

**Agar oligosaccharides improve metabolic health in HFD-fed *D. melanogaster*
through regulating gut homeostasis via the *Acetobacter persici*-acetic acid-
insulin/IGF-1 signaling axis**

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Abstract: Agar oligosaccharides (AOS) are marine oligosaccharides with anti-obesity potential, but the underlying mechanisms of action are unclear. In this study, high fat diet (HFD)-fed female *Drosophila melanogaster* (*D. melanogaster*) were used to investigate the role of AOS on physiological health with improved intestinal homeostasis found *via an Acetobacter* bacteria-SCFAs-insulin/IGF-1 (IIS) signaling axis. The results showed that supplementation with 0.25% AOS (average molecular weight: 1580000 g/mol) significantly extended *D. melanogaster* lifespan and reduced food intake, body weight, wing deformity, egg-laying, lipid levels, and improved intestinal homeostasis with decreased intestinal leakage and epithelial cell mortality. Furthermore, AOS significantly affected the intestinal IIS signaling pathway by upregulating the expression of AMPK α and S6K and downregulating the expression of InR, mTOR, PI3K, Akt, and FAS. The beneficial effects of AOS were lost in InR^{p5545}/TM3 mutant *D. melanogaster*. Integrated 16S rRNA sequencing, strain isolation, and *in vitro* fermentation revealed that AOS alleviated HFD-induced intestinal dysbiosis by significantly increasing the abundance of *Acetobacter persici* and the content of short-chain fatty acids (SCFAs), especially acetic acid in the intestine, as verified using axenic *D. melanogaster*. Collectively, AOS was beneficial for the metabolic health of HFD-fed *D. melanogaster* through regulating intestinal homeostasis *via the Acetobacter persici*-acetic acid-IIS signaling axis.

Keywords: Agar oligosaccharides; *D. melanogaster*; intestinal homeostasis; *Acetobacter persici*; acetic acid; IIS signaling pathway

1. Introduction

In recent years, the global prevalence of obesity has been on the rise, driven by changes in lifestyles, dietary habits, exercise patterns, and environmental factors [1]. High-fat diets (HFD) are closely associated with the development of obesity due to their high energy density and potential to stimulate overeating. When individuals consume a diet rich in fats, particularly saturated and trans fats, they are likely to take in more calories than their bodies can burn [2]. The excess calories are then stored as fat, leading to weight gain and eventually obesity if the pattern of consumption continues over time. The combination of increased calorie intake and the metabolic effects of dietary fats can create vicious cycles that result in increased risk of various health problems, including type 2 diabetes, insulin resistance, inflammation, and cancer [3].

The intestine is one of the largest immune organs in the body and serves as the main site of digestion and absorption of nutrients [4]. Intestinal homeostasis is underpinned by a dynamic and mutually beneficial relationship coexisting with the gut microbiota, which is thought to be a major determinant of health and disease [5-6]. Gut microbial-host interactions play a key regulatory role in multiple physiological processes, including the effective regulation of glucose metabolism, promotion of intestinal peptide secretion, immune system modulation, and potential effects on cancer cell proliferation [7]. HFD-induced obesity tends to induce gut microbiota dysbiosis, impairs intestinal barrier function [8], enhances intestinal inflammatory responses, disrupts the normal secretion of gut hormones, alters the metabolites of gut microbiota, and further leads to systemic immune dysregulation [9]. In patients with obesity, the

65 proportion of *Firmicutes* bacteria associated with complex carbohydrate metabolism is
66 significantly elevated, enabling the absorption of complex carbohydrates by the human
67 body and leading to excessive energy accumulation. Disrupted gut microbiota can cause
68 abnormal secretion of gut hormones such as GLP-1, leptin, and ghrelin, increasing
69 appetite and reducing satiety, leading to excessive energy intake and obesity [10]. A
70 balanced gut microbiota is associated with a properly regulated inflammatory response,
71 which is crucial for maintaining health and preventing chronic diseases. For example,
72 *Phascolarctobacterium faecium* can activate Toll-like receptor 2 signaling, promoting
73 the transformation of macrophages into anti-inflammatory M2 type, thereby reducing
74 systemic inflammation and improving glycolipid metabolism [11]. Moreover, intestinal
75 pathogens may damage DNA by inducing oxidative stress in the enteric mucosa,
76 enhancing epithelial inflammation, and disrupting the mucosal barrier, thereby
77 increasing the risk of tumorigenesis [12]. The gut microbiota has become a subject of
78 intense interest in the field of human health, with its metabolites being integral to
79 maintaining the overall health of the gut [13]. Dysbiosis can lead to pathogen-associated
80 molecular patterns such as lipopolysaccharide (LPS) entering the bloodstream,
81 activating the TLR4 signaling pathway, and triggering chronic inflammation and insulin
82 resistance [14]. Specific microbial metabolites can serve as key molecules for
83 suppressing obesity through immune control. Research has found that 4-
84 hydroxyphenylacetic acid level was negatively correlated with body fat accumulation,
85 with the mechanism involving the suppression of intestinal inflammation [15]. Short-
86 chain fatty acids (SCFAs), produced by specific bacteria (e.g., *Bifidobacteria*,

87 *Lactobacillus*, and *Acetobacter*) during fermentation of indigestible carbohydrates,
88 have a significant beneficial effect on gut health [16]. Notably, over 95% of SCFAs in
89 the intestine are acetic acid, propionic acid, and butyric acid, with carbon chain lengths
90 between C2 to C4. Functionally, SCFAs can regulate the pH of the gut environment,
91 bind to receptors such as GPR41/43, and serve as crucial substrates for subsequent
92 biochemical reactions [17]. Acetic acid serves as a pivotal energy substrate for the cells
93 of the intestinal mucosa, playing an indispensable role in upholding the structural
94 integrity of the intestinal barrier and mitigating inflammation within the intestinal tract
95 [18].

96 Several strategies have been proposed to improve gut homeostasis, including dietary
97 interventions, lifestyle modifications, probiotics, and fecal microbiota transplantation.
98 Certain functional oligosaccharides act as prebiotics, supporting the growth of
99 beneficial microorganisms and contributing to a healthy gut environment. For instance,
100 agar oligosaccharides (AOS) have been demonstrated to improve gut microbiota
101 stability and enhance the production of SCFAs, which play a crucial role in maintaining
102 gut homeostasis and regulating immune responses [19]. AOS are low-molecular-weight
103 derivatives of agar polysaccharides, which are natural polymers extracted from red
104 seaweed (e.g., *Gelidium* and *Gracilaria* species) via enzymatic, chemical, or physical
105 methods [20]. Structurally, AOS includes Gal (67.77%), Glc (20.41%), Xyl (11.08%),
106 Rib (10.01%), Glc-UA (0.25%), and Man-UA (0.48%), and is mainly composed of
107 agarose trisaccharides (38.15%), agarose pentasaccharides (25.25%), and agarose
108 heptasaccharides (15.80 %) [21]. AOS is suggested as a promising application prospect

to improve the gut homeostasis and quality of life of patients with obesity [22]. The insulin/insulin-like growth factor 1(IGF-1) signaling (IIS) pathway, a core regulatory axis, mediates essential physiological processes such as energy metabolism and colorectal cancer *via* downstream molecules like PI3K and Akt upon insulin/IGF-1 receptor binding. Key genes include InR, Chico, and dFOXO in *D. melanogaster*, and IR/IGF-1R, IRS, FOXO1/3 in mammals [23]. Disruption of the pathway or its genetic components frequently impairs systemic glycolipid homeostasis, promoting obesity and thereby connecting it to mechanisms that modulate gut homeostasis [24]. Despite the growing interest in AOS and its potential benefits, the specific effects and underlying mechanisms of its actions in gut homeostasis and obesity alleviation remain incompletely understood. To address this knowledge gap, *Drosophila melanogaster* (*D. melanogaster*), which has the advantages of fast reproduction, short life cycle, and easy observation was employed to investigate the effects of AOS on gut homeostasis and overall health with exposure to HFD. Firstly, a series of experiments on physicochemical parameters, intestinal damage, microflora profile, and SCFAs levels were performed using HFD-fed Canton-S *D. melanogaster*. Finally, the mechanism by which AOS improves the intestinal homeostasis and physiological health of *D. melanogaster* through gut microbiota-SCFAs-insulin/IGF-1 signaling (IIS) axis was elucidated by sterile and transgenic *D. melanogaster*. This study provides novel insights into the potential application of AOS in the prevention and improvement of obesity and related health problems.

2. Materials and methods

2.1. Experimental materials and reagents

Agar oligosaccharide (purity: $\geq 99\%$) (average molecular weight: 1580 kDa (1580000 g/mol) containing Galactose (67.77%), Glucose (20.41%), Xylose (11.08%), Ribose (10.01%), Glucuronic Acid (0.25%), and Mannuronic Acid (0.48%), as reported in our previous study [21], was obtained from Qingdao Bozhi Huili Biotechnology Co., Ltd., China. Triglyceride (TG) and total cholesterol (T-CHO) assay kits were purchased from Nanjing Jiancheng Bioengineering Co., Ltd. Sucrose (commercial), maize flour (commercial), AGAR powder (commercial), and yeast powder (commercial) were purchased from Hangzhou Best Biological Technology Co., Ltd. Other chemicals, such as 4',6-Diamidino-2-phenylindole (DAPI, from Beijing Solarbio Science & Technology Co., Ltd), 7-aminoactinomycin (7-AAD, from Beijing 4A Biotech Co., Ltd.), anhydrous ethanol (Shanghai Cloud Chemical Co., Ltd.), propionate and glacial acetic acid (Hangzhou Gaojing Fine Chemical Co., Ltd.) were of analytical grade.

2.2. Animal stocks

All animal experiments complied with the Animal Research Reporting of In-Vivo Experiments (ARRIVE) guidelines and were carried out in accordance the National Research Council's Guide for the Care and Use of Laboratory Animals (Supplemental Table 1). This study utilized two strains of *D. melanogaster*: wildtype Canton-S (male: n=150; female: n=150, the Drosophila Stock Center at the Shanghai Academy of Life Sciences, Chinese Academy of Sciences) and the InRp5545/TM3 mutant (male: n=100; female: n=100, the Bloomington Drosophila Stock Center #11661, Indiana University, Bloomington, IN, USA). The enclosed offspring female *D. melanogaster* were fed on

a cornmeal-yeast-agar standard medium as a control diet (CD) group. HFD was prepared by adding 5% lard to the CD. Different concentrations of AOS (0.125% and 0.25%) were added to the high-fat medium as HFD-AOSL and HFD-AOSH groups. The dosage was chosen following our previous studies [25]. All *D. melanogaster* were housed in a fully automatic biochemical incubator at a temperature of $(25.0 \pm 1.0) ^\circ\text{C}$, a relative humidity of 55%-65%, and a 12-hour light/12-hour dark cycle. After feeding, all *D. melanogaster* were anaesthetized by CO₂ and dissected.

2.3. Analysis of physiological indicators in HFD-fed *D. melanogaster*

Newly emerged female *D. melanogaster* (n=60 in each group) were exposed to different growth media within 12 hours. The medium was refreshed every 3-4 days, and dead *D. melanogaster* were counted every 6 days. Replacements from the reserve group were added in case of accidental deaths. Lifespan metrics including average, maximum, and median survival times were calculated, along with the lifespan extension rate. Furthermore, newly emerged female *D. melanogaster* were starved for 2 hours and then fed a dye-containing medium. After 12 hours, they were frozen, homogenized, and centrifuged, and the supernatant absorbance was measured at 625 nm to account for food intake. Moreover, *D. melanogaster* at 24-hour, 10-day, and 40-day time points were weighed to calculate average body weight. Wing defects were observed under a microscope to calculate the defect rate. A negative geotaxis experiment was used to measure locomotor activity at different time points. One male and one female from each group were mated after 24-hour, 10-day, and 40-day feeding. After removing the parents, the number of offspring was counted to evaluate reproductive ability.

2.4. Determination of total tissue lipid content in HFD-fed *D. melanogaster*

Female *D. melanogaster* maintained for 10 days and 40 days in each group (n=60 in each group) were collected and whole insects used for lipid determination. After fasting for 2 hours, the *D. melanogaster* were frozen and killed. According to the ratio of body weight (g): absolute ethanol (mL)=1:10, the samples were homogenized thoroughly and centrifuged (4°C, 11175 g) for 10 min. The supernatant was taken for the determination of triglyceride (TG) and total cholesterol (T-CHO) content according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Co., Ltd., China).

2.5. Determination of intestinal integrity and epithelial cells in HFD-fed *D. melanogaster*

The intestinal permeability was measured using the Smurf test according to our previous method [26]. The female *D. melanogaster* (n=60 in each group) were placed in a tube with filter paper saturated in 5 % sucrose solution for 72 hours, starved for 1 hour, and then maintained in another tube with filter paper saturated in 5 % sucrose solution containing 2.5 % eriochlorine disodium salt for 12 hours. The *D. melanogaster* were then observed under a microscope for the evaluation of Smurf leakage, and the leakage rate was calculated according to the percentage with Smurf leakage within the total population. Furthermore, following our previously reported procedures [26], the dissected guts of *D. melanogaster* were stained with 7-AAD to explore intestinal injury induced by HFD. The number of dead intestinal epithelial cells was determined based on the fluorescence intensity of 7-AAD using ImageJ software.

2.6. Detection of the IIS signaling-related genes in HFD-fed *D. melanogaster*

Female *D. melanogaster* (n=60 in each group) were maintained until they reached 40-day-old stages. Total RNA was extracted from the intestine, reverse-transcribed into cDNA, and subjected to a quantitative PCR reaction as previously reported [27]. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with normalization against the Rp49 gene. The primer sequences are listed in Supplemental Table 2.

2.7. Identification of the IIS Signaling in HFD-fed transgenic *D. melanogaster*

InR^{p5545}/TM3 mutant female *D. melanogaster* (n=60 in each group) were utilized in this study. This strain has a disrupted insulin/IGF-1 (IIS) signaling pathway, which leads to reduced pathway activity and also affects the PI3K-Akt signaling cascade [28]. Expression of genes related to the IIS signaling pathway were detected by qRT-PCR.

2.8. 16S rRNA sequencing of intestinal microbiota in HFD-fed *D. melanogaster*

The midgut of female *D. melanogaster* was used for 16S rDNA sequencing. Each group of *D. melanogaster* samples had 3 replicates (each replicate contained 30 midguts). According to the manufacturer's instructions, DNA was extracted from different samples using the E.Z.N.A.® Stool DNA Kit (D4015, Omega). The V3-V4 region of the prokaryotic small subunit (16S) rRNA gene was amplified using primers 343F (5'-TACGGRAGGCAGCAG-3') and 798R (5'-AGGGTATCTAATCCT-3'). The PCR products were confirmed by 1.5% agarose gel electrophoresis, purified using magnetic beads, and quantified using Qubit (Invitrogen). Sequencing was performed using the Illumina NovaSeq platform, analyzed using QIIME2, and graphs were plotted using R language (v3.5.2).

2.9. The isolation of *Acetobacter* bacteria

The intestines of 40-day-old female *D. melanogaster* (n=30 in each group) were dissected and placed in Dulbecco's phosphate-buffered saline (D-PBS) solution. The samples were homogenized and then subjected to serial dilution. The diluted homogenate was spread onto bromocresol purple-based chromogenic agar plates and incubated at 37 °C for 2 to 3 days. Single colonies with distinct yellow halos were selected from the plates. These colonies were further purified through multiple streak-plating on bromocresol purple chromogenic plates. Finally, single colonies with growth dominance and well-defined characteristics were picked and preserved according to the experimental requirements. The 16S rDNA sequencing was carried out by MK-Bio Technology Co., Ltd., Hangzhou, Zhejiang Province, China. Briefly, total genomic DNA from samples was extracted using the FastLee® DNA kit for Feces, monitored for concentration and purity on 1% agarose gels, and then diluted to 1ng/μL. Specific primers (16S V3-V4, 18S V9, ITS1) with barcodes were used to amplify 16S/18S rRNA genes in a 30μL PCR reaction with specific components and thermal cycling conditions. PCR products were mixed with 1X loading buffer containing SYBR green and run on a 2% agarose gel. Samples with a bright main product between 400-450bp were selected. PCR products were mixed in equidensity ratios and purified with the GeneJET Gel Extraction Kit. Sequencing libraries were prepared using the NEB Next®Ultra™DNA Library Prep Kit, index codes added, library quality assessed with Qubit® 2.0 Fluorometer and Agilent Bioanalyzer 2100 system, and then sequenced on an Illumina MiSeq platform to generate 250bp/300bp paired-end reads. Microbiome

bioinformatics were performed with QIIME2 2022.2. Raw sequence data were demultiplexed, quality filtered, denoised, merged, and chimeras removed using the DADA2 plugin. ASVs were aligned with MAFFT, and various diversity metrics were estimated after rarefying samples. Taxonomy was assigned to ASVs against silva138 reference sequences.

2.10. Detection of SCFAs in fermentation products

A nutrient broth (NB) medium was formulated. In a 10 mL vial, 5 mL of the liquid medium and 5 cooked meat particles were added. For the oligosaccharide-treated group, AOS was added at a concentration of 0.25% (0.0125 g). The medium was then sterilized. The isolated *Acetobacter persici* (1×10^9 CFU/200 μ L) was then inoculated into the fermentation medium (5 mL) and cultured under anaerobic conditions at 37 °C for 4 days. Samples were collected during the fermentation process as per the experimental design. The fermentation broth was centrifuged at 4 °C and 16342 g for 5 minutes. The supernatant was collected and acidified with a 5:1 v/v mixture of crotonic acid and metaphosphoric acid solution for over 24 hours. After acidification, the mixture was centrifuged again under the same conditions (4 °C, 16342 g, 5 min). The resulting supernatant was transferred into gas-chromatography vials for subsequent analysis. A gas chromatography-mass spectrometry (GC-MS) Agilent 5977B GC/MS-MSD system (Agilent Technologies Inc., USA) was employed to perform qualitative and quantitative analyses of the metabolites. The injector, ion source, quadrupole, and GC/MS interface temperatures were 260, 230, 150, and 280 °C, respectively. The flow rate of helium carrier gas was kept at 1 mL/min. 1 μ L of sample was injected with a 3 min of solvent

delay time and split ratio of 10:1. The initial column temperature was 50 °C and held 2 min, ramped to 150 °C at the rate of 15 °C/min and held 1 min, and then finally increased to 300 °C at the rate of 30 °C/min and kept at this temperature for 5 min. The ionization was carried out in the electron impact (EI) mode at 70 eV. The MS data were acquired in full scan mode from m/z 40-400 with an acquisition frequency of 12.8 scans per second. The identification of compounds was confirmed by comparison of the retention time and corresponding MS spectra. Data were analyzed using the MassHunter software package. The abundance of SCFAs were calculated with internal standard methods.

2.11. Preparation of axenic *D. melanogaster* and its stock, culture, and treatment

New eggs of *D. melanogaster* were collected and washed according to the following procedure: the eggs were treated with 33% Walch at rate of 3 times/min, and with 50% 84 disinfectant at rate of 1 times/min, and with 70% alcohol at rate of 2 times/2 min, and with sterile water at rate of 2 times/2 min in a sterile environment. The treated eggs were hatched and cultured into axenic flies on sterilized cornmeal-yeast-agar standard medium containing 5 mg/mL ampicillin, 500 mg/mL tetracycline and 2 mg/mL rifamycin. For 10-day-old axenic female *D. melanogaster* (n=60 in each group), parameters such as food intake, physical characteristics (body weight, locomotor activity, wing defect rate, and reproductive capacity), total tissue lipid content, intestinal integrity, and the number of dead intestinal epithelial cells were measured. Additionally, the relative expression level of fatty acid synthase (FAS), phosphatidylinositol 3-kinase (PI3K), and protein kinase B (Akt) was detected in whole body tissue.

2.12. Statistical analysis

All data were presented as the mean $\pm$ standard error of the mean (SEM). Statistical analysis and graphing were performed using GraphPad Prism 9 (GraphPad Software, San Diego, USA). For three groups, a one-way analysis of variance (ANOVA) was used to analyze the significance of differences. For two groups, a two-tailed unpaired t-test was used to analyze the significance of differences. A p-value <0.05 was considered as statistical significance.

3. Results

3.1. AOS improves the health status of HFD-fed *D. melanogaster*.

AOS was supplemented to a HFD and its effects on *D. melanogaster* survival determined. On the 40th day, the average lifespan of the group fed with 0.125% AOS was 1.22 times that of the HFD group ($p<0.05$). The average lifespan fed with 0.25% AOS was 1.32 times that of the HFD group ($p<0.001$). After AOS supplementation, maximum lifespan increased, with 0.125% and 0.25% AOS causing rises of 26.80% and 25.00% respectively (Fig. 1B). The median lifespan of the HFD-AH group was 28.85% longer than HFD group ($p<0.01$) (Fig. 1B). On the 10th and 40th day, AOS treatment reduced *D. melanogaster* food intake (Fig. 1C). Weight gain on HFD at day 40 was prevented in AOS-fed *D. melanogaster* (Fig. 1D). Locomotor activity of *D. melanogaster* did not differ among groups initially, but after 10 days was lower in HFD-fed *D. melanogaster* compared to the AOS group. At 40-day post-intervention, AOS had little effect on the locomotor activity compared to the HFD group (Fig. 1E). Moreover, no significant difference in reproductive ability was initially observed in *D.*

melanogaster, however, a decrease in offspring was observed after 10 and 40 days of AOS intervention compared with HFD group (Fig. 1F). Regarding the wing defect rate, a 10- and 40-day AOS intervention dramatically reduced residual rates (Fig. 1G). Regarding lipid level assays, after 10 days, AOS treatment reduced HFD-induced increase in TG content, close to the effect of CD group (Fig. 1H). The same phenomenon was also observed on the 40th day. Furthermore, HFD also increased T-CHO level which was prevented by AOS supplementation (Fig. 1I). Collectively, AOS had lipid-lowering effects on HFD-fed *D. melanogaster*, potentially influencing various physiological and metabolic aspects.

3.2. AOS protects the intestine of HFD-fed *D. melanogaster*

At day 10, the leakage rate of the gut, as determined by Smurf assay, was much higher in the HFD-fed *D. melanogaster* compared to the CD group, but AOS reversed this increase (Fig. 2A and 2C). The results at day 40 were similar (Fig. 2B and 2D). The number of dead intestinal epithelial cells reflects intestinal health. At day 10, the fluorescence intensity ratio of 7-AAD to DAPI in the CD group was determined to be 30.65 ± 1.27 (Fig. 2E and 2G). In contrast, the value in the HFD group was 64.39 ± 3.53 , which was 2.10-fold higher than that of the CD group (Fig. 2E and 2G). These results clearly demonstrate that the HFD significantly increased the number of apoptotic intestinal epithelial cells. After supplementation with AOS, the fluorescence intensity ratio of 7-AAD to DAPI was significantly decreased (Fig. 2E and 2G). On the 40th day, the fluorescence intensity ratio of 7-AAD to DAPI in intestinal epithelial cells of each group was measured (Fig. 2F and 2H). The ratio in the

HFD group was 80.39 ± 1.59 , which was 2.17-fold higher than that of the CD group (37.10 ± 2.34) and 1.67-fold higher than that of the HFD-AOS group (48.18 ± 2.78).

3.3. AOS regulated intestinal IIS signaling in HFD-fed *D. melanogaster*.

The effect of AOS on the expression of genes related to the insulin/insulin-like growth factor signaling (IIS) signaling in the intestines was investigated in HFD-fed *D. melanogaster* (40-day-old). AOS treatment significantly upregulated the mRNA expression of AMP-activated protein kinase (AMPK) α and S6K, downregulated the expression of insulin receptor (InR), PI3K, Akt, mammalian target of rapamycin (mTOR) and FAS, but showed no effect on the 4e-bp, dilp2, dilp3, dilp5, dilp6, and dFOXO (Fig. 3A). Importantly, mutation of the insulin receptor (InRp5545/TM3; Bloomington Drosophila Stock Center #11661, Indiana University, Bloomington, IN, USA) blocked the ability of AOS to mitigate the effects of HFD on lifespan (Fig. 3C), weight (Fig. 3D), food intake (Fig. 3H), lipid levels (Fig. 3J and 3K), intestinal health (Fig. 3I and 3M), or death of intestinal epithelial cells (Fig. 3L and 3N).

3.4. AOS modulates the intestinal microbiota in HFD-fed *D. melanogaster*

16s rRNA sequencing demonstrated no significant changes in Chao1 among groups (Fig. 4A), while AOS increased the Simpson index (Fig. 4B) and Shannon index (Fig. 4C) ($p < 0.05$). The Venn diagram directly shows the similarity and overlap of the number of ASVs in the three groups (Fig. 4D). Compared with the average number of ASVs (93) in the CD group, the average number of ASVs in the HFD group was 42, which significantly decreased by 54.84% ($p < 0.05$) (Fig. 4E). OPLS-DA analysis showed the samples of the CD group and the HFD-AOS group are relatively close to

each other (Fig. 4F), indicating that the microbial community structures of these two groups are more similar. At the phylum level, *Proteobacteria* was the main phyla (Fig. 4G). Furthermore, *Acetobacter*, a genus of *Proteobacteria* decreased by 86.45% in the HFD group compared with that in the CD group, whereas AOS significantly increased the abundance of *Acetobacter* (563.00%) compared with that in the HFD group (Fig. 4H & 4I) ($p<0.05$).

3.5. AOS promotes the production of SCFAs in HFD-fed *D. melanogaster*

As can be seen from Fig. 5A, since *Acetobacter* bacteria can oxidize ethanol to produce acetic acid, the pH around *Acetobacter* bacteria decreases, causing the acid-base indicator bromocresol purple in the plate to turn yellow. The colonies of *Acetobacter* bacteria isolated from the intestines of *D. melanogaster* are grayish white, with a smooth surface and a slippery feeling when touched. The phylogenetic analysis based on 16S rRNA gene sequences is shown in Fig. 5B. Compared by NCBI blast, the similarity between the strain C2502 isolated from the intestines of *D. melanogaster* and *Acetobacter persici* MK049956.1 reaches 100% (Supplemental Table 3). The sequence of *Acetobacter persici* MK049956.1 retrieved from GenBank also clustered with the corresponding species isolated in this study. Collectively, it is evident that the species of *Acetobacter* strains we isolated were identified as *Acetobacter persici*. The GC/MS analysis of the *in vitro* fermentation showed that SCFAs produced by *Acetobacter* bacteria mainly included acetic acid and propionic acid (Fig. 5C). Notably, AOS fermentation with *Acetobacter* bacteria could significantly increase the concentration of acetic acid by 1.78 times ($p<0.01$) (Fig. 5D).

3.6. The action of AOS to improve the health status was prevented in axenic HFD-fed *D. melanogaster*

In this study, axenic *D. melanogaster* were used to explore whether AOS-induced beneficial effects are mediated by the intestinal microbiota. After 24 hours of incubation, no growth of the strain was observed in the blank control (sterile water) and homogenized axenic *D. melanogaster* groups, while colonies grew on petri dishes coated with homogenized normal *D. melanogaster*, verifying the construction of axenic *D. melanogaster* (Fig. 6A). Importantly, in axenic *D. melanogaster*, AOS was unable to mitigate the effects of HFD on lifespan (Fig. 6B and 6C), body weight (Fig. 6D), locomotor activity (Fig. 6E), wing defect rate (Fig. 6F), reproductive ability (Fig. 6G), and food intake (Fig. 6H). For T-CHO (Fig. 6I) and TG (Fig. 6J) levels, no significant differences were observed between HFD and HFD-AOS groups in axenic *D. melanogaster*, and the same was true for intestinal leakage (Fig. 6K and 6L). Regarding the death of intestinal epithelial cells, the number of dead cells in the CD group was lower than in the HFD and HFD-AOS groups (Fig. 6M). The 7-AAD/DAPI ratio in the HFD group was 1.67 times that of the CD group ($p < 0.001$), and AOS did not alleviate the HFD-induced increase in cell death (Fig. 6N) in axenic *D. melanogaster*. Additionally, the effects of AOS on PI3K, Akt, and FAS expression were limited or absent in axenic *D. melanogaster* (Fig. 6O-Q). Therefore, our results in axenic *D. melanogaster* indicated that AOS supplementation, in the absence of gut microbiota, had no significant impact on longevity or other health indicators compared with the HFD group.

3.7. *Acetobacter persici* and acetic acid regulated the intestinal IIS signaling pathway in axenic HFD-fed *D. melanogaster*

As shown in Fig. 6O-Q, the effects of *Acetobacter persici* and acetic acid on PI3K, Akt, and FAS expression were investigated in axenic *D. melanogaster*. Treatment with *Acetobacter persici* or acetic acid significantly downregulated the expression of PI3K, Akt, and FAS, highlighting the significance of the regulation of the IIS signaling pathway by AOS through the intestinal microbiota (especially *Acetobacter persici*) and its metabolite acetic acid in influencing the metabolic health of *D. melanogaster*.

4. Discussion

Obesity is generally regarded as a negative factor affecting health and it is significantly reflected in physical signs and behavioral aspects such as body weight, exercise vitality, reproductive ability, and the rate of wing defects in *D. melanogaster* [29]. Under a long-term HFD, the resulting fat accumulation will lead to a significant increase in body weight, which has always been one of the important indicators for measuring obesity [30]. *D. melanogaster* is a valuable model for obesity research due to its rapid reproduction and short life cycle, which enables observation of generational obesity phenotypes within short time frames. They share conserved genes with higher organisms, particularly those regulating metabolism, providing key insights into complex biological systems [31]. Lifespan is one of the key indicators of the health status of *D. melanogaster* [32]. The function of the central nervous system to regulate food intake can be disrupted by sustained metabolic challenges such as HFD [33]. The increase in body weight and fat accumulation will increase the load on the movement

organs (e.g., wings) specifically manifested as limitations in flight and walking and a significant decrease in exercise vitality [34] and wing defects [35]. Additionally, TG and T-CHO are indicators of lipid metabolism and are the main components of blood lipids used to evaluate the obesity status and gut health of *D. melanogaster* [36]. Moreover, the determination of intestinal integrity primarily reflects the intestinal health status of the *D. melanogaster* [37]. Various studies have reported the negative effects of a HFD on the reproductive systems of male and female parents [38]. Here, AOS treatment significantly increased the survival rate, exercise vitality, and reduced body weight, wing defects and the level of TG and T-CHO. Furthermore, AOS treatment reduced the number of dead intestinal epithelial cells and mitigated the increase of permeability induced by HFD, suggesting a significant improvement in the intestinal health of AOS-treated *D. melanogaster*. In this research, enhanced reproductive ability of *D. melanogaster* was observed with HFD. It is speculated that the additional fat may provide an energy guarantee for the development and hatching of eggs. Alternatively, the HFD may affect the estrogen level of *D. melanogaster*, which in turn influences the number of eggs laid.

The IIS pathway plays an important role in energy balance, lipid metabolism, and gut health [39]. PI3K, a key downstream target of InR/IGF-1 signaling, generates phosphatidylinositol trisphosphate (PIP3) upon activation, thereby activating the Akt signaling pathway [40]. The PI3K-Akt pathway interacts closely with the AMPK and mTOR signaling pathways, jointly regulating energy metabolism, appetite, and intestinal barrier function [41]. The activation of the AMPK signaling pathway in the

intestine is an effective approach for treating obesity, diabetes, and inflammation, whereas overnutrition and obesity can lead to a decrease in AMPK activity [42-43]. Moreover, S6K, downstream of mTOR, is the regulator of goblet cell differentiation and intestinal epithelium homeostasis [44]. This PI3K-Akt pathway also regulates FAS. Overexpression of FAS in the intestine leads to lipid metabolism disorders, inflammation, immune dysfunction, and ultimately promotion of obesity [45-46]. Our study demonstrated that AOS inhibited HFD-induced increase in mTOR, InR, PI3K, Akt and FAS in the intestine. Furthermore, AOS also induced the expression of AMPK α . However, when the IIS- PI3K-Akt signaling pathway was interrupted, AOS failed to significantly improve the obesity and gut health-related indicators in InR^{p5545}/TM3 mutant *D. melanogaster*. This indicates that IIS signaling plays a crucial role in the mechanism of action of AOS-induced improvements in gut health and physiological status on HFD.

Richness of the gut microbiota is closely related to human health [47-48]. A decrease in the diversity of gut microbiota caused by HFD will negatively impact human health, increasing the risk of metabolic diseases including obesity and inflammation [49]. The gut microbiota can metabolize substrates (e.g., sugars and alcohols) available in the gut environment to generate SCFAs, especially acetic acid [50]. Importantly, acetic acid can exert indirect effects on the InR/IGF-1 signaling *via* its influence on gut hormone secretion and metabolite production [51-52]. For example, acetic acid-induced changes in the gut hormone glucagon-like peptide 1 (GLP-1) can in turn regulate insulin secretion and impact the activity of the InR/IGF-1 signaling [53]. Acetic acid is a key

461 metabolite produced by probiotic *Acetobacter* [54]. Production of SCFAs including
462 acetic acid by the gut microbiota through the fermentation of dietary fiber is an
463 important mechanism to promote intestinal health [55]. Some studies have observed a
464 decrease in the content of SCFAs (acetic acid, propionic acid, butyric acid) in HFD-fed
465 obese mice [56]. A variety of prebiotics (e.g., polysaccharides) are reported to resist
466 HFD-induced obesity by enhancing production of acetic acid, and this effect may be
467 related to gut microbiota regulation [57]. Analysis of microbe community structure
468 showed that AOS significantly increased the relative abundance of *Acetobacter* in the
469 gut microbiota of HFD-fed *D. melanogaster*. To identify bacterial strains promoted by
470 AOS, in vitro fermentation experiments were conducted using batch cultures inoculated
471 with intestinal contents from *D. melanogaster* under anaerobic conditions. A strain of
472 acetic acid bacteria-*Acetobacter persici* was isolated from the gut microbiota of *D.*
473 *melanogaster* and AOS promoted the production of acetic acid. Microorganisms in *D.*
474 *melanogaster* were analyzed by 16S rRNA sequencing and showed that *Acetobacter*
475 *persici* was significantly reduced in the high-sugar diet group compared to the control
476 diet group [58]. Analysis of the role of microbiota on *D. melanogaster* fat metabolism
477 showed a negative association between *Acetobacter persici* and triglyceride level [59].
478 We found that adding AOS during the fermentation process significantly increased the
479 *Acetobacter persici*-derived metabolite acetic acid. Thus, we provide evidence that
480 AOS regulates the gut microbiota (especially *Acetobacter* bacteria), increases the
481 production of SCFAs (especially acetic acid), and thereby exerts a health-improving
482 effect under a HFD regimen. Finally, axenic *D. melanogaster* models were used to

confirm the important role of the gut microbiota and its metabolites in the action of AOS. In an environment without the gut microbiota and its metabolites, AOS had no improvement effect on the physiological health and intestinal homeostasis indicators. Furthermore, HFD seriously affected the intestinal integrity of *D. melanogaster* and increased the mortality rate of intestinal epithelial cells, but AOS did not show a protective effect on intestinal health in axenic *D. melanogaster* experiments. Therefore, altered intestinal microflora profiles in HFD-fed *D. melanogaster* by AOS, especially the increase in the relative abundance of *Acetobacter*, evidence its positive effect on intestinal health is microbiota-dependent. The limitations of this study include that only the *Drosophila* model was used to study the overall prebiotic activity of AOS, and its monosaccharide components were not separated and purified to study the prebiotic activity of different components. In future studies, mice, which are more physiologically similar to humans, will be used as model organisms to further explore the improvement effect of AOS on obesity, intestinal homeostasis, and overall health in mammals. AOS will be fractionated according to molecular weight to identify the most effective agarose oligosaccharide structure or optimal molecular weight range. Moreover, animal experiments of fecal microbiota transplantation can be further performed. The isolation and purification of beneficial bacteria and metabolites can be introduced separately to further explore their roles in the regulation of intestinal and overall health by AOS.

5. Conclusion

AOS ameliorated HFD-induced lifespan reduction, dyslipidemia, and intestinal damage

in *D. melanogaster*, with the effects being more prominent at the dose of 0.25%. Its benefit in improving the negative impacts of HFD on health and intestinal homeostasis was achieved by modulating the gut microbiota. Importantly, AOS increased the abundance of *Acetobacter* bacteria (specifically *Acetobacter persici*) in the gut microbiota, increased the levels of metabolite SCFAs, particularly acetic acid, and inhibited the overactivation of the intestinal PI3K-Akt signaling by modulating the IIS pathway. Thus, AOS has the potential to improve obesity and gut health caused by high-calorie diet consumption, with the gut microbiota playing a key role.

Declarations of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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Figure Captions

Fig. 1. AOS improves the physicochemical parameters of HFD-fed *D. melanogaster*.

(A) Survival curves. (B) Average lifespan, maximum lifespan, and median lifespan. (C) Food intake. (D) Body weight. (E) Locomotor activity. (F) Reproductive ability. (G) Wing defect rate. (H) TG content. (I) T-CHO content. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$. $n = 60$.

Fig. 2. AOS promotes intestinal health of HFD-fed *D. melanogaster*. (A and B)

Intestinal leakage images at 10 and 40 days, respectively. (C and D) Statistical results of the leakage rate at 10 and 40 days, respectively. (E and F) 7-AAD staining, DAPI staining, and their merged images of the intestines at 10 days and 40 days, respectively. (G and H) Fluorescence intensity ratio of 7-AAD to DAPI at 10 and 40 days, respectively. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. $n = 60$.

Fig. 3. AOS regulates the expression of genes related to the intestinal IIS signaling pathway in HFD-fed *D. melanogaster*. (A) The mRNA relative expression levels of

the AMPK α , mTOR, 4e-bp, S6K, InR, dilp2, dilp3, dilp5, dilp6, PI3K, Akt, FAS, dFOXO genes in the intestines at 40 days of age. (B) Lifespan curves. (C) Average lifespan, maximum lifespan, and median lifespan. (D) Average body weight. (E) Locomotor activity. (F) Wing-defect rate. (G) Reproductive capacity. (H) Food intake. (I) Intestinal leakage images. (J) TG level. (K) T-CHO level. (L) 7-AAD staining, DAPI staining, and their merged images of the intestines. (M) Statistical results of the leakage rate. (N) Fluorescence intensity ratio of 7-AAD to DAPI in the intestines. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$. $n = 60$.

Fig. 4. AOS reshapes intestinal microbiota profiles in HFD-fed *D. melanogaster*.

(A-C) Chao1, Simpson, and Shannon indices, respectively. (D) Venn diagram. (E) ASV counts. (F) OPLS-DA analysis. (G) Bacterial phylum abundance and (H) Genus abundance. (I) Relative abundance of *Acetobacter*. * indicates $p < 0.05$, $n = 90$.

Fig. 5. Isolation and identification of *Acetobacter persici* and its metabolite via *in vitro* fermentation.

(A) The separated bacteria with yellow halos on the Bromocresol Purple Chromogenic Plate. (B) Phylogenetic tree of 16S rDNA gene sequences of acetic acid bacterial strains. (C) Detection of *in vitro* fermentation products of gut microbiota by GC-MS. (D) Concentration of acetic acid in the *in vitro* fermentation. ** indicates $p < 0.01$. $n = 30$.

Fig. 6. Effects of AOS, *Acetobacter persici*, and acetic acid on intestinal health in

axenic HFD-fed *D. melanogaster*. (A) Verification of axenic *D. melanogaster* constructs. (B) Lifespan curve. (C) Average lifespan, maximum lifespan, and median lifespan. (D) Average body weight. (E) Locomotor activity. (F) Wing-defect rate. (G) Reproductive capacity. (H) Food intake. (I) TG level. (J) T-CHO level. (K) Intestinal leakage images. (L) Statistical results of the leakage rate. (M) 7-AAD staining, DAPI staining, and the merged images of the intestines under a fluorescence microscope. (N) Fluorescence intensity ratio of 7-AAD to DAPI in the intestines. (O-Q) mRNA relative expression of PI3K, Akt, and FAS. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$. $n = 60$.

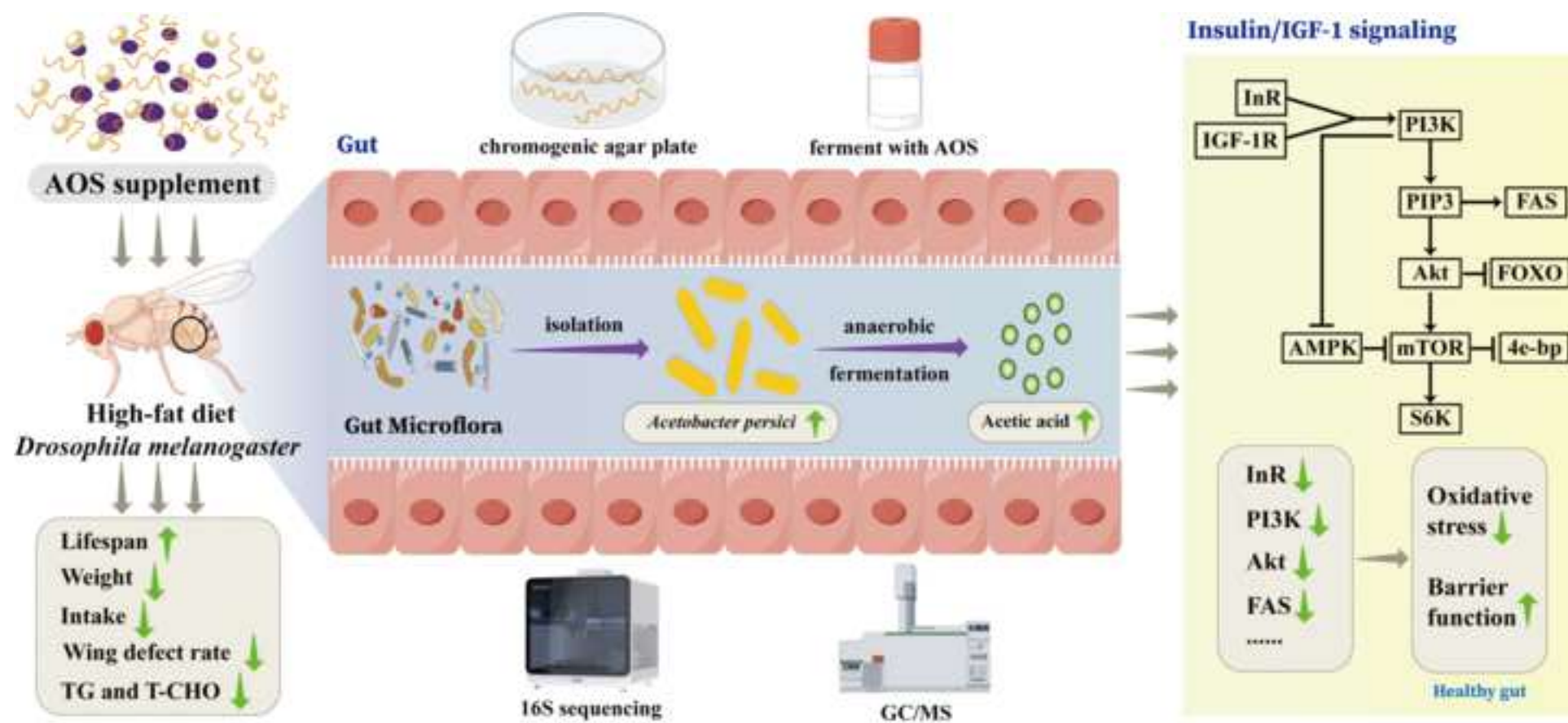
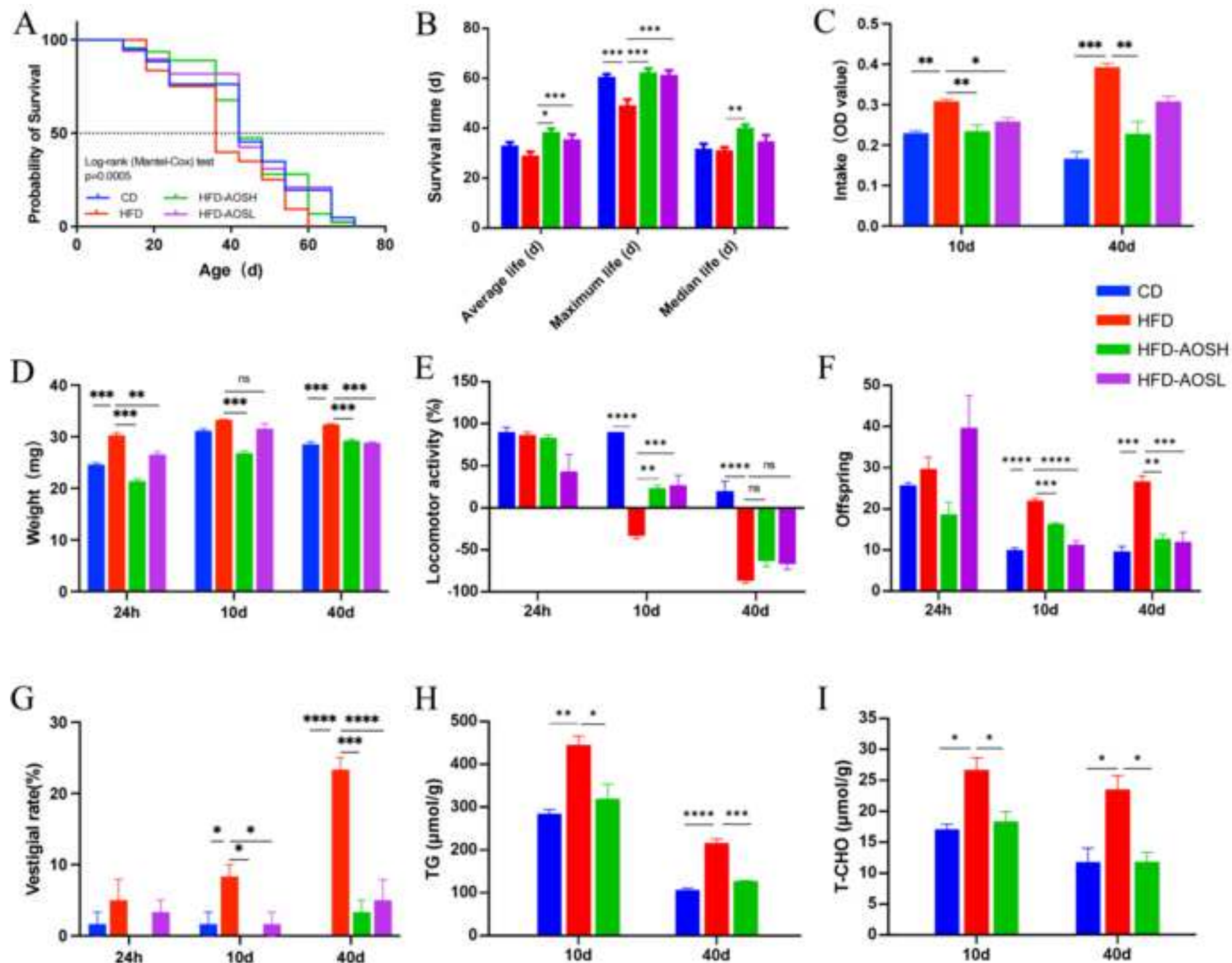


Fig.1

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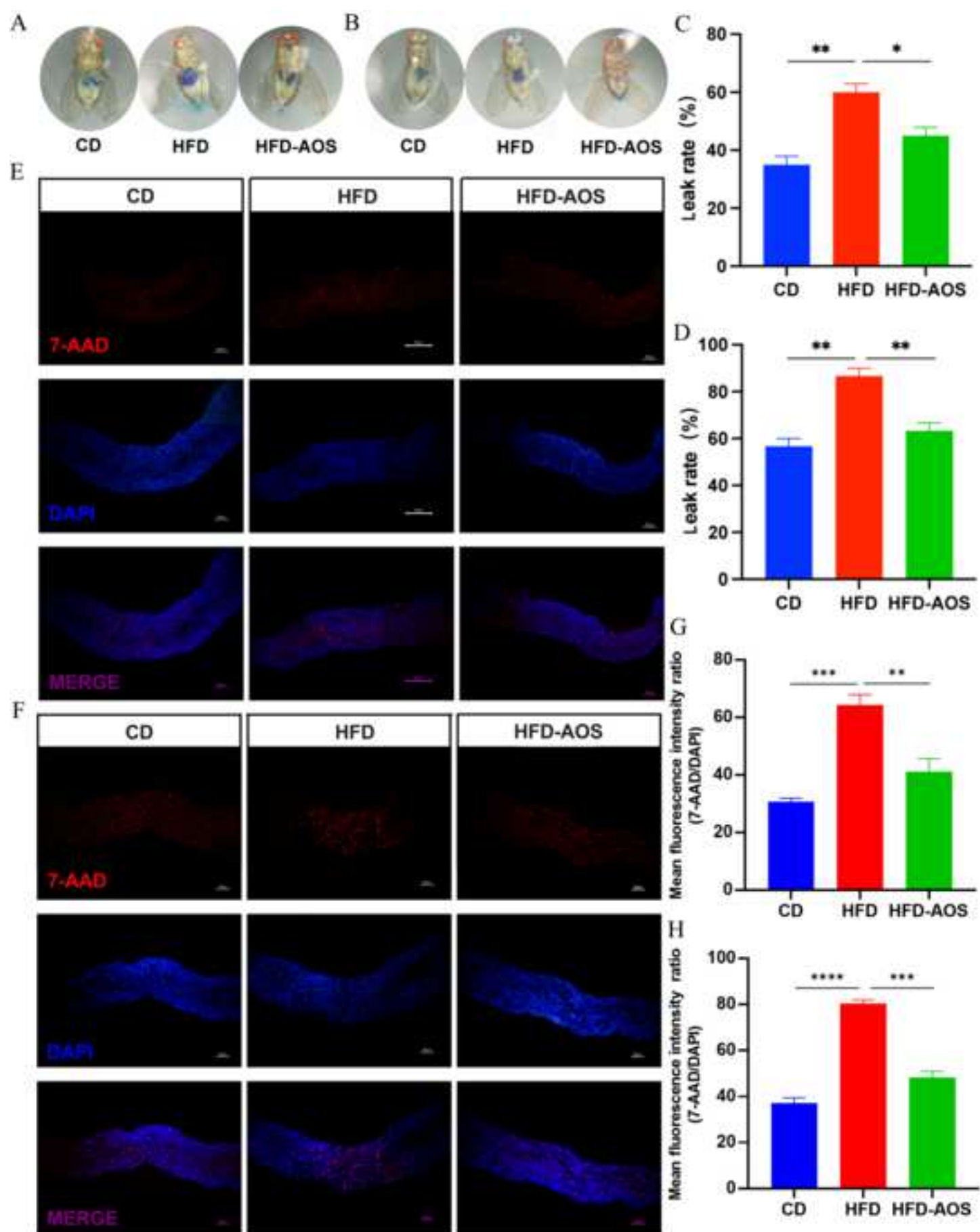


Fig.3

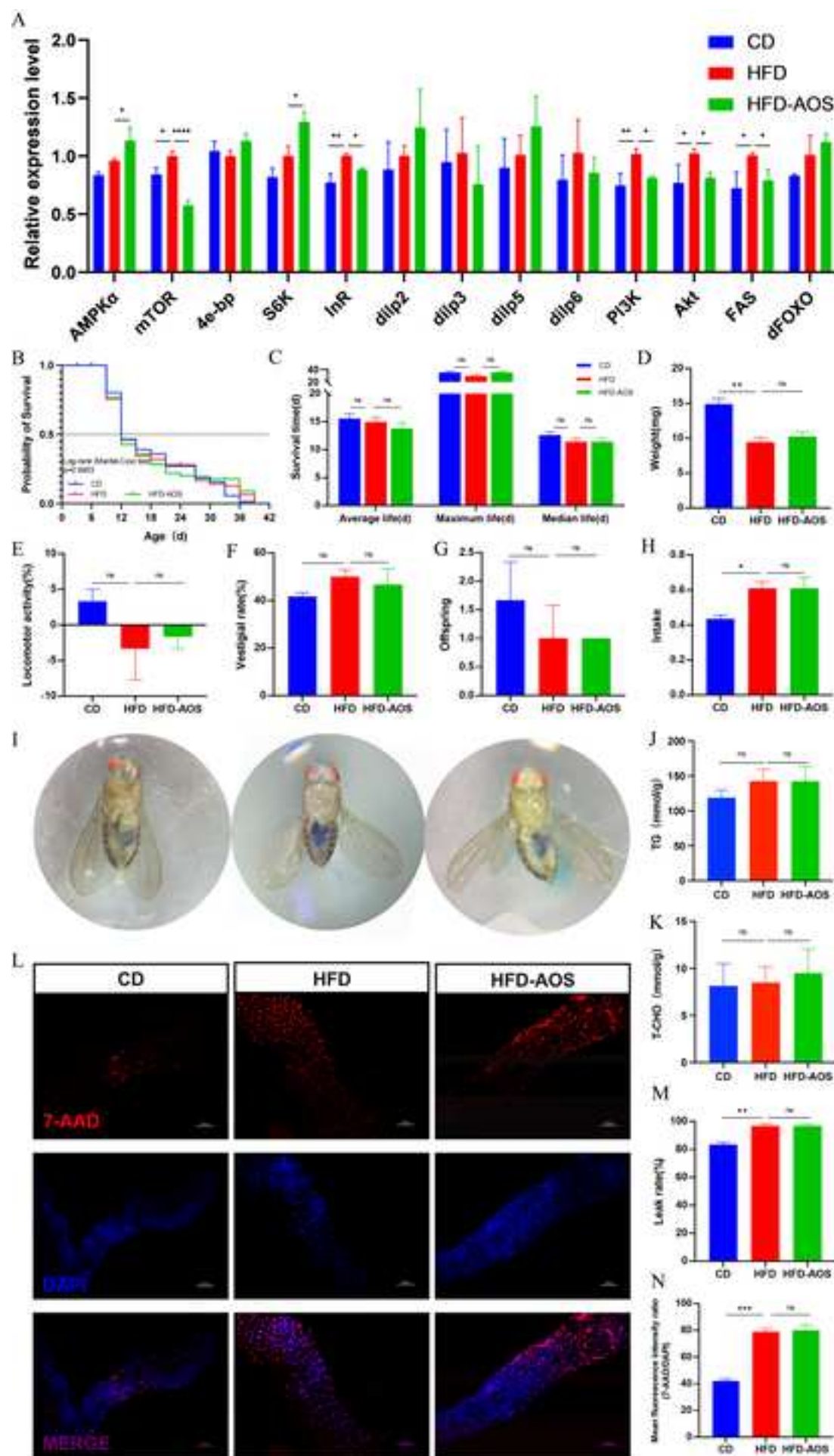
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Fig.4

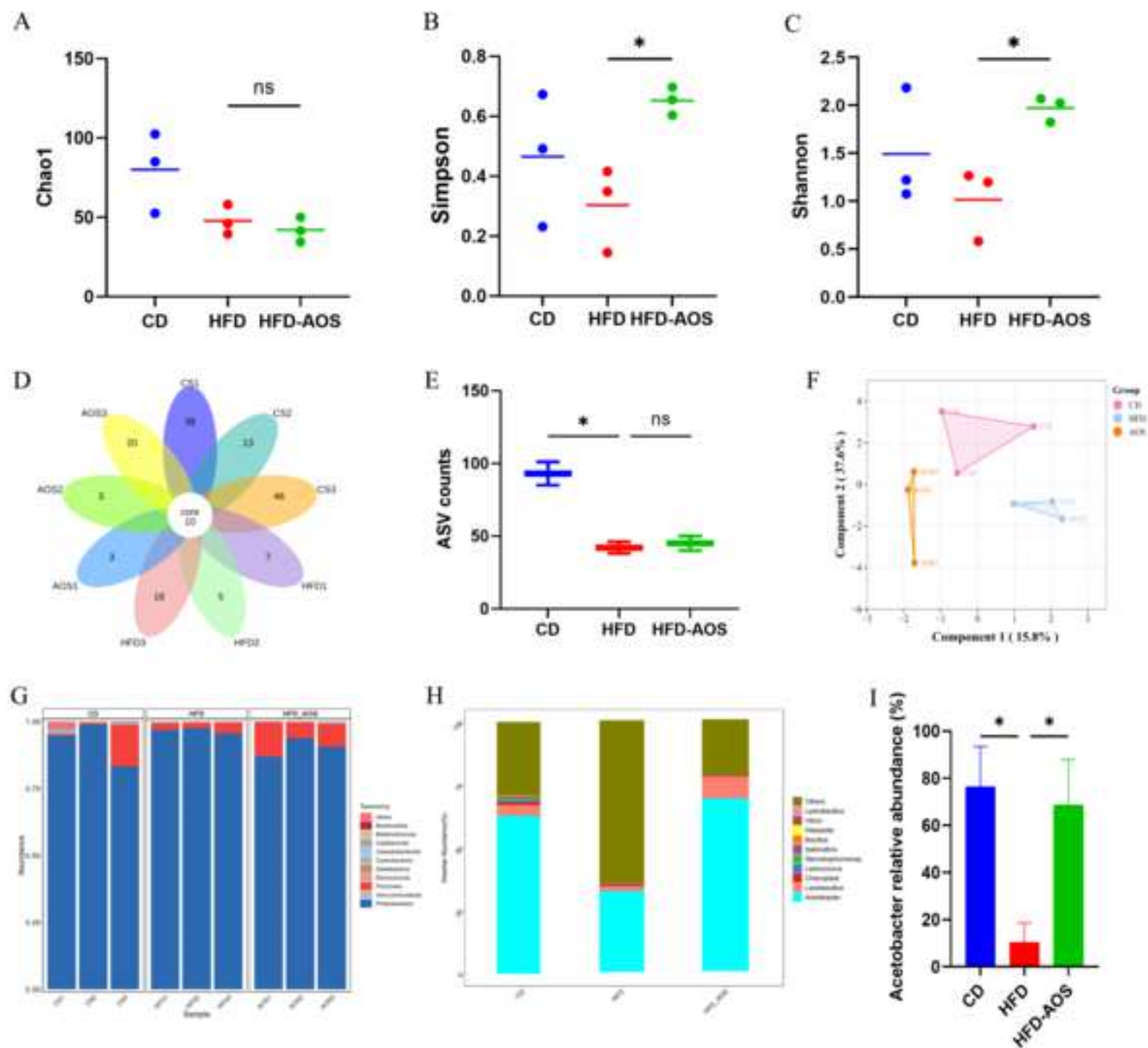
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Fig.5

