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Canine-derived *Weissella confusa* ZJUIDS-D034 and *Enterococcus faecalis* ZJUIDS-D016 combat aging by regulating gut microbiota

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ABSTRACT

Old age raises the susceptibility of age-related disease in domestic dogs. Discovering effective anti-aging interventions is key for mitigating age-related disease and conserving "healthspan" in pet dogs. In this study, 2 bacterial strains were isolated from canine feces. After screening and identifying the strains, *Weissella confusa* ZJUIDS-D034 and *Enterococcus faecalis* ZJUIDS-D016 were chosen to intervene during D-galactose-induced senescence in mice. We found that administering *Weissella confusa* ZJUIDS-D034 and *Enterococcus faecalis* ZJUIDS-D016 improved the aging phenotype of mice, including an increase in antioxidant activity, a decrease in pro-inflammatory cytokines, and the restoration of intestinal and liver tissue damage. In addition, *Weissella confusa* ZJUIDS-D034 and *Enterococcus faecalis* ZJUIDS-D016 lead to changes in the structure of intestinal microbiota in aging mice. Specifically, there was a decrease in the abundance of the *Cyanobacteria* and an increase in the abundance of *Akkermansia* and *Lactobacillus*. More importantly, there was a significant increase in acetic acid, a short-chain fatty acid, due to intervention with the 2 strains. This increase might be attributed to higher *Akkermansia*. We show that the modulation of gut microbiota and metabolism in aging mice may be a promising strategy through which *Weissella confusa* ZJUIDS-D034 and *Enterococcus faecalis* ZJUIDS-D016 might exert their anti-aging effects.

Introduction

With the transition of dogs from "property" to "family" more people have started raising pets, with pet dogs accounting for more than half of all pet ownership. According to an analysis by Packaged Facts, the proportion of American households owning pets and containing senior dogs aged seven years or above increased from 41.60 % to 53.50 % during 2012–2022. With advances in veterinary care, the lifespan of pet dogs is increasing, leading to a rise in aging-related industries within the pet sector.

Aging is a fundamental biological process, which involves a widespread and gradual decline in organ function. This leads to a lower capacity for adaptive regulation (De la Fuente and Miquel 2009). During aging in dogs, they become susceptible to age-related chronic diseases, such as obstetric complications, liver and kidney dysfunction, heart ailments, and cognitive impairment syndrome (Wang et al., 2023a). These disorders not only cause physical pain, but also emotional and economic pressure on their owners. Thus, in order to prevent age-related disease and maintain the healthspan in dogs, it is necessary to find effective anti-aging interventions (McKenzie et al. 2022).

As important aging characteristics we can define: (1) the degree of characteristics will change with the aging process; (2) promoting or strengthening this characteristic will accelerate aging or promote the occurrence of aging-related disease; (3) weakening or eliminating this characteristic can reverse or alleviate aging and related diseases. Each of these characteristics could be considered an entry point for anti-aging research (López-Otín et al. 2023). Many studies have shown that oxidative stress, chronic inflammation and gut microbial dysbiosis accompany aging. Marquez-Exposito et al. found that oxidative stress in mice increases with age. This was related to inactivation of the NRF2 antioxidant pathway in mice aged 4 months, 8 months, and 12 months (Marquez-Exposito et al. 2022). Wan.X et al. used green microalgalactose proteinosaccharides to mitigate oxidative stress in *C. elegans* and found that reactive oxygen species (ROS) content in *C. elegans* was

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decreased by 62.50 %, and malondialdehyde (MDA) content was decreased by 46.00 %, but superoxide dismutase (SOD) was increased by 27.50 %, and life of *C. elegans* was extended (Wan et al. 2021). These results suggested that enhancing the antioxidant capacity may be a viable approach to slow down aging. Systemic inflammation levels increase with age and chronic inflammation is a hallmark of aging (Thevaranjan et al. 2017). The activation of NF-κB (nuclear factor kappa-B) drives the inflammatory response and promotes the expression of pro-inflammatory factors and chemokines, which form part of the senescence-associated secretory phenotype (SASP) factors (Osorio et al. 2012; Zhu et al. 2023). Lei Zhang et al. discovered that by inhibiting NF-κB activation with a small molecule (SR12343), senescence markers were decreased and muscle fiber size was increased in WT mice aged 24 months (Zhang et al. 2021). Thus, influencing the level of organismal inflammation can potentially mitigate the aging process. Besides, the gut microbiome shows a correlation with age (Ghosh et al. 2022). As individuals age, there are increases and decreases in the abundance of *Enterobacteriaceae, Mycobacteriaceae*, and beneficial bacteria within the gut microbiome (Chen et al. 2023). The ratio of *Firmicutes/Bacteroidota* is also higher in the elderly compared to the young and centenarians (Wang et al. 2022). Han et al. utilized the model of *C. elegans* to knock out a single gene in *Escherichia coli*. They discovered that 29 mutants of *E. coli* significantly increased the lifespan of the host by 10.00 % to 40.00 % (Han et al. 2018) and that altering the gut microbiome can modulate the host's lifespan.

Probiotics are microorganisms that have beneficial effects on the host when consumed in moderation. Studies have shown that they can enhance the organism's antioxidant capacity (Paulino do Nascimento et al., 2022; Ho et al. 2019), reduce inflammation levels (Han et al. 2020), and regenerate gut microorganism of old organisms (Zhao et al. 2018), demonstrating their possible anti-aging properties. Probiotic preparations for dogs are rapidly advancing, but are mainly used for treatment against bacterial diarrhea (Wang et al., 2023a), treatment against inflammatory bowel disease (IBD) (Jang et al. 2021), and against acute intestinal disease (Schmitz et al., 2015). These probiotics are primarily derived from humans, pigs, and sheep, with fewer originating from dogs. Few studies have been conducted on canine anti-aging benefits from probiotics obtained from dogs. Developing anti-aging probiotics for dogs is crucial for promoting healthy aging in canines.

Previously, the probiotic strains *Enterococcus faecalis* ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034, which were derived from healthy canine feces, were isolated and screened. These strains were found to exhibit exceptional *in vitro* antioxidant effects (DPPH (2,2-Diphenyl-1-picrylhydrazyl), a hydroxyl radical scavenging effect, and a reducing effect on top of anti-retroviral properties (bile salt and acid tolerance). The anti-aging effect of 2 strains in a mouse model of *D-galactose*-induced aging and the effects on the gut microflora were explored in this study. Our findings may serve as the theoretical basis showing the repertoire of strains for developing 'anti-aging' probiotics derived from canines.

Materials and methods

Bacterial strains and culturing

Enterococcus faecalis ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034 were obtained from the feces of healthy dogs. Both strains were deposited in the General Microbiology Center of China Microbial Species Depository Administration Committee (CMSC), and the depository numbers of ZJUIDSD034 and ZJUIDS-D016 are CGMCC 27039 and CGMCC 27038, respectively; the Illumina sequencing data of the two strains have been uploaded to the NCBI Sequence Read Archive database, and the accession numbers of ZJUIDS-D016 and ZJUIDS-D034 are SRR29428242 and SRR29428477, respectively. The cultures were streaked onto MRS solid medium and grown for three generations. Single colonies were picked and inoculated into sterile MRS liquid medium, followed by incubation at 37 °C for 24 h. Afterwards, the bacteria were centrifuged at 4 °C for 10 min and 8000 rpm. The supernatant was discarded, and the microorganisms were rinsed with sterile phosphate-buffered saline (PBS, pH=7.2–7.4). The experiment was repeated twice, and the microorganisms were subsequently resuspended in sterile PBS to reach a concentration of 10^9 CFU/mL.

Animal experimental design

32 male Kunming mice (20 ~ 25 g) were purchased from Shanghai Sleekon Laboratory Animal Co Ltd [License No SCXK (Hu) 2022–0004]. After one week acclimatization, mice were randomly assigned to four groups (n = 8), including the NC, model, ZJUIDS-D016, and ZJUIDS-D034 group. From day 1 to day 42, the other three groups, apart from the NC group, were subjected to subcutaneous injections into the neck, with a daily dose of 1250 mg/(kg·d) of D-galactose. The D-galactose solution used had a concentration of 0.125 g/mL, and the volume of injection per mouse was 0.01 mL/g. The NC group was given a daily injection of 0.01 mL/g saline. From days 43 to 84, subcutaneous injections were given concomitantly with daily gavage of *Enterococcus faecalis* ZJUIDS-D06 and *Weissella confusa* ZJUIDS-D034 (10^9 CFU/mL) to the ZJUIDS-D016 and ZJUIDS-D034 groups. The NC and model groups were administered PBS by daily gavage of 0.01 mL/g.

Determination of hepatic antioxidant indices

Mouse liver tissues were homogenized and the levels of protein carbonyls, MDA, glutathione (GSH), and SOD contents were measured, following the instructions of the assay kit. The MDA assay kit was purchased from Beyotime, the SOD, GSH, protein carbonyl assay kit was purchased from Solarbio, Shenzhen, China.

Serum assay for inflammatory factor levels

Mouse blood samples were centrifuged at 4 °C and 3000 rpm for 10 min to obtain serum. IgG, IL-1β, IL-6, and TNF-α levels from the serum of each group were determined using the instructions provided by the kit which was purchased from Shanghai Fankel Industries Co.

Tissue hematoxylin-eosin staining (H&E)

Mouse liver and duodenum tissues were fixed with a 4 % solution of paraformaldehyde, subsequently dehydrated, embedded, and sectioned in preparation for hematoxylin-eosin staining.

Immunofluorescence staining of duodenum

Deparaffinization was performed on paraffin-embedded duodenal sections, which were then placed in repair cassettes containing 20 × TRIS-EDTA repair solution (pH 8.0) for antigen repair. Sections were incubated with 3 % hydrogen peroxide for 25 min at room temperature. They were then closed using PBS containing 3 % BSA. Samples were treated with primary antibodies against ZO-1 (1:800) and Occludin (1:500) at 4 °C (Proteintech Group, Co. Ltd., China), washed with PBS, and subsequently incubated with HRP-ultrasensitive goat-anti-rabbit secondary antibody (Haoke Biotech Co. Ltd. China) for 50 min at room temperature in darkness. The appropriate color amplification reagent was added in drops over 3–5 min. The aforementioned steps were repeated and subsequently the secondary and tertiary antibodies labeled with ZO-1 (red) and Occludin (green) were added. Finally, following restaining with DAPI, the samples and images were observed utilizing an inverted fluorescence microscope (Nikon Eclipse C1, Japan).

Western blot

Proteins extracted from ileum tissue of the duodenum were quantified utilizing a BCA protein assay kit (Yeasen) after RIPA lysis buffer

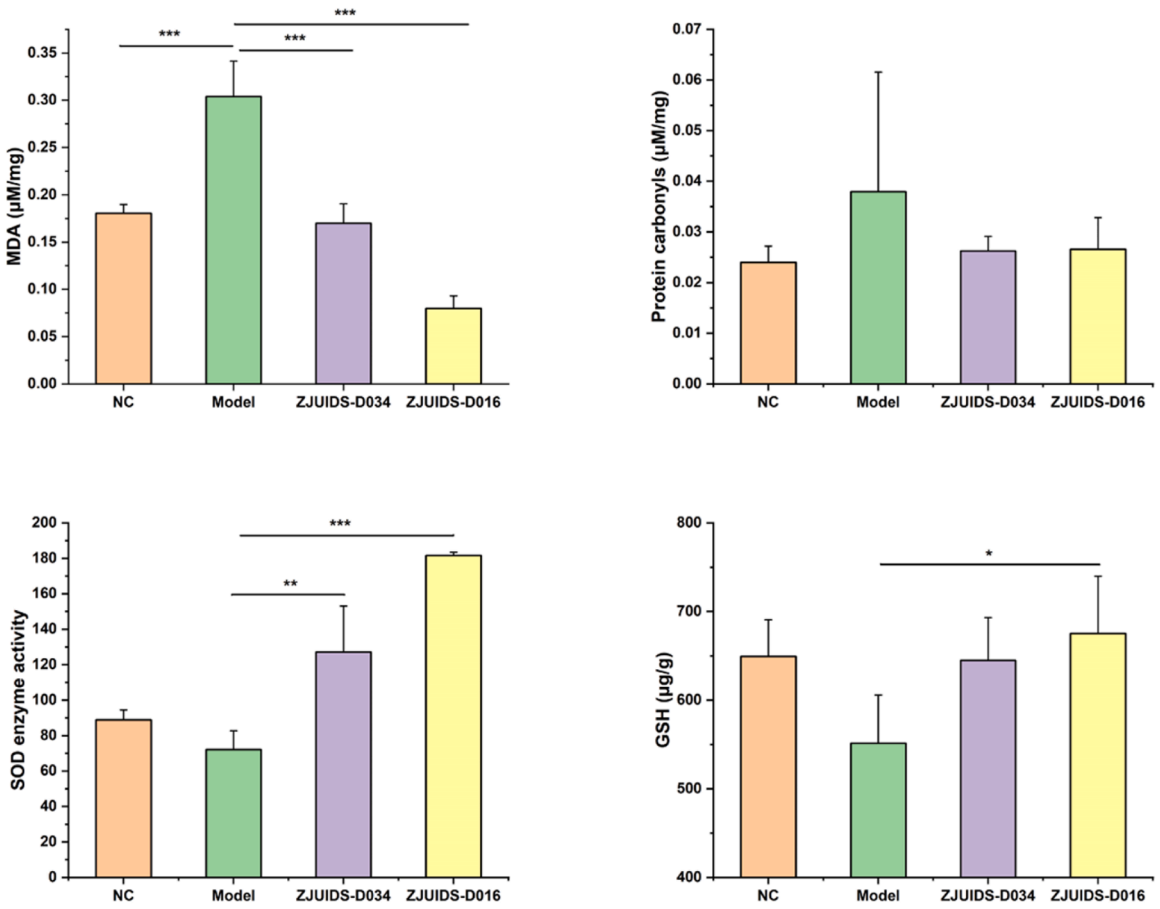

Fig. 1. Effects of ZJUIDS-D016 and ZJUIDS-D034 on liver antioxidant levels in aging mice. MDA, protein carbonyl, GSH, and SOD levels in the serum. All data are expressed as means ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(Beyotime) and containing phosphatase inhibitor (Sigma). GAPDH antibody was utilized as control. Antibody details were provided as follows: Occludin (1:5000, Proteintech,China); ZO-1 (1:5000, Proteintech, China); GAPDH (1:10000, Abcam,UK).

16S rRNA gene sequencing

Microbial DNA was extracted from the contents of mouse colons utilizing the FastLee Fecal DNA kit (Hangzhou Legenomics Bio-Pharm Technology Co, Ltd, China). After amplification of the 16S rDNA V3-V4 region with the use of universal primers 341F (CCTAYGGGRBG-CASCAG) and 806R (GGACTACHVGGGTWTCTAAT) from qualified DNA samples, sequencing was performed using the Illumina Novaseq platform (Beijing Novozymes Head Co., Ltd.).

Quantification of SCFAs (short-chain fatty acids)

Appendix contents were diluted fourfold with ultra-pure water and vortexed for five minutes. The suspension was then allowed to rest for another five minutes and then centrifuged at 4 °C, 10,000 rpm for 10 min. One milliliter of the supernatant was combined with 20 μL of chromatography-grade phosphoric acid (from Shanghai Aladdin Biochemical Technology Co., Ltd., China) and then injected into a meteorological vial for gas chromatography (GC) through a 0.45 μm membrane filter.

Statistical analysis

Statistical analyses were performed using SPSS 23.0. Data are expressed as means ± standard deviation (SD). Results were analyzed using a one-way analysis of variance (ANOVA). In all tests, p values of $<$ 0.05 were considered statistically significant.

Results

Effects of ZJUIDS-D016 and ZJUIDS-D034 on liver antioxidant levels in aging mice

Exposed to D-galactose treatment, mice in the experimental group showed increased levels of oxidative stress: a significant increase in MDA levels, $p < 0.001$ and an increase in protein carbonyls levels and decreased antioxidant capacity (decreased GSH and SOD levels). Treatment with *Enterococcus faecalis* ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034 resulted in the reversal of MDA, protein carbonyl, GSH and SOD levels in mice. Besides, the antioxidant effect of ZJUIDS-D016 was significantly better than that of ZJUIDS-D034. ZJUIDS-D016 had a significant effect on up-regulating GSH levels ($p < 0.05$), while ZJUIDS-D034 had no significant effect. (Fig.1).

Effects of ZJUIDS-D016 and ZJUIDS-D034 on serum inflammatory levels in aging mice

Compared with the NC group, the inflammation level of mice in the experimental group was significantly increased ($p < 0.001$). The levels of IL-6, IL-1β and TNF-α in aging mice were significantly reduced after treatment with *Enterococcus faecalis* ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034. In addition, the immunity of aging mice was significantly improved (increase in IgG levels, $p < 0.001$) and the anti-inflammatory

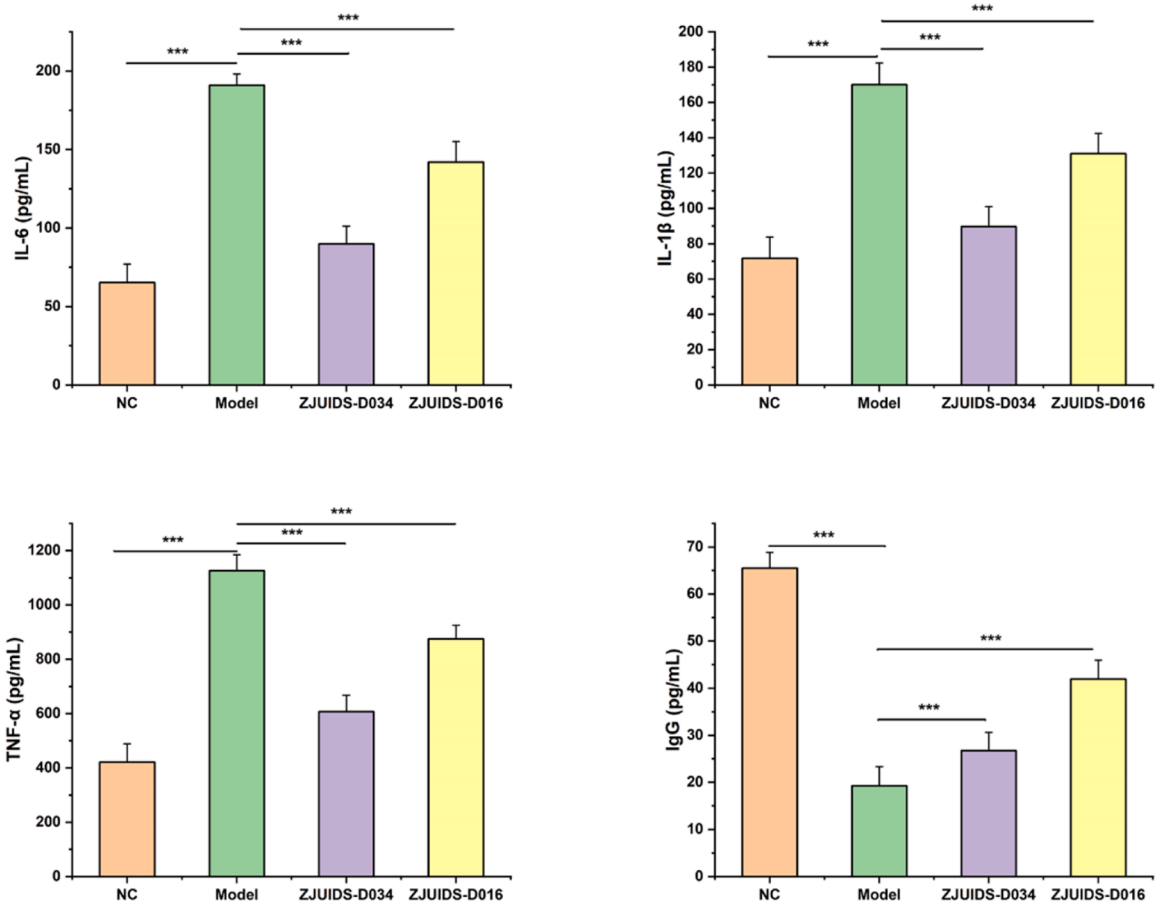

Fig. 2. Effects of ZJUIDS-D016 and ZJUIDS-D034 on serum inflammatory levels in aging mice. IL-6, IL-1β, TNF-α and IgG levels in serum, All data are expressed as means ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

effect caused by ZJUIDS-D034 was better than that of ZJUIDS-D016 (Fig.2).

Histomorphologic changes in liver and duodenum

Mouse liver tissues in the experimental group (Fig.3A) showed extensive hepatocyte steatosis with small round vacuoles in the cytoplasm and a small number of irregularly arranged hepatocyte cords, but only minor hepatocyte steatosis was seen in liver tissues of mice treated with *Enterococcus faecalis* ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034.

In the NC group, duodenal intestinal villi were longer, and mucosal epithelial cells at the tip of intestinal villi extended towards the intestinal lumen with irregular morphology; in the experimental group, the duodenum appeared damaged, intestinal villi became shorter and incomplete, and the local crypts appeared distorted. After gavage of *Enterococcus faecalis* ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034, duodenal morphology of mice improved, intestinal villi became longer, and the structure of the crypts partially recovered (Fig.3B).

Effects of ZJUIDS-D016 and ZJUIDS-D034 on the expression of intestinal barrier tight junction proteins ZO-1 and Occludin in aging mice

The expression of the intestinal barrier integrity related proteins ZO-1 and Occludin was decreased in the experimental group. WB results from the duodenum samples also confirmed this. After gavage of *Enterococcus faecalis* ZJUIDS-D016 and *Weissella confusa* ZJUIDS-D034, the expression of these 2 proteins was significantly increased in the duodenum of mice (Fig.4).

Changes in gut microbiota of aging experimental mice

To analyze species composition of mouse gut microbiota within each group, we clustered valid sequences from all samples into Operational Taxonomic Units (OTUs) with 97 % accordance. We found, illustrated in the Wayne diagram, 330 unique characteristic sequences in the NC group, 314 in the experimental group, 252 in the ZJUIDS-D016 group, and 269 in the ZJUIDS-D034 group. ZJUIDS-D034 and NC groups share 667 characteristic sequences and have greater similarity in gut microbiota of mice within the ZJUIDS-D034 group and the NC group (Fig.5a).

The α-diversity of gut microorganisms in all four groups of mice was assessed using the chao1, shannon, and simpson indices. Mice within the aging experimental group showed decreased α-diversity across all three indices. However, treatments with ZJUIDS-D016 and ZJUIDS-D034 ameliorated this reduction and increased richness and diversity of intestinal microbiota in aged mice (Fig.5b~Fig.5d).

β-diversity of gut microbiota from the groups was analyzed by UPGMA cluster analysis. The clustering tree depicts that gut microbiota structure of mice in the NC group was similar to that of the experimental group, while gut microbiota structure in the ZJUIDS-D034 group resembled that of the ZJUIDS-D016 group. At the same time, it is obvious that the samples of the experimental group were more scattered, indicating that aging caused changes in the structure of gut microbiota of mice, resulting in larger differences in the structure of gut microbiota of mice within the same group. The samples of the ZJUIDS-D016 group were more clustered, and the structure of gut microbiota of mice within the group resembled each other. This suggests that *Enterococcus faecalis* ZJUIDS-D016 had a regulatory effect on the structure of the gut microbiota of aging mice (Fig.5e~Fig.5f).

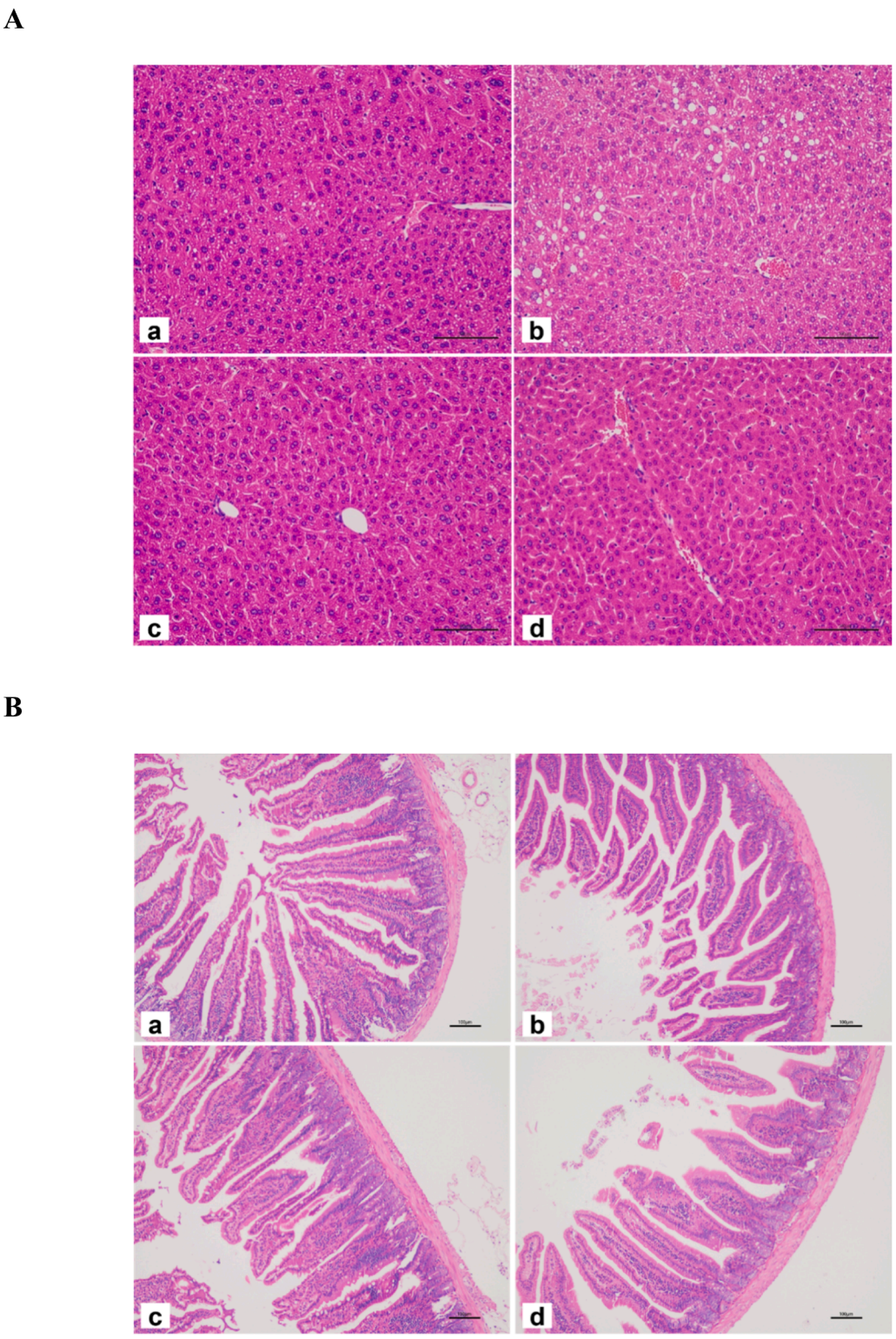

Fig. 3. HE staining of mouse liver and duodenum. A. HE staining of mouse liver; B. HE staining of mouse duodenum; a) Negative control group (NC); b) Aging experimental group (Model); c) ZJUIDS-D016 group; d) ZJUIDS-D034 group.

Fig. 6 displays the relative abundance of mouse gut microbiota at phylum level for each group. The dominant microbiota were still *Firmicutes, Bacteroidota, Verrucomicrobiota*, and *Actinobacteriota*, indicating that probiotic interventions did not affect the composition of the host's dominant microbial group. When further comparing the relative abundance changes of gut microbiota among different experimental groups, it was observed that in the aging experimental group, there was a decrease in the relative abundance of the *Firmicutes, Bacteroidota*, and *Actinobacteriota*, while an increase was noticed for *Verrucomicrobiota* and *Cyanobacteria*. Treatment with ZJUIDS-D016 and ZJUIDS-D034 was found to ameliorate these changes in the relative abundance of gut microbiota, bringing them closer to the negative control group. This indicates that these 2 strains of probiotics had a positive effect on modulating the structure of gut microbiota. However, ZJUIDS-D016 did

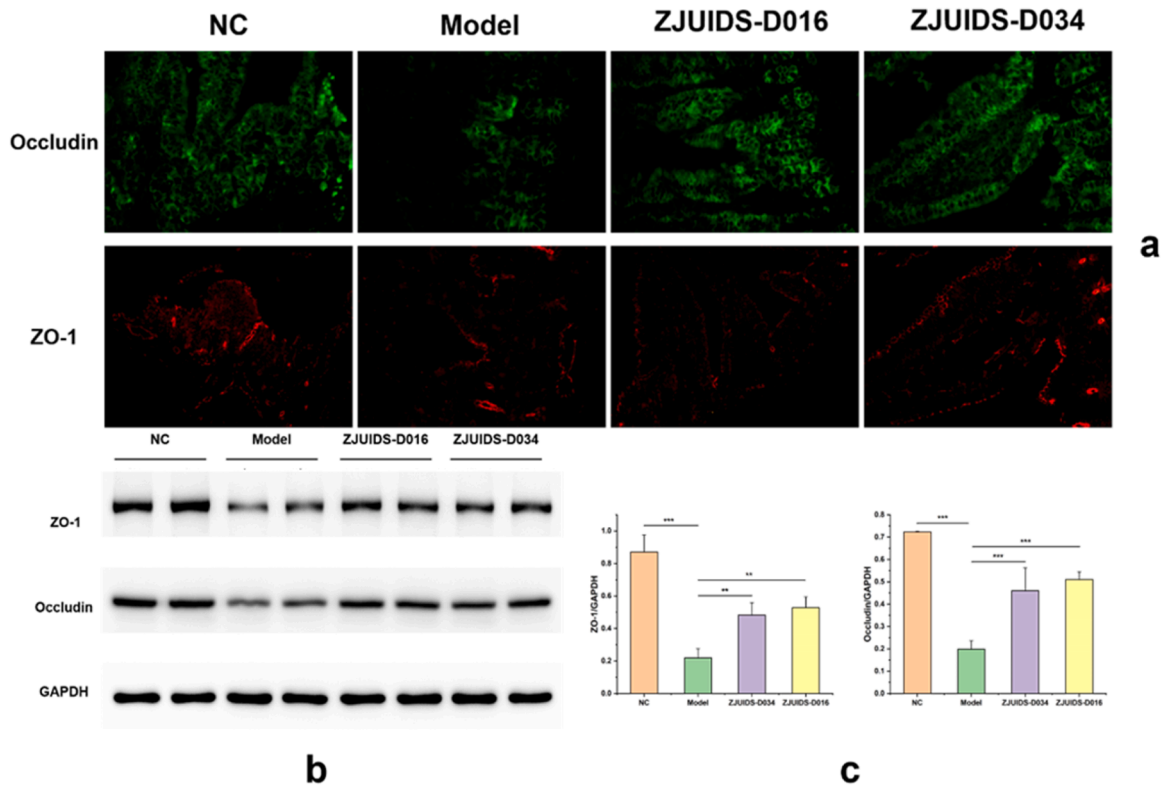

Fig. 4. Effects of ZJUIDS-D016 and ZJUIDS-D034 on the expression of intestinal barrier tight junction proteins ZO-1 and Occludin in aging experimental mice. a) Immunofluorescence staining of mouse duodenal tight junction protein; b) WB of mouse duodenal tight junction protein; c) Quantification of mouse duodenal tight junction protein. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

not exhibit an enhancing effect on relative abundance of *Actinobacteriota*.

Fig.7 shows that the relative abundance of *Akkermansia* and *Lactobacillus* decreased in the experimental group compared to the NC group. However, treatment with ZJUIDS-D016 and ZJUIDS-D034 resulted in an improvement in the relative abundance of *Akkermansia*. Following treatment with ZJUIDS-D034, there was a significant increase in the relative abundance of *Lactobacillus*, while ZJUIDS-D016 did not enhance the relative abundance of *Lactobacillus*.

Changes in short-chain fatty acids in mice

The experiment showed that the levels of acetic acid, propionic acid, butyric acid, and isovaleric acid in the colons of mice treated with D-galactose were significantly lower than those of mice in the NC group ($p < 0.01$). Additionally, the interventions of ZJUIDS-D016 and ZJUIDS-D034 resulted in elevated levels of major short-chain fatty acids, with the most significant increase observed in acetic acid ($p < 0.05$) (Fig.8).

Correlation analysis of anti-aging indicators with gut microorganism in aging mice

Fig.9 shows the results of Spearman's correlation analysis of physiological and biochemical indices of antioxidant and anti-inflammatory capacity of the 2 probiotic strains with gut microbiota. The effects of ZJUIDS-D016 and ZJUIDS-D034 on the α-diversity of gut microbiota were found to correlate significantly with antioxidant and anti-inflammatory indices in aging individuals (Fig.9a~Fig.9b). Specifically, the α-diversity index of gut microbiota of aging mice was found to correlate positively with antioxidant levels and negatively with inflammation in the aging mice. The α-diversity index of gut microbiota in aging mice correlated positively with antioxidant levels and negatively with inflammation levels in aging mice. In particular, the Chao1 index and Simpson index correlated significantly negative with MDA levels and significantly positive with SOD levels in aging mice. Conversely, Shannon's index correlated significantly positive with GSH and significantly negative with IL-6 (Fig.9a~Fig9b).

Specifically, the relative abundance of *Lactobacillus* in the ZJUIDS-D034 group correlated significantly negatively with the levels of inflammatory factors (IL-1β, IL-6, and TNF-α) and GSH levels. Furthermore, it was observed to be significantly positively correlated with the expression of the tight junction proteins ZO-1 and Occludin, and also significantly positively correlated with the levels of acetic acid in aging mice (Fig.9c); The relative abundance of *Akkermannsia* in the ZJUIDS-D016 group was found to be significantly negatively correlated with the levels of inflammatory factors (IL-1β, TNF-α) and GSH in aging mice. Additionally, a positive correlation, although not statistically significant, was observed between the relative abundance of *Akkermannsia* and the expression of the tight junction proteins ZO-1 and Occludin, as well as with the content of acetic acid (Fig.9d).

Discussion

Understanding the developmental patterns and mechanisms of aging is essential for developing interventions that can prolong lifespan and improve healthspan (Okoro et al. 2021). Aging is a complex process that involves the body's tolerance to endogenous stress, including oxidative stress and chronic inflammation. Effective anti-aging approaches involve prolonging the body's tolerance to endogenous stress and reducing the occurrence of aging-related diseases.

Oxidative stress is the result of excessive production or impaired clearance of ROS, which can cause oxidative damage to cells. This process is involved in the development of many age-related diseases. Chronic inflammation is the persistence of pro-inflammatory factors that damage cells and tissues. Its accumulation drives cellular and organismal aging, which is recognized as the leading cause of death in the

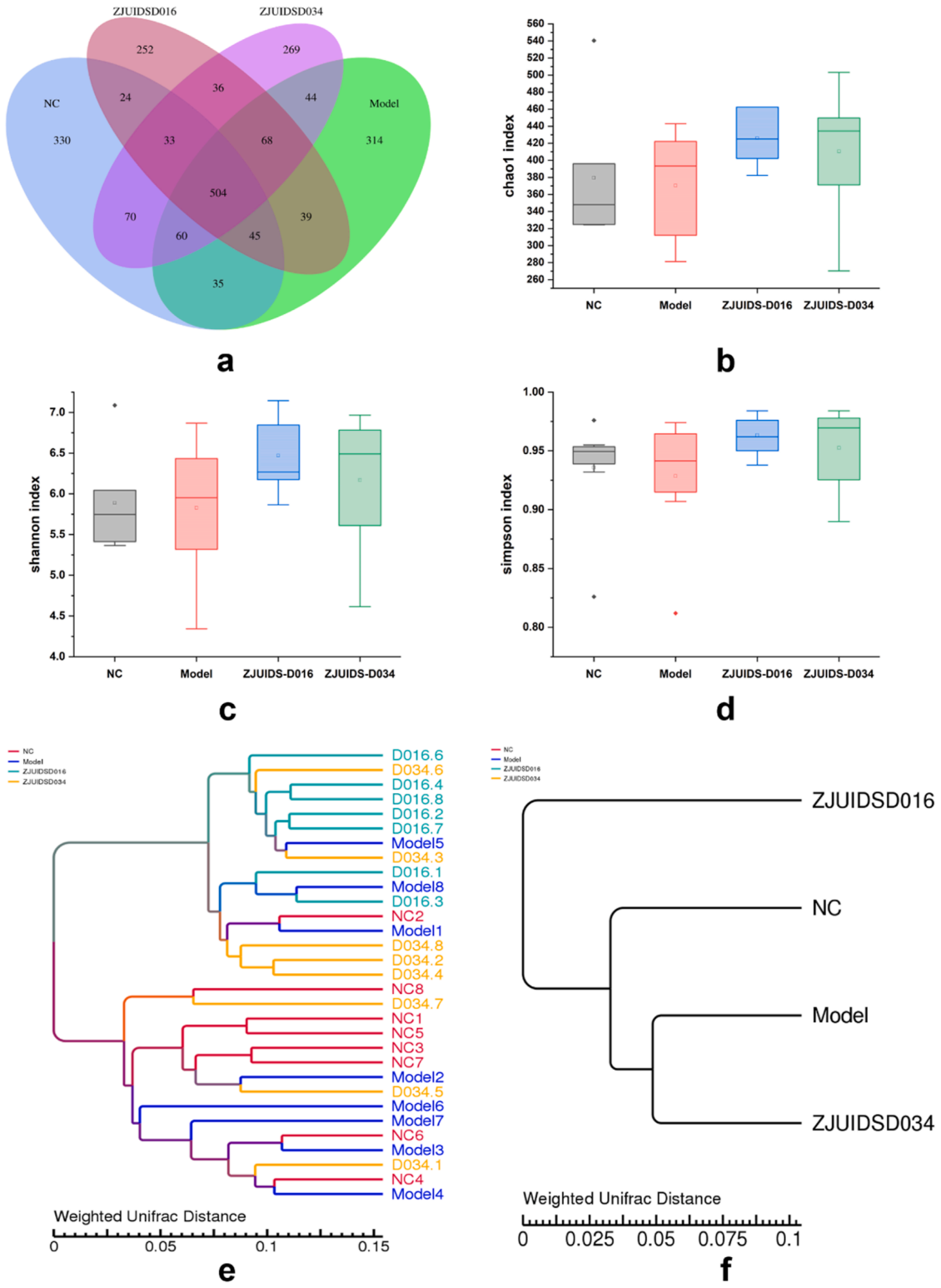

Fig. 5. Effects of ZJUIDS-D016 and ZJUIDS-D034 on gut microbial diversity in aging mice. a) OTUs Cluster Analysis Wayne Diagrams; b) Chao1 index; c) Shannon index; d) Simpson index; e)&f) UPGMA clustering tree of mouse gut microbiota portal levels.

world today (Furman et al. 2019). At the same time, these 2 endogenous stressors have to be differentiated. The Oxidation-Inflammation Theory of Aging (OITA), proposed by De la Fuente and Miquel (De la Fuente and Miquel 2009), links oxidative stress and inflammation. The hypothesis suggests that oxidation and inflammation are closely linked, and that excessive or uncontrolled free radical production induces an inflammatory response. Interventions improving both or one of them can be beneficial for improving health and longevity.

Our study aimed at evaluating the *in vivo* antioxidant and anti-inflammatory effects of 2 probiotic strains using the D-galactose-induced aging mouse model. That experimental model was used to assess the potential of the probiotics to resist aging. D-galactose injection in mice accelerates aging and oxidant production, accumulation of oxidative damage, changes in antioxidant enzyme activities,

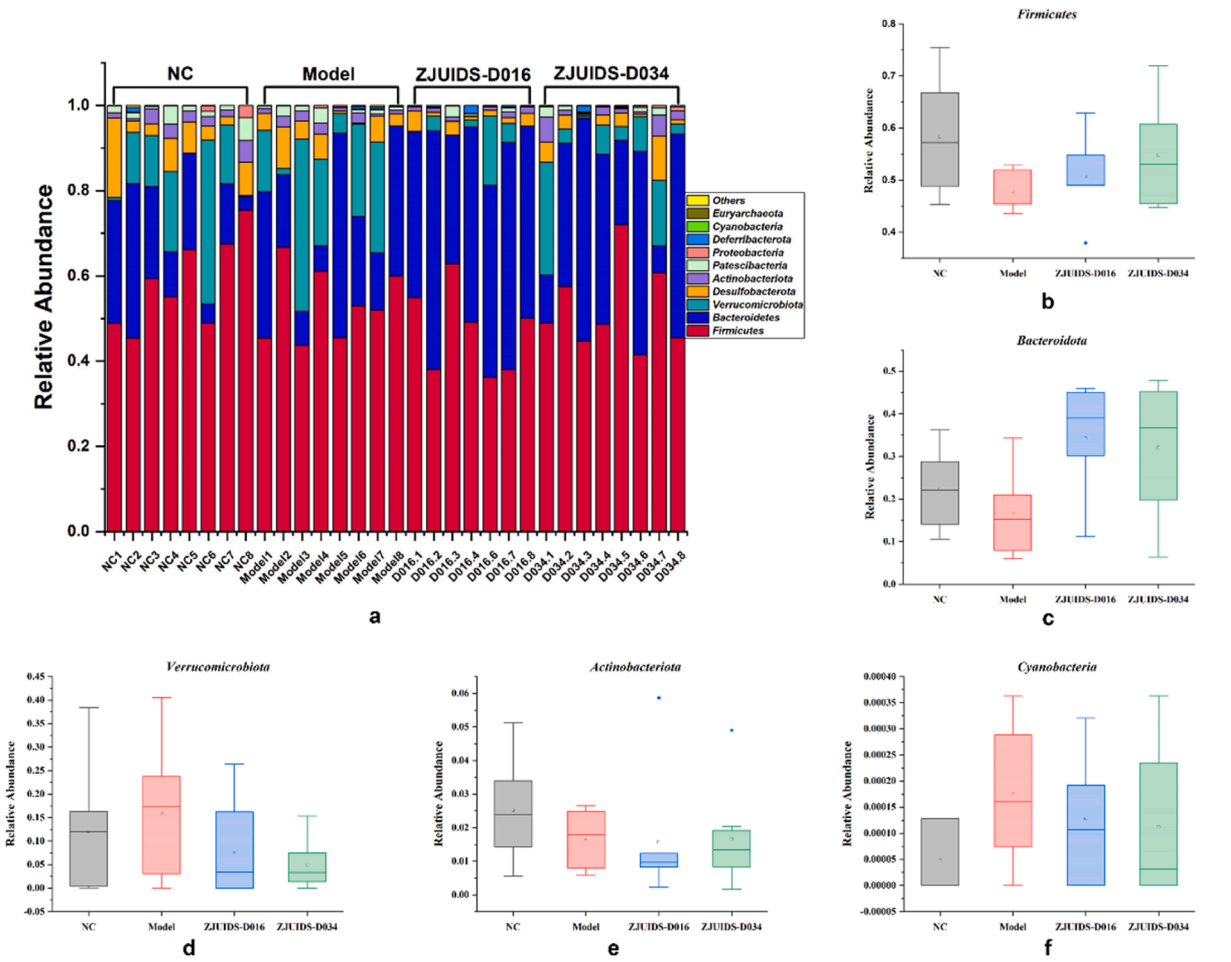

Fig. 6. Changes in relative abundance of species of gut microorganisms in aging mice by ZJUIDS-D016 and ZJUIDS-D034 (phylum level) a) Relative abundance of species at bar graph phylum level; b) Change in relative abundance of *Firmicutes*; c) Change in relative abundance of *Bacteroidota*; d) Change in relative abundance of *Verrucomicrobiota*; e) Change in relative abundance of *Actinobacteriota*; f) Change in relative abundance of *Cyanobacteria*.

inflammation, and tissue damage (Chelliah et al. 2021).

Reduced GSH is a non-enzymatic antioxidant in mammals. It acts directly to protect cells from free radicals and pro-oxidants and serves as a cofactor for antioxidant and detoxifying enzymes (Averill-Bates, 2022). SOD is involved in an antioxidant non-enzymatic defense mechanism. It is the only known enzyme that can directly scavenge free radicals by breaking down superoxide into oxygen and hydrogen peroxide with high specificity and efficiency (Borgstahl and Oberley-Deegan 2018). The levels of GSH and SOD can be used to assess the organism's antioxidant defense level. Higher levels of both enzymes indicate a higher level of antioxidant defense. MDA is a by-product of lipid peroxidation. Oxidative stress can increase lipid peroxidation in the body (Busch and Binder 2017). The level of oxidative stress in the body can be assessed by detecting MDA content. Protein carbonyl refers to the amino or imino group in the amino acid side chain of proteins that has been transformed by free radical attack. Protein glycosylation can reduce or eliminate the function of the original protein. The content of protein carbonyl can be used to characterize the strength of oxidative damage to proteins, and the level of protein carbonyl can serve as a marker of oxidative stress (Song et al. 2020). The levels of MDA and protein carbonyls can be used to assess the level of oxidative stress in the body. In this experiment, in terms of oxidative stress levels, D-galactose treatment resulted in increased oxidative stress levels (significant increase in MDA content, $p < 0.001$; increase in protein carbonyls) and decreased endogenous antioxidants (decrease in SOD and GSH content) in the mice of the experimental model group but this effect was partly combated by the strains ZJUIDS-D016 and ZJUIDS-D034.

An imbalance between pro- and anti-inflammatory cytokines in the body remains the main cause of dysregulated inflammation levels. This ultimately leads to an increase in the pro-inflammatory response. The most common pro-inflammatory factors are TNF-α, IL-6, and IL-1β. In humans it has been shown that the levels of IL-6 and TNF-α tend to be higher in older people compared to younger people, and the levels of IL-1β are very low in healthy young adults (Pawelec et al. 2014). Testing the levels of TNF-α, IL-6, and IL-1β in individuals can quantify inflammation and aging. Inflammation typically is an immune response, but persistent inflammation can damage the immune system, leading to a decline in the body's ability to fight disease. Assessing the IgG levels in the body can determine the strength of the immune system. In this experiment, serum levels of IL-6, IL-1β, and TNF-α in the D-galactose induced experimental aging group were significantly higher than those in the NC group mice ($p < 0.001$), indicating a high level of inflammation in the aging mice. Administration of the strains ZJUIDS-D016 and ZJUIDS-D034 significantly improved inflammation. With respect to body immunity, IgG content in serum of mice in the D-galactose induced aging experimental group was significantly lower than that of mice in the NC group ($p < 0.001$), indicating a decrease in immunity in aging mice. With the strains ZJUIDS-D016 and ZJUIDS-D034, the IgG level in the serum of aging mice significantly increased ($p < 0.001$), which suggests an improvement in their immunity.

The intestine is a key organ in the body. The duodenum is primarily responsible for absorbing nutrients, electrolytes, and water. The

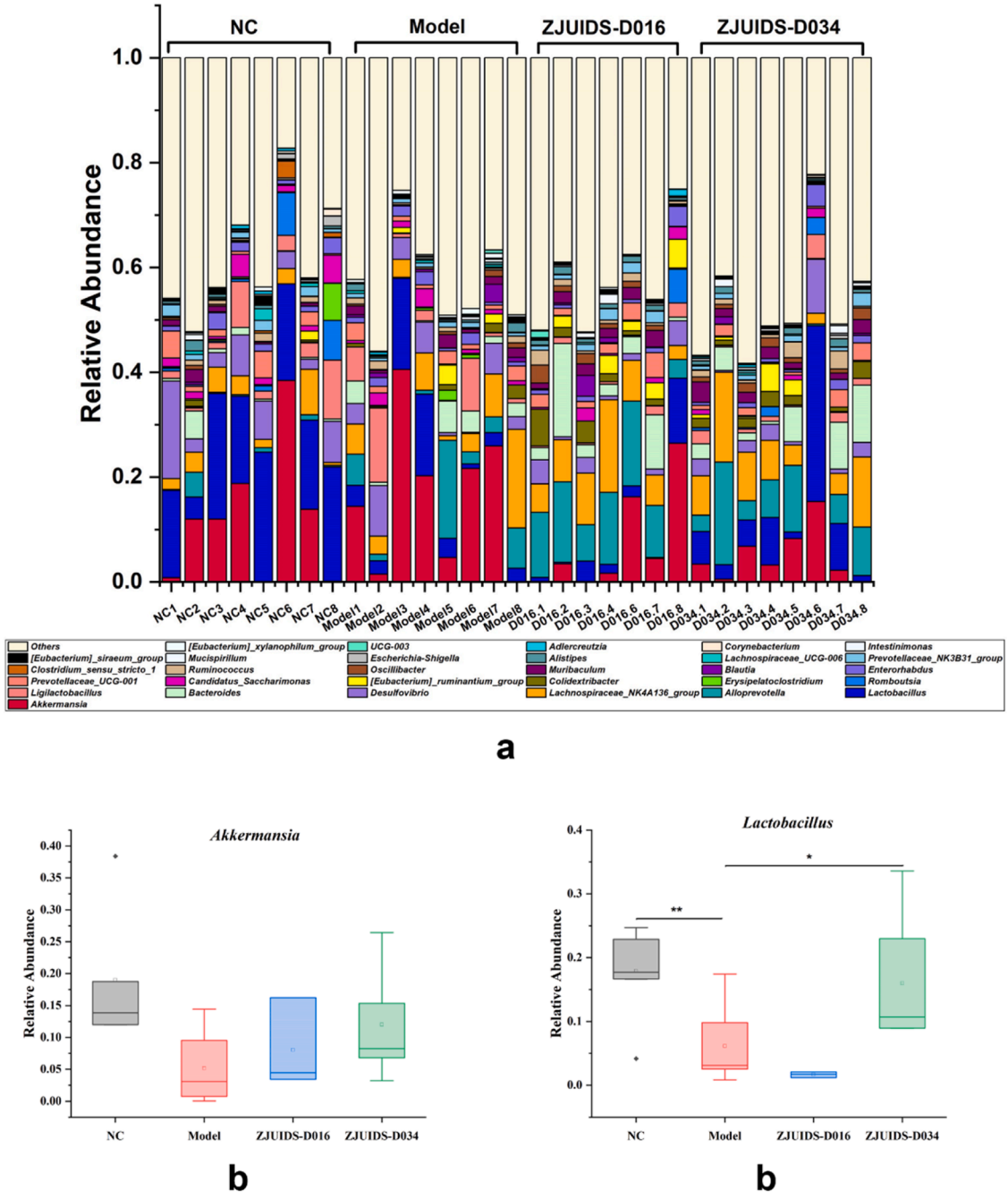

Fig. 7. Changes in relative abundance of species of gut microbiota in aging mice by ZJUIDS-D016 and ZJUIDS-D034 (Genus level). a) Species relative abundance bar chart genus level; b) Change in relative abundance of *Akkermansia*; c) Change in relative abundance of *Lactobacillus*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

intestinal barrier serves to protect the body from harmful compounds and microorganisms. When the intestinal barrier is compromised, harmful substances can enter the body due to increased intestinal permeability. The intestinal barrier function relies on tight junction proteins connecting adjacent intestinal epithelial cells and regulating the paracellular transport of substances through the lumen of the small and large intestine (Tran and Greenwood-Van Meerveld, 2013). Intestinal mucosal Occludin proteins improve adhesion between fibroblasts, and ZO-1 proteins, along with Occludin proteins and actin, they form a stable junction system that preserves the integrity of the intestinal barrier. This can impair nutrient absorption and in severe cases trigger a systemic inflammatory response in the body (Chen et al. 2017). Previous research has suggested that the integrity of the duodenal intestinal barrier is compromised during the aging process, leading to increased intestinal permeability. Restoring the integrity of the intestinal barrier may help to reduce inflammation. In our study, the intestinal barrier integrity of aging mice was damaged by D-galactose treatment, which was visible through decreased expression of the intestinal tight junction proteins ZO-1 and Occludin in the experimental group. However, after providing the probiotic strains ZJUIDS-D016 and ZJUIDS-D034, the expression of the 2 tight junction proteins increased, indicating repair of the damaged intestinal barrier.

Aging can lead to various age-related disorders, including liver disease (Georgieva et al. 2023). The liver is a vital metabolic organ performing several necessary functions, such as breaking down substances, metabolizing nutrients, filtering blood, and regulating hormones (Trefts et al. 2017). Aging is a significant contributor to the emergence of liver disease (Lozano et al. 2012). Duan et al. (Duan et al. 2023) demonstrated

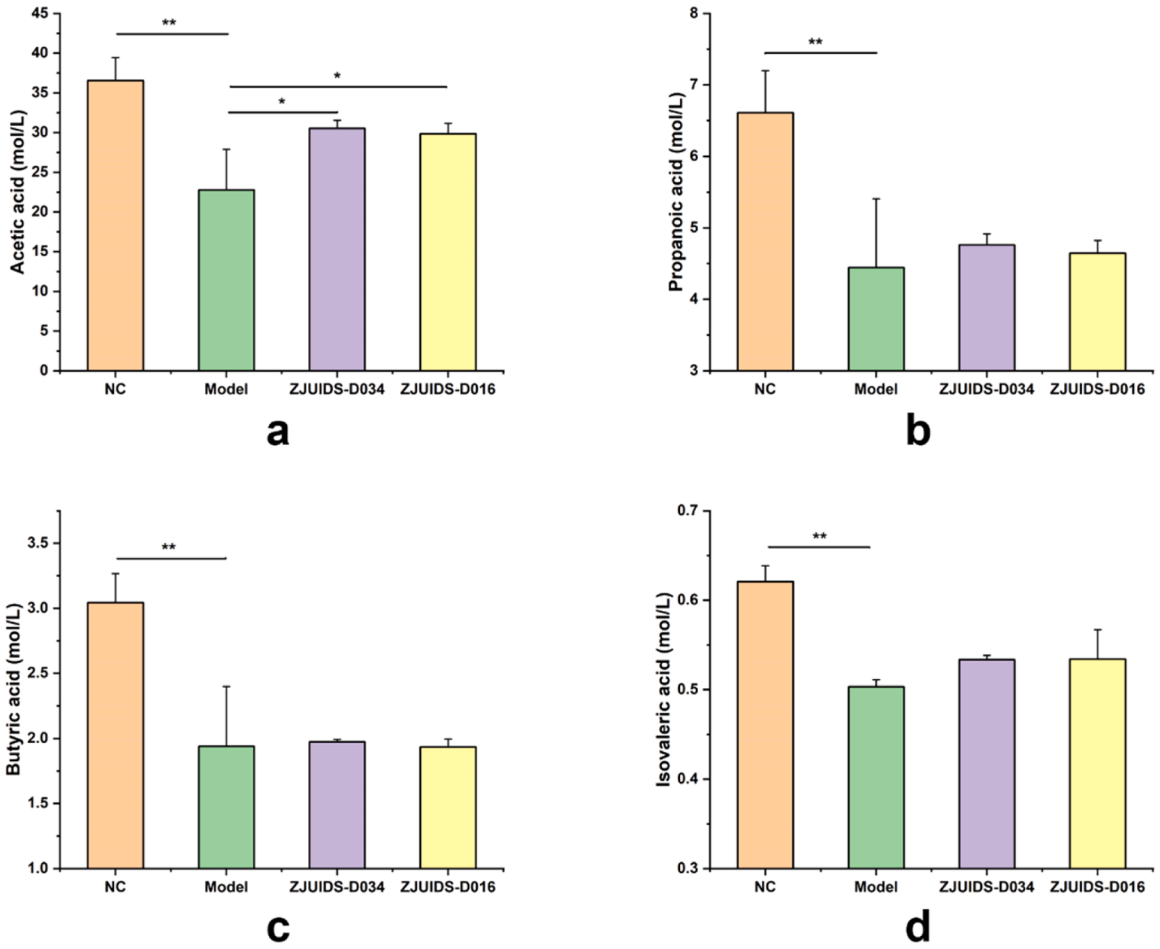

Fig. 8. Effects of ZJUIDS-D016 and ZJUIDS-D034 on short-chain fatty acid contents in colon tissue from aging mice. a) Acetic acid content; b) Propionic acid content; c) Butyric acid content; d) Isovaleric acid content. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

that aging hepatocytes and macrophages produce 13-HODE, which activates SREBP1 by directly inhibiting CAT activity. This activation gives rise to hepatic steatosis, leading to non-alcoholic fatty liver disease (NAFLD). Song et al. (Song et al. 2023) discovered that probiotic bacteria can reduce valproic acid (Depakene)-induced fatty liver degeneration and oxidative stress in mice. We found that ZJUIDS-D016 and ZJUIDS-D034 were effective in alleviating NAFLD in aging mice. The severity and poor prognosis of NAFLD are associated with aging (Puppala et al. 2023). The aging experimental group of mice showed increased hepatocellular steatosis in liver tissues after D-galactose treatment, but it was alleviated by ZJUIDS-D016 and ZJUIDS-D034. We conclude that both strains of probiotics had a positive effect on hepatic steatosis in aging mice.

Numerous studies have shown that dysbiosis of gut microbiota is associated with age-related chronic diseases. Thus, modulation of gut microbiota is expected to be an effective anti-aging intervention (Lim and Nam 2023). The administration of probiotics is a recognized method for modulating gut microbiota. Ai et al. gave *Lactobacillus plantarum* with oleic acid to mice and found that both alive and inactivated *Lactobacillus plantarum* reduced the proportions of *Bacteroidota* and *Firmicutes* in the gut. Additionally, relative abundance of *Staphylococcus* in the feces was reduced (Ai et al. 2024). Zeng et al. discovered that *Lactobacillus* can increase the relative abundance of *Firmicutes* and decrease the relative abundance of *Verrucomicrobiota* and *Actinobacteriota* in the feces of mice (Zeng et al., 2023a). Similarly, Du et al. found that *Bifidobacterium animalis* LKM512 could increase the abundance of beneficial bacteria in the intestines of zebrafish. The gastrointestinal tract certainly is the most densely colonized part of the body (Du et al., 2021). Short-chain fatty acids (SCFAs) are the main metabolites of fermentation by intestinal anaerobes (Vinolo et al. 2011) and are important mediators of gut microbial-host cell interactions. SCFAs are associated with many age-related diseases (Fernandes et al. 2020). The gastrointestinal tract contains mainly acetic, propionic, and butyric acids, with lower levels of valeric and isovaleric acids. In addition to regulating intestinal pH, short-chain fatty acids have specific functions. For example, propionic acid enhances muscle activity and promotes the proliferation of intestinal epithelial cells. Butyric acid, on the other hand, is the main energy source for colon cells and is related to the integrity of the intestinal barrier (Xiong et al. 2022). Short-chain fatty acid content in the colon of aging mice can be analyzed to identify the role of probiotics on inflammation and oxidation.

In our present study, two strains of probiotics significantly increased the content of acetic acid in the colon of aging mice ($p < 0.05$). Acetic acid is the most abundant short-chain fatty acid in the intestinal tract and has been shown to bring many benefits. A study conducted by Lyu et al. found that acetic acid regulates T-cell survival by controlling the level of acetylation of α-microtubulin, which in turn regulates the immune system (Lyu et al., 2023). Yang et al. found that short-chain fatty acids can act as signaling molecules to activate G protein-coupled receptors (GPCRs) on the cell surface and inhibit histone deacetylases (HDACs) via substrate transport proteins within the cell, leading to anti-inflammatory effects (Yang et al. 2023). Ma et al. also reported similar findings. It was discovered that acetic acid promotes longevity and health of the nematode, *Hydrocyteus vulgaris*, through DAF-16. Additionally, it could upregulate the expression of tight junction proteins ZO-1 and Occludin in aging mice. It reduces the concentration of

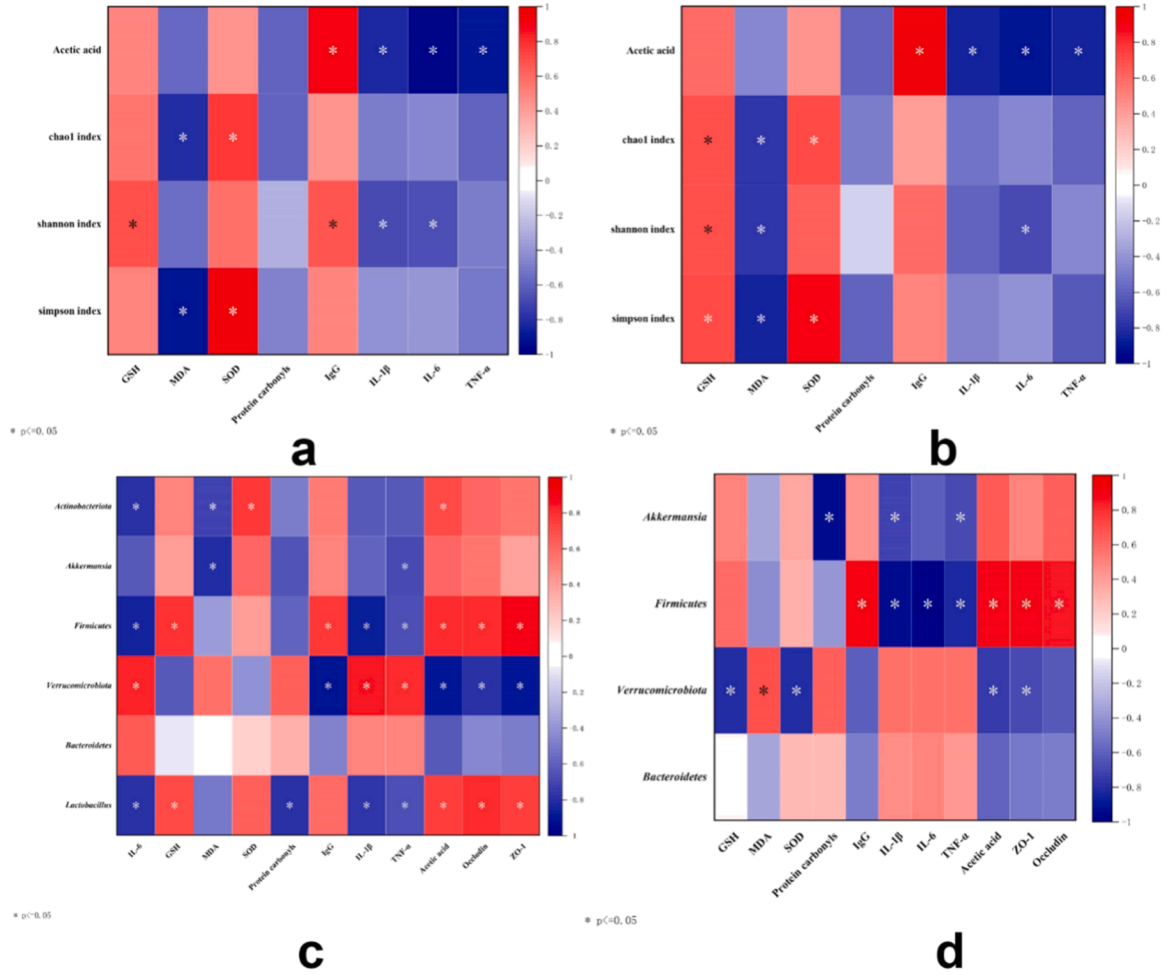

Fig. 9. Correlation analysis of anti-aging indicators with gut microbiota in aging mice. a) Correlation analysis between anti-aging indicators and α-diversity index of gut microbiota a in ZJUIDS-D034 group; b) Correlation analysis between anti-aging indicators and α-diversity index of intestinal flora in ZJUIDS-D016 group; c) Correlation analysis between anti-aging indicators and gut microbiota in ZJUIDS-D034 group; d) Correlation analysis between anti-aging indicators and gut microbiota in ZJUIDS-D016 group (* $p < 0.05$).

serum inflammatory factors and enhances antioxidants in aging mice (Ma et al. 2023). Thus, ZJUIDS-D016 and ZJUIDS-D034 promote the production of acetic acid in mice and may be the reason for their anti-aging effect.

We examined alterations in the diversity, structure, and relative abundance of gut microbiota in a senescence-induced mouse model. Two probiotic strains restored the α-diversity of gut microbiota. Experimental mice provided with ZJUIDS-D016 had similar gut microorganisms, suggesting that ZJUDIS-D016 could maintain the stability of gut microbiota. This could have long-term benefits for the health of hosts. Administration of ZJUIDS-D016 and ZJUIDS-D034 led to an increase in the abundance of beneficial bacteria such as *Firmicutes, Bacteroidota, Deferribacterota, Akkermansia* and *Lactobacillus*. Additionally, there was a decrease in the abundance of harmful bacteria, specifically *Cyanobacteria. Cyanobacteria* synthesize β-methionine-L-alanine (BMAA), a neurotoxic amino acid that has been linked to neurodegenerative diseases (Hu and Rzymski 2022). Lipopolysaccharides produced by *cyanobacteria* activate B-cells, which act as antigen-presenting cells and produce antibodies. This affects the host's innate immune response (Swanson-Mungerson et al. 2017). Additionally, the decrease in the relative abundance of *cyanobacteria* in experimental mice was beneficial for them. *Akkermansia* and *Lactobacillus* have been previously studied for their anti-aging properties. According to Zeng et al., the relative abundance of *Akkermansia* in gut microbiota decreases with age (Zeng et al., 2023a). However, healthy and long-lived elderly individuals have significantly higher levels of *Akkermansia* in their gastrointestinal tract. Additionally, fecal *Akkermansia* contents decrease with decreasing health of centenarians. *Akkermansia* may thus play a role in promoting healthy aging. Studies have also demonstrated that Akkermansia prevents age-related decline in the thickness of the colonic mucus layer (van der Lugt et al. 2019), reduces oxidative stress (Cerro et al. 2022), attenuates age-related inflammation and immune problems, and ameliorates muscle atrophy in aging mice (Shin et al. 2021). Altogether, *Akkermansia* can maybe extend healthy lifespan (Bárcena et al. 2019). Ma et al. found that *Akkermansia* significantly promoted acetic acid production and ameliorated systemic aging in old mice (Ma et al. 2023). Kumar et al. found that *Lactobacillus shortum* MTCC1750 could increase the antioxidant capacity and prolong the lifespan of C. elegans through the DAF-16 pathway (Kumar et al., 2022). Cheng et al. focused on *Lactobacillus*, but their findings did not confirm previous studies but found that *Lactobacillus paracasei* PS23 upregulated genes involved in neuroplasticity, oppressing inflammation, and assisting the antioxidative system, and also genes responsible for improving age-associated cognitive decline (Cheng et al. 2022). Thus, we suggest that increased abundance of *Akkermansia* and *Lactobacillus* by ZJUIDS-D034 may be one of the effects through which aging is combated, while ZJUIDS-D016 primarily increases the relative abundance of Akkermansia.

A correlation analysis showed the effects of ZJUIDS-D016 and ZJUIDS-D034 on the diversity of gut microbiota and the dominant

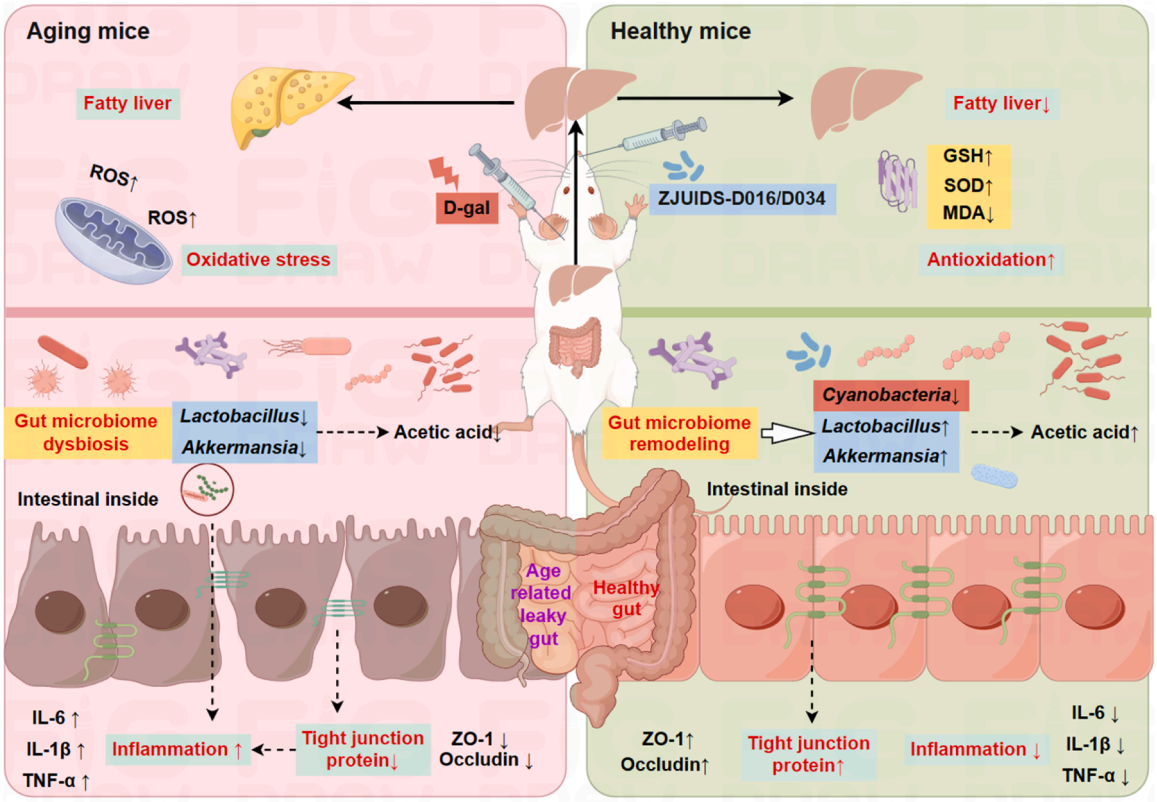

Fig.10. Diagram of ZJUIDS-D016 and ZJUIDS-D034 beneficial effects.

bacterial populations in aging mice, as well as the relationship between these changes and the levels of oxidative stress and inflammation. We observed a negative correlation between the diversity of gut microbiota and their levels of oxidative stress and inflammation .This implies that a decrease in gut microbiota diversity may be associated with an increase in oxidative stress and inflammation during the aging process. Also, the relative abundance of *Lactobacillus* and *Akkermansia* were negatively correlated with the levels of oxidative stress and inflammation in aging mice, and positively correlated with the content of acetic acid. ZJUIDS-D034 may exert its anti-aging effect by increasing the relative abundance of *Lactobacillus* and *Akkermansia*, thereby enhancing the production of acetic acid. Similarly, ZJUIDS-D016 might increase the relative abundance of *Akkermansia*, which may further drive acetic acid production.

In conclusion, the two probiotics, *Weissella confusa* ZJUIDS-D034 and *Enterococcus faecalis* ZJUIDS-D016, derived from dogs exhibit robust anti-aging effects. Both strains could repair the damaged intestinal barrier of aging mice by remodeling the structure of gut microbiota and increasing the content of metabolite acetic acid, as well as reducing the level of inflammation, oxidative stress and enhancing antioxidant capacity. At the same time, the two probiotics alleviated age-related liver disease of mice. The mechanism diagram is shown in Fig.10.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed

Declaration of competing interest

Authors declares NO conflict of interest

Data availability

Data will be made available on request.

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