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Identification of Indicative Gut Microbial Guilds in a Natural Aging Mouse Model

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ABSTRACT: Gut microbial dysbiosis during later life may contribute to health conditions, possibly due to an increase in intestinal permeability, immune changes, and systemic inflammation. Mouse models have been employed to determine the influence of gut microbes on aging; however, suitable gut microbial indicators are currently lacking. Therefore, this study aimed to determine the gut microbial indicators and their potential guilds in a natural aging mouse model. In agreement with previous studies, alpha diversity indices—including observed OTUs, ACE, Chao1, and Simpson—were significantly lower in aged mice than in younger mice. The results of beta diversity analysis revealed the compositional differences between young and aged mice, and the MRPP, ANOSIM, and Adonis tests indicated that the results were representative. By employing ANCOM and LEfSe analyses, *Bacteroides thetaiotaomicron* (*Bacteroides*) and *Anaeroplasma* were identified as the indicators of young and aged mice, respectively. Notably, these indicators were still present after 3 months. The result of network analysis confirmed the negative correlation of these genera in mice, and the potential guild members were identified based on the increased abundance of *Anaeroplasma* in aged mice. The gut microbes of aged mice tend to correspond to those involved in human diseases, selenocompound metabolism, and glycolysis/gluconeogenesis in functional predictions. In this study, the gut microbial indicators in aged mice have been identified, and it is envisaged that these findings could provide a new approach for future studies of antiaging.

INTRODUCTION

More than 100 trillion microbes are harbored in the human gastrointestinal tract to form a microbial community, known as the gut microbiota.[1] Previous studies have reported the involvement of gut microbiota in the etiology of chronic diseases and investigated the associated alterations in gut microbiota composition.[2] These diseases include obesity, type II diabetes, metabolic-related liver disease, and cardiovascular disease; most of these conditions are highly related to oxidative stress.[3] It is commonly suggested that the occurrence of aging is accompanied by the incidence of metabolic syndromes, such as insulin resistance, hypertension, dysglycemia, dyslipidemia, and obesity, and it has been concluded that the generation of reactive oxygen species (ROS) byproducts can accelerate the aging process.[3] Furthermore, the aging process is characterized by an imbalance of ROS production that results in loss of function in tissues and organs, which is known as the oxidative stress theory of aging.[4] Therefore, metabolic syndrome and aging may form a causal loop leading to unhealthy aging. As the link between gut microbial dysbiosis and metabolic disease has been widely reported, the relationship between microbial alteration and aging has also gained attention over the years.

The composition of the gut microbiota changes and diversifies rapidly in the early years of infancy. Indeed, it has been revealed that bacterial diversity is positively correlated with age, and gut microbial development can be significantly influenced by environmental factors such as diet, antimicrobial use, and exposure to animals in early life.[5] While the gut microbiota of children is established by the age of three, the composition may continuously change until adulthood; these changes are most commonly a reflection of lifestyle and differences in racial and geographical properties.[6] After the evolution of primary microbiota into a more stable and diverse composition, the factors affecting gut microbiota are more likely to be related to diet and antibiotic use. In other words, diet and lifestyle could have more influence on late-life gut microbial composition than other factors.

Change in gut microbiota occurs progressively and is observed to correlate with age.[6] It has been reported that gut dysbiosis occurs during aging and may cause unhealthy aging and a reduction in longevity.[7] Instability and variation of gut microbiota during aging could lead to the onset of disease or disease worsening.[8] Amamoto et al. (2021)[8] suggested that some Japanese elderly might experience substantial yearly changes in gut microbiota and that these subjects may benefit from the regular use of probiotics to stabilize gut microbiota. More specifically, gut microbial dysbiosis during later life that contributes to health conditions may be due to an increase in intestinal permeability, immune changes, and systemic inflammation.[9]

Gut microbiota has been widely studied in the fields of immunity, metabolism, and neuro-behavior,[10] and it plays a key role in multiple aspects of human health. The functional aspects

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of the gut microbiota, as reviewed by Jandhyala et al. (2015), include nutrient metabolism, xenobiotic and drug metabolism, antimicrobial protection, immunomodulation, and maintenance of gut barrier integrity.[11] Therefore, shaping gut microbiota could potentially provide benefits for individual robustness.

In terms of the influence of aging on gut microbiota, gut physiology changes that occur over time may contribute to age-related microbial dysbiosis.[9] However, it has been pointed out that the stability of gut microbiota correlates more closely with biological age than chronologic age, which indicates that physiological changes might have a greater effect on gut microbial composition than chronologic age. Therefore, it is believed that action taken against age-related changes in intestinal physiology could overcome changes that occur with chronologic age, potentially mitigating microbial dysbiosis or even extending lifespan.[9]

In the microbial community, up to 90% of the gut microbiota is composed of the phyla Firmicutes and Bacteroidetes, while Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia are other dominant gut microbial phyla that are commonly noted.[12] Among these phyla, Proteobacteria has been reported to comprise several members that, as human pathogens, could act as a possible microbial signature of human diseases—including metabolic disorders, inflammatory bowel disease, and lung disease.[13] Furthermore, it was reported that Proteobacteria was significantly increased in human gut microbiota in subjects over the age of 70,[12] and a higher level of Proteobacteria could also be an indicator of chronic low-grade inflammation associated with advanced aging, referred to as "inflammaging."[14] The gut microbial changes during aging and the relationships between aging fatigue, gut microbiota, and metabolites are not entirely clear. Several phenotypes have been suggested as biomarkers of aging and aging-related diseases, but the involvement of the gut microbiota as a potential indicator of aging has not been well studied.

In this study, a natural aging mouse model was designed to determine the differences in biomarkers between adult mice and old mice. By employing different tools, we aimed to identify as many distinct representative changes—in terms of microbiota composition—as possible, to provide evidence that microbiota could be used as a potential marker of aging. This approach proved successful, and biomarkers of aging were identified. The experiment was extended for 14 weeks to ensure the viability of the aging biomarkers over a prolonged time.

METHODS

Animal Care Protocol. Four-week-old and 48-week-old C57BL/6 male and female mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The experiment was designed with nine mice of each gender per group. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (NTU-107-EL-00018, IACUC, NTU). The mice were housed in a controlled atmosphere (25 ± 1 °C with a relative humidity of 50%) and a light/dark cycle of 12 h each (light on at 07:00 and light off at 19:00, every day). The mice were randomly assigned, and the experiment was separated into two parts. The mice's feces were collected at the end of 24 weeks to allow sampling of both adult mice and old mice. The timing for feces collection was selected according to the definition of life history phases of mice provided by Jackson Laboratory. According to the definition, a 28 week-old mouse corresponds to a human aged 30, while 72 weeks corresponds to a 56 year-old human. The second fecal collection time was 14 weeks after the first collection. At this particular time, the adult mice would reach middle age, which is equivalent to 38 years old in humans, while the aged mice would reach an equivalent age of 65 years old. Due to an upper life span of mice of 24 months, the survival rate of aged mice was expected to reduce to 50% within the subsequent 3 months. The aging markers in the natural aging model are presented in Figure S3 and as described in our previous study.[15]

Serum Biochemical Parameters. The blood was collected via submandibular blood collection with a Goldenrod Animal Lancet. Serums were obtained by centrifuging the collected blood at 3500 rpm at 4 °C for 10 min. Serum cholesterol and triglyceride were analyzed by using commercial triglycerides and cholesterol kits (Fortress Diagnostics), while SOD was determined by using an SOD assay kit (Cayman Chemical). Blood samples were harvested from the tail vein at 12 weeks, and the blood glucose was measured using a glucometer (Aurum Biomedical Technology, Taiwan).

Microbial DNA Isolation for Gut Microbiota Analysis. Gut microbial DNA was isolated and purified by using the InnuPREP Stool DNA kit, following the manufacturer's instructions with slight modifications. In brief, 100 mg of fecal sample was homogenized with 1 mL of the provided lysis solution for one to two cycles at 7.00 m/s. For lysis, the sample was heated to 95 °C for 20 min and vortexed every 5 min. Samples were then prefiltered using the provided filter at 10,000*g* centrifugation for 2 min. The sample solutions were collected in the receiver tube, and the material in the filters was discarded. A total of 25 μL of prepared protein kinase was added into each tube and mixed, and the samples were incubated in a 70 °C water bath for 20 min. The sample was then mixed with the provided binding solution at a ratio of half the volume of the sample solution and filtered under the conditions above.

The solution in the receiver tube was discarded, and the samples in the filter were washed twice with the washing solution containing ethanol. The DNA remaining in the filter was collected by adding 100 μL of preheated elution buffer and leaving it to stand for 5 min. The sample solution was centrifuged at 8000 rpm for 1 min, and the microbial DNA was collected. The quality of all the DNA samples was assessed using a NanoDrop 1000 spectrophotometer at wavelengths of 260 and 280 nm, where the ratio of the optical density of 260/280 should be in the range of 1.8− 2.0, while the DNA concentration should be higher than 30 ng/μL.

Next-Generation Sequencing and Gut Microbiota Compositional Analysis. The DNA samples were analyzed by BioTools using a 16S amplicon sequencing technique to determine the fecal microbial composition. For each DNA sample, PCR was performed to amplify the 16S rRNA gene, which includes 10 conserved regions (V3−V4) and 9 hypervariable regions (V1−V9). Amplified genes were sequenced by using the Illumina HiSeq2500 platform to generate the reads (250 bp). The effective tags obtained were clustered into different operational taxonomic units (OTUs) based on the sequence similarity at a 97% identity threshold, where each cluster represents a taxonomic unit of bacterial species or genus. Up to 367 mol % of the OTUs could be identified.

For the evaluation of alpha diversity, multiple indices were calculated, including the Shannon−Wiener diversity index, the Simpson diversity index, the Chao1 richness estimator, and the ACE index. Beta diversity analysis used quantified indices, such as principal component analysis (PCA), principal coordinates

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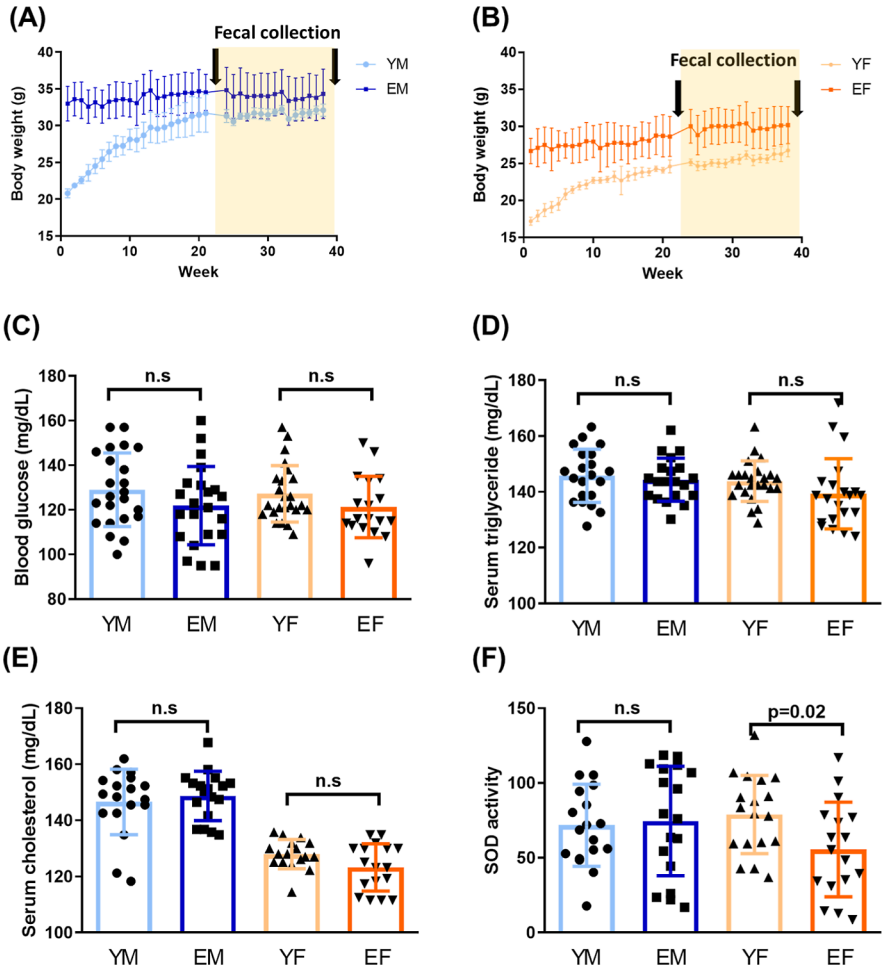

Figure 1. Body weight change and serum biochemical parameters are not indicative parameters for aged mice. Body weight change in (A) male and (B) female adult and aged mice. (C) Serum glucose level, (D) serum triglyceride level, (E) serum cholesterol level, and (F) serum SOD activity (n = 20−24 for serum glucose, triglyceride, and cholesterol levels, and n = 15−18 for SOD analysis). Two-tailed Student's t-test was conducted for the significance ($p < 0.05$) between young and aged mice of different genders.

analysis using either weighted or unweighted uniFrac, nonmetric multidimensional scaling (NMDS), and partial least squares discriminant analysis. Linear discriminate analysis effect size (LEfSe) was employed to identify the features most likely to account for the differences among samples using GraPhlAn to generate the cladograms. For the prediction of the gut microbiota functional profile, Tax4fun software was employed to provide outputs, using the SILVA database, as reference 16. To compare the difference between the two groups at each classification, Statistical Analysis of Metagenomic Profiles (STAMP) was applied.

Statistical Analysis. All data were presented as the mean ± SD value (standard deviation). The two-tailed Student's t-test or ANOVA/Tukey's test was employed to indicate significant differences ($p < 0.05$). To compare the difference of gut microbes at each level, Welch's t-test was used, and the statistical significance acceptance level was $p < 0.05$. For the LEfSe analysis, the nonparametric factorial Kruskal−Wallis sum-rank test was selected to find the significant species among the groups, and the values were shown as LDA scores (log 10).

RESULTS

Adult and Aged Mice Show a Distinct Gut Microbial Composition. As shown in Figure 1, there were no significant differences in serum biochemical parameters, except for serum SOD activity, between mice at the age of 28 weeks (adult mice) and 72 weeks (old mice) in both genders. Besides, body weights increase with age, which could not be a good indicator of aging. Therefore, it is important to find an indicative parameter between adult and old mice before sacrificing them.

The mice feces were collected at the age of 28 weeks (adult mice) and 72 weeks (old mice); the former is defined as the age of a mature adult, while the latter is defined as old age (at which point the senescent changes can be detected) (Figure 2). In the comparison of the abundance in the top 10 phyla, a significant increment was observed in Proteobacteria in the younger mice while the gut microbiota of old mice was rich in Tenericutes, Actinobacteria, and a comparatively higher abundance of Fusobacteria. Notably, more abundant Deferribacteres and Patescibacteria were detected in female old mice, while Verrucomicrobia was observably richer in male old mice as compared to their younger controls. The genera listed in bubble charts were the relatively more abundant dominant members found in gut microbiota, and the significance was indicated by a solid circle. As presented in Figure 2G,H, the genera *Parasutterella* and *Desulfovibrio* are the major members of the phylum Proteobacteria that are abundant in younger mice of both genders. *Bacteroides* from the phylum Bacteroidetes were significantly more abundant in the younger mice. In the gut

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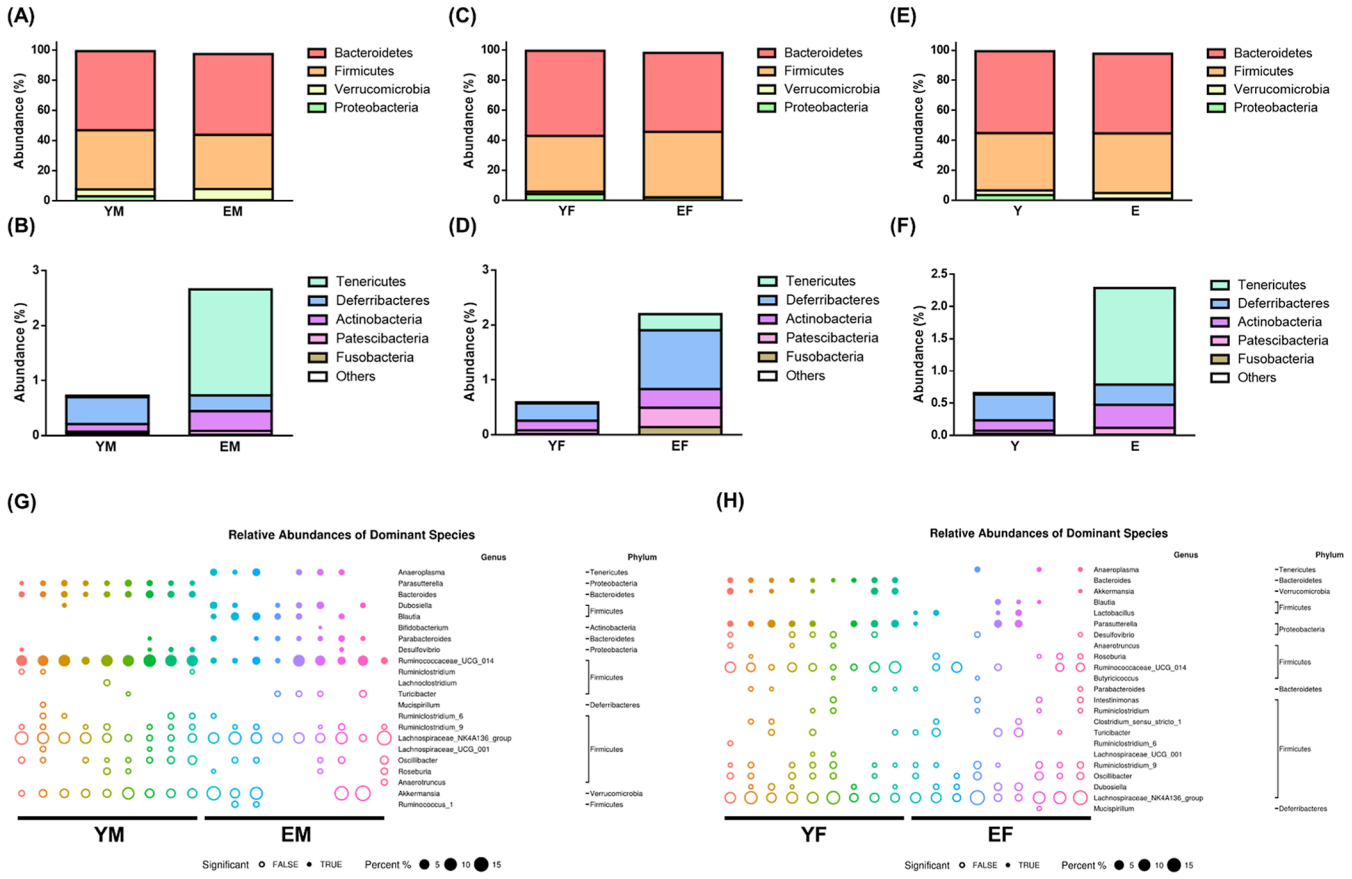

Figure 2. Relative abundance in phyla and dominant genera in adult and aged mice. Relative abundance in phyla and dominant genera in male mice (A,B,G); female mice (C,D,H); and in combination (E,F). (n = 9 for each gender).

microbiota of old mice, the genus *Anaeroplasma* from Tenericutes and *Blautia* from Firmicutes were the dominant colonic residents. Comparing the genders of old mice, it was observed that *Dubosiella* and *Bifidobacterium* were abundant in old male mice, while *Lactobacillus* was more abundant in old female mice.

Significant Changes in Diversity Indices Were Observed between the Gut Microbiota of Adult and Old Mice. Alpha diversity indices can be utilized for the analysis of species richness and evenness of gut microbiota. Each index is designed based on different assumptions, and therefore, more than one index will normally be adopted for a more descriptive analysis. First of all, the observed number of OTUs dramatically reduced in aged mice of both genders (Table S1). The Chao1 index suggested that the gut microbial richness was significantly higher in younger mice as compared to aged mice, and it was more observable in aged female mice. Moreover, a significant reduction in the Simpson index of aged male mice suggested a lower evenness of the gut microbial composition. Comparing the composition of gut microbiota, the result of PCA in Figure 3A,B indicated a distinct composition between young and aged mice of both genders. Similar results were obtained in other methods of beta diversity analysis, as presented in Figure 4. Specifically, some species correlated with compared groups. Further analysis was performed to determine the most significant species abundant between compared groups using a covariance matrix in PCA analysis. For instance, *Lactobacillus reuteri* (*Lactobacillus*) and *Lachnospiraceae bacterium* 609 (*Blautia*) positively correlated with the gut microbial composition of aged mice, while *Bacteroides thetaiotaomicron* and *Clostridium* sp. Culture 27 positively correlated with younger mice. These species represent the potential markers of aging that are focused on in later sections. The result was supported by the ANOSIM, MRPP, and Adonis indices, which showed a positive result on the reliability of the beta diversity (Table S2).

To provide further confirmation of the correlation between the aforementioned species with natural aging, the gut microbial composition of aged mice was compared with younger counterparts at all of the taxonomic ranks, and Welch's t-test was employed to determine significance. As shown in Figure 5A−D, all the members in the taxonomic rank of phylum and species showed significant differences between the compared groups. Similar to our previous observation, Proteobacteria and Tenericutes (phylum) could be the primary indicators of adult and aged mice. Elevation of Verrucomicrobia in young female but not male mice is an observation that could be investigated further. In the taxonomic rank of species, *L. bacterium* 609 and the members of Lactobacillus were significantly elevated in aged mice, while *B. thetaiotaomicron* and Clostridium species were more abundant in younger mice.

Distinct Gut Microbial Indicators in Adult and Aged Mice in Both Genders. To identify the most significant members between the compared groups and to factor out the influence of genders, analysis of the composition of microbiomes (ANCOM) and LEfSe were employed. As shown in Figure 6A−F, all the discriminative members between the groups [with statistical significance in the Wilcoxon rank-sum test ($p < 0.05$)] were shown as detected points with different colors. It was found that Tenericutes (in phyla), *Bacteroides* and *Anaeroplasma* (in

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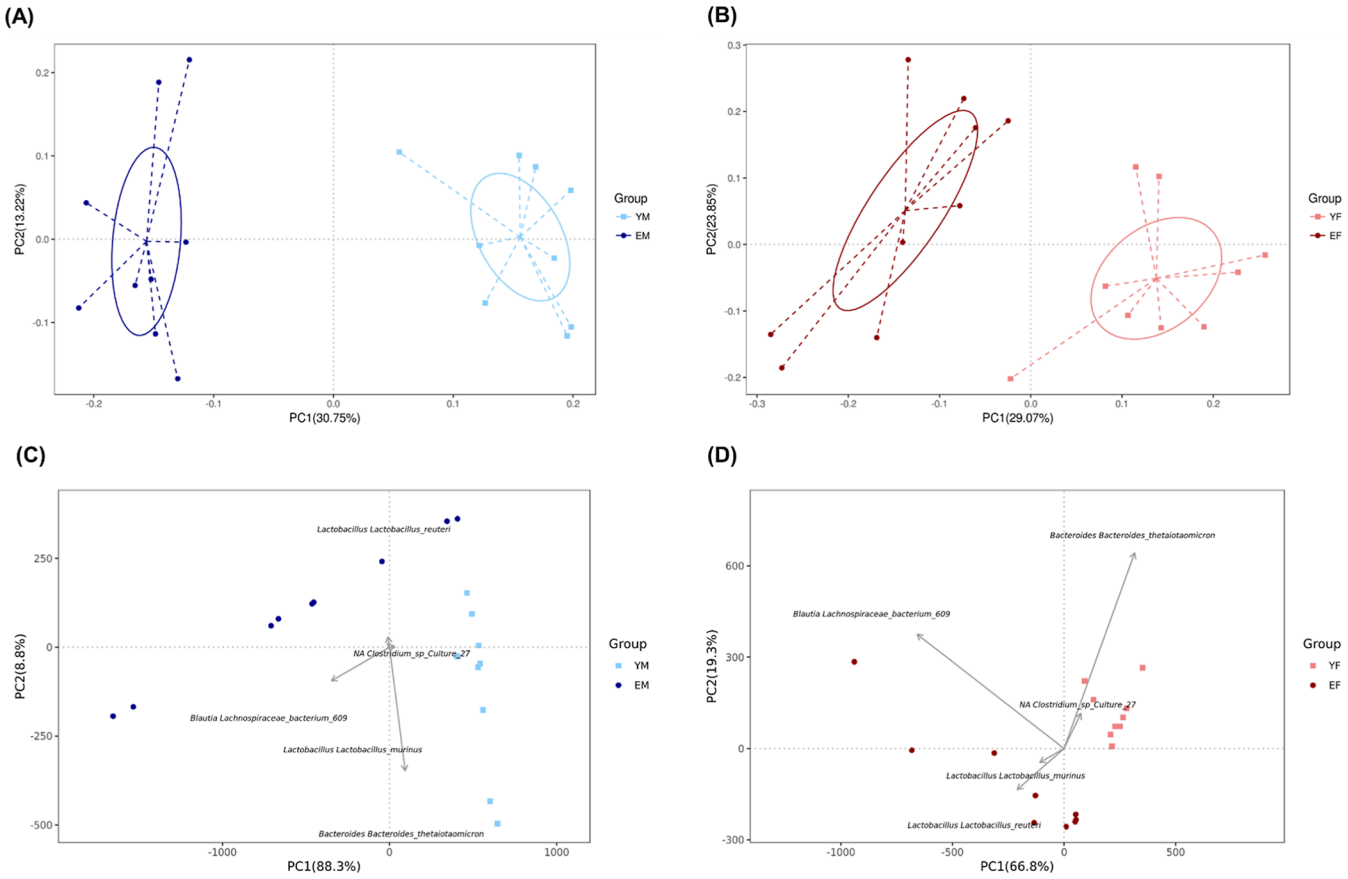

Figure 3. Adult mice and aged mice showed distinct gut microbial composition, in terms of β-diversity. PCA (Bray−Curtis) plotting of (A) male mice and (B) female mice; the species with higher contribution to the differences are shown in (C,D) (n = 9).

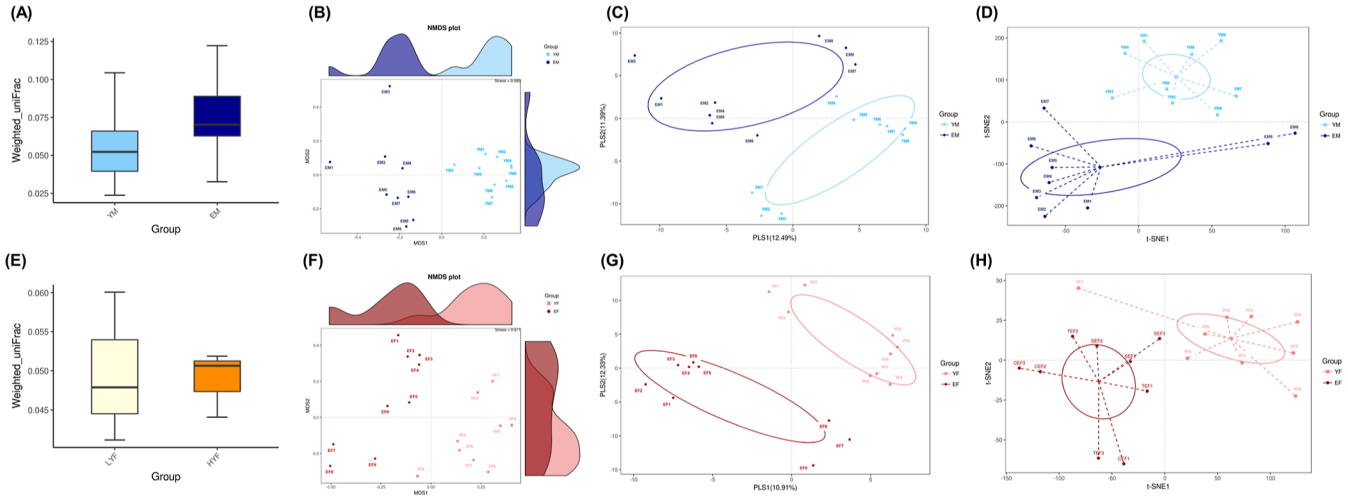

Figure 4. Result of β-diversity in the comparison of gut microbiota of adult and aged mice. Weighted uniFrac β-diversity of (A) male mice and (E) female mice; NMDS plotting of (B) male and (F) female mice; PLSDA plotting of (C) male and (G) female mice; t-SNE plotting of (D) male and (H) female mice (n = 9 for each gender).

the genera), and *B. thetaiotaomicron* showed the most pronounced significance among the compared groups, indicating their potential to act as indicators of aging in the mice model. LEfSe employed a nonparametric factorial Kruskal−Wallis sum-rank test to identify the microbial species with significant abundance, followed by linear discriminant analysis to estimate the impact of the abundance differences toward the difference between compared groups. This estimated impact could be used to indicate its viability as a potential biomarker. As listed in Figure 7C,D, Proteobacteria appear to be the most indicative biomarker of adult mice compared to aged mice in both genders (LDA score > 4). By lowering the threshold (LDA score > 3), the biomarkers of adult mice (in both genders) may include *Parasutterella* (genus) and *B. thetaiotaomicron* (species), while gut microbiota biomarkers of aged mice include *Lactobacillus* (genus), *Anaeroplasma* (genus), and *L. bacterium* 609 (species).

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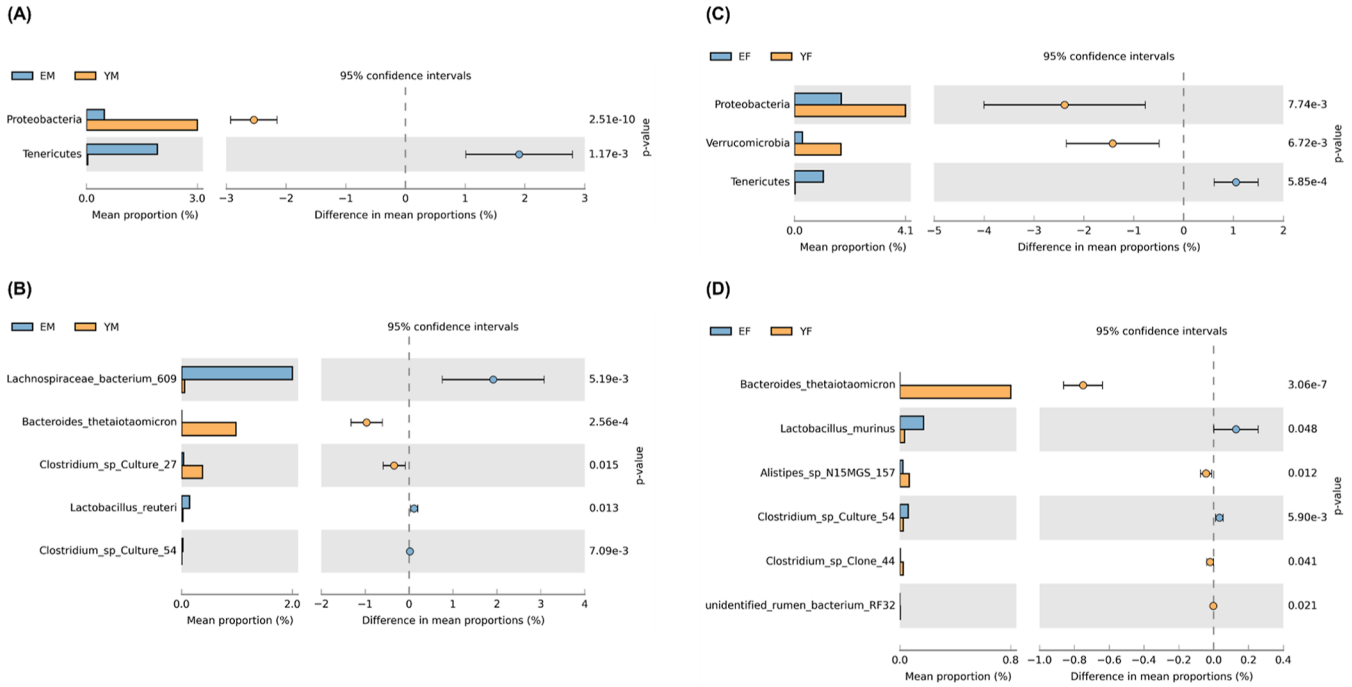

Figure 5. Gut microbial difference between the compared groups with STAMP. The statistical analysis was conducted at different taxonomic levels as follows: (A,C) phylum of male and female mice; and (B,D) species of male and female mice. Statistical significance was determined using Welch's test ($p < 0.05$) ($n = 9$).

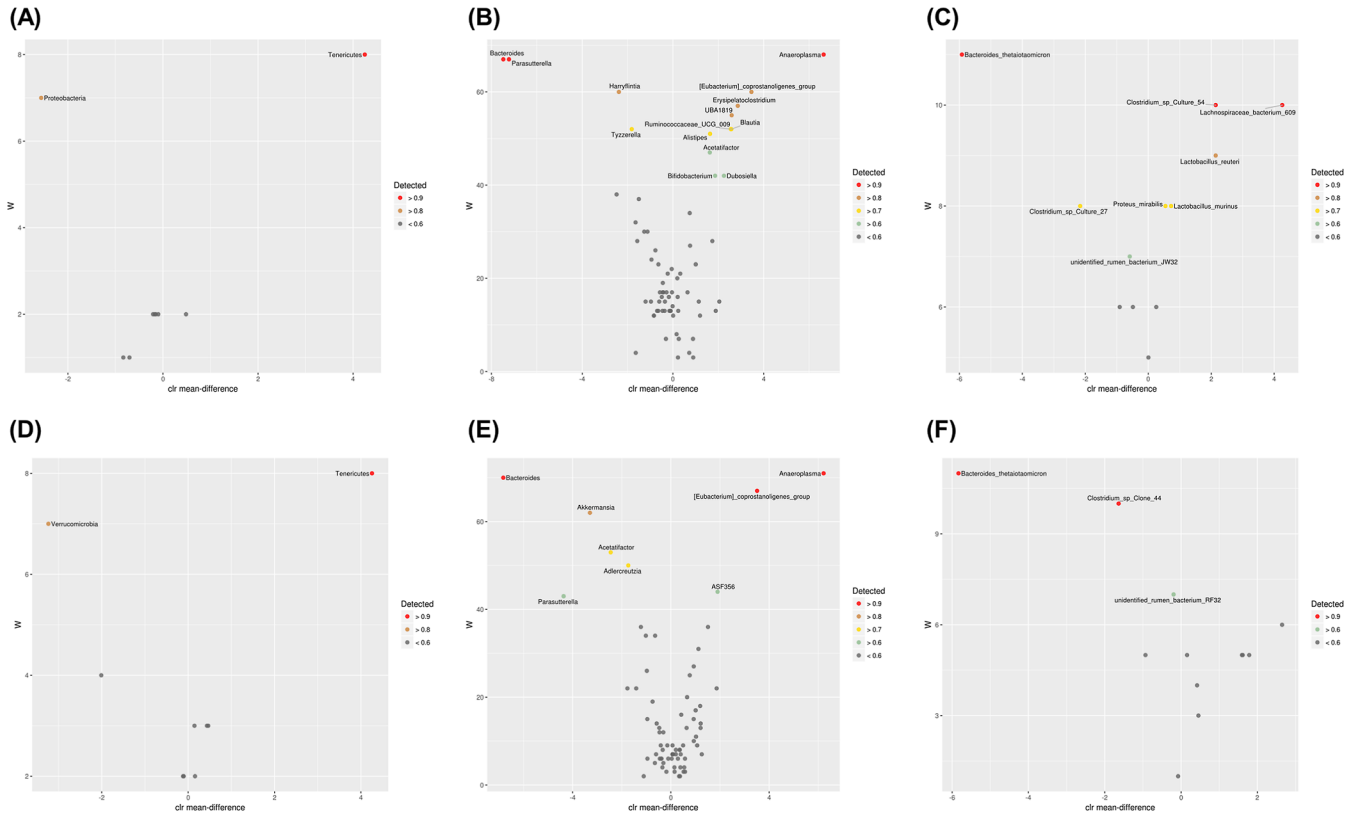

Figure 6. Result of the ANCOM identification of the significant species between the gut microbiota in the compared groups: (A,D) phylum in male and female mice; (B,E) genus in male and female mice; and (C,F) species in male and female mice, respectively. The statistical significance is indicated by using the Wilcoxon rank-sum test ($p < 0.05$) and adjusted by the Benjamini–Hochberg procedure. ($n = 9$).

Functionality Prediction of Gut Microbiota Varies between Adult and Aged Mice. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is a tool developed for metagenome functional prediction based on the marker genes identified. By comparison of the 16S rRNA sequences obtained with the Greengenes

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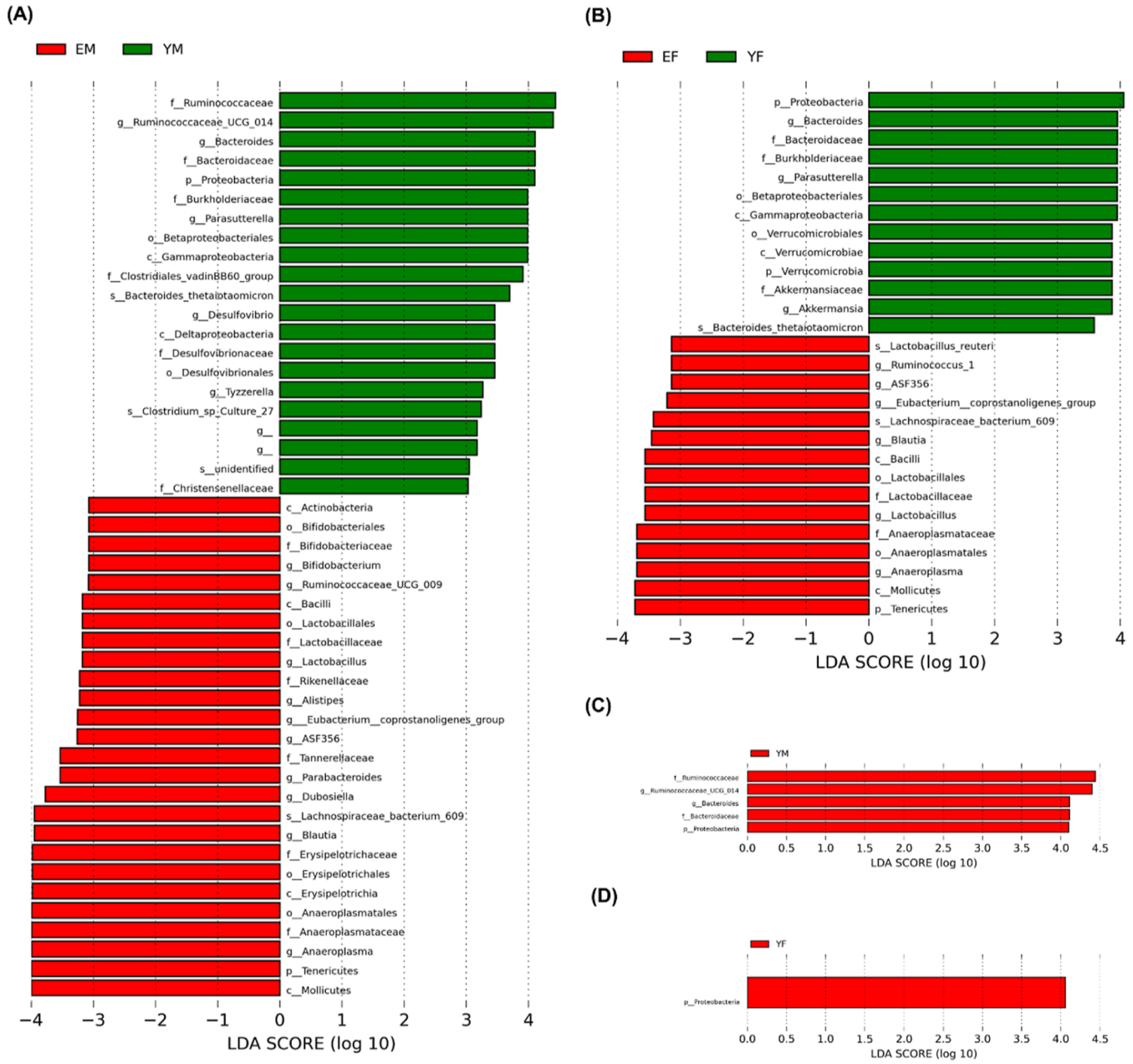

Figure 7. Gut microbial differences and identification of microbial indicators between compared groups (A,B) linear discriminant analysis (log 10 > 3.0) of male and female mice, and (C,D) linear discriminant analysis (log 10 > 4.0) of male and female mice, respectively, between the compared groups. (n = 9).

database, analyzed bacteria could be classified with related species that might exhibit similar functionalities. Combining the information obtained with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the functionalities and pathways of those genes involved could be forecast. Figure 8A−D shows the metabolic pathways and human diseases with significant differences between the compared groups. In detail, the gut microbes of aged mice might be more likely to be involved in selenocompound metabolism and glycolysis/gluconeogenesis in both genders but less so in toluene degradation and fatty acid biosynthesis. Among the human diseases, the microbiota of aged mice in both genders show a higher risk of *Staphylococcus aureus* infection. Figure 8 shows the NMDS plots based on the predicted results of the KEGG orthology level 4. The results suggest that there is indeed a difference, in terms of predicted functionalities, between the compared groups; the stress levels (lower than 0.05) in these plots are representative of the obtained plots.

Aging Indicators Remained the Same after 14 Weeks in Both Adult and Aged Mice. After 14 weeks, the 28-week-old mice reached 42 weeks of age, while the 72-week-old mice reached 86 weeks of age. The 42 week-old mice reached middle age from mature adults, while the 86 week-old mice (21.5 months old) nearly reached the upper limit of old age, and it was expected that survival would start to decrease and reach 50% at the 28th month. By employing Welch's t-test, it was found that *B. thetaiotaomicron* (from the genus *Bacteroides*) remained significantly higher in younger mice (Figure S1). From the LEfSe result, it was observed that *B. thetaiotaomicron* remained a potential biomarker even after the mice reached middle age. In

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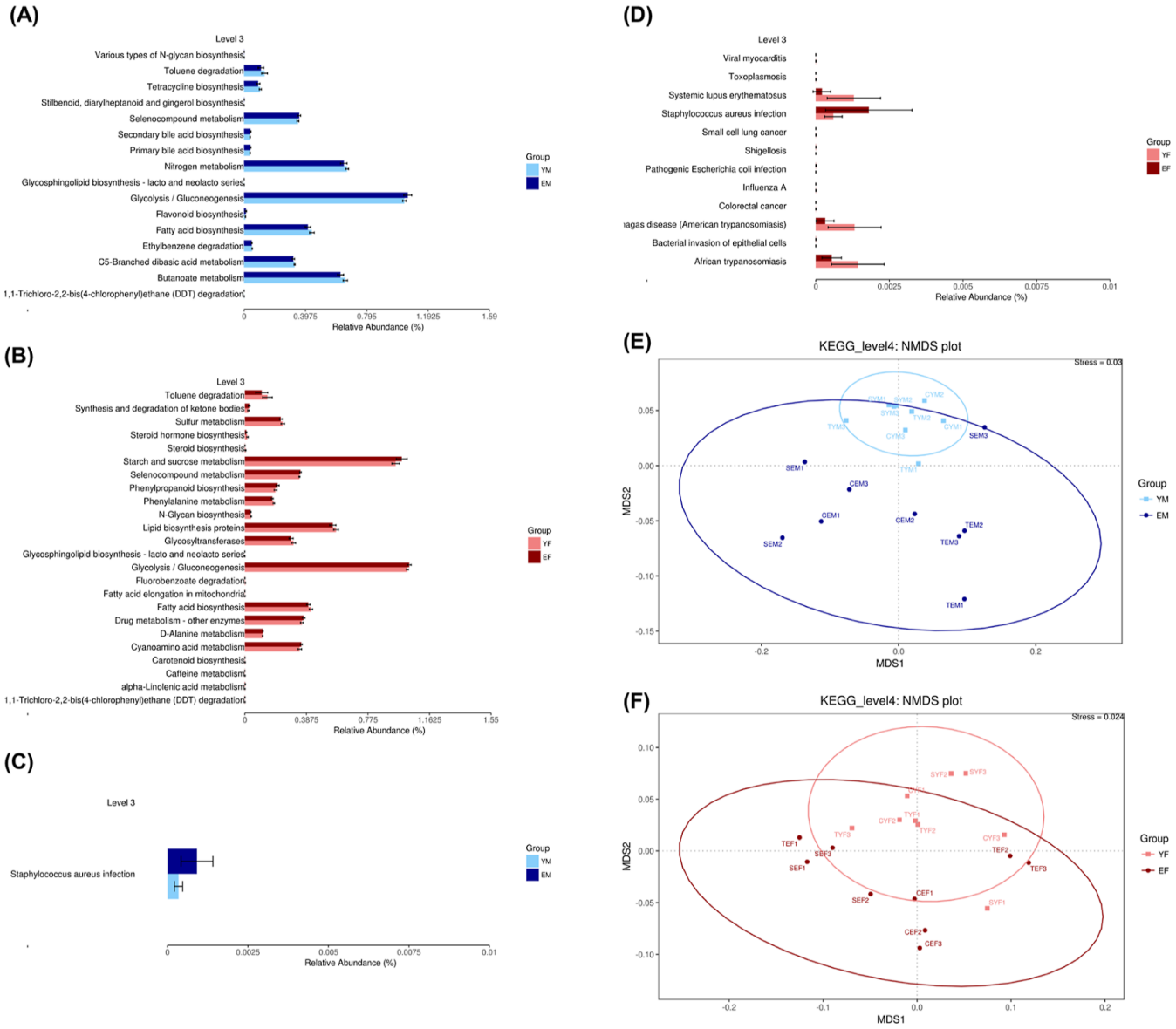

Figure 8. Comparison in level 3 KEGG functional prediction of adult and aged mice. The difference in functional prediction by using PICRUSt in (A,B) metabolism and (C,D) human diseases of male and female mice, respectively. (E,F) NMDS plotting based on the functional prediction using Tax4fun.

comparison, similar to the result before 14 weeks of feeding, the *Anaeroplasma* (genus) demonstrated potential as an indicator of aged mice at the age of 86 weeks. Our results show that *B. thetaiotaomicron* and *Anaeroplasma* are potential indicators of aging in mice models.

Gut Microbial Guild Correlated to Aging. The concept of a "guild" in the gut microbial study was defined by Wu et al. (2021) to explain the co-abundant behavior of gut microbes that contribute to the same ecological function.[17] In this study, the correlation between the gut microbes was determined (Figure S2). The results showed that *Ruminococcaceae_UCG_009*, *[Eubacterium]_coprostanoligenes_group*, and *Alistipes* were positively correlated to the abundance of the elderly indicator, *Anaeroplasma*, while they were negatively correlated to the abundance of *Bacteroides*. These direct correlations were observed in both genders of mice. Among those gut microbes with an elevated abundance of *Anaeroplasma*, the abundance of *Alistipes* and *[Eubacterium]_coprostanoligenes_group* elevated with the growth of UBA1819, while the *[Eubacterium]_coprostanoligenes_group* was negatively correlated with *Harryflintia*, which is positively correlated with *Bacteroides*. These results suggest that the guild related to aging consists of the genus *Anaeroplasma*, *Ruminococcaceae_UCG_009*, *[Eubacterium]_coprostanoligenes_group*, *UBA1819*, and *Alistipes*, while abundances of *Bacteroides* and *Harryflintia* form another guild that correlates with younger gut microbial communities.

DISCUSSION

Our results reveal that aged mice and adult mice exhibit distinct gut microbial compositions and that these differences are potential indicators of aging. These changes could function as references to be targeted in antiaging studies. By employing different analysis tools, our findings show distinct gut microbial compositions between adult and aged mice in terms of beta diversity. Furthermore, some gut microbial species were significantly different between the compared groups, and more

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importantly, the significance could be observed in both genders of mice. By further employing ANCOM and LEfSe, we successfully identified the most distinct species for use as biomarkers of adult and aged mice were successfully identified. Due to the differences in gut microbiota between the compared groups, the predicted functionalities of gut microbiota with significance were determined, which could be informative for future studies focusing on the natural aging mice model. Notably, some of the indicators remained the same after 14 weeks of rearing, indicating that the biomarkers could be used for at least 3 months in the aging study. Lastly, given that a guild of gut microbes may be responsible for and representative of aging progression and aging-related diseases, several correlated gut microbes were identified.

While the associations among gut microbial dysbiosis, the occurrence of metabolic syndrome, and the progression of aging are complicated, it is accepted that these factors mutually affect one another. Naturally occurring aging increases the susceptibility to metabolic issues and the occurrence of gut microbial dysbiosis, while, at the same time, the incidence of metabolic syndrome and dysregulation of gut microbiota accelerate the progression of aging. For example, Maffei et al. (2017) revealed that, compared to chronological age, biological age is more correlated to the changes in gut microbiota, in terms of diversity.[18] Therefore, these issues are inseparable during discussion. In a previous study, by segmenting 368 samples into 14 age-segment groups, Xu et al. (2019) successfully identified some genera that dramatically changed in elderly age groups and revealed that some beneficial gut microbes might increase with respect to aging but then reduce in centenarians.[19] Furthermore, some genera, such as *Bilophila*, *Odoribacter*, *Desulfovibrio*, and *Butyricimonas,* that are often linked to pathogenic responses, including inflammation, were also found to increase in the elderly. However, in contrast, it was suggested by Kong et al. (2016) that, unlike elderly subjects under 100 years old, centenarians should be considered healthy aging, as they have reached the limit of the human life span by surviving and delaying chronic diseases.[20] For example, the latter study revealed that, unlike most elderly people, the gut microbial diversities of centenarians were found to be greater than younger subjects. In addition, some gut microbial members were found to be elevated in centenarians, including *Blautia*, *Clostridium XIVa*, *Faecalibacterium*, *Escherichia-Shigella*, *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae*. Some of these were also identified in our study. Similarly, Ren et al. (2021) demonstrated that specific bacterial communities were observed in long-lived families, and it was suggested that their gut microbiota composition could be health-promoting during aging.[21] A Japanese cross-sectional study collected fecal samples from 367 healthy subjects, whose ages ranged from 0 to 104 years, to identify the bacterial co-abundance groups in each age group.[22] It was revealed that *Bacteroides*, *Eubacterium*, and *Clostridiaceae* were significantly abundant in the elderly, while *Enterobacteriaceae* was abundant in both infants and elderly but not in adults. These findings are similar to those of our study, suggesting that the mouse model could be suitable for preclinical antiaging research.

Furthermore, one study has shown that by transplanting fecal matter of long-living subjects into mice, greater alpha diversity and probiotic genera, including *Lactobacillus* and *Bifidobacterium*, and genera producing short-chain fatty acid (*Roseburia*, *Faecalibacterium*, *Ruminococcus*, and *Coprococcus*) were observed compared to elderly.[23] Another study demonstrated that besides a decrease in microbial diversity, elevation in the abundance of Proteobacteria and some potential bacterial pathogens—such as *Escherichia-Shigella*—was accompanied by a reduction in beneficial bacteria, like *Faecalibacterium* in long-living people of Chinese and Italian cohorts.[24] These studies suggested that even in centenarians, health could also be affected by the composition of gut microbiota. These studies also emphasized the issue of the influence of biological age and chronological age on gut microbiota changes and further demonstrated the importance of this study to identify the representative guilds or indicators in the natural aging mouse model.

As mentioned above, the diversity indices decreased with respect to increasing biological age.[18] In 2021, Li and colleagues found that the gut microbial composition of aged mice was significantly different from that of younger animals, in terms of the indices of alpha diversity and beta diversity and abundance of some gut microbes.[25] These changes were suggested to be associated with some phenotypes of aging, including oxidative stress, proinflammatory responses, and tight junction adhesion molecules. In our study, the reduction in the alpha diversity and the difference in the alpha diversity were in agreement with previous studies.

Regarding the correlation of certain biomarkers with gut microbiota, Ke et al. (2021) revealed that some hematological parameters that were significantly increased in aged mice—including mean corpuscular volume, neutrophil to lymphocyte ratio, and lymphocyte—were correlated to some gut microbes.[26] In the study of Maffei et al. (2017), the Frailty Index (FI_{34}) was found to be positively correlated with the abundance of *Coprobacillus* and *Dialister* and negatively correlated to *Paraprevotella*, *Sutterella,* and *Rikenellaceae* family OTU.[18] In the study of Fransen et al. (2017), by transferring the gut microbes of aged mice into germ-free mice, significant increases in TM7 bacteria and Proteobacteria and lower abundance of *Akkermansia*, accompanied by upregulation of TNF-α and TNFSF8 and higher sera LPS, suggested that the bacterial groups of older mice might be responsible for the inflammatory responses in germ-free mice.[14] The changes in the gut microbe compositions were similar to those in our study.

Aging is generally correlated to decreased gut microbial diversity due to changes in physiology and lifestyle, diet, medication, or antibiotic use.[27] Our results showed that gut microbial richness, in terms of Chao1 and evenness in the Simpson index, was significantly reduced in aged mice. It has been previously reported by Leite et al. (2021) that decreased microbial diversity in the human duodenum was associated with chronological age, concomitant diseases such as sarcopenia, medication, and coliform numbers.[28,29] More importantly, it was suggested that the abundance of the genus *Lactobacillus* was positively correlated with chronological age, which is in agreement with our result showing an increased abundance of *L. reuteri* in aged mice. Furthermore, our findings were also similar to a study in which fecal matter of the elderly was transplanted into mice, resulting in an increased abundance of *Lactobacillus*, *Bifidobacterium*, and *Blautia*.[23] These studies shed light on the influences of natural aging on changes in the microbial composition as compared with adult mice.

In our study, Proteobacteria was found to be a dominant phylum that was more abundant in adult mice compared to aged mice. In detail, *Parasutterella* and *Desulfovibrio* were found to be the major genera of phylum Proteobacteria in these adult mice. A study done by Ju et al. (2019) suggested that colonization of *Parasutterella* in mice could lead to significant changes in the

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metabolism of aromatic amino acids, including catabolism of tryptophan and tyrosine.[30] Activation of tryptophan metabolism has been previously suggested to prevent hyperinflammation caused by age-related decline.[31] Another study revealed that levels of tyrosine significantly increased in long-lived *Drosophila melanogaster*.[32] In addition, *Parasutterella* is associated with alterations in bile acid and supports the regulation of FXR signaling to enhance bile acid synthesis via *Cyp7a1* expression.[31] These studies indicate the benefits of *Parasutterella* and the impacts brought about by the loss of its abundance during aging. In our study, another member of Proteobacteria found to be abundant in adult mice, but less so in aged mice, was *Desulfovibrio*. The abundance of *Desulfovibrio* was previously reported to be associated with the incidence of colitis[33] and Parkinson's disease.[34] However, a recent study by Hong et al. (2021) suggested that *Desulfovibrio vulgaris* from the *Desulfovibrio* genus is a beneficial species that showed a preventive effect on nonalcoholic fatty liver disease via its involvement in acetic acid production.[35] Therefore, the role of *Desulfovibrio* in the progression of aging should be further investigated in the future.

In comparison, the abundance of *L. bacterium* 609 and *L. reuteri* was found to be positively correlated with aging. A study in 2012 suggested that the abundance of *L. bacterium* 609 positively correlated with the development of nonalcoholic fatty liver disease in subjects fed with a high-fat diet.[36] However, *L. bacterium* 609 has not been widely studied previously, and therefore, further studies are needed to confirm its association with aging. Unlike *L. bacterium* 609, *L. reuteri* is a species that has been well studied, and some strains of this species have been developed as a probiotic against age-related bone loss,[37] inflammation,[38] and immune-modulation.[39] However, as discussed above, the genus *Lactobacillus* has been reported in aged mice or the elderly in several studies.[23,28] Besides *L. bacterium* 609 and *L. reuteri*, our study revealed that *Anaeroplasma* could be a potential biomarker in both 72-week-old and 86-week-old mice. However, there are only a few studies that have discussed *Anaeroplasma* previously. One study demonstrated that the relative abundance of *Anaeroplasma* was significantly reduced in postoperative cognitive dysfunction in elderly patients.[40] In another study, it was found that the genus *Anaeroplasma* was significantly increased in postmenopausal osteoporosis mice, concomitant with increased fecal hydrodeoxycholic acid.[41] The genus *Anaeroplasma* is a member of the family Anaplasmataceae, which includes other members that are responsible for anaplasmosis in both humans and rodents and monocytic ehrlichiosis in humans.[42] Although there is limited information regarding the involvement of *Anaeroplasma* in human diseases, the abundance of *Anaeroplasma* in aged mice might be relevant in a natural aging mice model as an indicator of aging. Notably, the *Alistipes* genus from the Rikenellaceae family was identified as a potential representative species that is more abundant in middle-aged (around 84-week-old) and older female mice (122-week-old).[43] However, *Alistipes* was only found as a biomarker in 72 week-old to 86 week-old male mice but not in female mice. Although our results were not as consistent as those with the *Anaeroplasma* study, they provide evidence that *Alistipes* from the Rikenellaceae family could be a potential indicator of a natural aging mice model.

Lastly, our findings showed that *B. thetaiotaomicron* was a biomarker of adult mice of both genders that was found to be sharply decreased after aging. In the colon, *B. thetaiotaomicron* is responsible for the utilization of carbohydrates, including fiber and glycans, and murine gut colonization.[44] In addition, *B. thetaiotaomicron* is also known to be a producer of short-chain and organic acids via fermentation.[45] *B. thetaiotaomicron* presents in up to 46% of humans and, due to its stability in human gut microbiota, it is potentially one of the most useful candidates for therapeutics in the gastrointestinal tract.[46] One example is the study by Charlet et al. (2020) who demonstrated the modulatory effect of *B. thetaiotaomicron* intestinal inflammation in the DSS-induced colitis model.[47,48] A study showed that *B. thetaiotaomicron* and *Faecalibacterium prausnitzii*, both commensal bacteria, could contribute to epithelial homeostasis establishment via goblet cell development and mucus glycan production.[49] A recent study showed that Mediterranean diet intervention improved the health status of prefrail subjects by altering gut microbiota.[50] The authors revealed that the abundance of *B. thetaiotaomicron* was positively associated with a Mediterranean diet intervention. Therefore, a reduction in the abundance of *B. thetaiotaomicron* in aged mice could be a reasonable indicator of aging, and its preservation could be a potential strategy for retarding aging or achieving healthy aging. Furthermore, supplementation with *B. thetaiotaomicron* may alleviate aging-related concomitant diseases.

CONCLUSIONS

Some studies have reported the differences in gut microbiota composition between young and aging animals and between healthy and unhealthy aging and demonstrated the importance and involvement of gut microbiota in unhealthy aging. However, they showed very different results, in terms of the changes in the abundance of gut microbiota, and few studies looked for indicative guilds as supportive information for the development of the natural aging mouse model. Therefore, our study aimed to identify potential biomarkers of natural aging in the mouse model, and fortunately, some species were identified as potential candidates. Determining these indicators is important, as this knowledge creates the potential to rule out confounding factors such as dietary and environmental effects in the experimental mouse model. It is envisaged that the findings of this study will provide useful information for future antiaging research.

LIMITATIONS AND FUTURE PERSPECTIVE

In this study, new indicators of natural aging in mice have been identified. However, there are some limitations that must be overcome in future studies. One of the major functions of gut microbiota is their involvement in metabolism. Therefore, information on the gut microbial metabolites, such as short-chain fatty acids[51] and amino acids,[52] could serve as supportive information to be combined with our findings on gut microbiota. Some hints could be obtained from the results of KEGG of our studies. Moreover, the correlation of these metabolites with the identified gut microbial guild could be clarified in future studies. Hopefully, a more precise and informative study of natural aging models will be developed in the future.

COMPLIANCE WITH ETHICAL STANDARDS

The experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (NTU-107-EL-00018, IACUC, NTU), and the guidelines for the care and use of the animals were followed.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05949.

Gut microbial differences and identification of microbial indicators between compared groups; potential guilds formed in young and aged mice; alpha diversity indices between adult and aged male and female mice; and ANOSIM, MRPP, and Adonis indices for the degree of separation between tested groups (PDF)

The highlights of the study (PDF)

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Y.-C.H. and L.-H.K. contributed equally. M.H.P. and M.W. conceived the idea and designed the experiments; Y.C.K. and L.H.K. carried out the experiments; Y.C.K. wrote the manuscript; and M.H.P., M.W., and Y.C.T. reviewed the manuscript. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

Declaration: I, as the corresponding author, declare that the results/data/figures in this manuscript have not been published, nor are they under consideration for publication elsewhere. I certify that the above information is true and correct. All the authors contributed to the study and the manuscript.

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