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Lignan-enriched nutmeg extract changes gut microbiota associated with aging in C57BL/6 mice

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ABSTRACT

As the aging population grows, increasing interest in promoting healthy aging through dietary interventions is growing. Gut microbiota play a key role in age-related physiological decline and longevity. We investigated whether lignan-enriched nutmeg extract (LNX) could modulate the gut microbiota of aged mice. C57BL/6 mice (27 months old) were orally administered LNX for 5 weeks and compared to young (3 months) and aged controls. LNX significantly reduced body weight without affecting food intake. 16S rRNA sequencing showed that age-related microbial shifts, including decreased *Firmicutes* and increased *Bacteroidota*, were partially reversed by LNX. While microbial diversity was unchanged, LNX increased *Bifidobacterium*, *Blautia*, and *Acetatifactor muris*, a taxon previously associated with healthy aging, and reduced *Turicibacter sanguinis*, linked to normal aging. These findings suggest that LNX modulates specific microbial taxa without broadly restoring a youthful microbiota composition.

1. Introduction

The number of global elderly population continues to increase (WHO, 2022). Demographic changes are directly linked to aging, which in turn has various implications on health and is associated with several diseases. Furthermore, the rise in healthcare costs and social expenses due to increased aging population is recognized as a worldwide societal issue. As aging societies expand, there is a growing interest in healthy aging alongside an increase in lifespan. Clinical research on healthy aging, as well as lifestyle choices and diet in centenarians, has brought attention to the significance of gut microbiota in relation to aging process. Human gut microbiota exhibits variations based on the degree of aging and environmental factors, such as dietary habits and lifestyle patterns (Yatsunenko et al., 2012).

Aging is a complex biological process with multifaceted effects on gut microbiota, involving genetic, proteomic, metabolic, and immunological dimensions (López-Otín et al., 2013). Functional changes associated with aging lead to organ and systemic functional decline and, ultimately, mortality. Maintaining the functional homeostasis of the body involves a crucial role for gut microbiota, which interacts with the host's physiological functions and processes (Marchesi et al., 2016). Gut microbiota is influenced by factors related to the host and external

Abbreviations: ACE, abundance-based coverage estimators; AMPK, AMP-activated protein kinase; GI, gastrointestinal; LNX, lignan-enriched nutmeg extract; MDS, multidimensional scaling; NMDS, non-metric multidimensional scaling; OTUs, operational taxonomic units.

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environment, including lifestyle, diet, antibiotic use, genetic history, and age (Rothschild et al., 2018). Furthermore, alterations in gut microbiota reciprocally influence the host's physiological functions and are linked to immune disorders, diabetes, obesity, cardiovascular diseases, and neurodegenerative disorders. The gut microbiota of the elderly differs from that of adults (Claesson et al., 2012) and is associated with factors such as intestinal motility, inflammation, and disruption of the intestinal epithelial barrier. However, further research is needed to fully understand the changes in gut microbiota with aging.

Calorie restriction (CR) has been reported to delay aging-related diseases and extend lifespan as a temporary restriction of dietary consumption, without leading to malnutrition (Bartke et al., 2001). CR alters host physiology and induces changes in gut microbiota in both humans and model animals (Fontana & Partridge, 2015; Fraumene et al., 2018). Therefore, the changes in microbiota due to dietary restriction can be considered as contributing to increased lifespan and healthy aging by delaying the onset of aging-related disorders. This study aims to investigate the effect of dietary compounds that may suppress aging on the changes in gut microbiota. In this study, nutmeg seed extract is used as a candidate substance to inhibit aging.

Myristica fragrans, commonly known as nutmeg, has been found to have various anti-aging effects (Lestari et al., 2012). Nutmeg is native to Indonesia and has been cultivated in several regions, including South China and India, for its use as a spice to add the flavor of food. In addition nutmeg has been used as a traditional remedy to alleviate symptoms like indigestion, abdominal pain, vomiting, and appetite loss. However, nutmeg seeds contain a toxic substance called myristicin, which can induce hallucinations (Maeda et al., 2008). Nevertheless, previous research has developed methods to diminish myristicin and enhance the active compound, lignan, which improves muscle function and exercise performance related to aging (Lee et al., 2023). Lignan-enriched nutmeg extract (abbreviated as LNX) with enhanced lignan components is reported to have anti-obesity effects as well (Perumal et al., 2023). Among the lignan components, Nectandrin B is particularly effective in inhibiting aging process by activating AMP-activated protein kinase (AMPK) (Jang et al., 2019). Moreover, the active components of nutmeg are known to regulate gut microbiota, with anti-inflammatory and anti-obesity effects (Zhao et al., 2020). Moreover, recent studies have further demonstrated that nutmeg extracts could reduce hepatic lipid accumulation and improve non-alcoholic fatty liver disease (NAFLD) through modulation of inflammatory pathways such as NF-κB and AhR-FAS (Zhao et al., 2022). In addition to these systemic metabolic effects, nutmeg-derived compounds have been shown to modulate the metabolites by commensal bacteria, including short-chain fatty acids (SCFAs) and antimicrobial peptides (Oo et al., 2024). This suggests that *Myristica fragrans* may exert its health-promoting effects not only by directly affecting host pathways but also by shaping the metabolic output of the microbiota, thereby supporting host defense and metabolic balance.

The microbiome consists of symbiotic microorganisms that reside in various parts of the host's body and is ten times more abundant than the host's cells (about 60 trillion in humans). Human microbiome exists in the oral cavity, skin, reproductive organs, and the digestive system, among other places (Ma et al., 2024). Particularly, gut microbiota is often referred to as the "second genome" and regulates the host's health, immune system, and even conditions such as mental health disorders by the so-called 'Gut-Brain axis' (Carabotti et al., 2015; Grice & Segre, 2012). Recent reviews and meta-analyses have highlighted that the composition and diversity of the gut microbiota change markedly with age, typically showing a decline in beneficial commensals and a relative increase in potentially harmful bacteria (Ghosh et al., 2022). These age-related shifts in microbial communities have been associated with chronic low-grade inflammation, metabolic disorders, and increased vulnerability to age-related diseases (Kanimozhi & Sukumar, 2025). In particular, SCFAs, key metabolites produced by gut microbiome, also decline with aging, affecting inflammation and metabolic health (Liu et al., 2024). In response to these findings, recent studies have explored microbiome-targeted interventions – including the use of probiotics, prebiotics, and plant-derived bioactives – as promising strategies to mitigate age-related dysbiosis and promote healthy aging (Kanimozhi & Sukumar, 2025). Building upon this emerging evidence, and our own recent work demonstrating that nectandrin B, a major lignan component of LNX, could extend lifespan in *Drosophila melanogaster* (Ahn et al., 2023), we hypothesized that LNX supplementation could modulate the gut microbiota in aged mice. To test this, we compared the gut microbiota of aged mice (27 months old) that consumed LNX for 5 weeks with that of young mice (3 months old) and untreated aged controls using 16S rRNA sequencing. In particular, we focused on microbial taxa known to disrupt gut microbial balance, degrade short-chain fatty acids (SCFAs), or influence aging, such as *Clostridia*, *Akkermansia*, *Bifidobacteria*, and *Prevotella* (Lim & Nam, 2023; Ramos Meyers et al., 2022). This study is expected to provide essential insights for the development of microbiota-based interventions aimed at promoting healthy aging.

2. Materials and methods

2.1. Experimental animal

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Korea Basic Science Institute (KBSI-IACUC-23-12) and conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Male C57BL/6 mice aged 3 months (Young group) and 27 months (Old group and Old_LNX group) were obtained from the Animal Facility of Aging Science (KJ309), Korea Basic Science Institute (Gwangju, Korea). Only male animals were used in this study to minimize sex-related variability in physiological and metabolic parameters. The mice were housed individually in a specific pathogen-free (SPF) facility under controlled environmental conditions (temperature: 22 ± 2 °C; humidity: 50 ± 5 %; 12-h light/dark cycle). Environmental enrichment was provided in all cages, including autoclaved paper bedding, nesting materials, and climbing frames, in accordance with animal welfare standards. Sterilized food and water were supplied ad libitum. The SPF status of the animal room was regularly monitored using a laser scanning confocal microscope (Leica TCS SP5 AOBS/Tandem, KJ302) at the Honam Regional Center of KBSI. All mice were acclimated to the housing conditions for 7 days prior to the start of the experiment. Body weight and food intake were recorded weekly at fixed times (10:00–12:00 AM) to minimize the influence of circadian variation. Sampling procedures, including fecal and tissue collection, were also performed at consistent time points within this window across all groups. The study initially consisted of 8 mice per group: Young, Old (aged control), and Old_LNX (aged with LNX treatment). Animals in the Old and Old_LNX groups were randomized after stratification to ensure similar mean body weights. Blinding was not applied during treatment or outcome assessment due to practical constraints, which is acknowledged as a limitation. During the 5-week experimental period, several animals in the aged groups died due to age-related health deterioration unrelated to treatment. The final sample sizes were Young ($n = 8$), Old ($n = 4$), and Old_LNX ($n = 7$). No additional animals were introduced. Animals that died or were removed for humane reasons prior to the experimental endpoint were excluded from analysis. All procedures and observations were conducted under ethical oversight.

2.2. Dietary condition

The mice in this experiment were allowed to freely consume Purina Chow standard diet (LabDiet #5001, St. Louis, MO, USA) ad libitum. The test diet consisted of dried lignin-enriched nutmeg extract (LNX), dissolved in water to provide 100 mg per kg of body weight. LNX was prepared through a two-step ethanol extraction process, which enriched bioactive lignans such as nectandrin B while effectively reducing the

content of potentially toxic compounds like myristicin to 0.04 %, as confirmed by HPLC analysis (Lee et al., 2023). Although LNX contains lipophilic components, it was suspended in distilled water using brief vortexing and sonication prior to oral gavage, allowing for uniform dispersion. Water was selected as a vehicle to reflect practical dietary supplement intake and minimize additional variables from solubilizers or oils. The LNX was orally administered and continued for five weeks. Oral administration was performed daily between 10 AM and 11 AM to maintain a consistent daily rhythm.

2.3. Analyses of biochemical parameters

Blood glucose concentrations were measured using glucose test strips and a handheld glucometer (Accu-Chek Active; Roche Diagnostic GmbH, Mannheim, Germany). Serum levels of total cholesterol (TCHO), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-CHO) were quantified by enzymatic colorimetric assays with commercial kits (Asan Pharmaceutical Co., Seoul, Republic of Korea), following the manufacturer's instructions. Low-density lipoprotein cholesterol (LDL-CHO) concentrations were subsequently estimated using the Friedewald equation: LDL-CHO = TCHO – (HDL-CHO + TG/5).

2.4. Collection of fecal samples

To prevent coprophagy, the experimental animals were transferred to a new, sterile cage at each occurrence. After the five-week LNX consumption period, mice were euthanized, and their gastrointestinal (GI) tracts were immediately excised under sterile conditions. To obtain representative gut microbiota, the luminal contents – including fecal matter within the intestinal lumen – were carefully extracted by gently scraping the GI tract using sterile tools. This approach enables the collection of fecal material enriched with both luminal and partially mucosal-associated microbiota. All samples were immediately weighed, flash-frozen in liquid nitrogen, and stored at −80 °C until genomic DNA extraction.

2.5. Microbiota DNA extraction

Genomic DNA extraction from fecal samples of each experimental animal was performed using the phenol-chloroform-isoamyl alcohol extraction method, as previously described (Kannan et al., 2015). In brief, samples were homogenized in a lysis buffer (200 mM NaCl, 200 mM Tris-HCl pH 8.0, 20 mM EDTA) and extracted by bead-beating. The extracted genomic DNA was then purified by phenol:chloroform:isoamyl alcohol extraction, followed by precipitation with 3 M sodium acetate and further purification with isoamyl alcohol. After a 70 % ethanol wash, the DNA pellet was air-dried and resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Bacterial DNA was quantified using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Kyoto, Japan).

2.6. Bacterial 16S rRNA gene sequence analysis

DNA base pair sequencing was carried out according to the Illumina 16S rRNA Metagenomic Sequencing Library protocol. The 16S rRNA gene was amplified using 16S rRNA V3-V4 primers (16S rRNA Amplicon PCR Forward Primer: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′; Reverse Primer: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′). The initial gDNA was further amplified with 16S rRNA V3-V4 primers, and a limited cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters (Klindworth et al., 2013). The final products were normalized using PicoGreen and pooled out for the next step. The library size was confirmed using TapeStation DNA screentape D1000 (Agilent Technologies, Santa Clara, CA). DNA base pair sequencing was performed on the MiSeq™ platform (Illumina, San Diego, CA) according to standard protocols, with 2 × 300 bp read lengths.

2.7. Analysis of sequence data

To improve the assembly of paired-end Next Generation Sequencing (NGS) reads, terminal NGS sequence fragments were combined using FLASH (Fast Length Adjustment of SHort reads) (Magoč & Salzberg, 2011). Amplicon errors were modeled and noise sequences were filtered out using the DADA2 software, correcting errors within the surrounding sequences and removing chimeric and singleton sequences (Callahan, McMurdie, et al., 2016). Subsequently, the reconstructed sequence data underwent noise removal with default parameters through a Q2-Feature classifier trained on the SILVA reference (region V3-V4) database (https://www.arb-silva.de/) (Bokulich et al., 2018). Q2-diversity calculations were carried out with the sampling depth option for diversity analysis, and statistical tests were performed (Bolyen et al., 2019). After inspecting the final "table.qzv" file, features were filtered out based on threshold counts according to the QIIME2 experiment.

2.8. Diversity analysis for relative abundance evaluation

Metagenomic sequence data for each sample were analyzed using the phyloseq package (1.28.0) in R version 3.6.1 (McMurdie & Holmes, 2013). The taxonomic classification table (OTU) and metadata were sent to the phyloseq package. OTUs not present in at least one sample were removed and considered as sequencing errors. Data were normalized by cumulative sum scaling (CSS) using the metagenomeSEq. (1.16.0) package from Bioconductor software (Callahan, Sankaran, et al., 2016). Additional analyses and visualizations were performed using the phyloseq package.

Alpha-diversity analysis for relative species abundance was determined as previously described (Ahn, Kim, et al., 2025). Alpha-diversity metrics, including the Shannon index and Abundance-based Coverage Estimators (ACE), were calculated using values without filtering from the phyloseq package. Differences between alpha-diversity and richness among groups were measured using core matrix analysis. Normalization by cumulative sum scaling (CSS) was performed on metagenomic sequences before converting to relative frequencies.

Beta-diversity was calculated using the Bray-Curtis dissimilarity on log-transformed OTU data, as previously described (Ahn, Kim, et al., 2025), to compute non-metric multidimensional scaling (NMDS) from Bray-Curtis floating matrices. NMDS was performed using the metaMDS function within the package to obtain as much information as possible about relationships between samples while reducing dimensionality.

2.9. Linear discriminant analysis effect size (LEfSe) method

Differentially abundant analysis of gut microbiota across experimental groups was performed using the LEfSe pipeline, as previously described (Ahn, Lee, et al., 2025; Segata et al., 2011). The method first applies the non-parametric Kruskal-Wallis rank sum test to detect taxa with significant differences in abundance, followed by pairwise Wilcoxon tests and linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant feature. Taxa with an LDA score greater than 3 and $P < 0.05$ were considered statistically significant.

2.10. Heatmap and phylogenetic tree construction

Heatmaps and cluster analysis were performed using the Heatplus package (version 2.30.0) from Bioconductor and the vegan package in R (version 4.3.3), based on all OTU values or key abundant OTU values as previously described (Ahn, Kim, et al., 2025). Average linkage clustering was employed for cluster analysis. Quantitative analysis using Bray-Curtis distances was used for heatmap generation. Phylogenetic trees for each sample were constructed directly from raw sequence data

without any filtering to visualize the abundance of taxa associated with the taxonomic hierarchy. The 16S rRNA sequences from each sample were aligned using ClustalW with default parameter settings. These results were arranged for building maximum similarity phylogenetic trees (MEGAX). All phylogenetic trees were created as images in iTOL.

2.11. Differential analysis

To identify bacterial species with significant changes in abundance, we used the DESeq2 package in R (Paulson et al., 2013). Raw read count data were normalized using the median of ratio method, where counts were adjusted by sample-specific size factors based on the median ratio

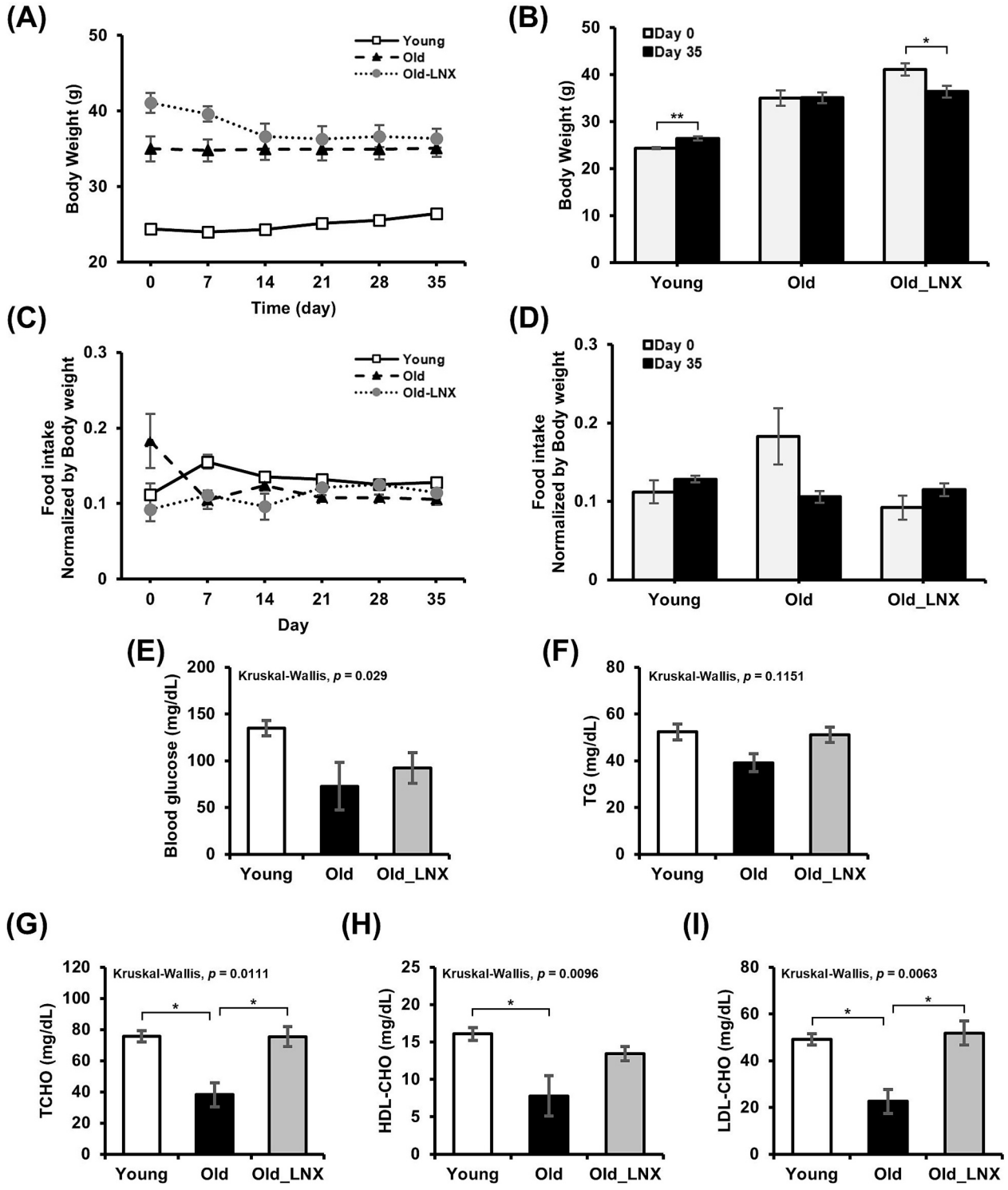

Fig. 1. LNX consumption induces weight loss. (**A**) Body weight of mice in each experimental group was measured every 7 days. (**B**) Body weight of mice in each experimental group before and after LNX consumption. (**C**) Food intake normalized by body weight in each experimental group was measured every 7 days. (**D**) Food intake normalized by body weight in each experimental group before and after LNX consumption. (***E*-I**) After 35 days of LNX consumption, (**E**) blood glucose, (**F**) triglycerides, (**G**) total cholesterol, (**H**) HDL-cholesterol, and (**I**) LDL-cholesterol of each group of mice were measured. Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post hoc test. *$p < 0.05$; **$p < 0.01$.

of species counts relative to the geometric mean per species. Differential abundance analysis was performed using DESeq2 package, comparing the two groups: Old vs. Old_LNX. Bacteria with an adjusted *P*-value (padj) < 0.05 were considered significantly different.

2.12. Statistical analyses

All statistical analyses were performed using R software. For multi-group comparisons, the Kruskal-Wallis test was used, followed by Dunn's post-hoc test for pairwise comparisons. Statistical significance was determined at a threshold of $P < 0.05$. In the case of differential abundance analysis performed with DESeq2, the Benjamini-Hochberg false discovery rate (FDR) correction was applied to adjust for multiple testing, and taxa with adjusted *P*-values (padj) < 0.05 were considered statistically significant. All results are presented as mean ± SEM, and all graphs were generated using R software.

3. Results

3.1. LNX consumption induces weight loss

Throughout the 5-week study period, body weight and food intake were measured at one-week intervals in the Young ($n = 8$), Old ($n = 4$), and Old_LNX ($n = 7$) groups. As shown in Fig. 1A and B, the Young group showed a gradual increase in body weight over time, resulting in an average increase of 8.2 % by week 5 ($P < 0.01$). In contrast, the Old group did not exhibit a statistically significant change in body weight during the study period. Remarkably, the Old_LNX group demonstrated a significant reduction in body weight, with an average decrease of 11.4 % at the end of the 5 weeks ($P < 0.05$). Notably, this reduction was most pronounced during the second week of treatment. To investigate whether weight loss was associated with reduced food intake, food consumption was monitored in parallel. While food intake differed significantly among the groups during the first week ($P < 0.05$), no significant differences were observed in subsequent weeks (Fig. 1C,D). These results suggest that the observed weight loss in the Old_LNX group is unlikely to be solely attributable to reduced food intake.

To further investigate metabolic effects associated with the observed weight loss, blood glucose and lipid levels were analyzed (Fig. 1E–I). Compared to the Young group, the Old group exhibited reduced total cholesterol (TCHO), triglycerides (TG), HDL-cholesterol (HDL-CHO), and LDL-cholesterol (LDL-CHO), consistent with previous reports of age-related decline in hepatic lipid synthesis and secretion (Timchenko, 2009; Van Liew et al., 1993). Interestingly, LNX administration in aged mice led to an increase in TCHO, TG, HDL-CHO, and LDL-CHO compared to the Old group. Fasting blood glucose levels also showed a slight increase in the Old_LNX group, although differences were not statistically significant. These results suggest that LNX supplementation may partially restore age-suppressed lipid metabolism.

3.2. Phylogenetic distribution of mouse microbiota at phylum level

The diversity of gut microbiota from mouse feces was analyzed using 16S rRNA metagenome analysis. Identification of species was carried out from metagenome sequence information using the V3-V4 region of 16S rRNA genes on the MiSeqTM genome sequencer (Illumina). The total reads for 16S rRNA amplicon sequences were an average of 25,294 for the Young group, 26,282 for the Old group, and 22,878 for the Old_LNX group (Supplemental Table S1). The variation range of OTU values showed no issues for statistical analysis, with 3.91 % difference between the Young and Old group and 12.95 % difference between the Old and Old_LNX group. A total of 10 phyla were identified from the entire 16S rRNA sequences (Supplemental Table S2). In all three groups, *Bacteroidota* (48.6 % in the Young group, 57.1 % in the Old group, and 56.5 % in the Old_LNX group) formed the largest phylum, followed by *Firmicutes* (44.3 % in the Young group, 36.4 % in the Old group, and 38.2 % in the Old_LNX group). The third phylum was *Verrucomicrobiota* (2.7 %) in the Young group, whereas it was *Patescibacteria* (4.9 %) in the Old group. Particularly, LNX consumption reduced *Patescibacteria* to 3.7 % in the Old_LNX group, but *Actinobacteriota* increased about 2-fold to 0.8 % in the Old_LNX group. Interestingly, *Desulfobacteriota* was found to be 2.2 % in the Young group but was low in the Old group and Old_LNX group, at 0.6 % and 0.5 %, respectively (Fig. 2A). Another notable aspect was the ratio of the major phyla, namely *Firmicutes* to *Bacteroidota*, which decreased from 0.92 in the Young group to 0.65 in the Old group ($P < 0.05$) and slightly increased to 0.68 in the Old_LNX group, but without statistical significance (Fig. 2B).

The gut microbiota of experimental mice were compared at various taxonomic levels, including Phylum, Class, Order, Family, Genus, and Species (Supplemental Fig. S1 and Supplemental Table S2–S7). At the class level, the Old group showed an 8.5 % increase in *Bacteroidia* and a 10.9 % decrease in *Clostridia* compared to the Young group, while these changes were less pronounced in the Old_LNX group (approximately 1–3 %) (Supplemental Table S3). Notably, *Saccharimonadia*, *Bacilli*, and *Gammaproteobacteria*, which increased with aging, were reduced in the Old_LNX group. At the order level, *Bacteroidales* increased in the Old group but showed a smaller increase in the Old_LNX group, whereas *Lachnospirales*, which had decreased in the Old group, increased again in the Old_LNX group to levels similar to the Young group (Supplemental Table S4). Similarly, at the family level, *Lachnospiraceae* decreased with aging but was restored by LNX intake (Supplemental Table S5). The overall genus and species-level distributions followed a similar trend, with some individual variation, as detailed in Supplemental Tables S6 and S7.

3.3. Identification of LNX-associated gut microbial taxa by LEfSe analysis

The explore specific microbial taxa associated with LNX supplementation, we performed linear discriminant analysis effect size (LEfSe) across all taxonomic levels to compare bacterial abundance among the three groups (Fig. 3A). A total of 34 taxa were identified as significantly different, based on multiple testing-corrected Kruskal-Wallis tests ($p < 0.05$) and effect size thresholds (LDA score > 4). Among these, 17 taxa, including *c_Clostridia* and *o_Oscillospirales*, were enriched in the Young group, whereas 14 taxa, including *o_Bacteroidales*, were enriched in the Old group. Notably, the Old_LNX group showed enrichment of three taxa, including *f_Muribaculaceae*. For a more focused comparison at the genus level, pairwise LEfSe analysis (LDA score > 3) were conducted. In the comparison between Young and Old groups, the Old group showed significantly higher abundance of *f_tannerellaceae_.*, *g_ASF356*, *g_Lactobacillus*, and *g_[Eubacterium]_nodatum_group* (Fig. 3B). In contrast, comparing Old and Old_LNX groups revealed that *g_Lachnospiraceae_UCG_006* was significantly enriched in the Old_LNX group (Fig. 3C). As members of the *Lachnospiraceae* family are well-known butyrate producers (Vital et al., 2014), this shift suggests a potential functional improvement in colonic short-chain fatty acid (SCFA) profiles following LNX supplementation.

3.4. Alpha-diversity analysis of mouse gut microbiota

To evaluate within group microbial diversity, we analyzed six alpha-diversity indices, including ACE, Shannon, Fisher's alpha, InvSimpson, Simpson, and species evenness (Evenness). The Kruskal-Wallis test revealed significant group differences for all indices except Evenness ($p < 0.05$, Fig. 4 and Supplemental Table S8). Compared to the Young group, the Old group showed a marked reduction in species richness. Fisher's alpha was significantly decreased by 31.2 % ($p = 0.0453$), while ACE also declined by 26.2 %, although this did not reach statistical significance ($p = 0.0608$). Although the Shannon and Simpson indices showed a decreasing trend in the Old group, these differences were not statistically significant. Fisher's alpha was significantly reduced in the Old group compared to the Young group ($p = 0.0453$), while InvSimpson

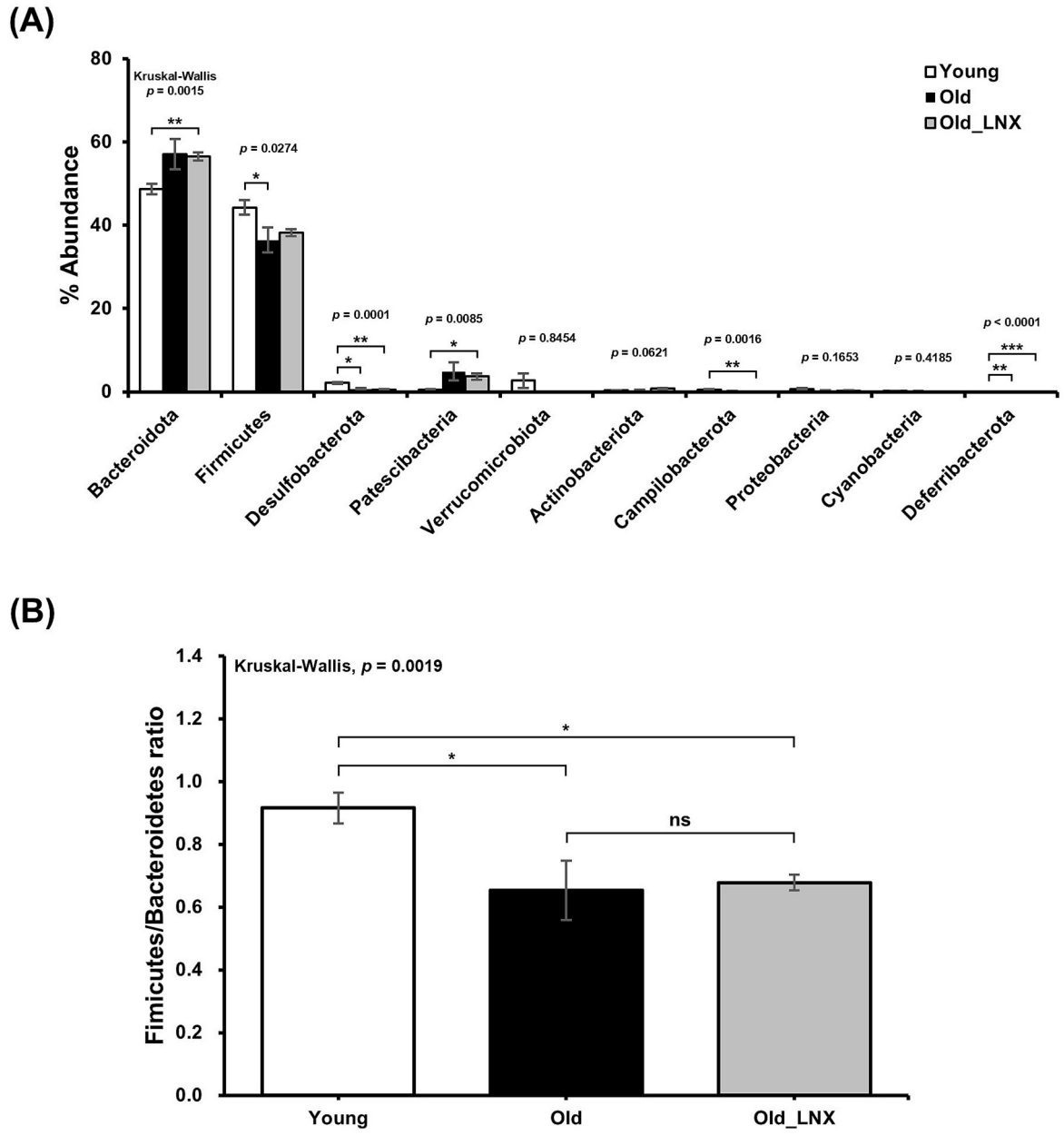

Fig. 2. LNX consumption alters the composition of gut microbiota at the phylum level. (**A**) % Abundance each phylum in each experimental group are shown. (**B**) *Firmicutes/Bacteroidetes* ratio in each experimental group are shown. Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

was significantly lower only between the Young and Old_LNX groups ($p = 0.0384$), not between the Young and Old. Dunn's post-hoc analysis demonstrated that microbial diversity was significantly reduced in the Old_LNX group compared to the Young group in terms of ACE, Fisher, Shannon, Simpson, and InvSimpson indices ($p < 0.05$), whereas no significant differences were observed in Evenness among the groups. No significant differences were found between the Old and Old_LNX groups for any alpha-diversity measure. These results suggest that aging leads to a reduction in gut microbial diversity, and that LNX supplementation fails to reverse this decline.

3.5. Beta-diversity analysis of mouse gut microbiota

The beta-diversity, which represents taxonomic diversity between different groups, was analyzed. Non-metric Multidimensional Scaling (NMDS) and Metric Multidimensional Scaling (MDS) plots revealed a substantial difference in microbial communities between the Young and Old groups. Moreover, LNX supplementation induced a shift in microbial composition, leading to a distinct separation between the Old and Old_LNX groups (Fig. 5A,B). Similarly, in Detrended Correspondence Analysis (DCA), Redundancy Analysis (RDA), and Canonical Correspondence Analysis (CCA) plots, the Young group was positioned toward the left, whereas the Old group was shifted to the right, indicating a distinct separation in microbial composition between young and aged individuals (Fig. 5C,D,E). Additionally, the Old group exhibited a widely dispersed distribution, reflecting high inter-individual variation in microbial composition. In contrast, the Old_LNX group formed a more compact cluster within the Old group, suggesting that LNX supplementation reduced microbial community variability and promoted a more uniform microbiota structure (Fig. 5C–E). These results indicate that LNX intake may help stabilize gut microbiota composition in aged individuals by mitigating the increased diversity in microbial profiles

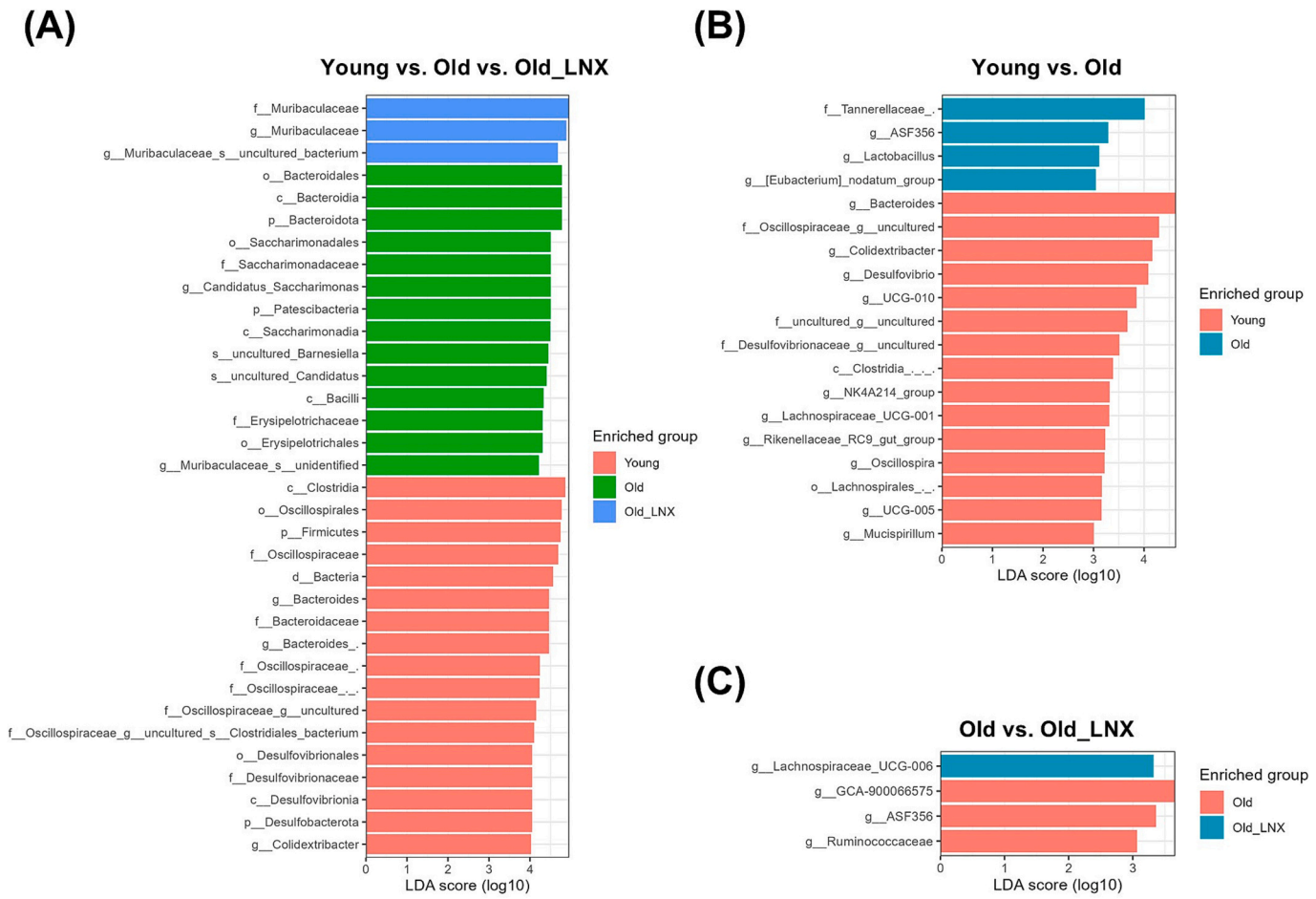

Fig. 3. Identification of differentially abundant taxa by LEfSe analysis. (**A**) Overview of taxa significantly enriched among the three groups identified by LEfSe analysis (Kruskal–Wallis test $p < 0.05$; LDA score > 4). (**B**) Pairwise LEfSe comparison between the Young and Old groups (Wilcoxon test $p < 0.05$; LDA score > 3). (**C**) Pairwise LEfSe comparison between the Old and Old_LNX groups (Wilcoxon test $p < 0.05$; LDA score > 3).

observed in the Old group.

3.6. Phylogenetic correlation analysis of mouse gut microbiota

Phylogenetic heatmap analysis was conducted on a total of 19 individuals from the three mouse groups. As seen in Supplemental Fig. S2, the young group is clearly distinguishable from the old group at all taxonomic levels from phylum to species. However, the old and LNX-old groups are somewhat mixed from phylum to Order but show differentiation at the Genus and Species levels. A circular cladogram representing the phylogenetic tree revealed significant differences in gut microbiota between the three groups (Fig. 6). Analysis of microbial diversity at the taxonomic levels of phylum and family revealed a decreasing trend in the number of identified taxa following LNX supplementation. Specifically, the Young group exhibited 11 distinct phyla, while the Old group displayed 10, and the Old_LNX group showed a further reduction to 9 phyla. A similar trend was observed at the family level, with 45 families detected in the Young group, 42 in the Old group, and a decreased count of 37 families in the Old_LNX group. Considering the observed reduction in diversity alongside the previously noted health-beneficial effects, these findings suggest that LNX supplementation may have influenced the formation of a gut microbiota composition specific to beneficial bacteria.

3.7. Identification of gut bacterial species associated with aging retardation

To investigate specific taxa affected by LNX supplementation, differential analysis was performed using the DESeq2 program based on the read count values of each operational taxonomic unit (OTU). This analysis identified a total of 12 OTUs that significantly differed between the Old and Old_LNX groups (Supplemental Fig. S3A and Supplemental Table S9). Taxonomic classification of these OTUs revealed that species belonging to the *Muribaculaceae* family (OTU0047, OTU0024) were more abundant in the Old group, whereas species from the *Clostridia* family (OTU0145, OTU0202, OTU0302, OTU0355) were more abundant in the Old_LNX group (Supplemental Fig. S3A). Analyzing the patterns of changes and conducting partial clustering analysis at the individual level, OTU0024 and OTU0405 decreased in over half of the individuals in response to LNX, while OTU0011 and OTU0086 increased in over half of the individuals (Supplemental Fig. S3B).

To further investigate how LNX supplementation affects gut microbiota changes previously associated with aging, we examined the abundance of specific bacterial taxa. *Muribaculum intestinale* has been identified as a common gut microbial feature in both healthy and normal aging mice, exhibiting increased abundance in both groups (Ke et al., 2021). Consistently, our results showed an increase in *M. intestinale* in the Old group, with no significant changes following LNX supplementation (Fig. 7A). Previous studies reported that *Turicibacter sanguinis* and *Enterohabdus* were more abundant in normal aging mice compared to healthy aging mice (Ke et al., 2021). Our findings similarly

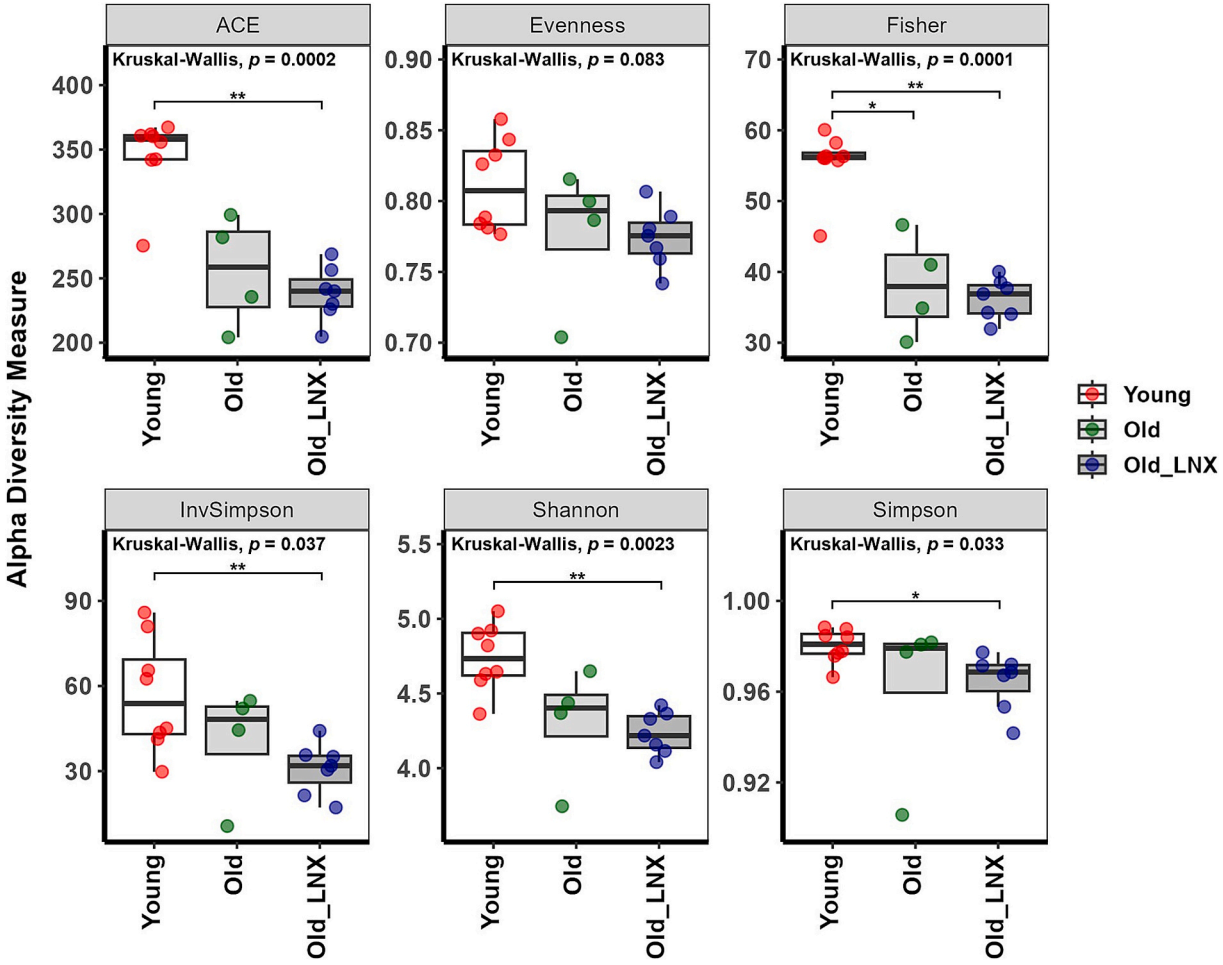

Fig. 4. Alpha-diversity of gut microbiota is reduced by aging and modulated by LNX consumption. Comparisons of α-diversity indices of the gut microbiome in each group, measured using ACE richness, Evenness, Fisher, InvSimpson, Shannon and Simpson methods, are shown in box plots. Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post hoc test. *$p < 0.05$; **$p < 0.01$.

demonstrated that *T. sanguinis* was increased in the Old group relative to the Young group, but its abundance was reduced to Young group levels following LNX supplementation (Fig. 7B). However, *Enterohabdus* abundance showed no significant differences among the Young, Old, and Old_LNX groups (Fig. 7C). Conversely, prior research has indicated that *Acetatifactor muris*, *Akkermansia muciniphila*, and *Ruminococcaceae* were more prevalent in healthy aging mice compared to normal aging mice (Ke et al., 2021). In our study, *A. muris* abundance was reduced in the Old group relative to the Young group, but LNX supplementation restored it to Young group levels (Fig. 7D). In contrast, *A. muciniphila* abundance remained unchanged across all three groups (Fig. 7E), while *Ruminococcaceae* showed a slight increase in the Old group but was nearly undetectable in the Old_LNX group (Fig. 7F). Additionally, previous studies have reported an inverse correlation between the frailty index and *Phocea massiliensis* (Ke et al., 2021). Our results revealed a decrease in *P. massiliensis* abundance in the Old group, with LNX supplementation further reducing its levels beyond those observed in the Old group (Fig. 7G). Human gut microbiome studies have shown that *Blautia* and *Bifidobacterium* decrease with aging (Lim et al., 2021; Yan et al., 2022; Zhang et al., 2021). In agreement with these findings, our results indicated that *Blautia* abundance was reduced in the Old group compared to the Young group but was restored to Young group levels following LNX supplementation (Fig. 7H). Meanwhile, *Bifidobacterium* abundance remained relatively unchanged between the Young and Old groups; however, LNX supplementation increased its abundance in Old mice (Fig. 7I). Collectively, these findings suggest that LNX supplementation modulates the gut microbiota of aging mice in a manner potentially beneficial to health by increasing the abundance of *A. muris*, *Blautia*, and *Bifidobacterium*, while decreasing *T. sanguinis*.

4. Discussion

Lignan-enriched nutmeg extract (LNX) is an effective substance that acts as a prebiotic or modulator of gut health, influencing probiotic gut microbes (Zhao et al., 2020). Within lignan-enriched nutmeg extract, various lignan compounds exist, altering gut microbiota and regulating non-alcoholic fatty liver by inhibiting hepatic fat accumulation and enhancing liver function. Many natural substances exhibit liver protective and weight loss effects. However, in many cases, they are not easily consumed orally but rather modulate gut microbiota through the digestive tract (Yang et al., 2022). Hence, this study aimed to test the hypothesis that oral supplementation of concentrated LNX would induce short-term changes in the gut microbiota of aging mice by selectively promoting beneficial microbial taxa. Our findings confirmed this hypothesis, as LNX supplementation led to increased abundance of health-associated genera such as *Bifidobacterium*, *Blautia*, and *Acetatifactor muris*, while reducing potentially harmful taxa like *Turicibacter sanguinis*. These results suggest that LNX may contribute to partial shifts toward a gut microbial profile more commonly observed in younger or healthier aging mice, although further studies are required to establish functional outcomes.

When LNX was administered orally for 5 weeks, although previous

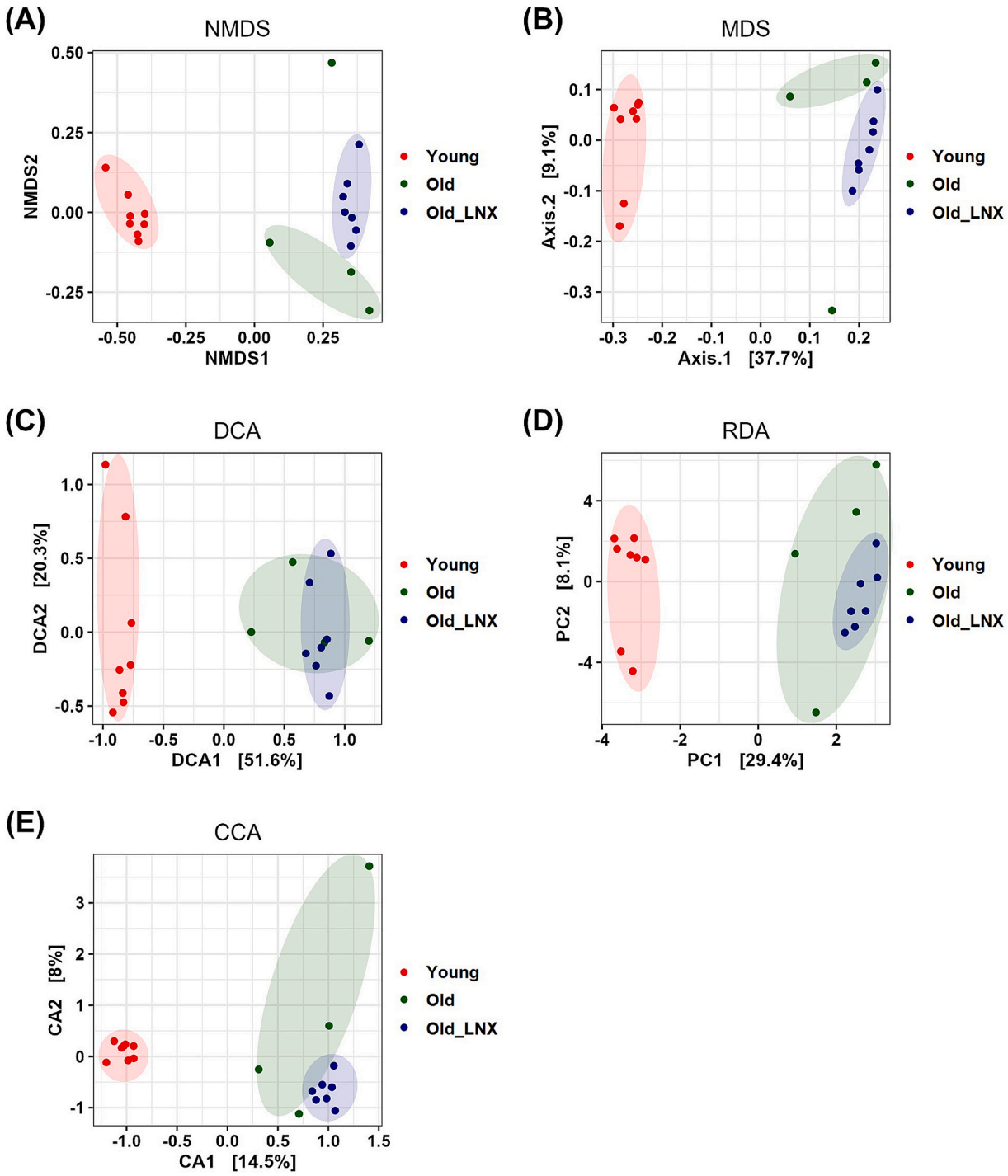

Fig. 5. LNX consumption shifts and stabilizes gut microbial community structure in aged mice. The β-diversity plots of the gut microbiome in each group, measured using nonmetric multidimensional scaling (NMDS) plot (**A**), multidimensional scaling (MDS) plot (**B**), detrended correspondence analysis (DCA) plot (**C**), redundancy analysis (RDA) plot (**D**), and canonical correspondence analysis (CCA) plot (**E**).

studies have reported that LNX supplementation can improve muscle mass and function in aged mice (Lee et al., 2023), the current study did not directly measure these physiological outcomes. Thus, while gut microbial changes may be related, their causal role in these effects remains speculative and warrants further investigation.

LNX intake over 5 weeks led to significant weight loss in aged mice, which did not correlate with reduced dietary intake (Fig. 1A–D). This suggests that LNX likely increased the metabolic rate of aged mice. Previous study has reported a similar outcome, where the administration of nutmeg extract to 20-month-old Wistar aging rats led to a 10 % weight loss despite increased food consumption (Pratiwi et al., 2018). The physiological activity of nutmeg extract compounds is presumed to inhibit autophagy through the IGF1-Akt-mTOR pathway, where weight loss could result from reducing calorie intake or activating AMPK to inhibit glucose formation and suppress fatty acid synthesis (Yang et al., 2016). In fact, LNX contains a major lignan component, nectandrin B, which activates AMPK, reduces intracellular ROS levels in aging human skin cells, and helps prevent aging (Jang et al., 2019). Nectandrin B is known to act on AMPK, Sirt1, and the mTOR signaling pathway, promoting anti-aging or rejuvenating effects, with AMPK and Sirt1 acting as

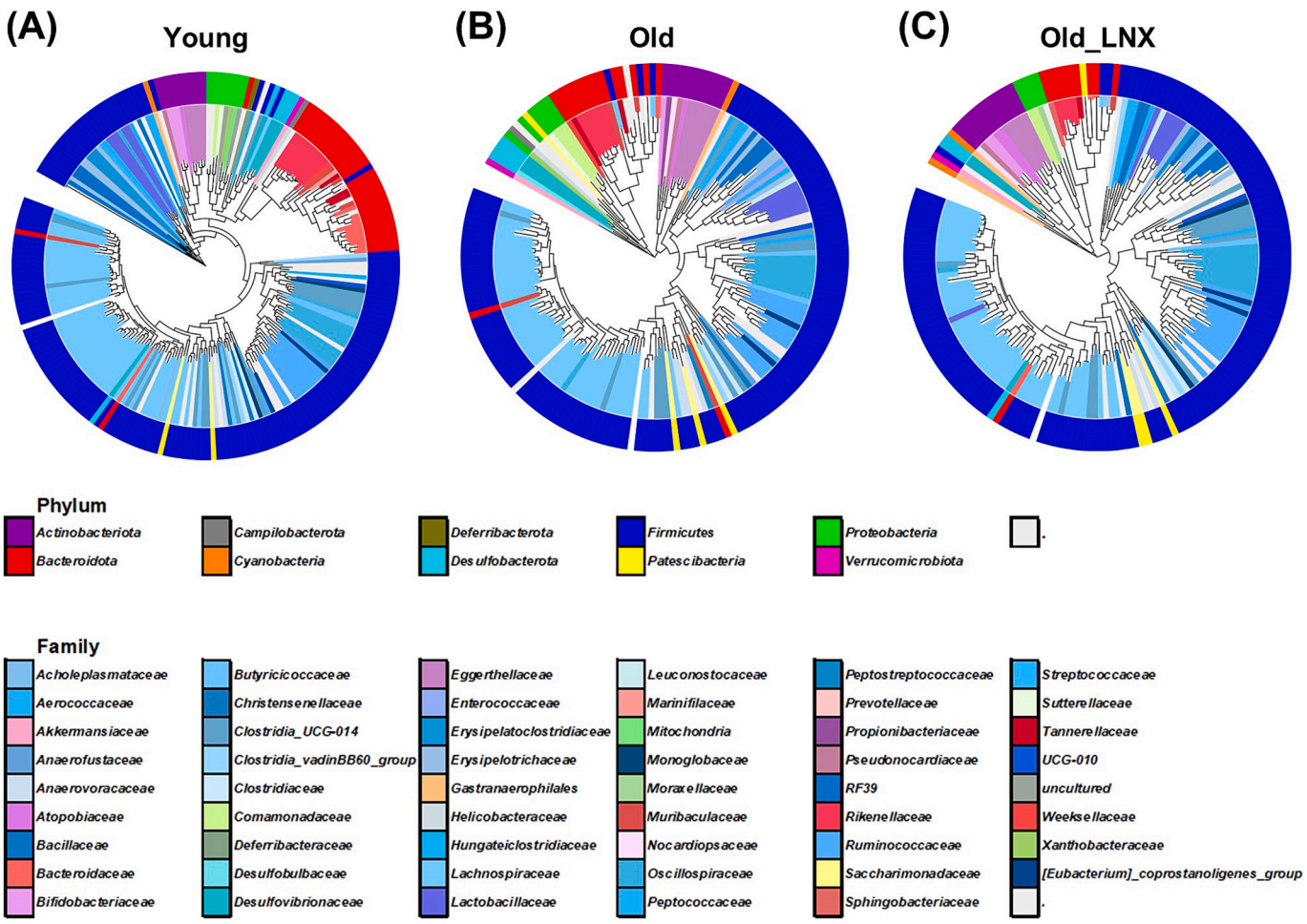

Fig. 6. LNX consumption alters phylogenetic structure and reduces taxonomic diversity of gut microbiota in aged mice. Maximum-likelihood phylogenetic tree comprising taxa of the gut microbiome in the Young group (**A**), the Old group (**B**), and the Old with LNX consumption group (**C**). The circular dendrogram rings represent phylum levels, with corresponding taxa depicted in the inner layer.

key partners (Ruderman et al., 2010). When fruit flies freely consumed nectandrin B, they exhibited increased exercise performance and extended lifespan (Ahn, Mahbub, et al., 2023). Furthermore, recent reports indicate that LNX acts on mouse adipocytes, inhibiting STAT3 phosphorylation and FAS expression, thereby suppressing fat accumulation and having an anti-obesity effect (Perumal et al., 2023). Therefore, the weight loss observed in aged mice due to LNX intake aligns with previous findings. Despite this consistency, it is important to note that the reduced sample size due to natural mortality in aged mice may limit the statistical power of this observation. Although appropriate statistical methods for small and unbalanced group sizes were applied, future studies with larger, power-calculated sample sizes will be necessary to confirm these findings with greater robustness. In addition, although animals were randomly allocated into treatment groups after body weight stratification, we were not blinded to group assignment due to logistical constraints. This lack of blinding is a limitation of the current study and should be addressed in future experimental designs to reduce potential bias.

Furthermore, to explore potential physiological correlates of the observed weight loss, we measured additional serum metabolic markers (blood glucose, TCHO, TG, HDL-CHO, LDL-CHO) (Fig. 1E–I). Interestingly, aged mice showed lower levels of these markers compared to young mice, while LNX-treated aged mice exhibited increases in all measured lipid and glucose levels despite reduced body weight. This paradoxical finding suggests that LNX may enhance overall metabolic turnover or restore aspects of age-related metabolic decline, rather than simply decreasing adiposity. However, since we did not assess energy expenditure (e.g., via indirect calorimetry), nutrient absorption, or adipose tissue histology, the mechanistic interpretation remains speculative. Future studies combining metabolic phenotyping with functional microbiota analysis will be needed to clarify these effects.

Next, this study aimed to investigate the changes in gut microbiota resulting from LNX consumption. It has applied the methods used in the previous gut microbiota experiments to examine and analyze these changes (Lkhagva et al., 2021). In summary, mice aged 3 and 27 months were group-housed in SPF animal facilities. They were orally administered LNX daily for 5 weeks. After 5 weeks, fresh fecal samples were collected, and 16S rRNA was sequenced to observe changes in gut microbiota. The gut microbiota in the gastrointestinal tract, especially the large intestine, can differ from fecal microbiota due to variations in pH and moisture content (Ahn et al., 2023). However, rough estimates can be made. It was well established from previous studies that major changes in the phylum-level composition of gut microbiota are associated with obesity (Turnbaugh et al., 2009). In this experiment, regardless of age or LNX intake, the relative distribution of gut microbiota at the phylum level was primarily composed of *Bacteroidetes* and *Firmicutes*, making up approximately 93–95 % (Supplemental Table S2, Fig. 2). Typically, during aging, there is a decrease in the major phylum Bacteroidetes and an increase in the *Firmicutes* to *Bacteroidetes* (F/B) ratio, shifting toward an obesity-associated composition (Binyamin et al., 2020). However, in this experiment, even though the very old (27 months) mice, equivalent to humans being nearly 90 years old,

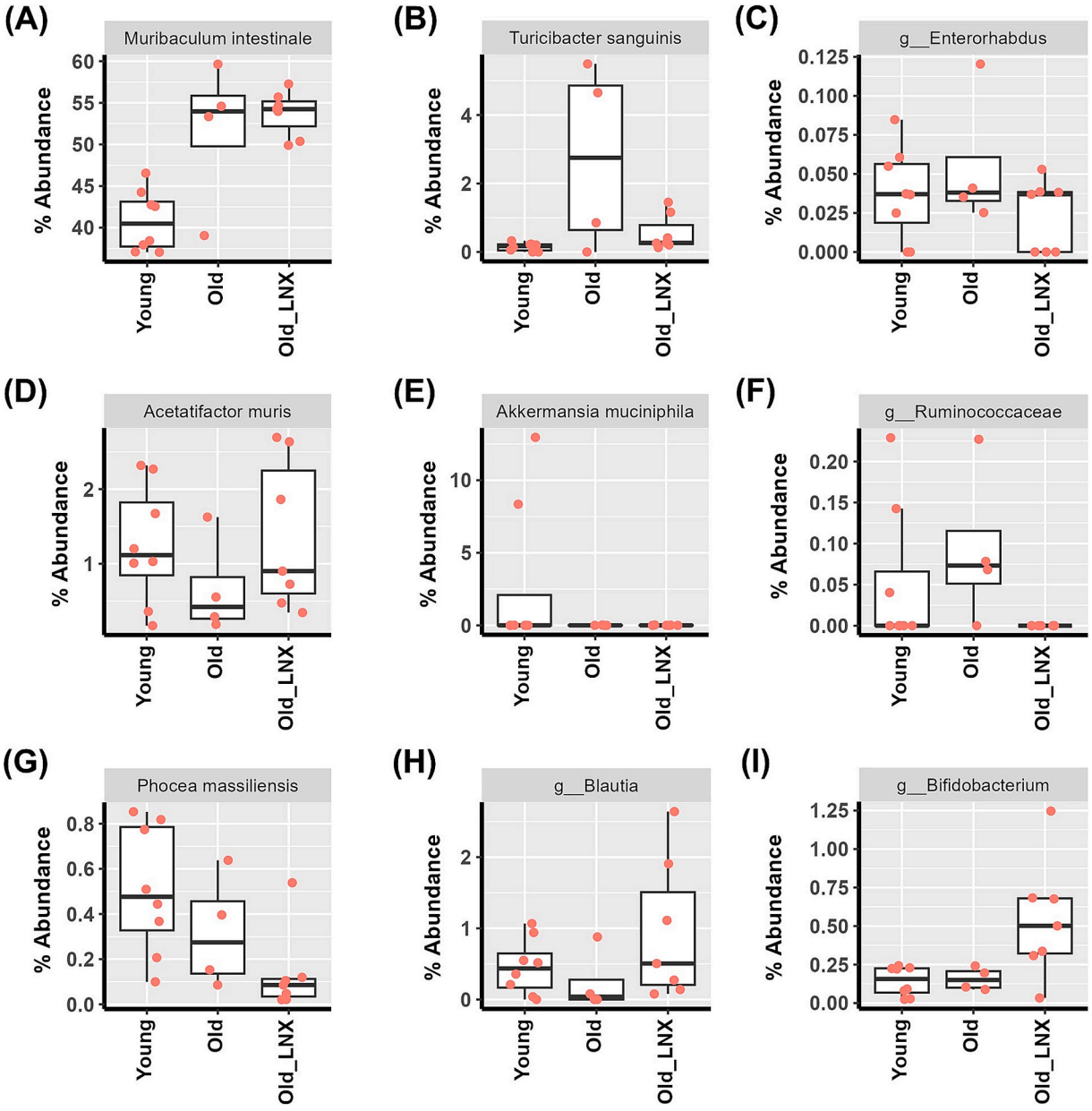

Fig. 7. Modulation of aging-related gut microbial species by LNX consumption in aged mice. Comparison of the abundance of *Muribaculum intestinale* (**A**), which is known to increase in aging mice, in each group. (**B,C**) Comparison of the abundance of *Turicibacter sanguinis* (**B**) and *Enterohabdus* (**C**), which are known to be more abundant in normal aging mice than in healthy aging mice, in each group. (**D**—**F**) Comparison of the abundance of *Acetatifactor muris* (**D**), *Akkermansia muciniphila* (**E**), and *Ruminococcaceae* (**F**), which are known to be more abundant in healthy aging mice than in normal aging mice, in each group. Comparison of the abundance of *Phocea massiliensis* (**G**), which is known to be inversely correlated with the senescence index, in each group. (**H,I**) Comparison of the abundance of *Blautia* (**H**) and *Bifidobacterium* (**I**), which are known to decrease with aging in humans, in each group.

experienced weight loss due to LNX intake, the Firmicutes, a microbial group often associated with obesity, decreased similarly (Ley et al., 2006). Similarly, a study on the F/B ratio found that as humans transition from infancy (under 1 year) to adulthood (24–45 years), the ratio increases, but in the elderly (70–90 years), it decreases (Mariat et al., 2009). Additionally, clinical fecal trials in the United States reported lower F/B ratios in the elderly compared to young adults (Claesson et al., 2011). Therefore, changes in the F/B ratio can vary with lifestyle, patterns, diet, and the aging process.

An intriguing observation is that *Clostridia* (*Clostridiales* order, *Clostridiaceae* family, *Clostridia_UCG-014* genus) increase from the order level to the genus level in aged mice and are further enhanced by LNX consumption (Supplemental Tables S4–S6). Clostridium is associated with a high-fat diet or obesity phenotype (Woting et al., 2014). Moreover, *Bifidobacteria* (*Bifidobacteriales* order, *Bifidobacteriaceae* family, *Bifidobacterium* genus) increase at the order level to the family level in aged mice and LNX intake enhances this increase (Supplemental Tables S4–S6). These microbes are also beneficial bacteria observed in high-fat diets (Volynets et al., 2017). Therefore, it might not be sufficient to explain the weight loss (anti-obesity effect) simply as a result of a few specific microbial changes. Besides specific microbial changes, it's essential to pay attention to the analysis of microbial diversity. Generally, the loss of diversity in gut microbiota signifies an important characteristic of gut dysbiosis (Mosca et al., 2016). In centenarians, humans show a decrease in *Lachnospiraceae*, *Prevotellaceae*, *Akkermansiaceae*, and *Ruminococcaceae* at the family level (Kim et al., 2019), and similar results were seen in very old mice (Supplemental Tables S5). Interestingly, LNX consumption increased the relative distribution percentages of

Lachnospiraceae and *Eubacterium* and enhanced *Bifidobacteriaceae*, aligning with previous reports (Wang et al., 2015). Additionally, it is known that *Lachnospiraceae* decrease with increasing frailty (senility) (Claesson et al., 2012; van Tongeren et al., 2005), but LNX increased the relative distribution percentages of this microbial family and genus, contrary to the typical trend (Supplemental Tables S5,S6).

This study observed a decrease in species diversity with aging, and even LNX consumption did not restore the gut microbiota diversity in aging mice (Fig. 4). Reduced alpha-diversity of species richness (ACE), evenness, and concentration (Shannon) is typically associated with aging (Giri et al., 2022). Alpha diversity is expressed in terms of species richness (ACE), evenness, and concentration (Shannon), and beta diversity measures the distance between groups using the Bray-Curtis distance index. Aging is usually reported to increase diversity, but it decreases significantly in very old aging individuals (Xu et al., 2019). The 27-month-old mice used in this experiment can be compared to humans aged 80–90 years, aligning somewhat with these findings. The beta-diversity of gut microbiota showed group separation between young mice, old mice, and LNX-fed old mice, indicating that LNX caused specific changes in certain species (Fig. 5). However, ecological multivariate analysis did not display significant group differences due to LNX consumption. Similarly, *Lycium barbarum* extract intake showed partial differentiation in beta diversity in the gut microbiota of high-fat diet mice (Tian et al., 2020). This aligns with the fact that the components of *Lycium barbarum* extract act as prebiotics that can regulate gut microbiota.

Finally, to pinpoint changes in specific species rather than the overall changes in the gut microbiota in very old mice, microbial identification was performed at the species level. Among the microbial species identified from 16S rRNA gene sequences in the microbial database, *Akkermansia muciniphila*, *Ruminococcaceae*, and *Acetatifactor muris* increase in very old mice (Ke et al., 2021). Particularly, *A. muciniphila* is known to correlate with the thinning of the mucous layer in the gut epithelium with aging (van der Lugt et al., 2019), but LNX consumption didn't provide protection in very old mice (Fig. 7). However, interestingly, *Acetatifactor muris* exhibited remarkable decreasing in very old aging and recovering to levels similar to young mice after LNX intake. *Turicibacter sanguinis* has recently been identified as beneficial gut microorganism for fat metabolism in aging mice (Lynch et al., 2023). *Turicibacter sanguinis* also showed an increasing trend with aging, matching previous findings, and LNX consumption provided clues for rejuvenation. Moreover, LNX increased *Blautia*, which is consistent with previous findings (Zhao et al., 2022). LNX increased the abundance of *Bifidobacterium* in aged mice. While *Bifidobacterium* is known to decrease with aging, it has been reported to be highly prevalent in centenarians, suggesting a potential link to longevity (Ku et al., 2024). In conclusion, it is crucial to emphasize that the four gut microbial species (*T. sanguinis*, *A. muris*, *Blautia*, and *Bifidobacterium*) were modulated in a manner consistent with previously reported healthy aging profiles. Rather than fully rejuvenating or restoring a youthful microbiota, LNX may contribute to partial modulation of specific taxa linked to healthier aging trajectories. Further studies are needed to validate the functional roles of these taxa, and to explore their potential in microbiota-targeted interventions. In addition, although this study focused on three major components of LNX, a full quantitative analysis of its phytochemical composition remains incomplete. Further profiling using analytical techniques such as LC-MS/MS or GC–MS is underway and will be addressed in future studies.

5. Conclusions

This study demonstrates that LNX influences gut microbiota composition in aged mice, potentially contributing to weight loss and metabolic regulation. Our findings revealed that LNX consumption led to a notable reduction in body weight in aged mice without a clear correlation with food intake, suggesting additional metabolic influences. At the phylum level, the *Firmicutes-Bacteroidota* ratio, typically associated with aging and obesity, decreased with aging but showed a slight increase following LNX consumption, albeit not statistically significant. Microbial diversity analyses revealed that aging leads to a reduction in species richness and evenness, and while LNX did not restore overall diversity. Beta-diversity analysis indicated distinct clustering between young and aged mice, with LNX-fed aged mice showing specific microbial changes. Notably, *Turicibacter sanguinis*, *Acetatifactor muris*, *Blautia*, and *Bifidobacterium* were altered in a manner consistent with previously reported healthy aging profiles. Rather than restoring a youthful microbiota, these results suggest that LNX may promote partial modulation of specific microbial taxa associated with age-related gut changes. Future studies should explore the functional roles of these taxa and the long-term effects of LNX.

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CRediT authorship contribution statement

Ji-Seon Ahn: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Jae Uk Kim:** Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation. **Je-Ho Lee:** Validation, Project administration, Investigation. **Gyung-Tae Ban:** Validation, Investigation. **Yoosik Yoon:** Supervision, Methodology. **Hwa-Seung Yoo:** Supervision, Resources, Conceptualization. **Jong-Soon Choi:** Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Hea-Jong Chung:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hea-Jong Chung reports financial support was provided by Korea Basic Science Institute. Jong-Soon Choi reports financial support was provided by Korea Basic Science Institute. Hea-Jong Chung reports financial support was provided by National Research Foundation of Korea. Je-Ho Lee reports a relationship with Geron Biotech Ltd. that includes: employment and non-financial support. Gyung-Tae Ban reports a relationship with Geron Biotech Ltd. that includes: employment and non-financial support. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2025.106966.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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