

Lactiplantibacillus brownii strain MH-1 prolongs healthspan and improves stress resistance in *Caenorhabditis elegans*

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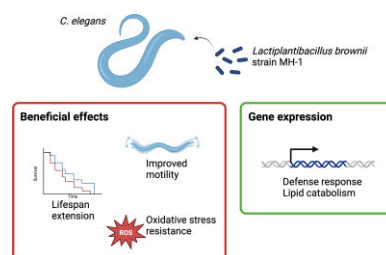
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Abstract

Lactiplantibacillus brownii was first discovered in 2023. We previously isolated *L. brownii* strain MH-1 from homemade pickled Chinese cabbage in Hiroshima Prefecture, Japan, and performed whole-genome sequencing. In this study, we examined the beneficial effects of *L. brownii* strain MH-1 on a host using *C. elegans*. MH-1 and heat-killed MH-1 extended the lifespan and also alleviated motility decline during aging in worms. Feeding MH-1 to nematodes indicated its presence in the intestinal tract with no change in body or brood sizes, suggesting that dietary restrictions did not occur. There were no significant differences in intestinal barrier enhancement or fat accumulation in MH-1-fed *C. elegans*. The stress response of *C. elegans* was enhanced by MH-1 for oxidative stress tolerance. MH-1 increased the expression of genes involved in defense response and lipid catabolism. These results demonstrate the beneficial effects of MH-1 on nematodes and its potential use as a probiotic.

Keywords: *Lactiplantibacillus brownii*, *Caenorhabditis elegans*, healthspan, motility, oxidative stress resistance

Graphical abstract



Lactiplantibacillus brownii strain MH-1 exerts beneficial effects including the extension of the lifespan and healthspan in the model animal *C. elegans*.

Many traditional foods worldwide have been developed through fermentation to delay spoilage and ensure preservation. Many fermented foods contain lactic acid bacteria (LAB), and many strains have been isolated from these foods. Fermented foods containing LAB are recognized as foods that are beneficial to human health.

The genus *Lactobacillus*, a LAB, is composed of extremely diverse species at the genotype level, and *Lactiplantibacillus* was defined as a new genus in 2020 (Zheng et al. 2020). This genus currently includes 20 species, and the type species is *Lactiplantibacillus plantarum*. Some *Lactiplantibacillus* strains possess beneficial properties. *Lactiplantibacillus plantarum* OLL2712 ingestion has protective effects against memory function decline in older adults (Sakurai et al. 2022). Oral administration of *Lactiplantibacillus pentosus* P2020 significantly decreased serum uric acid levels and reduced renal inflammatory response in mice (Wang et al. 2023). Heat-killed

Lactiplantibacillus argenteratensis BBLB001 significantly ameliorated dextran sodium sulfate-induced colitis in mice (Itoh et al. 2024).

Lactiplantibacillus brownii is a new species discovered in sauerkraut (fermented cabbage) collected from local markets in Moscow, Russia, and was first reported in 2023 (Heng et al. 2023). Previously, we isolated the *L. brownii* strain MH-1 from homemade pickled Chinese cabbage in Hiroshima Prefecture, Japan, and performed whole-genome sequencing (Hashimoto et al. 2024).

Caenorhabditis elegans has been extensively utilized as an animal model for aging research because of its many benefits, such as a short lifespan, a completely sequenced genome, and gene homologs to 74% of human genes (*C. elegans* Sequencing Consortium 1998). Additionally, *C. elegans* has been used to evaluate the effectiveness of probiotics, to assess pathogenicity and elucidate its mechanisms, and as a model for neurodegenerative dis-

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eases and obesity research (Alegado et al. 2003; Caldwell, Willcott and Caldwell 2020; Poupet et al. 2020; Yue et al. 2021). We have previously explored the beneficial effects and mechanisms of prospective probiotics using *C. elegans* as the model host. *Lactococcus kimuchii* alleviates motility decline via the insulin signaling pathway (Takeuchi et al. 2024). *Bacillus subtilis* var. *natto* increased stress resistance (Teramoto et al. 2023). *Clostridium butyricum* improved locomotion and resistance to stressors (Kato et al. 2018). All the above bacteria also exhibited extended lifespans in *C. elegans*.

With the short time since the recognition of *L. brownii* as a new species, its effects on *C. elegans* are unknown. In this study, we aimed to examine the beneficial effects of *L. brownii* strain MH-1 on a host, using *C. elegans* as the model.

Materials and methods

Bacterial strains and culture conditions

Escherichia coli OP50 (OP) (used as the standard diet for *Caenorhabditis elegans*) was cultured on tryptone soya medium (Nissui Pharmaceutical, Tokyo, Japan) at 37 °C overnight in static culture. *Lactiplantibacillus brownii* strain MH-1 (MH-1) isolated from homemade pickled Chinese cabbage made in Japan (Hashimoto et al. 2024) was cultured on Lactobacilli de Man, Rogosa, and Sharpe (MRS) agar (Difco BD, Sparks, MD) for 48 h anaerobically and Lactobacilli MRS broth (Difco BD) overnight in static culture at 30 °C. Each bacterium cultured on medium plates was scrapped and suspended in M9 buffer (3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 5 g/L NaCl, 1 mM MgSO₄) (10 mg wet weight/50 µL M9 buffer). Precisely 50 µL of the bacterial suspension was spread on peptone-free modified nematode growth media (mNGM, NGM without peptone) plates in 5.5-cm-diameter plates to feed the worms.

Caenorhabditis elegans strains and culture conditions

Caenorhabditis elegans strain Bristol N2 (wild-type) was obtained from the Caenorhabditis Genetics Center, University of Minnesota. The nematodes were maintained using standard techniques (Brenner 1974). The mature nematodes were exposed to a mixture of sodium hypochlorite and sodium hydroxide to obtain their eggs. The egg suspension was incubated in an M9 buffer at 25 °C for 1 day to encourage hatching and synchronization. A suspension of synchronized L1-stage worms was centrifuged at 156× *g* for 1 min. After aspirating the supernatant, the remaining larvae were transferred to mNGM plates covered with 10 mg of bacteria and used for experimental assays. All *C. elegans* experiments were conducted at 25 °C.

Lifespan assay

Synchronized L1-stage worms were cultured on mNGM plates covered with 10 mg OP for 2 days until they reached 3 days old. The 3-day-old worms were then placed on 5.5-cm mNGM plates covered with either 10 mg OP alone, a mixture of 3 mg OP and 7 mg MH-1, or heat-killed MH-1 (HK-MH-1) (35 worms per plate). HK-MH-1 was prepared by heating MH-1 at 90 °C for 10 min in a water bath. The worms were transferred to fresh plates daily during the reproductive period and every other day thereafter. The numbers of live and dead worms were counted daily. Worms were considered dead if they failed to respond to gentle touch with the worm picker. Worms that disappeared from the plate or died during internal hatching were considered lost and excluded from the analysis. The assay was performed in duplicate, and the replicated

data were merged and presented. Worm survival was calculated using the Kaplan–Meier method.

The mean lifespan (MLS) was estimated using the following formula (Wu et al. 2006):

$$MLS = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} d_j$$

where d_j is the number of worms that died in the age interval (x_j to x_{j+1}), and N is the total number of worms. The standard error (SE) of the estimated MLS was calculated using the following equation:

$$SE = \sqrt{\frac{1}{N(N-1)} \sum_j \left(\frac{x_j + x_{j+1}}{2} - MLS \right)^2 d_j}$$

The maximum lifespan was determined as the MLS of the longest-living 15% of worms in each group.

Measurement of locomotion

The 3-day-old worms cultured on OP plates were placed on mNGM plates covered with OP alone or with a 3:7 mixture of OP and MH-1 (or HK-MH-1). Worm motility was examined daily and scored using a previously described method (Gruber et al. 2011; Komura et al. 2013). Briefly, worms were categorized as “class A” when they moved vigorously or spontaneously in response to prodding, “class B” when they had uncoordinated movement or did not move unless prodded, and “class C” when they only moved their heads and/or tails in response to prodding. Dead worms were categorized as “class D.” The assay was performed in duplicate or triplicate, and the merged data were statistically analyzed.

Observation of intestinal MH-1 in C. elegans

MH-1 in the intestinal tract of *C. elegans* was observed using a previously described method with slight modifications (Takeuchi et al. 2024). Briefly, MH-1 cells scrapped from MRS agar were suspended in 10 times of phosphate-buffered saline (PBS) containing 0.01% fluorescein isothiocyanate (FITC; Dojindo, Kumamoto, Japan) and incubated for 1 h at 30 °C. The cells were washed 5 times with PBS and resuspended in M9 buffer (10 mg wet weight/50 µL M9 buffer). The 3-day-old worms cultured on OP plates were placed on mNGM plates covered with either OP alone, FITC solution, or a 3:7 mixture of OP and FITC-labeled MH-1. The plates were incubated for 1 h, and fluorescence was observed using a BX53 microscope (Olympus, Tokyo, Japan).

Measurement of body size

The 3-day-old worms cultured on OP plates were placed on mNGM plates covered with either OP alone or a 3:7 mixture of OP and MH-1. The body sizes of the worms were measured at the ages of 5, 7, and 11 days. Images of mature worms were captured using a BZ-X800 microscope (Keyence, Tokyo, Japan), and the area of worms was measured using Hybrid Cell Count software (BZ-H3C, Keyence). The projection area of the worm was used as an index of body size.

Measurement of brood size

The 3-day-old worms cultured on OP plates were placed on mNGM plates covered with OP alone or with a 3:7 mixture of OP and MH-1. The parent worms were transferred to fresh mNGM plates daily until the end of the reproductive period. The eggs remaining on the plates were hatched, and the number of progenies was counted.

Smurf assay for intestinal barrier function

The integrity of the intestinal barrier function was examined using a previously described method (Kim et al. 2020) with some modifications. The 3-day-old worms cultivated on OP plates were placed on mNGM plates covered with OP alone or a 3:7 mixture of OP and MH-1 until they reached 11 days. Then, the worms were transferred to an NGM liquid medium containing HK-OP50 mixed with 5% acid blue 9 as a blue food dye (Tokyo Chemical Industry Co., Tokyo, Japan) at 25 °C for 3 h. The worms were washed with M9 buffer until the blue color of the dye was invisible. The worms were observed on a glass slide with 50 mM sodium azide in the presence of a blue dye in the body cavity using a BX53 microscope (Olympus). Subsequently, the stained worms were counted to determine the body cavity leakage.

Lipid staining

Lipid staining was performed using a previously described method with some modifications (Gusarov et al. 2017). Briefly, 3-day-old worms cultured on OP plates were placed on mNGM plates covered with OP alone or a 3:7 mixture of OP and MH-1 until they became 7 days old. Subsequently, the worms were washed with M9 buffer and fixed with 50% isopropanol in PBS for 15 min on ice. Oil red O stock solution (0.5 g/100 mL in isopropanol; Sigma-Aldrich, St. Louis, MO) was diluted in distilled water to a 60% working solution and filtered through a 0.2 µm membrane filter. Fixed worms were incubated in the working solution at 25 °C for 20 min. After removing the working solution, the stained worms were washed thrice with M9 buffer containing 0.5% Triton X-100. The worms were then placed on glass slides and imaged using a BX53 microscope (Olympus). The dye intensity was measured using ImageJ software (Yen et al. 2010).

Oxidative stress resistance assay

The 3-day-old worms cultured on OP plates were placed on mNGM plates covered with OP alone or a 3:7 mixture of OP and MH-1 until they reached 9 days old, after which they were subjected to a stress-resistance assay. For the oxidative stress assay, 9-day-old worms were transferred to mNGM plates containing 2 mM paraquat. The number of live and dead worms was counted daily. Similarly, the worms were transferred to M9 buffer containing 2 mM hydrogen peroxide and scored for viability hourly. Worms were considered dead if they failed to respond to repeated touches with a worm picker.

RNA extraction and sequencing

The 3-day-old worms cultured on OP plates were placed on mNGM plates covered with OP alone or a 3:7 mixture of OP and MH-1 for 1 day until they reached 4 days old. Approximately 200 worms per group ($n = 3$) were collected and washed at least thrice with an M9 buffer containing 0.2% gelatin. The worms were soaked in RNeasy Protect Tissue Reagent (Qiagen, Hilden, Germany) and stored at -80 °C until RNA extraction. Thawed samples were ground using a microtube pestle (Scientific Specialties, Inc., Lodi, CA) and homogenized using TRIzol (Thermo Fisher Scientific, Waltham, MA). Total RNA was isolated using an RNeasy Mini Kit (Qiagen). RNA sequencing was performed by DANA FORM (Kanagawa, Japan) using 2 out of every 3 samples as follows: RNA-seq libraries prepared using the SMARTer Stranded RNA-Seq Kit (Clontech) were run on a NovaSeq 6000 System (Illumina Inc., San Diego, CA) with 150 bp paired-end reads. The quality of RNA-seq results was assessed using FastQC (version 0.11.9). The raw

reads obtained were trimmed and quality-filtered using Trim Galore! (version 0.6.7), Trimmomatic (version 0.39) (Bolger, Lohse and Usadel 2014), and Cutadapt (version 3.7). Trimmed reads were mapped to the N2 *C. elegans* reference genome cell (WBcel235.105) using STAR (version 2.7.9a) (Dobin et al. 2013). Reads on the annotated genes were counted using featureCounts (version 2.0.1) (Liao, Smyth and Shi 2014). Quantitative differential expression analysis between conditions was performed using the RNA-seq Analysis Portal (Qiagen) to compare OP-fed and OP + MH-1-fed groups. The upregulated genes were defined as fold change >2 and FDR P -value < .05 (Table S1); the downregulated genes were defined as fold change < -2 and FDR P -value < .05 (Table S2). Heat maps were generated with these genes using RNA-seq Analysis Portal (Qiagen). The upregulated genes were subjected to enrichment analysis based on their gene ontology (GO) categories for biological process (BP), molecular function, and cellular components using Metascape (Zhou et al. 2019) (Table S3). The dataset is accessible through the Gene Expression Omnibus with the accession number GSE261495.

Reverse transcription and real-time polymerase chain reaction (PCR)

Removal of genomic DNA and cDNA synthesis were performed using a QuantiTect Reverse Transcription Kit (Qiagen) with the RNA obtained as described above. Real-time quantitative polymerase chain reaction (PCR) was conducted using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) with the following parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples from three biological replicates were analyzed. Relative mRNA expression was determined using the cycle threshold ($\Delta\Delta C_t$) method (Livak and Schmittgen 2001) and normalized by the expression of the housekeeping genes *cyc-1*, *tba-1*, and *act-1*. The primers used for real-time PCR are listed in Table S4.

Statistical analysis

The log-rank (Mantel-Cox) test and unpaired 2-tailed t -test were used for statistical analyses using GraphPad Prism 8 software (Ver. 8.4.3, GraphPad, San Diego, CA).

Results

MH-1 increased the motility of *C. elegans*

We first examined whether MH-1 feeding not only prolonged lifespan but also alleviated the motility decline during aging in *C. elegans*. Previous reports revealed that feeding *C. elegans* MH-1 alone had no effect on lifespan extension and that *C. elegans* avoided MH-1 lawn. To minimize the effect of OP in the mixed feeding of OP and MH-1, 10 mg OP and 3 mg OP feeding were compared and no difference in lifespan was observed (Hashimoto and Shimizu 2020). Therefore, the worms were fed a 3:7 mixture of 3 mg OP and 7 mg MH-1 (OP + MH-1). The synchronized worms were fed OP until they matured into 3-day-old adult worms, which were then divided into 2 groups that were fed OP alone or OP + MH-1 (Figure 1a). Worms fed OP + MH-1 had extended lifespans compared to those fed OP alone (Figure 1b). The motility scores of worms in the OP + MH-1 group were higher than those in the OP group (Figure 1c and d). The proportion of class A worms that exhibited vigorous or spontaneous locomotion was significantly higher in the OP + MH-1 group than in the OP group (Figure 1e).

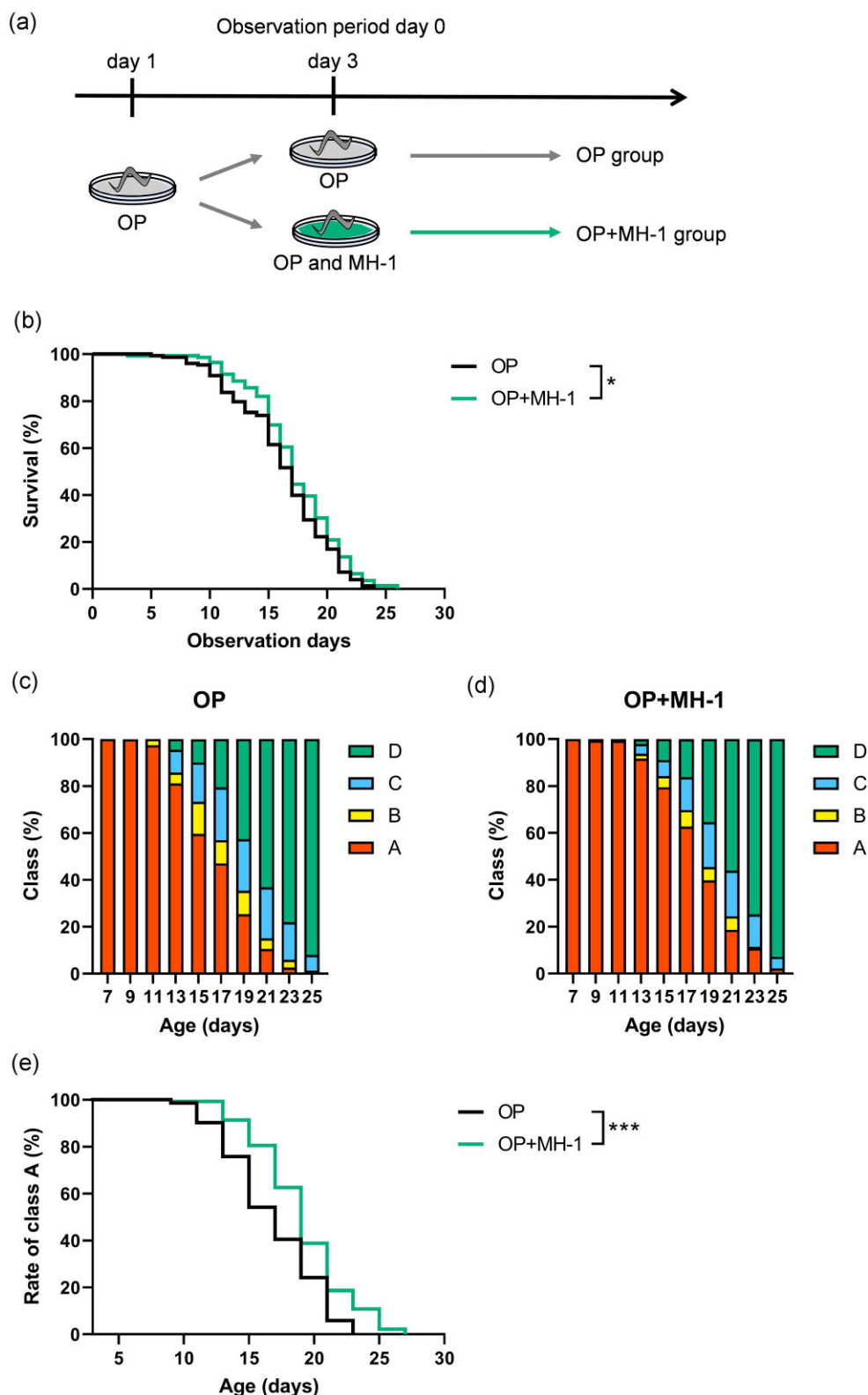


Figure 1. MH-1 feeding increased the lifespan and reduced the motility decline during aging in *Caenorhabditis elegans*. (a) Diagram illustrating the survival assay and locomotor activity test. The synchronized worms were fed *Escherichia coli* OP50 (OP) until they matured into 3-day-old adult worms. The worms were then divided into 2 groups: one group was fed OP alone, while the other group was fed a 3:7 mixture of OP and *Lactiplantibacillus brownii* MH-1 (OP + MH-1). (b) Survival curves of *C. elegans* fed OP and OP + MH-1. Day 0 of the observation period was set as the day the worms were 3 days old. Survival rates were calculated using the Kaplan–Meier method and compared with the control OP group using the log-rank test. * $P < .05$; OP, $n = 153$; OP + MH-1, $n = 139$. Table S5 provides detailed lifespan data and statistics. (c, d) Motility of *C. elegans* fed OP (c) and OP + MH-1 (d). Worms were grouped into four classes based on their locomotion: class A, robust and coordinated sinusoidal locomotion (red bars); class B, uncoordinated and/or sluggish movement (yellow bars); class C, no forward or backward movement but head movements or shuddering in response to prodding (blue bars); and class D, dead worms (green bars). (e) Healthspan curves of *C. elegans* fed OP and OP + MH-1. The proportion of class A worms was plotted. Class A proportions were calculated using the Kaplan–Meier method and compared with the control OP group using the log-rank test. *** $P < .001$.

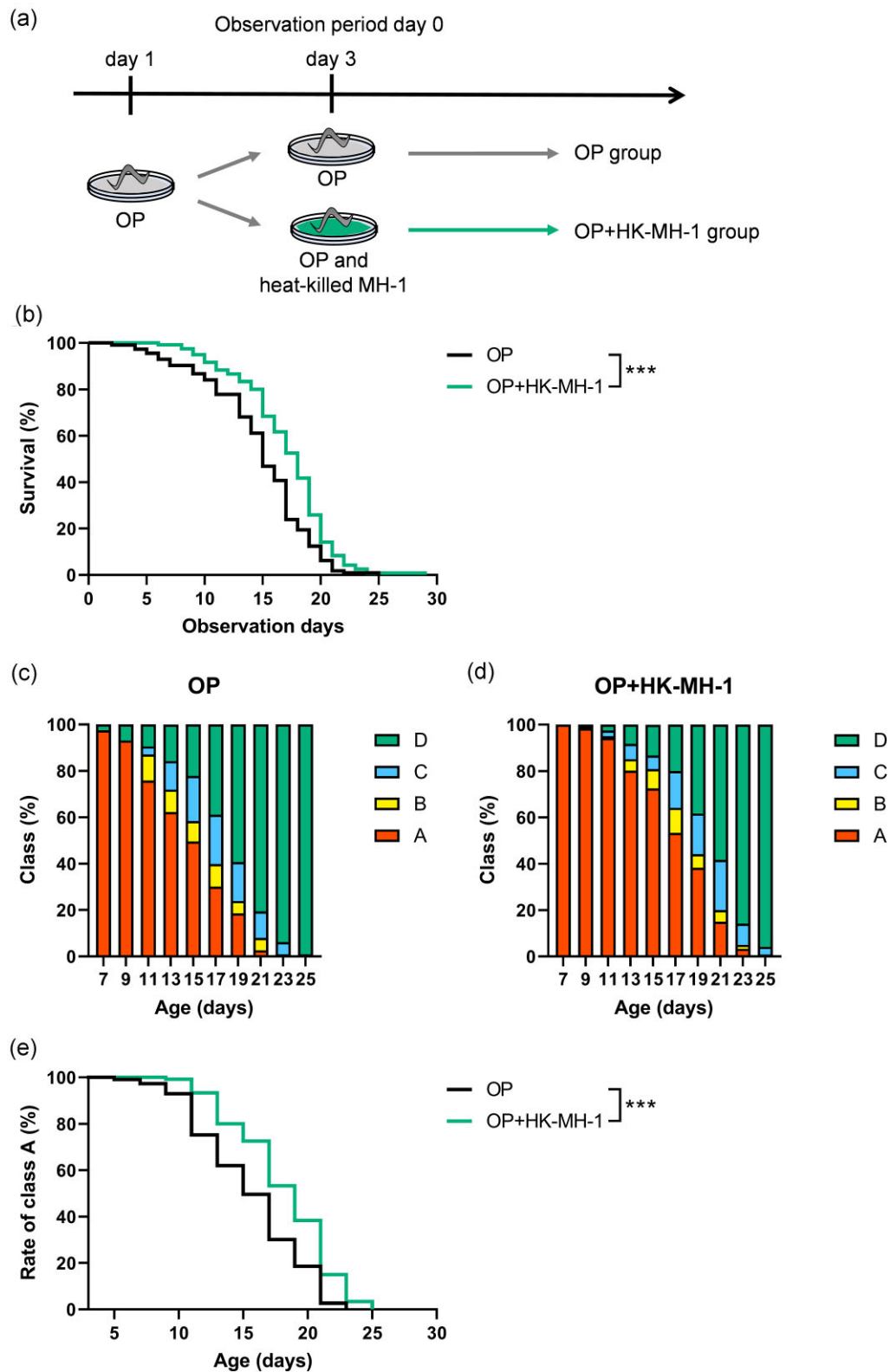


Figure 2. Heat-killed MH-1 (HK-MH-1) extended the lifespan and healthspan of *C. elegans*. (a) Diagram illustrating the survival assay and locomotor activity test. The synchronized worms were fed OP until they matured to 3-day-old adult worms and were subsequently fed OP alone or a 3:7 mixture of OP and HK-MH-1. (b) Survival curves of *C. elegans* fed OP and OP + HK-MH-1. Day 0 of the observation period was set as the day the worms were 3 days old. Survival rates were calculated using the Kaplan–Meier method and compared with the control OP group using the log-rank test. ***P < .001; OP, n = 113; OP + HK-MH-1, n = 120. [Table S5](#) provides detailed lifespan data and statistics. (c, d) Motility classification of *C. elegans* fed OP (c) and OP + HK-MH-1 (d). (e) Healthspan curves of *C. elegans* fed OP and OP + HK-MH-1. The proportion of class A worms was plotted. Class A proportions were calculated using the Kaplan–Meier method and compared with the control OP group using the log-rank test. ***P < .001.

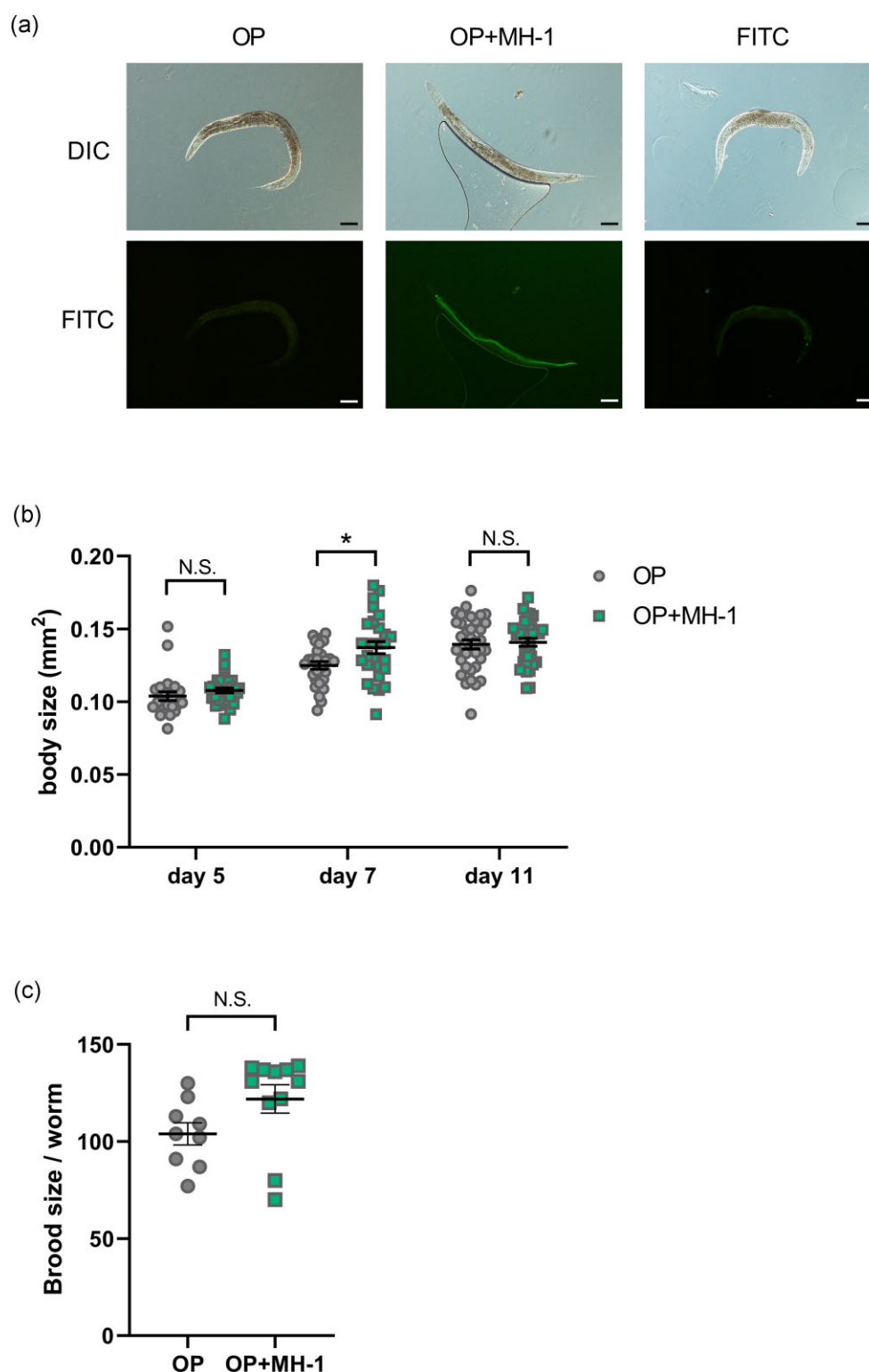


Figure 3. MH-1 did not induce negative effects caused by dietary restriction in *C. elegans*. (a) Microscopy images of *C. elegans* fed OP, OP + FITC-labeled MH-1, or FITC solution only. Scale bars, 100 μ m; DIC, differential interference contrast. (b) Body sizes of worms fed OP or OP + MH-1 at 5, 7, and 11 days. Day 5: OP, $n = 24$; OP + MH-1, $n = 32$. Day 7: OP, $n = 30$; OP + MH-1, $n = 28$. Day 11: OP, $n = 36$; OP + MH-1, $n = 33$. (c) Brood size of worms fed OP or OP + MH-1. OP, $n = 9$; OP + MH-1, $n = 11$. All values are presented as individual plots and means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using an unpaired 2-tailed t -test. * $P < .05$; N.S., not significant.

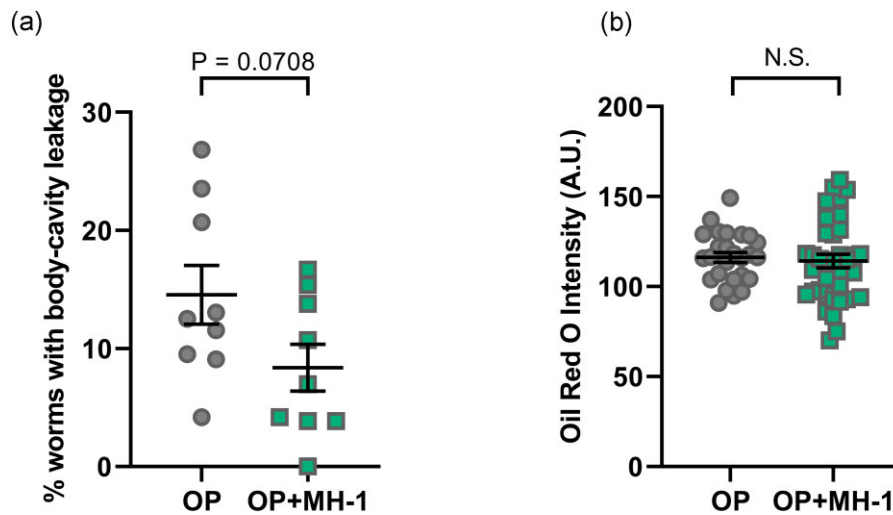


Figure 4. Effect of MH-1 feeding on intestinal barrier function and fat accumulation in *C. elegans*. (a) Percentage of worms with body-cavity leakage fed OP or OP + MH-1 at 11 days. (b) Quantification of red dye intensity based on the projection area of the worm body fed OP or OP + MH-1 at 7 days. OP, $n = 26$; OP + MH-1, $n = 37$. All results are presented as individual plots and means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using an unpaired 2-tailed t-test. N.S., not significant.

Lifespan and motility of *C. elegans* fed HK-MH-1

Next, we investigated the effects of HK-MH-1 on lifespan and motility (Figure 2a). Worms fed OP + HK-MH-1 had longer lifespans compared to those fed OP alone (Figure 2b). The proportion of class A worms was significantly higher in the OP + HK-MH-1 group than in the OP group (Figure 2c-e).

Nutritional status of *C. elegans* following feeding with MH-1

To determine whether MH-1 induces negative effects that are typically caused by dietary restriction, we examined the dietary intake, body size, and brood size of the worms. FITC-labeled MH-1 was fed to *C. elegans* to identify intestinal MH-1 in the gut. While FITC fluorescence was detected in the intestines of worms fed OP + FITC-labeled MH-1, little fluorescence was detected in the intestines of worms fed OP only and FITC solution only (Figure 3a). The body size of worms fed OP + MH-1 was larger than that of worms fed OP alone at 7 days. In contrast, there was no significant difference between the OP and OP + MH-1 groups at 5 and 11 days (Figure 3b). The brood size of the worms fed OP + MH-1 did not significantly differ from that of worms fed OP alone (Figure 3c).

Integrity of intestinal barrier function with MH-1 feeding

We investigated whether MH-1 improves the intestinal barrier function in worms. The percentage of worms fed OP + MH-1 with body-cavity leakage was not significantly different from that of the control group; however, there was a decreasing trend (Figure 4a).

Lipid accumulation in *C. elegans* fed MH-1

We examined lipid accumulation in worms fed MH-1. Oil Red O staining showed no significant difference in dye intensity between worms fed OP + MH-1 and those fed OP alone (Figure 4b).

Stress resistance in *C. elegans* fed MH-1

To investigate the effect of MH-1 feeding on stress resistance, the worms were exposed to oxidative stress (Figure 5a). Worms fed OP + MH-1 lived significantly longer than those fed OP alone in the presence of paraquat or H_2O_2 , which produced reactive oxygen species in the cells (Figure 5b and c).

Analysis of gene expressions regulated by MH-1 feeding

We performed RNA sequencing to identify the genes regulated by MH-1. A total of 54 genes were upregulated in the OP + MH-1 group compared with the OP group, with fold change >2 and FDR P -value $<.05$ (Table S1), whereas 317 genes were downregulated in the OP + MH-1 group, with fold change <-2 and FDR P -value $<.05$ (Table S2 and Figure 6a). The GO term for biological processes that were enriched in genes upregulated in the OP + MH-1 group was “lipid catabolic process,” “defense response to other organisms,” and “fatty acid metabolic process” (Figure 6b and Table S3). The expression levels of genes related to these 3 GO terms were evaluated using real-time PCR (Figure S1). The mRNA levels of *C32H11.4*, *cpt-5*, and *ech-1.1* were significantly higher in the OP + MH-1 group than in the OP group among the 20 genes (Figure 6c).

Discussion

In this study, we investigated the potential beneficial effects of the *Lactiplantibacillus brownii* strain MH-1, isolated from Japanese pickles, on *C. elegans* as a model host.

MH-1 and HK-MH-1 extended the lifespan and alleviated the motility decline during aging in worms. Because nematodes avoided MH-1 lawn alone, we evaluated it by mixed feeding with OP. Reducing OP to 3 mg had no effect on nematode longevity (Hashimoto and Shimizu 2020). We used peptone-free modified NGM, which does not induce much OP pathogenicity. In fact, we found no difference between viable and dead OP in our previous report (Kato et al. 2018). These reports suggest that the virulence

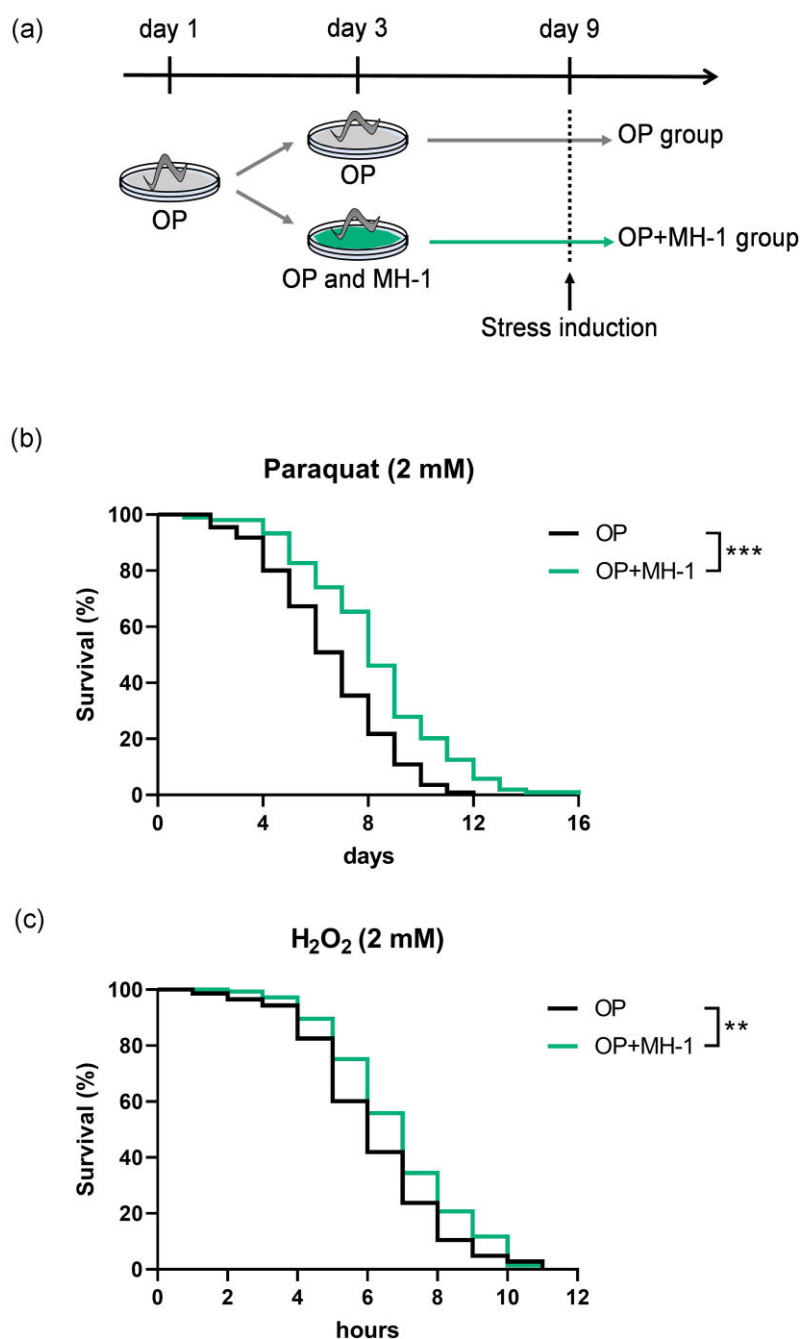


Figure 5. Evaluation of stress resistance in *C. elegans* fed MH-1. (a) Diagram illustrating the stress resistance assay. The synchronized worms were fed OP until they matured to 3-day-old adult worms and were subsequently fed OP alone or a 3:7 mixture of OP and MH-1 for 6 days until they were 9 days old. The 9-day-old worms were then exposed to stress. Day 0 of the graph was set as the first day that worms were exposed to stress. (b, c) Susceptibility of worms fed MH-1 to paraquat (b), hydrogen peroxide (c). (b) OP, $n = 110$; OP + MH-1, $n = 104$. (c) OP, $n = 143$; OP + MH-1, $n = 145$. Survival rates were calculated using the Kaplan–Meier method, and differences in survival were tested using the log-rank test. *** $P < .001$; ** $P < .01$. Table S5 provides detailed lifespan data and statistics.

impact of OP on nematodes is minimal in this study. Increasing the healthspan, one common definition of which is the period of life spent in good health, free from the chronic diseases and disabilities of aging, is considered to be crucial. Locomotor capacity indicates the muscle degradation rate and is used as a healthspan parameter in *C. elegans* (Rollins et al. 2017; Loo et al. 2023). Our results suggest that MH-1 and HK-MH-1 prolong not only the lifespan but also the healthspan. In addition to MH-1, *Clostridium butyricum* MIYAIRI 588 and *Lactococcus kimchii* NBRC113348 extend the lifespan and healthspan of *C. elegans* (Kato et al. 2018; Takeuchi

et al. 2024). In contrast, *Lactococcus lactis* subsp. *lactis* did not extend the lifespan of the nematodes but only extended their healthspan (Ali et al. 2023), suggesting that an increased healthspan does not necessarily correlate with an increased lifespan.

Probiotics are living microorganisms that confer health benefits to the host when administered in adequate amounts (Sanders 2008). We found that MH-1 did not necessarily have to be viable to extend the lifespan and healthspan of worms. *Levilactobacillus brevis* strain MKAK9 also exerted longevity effects on *C. elegans*, regardless of whether they were alive or dead (Kumar et al. 2024).

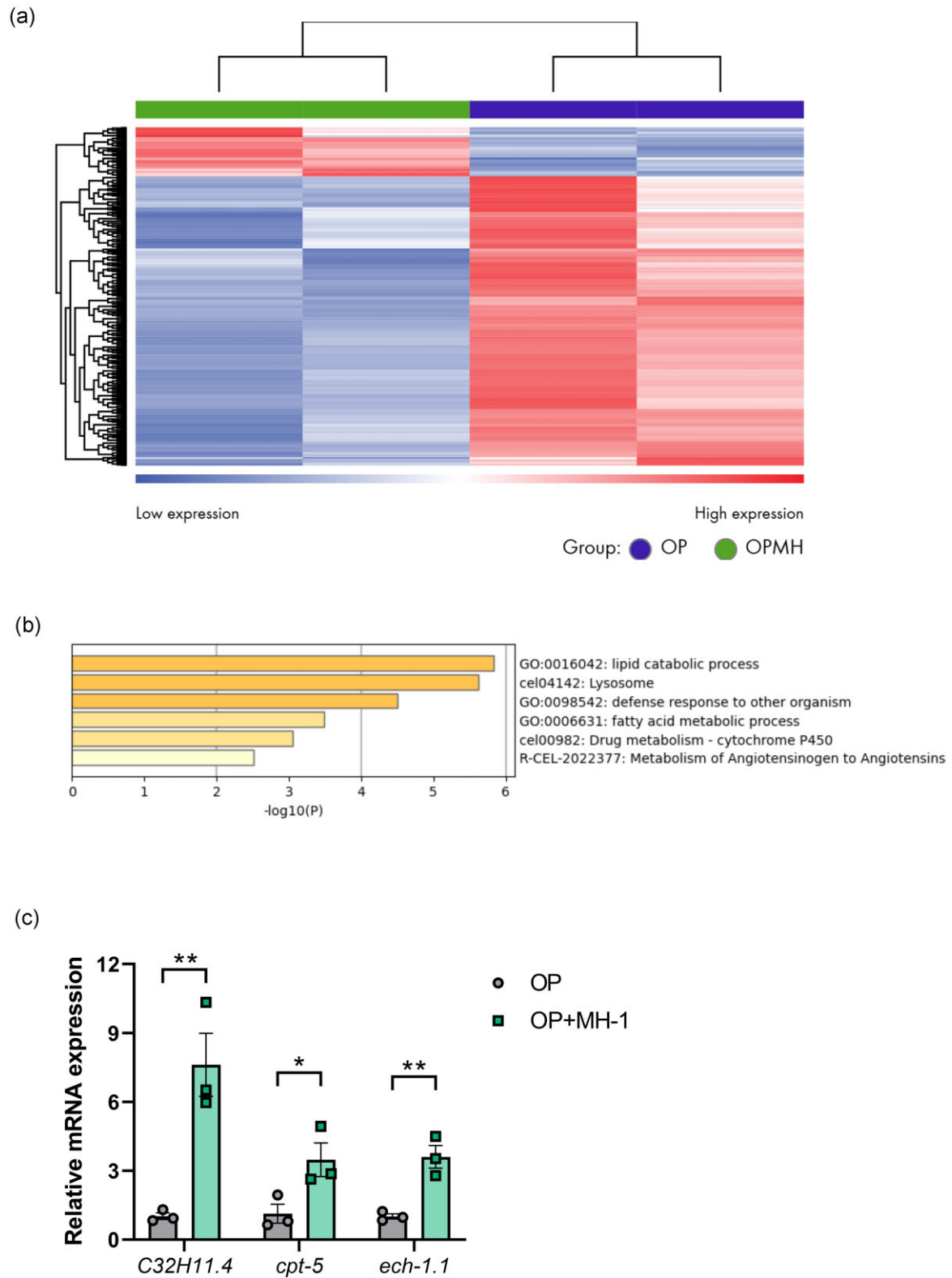


Figure 6. Significant expression of genes regulated by MH-1 feeding in *C. elegans*. (a) Heat map of gene expression for worms fed OP and OP + MH-1, with fold change >2 and <-2 and FDR P -value $<.05$. (b) The top-level gene ontology (GO) terms for biological processes that were significantly enriched in genes upregulated by OP + MH-1 feeding. (c) Expression of genes related to GO terms quantified using real-time polymerase chain reaction (PCR). Relative mRNA levels (normalized to *cyc-1*, *tba-1*, and *act-1*) in the worms fed OP + MH-1 compared with those fed OP alone (control). Values are presented as individual plots and means $\pm$ SEM of 3 independent experiments. Statistical analysis was performed using an unpaired 2-tailed t-test. $**P < .01$; $*P < .05$.

Additionally, the study above revealed that the cell wall and its associated exopolysaccharide components of the heat-killed *L. brevis* strain MKAK9 significantly increased worm longevity (Kumar et al. 2024). In contrast, lipoteichoic acid purified from *Bifidobacterium animalis* subsp. *lactis* BPL1 prolongs longevity in *C. elegans* (Balaguer et al. 2023). For MH-1, it is possible that the effect of longevity on *C. elegans* was caused by its bacterial components; however, this could not be determined in this study and further research is needed.

Dietary restriction extends the lifespan of several organisms, including *C. elegans* (Fontana, Partridge and Longo 2010). The body and brood sizes of worms decrease when dietary restriction occurs (Tain et al. 2008; Bar et al. 2016). We detected FITC-labeled MH-1 in the intestines of worms, indicating that the worms ingested MH-1 as food. The body size of worms fed with MH-1 did not decrease compared to that of worms fed with OP, and the brood size of worms did not differ between the two groups. These results indicate that the longevity effects of MH-1 on *C. elegans* were not due to dietary restriction.

Subsequently, we investigated the effects of MH-1 feeding on intestinal barrier function in *C. elegans*. The intestinal barrier function of aging worms was not altered by MH-1. The protective effect of lactobacilli against leaky gut has been reported in obesity and inflammatory conditions (Wang et al. 2020; Ondee et al. 2023). In this study, we examined the intestinal barrier function in healthy worms. Thus, it should be noted that different results may be obtained when worms are infected with pathogenic bacteria or fed a high-glucose diet that causes obesity.

Generally, excessive fat accumulation leads to poor health. The lifespan, motility, and reproductive capacity of high-fat *C. elegans* were significantly lower than those of normal nematodes (Yu et al. 2024). Fat accumulation tended to decrease with *Lactobacillus* supplementation (Gu et al. 2022; Kumar et al. 2022; Bouasker, Nodland and Millette 2024). However, MH-1 did not alter fat accumulation in *C. elegans*, suggesting that MH-1 has no fat-reducing effect on worms.

Stress resistance is closely correlated with longevity in *C. elegans* (Soo et al. 2023). *Lactobacillus rhamnosus* CNCM I-3690 has been found to extend the lifespan and improve the oxidative stress resistance of *C. elegans* (Grompone et al. 2012). Accordingly, we investigated whether MH-1 enhances stress resistance in worms. MH-1 increased stress resistance to oxidative stress. The oxidative stress tolerance induced by MH-1 feeding likely contributed to extended lifespan. *Bacillus subtilis* NCBI3610 biofilm enhances oxidative stress tolerance induced by juglone (Smolentseva et al. 2017). When *C. elegans* are previously exposed to pathogenic bacterial odors, they become tolerant against pathogen infection by enhancing heat shock factor-1 dependent molecular chaperone gene expression (Ooi and Prahlad 2017). Therefore, biofilms and odorants produced by bacteria may enhance stress tolerance.

Finally, we identified the genes that were upregulated by MH-1 feeding. Based on the transcriptome analysis, the enriched GO terms were “lipid catabolic process,” “defense response to other organisms,” and “fatty acid metabolic process.” The enriched GO term and real-time PCR analysis indicated that *cpt-5* and *ech-1.1* in the “lipid catabolic process” and “fatty acid metabolic process” were significantly upregulated compared to the control group. *cpt-5* and *ech-1.1* encode carnitine palmitoyl transferase and enoyl CoA hydratase, respectively, that participate in mitochondrial β -oxidation (Ratnapan et al. 2014). Mitochondrial β -oxidation facilitates the degradation of stored fat for energy production. Aspirin increases the expression of *cpt-5* and reduces fat storage

in *C. elegans* (Huang et al. 2017). CEBP-2 inactivation leads to low-fat content in *C. elegans*, and *cebp-2* suppresses the expression of *ech-1.1* (Xu et al. 2015). Increased expression of these two genes was inferred to cause fat loss in *C. elegans*; however, MH-1 did not affect fat accumulation. It is possible that these two genes alone are not sufficient for fat loss and that a balance with other genes is involved. The *C32H11.4* gene involved in defense responses was upregulated in *C. elegans* fed with MH-1. This gene is thought to have CUB-like domains; however, its functions in *C. elegans* are largely unknown (Alper et al. 2007; Oliveira et al. 2009). *C32H11.4* is regulated downstream of SKN-1, a transcription factor that acts in multiple lifespan pathways (Oliveira et al. 2009). Furthermore, RNAi of *C32H11.4* reduced oxidative stress resistance (Oliveira et al. 2009). RNA-seq analysis showed that MH-1 feeding increased the expression of genes encoding a glutathione S-transferase (*gst-14*), which is involved in the protection against oxidative stress via SKN-1 (Park, Tedesco and Johnson 2009). Oxidative stress tolerance conferred by MH-1 feeding likely contributes to the lifespan extension. In this study, it was not possible to determine the host biological pathways that are required for the MH-1-induced healthspan extension of *C. elegans*. Nevertheless, the findings of this study pave the way for the use of *L. brownii* as a probiotic.

Many gene expression mechanisms, one of which is the insulin/IGF-1 signaling (IIS) pathway, have been reported to extend the lifespan of *C. elegans* (Roselli et al. 2019). Bacteria involved in the IIS pathway and prolonging nematode lifespan, including *Bacillus subtilis* biofilms via *daf-2/daf-16* (Donato et al. 2017), *Clostridium butyricum* MIYAIRI 588 via *daf-16* (Kato et al. 2018), and *Lactococcus kimchii* NBRC113348 via *daf-2* and *ins-20* (insulin-like peptide) (Takeuchi et al. 2024), have been reported. Many genes directly regulated by *daf-2* are associated with *C. elegans* longevity (Murphy et al. 2003), and one of these genes, *fat-7*, contributed to the gene upregulation in *C. elegans* fed MH-1. These results suggest that the longevity effect of MH-1 on *C. elegans* may be partially mediated by the IIS pathway, and further studies using loss-of-function mutants are needed.

In conclusion, we demonstrated that *L. brownii* strain MH-1 and heat-killed MH-1 extended the lifespan and reduced the motility decline in a *C. elegans* model for the first time. MH-1 did not alter the integrity of the intestinal barrier function and the fat accumulation with age. In contrast, MH-1 conferred resistance to oxidative stress in worms. MH-1 is expected to be used as a novel probiotic strain.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data supporting the findings of this study can be obtained from the Gene Expression Omnibus (GEO; GSE261495).

Author contribution

Conceptualization: M.H. and E.K.-N.; investigation: M.H.; statistics: M.H. and Y.T.; writing—original draft preparation: M.H., Y.T., and E.K.-N.; writing—review and editing: M.H., Y.T., and E.K.-N.; supervision: E.K.-N. All authors have read and approved the manuscript for publication.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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