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Original Article

Glucomannan Hydrolysate Promotes Gut Proliferative Homeostasis and Extends Life Span in *Drosophila melanogaster*

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Abstract

Dietary supplementation of glucomannan has been shown to have multiple health benefits, but its effect on life span has not been investigated. Here, we show that glucomannan hydrolysate (GMH) treatment extends mean life span of the model organism *Drosophila melanogaster*. To unravel the underlying mechanisms, we first examined the effect of GMH on the gut microbiota. We found that GMH treatment is associated with an elevated bacterial load in aged flies but overall has limited effects on the relative microbiota composition. We also demonstrated that GMH inhibits age-associated hyperproliferation of intestinal stem cells and thus delays the deterioration of gut integrity. Further analysis of the midgut transcriptome revealed that both EGFR/MAPK and JAK/STAT signaling pathways are suppressed in GMH groups. Multiple key regulators or effectors of EGFR/MAPK pathway, *Ets21c*, *Mkp3*, and *Rho*, are downregulated by GMH treatment. In the JAK/STAT pathway, major ligands (eg, *Upd2* and *Upd3*) and negative feedback inhibitors (eg, *Socs36e*) are all significantly downregulated. Additionally, the expression of genes encoding antimicrobial peptides is elevated by GMH treatment. Taken together, our study shows that dietary supplementation of GMH can prolong life span, possibly through regulating gut proliferative homeostasis.

Keywords: Nutrition, Transcriptomics, Stem cells

Aging is a natural process that leads to irreversible impairment of physiological functions and increased vulnerability to death. Great efforts have been spent on investigating cellular and molecular mechanisms underlying the aging process, with the ultimate goal of developing effective interventions to delay the onset of aging. It is well known that some drug treatments and nutrient manipulations extend life span (1–3). However, most drugs, such as rapamycin (3) and metformin (4), have high risks of side effects, while nutrient manipulations like dietary restriction have a poor dietary adherence (5). Therefore, it would be desirable to discover new dietary intervention strategies that have high efficiency and low risks.

Konjac glucomannan is a natural, odorless fiber extracted from the root of the *Amorphophallus konjac* plant, which is a common food ingredient in Asia. Konjac glucomannan is composed of d-mannose and d-glucose monomers with a ratio of 1.6:1. It can be hydrolyzed by acids or enzymes into konjac glucomannan hydrolysates (GMH). Both forms of glucomannan have been frequently consumed and widely studied (6–9). Konjac glucomannan has a special feature that it swells and jellifies in the gut, thus helping with weight loss by increasing gastric satiety (6). When hydrolyzed into GMH, the solubility of depolymerized glucomannan is significantly improved, which enhances its efficacy both locally and systemically (6). GMH has been demonstrated to have prebiotic activity, promoting the growth of probiotic bacteria (7–10). It stimulates the immune system both in the gut and on the skin (7,10,11) and even reduces the levels of serum cholesterol and glucose in diabetic mice (7,12). Despite these beneficial effects of GMH, no studies have investigated its impact on life span. To examine whether GMH supplementation extends life span, we used *Drosophila melanogaster* as a model organism. *Drosophila* is ideal for aging studies because of

its relatively short life span and ease of environmental and genetic manipulations (13,14).

The digestive tract is at the frontline of responses to dietary supplementations and a key organ involved in aging (13). It is not only responsible for physiological functions like nutrient absorption but also acts as the defense barrier to control both commensal and pathogenic microbes (15). In the aging intestine, there are dramatic changes in both the quantity and the composition of gut-associated microbes, termed as dysbiosis (16,17). In a variety of organisms, including humans, changes in the gut microbiota have been associated with disorders like obesity, cancer, and chronic inflammation, potentially due to the dysregulation of the frequent interaction between epithelial cells and commensal bacteria (18). Therefore, it has been proposed that manipulating this interaction may be a viable intervention for healthy aging (19). Dietary supplementation of prebiotics that promotes the growth of beneficial bacteria may be a promising approach. Besides the control of commensal bacteria, it is also critical for organisms to maintain gut homeostasis through regenerative processes. As a high-turnover tissue, the intestine undergoes constant regeneration sustained by intestinal stem cells (ISCs), which are the only dividing cells in the *Drosophila* intestine (15,20). In the aging intestine, dysregulated ISC proliferation and abnormal differentiation both lead to the accumulation of mis-differentiated cells in the epithelium (21,22), ultimately leading to epithelial dysplasia and premature death (23). It has been shown that limiting the rate of ISC proliferation in aged flies is sufficient to extend life span (21,24). In *Drosophila*, the proliferation rate of ISCs is regulated by several stress- and growth-signaling pathways such as JAK/STAT, MAPK, and EGFR pathways (25–27). It has been suggested that the fundamental cause of the aging-associated loss of gut proliferative homeostasis is the dysregulation of interactions between intestinal epithelium and commensal bacteria, which leads to chronically and excessively elevated proliferation of ISCs (16).

In this article, we present evidence for the effect of GMH on extending life span in *Drosophila*. Combining longitudinal transcriptomic and metagenomic analysis with biochemical assays, we show that the life-span–extending effect of GMH is likely through regulating gut proliferative homeostasis.

Materials and Methods

Fly Stocks and Husbandry

All flies were reared under standard laboratory conditions with a 12-hour light/dark cycle at 25°C in vials containing agar–dextrose–yeast medium. Nine different strains of wild-type flies were used in this study. Besides Canton-S and Oregon-R, we included one strain (B18) from the Global Diversity Lines and six strains (DGRP-21, DGRP-38, DGRP-40, DGRP-85, DGRP-105, DGRP-136) from Drosophila Genetic Reference Panel (DGRP) (28). To examine whether *Wolbachia* infection status modulates the life-span–extending effect, we chose three *Wolbachia*-positive strains (DGRP-21, DGRP-40, DGRP-136) and three negative strains (DGRP-38, DGRP-85, DGRP-105) (28). Flies were maintained on an agar–dextrose–yeast medium, whose ingredients include 15 g agar, 50 g sucrose, 100 g Brewer's yeast, 3 mL propionic acid, 3 g *p*-hydroxybenzoic acid methyl ester, and distilled water to make a total volume of 1 L.

GMH Supplementation and Survival Assay

The GMH powder was kindly provided by Chengdu Yongan Yuanhe Biotechnology Co as a gift. The GMH-supplemented medium was prepared by adding the GMH powder into the control agar–dextrose–yeast medium during the regular food preparation process at a concentration of 0.25% w/v. Survival assays were conducted on mated females of multiple fly strains and on mated females, mated males, and virgin females for one strain (B18). Virgin female flies were collected right after eclosion. Assays on mated females were conducted with the presence of a few male flies, while assays on mated females and mated males of B18 were conducted simultaneously with mixed sexes of similar numbers in the same vial. Flies were housed at a density of 20–30 flies per vial, and a minimum of 120 flies (6 vials) were tested for each condition. Flies were transferred to fresh medium every 2 days for mated females and every 3–5 days for virgin females and males. During each transfer, dead flies in each vial were counted. Flies that escaped or died accidentally were recorded as missing. All data were analyzed with log-rank test using the online application for the survival analysis of life-span assays (29). Survival assays for all conditions were done once with the exception of virgin females of B18, for which we conducted an extra independent replicate. Besides survival assays, we used mated females of B18 to conduct independent experiments of GMH treatment, always with a concurrent control group, for each of the mechanistic studies described below.

Long-term Feeding Assay

Both control medium and GMH-supplemented medium were prepared with the addition of a blue indigestible dye, FD&C blue No.1, at a concentration of 1% w/v. Dyed growth medium was poured into the cap of an aerated 50 mL Falcon centrifuge tube. Mated females of strain B18 were transferred into Falcon tubes with medium-containing cap on. Falcon tubes were placed upside down (cap-side down) in incubators for 2 days. Flies were then removed from the tube, and 2 mL of phosphate-buffered saline (PBS) was added into the Falcon tube to wash colored-feces off the sides of tubes. Feces on the surface of the food were not included. With an assumption that diet does not affect the fly's preference to secrete on the sides of the tube and on the food surface, we can estimate and compare the relative amount of food consumed across the 2-day period. The relative food intake was measured as a function of the amount of blue dye in the feces and calculated from the optical density of a serial dilution of FD&C blue No.1 solution. We conducted three biological replicates with 20 individual flies per condition per replicate.

Gut Microbiota Sequencing and Analysis

Guts of flies were dissected in PBS after 10, 20, 30, and 40 days of treatments. DNA was extracted from a pool of 20 guts for each sample using phenol–chloroform extraction method. According to Illumina 16S metagenome library preparation guide, 16S ribosomal RNA gene amplicons were prepared for the Illumina MiSeq System. Briefly, two rounds of PCR reactions were carried out to first amplify the hypervariable regions (V3 and V4) of the bacterial 16S rRNA genes and then to add adaptors and barcodes for Illumina sequencing. Three biological replicates were conducted.

Microbiota-derived reads were analyzed following a Bioconductor workflow based on dada2, phyloseq, and edgeR (30–32). Low-quality reads were filtered: those containing N, with base quality score ≤2, or with more than two expected errors. Additionally, the first 10 bases and all bases after position 230 were trimmed. Dereplication was performed to combine identical reads while keeping track of their abundance. The core dada2 ribosomal sequence variants inference algorithm was applied to the dereplicated data to

infer sample sequences exactly and resolve differences of as little as one nucleotide by modeling and correcting sample-specific sequencing errors (30). We then merged the inferred forward and reverse sequences, removing paired sequences that do not overlap perfectly. Chimera sequences were further removed. The taxonomy of ribosomal sequence variants was assigned based on the SILVA database (version 128) (33). Sequences assigned to the genus of *Wolbachia* were removed. Principal coordinates analysis was performed on log-transformed abundance data using Bray–Curtis dissimilarity. Constrained correspondence analysis was performed to evaluate the contribution of diet and treatment length to the variation in microbiota composition. Alpha diversity was measured with the Shannon index and the Simpson index. Differential abundance across control and supplementation groups was tested with edgeR (32,34).

Quantification of Bacterial Load

Flies were washed with 70% ethanol once and with PBS three times in succession. Surface-sterilized flies were individually homogenized in 500 µL of sterile PBS using bead beating with a tissue homogenizer (OPS Diagnostics). The original or diluted homogenates were plated on de Man, Rogosa and Sharpe agar plates with a WASP II autoplate spiral plater (Microbiology International). Plates were incubated at 29°C for 1–2 days to achieve the optimal colony size, and the colony-forming units were counted by the software Protocol3. Colonies were identified based on their distinct morphologies (35). Three biological replicates were conducted with five fly individuals per condition per replicate. Results were analyzed using a mixed-effects model in R.

Gut RNA-Seq and Data Analysis

After 10, 30, and 50 days of GMH treatment, 50 guts were dissected in sterile PBS and pooled for each replicate. Two biological replicates per condition were used. Total RNA was extracted using Trizol (Invitrogen) and RNeasy mini plus kit (Qiagen). mRNA was isolated using magnetic mRNA isolation kit (NEB). KAPA-stranded RNA-Seq library preparation kit was used to construct libraries for Illumina sequencing.

Raw-sequencing reads were first processed with Trimmomatic (version 0.33) (36) to trim adaptor sequences and low-quality bases and then mapped to the reference genome of *D melanogaster* (FlyBase Dmel Release 6.09) with STAR (version 2.5.1b) (37). Differentially expressed genes were identified with edgeR (32). Only genes expressed (defined as count per million > 1) in at least two out of four samples were included in analysis. Significant differentially expressed genes were defined with FDR < 0.05 and $\log_2$ (fold change) ≥ or ≤ 0.5. Pathway enrichment of differentially expressed genes was evaluated using DAVID 6.8 (38).

ISC Proliferation and Gut Length Measurement

Immunostaining and length measurement of midguts were performed as described before (35). Guts were dissected in PBS and fixed for 30 minutes in PBS with 0.1% Tween 20 (PBT) and 4% paraformaldehyde. They were subsequently rinsed in PBT and incubated with primary antibodies (1/1,000 anti-PH3 [Upstate/Millipore] or 1/1,000 anti-GFP, Roche) in PBT plus 1% bovine serum albumin. Staining was revealed after a second incubation with Alexa488- or Alexa594-coupled anti-mouse antibodies (Invitrogen), and nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). Guts were then scanned with an Axioplot imager (Zeiss) and recomposed using the software program MosaiX (Zeiss). Tiled images of guts for length measurements were acquired with fluorescence at ×10 magnification and assembled into one single image with Zen imaging software (Zeiss). Midgut length was measured by tracing from the middle of the proventriculus along the midgut to the midgut–hindgut junction, as indicated by the branching of the Malpighian tubules. Measurements to the nearest micrometer were obtained with ImageJ (FIJI package). Three biological replicates with 10 fly individuals per condition per replicate were used.

Results

GMH Supplementation Extends *D melanogaster* Life Span

We first tested whether GMH supplementation affects the life span of *D melanogaster* while taking into account sex, genetic background, and mating status. Survival tests in mated male and female flies of strain B18 (a wild-type stock) both showed significant life-span extension (Figure 1A and B). In the control groups, the mean life spans were 57.39 days for male flies and 58.05 days for female flies. In the GMH supplementation groups, the mean life span was increased by 20.2% in males (p = .0e + 00) and by 14.88% in females (p = 6.2e − 07). As mating status is known to markedly affect life span, we also examined the effect of GMH supplementation on virgin B18 females and observed an increase in the mean life span by 27.48% (p = .0e + 00; Supplementary Figure 1). To evaluate the life-span–extending effect of GMH under different genetic backgrounds, we repeated the survival test in two additional wild-type strains (Oregon-R and Canton-S) and six DGRP strains using mated female flies. GMH supplementation increased the mean life span by 11.7% in Oregon-R (p = .01; Figure 1C) and 17.66% in Canton-S (p = 9.0e − 09; Figure 1D). Five out of six DGRP strains also showed significant results, with a 13.4% increase of the mean life span in strain 21 (Figure 2A, p = .0032), 10.1% in strain 38 (Figure 2B, p = 2.2e − 05), 4.6% in strain 40 (Figure 2C, p = .050),

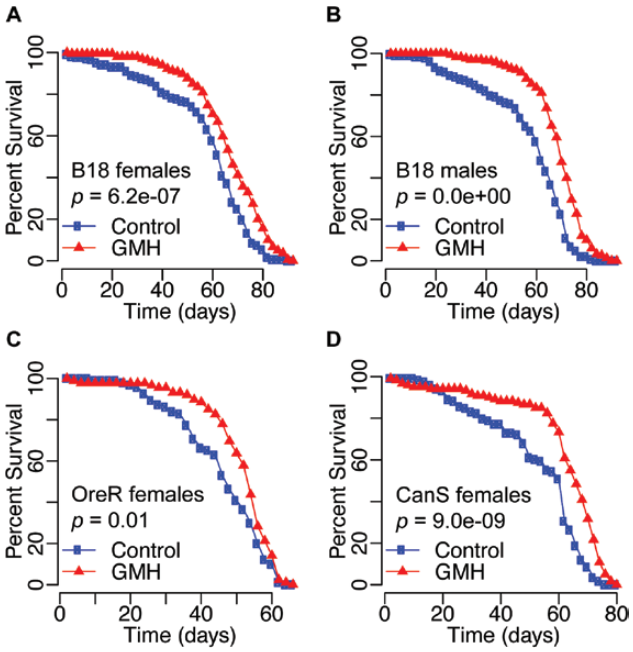

Figure 1. Life span extension effect of GMH is observed in strains of B18, Oregon-R, and Canton-S. GMH supplementation increase the mean life span of (**A**) mated female flies of strain B18; (**B**) mated male flies of strain B18; (**C**) mated female flies of strain Oregon-R; (**D**) mated female flies of strain Canton-S. Abbreviation: GMH = glucomannan hydrolysate.

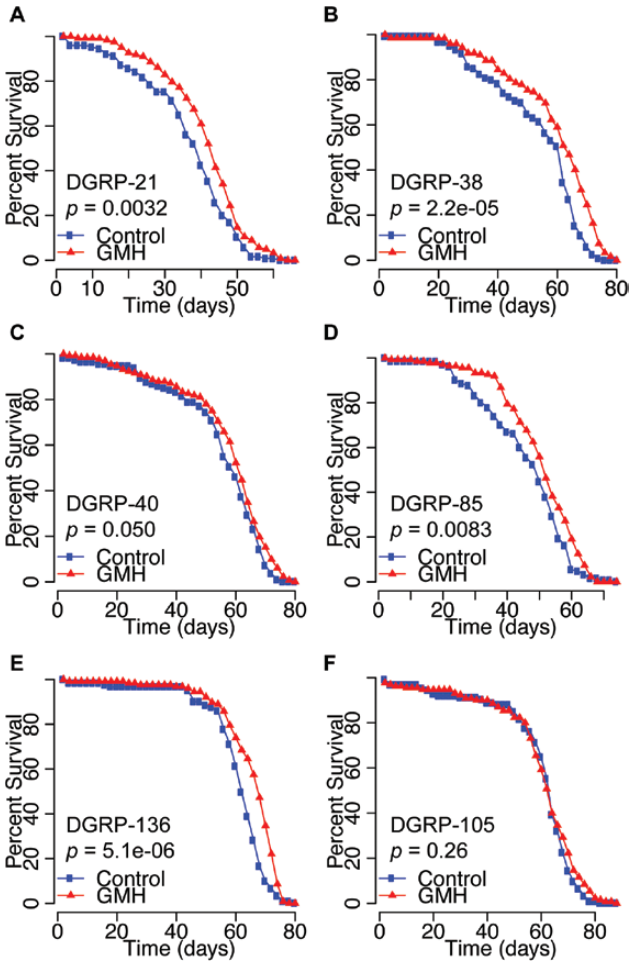

Figure 2. The response to GMH treatment is different in six DGRP strains. GMH significantly extend the mean life span of mated female flies of (**A**) strain DGRP-21; (**B**) strain DGRP-38; (**C**) strain DGRP-40; (**D**) strain DGRP-85; (**E**) strain DGRP-136 but GMH does not extend the life span of flies of (**F**) strain DGRP-105. Abbreviations: DGRP = Drosophila Genetic Reference Panel; GMH = glucomannan hydrolysate.

10.0% in strain 85 (Figure 2D, p = .0083), and 7.3% for strain 136 (Figure 2E, p = 5.1e − 06). Only strain 105 did not respond to GMH supplementation (Figure 2F). These findings suggest that GMH supplementation can promote longevity in both sexes across different genetic backgrounds, regardless of mating status.

GMH Supplementation Is Associated With Elevated Bacterial Load in Aged Flies

To explore mechanisms underlying the life-span–extending effect of GMH supplementation, we first evaluated its impact on feeding behavior. It is possible that GMH supplementation results in dietary restriction, which is an effective way to extend life span (14). With long-term feeding assays (Supplementary Figure 2), we found no significant differences in the relative food intakes of control and GMH treatment groups, suggesting that dietary restriction is probably not involved. From existing studies, we know that GMH can function as an effective prebiotic in mice (7). To test whether GMH extends life span through its potential impact on the gut microbiota, we quantified bacterial load (ie, the number of bacterial cells per gut) by plating the microbiota of surface-sterilized flies on solid medium (35). We further characterized the overall gut microbiota composition with 16S rRNA sequencing. Bacterial load results showed that the number of representative colony-forming units in the gut increased exponentially in the process of aging in both control and treatment groups (Figure 3A), as previously shown (16,17,35,39). However, GMH-supplemented group has significantly more colony-forming units across the three sampling time points (p = .031), specifically on Day 30 (p =.044) and Day 50 (p = .053), but not on Day 10 (p = .74). Examining colony morphologies revealed that *Acetobacter* species were dominant in both GMH-supplemented and control groups.

Our 16S rRNA sequencing experiment at four time points (Days 10, 20, 30 and 40) also unraveled an apparent effect of age on gut microbiota composition, with *Acetobacter* species dramatically increasing over time (Figure 3B). The principal coordinates analysis clearly separated samples by sampling times (Figure 3C). However, no apparent clustering was observed for either control or GMH groups. Constrained correspondence analysis further confirmed that the sampling time explains 64.57% (p < .001) of the variance in microbiome composition, while the type of diet explains only 6.17% (p = .255). Differential abundance analysis at the genus level revealed only one genus, *Lactobacillus*, has a significantly lower abundance in GMH group on Day 40 (Supplementary Figure 3A). For *Acetobacter*, the trend was consistent with the result of bacterial load measurement, suggesting that the GMH group has higher abundance, but this was not significant (Supplementary Figure 3B). Altogether, our results suggest that GMH might regulate the bacterial load but overall has limited effects on the relative microbiota composition.

GMH Supplementation Delays the Deterioration of Gut Integrity

Recent studies have shown that the gut microbiota has a profound influence on host physiology, especially on digestive and immune functions (15,35,40). After observing the impact of GMH on bacterial load, we evaluated its effect on gut epithelial homeostasis. As the gut is a tissue with high turnover rate, the proliferative activity is critical in maintaining gut integrity (21). Therefore, we measured the rate of stem cell proliferation in the gut by performing immunostaining with an anti-phosphohistone H3 (anti-PH3) antibody, which labels dividing cells. Consistent with previous studies (20,41), low levels of homeostatic proliferation were observed in young and healthy guts on Days 10 and 30. On Day 50, we detected a dramatic increase in the number of PH3-positive cells in both control and GMH groups, but GMH treatment group had significantly fewer dividing cells than the control group (p = .00035; Figure 4A–C). We also measured gut length as another parameter of the homeostatic status of the gut. Both groups showed significant shortening in gut length along the process of aging, a known phenomenon (42). However, on Day 50, guts from GMH-treated flies were significantly longer compared with the ones from the control group (p = .0024; Figure 4D). In summary, GMH supplementation delays two processes associated with aging—gut stem cell hyperproliferation and gut shortening.

GMH Supplementation Decreases EGFR/MAPK and JAK/STAT Pathways

To search for hints of mechanisms underlying the life-span–extending effect of GMH supplementation, we set out to identify differentially expressed genes between control and supplementation groups.

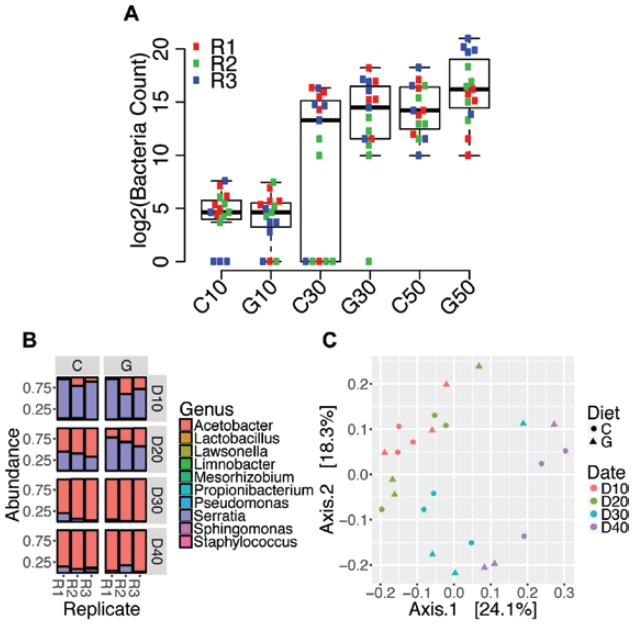

Figure 3. The limited effect of GMH supplementation on gut microbiome. (**A**) The total bacterial load given as the log number of CFUs. There are three biological replicates with five individuals per replicate; (**B**) Each bar represents average relative abundance of each bacterial taxon (top 10 taxa) within a group at genus level; (**C**) Principal coordinate analysis of gut microbiome compositions in control and GMH groups at different time points. Abbreviations: CFUs = colony-forming units; GMH = glucomannan hydrolysate.

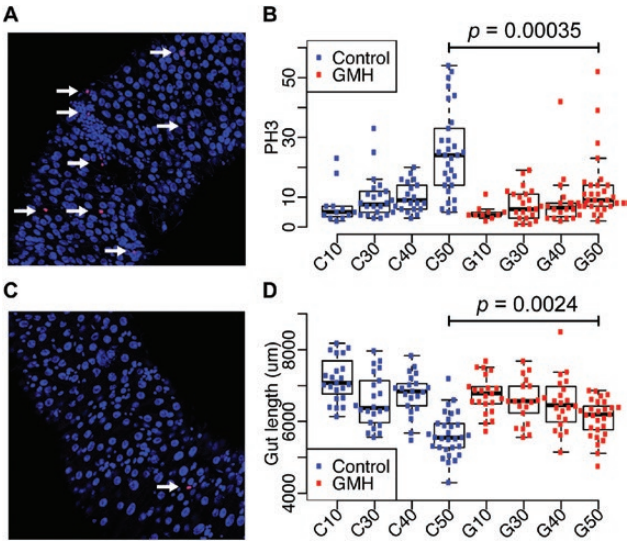

Figure 4. GMH supplementation delays the deterioration of gut integrity. (**A**) Immunostaining of PH3, a mitosis biomarker, is shown in red (indicated by arrows, 4′,6-diamidino-2-phenylindole in blue). More PH3 positive cells are observed in flies fed on control diet than (**C**) the flies fed on GMH-supplemented diet at Day 50; (**B**) Quantification of PH3 positive cells in midguts from flies fed on either control or GMH-supplemented food at different time points. (**D**) Measurement of the midgut length from flies fed on either control or GMH-supplemented food at different time points. Flies used are mated females of strain B18. For panel (B) and (D), $n = 3$ biological replicates with 10 individual flies per replicate. Abbreviation: GMH = glucomannan hydrolysate.

We carried out a mRNA-sequencing experiment using RNA isolated from the midguts of flies fed on control or GMH food for 10, 30, and 50 days. Overall, we observed significant effects of both age and diet on gene expression (Supplementary Figure 4). Regarding the effect of the diet, we found 83 genes differentially expressed in guts on Day 10 (Supplementary Figure 5A), 109 genes on Day 30 (Figure 5A), and 50 genes on Day 50 (Supplementary Figure 5B). Only a small proportion of the differentially expressed genes overlapped across different time points (Figure 5C). It is known that several growth and stress-signaling pathways are involved in regulating ISCs proliferation rate, including MAPK, EGFR, JNK, and JAK/STAT pathways. Therefore, we mainly focused on genes related to these pathways. In accordance with previous studies (43,44), we found significant upregulation of stress-signaling pathways from Days 30 to 50 in both control and GMH groups (Supplementary Figure 6), confirming the age-associated loss of gut homeostasis. However, GMH supplementation slowed down this overall trend. In comparison with controls, multiple genes in both JAK/STAT and EGFR/MAPK pathways were downregulated in GMH groups (Figure 5B, Supplementary Table 1). Genes that encode two key ligands in the JAK/STAT pathway, unpaired2 (*Upd2*) and unpaired3 (*Upd3*), were both significantly downregulated on Day 50. *Upd3* was also significantly downregulated on Day 30. The negative feedback regulator of JAK/STAT pathway, *Socs36E*, was downregulated at all three time points. Moreover, *Ets21c*, a downstream effector of the EGFR/MAPK pathway, was downregulated in GMH groups at both Days 10 and 30. The phosphatase MAPK phosphatase 3 (*Mkp3*), a negative regulator of the EGFR pathway, was downregulated at Day 10. Another positive regulator of EGFR pathway, rhomboid (*Rho*), was downregulated at both Days

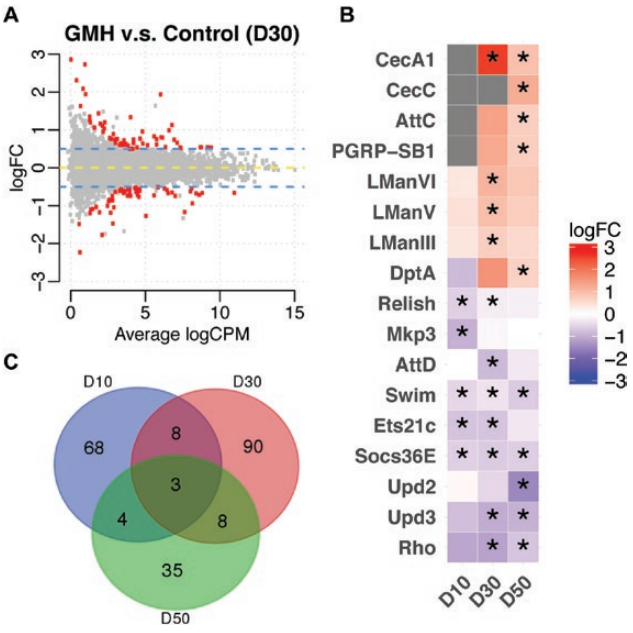

Figure 5. Midgut transcriptome analysis shows differences in gene expression levels between control and GMH treatment groups. (**A**) MA plot shows the significantly differentially expressed genes between control and GMH groups on Day 30; (**B**) heat map shows expression levels of selected genes of interest across three time points, * indicates FDR < 0.05. Genes with low expression levels in half of the samples or more were not included in the differential expression analysis and indicated as grey here; (**C**) Venn diagram of differentially expressed genes at three time points. Flies used are mated females of strain B18. Abbreviation: GMH = glucomannan hydrolysate.

30 and 50. Taken together, the suppression of both JAK/STAT and EGFR/MAPK pathways might explain our observation of reduced ISC proliferation rate in GMH-treated flies. Furthermore, consistent with our observations of elevated gut bacterial load, several antimicrobial peptide (AMP)-encoded genes were upregulated in GMH groups at Days 30 or 50, including Cecropin A1 (*CecA1*), Cecropin C (*CecC*), Attacin C (*AttC*), Diptericin A (*DptA*), with only one exception, Attacin D (*AttD*), which was downregulated at Day 30 (Figure 5B). Additionally, as GMH is composed of glucose and mannose monomers, three genes that encode lysosomal α-mannosidase were all upregulated on Day 30, further suggesting that GMH or its metabolites were absorbed by the gut. For pathway enrichment analysis, although only one pathway survived Bonferroni correction, it showed a consistent pattern that differentially expressed genes are enriched in the above-mentioned pathways (Supplementary Table 2). Overall, the gut RNA-Seq results revealed that GMH supplementation decreases both JAK/STAT and EGFR pathways and promotes pathways in AMP-involved immune responses, suggesting an impact on gut homeostasis.

Discussion

In this study, we investigated the effect of life-long GMH supplementation on the life span of *D melanogaster*. We found that in the genetic background of B18, GMH can significantly extend the life span of virgin female flies, mated female flies, and mated male flies, suggesting that the effect of GMH in B18 is not affected by sex or mating status. To examine whether genetic backgrounds have influences on GMH's effect, we repeated the experiment using mated females from different wild-type strains, including Oregon-R, Canton-S, and six strains from the DGRP. GMH had a life-extending effect on all but one strains. Although the effect of GMH is statistically significant across most conditions tested, we want to stress that the exact numbers of percent life span extended might not be accurate estimates because each of them was from a single survival assay. Indeed, one independent survival assay using virgin B18 females yielded a significant but smaller life-span extension (17.44%) than the previous estimate (27.48%, Supplementary Figure 1).

As the rate of aging is determined by both environmental and genetic factors in *Drosophila* (14), we will discuss the life-span–extending effect of GMH from both perspectives. First, environmental factors such as diet, mating status, and fly density are well controlled in our experiment. To further control for *Wolbachia* infection status (14), we chose three *Wolbachia*-positive and three *Wolbachia*-negative strains from DGRP. Among the five strains that were significantly responsive to GMH treatment, three were *Wolbachia*-positive and the other two were not. Therefore, *Wolbachia* infection status does not explain the differences we observed. Second, although the six DGRP strains are from the same species, they differ genetically from each other. Each homozygous strain in DGRP captures a different genome sampled in the natural population (28). The varying response to GMH supplementation suggests the presence of genetic variations moderating its life-span–extending effect.

We found that GMH treatment is associated with elevated bacterial load of *Acetobacter* in comparison with the control group, suggesting that it may have prebiotic potentials, although additional confirmative evidence is needed. In young and healthy intestines of *D melanogaster*, the composition of the gut microbiota is relatively simple with only 5–20 species (43). Major members of the microbiota are benign microbes like *Acetobacter*. Multiple lines of evidence have suggested that *D melanogaster* has evolved the ability to distinguish beneficial microbes from pathogenic ones so that they can prevent the deleterious induction of immune responses under basal conditions (22,44). In the aging intestine, there is an increase of both the number and the diversity of microorganisms (17,39), termed dysbiosis. This dysbiosis may further exacerbate the age-associated loss of gut homeostasis by chronic activation of stress-signaling pathways and lead to life-span shortening (13). However, unlike pathogenic microbes which trigger the stress-signaling pathways and overly stimulate ISCs proliferation, *Acetobacter* only activates the immune responses at a basal level (43). Increased abundance of *Acetobacter* may also outcompete the growth of pathogenic species. Therefore, GMH-mediated increase in the abundance of *Acetobacter* may exert beneficial effects on life span by reducing the pressure from age-associated dysbiosis. At the same time, we also implemented 16S rRNA sequencing to investigate the relative composition pattern of the overall gut microbiome after GMH treatment. Apparently, time has a profound effect on gut microbiota composition, with a shift toward higher relative abundance of *Acetobacter* in older flies of both control and GMH treatment groups. This result is consistent with what we observed in the bacterial load experiment and also with previous studies (45). It was hypothesized that the gut becomes more oxic in old flies, promoting the growth of aerobic bacteria such as *Acetobactor*, but not anaerobic bacteria like *Lactobacillus* (45). However, GMH only has limited effects on the overall gut microbiota composition, suggesting that the impact of GMH might mainly be on the absolute quantity of gut microbes. It is well known that *Drosophila* gut microbiota is greatly influenced by microbes in the environment, especially food (35,39,46). Therefore, there are multiple possible mechanisms underlying the GMH-associated bacterial load increase, including bacterial growth in the gut, preferential ingestion or retention of specific bacteria, and increased bacterial growth on the food. Further experiments, especially those with axenic or gnotobiotic flies, are needed to confirm and to elucidate the effect of GMH on gut microbiota.

Our results suggest that GMH supplementation has a significant impact on ISC proliferation. As the gut undergoes constant self-renewal, ISC proliferation is required for both normal tissue turnover and epithelial recoveries after damage or infection to maintain intestinal homeostasis in *Drosophila* (47). Generally, low levels of ISC proliferation are observed in young and healthy guts, while strong regenerative activity is more common when epithelial cells are faced with increased stress and damage at later life stages. As a result of ISCs hyperproliferation in the aging intestine, disruption and perturbation of normal intestinal organization and function may ultimately lead to death of the animals. Therefore, the life-span–extending effect of GMH supplementation is at least partially via the preservation of gut proliferative homeostasis in *Drosophila*. However, the underlying mechanism is still unknown. It is well established that ISC proliferation rates are regulated by several growth and stress-signaling pathways, including MAPK, EGFR, JAK/STAT, and JNK pathways. From the result of global gene expression analysis of *Drosophila* midgut, we found that both growth and stress-signaling pathways are altered after GMH supplementation. EGFR and MAPK pathways are essential in controlling the rate of ISC proliferation in homeostatic conditions (27). Our results showed that genes in EGFR/MAPK pathways are downregulated in GMH groups, suggesting that ISC proliferation is inhibited in homeostatic conditions. Under stress conditions, JAK/STAT pathway is activated to promote the differentiation and proliferation of ISCs (22,25). Our results revealed that genes in JAK/STAT pathway are also downregulated in GMH groups, suggesting that there is less stress and damage in the

intestines of flies with GMH supplementations or that the activity of these pathways is directly targeted by GMH. Accordingly, lower stress levels in GMH-supplemented flies may explain the reduced shrinking of guts in these flies. It is hypothesized that the deregulation of the interaction between intestinal epithelium and the gut microbiome causes age-related decline of proliferative homeostasis. Therefore, the suppression of JAK/STAT pathway could be a secondary consequence of the increased abundance of *Acetobacter* in the aging intestine, which would prevent overgrowth of more damaging bacteria. Considered together, the improving effect of GMH on gut proliferative homeostasis is likely through both growth and stress-signaling pathways. In this study, we are unable to elucidate the relationship between improved proliferative homeostasis and regulated bacterial load. Future experiments to disentangle their interactions should evaluate the life-span–extending effect of GMH under axenic or germ-free conditions.

Beyond the tightly controlled ISC proliferation, other factors like host immune homeostasis also play a vital role in maintaining intestine homeostasis (13,44). Evidences from RNA-Seq suggested that genes that encode AMPs are significantly upregulated in GMH groups when flies grow old. Both our study and previous studies showed that the number of bacteria found in the gut increases significantly in old flies (39), and the impairment of the ability to manage the overgrowth of bacteria in aged flies appears to be another potential cause of death (16). One of the strategies to control the growth of the gut microbiota and pathogens is to activate the Imd (immune deficiency) pathway to induce the expression of AMPs (22,48). It is noteworthy that other studies also demonstrated that GMH has the ability to enhance immune systems directly by stimulating the gut-associated lymphoid tissue system (49) or indirectly by inhibiting the adhesion of pathogens to epithelial cells (50). Given the fact that we observed increased bacterial load in the GMH treatment group, it is possible that elevated expression level of AMPs is related with the changes in gut microbiome. However, further studies are still needed to elucidate the interaction between gut microbiome and immune responses after GMH treatment. While our study has focused on the homeostasis of gut renewal, microbiota, and immune responses, other potential life-span–extending mechanisms, such as nutrient absorption, are worth exploring in the future. Although our long-term feeding assay suggests that flies have similar relative food intake under control and GMH-supplemented diets, due to the limit of our method in measuring only relative intake, rather than absolute intake, and the small sample size in our assay, additional experiments with separate methods and a larger sample size are also needed to evaluate the role of dietary restriction (14).

As a natural compound, GMH has been shown to exert beneficial effects both locally, by promoting the growth of probiotics, and systematically, by lowering serum cholesterol and glucose levels. For the first time, we demonstrate that GMH supplementation can extend the life span of *D melanogaster*. Furthermore, our study indicates that GMH intervention prolongs life span by preserving gut proliferative homeostasis at later life stages. This study provides insights for future studies to investigate the life-span extension effect of GMH in other organisms.

Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None reported.

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