarkable microbial community changes associated with age.

## *The top abundant gut microbial genera in the four age groups*

We then focused on the most abundant genera. Our results showed a trend of age-associated changes in top abundant genera, similar to that of the beta diversity. The heatmap in **Fig. 2a** showed the top 20 abundant genera from each of the age groups, which were mainly commensals (**Fig. 2b**). Half of these genera were shared by all age groups (**Fig. 2c**), including four genera from family Ruminococcaceae (*Ruminococcus 1*, *Ruminococcaceae UCG-005*, *Ruminococcaceae UCG-014*, and *Subdoligranulum*), three genera from family Prevotellaceae (*Prevotella 9*, *Prevotella 2*, and *Prevotellaceae UCG-003*), *Lactobacillus*, *Blautia*, and *Dialister*.

We also looked into *Bacteroides*, which had been reported to be abundant in gut microbiota of humans living in developed countries [22]. However, the genus show a low mean abundance less than 0.1% in our captive macaques (data not shown).

### *Correlation between differentially abundant gut microbes and age*

To further determine age-associated gut microbes, we then identified OTUs with different abundance among age groups using STAMP (**Fig. S3 and S4**). The alluvial plots in **Fig. 3a, 3b, 3c, 3d** and **3e** clearly illustrated age-associated shifts of these taxa at different phylogenetic levels. We further explored their correlation with age using Spearman correlation. At the phylum level (**Fig. 3e and S4**), Epsilonbacteraeota, Deferribacteres, Fusobacteria, Bacteroidetes, Patescibacteria, and Cyanobacteria were negatively associated with age, while Actinobacteria, Kiritimatiellaeota, Lentisphaerae, Firmicutes, WPS-2, Spirochaetes, Planctomycetes, Euryarchaeota, and Tenericutes were negatively associated with age. At the genus level, in total 115 genera were significantly associated with age, with 29 and 18 from family Lachnospiraceae and Ruminococcaceae respectively (**Fig. S6**). A large proportion of the genera negatively associated with age were from family Lachnospiraceae. The top 40 genera with the strongest correlations with age were shown in **Fig. 3g**. Among these microbes, 23 genera were negatively associated with age, most of which were potential commensals. These microbes includes night genera from family Lachnospiraceae (*Lachnospiraceae UCG-001*, *Lachnospiraceae UCG-003*, *Lachnospiraceae UCG-004*, *Lachnospiraceae UCG-008*, *[Eubacterium] ventriosum* group, *Fusicatenibacter*, *GCA-900066575*, *[Ruminococcus] torques* group, and *Roseburia*), two genera from family Prevotellaceae (*Alloprevotella* and *Prevotella 2*), two genera from family Ruminococcaceae (*Faecalibacterium*, and *Fournierella*), *Actinobacillus*, *Campylobacter*, *Helicobacter*, *Mucispirillum*, *Veillonella*, *Cetobacterium*, *Brachyspira*, and *Gemella*. These top age-associated genera also included seventeen genera positively associated with age, including six from the Ruminococcaceae family (*Ruminococcaceae UCG-002*, *Ruminococcaceae UCG-010*, *Ruminococcaceae UCG-013*, *Ruminococcaceae NK4A214*, *CAG-352*, and *[Candidatus] Soleaferrea* group), *Treponema 2*, *Methanobrevibacter*, the Rikenellaceae *RC9* gut group, *Christensenellaceae R-7* group, *[Eubacterium] coprostanoligenes* group, *Lachnospiraceae UCG-007*, *Libanicoccus*, *Oscillibacter*, *Mogibacterium*, and *Stenotrophomonas*.

In addition, we also found significantly correlation of with age in lactic acid bacteria known as probiotics in humans (**Fig. S6**). *Bifidobacterium*, which is important in breastfeeding, decreased with age ( $r = 0.34$ ,  $P = 4.2 \times 10^{-4}$ ), whereas *Lactobacillus* increased with age ( $r = 0.29$ ,  $P = 0.0025$ ).

### *Differential taxa of gut microbiota enriched in the four age groups*

We then utilized LEfSe to identify differential taxa exclusively enriched in each of the four age groups. At the phylum level, *Epsilonbacteraeota* and *Cyanobacteria* were enriched in infants, Firmicutes, Actinobacteria, and Kiritimatiellaeota were enriched in the middle-aged, while Proteobacteria and Euryarchaeota were enriched in the elderly (**Fig 4a**). No phylum was enriched in young adults. At the genus level, the largest number (night-teen) of enriched genera were observed in infants, with *helicobacter* as the most enriched one (**Fig. 4b**). Other infant-enriched genera included seven genera from family Lachnospiraceae (*Anaerostipes*, *Blautia*, *Dorea*, *Fusicatenibacter*, *Lachnospiraceae UCG-001*, *Lachnospiraceae UCG-004*, and *Roseburia*), three genera from family Prevotellaceae (*Alloprevotella*, *Prevotella 2*, and *Prevotellaceae UCG-001*), five from family Ruminococcaceae (*Butyricoccus*,

*Faecalibacterium*, *Fournierella*, *Ruminococcaceae* UCG-008, and *Subdoligranulum*), *Holdemanella*, *Phascolarctobacterium*, and *Sutterella*. In contrast, *Lactobacillus* as the only one genus enriched in young adults. The middle-aged and the elderly had intermediate numbers of enriched genera. Seven genera were enriched in the middle-aged, including three genera from family Ruminococcaceae (*Ruminococcaceae* NK4A214 group, *Ruminococcaceae* UCG-002, and *Ruminococcaceae* UCG-010), *Treponema* 2, *Rikenellaceae* RC9 gut group, *Christensenellaceae* R-7 group, and *Lachnospiraceae* FCS020 group. Six genera were enriched in the elderly, including *Prevotellaceae* UCG\_003, *Megasphaera*, *Ruminococcaceae* UCG-013, *Coproccoccus* 3, and *Desulfovibrio*.

#### Age-associated gut microbiota networks and key driver genera

We then further used the Sparse Compositional Correlation (SparCC) analysis to explore the interaction among gut microbes in the four age groups (**Fig. 5**). All genera with relative abundance  $\geq 0.1\%$  were included in the networks. Surprisingly, although not preferentially selected, the age-associated genera were found to be the major components of these networks. The gut microbiota network in infants had the lowest connectivity of interactive in infants, as indicated by small Maximal Clique Centrality (MCC) scores (total MCC score = 56) (**Fig. 5a and 6a**). The network developed into a more mature stage in young adults (total MCC score = 274) (**Fig. 5b and 6a**), and had the highest connectivity in the middle-aged (total MCC score = 3688) (**Fig. 5c and 6a**). Unexpectedly, although similar gut microbiota diversities were found between the elderly and middle-aged, the network connectivity dramatically decreased in the elderly (total MCC score = 83) (**Fig. 5d and 6a**).

We then utilized cytoHubba to analyze hub genera, which were supposed to identified by ranking their centralities MCC and EcCentricity (EPC) scores. Among the hub genera shown in **Fig. 6a**, *Prevotella* 9 was the only one shared by all four age groups as well as the network constructed using all samples (**Fig. 6a and 6b**). The inter-genera interactions mediated by *Prevotella* 9 could be of potential importance. The strongest positive interactions in the microbial communities were found in *Prevotella* 2 and *Alloprevotella* with *Prevotella* 9 in infants. In addition to *Prevotella* 9, *Helicobacter* and *Prevotella* 2 were another two important hub genera in infants. The role of such interactions mediated by these genera, in particular *Prevotella* 9, gradually diminished with age, and were in part replaced by interactions mediated by hub genera negatively associated with age, such as *Ruminococcaceae* UCG-002 and *Rikenellaceae* RC9 gut group.

Moreover, we used NetShift analysis to detect rewiring between microbiota networks, and identified key driver microbes responsible for the changes (**Fig. 6c and Table. S3**). *Prevotella* 9 was found to be the only driver genus responsible for the microbial changes between infants and young adults. Novel interactions with *Prevotella* 9 were established in the gut microbiota of young adults compared to that of infants. As for adults, multiple potential drivers were identified. Among these drivers, *Rikenellaceae* RC9 gut group and *Megasphaera* are the two key driver genera that contribute to the long-term development of gut microbiota in adults. Another five genera including *Dialister*, *Christensenellaceae* R-7 group, *[Eubacterium]* coprostanoligenes group, *Ruminococcaceae* UCG-005 and *Ruminococcaceae* UCG-002 group are

involved in the change of gut microbiota between young adults and the middle-aged. Another five genera including *Ruminococcaceae UCG-014*, *Holdemanella*, *Succinivibrio*, *Alloprevotella*, *Lachnospiraceae UCG-007*, and *Prevotella 2* are involved in the change of gut microbiota between the middle-aged and the elderly.

### *Age-associated microbial phenotypes and functions and their correlations with gut microbiota*

To understand the potential function impact of age-associated taxonomic changes in gut microbiota, the microbial phenotypes were predicted using BugBase and compared among age groups. Anaerobic and Gram-positive phenotypes was significantly up-regulated, whereas facultative anaerobic and Gram-negative phenotypes were down-regulated in the middle-aged and elderly groups compared to infants (all  $P < 0.01$ ) (**Fig. 7a**). In line with these findings, Spearman correlation analysis showed that, the anaerobic and Gram-negative phenotypes significantly decreased ( $r = -0.37$ ,  $P = 1.2 \times 10^{-4}$  and  $r = -0.34$ ,  $P = 4.3 \times 10^{-4}$  respectively) with age, whereas the facultative anaerobic and Gram-positive phenotypes significantly increased with age ( $r = 0.42$ ,  $P = 8.7 \times 10^{-6}$  and  $r = 0.34$ ,  $P = 4.3 \times 10^{-4}$  respectively) (**Fig S6**).

We also determined age-associated changes in gut microbial function using the software Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and identified 152 Kyoto Encyclopedia of Genes and Genomes (KEGG) modules to be significantly associated with age (**Table. S2**). The principle component analysis (PCA) plot derived from the abundance of KEGG modules revealed remarkable differences in microbial functions among age groups, showing a similar pattern with beta diversity (**Fig. 7b**). We observed significant correlation between these microbial functions and age. As shown in the heatmap in **Fig. 7c**, metabolic pathways that were the most positively associated with age were mainly involved in biosynthesis and metabolism of lipids and carbohydrates. And metabolic pathways that were the most negatively associated with age were mainly involved in biosynthesis of immunomodulating metabolites such as lipopolysaccharides, and metabolism of polyunsaturated fatty acids and vitamins such as folate and riboflavin. Noteworthy, strong correlations were found between these age-associated microbial functions and gut microbes, in particular the hub genera and drivers (**Fig. S8**). *Prevotella 9*, was the core genus that was involved in these functions.

## Discussion

By using the NHP model of captive crab-eating macaques, we revealed remarkable lifelong age-associated changes in gut microbial composition and functions. Moreover, our study identified hub and driver microbes that holds a potential significance in the age-associated microbial interplay. Given the similarities between the captive crab-eating and humans, these findings could provide better understanding of age-associated changes in the human gut microbiota.

The gut microbiota of captive macaques in this study showed similarities to that of humans, especially those in developing countries [12, 21, 23, 24]. In line with human and other NHPs, the gut microbiota of our captive crab-eating macaques was dominated by Firmicutes and Bacteroidetes across all ages

(Fig. 1a) [1, 25]. Most of the common genera with high abundance across all ages, are potentially commensals from the Ruminococcaceae and Prevotellaceae families such as Prevotella 9 (Fig. 2b). In contrast, Bacteroides had very low abundance. Gut microbial communities of individuals from developing countries has been reported to be dominated by Prevotella [24], while those from developed countries was highly abundant in Bacteroides [26]. Plant-based diets with low fat could be involved in the higher similarities between the gut microbiota of and captive macaques and humans living in developing countries [22].

The lack of significant change in alpha diversity might indicate the important of studies in captive NHPs (Fig. 1). Yatsunenکو et al. reported that observed OTUs increased with age in all three populations [12]. In a recent study of non-captive rhesus macaques Chen et al. reported that male adults had significant higher Shannon index than male juvenile [27]. However, under a well-controlled environment provided by captivity, alpha diversity changes are probably smoothed out. By age of 1–2 years old, infant gut microbiota had gained more than 94% of OTUs observed in adults (Fig. 2a). Age-associated factors, such as diets and life styles, rather than age itself, might actually contribute to the increase of alpha diversity in human populations.

Nevertheless, the remarkable age-associated changes including the F/B ratio and beta diversity as well as network topology emphasized actual effects of age on the gut microbiota in captive macaques (Fig. 1b). The F/B ratio is considered as an indicator of maturation and development of gut microbiota [28], and has been reported to be involved in health-related conditions or diseases such as obesity [29]. In the current study the F/B ratio increased in adult macaques, and decreased in elderly macaques (Fig. 1b), resembling observation in humans [28, 30]. It could be due to increased Firmicutes and decreased Bacteroidetes with age (Fig. 3f). Interestingly, although middle-aged and elderly macaques had similar beta diversity, evident reduction of connectivity in elderly macaques, indicating a decline of microbial interactions. Such findings suggest that, network connectivity could be more sensitive than the F/B ratio and biological diversity to detect age-associated changes in the gut microbiota.

Moreover, the age-associated microbes identified in captive macaques could be involved in the host's development and aging in good health (Figs. 3 and 4). These microbes could play distinct roles dependent of their direction of age-correlation. A large proportion of these age-associated genera decreased with age, including those enriched in infants. The composition and activities in the infant gut microbiota has been engaged in the host's early development and a variety of diseases, such as allergy and autisms [5, 31, 32]. These genera negatively associated with age in fact consisted of at least two distinct groups. First, these genera contained potential commensals, which were active players in the early development of gut microbiota (Fig. 4b, S4, and S6). The interplay between these commensals and the host intestinal barriers are important to the postnatal development of host metabolism, immunity and mucosal barrier [33–35]. Commensals could benefit the host by producing metabolites such as short chain fatty acids [36]. A number of the age-associated commensals in the current study are butyrate-producing bacteria in the host colon, including Faecalibacterium (such as Faecalibacterium prausnitzii), Roseburia (such as Roseburia faecis), Anaerostipes (such as Anaerostipes butyraticus), and

Butyricococcus (*Butyricococcus pullicaecorum*) [37]. These commensals include anti-inflammatory bacteria, and outcompete pathogens to protect the host, and abnormal alteration of them has been reported in various human diseases [38–42]. For example, *Faecalibacterium prausnitzii* is one of the most abundant anti-inflammatory commensal bacteria in the colon, and was reduced in Crohn disease patients [40]. We also notice that *Bifidobacterium*, the key probiotics for the metabolism of oligosaccharides in breast milk [43], reduced with age. The abundance of *Bifidobacterium* was lower in our post-weaning infant macaques than that in lactating macaques reported by Rhoades et al. [9].

Second, these bacteria negative associated with age also contained a number of suspicious pathogens, especially enteropathogens (Fig. 4b, S4, and S6). *Campylobacter* and *Actinobacillus* are causes of infectious diseases in humans, *Campylobacteriosis* and *Actinobacillosis* [44]. Species from the genus *Brachyspira* are known pathogens causing diarrhea in animals and human [45]. Bacteria from the *Gemella* genus are involved in endocarditis [46]. *Anaerobiospirillum succiniciproducens* from the genus *Anaerobiospirillum* has been found to be associated with has diarrhea and bacteremia [46]. It was noted that *Helicobacter* was identified as a hub genus with high abundance in infant gut microbiota, but its role remained largely unclear. *Helicobacter macacae* from the genus have been reported to be frequently detected in rhesus monkeys without a diarrheal history [47]. Rhoades et al. report that 8-month infant remained asymptomatic for diarrhea were enriched for the species [9]. It should be taken into account that all macaques in the current study were in good health. Therefore, the gradual decrease of these suspicious pathogens with age might associated with the maturation of gut mucosal barrier. In addition, recent studies have reported possible effects of pathogens protecting the host against allergic sensitization [48, 49]. In our captive macaques the suspicious pathogens with their abundance under control might allow “good” exposure for the proper training of the host’s immune system. In line with such findings, biosynthesis of bacterial toxins was also negatively associated with age, further suggesting a potential role of these age-associated microbes in modulation of the host’s immunity.

While the roles of the microbes negatively associated with age remained largely unclear, they could be related to the host’s healthy aging (Fig. 4b, S4, and S6). A subset of these microbes has been implicated to be involved in diets and energy metabolism, especially lipid metabolism, as well as diseases. Importantly, the genus *Lactobacillus*, which was highly abundant increased in adult macaques, are widely used probiotics with potential effects on lipid metabolism [50]. *Eubacterium coprostanoligenes* was identified as a cholesterol-reducing anaerobe [51]. In addition, recently a population-based study linked Genera *Christensenellaceae* R-7 group, *Ruminococcaceae* (UCG-002, and UCG-010), and *Lachnospiraceae* FCS020 group with circulating metabolites related to blood lipids [52]. *Candidatus soleiferrea* was increased in a randomized controlled trial of hypocaloric diet with Hass avocado [53]. In addition, *Treponema* 2, *Rikenellaceae* RC9 gut group, *Prevotellaceae* UCG-003 were increased in rats with isoproterenol-induced acute myocardial ischemia [54], whereas in a meta-analysis *Christensenellaceae* R-7 group was found to be reduced in patients affected by intestinal diseases [55]. In line with these findings, changes of microbial functions related to metabolisms of lipids and carbohydrates increased with age (Fig. 7b). Intriguingly, the archaea *Methanobrevibacter* increased with age in the gut microbiota of our macaques. Although the reported role of these methanogen in host health remain controversial, our

results indicated that such increase of *Methanobrevibacter* abundance with age might not necessarily affect the health of the host.

This study further highlights the substantial role of driver microbes in age-associated changes of the gut microbiota (Figs. 5 and 6). Genus *Prevotella* 9, with a high abundance in our captive macaques, was identified as the most important hub mediating large proportion of microbial interactions in gut microbiotas across all ages. And it acted as the key driver responsible for the gut microbiota maturation from infants to young adults. The exact biological significance of *Prevotella* 9 in the context of integrative bacterial community and microbiota development has yet to be further elucidated. A recent reanalysis of existing gut metagenomes from NHPs and humans reported that *Prevotella* were prevalent in primate gut microbiota of different host species [20]. In line with such finding, the *Prevotella* 9 genus was highly abundant across all ages with gradual age-associated decrease in our captive macaques. The high abundance of the genus in primates could be strongly associated with plant-based, low-fat diets [22]. In addition, the high abundance of *Prevotella* in humans and NHPs might also have possible implications for host-microbiota coevolution [56]. Although *Prevotella* 9 remained abundant in adult macaques, its level decreased with age, and possibly freed up space for other microbes that were necessary for further microbiota development, such as Rikenellaceae RC9 gut group and *Megasphaera*. Such shift of driver microbes could in turn impact the changes of gut microbiota phenotypes and functions.

## Conclusions

In summary, by using captive crab-eating macaques to control confounding factors, the current study demonstrates evident age-associated structural and functional changes in the healthy gut microbiota during the host's development and aging. Our key findings of age-associated microbes, composed of both commensals and suspicious pathogens, highlight the potential importance of appropriate bacterial exposure and community balance for the host. Moreover, the hub genera and drivers identified by network topology analysis probably play a pivotal role as core microbes in the microbial communities, and are responsible for the maturation and development of primate gut microbiota. By characterizing the age-associated healthy gut microbiota, the current study also provides an important baseline for better comparison and understanding of disease-related changes in the primate gut microbiota.

## Methods

### *Animals in the study*

A total of 104 male crab-eating macaques from Guangdong Xiangguan Biotechnology Co. Ltd. (Guangzhou, China) were included in the current study. All of the animals were confirmed to be in good health by records and veterinary examination prior to the study. These animals were composed of four different age-groups (N=26 for each group), including infant (1-2 years old), young adult (4-6 years old), middle-aged group (8-10 years old), and an elderly macaques ( $\geq 13$  years old). Post-weaning infant

macaques were selected to reduce possible effects of breastfeeding. All animals were kept in a well-controlled environment with moderate room temperature (16-28 °C) and relative humidity of 40%-70%, as well as a 12/12-hour light-dark cycle. The study complied with protocols approved by the Animal Ethics Committees of Guangdong Institute of Applied Biological Resources, and were in compliance with the Guide for the Care and Use of Laboratory Animals [57].

#### *Stool sample collection and DNA extraction*

Rectal swab samples were freshly collected from each monkey, and stored at -80 °C immediately until DNA extraction. Microbial DNA was extracted using TIANamp Stool DNA kit (Cat.#DP328, Tiangen, China) according to the manufacturer's instructions, and its concentration and quality were assessed using a Nanodrop One Microvolume UV Spectrophotometer (ThermoFisher, U.S.).

#### *16S rRNA gene sequencing*

The hypervariable V4 regions of bacterial/archaeal 16s rRNA genes were amplified using polymerase chain reaction and V4-specific primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR products between 400 and 450 bp were checked using the 2% agarose gel, purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA), and sequenced on an Ion S5XL sequencer with a single-end 400-bp read length configuration.

#### *Processing of 16S rRNA gene sequencing data*

Bioinformatic analysis of the 16S rRNA gene sequencing data was performed using the QIIME2 (version 2018.6.0) analysis pipeline [58]. Briefly, sequencing data were processed by the dada2 program to filter low-quality and chimeric sequences, and generate unique feature tables equivalent to OTU tables at exact match or 100% sequence similarity. Taxonomy was then assigned to these features using the q2-feature-classifier against the full-length SILVA database (release r132) at 99% similarity cutoff [59]. Analysis of microbiota diversities were conducted in QIIME2: alpha diversity metrics including Pielou's evenness, phylogenetic diversity, observed OTUs, Shannon and Simpson's indices, and beta diversity including weighted/unweighted UniFrac distances, and Bray-Curtis dissimilarity. Comparison of beta diversity was performed using the nonparametric method PERMANOVA. Abundance of OTUs were compared among groups by using STAMP [59]. the Linear discriminant analysis (LDA) Effect Size (LEfSe) algorithm was used with a log (LDA) score cutoff of 2 to identify taxa specifically enriched in particular age groups [60]. Phylogenetic cladograms of LEfSe results were visualized using the GraPhlAn tool (<https://bitbucket.org/nsegata/graphlan>).

#### *Microbial interactive network construction and analysis*

The SparCC (<https://bitbucket.org/yonatanf/sparcc>) algorithm was used to estimate the correlations among gut microbes [61]. 100 bootstrap replicates were used to calculate the pseudo *P*-values in the SparCC analysis, and correlations with | correlation coefficient (*r*) | >0.2 and *P* < 0.01 were considered significant. For each OTU with significant SparCC correlation, a weighted node connectivity score was

calculated as an indicator of its weight in the network, by summing up its  $|r|$  with all of its first neighbors [62]. The constructed gut microbial interactive network was further visualized using Cytoscape version 3.7.0 [63]. The cytoHubba plugin was used to identify hub genera in the networks [64]. Two node ranking methods including a local-based method MCC and a global-based method EPC were used to evaluate importance of genera. In addition, NetShift (<https://web.mriapps.net/netshift/>) was used to evaluate potential driver microbes using a case-control strategy to compare a pair of networks as described [64, 65]. Neighbor Shift (NESH) Scores were calculated to quantify enriched interaction in the case over the control.

### *Prediction of microbial phenotypes and function profiles*

The BugBase (<https://bugbase.cs.umn.edu/>) analysis tool was utilized to predict high-level phenotypes in fecal microbiome samples. PICRUSt version 1.1.4 was used to predict microbial functions from the 16S rRNA gene sequencing data, which were further categorized using the BRITE hierarchy of the KEGG database [66]. PCA based on KEGG module abundance was conducted using STAMP.

### *Statistical analysis*

Statistical analysis was performed using GraphPad Prism V.7.0a (GraphPad Software, USA) and the R statistical language (version 3.6.0). Abundance of OTUs and KEGG modules among groups were compared using the non-parametric Kruskal-Wallis test, and evaluated for pair-wise inter-group differences with Tukey's post hoc test if overall significance was found. The Benjamini-Hochberg false discovery rate (FDR) correction was applied for multiple testing. Correlations of OTUs, microbial phenotypes and KEGG functions with age were determined using Spearman's correlation analysis. Differences in the taxa were analyzed by LEfSe with default settings.

## **Abbreviations**

EPC: EcCentricity; FDR: false discovery rate; KEGG: Kyoto Encyclopedia of Genes and Genomes; LDA: the Linear discriminant analysis; LEfSe: the Linear discriminant analysis Effect Size; MCC: Maximal Clique Centrality; NESH: Neighbor Shift; NHPs: non-human primates; OUT: operational taxonomic unit; PCoA: principle coordination analysis; PERMANOVA: permutational multivariate analysis of variance; PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; SparCC: Sparse Compositional Correlation

## **Declarations**

### **Ethics approval and consent to participate**

The study complied with protocols approved by the Animal Ethics Committees of Guangdong Institute of Applied Biological Resources, and were in compliance with the Guide for the Care and Use of Laboratory Animals.

## Consent for publication

Not applicable.

## Availability of data and materials

The raw datasets of 16s rRNA gene amplicon sequencing in the current study are deposited and available in the BioProject (<https://www.ncbi.nlm.nih.gov/bioproject>) repository under the accession number PRJNA598010.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Z-YW and J-HR contributed equally to this work. J-HR and J-HC conceived the project and planned the experiments. J-HR, B-HL and M-TT collected the fecal samples. Z-YW, M-TT, G-AZ, Q-CL, L-MW, B-QX performed the experiments. Z-YW, G-AZ, X-YL and J-HC analyzed and interpreted the experiment data. Z-YW, X-YL and J-HC drafted the manuscript. All authors read and approved the final manuscript.

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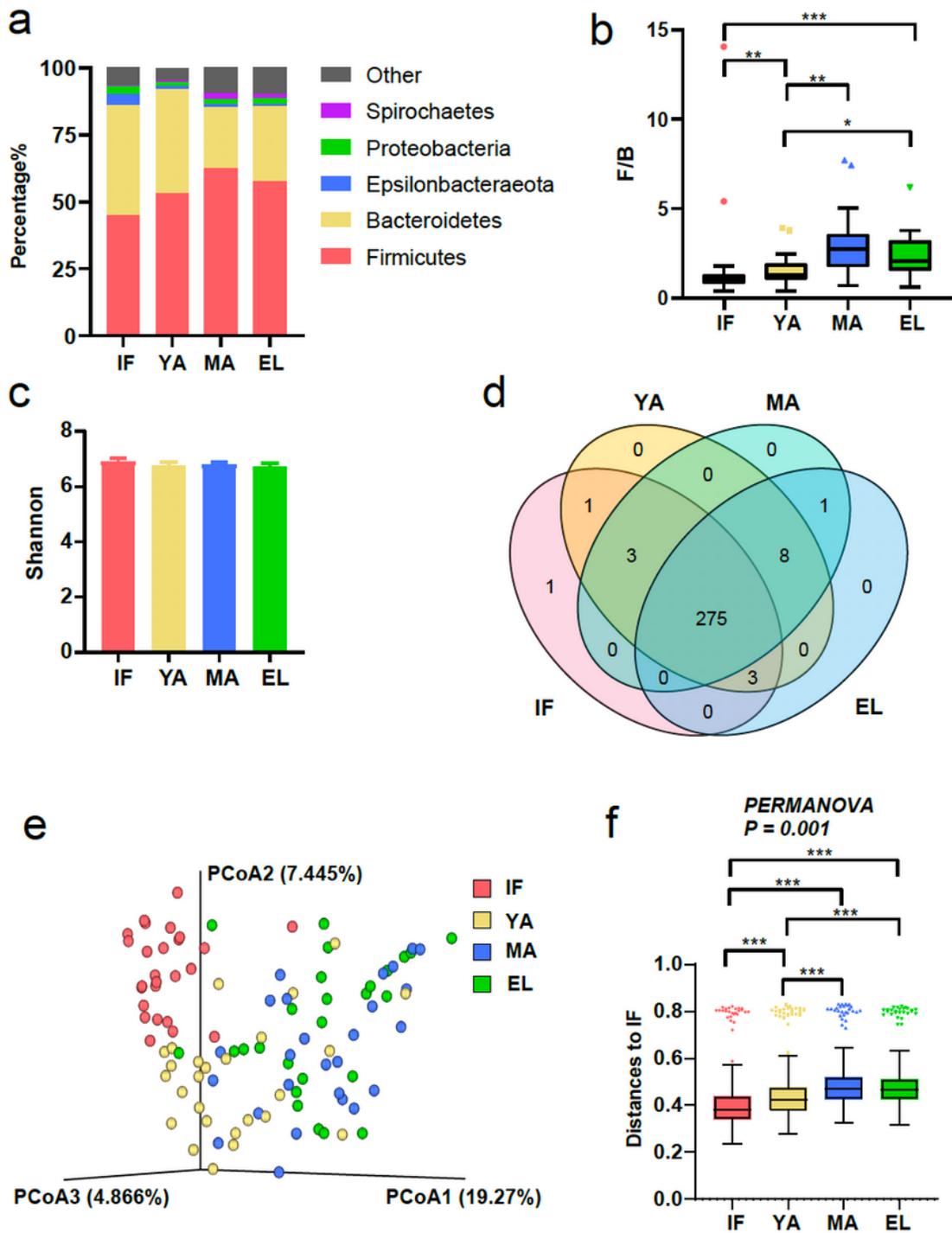
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## Figures



**Figure 1**

Firmicutes to Bacteroidetes ratio and beta diversity in gut microbiota in different age groups of captive crab-eating macaques. (a) Composition of gut microbiota at the phylum level in the age four groups. (b) The relative proportion of Firmicutes to Bacteroidetes (F/B) ratio. (c) PCoA plot based on the Bray-Curtis distance matrix of all fecal samples. (d) Venn plot illustrating overlap of gut microbial genera among age groups. Genera detected in more than 6 fecal samples are included. (e) PCoA analysis of all fecal

samples based on taxonomic profiles. (f) Unweighted Unifrac distance of gut microbiota between the three adult groups and the infant group. Pairwise P-values are calculated using nonparametric Kruskal-Wallis test with Tukey post-hoc test. IF, infants; YA, young adults; MA, the middle-aged; EL, the elderly. \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .

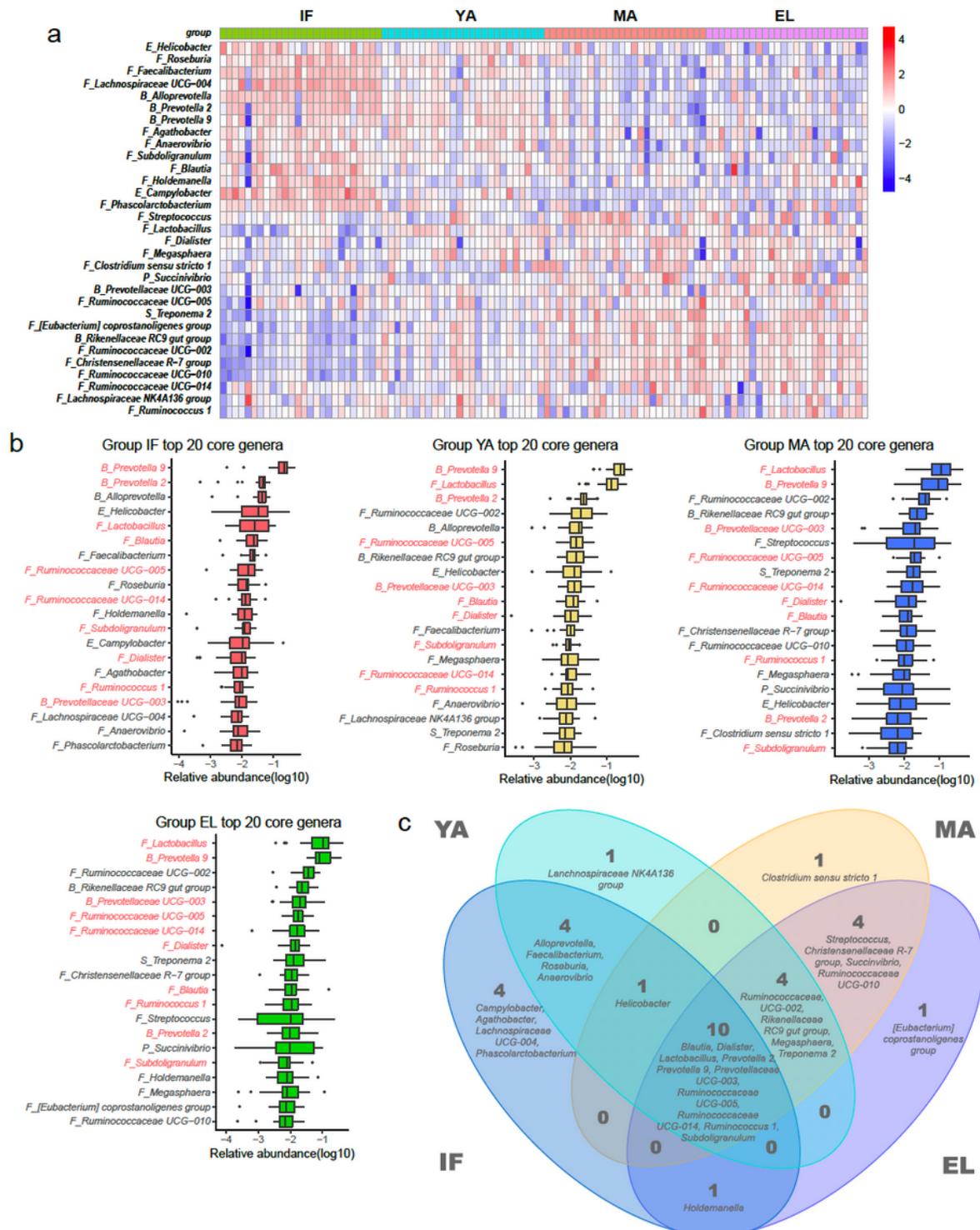


Figure 2

The most abundant genera of gut microbiota in different age groups. (a) Heatmap showing the most abundant genera in gut microbiota of the four age groups. (b) Box plots showing ranking of top 20 abundant genera in infants, young adults, the middle-aged and elderly. (c) Venn plot illustrating overlap of top 20 abundant genera among age groups. Single letters in front of genus names indicate the phylum which the genera belong to: B, Bacteroidetes; F, Firmicutes; E, Epsilonbacteraeota; P, Proteobacteria; S, Spirochaetes. IF, infants; YA, young adults; MA, the middle-aged; EL, the elderly.

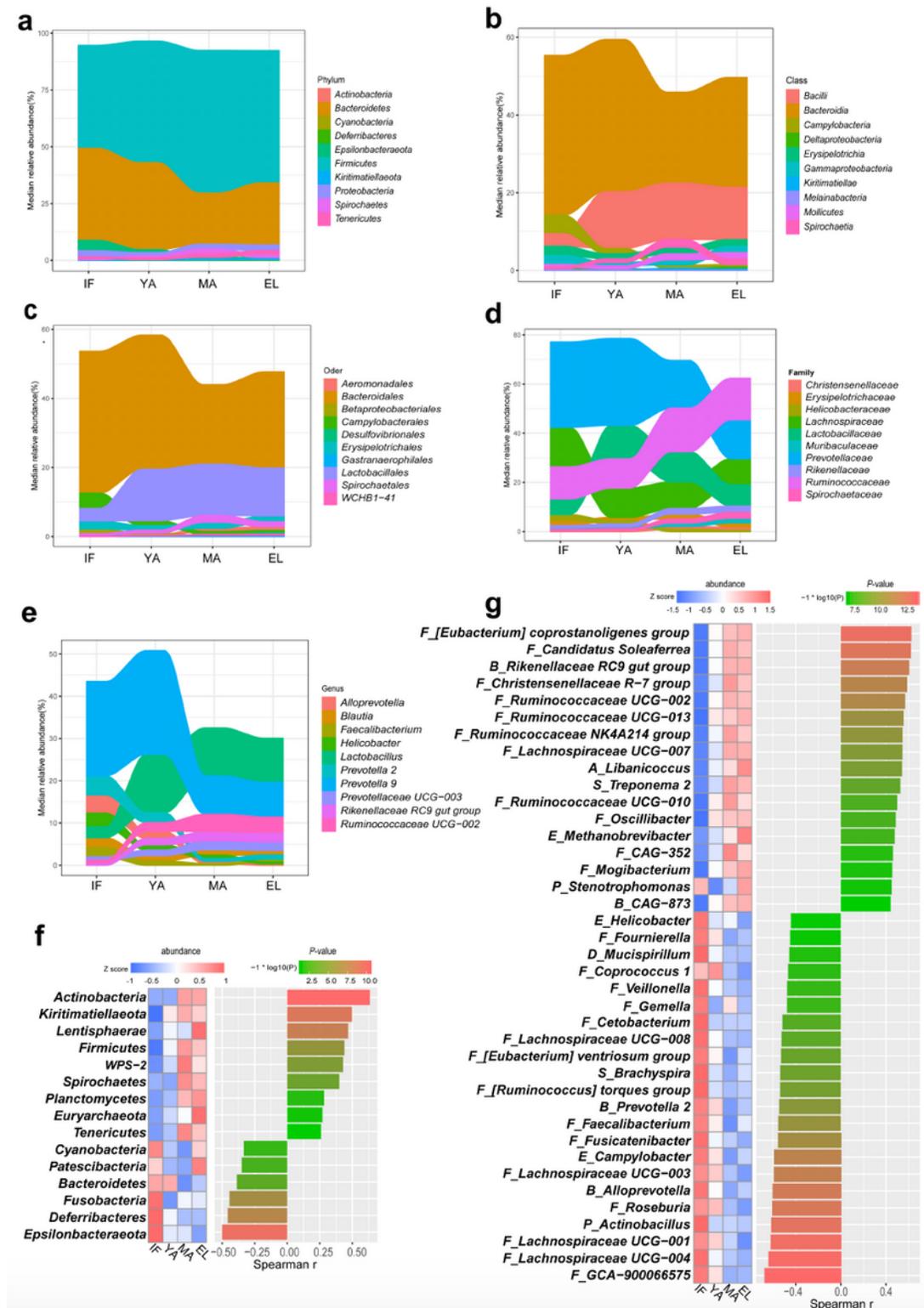
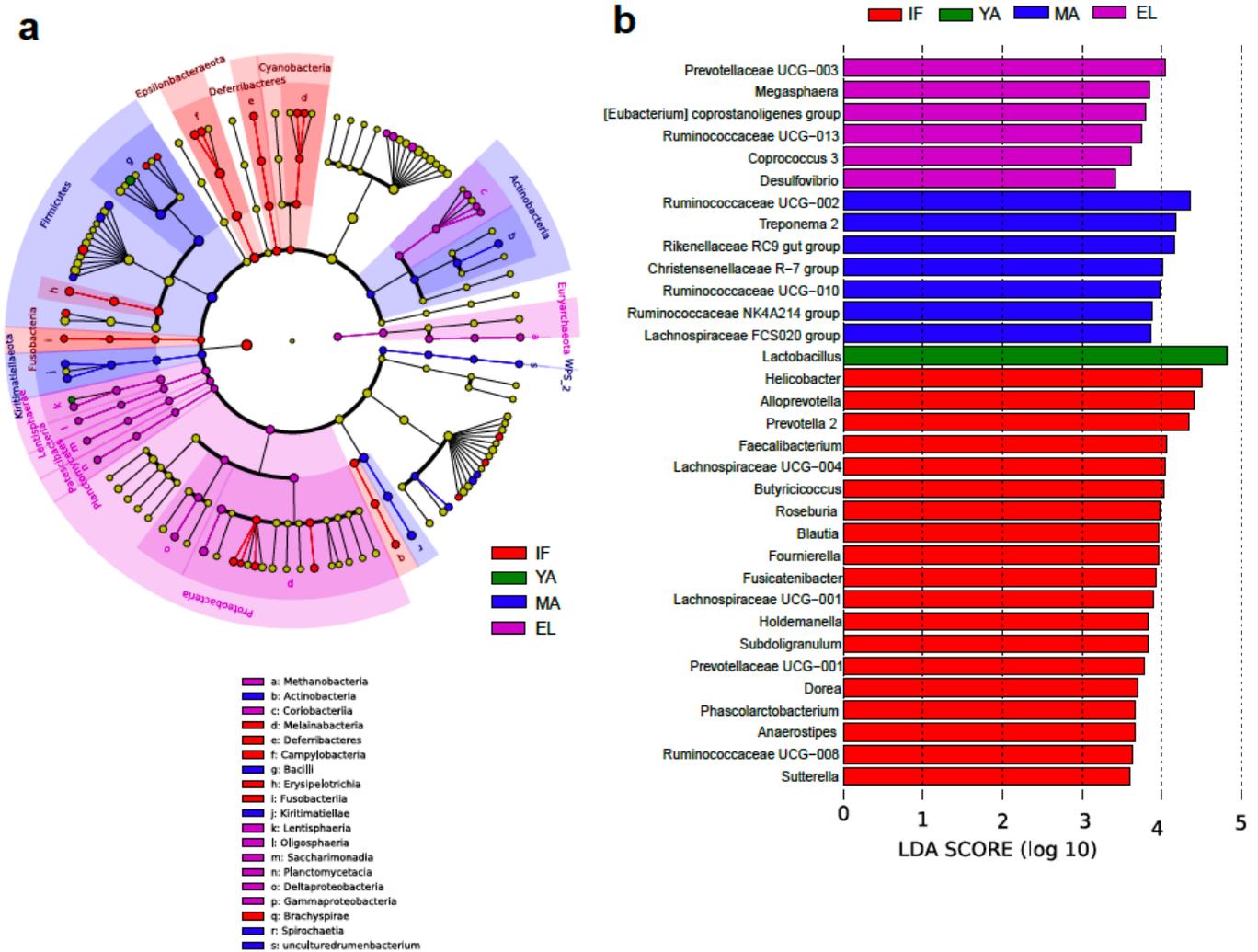


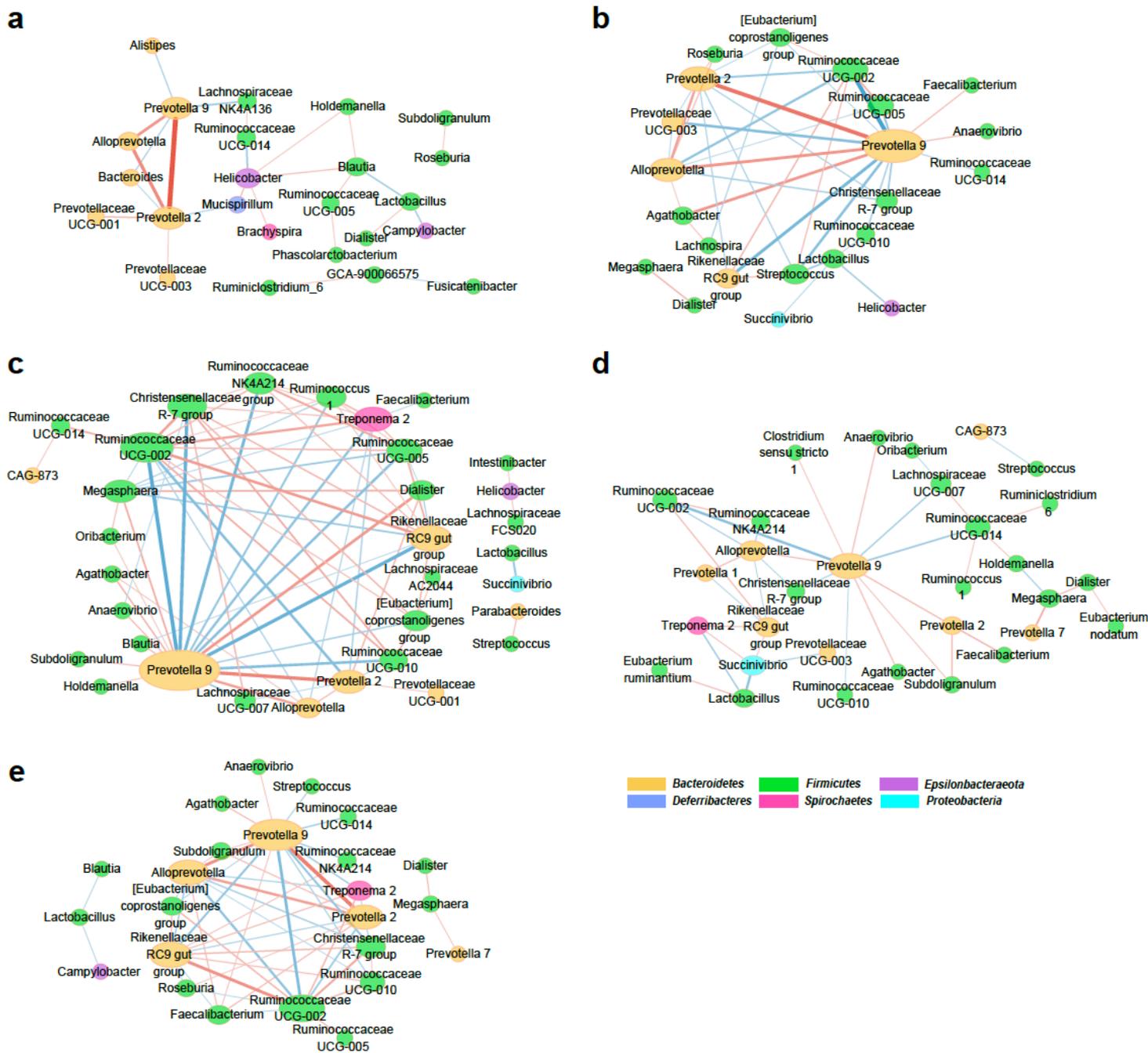
Figure 3

Correlation between differentially abundant gut microbes and age. Alluvial plots illustrating host age-associated phylogenetic shifts of the top 10 differentially abundant taxa at the phylum (a), class (b), order (c), family (d) and genus levels (e). Differentially abundant taxa are ranked by their median of abundance. Heatmaps showing significant age correlations for differentially abundant phyla (f) and genera (g). P-values are derived from Spearman correlation test. For genera, only the top 40 genera ranked by  $|r|$  are shown.



**Figure 4**

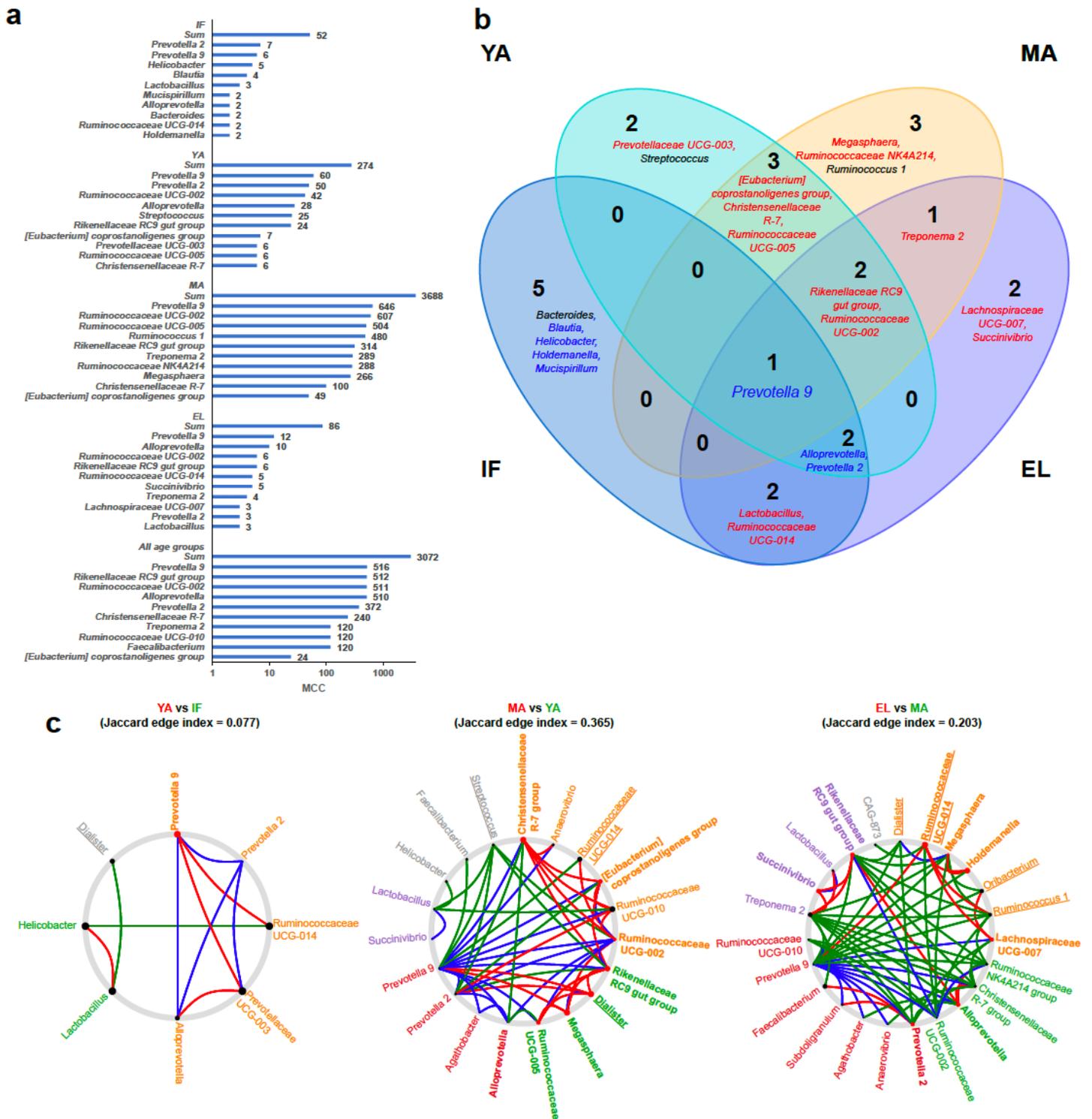
Differentially abundant taxa enriched the four age groups from LfSe analysis. (a) Phylogenetic cladogram showing differentially abundant taxa from kingdom to family levels. Microbial classes are indicated with letters. (b) Bar chart showing differentially abundant with average abundance > 0.1%.



**Figure 5**

The interactive networks of gut microbiota. Microbial interactive networks in infants (a), young adults (b), the middle-aged (c), the elderly (EL, d) and all samples (e) are constructed from SparCC results, and visualized using Cytoscape. Genera with average abundance > 0.1%, correlation  $|r| > 0.2$  and  $P < 0.05$  are included in the networks. Node colors denote the phylum of the genera. Node sizes represent weighted

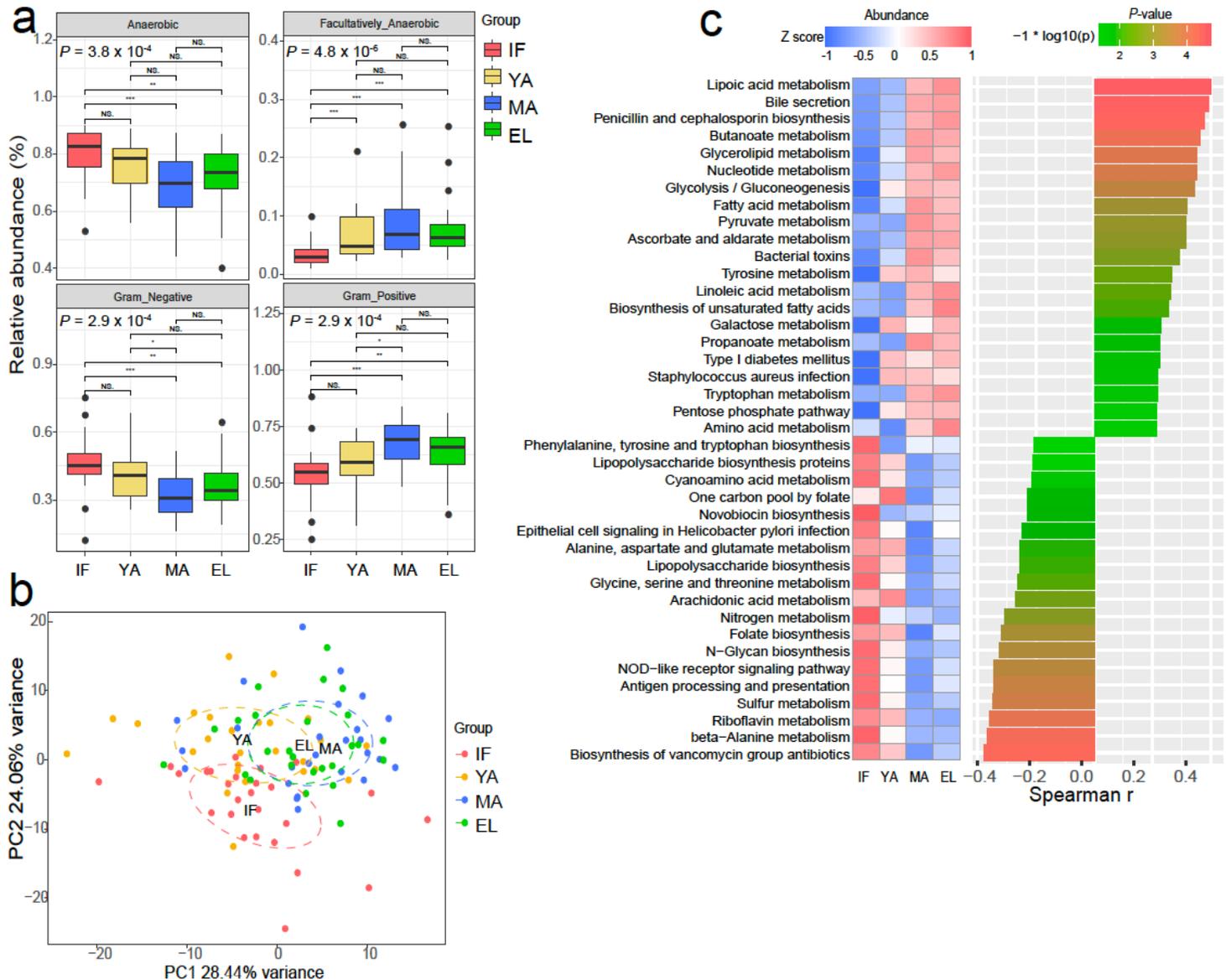
node connectivity. Edge colors and thickness represent correlation  $r$ . IF, infants; YA, young adults; MA, the middle-aged; EL, the elderly.



**Figure 6**

Topological analysis identifies hub and driver genera in the microbiota SparCC networks. (a) MCC scores from the whole network and top 10 hub genera in the SparCC networks. (b) Venn plot showing the overlap of hub genera in the four ages groups. Genera are colored blue if negatively associated with age, and red

if positively associated with age. (c) NetShift common sub-networks based on the SparCC networks with highlighted driver genera. Node sizes are in proportion to their NESH scores, and potential drivers are highlighted red. Edges present only in case are colored red, green only in control, and blue in both. Node names without underlines denote age-associated genera.



**Figure 7**

Age-associated gut microbial phenotypes and functional profiles. (a) Comparison of gut microbial phenotypes predicted by BugBase among the four age groups. P-values for group comparisons are derived from nonparametric Kruskal-Wallis test with Tukey post-hoc test. (b) PCA plot based on microbial function profiles predicted by PICRUSt. (c) Heatmap showing abundance and age correlation of gut microbial functions. P-values are derived from Spearman correlation test. \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .

## Supplementary Files

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