

Original Article

Lifelong Consumption of Freeze-Dried *Parabacteroides Distasonis* Improves Gut Barrier Integrity and Extends Lifespan and Health Span in *Drosophila Melanogaster*.

Sarah Shnayder^{1#}, Vicki Do^{1#}, Junyue Wang^{2#}, Luana Machado^{1#}, Sofia Rosa¹, Lora Kusaku¹, Benjamin Kuo¹, Jill Hutchinson¹, Grace Ogden¹, Neja Majeti^{3&}, Weimin Guo², Samuel Beck⁴, Mitch McVey^{1*}, Jimmy W. Crott^{2, 5*}

¹Department of Biology, Tufts University, Medford, MA, USA. ²Department of Pathology and Laboratory Medicine, Boston University Chobanian & Avedisian School of Medicine, Boston, MA, USA. ³Drexel University, Philadelphia, PA, USA. ⁴Department of Dermatology, Boston University Chobanian & Avedisian School of Medicine, Boston, MA, USA.

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ABSTRACT: Chronic inflammation has been shown to be associated with aging. In *Drosophila melanogaster*, age-related inflammation in the gut results in a disruption of gut barrier integrity, which can lead to reduced intestinal function and death. The anaerobic bacterium *Parabacteroides distasonis* (*Pd*) has anti-inflammatory properties and can attenuate tumorigenesis and promote intestinal barrier integrity in a mouse model of colorectal cancer. Here, we show that administration of *Pd* to adult *Drosophila* significantly increases median lifespan and promotes maintenance of climbing ability throughout adult life. Furthermore, *Pd* supplementation enhances gut barrier integrity in aged adults. RNA-Seq analysis of adult gut tissue revealed that the elevated healthspan in *Pd* supplemented flies is accompanied by transcriptional upregulation of genes involved in DNA replication and maintenance of genome integrity. These results suggest that *Pd* supplementation may induce a transcriptional shift towards augmented DNA repair in the aging gut that prevents the breakdown of gut barrier integrity and promotes healthy aging.

Keywords: parabacteroides distasonis, gut barrier integrity, lifespan

INTRODUCTION

Aging is accompanied by chronic low-grade inflammation characterized by elevated plasma IL-6 and C-reactive protein – a phenomenon aptly termed inflammaging [1]. Chronic inflammation is thought to play a causal role in many age-related diseases as well as decreased lifespan in multiple organisms [2]. In humans, individuals with above average inflammatory markers, especially IL-6 and TNF α , have higher all-cause mortality rates [3] and are more likely to be frailer [4], less

independent [5], and suffer from conditions like dementia [6] and Alzheimer's disease [7].

Related to increased inflammation, advanced age has been associated with increased gut permeability in various species including rats [8], mice [9] and baboons [10]. Similar findings have been reported in *Drosophila* [11–13], and findings that young flies with elevated permeability had significantly shorter lifespan directly linked barrier integrity to lifespan for the first time [14].

Collectively these studies established that aging impairs the intestinal epithelial barrier, resulting in a

*Correspondence should be addressed to: Dr. Jimmy W. Crott, Boston University Chobanian & Avedisian School of Medicine, Boston, MA, USA. Email: jcrott@bu.edu; Dr. Mitch McVey, Department of Biology, Tufts University, Medford, MA, USA. Email: mitch.mcvey@tufts.edu. # These authors contributed equally to this work. &Present address: Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA. ⁵Parts of this work was completed at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA.

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leakage of microbial components into the lamina propria and blood stream where they can promote inflammation and immune dysregulation [15, 16]. Consequently, intestinal permeability is now considered a promising target for disease prevention and therapeutic intervention [17, 18].

Gut barrier integrity and the gut microbiome are closely intertwined. For example, germ-free mice display reduced gut permeability, lower plasma IL-6, and increased longevity compared to conventionally colonized mice [9]. Moreover, numerous studies document age-related changes in gut microbiota composition that correlate with markers of inflammation, suggesting that gut dysbiosis might drive inflamm-aging [9, 19–22]. Supporting a causal role for microbiota in inflammaging, transferring gut bacteria from old to young mice increased inflammatory markers in the recipients, establishing that an age-altered gut microbiome alone is sufficient to induce an inflammatory state [23, 24]. Additionally, data showing that Toll-like Receptor 4 (TLR4) knockdown protects aging mice from elevated IL-6, TNF α , and MCP-1 levels highlight this receptor as a critical mediator of inflamm-aging [25].

Amongst the whole community changes, specific microbes may also have a bearing on aging and longevity. For example, Barcena *et al* compared fecal microbiome of control and progeroid (*Lmna*^{G609G/G609G}) mice and observed that the genera *Akkermansia* and *Allobaculum* were higher in controls while *Prevotella* and *Bacteroides* were higher in progeroid mice. Interestingly, transplanting stool from controls increased lifespan in progeroid recipients – an effect that could be recapitulated by gavage with *Akkermansia muciniphila* alone [26].

Similarly, while the gut microbiota is a major source of antigens capable of breaching the epithelium and inciting inflammation, it also includes specific members that support or enhance barrier integrity. For instance, after observing that *Lactobacillus paracasei* D3-5 increased lifespan and reduced functional decline in *C. elegans*, Wang *et al.* demonstrated that feeding this bacterium to old (75–80 week) obese mice also increased several metrics of physical performance and cognition and decreased systemic inflammation and gut permeability [27]. Similarly, Matsumoto *et al* fed 10-month-old mice *Bifidobacterium animalis* subsp. Lactis LKM512 for 11 months and observed an increase in lifespan coupled with decreased gut permeability and serum TNF [28]. In *Drosophila*, increased microbial load can decrease lifespan through intestinal barrier dysfunction [29, 30]. Furthermore, intestinal barrier dysfunction due to age-related changes in the microbiome is a primary driver of mortality in *Drosophila* [31], and elimination of microbes in flies with impaired gut barrier function can increase lifespan [30].

In our own work, we found that administration of postbiotic *Parabacteroides distasonis* (*Pd*), a gram-negative anaerobe, strengthened barrier integrity in mice. Moreover, *Pd* extracts increased transepithelial electrical resistance (TEER) in Caco-2 cells, coupled with upregulation of key tight junction proteins in both models [32]. We also observed inverse associations between fecal *Pd* abundance and colonic tumor burden and IL-1 β levels in a screening study [33]. Further, *Pd* administration suppressed colon tumor formation, pro-inflammatory TLR4 signaling, and cytokine production in mice [32, 34]. Given the robust anti-inflammatory and gut barrier-enhancing effect of *Pd*, we hypothesized that *Pd* may promote gut barrier integrity, extend healthspan, and increase lifespan in *Drosophila melanogaster*. Using two common laboratory strains (*w*¹¹¹⁸ and *Oregon-R*), we fed flies a solution containing 0.1% (w/w) *Pd* beginning at two days after adult eclosion. This treatment robustly increased median lifespan and slowed the age-related decline in physical activity and gut barrier integrity. Gene expression analysis of aged control and *Pd*-supplemented flies revealed an upregulation of several septate and adherens junction genes, which fits well with reduced permeability and with previous observations in mice. Additional transcriptomic changes are indicative of an innate immune response involving upregulation of enzymes that produce reactive oxygen species (ROS) and members of the Toll and Immune deficiency (*Imd*) pathways that culminate in the expression of various antimicrobial peptides (AMPs). Concomitant elevations in gene networks that promote gut proliferation and DNA repair are predicted to enhance renewal of the gut lining and genome stability in gut stem cells. Although the precise mechanisms by which *Pd* promotes lifespan and healthspan could not be fully elucidated, key changes speak to activations of mechanisms that both mimic and differ from the murine situation. More work is required to parse the relative impacts of the many changes observed.

METHODS

Pd culture

Pd was cultured as described previously [35]. Briefly, *Pd* 8503 was purchased from ATCC (Manassas, VA), and streaked onto chocolate agar plates (Remel/Thermo Fisher Scientific, Waltham, MA), then incubated in a gas pack (Remel). Single colonies were picked and inoculated into brain heart infusion (BHI) media (Gibco/Thermo), supplemented with hemin (Thermo) and vitamin K (Thermo). Bacteria were harvested by centrifugation, washed with PBS, freeze dried and stored at –80°C until use. The identity of cultured bacteria was confirmed through Gram staining (negative and rod-shaped), PCR

for *Pd* specific sequences, and periodic bacterial sequencing.

Fly feeding and maintenance

w¹¹¹⁸ and *Oregon-R* strains were acquired from the Bloomington Drosophila Stock center (Bloomington, IN). Flies were raised in bottles that were kept in a humidified, temperature-controlled incubator at 25°C with a 12:12 hour on/off light cycle. All stocks were reared on Bloomington Drosophila Stock Center standard cornmeal food and were transferred into new bottles every 2-3 days.

Lifespan analysis

Prior to starting the lifespans, 20 females and 15 males were placed into bottles and allowed to lay eggs for 2 days, after which they were removed. Unmated female and male progeny from these bottles were collected within 8 hours of eclosion and raised in separate sex cohorts of 25 flies per vial. Freeze dried *Pd* bacteria were resuspended in ddH₂O to a final concentration of 0.1% (w/v). The food (standard cornmeal) in each lifespan vial was overlaid with 100 µL of either 0.1% *Pd* or 100 µL of ddH₂O as a control, and the solution was allowed to soak into the food overnight. Flies were transferred every 2 days to new *Pd* or control vials. Deaths were scored every day. An additional experiment was conducted with heat treated *Pd* (95°C for 10 min), which was similarly overlaid on standard cornmeal food.

Negative Geotaxis Assay

Cohorts of 25 flies were transferred into clear vials without anesthetization. Flies were firmly tapped to the bottom of the vials three times and then allowed to climb up the sides of the vials. Videos of the climbing were recorded for 15 seconds, at which time the distance from the bottom of the vial was recorded for each fly and values were reported as a percentage of the total height of the vial. Assays were repeated with three biological replicates.

Smurf assay

Smurf assays were conducted using the method of Rera *et al* [14]. *w¹¹¹⁸* females were fed diets supplemented with 0 or 0.1% (w/v) freeze-dried *Pd* as above. Flies were placed on food overlaid with 200 µL 2.5% (w/v) of FD&C blue dye #1 (Sigma-Aldrich, St. Louis, MO) and allowed to feed for 16 hours overnight. The next morning, they were anesthetized and scored for dye leakage outside of the gut into the abdomen and/or thorax (classified as “Smurfs”).

CAFE Assay

Food consumption was measured using the capillary feeder (CAFE) assay [36]. The CAFE apparatus consisted of an empty plastic vial fitted with two vial plugs at the bottom and a third plug at the top containing a hole to accommodate a 200 µL pipette tip. A 5 µL glass capillary tube was inserted into the pipette tip and suspended into the vial, allowing flies to feed from the open end of the tube. Tubes were filled by capillary action with a 5% (w/v) sucrose solution prepared in ddH₂O, either alone or supplemented with 0.1% (w/v) *Pd*. Cohorts of 10 female flies were placed into each vial and allowed to feed for 4 hours. The initial and final liquid levels in each capillary tube were marked, and food consumption was calculated as the distance in centimeters between the two marks. To control for evaporation, identical CAFE apparatuses containing each solution and no flies were run in parallel, and the volume of liquid lost was subtracted from experimental measurements. The assay was performed using three biological replicates.

Gut dissections and staining

Intestines were dissected from *w¹¹¹⁸* females into 1ml of 3.7% formaldehyde in PBST. Following a 30 min incubation (room temp), guts were washed 5X for 5 min with 1 mL PBSTx (PBST + 0.3% Triton-X). They were then blocked for 1 hr in 1 mL of PBSTx + 0.5% BSA at RT and incubated overnight on a rocking platform with 1:1000 dilution of anti-phospho histone H3 S10 (#9701S, Cell Signaling Technology) or anti-DLG (4F3, Developmental Studies Hybridoma Bank, 1:500 dilution). The next morning, guts were washed 4X for 5 min with 1 mL PBST and incubated for 2 hrs at RT with 1:1000 dilution of goat anti-mouse Alexa Fluor 488 (# ab150113, Abcam, Waltham, MA) + DAPI (0.1 µg/mL). Additional control slides treated only with secondary antibody were prepared to ensure signal specificity. Guts were washed 5X for 5 min with 1 mL PBST and transferred to slides with 30 µL of Vectashield (Vector Laboratories, Newark, CA). A cover slip was placed over the guts, and the edges were sealed with clear nail polish. Stained guts were visualized on a Leica (Waltham, MA) DMi8 Thunder Fluorescent Microscope.

Gut gene expression

Female *w¹¹¹⁸* fed control or *Pd* diets were harvested at 40 days of age and dissected guts were immediately placed into RNeasy lysis buffer (Qiagen). Total RNA was isolated from individual guts using RNeasy spin columns (Qiagen) with glycogen to enhance yield. RNA was quantified and quality checked using a Fragment Analyzer (Agilent,

Santa Clara, CA). Fifty ng of RNA was used as input library preparation using a Stranded Total RNA Prep with Ribo-Zero Plus kit following manufacturer instructions (Illumina, San Diego, CA). The resulting libraries were then quantified, mixed in equimolar concentrations, and sequenced on an Illumina NovaSeq X Plua using a 1.5B 100-cycle kit using single read 100 bases format. Base calling and demultiplex was then performed on an Illumina DRAGEN server using bclconvert.

Raw single-end sequencing reads were received adapter-trimmed from the core facility. Read quality was assessed using FastQC (v0.12.1), and aggregate quality metrics were compiled using MultiQC (v1.30). Reads were aligned to the *Drosophila melanogaster* reference genome (BDGP6.54, Ensembl Metazoa) using STAR (v2.7.11b). Gene annotations were assigned using the Ensembl GTF file (*Drosophila melanogaster*.BDGP6.54.gtf). Gene-level read counts were generated using featureCounts from the Subread package (v2.1.0).

RNA-seq was performed on 10 Ctrl and 8 *Pd* gut samples. Differential expression analysis was conducted in R (v4.4.2) using DESeq2 (v1.44.0). Genes with fewer than 10 total counts were filtered prior to analysis. Gene identifiers were converted from Ensembl IDs to gene symbols using the rtracklayer package (v1.62.0).

Significantly differentially expressed genes (DEGs) were defined as those with an absolute $\log_2(\text{fold change}) > 1$ and an adjusted $p\text{-value} < 0.01$. PCA was performed on variance-stabilized transformed data.

Gene Ontology (GO) enrichment analysis for Biological Process, Molecular Function, and Cellular Component ontologies was performed using the clusterProfiler package (v4.12.6) with the *Drosophila* annotation database org.Dm.eg.db (v3.19.1). KEGG pathway enrichment was conducted using clusterProfiler following conversion of gene identifiers to KEGG-compatible annotation symbols via the FlyBase ID Converter (www.biotoools.fr/drosophila/fbgn_converter). Reactome pathway analysis was conducted using the ReactomePA package (v1.48.0) with Entrez gene identifiers. WikiPathways enrichment utilized a custom gene set derived from the WikiPathways GMT file for *Drosophila melanogaster* (wikipathways-20250810-gmt-*Drosophila melanogaster*.gmt). All enrichment analyses were conducted separately on upregulated and downregulated gene sets using the Benjamini-Hochberg correction method with an adjusted $p\text{-value} < 0.05$. All data visualizations, including PCA plots, volcano plots, and enrichment dot plots, were generated using ggplot2 (v4.0.0) in R.

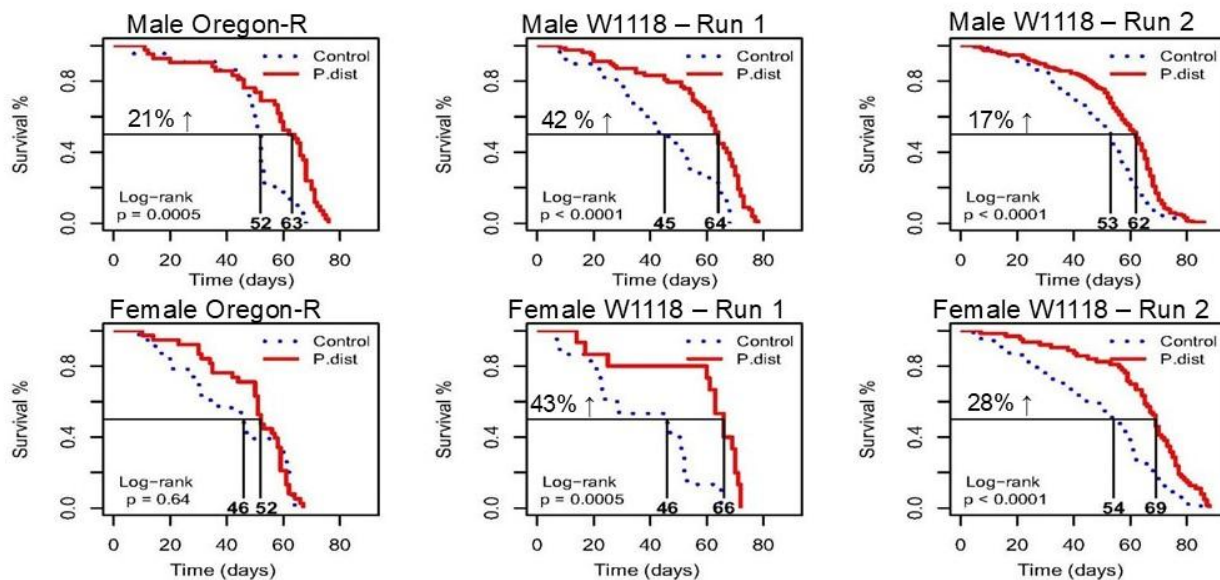


Figure 1. *Pd* supplementation extends *Drosophila* lifespan. Kaplan-Meier plots of survival of *Oregon-R* and *w¹¹¹⁸* flies raised on standard food with an overlay of 0.1% freeze-dried *Pd* or water starting 8 hours after adult eclosion. $n > 150$ individuals for all trials. Survival curves were compared by log-rank test and significance accepted when $P < 0.05$.

Several genes were selected for verification using RT-PCR. Briefly, 250 ng of total RNA was reverse transcribed using the Verso cDNA Synthesis Kit (Thermo) and amplified using various primer pairs (See

Table S1 for primer sequences) and PowerUp SYBR Green Master mix (Applied Biosystems) on a Quantstudio3 Real-Time PCR system (Applied Biosystems). The bTub56D gene was used as an internal

control as described by Zhang et al [37]: importantly the number of reads for this gene did not differ between groups ($P=0.61$). Relative gene expression was calculated using the equation $2^{-\Delta\Delta Ct}$.

Statistical analysis

All data are presented as mean $\pm$ SEM. RNA seq data analysis was conducted in R as described above. All other analyses were conducted in GraphPad Prism (v10.6.1, San Diego, CA). Survival curves were compared by Log-Rank test with significance accepted when $P<0.05$. Data distribution normality was assessed by Shapiro-Wilk test. Smurf and climbing data were analyzed by linear regression. Climbing data were also analyzed by unpaired t tests. Immunofluorescence endpoints compared by one-way ANOVA or Kruskal-Wallis test. Significance was accepted when $P<0.05$. Individual gene expression data were analyzed by Unpaired t test and Mann-Whitney tests with False Discovery Rate correction set at 5%.

RESULTS

Pd increases median lifespan and improves healthspan

To determine the effect of *Pd* supplementation on adult lifespan in *Drosophila*, we collected virgin females and males from two genetically distinct wild-type stocks and

passed them every two days on standard cornmeal diet overlaid with a solution of *Pd* or water. We observed a consistent extension of median lifespan for *Pd* supplemented adults, with increases ranging from 13% to 43% (Fig. 1). Interestingly, *Pd* supplementation extended the median lifespan of both males and females by roughly equal proportions in two trials using the *w¹¹¹⁸* stock and had similar effects in the *Oregon-R* background, indicating that the effect is not sex-specific. Heat treating *Pd* destroyed its lifespan-extending capacity (Fig. S1)

One possible explanation for the increased lifespan could be that *Pd* is distasteful to *Drosophila* and induces caloric restriction (CR), which is known to extend longevity [35]. To test this, we determined whether flies consume less food containing *Pd* using the CAFE assay [33]. We observed no difference in food consumption when *Pd* is present (Fig. S2) and therefore conclude that mean lifespan extension induced by *Pd* supplementation likely occurs by a non-CR mechanism.

Pd supplemented *Drosophila* appeared qualitatively more active throughout their lifespan. To quantify this effect, we used the negative geotaxis assay, which measures the animals' climbing ability. As previously shown, males climb at a faster rate than females and climbing ability degrades with age [36]. *Pd* supplementation slowed the decrease in climbing ability in both males and females, and this effect became statistically significant in both sexes by day 47 (Fig. 2).

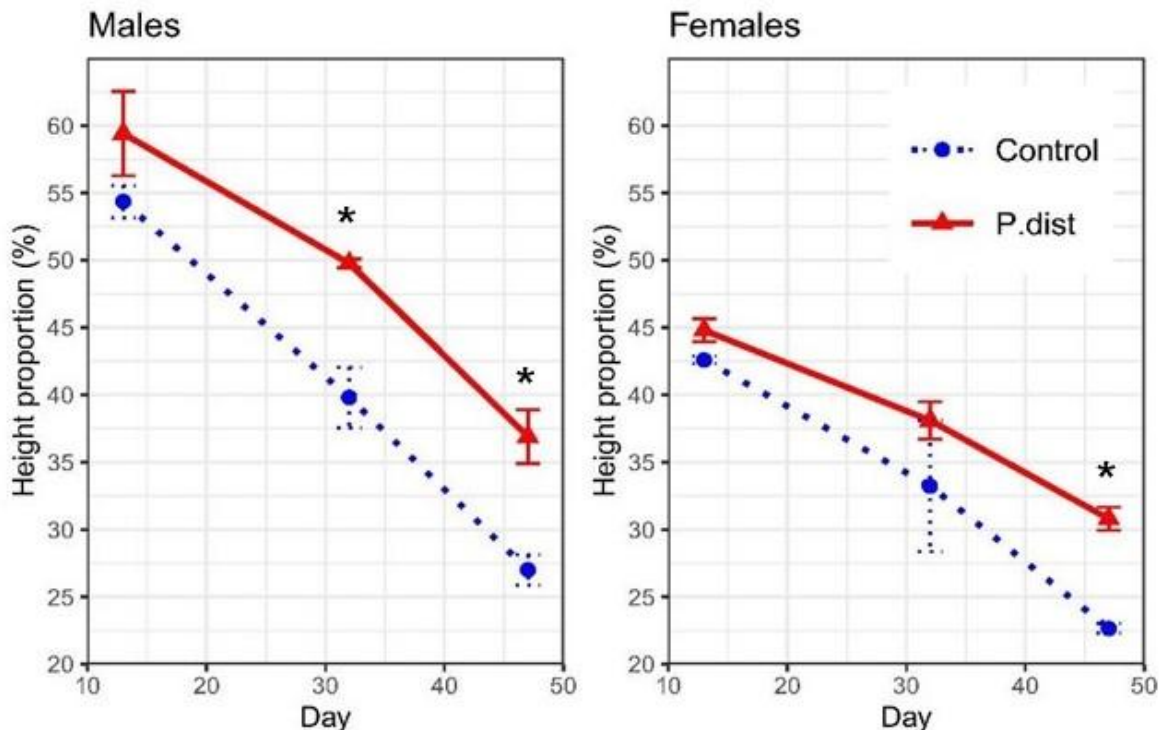


Figure 2. Results of negative geotaxis assays using *w¹¹¹⁸* flies. Climbing ability is quantified as the distance each fly climbed in 15 seconds as a proportion of the total vial height. Each point represents the average of three biological replicates of 25 flies. Groups compared by t test. * $P<0.05$.

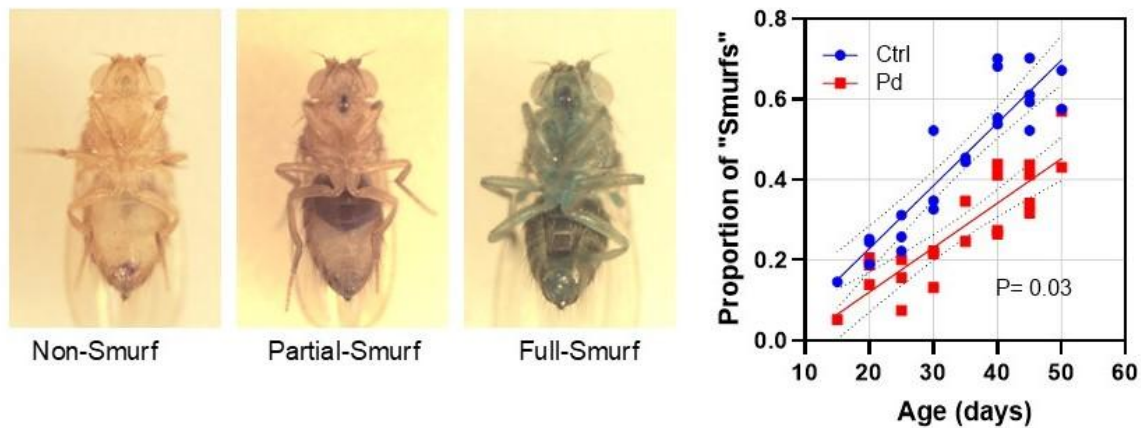


Figure 3. Pd supplementation promotes gut barrier integrity. Female adult flies raised on food overlaid with Pd or water were moved to food containing blue dye for 16 hours, after which they were characterized as non-Smurfs or partial/full Smurfs based on the diffusion of the dye throughout their bodies. $n = 2-4$ for all days except day 15, where $n = 1$, with at least 35 flies per trial. Linear regression lines and 95% confidence intervals are shown. Slopes compared by linear regression: $P(\text{regression}) = 0.03$.

Pd promotes maintenance of gut barrier integrity

As *Drosophila* age, their gut barrier integrity decreases, resulting in leakage of gut contents into the abdomen and activation of inflammatory pathways. We assessed gut integrity using the Smurf assay on adult females aged 15–50 days. We observed an increasing number of Smurfs in both populations with age, although the increase was attenuated in *Pd* supplemented flies (Fig. 3). By day 40, more than 50% of the females in the control group were classified as Smurfs, while only 35% of *Pd* supplemented females showed any evidence of gut leakage ($P = 0.03$).

As flies age, damage to the polyploid enterocyte cells can compromise barrier integrity. To deal with this damage, intestinal stem cells can undergo proliferation and differentiation into both enteroendocrine cells and enterocytes. Given the enhanced gut barrier integrity that we observed with *Pd* treatment, we wondered if this might be due to enhanced tissue renewal. To test this, we dissected midguts from 40-day old females and stained for phospho-histone H3, a mitotic cell marker. As expected, the number of mitotic cells increased in older flies (Fig. 4). Strikingly, in *Pd* treated guts, the mean number of mitotic cells in the midgut nearly tripled compared to control flies.

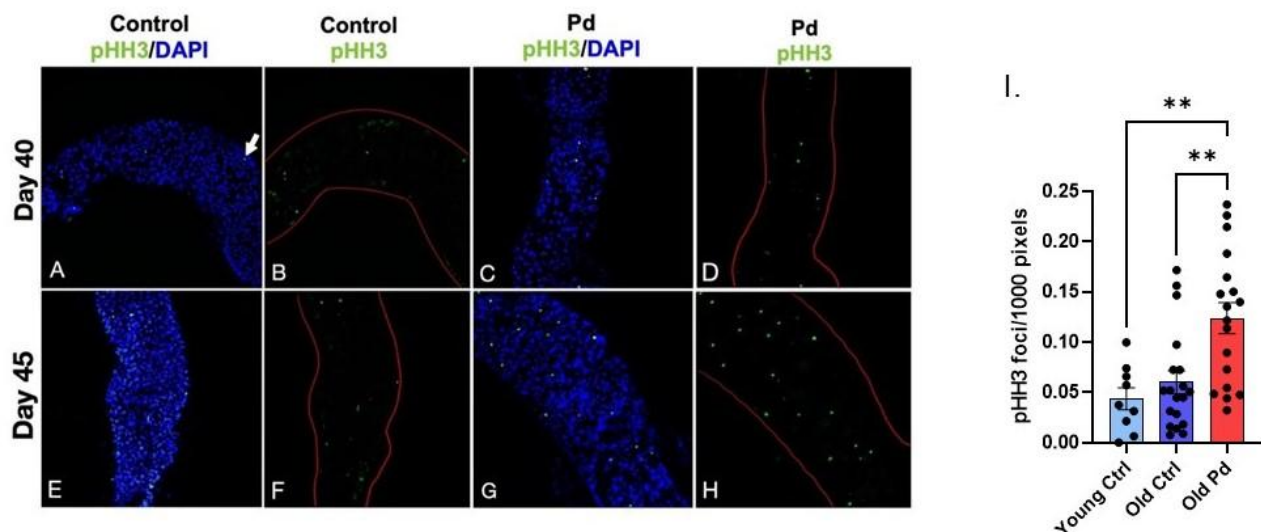


Figure 4. Pd supplementation increases cell proliferation in the midgut. Female flies were treated with water or 0.1% *Pd*. Guts were dissected between ages 40–45 days and stained for mitotic cells using an antibody against phospho-histone H3 (pHH3). pHH3 foci were counted and normalized to total gut area as measured by DAPI staining. Young (3 days) $n = 9$ guts, old control $n = 19$ guts, old *Pd* $n = 18$ guts. (A–H) Fluorescent micrographs of Ctrl and *Pd* supplemented flies stained with pHH3 or DAPI. Representative images were selected for having a pHH3 foci count close to the mean for the group. (I) pHH3 quantification. Groups compared by Kruskal-Wallis test. * adjusted $P < 0.05$.

While renewal of gut cells is necessary for proper gut homeostasis, hyperproliferation can result in dysplasia and loss of function. To determine whether this might be the case in the *Pd* treated flies, we dissected guts from young and old females and stained with an antibody against *discs large* (*dlg*), a vital component of the smooth septate junction that promotes gut barrier integrity. In young flies, midgut cells were uniformly large and regularly hexagonal or pentagonal shaped (Fig. S3A). *Dlg* membrane staining in young guts was concentrated at cellular vertices. In older control flies, the *dlg* membrane staining became more diffuse, suggesting that smooth septate junctions were less uniform. In addition, average cell size became smaller, and the overall shape of the cells became more elongated, as measured by the axis ratio and the degree of eccentricity (Fig. S3C,D). *Pd* treatment partially reversed these aging-related phenotypes, restoring *dlg* staining to a younger phenotype and altering cell shape so that it more resembled that of young guts. Taken together, these data suggest that *Pd* can restore midgut barrier function to levels that are more youthful, resulting in improved gut integrity as flies age.

***Pd* administration dramatically alters the gut transcriptome**

To gain insight into gene expression changes that might be responsible for the improvement in gut barrier integrity, we isolated full-length guts from 45-day old control and *Pd* treated *w¹¹¹⁸* adult flies. Eighteen gut RNA samples were successfully sequenced (10 Ctrl and 8 *Pd*) and yielded an average of 40.7×10^6 reads ($18.6 - 80.6 \times 10^6$) per sample. Principal Component Analysis (PCA) revealed a robust separation in the gene expression of Ctrl and *Pd* flies (Fig. 5A). DESeq2 analysis identified 1569 genes for which expression was significantly higher and 1204 for which expression was significantly lower in the *Pd* fed compared to control flies (Fig. 5B. Table S2A).

To determine the functional consequences of genes showing differential expression, we examined their association with established pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [38], the Reactome Pathway Knowledgebase [39], and three sub-ontologies from the Gene Ontology knowledgebase [40]: metabolic functions, cellular compartments, and biological processes (Table S3, Fig. S4).

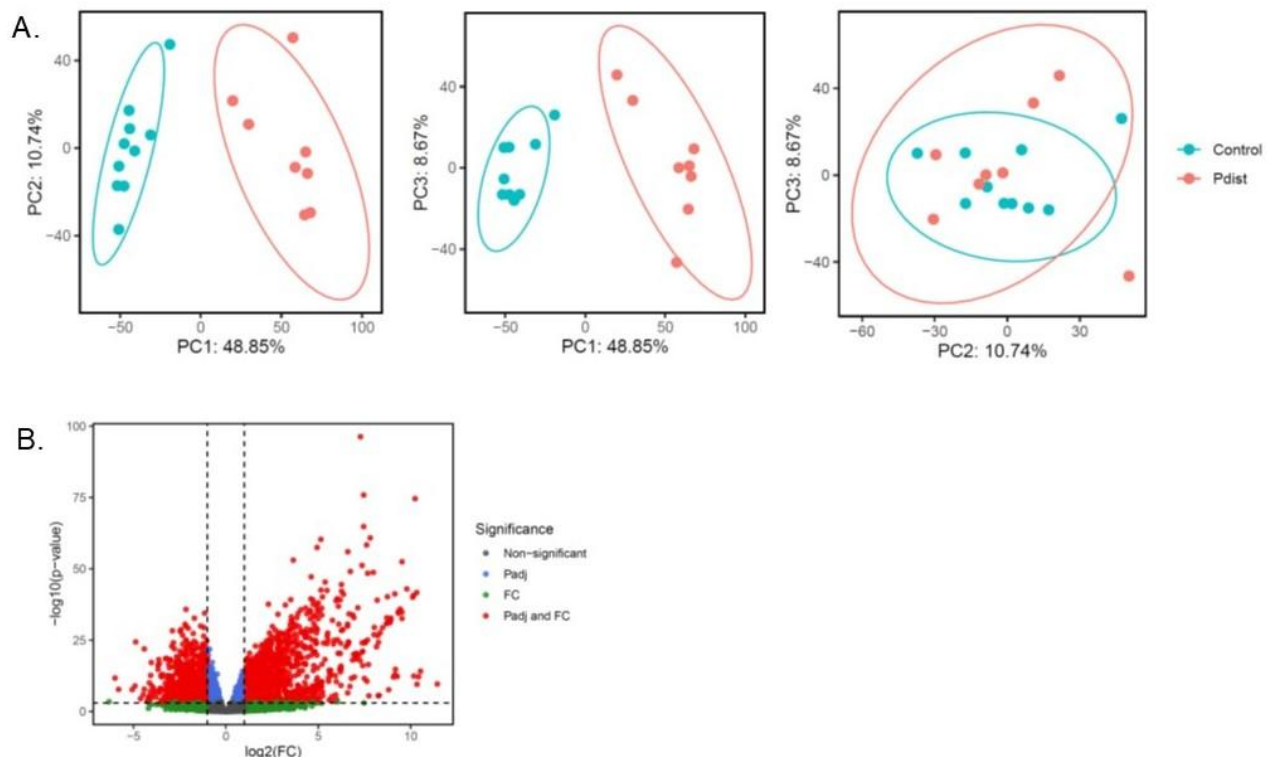


Figure 5. *Pd* supplementation dramatically alters midgut gene expression. (A) Principal Components Analysis showing clear separation of expression profiles according to treatment. (B) Volcano plot showing output of DESeq2 analysis: genes with a $\log_2\text{foldchange} > 1.0$ or < -1.0 and an adjusted $P < 0.01$ are highlighted in red. $N = 10$ and 8 per gp for Ctrl and *Pd* respectively.

Amongst the genes elevated in *Pd* treated guts there was a striking enrichment of genes related to DNA replication, cell proliferation, and DNA repair.

Specifically, in each of the categories and databases searched, the top 10 hits were all related to DNA replication and to a lesser extent DNA repair (Table S3A,

Fig. S4). Elevated expression of cell cycle genes *CycE* and *Cdk1* in *Pd* flies as well as DNA repair genes *Brca2*, *Msh6*, *Mlh1*, *Blm*, *Xrcc2*, and *Fancc2* (Table S2B) were confirmed by RT-PCR (Fig. 6).

Although not among the top 10 most enriched GO Biological Processes, genes associated with the function of cell-cell adhesion (GO:0098609) were significantly enriched in *Pd* fed flies (Table S3A). *Pd* feeding also induced a modest elevation of the septate and adherens junction genes *crooked* (*crok*) and *coiled* (*cold*) and to a lesser extent *polycatoid* (*pyd*) and *kune* ($\log_2FC = 0.81$ and 0.52 respectively (Table S2B) – all changes confirmed by RT-PCR (Fig. 6). Interestingly, other

components of septate junction genes, including *Snakeskin* (*Ssk*), *Tetraspanin 2A* (*Tsp2A*), and *mesh* tended to be reduced in *Pd* treated flies (Table S2B).

Amongst the set of genes expressed at lower levels in *Pd* there was an enrichment of genes related to energy metabolism, especially oxidative phosphorylation (Table S3B). Finally, manual review of the differentially expressed genes revealed several genes relevant to aging and gut tissue homeostasis. Elevated expressions of *Notch* (*N*), *Toll*, *spatzle* (*Spz*), *frizzled* (*Fz*), and *Sirt6*, and downregulation of *Toll-9* were all confirmed by RT-PCR (Table S2B, Fig. 6).

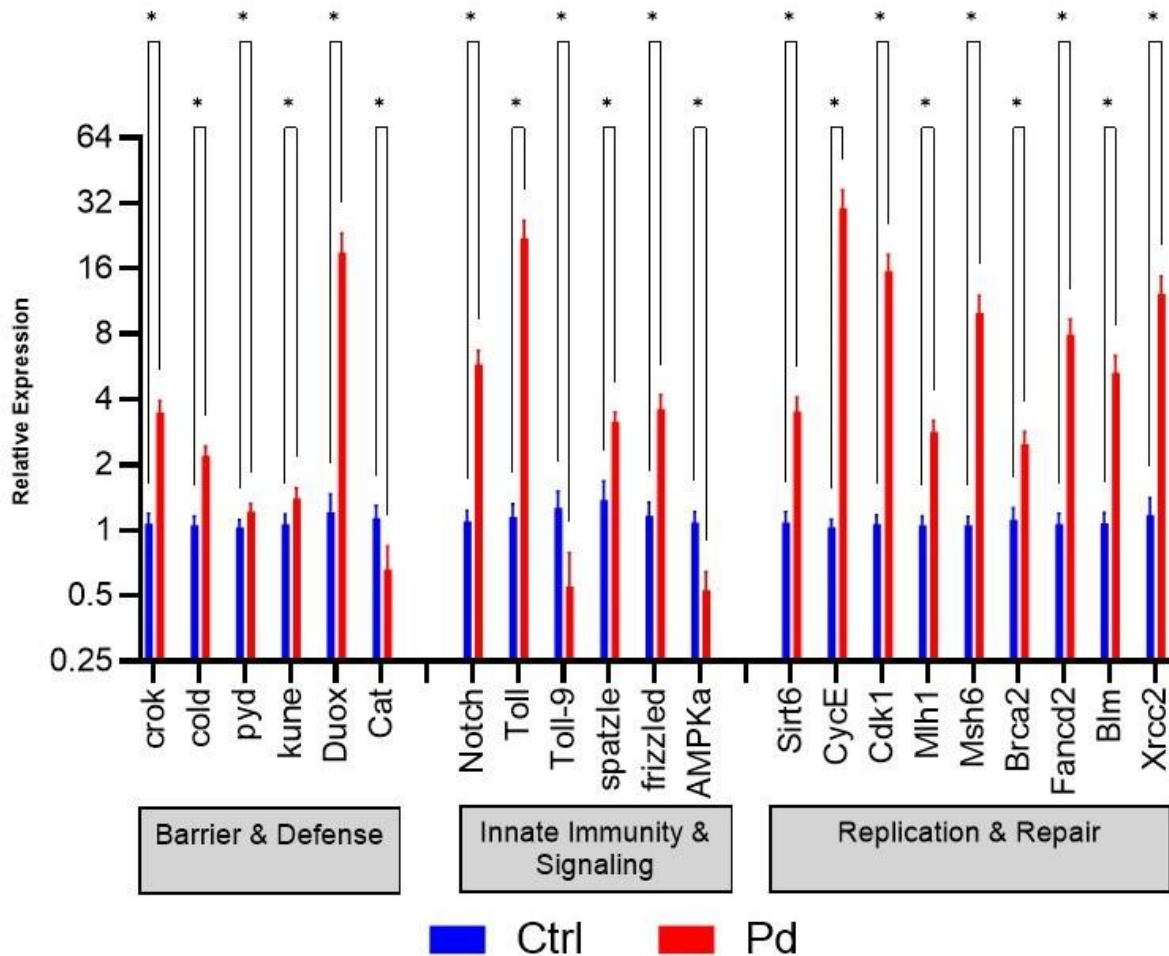


Figure 6. *Pd* supplementation alters expression of barrier, signaling and DNA replication and repair genes in the fly gut. Relative gene expression was assessed by RT-PCR using β Tub56D as the internal control and the equation $2^{-\Delta\Delta Ct}$. $N=10$ and 8 per group for control and *Pd* groups respectively. Data = mean $\pm$ SEM. Data distribution was normal for 7 of 21 genes. Groups were compared by t test (parametric) and Mann-Whitney tests (non-parametric). * $Q < 0.05$ for both tests.

DISCUSSION

In this study, we observed that supplementing with freeze dried *Pd* robustly extended the median lifespan of male and female *w¹¹¹⁸* flies and male *Oregon-R* flies. In

addition, *Pd* supplemented *w¹¹¹⁸* flies demonstrated a reduced loss of climbing ability and slower loss of gut barrier integrity with age. Elegant studies of Rera *et al* [14] demonstrated that diverse interventions and genotypes that compromised barrier integrity also

shortened lifespan, and that early age loss of barrier integrity predicts shorter lifespan in *Drosophila*. Based on those findings, we suggest that the preservation of barrier integrity likely underpins the observed extension of median lifespan seen amongst *Pd* supplemented flies.

We previously found that supplementation of mice with freeze dried *Pd* resulted in the upregulation of the tight junction proteins Zol1 (Tjp1) and occludin (Ocln) in the colonic epithelium concomitant with reduced blood endotoxin and markers on inflammation. Similarly, we observed Zo-1 upregulation in response to treatment of HT29, SW480 and Caco2 cells with *Pd* membrane protein extract [32]. The *Drosophila* orthologue of Zol1 is polychaetoid (*pyd*), a component of adherens junctions. Here, *pyd* expression was modestly upregulated by *Pd*, and the septate junction assembly genes *coiled* (*cold*) and *crooked* (*crok*) were robustly increased (Table S2, Fig. 6). A total of 33 genes related to the biological process of “cell-cell adhesion” (GO:0098609) were also elevated in *Pd* supplemented flies (Table S3A). The upregulation of these barrier related transcripts fits well with the slowed acquisition of the Smurf phenotype amongst *Pd* treated flies (Fig. 3).

Related to intestinal barrier function is anti-microbial defense. In the absence of adaptive immunity, the Toll and Imd pathways are the major immune pathways in *Drosophila* [41]. In general, the Toll pathway is activated upon recognition of peptidoglycans from Gram-positive bacteria and fungi by PGRP-SA. A cascade of events ensues whereby spätzle (*spz*) is cleaved and then binds to Toll (TL), culminating in the expression of anti-microbial peptide (AMP) genes including drosomycin. The Imd pathway, activated upon recognition of peptidoglycans from gram negative bacteria by PGRP-LE/LC and through a cascade involving Imd and Relish (Rel), results in the production of the AMPs Attacin-D and Diptericin [41]. Interestingly, *Pd* supplemented flies displayed higher expression of key members of both pathways. Specifically, Toll members *Tl*, *Spz* and *PGRP-SA* were all elevated while key Imd members Diptericin A and B, Attacin-D and Cecropin A1 were also elevated. Moreover, the Imd pathway is negatively regulated by *PGRP-SC1* and *PGRP-SC2* [42] so the downregulation of these in *Pd* supplemented (Table S2) could de-repress the pathway. Additional AMPs *Def*, *Mtk*, *AttB*, and *AttC* were also elevated (Table S2).

Rera *et al* [14] found that Smurf flies with increased gut permeability also had elevated expression of the AMPs drosocin, drosomycin, diptericin, thor, InR, and ImpL2, as well as significantly elevated bacterial load. Interestingly, we show that *Pd* supplementation, which slowed the acquisition of the Smurf phenotype, increased expression of diptericin (*dptA* and *B*) but not the other AMPs. The observed gut response to long-term *Pd*

consumption is somewhat reminiscent of the short-term response reported for infection with the Gram-negative bacterium *Erwinia carotovora carotovora* 15 (*Ecc15*). Specifically, many of the same genes were upregulated, including the AMPs *AttB/C/D*, *Mtk*, *Def*, and *LysX* (Table S2). Moreover, like *Pd*, *Ecc15* is reported to promote proliferation of intestinal stem cells [43].

Another key antimicrobial defense mechanism in the fly gut involves the production of reactive oxygen species by Dual Oxidase (*Duox*) and NADPH oxidase (*Nox*). Both genes were robustly higher in *Pd* supplemented flies compared to controls (Table S2; *Duox* confirmed by RT-PCR, Fig 6). These findings fit well with reports that intestinal or whole-body knockdown of *Duox* drastically reduces fly longevity and the fly’s ability to clear intestinal *Ecc15* infection [44]. Importantly *Duox* and *Nox* upregulation was not balanced by the antioxidant enzymes superoxide dismutase and catalase, which were downregulated (Table S2, Fig. 6). Clues as to the mechanism for *Duox* upregulation may come from the work of Lee *et al.* who demonstrated that intestinal *Duox* upregulation, and *Duox* mediated production of H₂O₂, is activated by bacterially derived uracil and requires Hedgehog signaling via smoothened (*smo*) and *Cad99C*, both of which we also found to be elevated (Table S2) [45, 46]. In the absence of a real threat from *Pd* (which is typically missing from the normal *Drosophila* gut and was given in a non-viable preparation), these heightened defenses could serve to protect against infections by other bacteria or fungi.

Another notable response to *Pd* supplementation we observed was an elevation of proliferation in the gut as measured by the mitotic marker phospho-Histone H3 (Fig. 4). This effect was corroborated by RNA-Seq data, which showed a striking upregulation of dozens of genes related to DNA replication, especially *Cyclins A, B, D, and E* (Tables S2,3 & Fig. 6) in *Pd* supplemented flies. Buchon *et al* [47] report that elevated intestinal proliferation is also observed in response to *Ecc15* infection and furthermore that the effect was abrogated by knockdown of *Duox* or overexpression of catalase and recapitulated by feeding flies 0.1% H₂O₂. Others have shown that gut hyperproliferation in response to *Lactobacillus plantarum* is dependent on ROS formed by *Nox* [48]. Indeed, ROS are now recognized as critical intracellular messengers during cell growth and differentiation, especially in the intestine [49]. Taken together, these findings suggest that ROS produced by *Duox* and *Nox* could be critical signaling mediators driving the elevated proliferation observed in *Pd* supplemented flies. Notch signaling may also participate here, given its established role in supporting ISC proliferation and differentiation into enteroendocrine cells [50] and the higher expression of the Notch receptor (N)

as well as downstream genes *deltex* and *Su(H)* amongst *Pd* flies (Table S2, Fig. 6). Importantly, elevated proliferation was not associated with any overt morphological abnormalities in the gut (Fig. S3).

Pd supplemented flies also displayed higher expression of genes involved in various modalities of DNA repair including mismatch repair, base excision repair, nucleotide excision repair, homologous recombination, and the Fanconi Anemia (interstrand crosslink repair) pathway (Tables S2 and S3), key members of which were confirmed by RT-PCR (Fig. 6). We suggest that this broad upregulation of DNA repair could be driven by elevated Sirt6 (Table S2, Fig. 6) which is a DNA damage sensor [51] and master regulator of DNA repair [52]. Importantly, Sirt6 knockdown is reported to reduce, while overexpression extends lifespan in *Drosophila* [53], consistent with our observed lifespan extension.

Although not quantified here, robust elevation of Sirt6 and many DNA repair genes, may be due to elevated rates of collateral DNA damage formation. One potential mediator is H_2O_2 formed by Duox and Nox (via superoxide O_2^-). The importance of H_2O_2 is supported by findings that catalase mutant flies display elevated DNA double-strand breaks (γ H2AvD) and oxidative adducts (8-oxo-dG) [54]. As described above, we saw down regulation of catalase plus the upregulation of Duox, changes expected to compound the elevation of cellular H_2O_2 in *Pd* supplemented flies.

Elevated DNA double strand breakage has also been observed in response to ingestion of *Ecc15*, and other pathogenic bacteria *Serratia marcescens*, *Vibrio cholerae*, and *Psuedomonas entomophila* by *Drosophila* but not *Acetobacter tropicalis*, *Lactobacillus brevis*, or *Lactobacillus plantarum*. A concomitant elevation of DNA damage genes (GO:0006281) was also observed and the upregulation of γ -H2Av was dependent on ATM (*tefu*) and Nox, but not ATR (*mei-41*) or Duox [55]. Here we also see a robust upregulation of both *tefu* and *mei-41* in *Pd* supplemented flies (Table S2). While these observed molecular and cellular responses are internally consistent and match what has been reported in the literature, a predicted elevation in DNA damage seems somewhat paradoxical to the observed improvement in barrier integrity and longevity seen with *Pd*. One way to reconcile this is that an elevated baseline level of damage may induce a hormetic or protective repair and regenerative response, as described by Ristow [56], with the net effect being a stronger gut barrier, although this model is clearly speculative.

Scrutiny of the genes with lower expression in *Pd* supplemented flies reveals an enrichment of energy metabolism genes (Table S2). Specifically, multiple subunits of each of the five complexes of the electron

chain, both nuclear and mitochondrial encoded, were found to be lower in *Pd* flies (Tables S2,3). One obvious interpretation this vast unidirectional change is a reduction in Mt biogenesis which is normally associated with aging and reduced function (reviewed in [57]). One gene expression change of relevance here was the down regulation of AMP kinase alpha (AMPK α) in *Pd* flies (Table S2, Fig 6). AMP kinase is an energy sensor that regulates cellular energy metabolism [58], consisting of AMPK α , SNFAG and *alc* in *Drosophila* [59]. All three components were downregulated due to *Pd* supplementation (Table S2). AMPK is regulated at the protein level by kinases like Lkb1 kinase (Lkb1), which phosphorylates other proteins like Glycogen synthase (Glys) to inhibit them. Although we did not measure phospho AMPK levels, if we assume that low AMPK component mRNAs translates to lower active AMPK complex, this might lower activation of energy producing pathways and de-repress energy consuming programs like cell growth and proliferation. Unravelling this mechanism will require dissection of the pathway at the protein level as well as measurement of mitochondrial abundance and respiration.

Interestingly, heat treating the *Pd* membrane fraction destroyed its lifespan extending properties (Fig. S1). Because *Pd* was administered as a non-viable “post-biotic” preparation, this suggests that the active component of *Pd* is heat sensitive, perhaps a protein. We recently identified an anti-inflammatory membrane protein from *Pd* that sequesters bacterial lipopolysaccharide, thus preventing it from binding to, and activating, host TLR4 signaling [60]. It is unclear whether a similar mechanism is at play here and in fact *Pd* contains its own LPS molecules. Further work is required to identify the heat sensitive active component(s) of *Pd* that are responsible for lifespan extension in *Drosophila* as well as its target molecule(s).

Several questions associated with this study remain unresolved, mostly regarding fully unravelling the complex mechanism of action of *Pd*. Certainly, profiling ROS, DNA damage markers, and abundance of key intermediaries at the protein level, as well as the use of genetic analyses that determine whether knocking down those intermediaries recapitulates or destroys the life extending and barrier enhancing effects of *Pd* supplementation would complete the picture and will be the focus of future work. Nevertheless, at the organism level we have shown that *Pd* supplementation lengthens *Drosophila* median lifespan, slows loss of age-related climbing activity and strengthens gut barrier integrity concomitant with increased intestinal proliferation and the upregulation of several septate junction and adherens junction genes. Our working model, informed by RNAseq analysis, is that *Pd* activates immune defenses including

Toll and Imd pathways and upregulates signaling ROS via Duox and Nox to drive intestinal proliferation and repair. The relative contribution of each of these potential mediators to the improved healthspan phenotype remains unclear and requires further investigation.

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Data Availability Statement.

The RNA seq data are deposited at the NIH Gene Expression Omnibus (GEO) under accession # GSE315176. *Tables S2 and S3 are available at Boston University Library at <https://hdl.handle.net/2144/53133>

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.1584

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