The effect of axenic dietary restriction on the age-related changes in *C. elegans*

Ping Wu1\*, PhD; Lieselot Vandemeulebroucke1\*, PhD; Myriam Claeys2, BSc; Wim Bert2, PhD; Bart P. Braeckman, PhD1.

1. Laboratory of Aging Physiology and Molecular Evolution, Department of Biology, Ghent University, 9000 Ghent, Belgium.
2. Nematology Research Unit, Department of Biology, Ghent University, 9000 Ghent, Belgium.

\* These authors contributed equally to this work.

Corresponding author: Name: Bart P. Braeckman, PhD. Email: bart.braeckman@ugent.be

**Abstract**

Axenic dietary restriction (ADR) is highly effective in extending lifespan of *C. elegans* but its effects on healthspan improvement is less well characterized. Using transmission electron microscopy, morphometric analyses, and functional assays, we found ADR can preserve tissue ultrastructure, including the cuticle, epidermis, and intestinal lumen, and reduce age-associated pathologies like gonad degeneration, uterine tumor clusters, pharyngeal deterioration, and intestinal atrophy. However, there was no notable improvement in behavioral and functional metrics. Our results underscore that lifespan extension through ADR does not inherently translate to broad healthspan improvements.

**Keywords**: axenic dietary restriction, lifespan, healthspan, *C. elegans*.

# Introduction

In humans, aging is associated with external indications of general decline and loss of health, such as unintentional weight loss, slow walking speed, and low physical activity (1). In a similar vein, aging in the model organism *Caenorhabditis elegans* also manifests itself in a set of corresponding characteristics, including a decline in muscle function akin to human sarcopenia (2), coupled to diminishing locomotive abilities, and an increase in organ deterioration and functional decline of neurons (3, 4). Given these features shared between human and *C. elegans*, a greater understanding of the ageing process in *C. elegans* could reveal new avenues for investigating age-related changes in humans.

Over the past thirty-five years, pioneering efforts have yielded significant breakthroughs in the pursuit of maximizing lifespan in *Caenorhabditis elegans*. These milestones encompass the discovery and exploration of long-lived mutants within the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway, modulation of protein translation and mitochondrial activity, reproductive interventions, and implementation of dietary restriction regimens (5, 6). These discoveries have demonstrated that it is possible to effectively postpone a series of age-related degenerative changes. For instance, the motility of worms is prolonged in both long-lived *daf-2* and *eat-2* mutants compared to wild type animals (7, 8). Additionally, *daf-2* mutants exhibit increased resilience against bacterial colonization, a factor that can cause premature mortality in *C. elegans* (9, 10). Moreover, *daf-2* can also delay age-dependent increases in neural abnormalities and maintain neuronal functions, such as learning ability, for a longer time (11). Dietary restriction (DR) is a well-established nongenetic means to improve healthspan across a variety of species, ranging from budding yeast, fruit flies, to rats, mice, and even humans (6, 12, 13). This intervention has demonstrated its ability to prevent obesity, delay the onset of age-related conditions such as sarcopenia, presbycusis, and brain atrophy, as observed in Rhesus monkeys (14, 15). In rodents, intermittent fasting has also shown promise by reducing the incidence of numerous chronic diseases, including stroke, cardiomyopathy, hypertension, diabetes, and several neurodegenerative diseases (16). However, recent work has raised questions regarding whether DR-induced lifespan extension truly correlates with delayed age-related degenerative changes. The *C. elegans* DR mimetic strain *eat-2* does not show improvements in health parameters such as resistance to oxidative stress, heat tolerance, and overall movement, suggesting an uncoupling of lifespan and healthspan in this context (17). In addition, age-related changes are widespread and multifaceted. Therefore, it is necessary to assess multiple age-associated changes and reveal whether any of them serve as critical factors limiting lifespan and, ultimately, to identify effective strategies for potential anti-aging interventions.

The lifespan of *C. elegans* is notably flexible and is influenced by environmental conditions, nutrient availability, and genetics. One of the most robust longevity effects in *C. elegans* is observed when worms are cultured in axenic media (18). In this regimen, coined axenic dietary restriction (ADR), worms are cultured in the absence of their usual laboratory food source, *Escherichia coli*, instead feeding on a nutrient-rich sterile medium (19). Under ADR conditions, worms exhibit an impressive doubling of lifespan, higher oxidative and heat stress resistance, and increased metabolic activity, compared to bacteria-fed worms (19). ADR can also increase mitochondrial efficiency by increased electron transport chain (ETC) capacity, while concurrently repressing the age-related rise in leak respiration and decline in coupling efficiency (20). These remarkable responses to ADR are independent of well-studied transcription factors governing stress responses (e.g., *daf-16*, *skn-1*, *pha-1*, *hif-1*, and *hsf-1*) and unfolded protein responses (e.g., *atfs-1*, *dct-1*, *ubl-5, hsp-6, hsp-60, hsp-4*) (21, 22). Despite these molecular insights, a comprehensive understanding of age-dependent alterations at the tissue level in ADR animals remains relatively unexplored. To address this gap, we used ultrastructural and morphometric tissue analysis to analyze whether ADR can effectively mitigate the onset of age-related pathologies.

Aged *C. elegans* exhibit a multitude of pathologies during senescence which are evident at the anatomical and functional level, including degeneration of the pharynx, atrophy of the intestine and gonad, and a redistribution of steatotic lipoprotein (23). Throughout the aging process, the hermaphrodite gonad undergoes significant morphological deterioration particularly degeneration and disintegration of the distal side (24). Morphological alterations also occur in the proximal gonad with advancing age. Notably, post-sperm depletion and accumulation of unfertilized oocytes within the uterus which results in tumor formation in the midbody (25). These tumors can exert pressure on the intestine and other body tissues, potentially contributing to structural compromise. Another feature is the decline in pharyngeal pumping rate with age, with bacterial colonization in the posterior bulb being linked to early demise in *C. elegans*, hinting at the potential life-limiting nature of pharyngeal pathologies (10). Additionally, the intestine also shows a progressive decline including the degeneration of intestinal microvilli, thinner and tortuous intestinal lumen, atrophy, and altered cell morphology caused by ingested bacteria (23, 26). It seems that bacterial food holds the potential to significantly impact the aging process of various tissues. We wonder whether nutrient limitation through ADR can delay the onset of many of these pathologies.

# Materials and methods

## *C. elegans* strains and *E. coli* RNAi strains

The wild-type strain used was Bristol N2 male stock (CGC, Caenorhabditis Genetics Center). The OD95 *unc-119(ed3)III;* *ltIs37 [pie-1p::mCherry::his-58 + unc-119(+)] IV; ltIs38 [pie-1p::GFP::PH(PLC1delta1) + unc-119(+)]* strain was used to image the reproductive system. *C. elegans* strains were maintained at 20°C on nutrient agar (NA) (Oxoid Ltd. Thermo Fisher Scientific Inc., USA) plates seeded with *Escherichia coli* OP50 as a food source.

## *C. elegans* maintenance

For the standard fully fed (FF) cultures, a batch of eggs was produced by subjecting gravid adults to hypochlorite treatment. These eggs were then allowed to hatch overnight in salt buffer (S-buffer: 0.1 M NaCl + 1 M KH2PO4 + 1 M K2HPO4) and the L1 larvae were subsequently introduced onto nutrient agar (NA) or nematode growth medium (NGM) plates that had been pre-seeded with *E. coli* OP50 bacteria. For ADR, standard undefined axenic medium was used consisting of 3% soy peptone (Sigma-Aldrich, St. Louis, MO) and 3% yeast extract (Becton-Dickinson, Franklin Lake, NJ), supplemented with 0.05% haemoglobin (bovine; Serve, Heidelberg, Germany) diluted from a 100x stock in 0.1 M KOH (autoclaved for 10 minutes). To avoid contamination, all equipment and preparatory procedures were conducted within a controlled laminar flow cabinet.

***C. elegans* development**

To ensure the accuracy and reproducibility of our experiments, we employed three different methods to avoid contamination during the preparation of *C. elegans* for our studies (Figure S1). (a) microbleaching. Briefly, approximately ten gravid worms were placed in a drop of 10 μL sterile distilled water in a 5-cm petri dish, 10 μL of a concentrated bleach solution (comprising 13.5% hypochlorite and 1 M NaOH) was added and left to incubate until all adults were dissolved or for a maximum of ten minutes. Next, 5 mL of axenic medium containing 20% sterile skimmed milk was added. The eggs were allowed to hatch and were incubated at 20°C until adulthood. For monoxenic and axenic cultures, it took 2 and 4 days on average to reach L4 stage at 20°C, respectively. (b) NGM plates seeded with paraformaldehyde (PFA) killed *E. coli* bacteria (27). An overnight culture of *E. coli* OP50 was killed by adding 32% PFA to a 0.5% final concentration. PFA-treated bacteria were shaken at 37°C for 1 h at 150 rpm, transferred to 50-mL conical tubes, and centrifuged at 3000×g for 20 mins. Supernatant was removed and washed with LB five times to remove residual PFA. After washing, the PFA treated bacteria were concentrated five times before seeding onto NGM plates. (c) NGM plates seeded with UV-killed *E. coli* OP50. Briefly, gravid adults were synchronized by microbleaching to eliminate live bacteria. Collected eggs were then allowed to hatch overnight in S-buffer and the L1 larvae were subsequently introduced onto UV-treated NGM plates to develop into L4 stage. L4 worms were collected and cleaned five times with S-buffer. Next, worms were transferred to both monoxenic and axenic conditions for sampling at different timepoints throughout lifespan. For the lifespan assays, we used all three methods. For detailed pathology measurements, we employed methods (a) and (b). For transmission electron microscopy and locomotory tests, we utilized method (c) to maintain a sterile environment throughout the experiments.

## Transmission electron microscopy

Worms were sampled from experimental conditions at different ages and placed on ice for a few minutes to relax and straighten. Subsequently, they were primary fixed at 4°C in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) supplemented with 0.25 mg/ml MgCl2. After 30 minutes, the head and tail tip were removed, and the remaining parts were cut in the middle. The upper and lower parts were treated separately. Overnight fixation was carried out in fresh primary fixer under rotation (Carl Roth, Karlsruhe, Germany) at 4°C. This was followed by two hours of post-fixation in 2% osmium tetroxide in the same buffer at room temperature (RT). After post-fixation, the specimens were stained “en bloc” for 1 h in a 1% solution of uranyl acetate in distilled water, dehydrated in ethanol and isopropanol series and embedded in low viscosity embedding medium Spurr resin (EMS, Hatfield, UK). Ultrathin (60 nm) sections were cut using a Leica Ultracut UC7 ultramicrotome (Leica, Vienna, Austria) with a diamond knife (Diatome, Ltd., Biel, Switzerland) and collected on formvar-coated copper single slot grids (Agar Scientific, Stansed, UK). After post-staining for 25 min with uranyl acetate at 37°C and 4 min with lead citrate at RT, sections were examined with a JEOL JEM 1010 (Jeol, Ltd, Tokyo, Japan) transmission electron microscope equipped with a side-mounted CCD Veleta camera. Measurements of cuticle width were made using Radius 2.1 software (Emsis GmbH, Münster, Germany).

## Thrashing assay

The number of thrashing movements was measured by transferring individual adults into a drop of S-basal on a microscope slide at room temperature. After equilibrating for at least 30 s, the number of body bends was counted over a period of 30 s. One single thrash was defined as a complete change in the direction of bending at the mid body.

## Behavioral analysis

Worms were transferred from the experimental conditions to NGM plates that were previously seeded with a 20-μL drop of fresh OP50 and dried. Worms were allowed to habituate on the plates for 30 minutes. Movement of the worms was recorded for 10 minutes without perturbation, using a Nikon AF Micro Nikkor 55 mm lens connected to a ToupTek XCAM 1080 PHA digital camera (ToupTek Photonics, China), mounted on a fixed height stand. Three videos were made for each sampling time for both dietary conditions, each filming approximately twenty worms.

Videos were analyzed to extract behavioral features using Tierpsy (28). This worm tracking software is freely available at https://github.com/Tierpsy/tierpsy-tracker and more information on extracted features can be found via https://github.com/celegans-ulb/MultidimensionalPhenotyping. Per video, a set of 4539 features were extracted, which represent parameter means calculated for all worms imaged in the video. These features contain information on worm posture, locomotion, morphology, and behavior. After feature extraction, worms containing more than 25% of missing values were removed from the dataset, along with features that show no variance.

## Pathology morphometrics

For the light microscopical preparations, 20 μL of nematode suspension was added to microscope slides and worms were anesthetized with 10 mM levamisole. Nomarski (differential interference contrast or DIC) microscopy images were acquired with a Bresser Optik MikroCam PRO digital camera mounted on a Reichert Jung POLYVAR microscope. Worm images were analyzed blindly by using blinder software (29). For gonad, pharynx, and uterine tumor pathologies, images were randomized and scored on a scale from 1 to 5. Intestinal atrophy was quantified by measuring relative intestinal width at a point posterior to the uterine tumors. The relative intestinal width was calculated as the total width minus the width of the lumen, divided by the body width (23).

## Lifespan assay

One hundred of L4 larval stage worms were placed into small screw-cap tubes (three to five worms per tube) containing 0.3 mL of axenic medium (3% yeast extract + 3% soy peptone + 0.05% hemoglobin + 5 μg/mL cholesterol), and around hundred worms were placed on small NGM plates (10 per plate) seeded with *E. coli* OP50 as fully fed (FF) control. To prevent progeny production, 100 μM final concentration of FUdR was added. Survival was scored every other day. Worms on NGM plates were considered dead if they did not respond to gentle prodding with a platinum wire. In liquid conditions, worms were scored dead if no movement was detectable, even after the tubes were gently tapped. Worms that died of protruding vulva or crawling off the plates were censored. All lifespan experiments were carried out at a temperature of 20°C.

## Statistical analysis

This analysis was conducted by examining the impact of these diets on both chronological age and relative age (physiological age). For physiological age, chronological age was normalized to mean lifespan within the respective diet to ensure stability and reduce susceptibility to outliers. Various aging parameters in animals on FF or ADR diets were analyzed using appropriate regression models: cuticle thickness and worm length were analyzed using simple linear regression, while gonad, uterine, and pharynx scoring values were modeled using an asymmetric sigmoidal 5-parameter logistic (5PL) regression model; other parameters were evaluated using an exponential model. The regression performance was assessed using the adjusted squared correlation (R2). Statistical comparison of the inflection points and/or slope rate were done using a Welch *t*-test. All statistical analyses were conducted using GraphPad Prism version 10 software.

# Results

## Cuticle and epidermis senescence is delayed in ADR worms

The cuticle is a tough flexible layer, secreted by the epithelial cells, that protects the worm from the environment and enables locomotion as it acts as an external skeleton under hydrostatic pressure (Figure 1A). Previous research indicated that the basal and cortical zones of the cuticle progressively grow thicker during aging, most likely because of unregulated biosynthesis of cuticle-related proteins and the absence of post-reproductive shut-down of overall expression (3). However, our data shows that cuticular thickness does not significantly increase with chronological or relative age in monoxenically (*P*=0.6504) or axenically (*P*=0.7154) cultured worms (Figure 1B, C). Hence, the slopes of the linear regressions for both diets do not differ significantly (*P*=0.5340), suggesting cuticle thickness remains unaffected over time and with diet. However, as worms age, the delineation of the five major layers becomes less clear and the fluid-filled space supported by the collagen struts becomes more electron dense, which indicates that the cuticle loses its firmness with aging. We note that this phenomenon appears to manifest itself at a slower rate in axenically cultured worms. Furthermore, the quality of the collagen struts is maintained for a longer time in ADR worms (Figure 1D). In conclusion, we found that cuticular thickness does not increase with age in both dietary regimes, but cuticle senescence is delayed in ADR worms.

Underneath the cuticle lies the epidermis, also referred to as hypodermis, which is a single epithelial layer that encloses the pseudocoelomic fluid-filled cavity housing the main organs. As fully fed worms age, the epidermal cylinder becomes exceedingly thin in all regions and the capacity to maintain its shape is gradually lost. In addition, the cytoplasm contains fewer cell organelles and those that are present often appear damaged. The cytosol becomes progressively less electron dense (3). We observed comparable patterns of age-dependent epidermal deterioration in our ad libitum fed worms. Although similar observations were made for axenically cultured worms, these changes seem to appear at a slower rate: the epidermis maintains its shape and the cell organelles remain intact for a longer period (Figure 1E). A striking difference is the higher number of lipid droplets in the epidermis of ADR worms. As the epidermis is one of the major fat storage tissues besides the intestine, this observation alludes to altered fat metabolism or fat storage dynamics under ADR.

## Locomotory function is not improved in ADR worms, despite delayed decay of body wall muscles

*C. elegans* has 95 obliquely striated body wall muscles that are arranged in staggered pairs in four longitudinal bundles that run from head to tail, arranged in two dorsal and two ventral quadrants. Structural and functional decline of muscles is an imminent consequence of aging in a wide variety of species. *C. elegans* develops sarcopenia during aging, which is the progressive loss of muscle mass characterized by a decline in muscle quality and quantity with advancing age. The muscular loss starts in midlife and magnifies with age, leading to reduced mobility (30). The changes in mobility are not caused by neuropathology during aging, as the nervous system appears to remain intact in old worms, whereas muscle cells shrink overall, sarcomeres and myofibrils become progressively disorganized and myosin filaments are gradually lost (3, 30). Here, we confirm these findings for both axenically and monoxenically cultured worms, although maintenance of sarcomere structure and myofibrils appear to persist for a longer time in ADR worms (Figure 2A).

The main function of body wall muscles is locomotion, and locomotory function is a parameter often used for healthspan assessment in *C. elegans* (7). Spontaneous locomotion, measured as the average distance moved within one hour, declines similarly with age in bacterially deprived and ad libitum fed worms(31), which suggests that this healthspan parameter may not be improved in DR conditions. As most ADR assays are performed in liquid axenic medium, we chose to observe thrashing movement. Our data shows that thrashing movement is significantly lower in young axenically cultured worms compared to monoxenically cultured individuals, but the decline in thrashing rate does not significantly differ for both dietary regimens across chronological (*P*=0.4230) and relative age (*P*=0.7913) (Figure 2B). This contrasts with the findings of (32), which show that worms grown on dietary restriction plates have a higher thrashing frequency compared to fully fed animals.

To gain more insight in what aspects of locomotion might be affected by ADR, behavioral features of worms freely crawling on an agar plate were analyzed in detail using Tierpsy tracking (28). We first focused on the curvature and angular velocity of different body segments over aging under FF and ADR diets as these features were found to significantly alter with age(33). Unexpectedly, no significant differences could be found for any of these features between FF and ADR across chronological and physiological age (Figure 2C-E, Figure S2). In addition, other key parameters that may relate to health, such as worm size (length 90th), speed (speed 90th) and path coverage in the midbody, were compared as well. The worm size (length 90th)parameter slightly increases in both FF and ADR with age at a similar rate (*P*=0.7408) (Figure 2F). The speed 90th parameter declines also at a similar rate with age (*P*=0.9747) in both dietary regimens (Figure 2G). The midbody path coverage refers to the cumulative distance covered by the central part of the worms along its path. A more gradual decrease in midbody path coverage was observed for aging ADR worms compared to fully fed control, suggesting axenically cultured worms retain their ability to actively explore their surroundings much longer. However, the midbody coverage declines at similar rate in FF and ADR diets in both chronological age (*P*=0.0973) and relative age (*P*=0.4054) (Figure 2H). Integrating these findings, it becomes evident that although ADR has the capability to decelerate the degradation of body wall muscle (TEM observations), it does not clearly translate into enhanced locomotory functionality in axenically cultured worms.

## ADR delays age-related changes in the hermaphrodite germline across chronological age

The germline of *C. elegans* undergoes notable morphological deterioration during the aging process (34). As the worms age, the distal gonad undergoes degeneration characterized by the emergence of cavities, graininess, and cellular changes. Simultaneously, in the proximal gonad, oocytes experience shrinkage and fusion, resulting in the formation of large clusters resembling uterine tumors. Like other dietary restricted animals, axenically cultured worms reduce brood size and prolong their reproductive period(19). The gonadal arm of ADR worms is substantially shorter and contains less matured oocytes in the proximal gonad compared to that of FF worms (Figure S3). Although gonadal shrinkage is observed in FF and ADR worms, we wondered whether it is proportionally delayed in the long-lived ADR worms. To explore this, we semi-quantitatively assessed the dynamics of gonad atrophy: gonad morphology was categorized into five classes, each with distinctive features (24) (Figure S4). Class 1 worms have full-sized and youthful gonads; class 2 worms have gonads that are still intact but display slight signs of atrophy and irregular borders; for class 3 worms, we observe a reduced gonadal diameter, accompanied by initial signs of fragmentation, yet the last oocyte remains discernible; in class 4 worms fragmented gonads become more prominent; finally, in class 5, gonad remnants are barely recognizable. We found that, for both diet regimens, senescent pathologies of the gonad become evident at an early stage, around the time of sperm depletion (day 5 of adulthood) and reach their peak severity just prior to the median lifespan (Figure 3A). During chronological aging, gonad deterioration in ADR worms occurred at a reduced pace compared to FF worms (*P*=0.0375). However, when the data are adjusted for relative age, the rate of gonad integrity decline in ADR worms is similar with FF (*P*=0.1279), and the emergence of accelerated gonad atrophy appears earlier in ADR worms (Figure 3B).

We also assessed uterine tumor formation using semi-quantitative scoring. Class 1 represents a normal uterus containing fertilized eggs; class 2 indicates a slightly abnormal uterine content, without visible tumors but unfertilized oocytes have an abnormal appearance; class 3 to 5 worms have uterine tumors of different size (Figure S4). Specifically, class 3 denotes the presence of small tumors (occupying half of the worm's width), class 4 encompasses medium-sized tumors (2/3 of the worm's diameter), and in class 5 worms, large tumors occur that fill the body cavity and exert pressure on the intestine. The ADR group demonstrates a slower rate of uterine aging compared to the FF group (*P*=0.0286), and before reaching their median lifespan, both FF and ADR groups reach a plateau of maximal uterine tumor formation (Figure 3C). Furthermore, uterine tumor formation is significantly delayed when considering the inflection point as a function of chronological age (*P*=0.0319) (Figure 3C). However, at a relative age scale, ADR leads to earlier uterine tumor formation, but the relative rate at which tumor clusters are formed is comparable to that of the FF group (Figure 3D). Notably, uterine pathology manifests itself earlier, peaking around day 10 of adulthood, compared to gonadal atrophy which peaks around day 17 in FF diets.

The effect of ADR on delaying germline age is not limited to specific dietary conditions, as demonstrated by similar outcomes when worms are developed under FF conditions using PFA-killed bacteria (Figure S5A, B). Collectively, these data indicate that while ADR may retard germline aging in terms of chronological age, this effect is not observed when considering relative age. Moreover, these pathological markers appear to be independent of the worms' dietary history prior to reaching maturity.

## ADR slows down pharyngeal degeneration but not the age-dependent decrease in pumping rate

 *C. elegans* is a bacterivore filter feeder, with its pharynx serving as the primary organ responsible for acquiring and processing food from the environment. The main food source for *C. elegans* maintained in the lab is *E. coli* bacteria. Larvae and young adults exhibit efficient consumption of this food source, and intact bacterial cells are seldomly observed beyond the anterior portions of the pharynx lumen (10, 35, 36). As the worms age, the presence of intact bacteria becomes more prevalent within the pharyngeal lumen, and in some instances, even within the cells of the posterior pharynx, leading to swelling of the lumen and pharyngeal muscle cells. This is often accompanied with other age-dependent degenerative structural changes, such as darkening of the pharyngeal regions (35, 36). Notably, certain long-lived animals, such as *daf-2* mutants, exhibit relatively sustained youthful pharynx function at advanced ages compared to wild type or shorter-lived mutants (37). Hence, we hypothesize that long-lived axenically cultured worms, absent of bacteria, may also exhibit enhanced maintenance of pharyngeal integrity and function. To examine this, we scored pharynx structure with age under FF and ADR conditions (Figure S4). Class 1 worms show a healthy pharynx with smooth borders and intact radial muscle striations; class 2 worms have a pharynx with slightly uneven borders, still discernible; class 3 worms are characterized by non-circular muscle striations, or intra-pharyngeal bulb cavities or vacuoles; and class 4 worms have a pharynx in which the bulb is hardly recognizable. In line with our expectation, ADR effectively preserves the pharyngeal structure of *C. elegans*, with a markedly slower rate of degradation compared to FF counterparts across chronological age (Figure 4A). However, when considering relative age, the degeneration of the pharynx in ADR worms occurs at a comparable rate to that of FF worms (Figure 4B). This result is consistent with the outcomes when worms are developed under FF conditions using PFA-killed bacteria (Figure S5C). These patterns of structural decay are not reflected at the functional level: although FF worms exhibit higher pumping rates than their ADR-treated counterparts, the rate of decline is similar for both dietary regimens, both in terms of chronological (*P*=0.3754) and relative age (*P*=0.7861) (Figure 4C, D). In summary, while ADR effectively slows the deterioration of pharyngeal structure as a function of chronological age, it does not clearly prevent the age-related functional decline.

## ADR decelerates intestinal atrophy but not the deterioration of microvilli

The intestine of *C. elegans* serves as a metabolic organ, responsible for the breakdown of ingested nutrients and their subsequent distribution to other organs (38). Furthermore, the intestine undertakes vital roles such as the synthesis and storage of macromolecules, initiation of immune responses, and provisioning germ cells with yolk (38, 39). The progressive deterioration of the intestine with age manifests itself in various pathologies, including the deterioration of intestinal microvilli and nuclei, aberrations in the intestinal lumen, as well as alterations in shape, size, and the composition of cytoplasmic content (3, 26). Prior studies conducted in *C. elegans* have underscored the important role of the intestine in fitness maintenance and lifespan extension. For instance, the age-related decline in the intestinal barrier function is reduced in *eat-2* mutants due to an increased turnover of autophagosomes (40). Aligning with this observation, *daf-2* mutation not only extends lifespan but also mitigates indicators of senescence, including the accumulation of pseudocoelomic lipoprotein pools, alterations in lipid distribution, and the occurrence of intestinal atrophy (23). Based on these findings, we wonder whether ADR would also mitigate the gradual deterioration of the intestine. We initially studied changes in the intestinal lumen and microvilli in response to both FF and ADR diets using transmission electron micrographs (TEM). In young animals, the intestinal lumen is uniformly sized with consistent microvilli anatomy across cells. However, in older animals, it becomes thinner and more irregular, with microvilli experiencing degradation (26). Similar patterns of aging-related changes in the lumen and microvilli were observed in FF and ADR worms, but the lumen of ADR worms appear to remain uniformly sized for a longer time (Figure 5A). Notably, some of the older axenically cultured worms exhibited patches where the microvilli had flattened. To further analyze these changes, we quantified the length of microvilli in TEM micrographs of both FF and ADR animals. We found no significant difference in the rate of microvilli shortening between the two dietary conditions (*P*=0.8451) (Figure 5B, C). We also observed patches in some old ADR cultured individuals where most of the villi had degraded, and the terminal web had lost its structural integrity (Figure 5D). This suggests that the barrier between the intestinal lumen and the gut cell cytoplasm was compromised. Finally, we quantified the relative intestinal width as a measure of intestinal atrophy during aging (23). Our data showed that ADR animals had a higher relative intestinal width than age-matched FF worms, and the ADR diet causes a significantly reduced rate of intestinal atrophy over chronological (*P*=0.0087) and relative age (*P*=0.0415) (Figure 6E, F). These results indicate that intestinal atrophy is delayed by ADR, but it does not impede the deterioration of microvilli.

## Healthspan is shortened in long-lived ADR worms

To accurately assess animal health, we divide the animal's lifespan into a period of healthspan and sickspan (time spent in a state of morbidity). In this study, healthspan is defined as the period during which animals maintain over 50% of their maximal functional capacity on a particular diet. This assessment encompasses pumping rate, behavioral parameters, intestinal border size, and intestinal atrophy. For the gonads, and uterus healthspan is determined as the period during which a particular scoring value remains below 3, and healthy pharynx is scored no less than 2. These thresholds balance the early and late stages of aging, identifying a period where aging is apparent but not in its advanced stage. Using these definitions, we chronologically and physiologically analyzed healthspan data, quantifying the number of days or relative age in healthspan or sickspan for both diets across each assay. Ideally, long-lived ADR worms would show extended healthspan and reduced sickspan. Chronologically, ADR appears to marginally increase the duration of healthspan across most parameters (Figure S6A). However, physiologically recalibrated data reveals no proportional healthspan increase in long-lived ADR worms. Instead, there is a tendency towards reduced healthspan, suggesting an elongated sickspan or frailty period in ADR worms (Figure S6B).

# Discussion

*C. elegans* exhibits a wide array of age-associated changes including declines in physiological processes (e.g. locomotion, pharyngeal pumping, and defecation frequency) as well as deterioration in appearance (e.g. muscle integrity and redistribution of yolk protein)(3). Maintenance of pharyngeal pumping rate and body movement are positively correlated with lifespan and therefore suggested to be used to predict lifespan (8). However, long-lived mutants such as *daf-2(e1370)* and *eat-2(ad1113)* spend a higher proportion of their lifespan in a frail state (17), despite observations that *daf-2* mutants live healthier compared to wild-type counterparts (9). These conflicting results have led us to investigate whether ADR, a lifespan-doubling treatment, can also increase healthspan by using various aging biomarkers. Our study evaluated a range of parameters in *C. elegans* via ultrastructural (TEM) observation, morphometric analysis, and functional tests. Our findings revealed enhanced ultrastructural preservation in major tissues of ADR worms as they aged chronologically, suggesting that the aging process is delayed in axenically cultured individuals. Most morphometric parameters, including gonadal and intestinal atrophy, uterine tumor development, and pharynx deterioration exhibit significant delays in ADR worms over chronological age. This aligns with recent findings that culturing animals on solid axenic medium plates at adulthood can almost completely suppress these pathologies (41). Conversely, on a physiological time scale, long-lived ADR worms displayed a steeper decline of these morphological parameters except for intestinal deterioration compared to FF worms (Figure S7). Intestinal atrophy consistently showed a delay in both chronological and relative age. Other functional parameters like pumping rate and some locomotive features in ADR worms displayed a similar chronological decline as FF cultured worms. However, at a relative time scale, they exhibited these declines earlier. These results highlight that interventions extending lifespan do not invariably correlate with improved health outcomes. Notably, in line with previous research (23), our study reveals that the morphometric parameters of senescence reach maximal severity before the population mean lifespan, peaking at approximately 25% mortality in both FF and ADR conditions (Figure S7). Furthermore, uterine tumor formation appears to reach its severity earlier than other aging pathologies like gonad atrophy. This is likely because tumor formation is directly linked to the accumulation of unfertilized oocytes in the uterus. In *C. elegans*, the reproductive strategy involves continuous oocyte production throughout the reproductive period. After the reproductive phase concludes, the absence of fertilization leads to the buildup of unfertilized oocytes, which can induce tumorigenesis. This process is a direct consequence of the reproductive strategy, as the organism's reproductive system remains active in oocyte production even when fertilization is no longer occurring. Consequently, the presence of these unfertilized oocytes rapidly triggers pathological changes, resulting in earlier and more pronounced tumor formation. In contrast, gonad atrophy is not an immediate consequence of the reproductive strategy but rather a gradual process reflecting the general aging of the reproductive organs. Gonad atrophy is characterized by a progressive decline in cellular function and structural maintenance within the gonads, which develops over an extended period. Unlike the rapid and direct pathological response caused by uterine tumor pathology, gonad atrophy represents a slower degenerative process. It is associated with the overall aging of the organism rather than a direct response to the cessation of reproductive activity. Additionally, locomotory decline may be associated with muscle deterioration (sarcopenia), which is a feature of aging in *C. elegans* and other organisms (42). However, we here have shown that even when the body wall muscles are preserved, none of the locomotive parameters are significantly improved under ADR conditions. This discrepancy points to the significance of factors beyond muscle structure, such as neural control and neuromuscular communication(43), and energy availability/allocation in dictating locomotive function. In addition, while our automated behavioral analysis primarily reflects voluntary movement, it is important to consider the implications of thrashing rates in a liquid environment. This scenario might better indicate muscle function and endurance, particularly in aging worms. The data suggest that thrashing rates in FF worms decline more rapidly with age compared to ADR worms, indicating better muscle preservation in the latter. This observation aligns with the hypothesis that ADR can enhance muscle resilience by possibly reducing age-related muscle degradation.

Recent research has demonstrated that longevity mutants *eat-2*, *glp-1*, and *daf-2* exhibit a delayed decline in maximum force and dynamic power during aging compared to their wild-type counterparts (44). This delay, however, does not result in a reduced morbidity period, but rather to a proportional extension of healthspan relative to their increased lifespan(44). However, our findings contrast with these, as we observed that long-lived ADR worms spend a larger proportion of life in a frail state compared to FF worms (decreased healthspan-to-sickspan ratio). This suggests that lifespan extending interventions such ADR do not necessarily translate into healthspan gains. This was also found for some long-lived mutants except for *daf-2* (7, 17). Mixed reports on the effect of DR on the healthspan-to-sickspan ratio also occur in other species. Caloric restriction in mouse lemurs extends survival by 50% but compromises brain gray matter integrity (45). Similarly, mTOR downregulation prolongs mouse lifespan but disrupts glucose homeostasis, potentially implicating type 2 diabetes (46). Conversely, DR not only increases lifespan but also offers protection against diabetes, cancer, and cardiovascular disease in rhesus monkeys, and induces protective changes against age-related pathologies in humans (6, 14). These findings underscore the complexity and variability inherent in the biological trade-offs between lifespan and healthspan extension. Evidently, healthspan extension is the prime goal for improving wellbeing in humanity. However, evidence suggests that currently, particularly for females, medical advances have extended longevity mainly by prolonging the period of late-life morbidity, with minimal impact on the duration of disability-free life (47).

# Author Contributions

**Ping Wu**: Conceptualization, Methodology, Investigation, Data curation, Visualization, Formal Analysis, Writing- Original draft preparation. **Lieselot Vandemeulebroucke**: Conceptualization and Methodology. **Myriam Claeys**: technical support and interpretation of electron microscopical images. **Wim Bert**: interpretation of electron microscopical images and reviewing. **Bart P. Braeckman**: Conceptualization, Supervision, Resources, Writing- Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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**Figure legends**

**Figure 1**. Age-associated changes in *C. elegans* cuticle and epidermis under fully fed (FF) and axenic dietary restriction (ADR) conditions. (A) diagram showing orientation of the TEM sections. Mean cuticle thickness of worms under FF and ADR conditions over chorological age (B) and relative age (C). At least 10 worms were measured per timepoint for at least two biological replicates. Error bars are SD and a linear regression model was fitted to the data. Slopes were compared using a Welch *t*-test. (D) representative TEM micrographs of the cuticle of FF and ADR worms taken at different timepoints. (E) representative TEM micrographs of epidermis in FF and ADR conditions at different timepoints. cuticle (C), epidermis (e), muscle sarcomere (mu), nucleus (N), mitochondrion (m), lipid droplet (L), and collagen strut (line segment with endpoints).

**Figure 2**. Locomotory function decreased both in monoxenically (FF) and axenically (ADR) cultured worms. (A) representative TEM micrographs of the body wall muscle sarcomeres of monoxenically (FF) and axenically (ADR) cultured worms at different timepoints. Cuticle (C), mitochondrion (m), epidermis (e), yolk (Y), asterisk (dense body) and collagen strut (line segment with endpoints). (B) Thrashing rate of FF and ADR worms over chronological and relative age. (C-E) Age-dependent decrease of the curvature of different body segments (hips, midbody, and neck) in FF and ADR worms as quantified by Tierpsy over chronological and relative age. Worm size (F), speed (G) and midbody path coverage (H) in FF and ADR worms over chronological and relative age, quantified by Tierpsy video analysis. Six worms were measured per timepoint over two biological replicates. Error bars are SD. An exponential model was used to fit a trendline to the data of the thrashing rates, the curvature of different body segments, worm speed and midbody path coverage. Worm length was modeled by linear regression. Statistical comparison of the rate constant or slope were done using a Welch *t*-test.

**Figure 3**. Aging pathology in the *C. elegans* hermaphrodite gonad and uterine. Age-dependent increase in gonad deterioration under fully fed (FF) and axenic dietary restriction (ADR) conditions using chronological age (A) and relative age (B). Uterine tumor development during ageing under FF and ADR conditions using chronological age (C) and relative age (D). At least 20 worms were scored per sample for two biological replicates. Error bars are SD. An asymmetric sigmoidal 5-parameter logistic (5PL) regression model was applied for the trendlines. Statistical comparison of the inflection points and/or hillslope were done using a Welch *t*-test.

**Figure 4**. Pharyngeal aging in the *C. elegans* hermaphrodite. Progression of pharyngeal aging in response to FF and ADR diets, plotted against chronological age (A) and relative age (B). At least 20 worms were scored per sample for two biological replicates. Error bars are SD. An asymmetric sigmoidal 5-parameter logistic (5PL) regression model was applied. Statistical comparison of the inflection points and/or hillslope were done using a Welch *t*-test. Pharyngeal pumping rates for FF and ADR-treated worms as a function of chronological (C) and relative age (D), respectively. Ten worms were counted for three biological replicates. Error bars are SD. An exponential model was utilized for calculating the trendlines. Statistical comparison of the rate constant was done using a Welch *t*-test.

**Figure 5**. Intestinal integrity of FF and ADR worms. (A) representative TEM images of microvilli and intestinal lumen of FF and ADR worms at different ages. Lumen (Lu), microvilli (mv), bacterium (b), terminal web (arrowheads). (B-C) Length of microvilli in FF and ADR animals across chronological (B) and relative age (C). Six worms were measured per timepoint over two biological replicates. (D) section of a 50-day old ADR worm showing flattened microvilli and patches of reduced microvillar density. Arrows indicate regions with flattened microvilli. Relative intestinal width under FF and ADR conditions across chronological (E) and relative age (F). Twenty worms were scored per sample over two biological replicates. Error bars are SD. Trendlines were calculated using an exponential model. Statistical comparison of the rate constant was done using a Welch *t*-test.