InVivo Biosystems

Vitality & Antioxidant Study Report

for

Thaena, Inc.

June 1, 2022

Customer	**Thaena, Inc.**
Provider	**InVivo Biosystems** 1505 Westec Drive Eugene, OR 97402
Customer Contact	Andrea McBeth Chief Executive Officer andrea@thaena.com
Project Code	Thaena-001a
Project Scope	1. Identify and measure the life-extending capabilities of ThaenaBiotic. 2. Assess and measure mitigation of healthspan decline with ThaenaBiotic.

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Introduction

Rationale

Thaena wishes to leverage the InVivo Longevity platform to produce data for IP filing and marketing claims on the efficacy of their product ThaenaBiotic.

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Executive Summary

Thaena's product ThaenaBiotic was tested for activity on the Vitality Platform, which uses the animal model *C. elegans* to simultaneously generate data about lifespan and healthspan.

The Vitality Platform was used to monitor the lifespan and healthspan of animals treated with ThaenaBiotic. **The lifespan of the animals treated with 0.05mg/mL of ThaenaBiotic was significantly increased relative to control ($p<0.0001$).**

Healthspan of the animals was measured by tracking their movements for the duration of their lifespan. **Animals treated with 0.05mg/mL of ThaenaBiotic showed higher levels of activity especially in the late lifespan**.

Results

1. Dosage and toxicity

Background

The ideal dose of a lifespan-active drug will balance providing a high enough dose to be effective while avoiding doses high enough to be either toxic or aversive to the animals. Because worms are strongly physically resistant to environmental chemicals, and worm physiology differs from humans, cultured cells and other animal models, we ran a series of experiments to empirically determine an ideal dosage and delivery strategy for a treatment.

1.1. Solubility and test article delivery results

The body of *C. elegans* is encased in a selectively permeable cuticle that only permits some compounds to be absorbed efficiently through the skin, so the most reliable mechanism for delivering compounds to the worms is through ingestion. Water-soluble compounds permeate the media and food and are readily taken up by the worms. Less soluble compounds require a vehicle such as DMSO and work best when combined directly with food. The first step is to check the solubility of the test article and determine the best delivery method.

Solubilization: Thaenabiotic was a heterogeneous mixture that was dissolved readily into water.

Delivery strategy: The indicated compound dosage is based on the total volume of the plates with the assumption that the water-soluble compound diffuses throughout the agar. The compound is dissolved in a working solution and then combined directly with the food bacteria before seeding on agar plates. The food spots are dried slowly, allowing the compound to diffuse into the food bacteria and the agar for at least 24 hours before worms are introduced.

1.2. Growth and development assay results

High-resolution imaging and automated detection are used to precisely measure the growth rate of animals from hatching to the first day of adulthood (total of 4 days). The *C. elegans* growth and development assay is highly sensitive and widely used in toxicology studies. Performing this test over a range of doses helps to identify a set of doses that have a physiological impact and exclude dose ranges that are likely too toxic to benefit lifespan.

Results

Thaenabiotic was mostly benign and did not produce any observed adverse effect on growth exposed from hatchling to adult. Representative images of the worms exposed to multiple dosages are shown in Figure 1.1 and their size (area) is plotted in Figure 1.2. At concentrations up to 0.42

mg/mL, the treatment had the unusual trend of actually increasing the worm size with concentration. This suggests that ThaenaBiotic might be introducing supplemental nutrients that could impact the subsequent lifespan assay. At 1.25 and 3.8 mg/mL the size of the worms decreased back to a level similar to controls suggesting some mild toxicity at this concentration. The highest no observed adverse effect level (NOAEL) for this assay was 0.42 mg/mL.

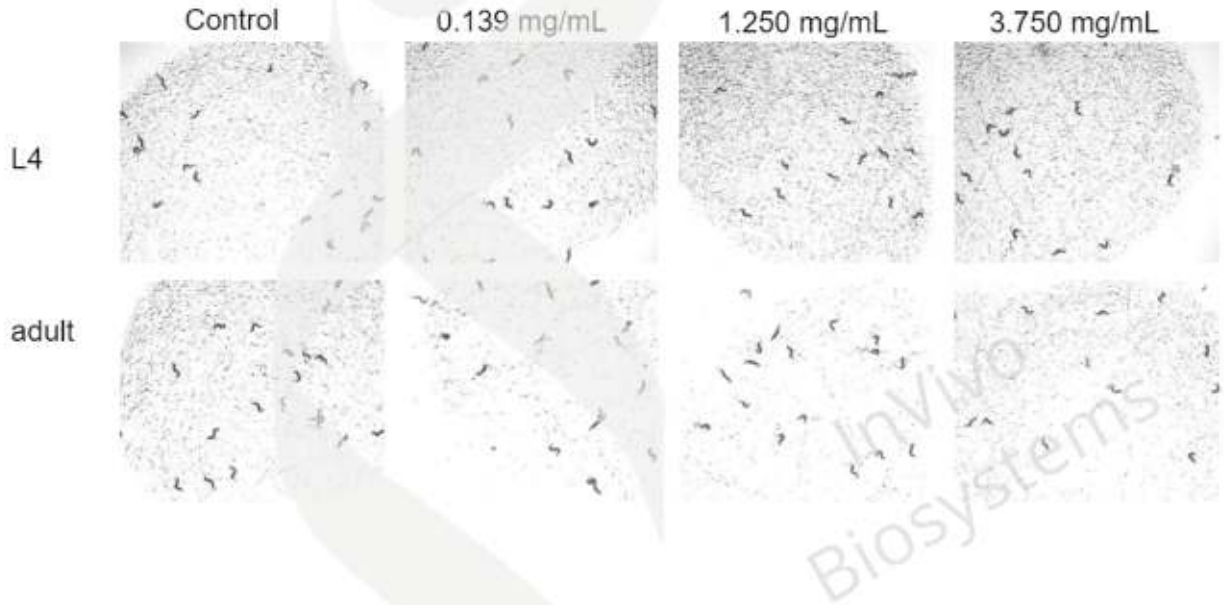

Figure 1.1. Growth and toxicity assay. Each panel is one representative image from three replicates used for measuring worm size and growth with Thaenabiotic exposure. Concentrations indicated above the top row were selected to span and highlight the transition from a non-toxic to a toxic dose. Images were acquired using WormLab imager and measured using automated detection and measurement.

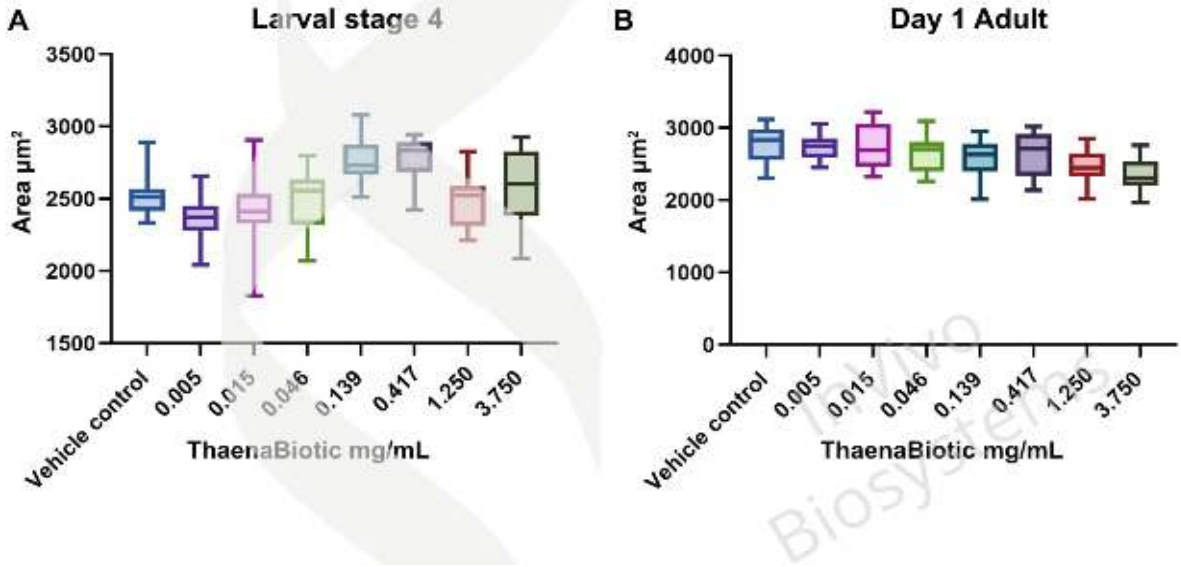

Figure 1.2 Growth and toxicity assay plots of worm size data obtained from high resolution imaging and automated detection and tracking software. Line = mean length or area, box = 25th to 75th percentile, whiskers = minimum and maximum..

1.3. Acute toxicity assay results

The acute toxicity assay tests for toxicity in adult worms by simulating the actual conditions of the lifespan assay. This accomplishes two things. First, in the early stages, it determines doses that are acutely toxic to adult worms. Second, over time worm deaths are scored to rule out doses that will likely have a negative impact on lifespan despite lacking immediate toxicity. Worms are plated on the exact media and sealed plates that will be used in the lifespan assay and then incubated at 25°C to provide a pilot lifespan that helps catch any other dosing and delivery pitfalls early on. Adult worms were monitored in the days immediately following exposure for early lethality or any other obvious defects.

Results:

In the early stages of the acute toxicity assay, no immediate adverse effects were observed at any concentration of ThaenaBiotic. However, after a few days, the highest concentration, 3.8mg/mL, showed a clear decline in survival. Unlikely to have benefit in a full lifespan assay, the 3.8 mg/mL concentration was removed from consideration for the full lifespan assay. The next highest dose, 1.25 mg/mL ultimately showed a slight decrease in early survival. All lower concentrations of ThaenaBiotic showed similar survival in an accelerated lifespan assay.

1.4. Dosage and toxicity conclusions

ThaenaBiotic was mostly benign in the growth and toxicity assay except that the maximum concentration of 3.75mg/mL showed a clear early decline in survival. Whereas a single compound drug might have a well-defined dose-response curve, natural product formulations are generally less toxic overall. Because of this, it is useful to test over a broad range of concentrations. Starting with the highest NOAEL concentration, we proposed the following series of three concentrations for the lifespan assay, which were approved by the client:

0.05 mg/mL
0.25 mg/mL
1.25 mg/mL

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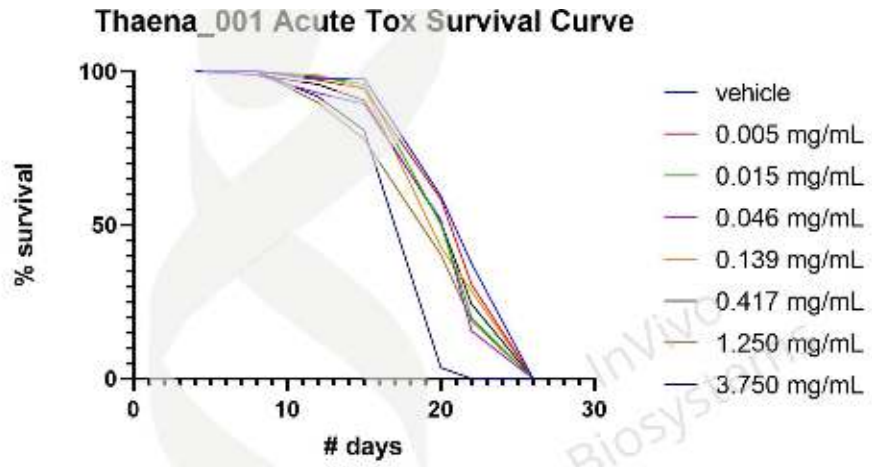

Figure 1.3 Acute toxicity assay. L4 stage worms were plated on solid media identical to that of the actual lifespan assay. Worms were examined for immediate toxic effects and then scored intermittently for survival until the start of Step 2 lifespan experiment. Concentrations shown are the final concentration of ThaenaBiotic in the solid media.

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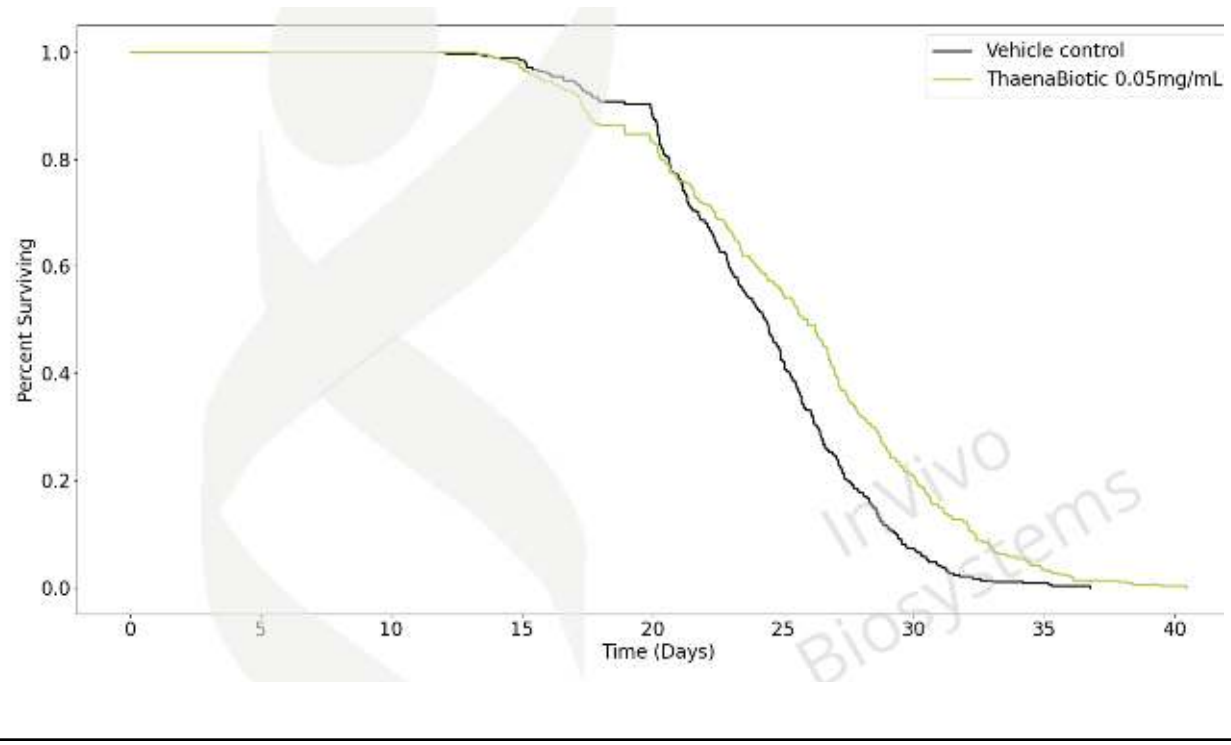
2. Vitality Study

- **Purpose:** Compare the lifespan and healthspan of three treatment groups monitoring the adult animal's survival and movement. The healthspan metrics are extracted from the same dataset as the lifespan readings.
- **Approach:** The lifespan assay is adapted from a standard protocol employed and published by the *Caenorhabditis* Intervention Testing Program (CITP). Conditions as determined in the previous experiment:
    - **Negative control**: 0.05% DMSO.
    - **Reference Compounds**: Resveratrol at 100 µM in 0.05% DMSO, Rapamycin at 50 µM in 0.05% DMSO.
    - **ThaenaBiotic test**: Whereas a single compound drug might have a well-defined dose-response curve, natural product formulations are complex and may have multiple effects on the animal's physiology; therefore, in order to discover positive effects on lifespan and healthspan it is useful to test over a broad range of concentrations. NOAEL and appropriate dosing was determined in the previous project.
        - NOAEL at concentrations of 1.25, 0.25, and 0.05 mg/mL in 0.05% DMSO.
- **Deliverables:**
    - Kaplan-Meier estimate of survival function (Figure 1)
    - Descriptive statistics and tests for differences between groups (Tables 1- 3)

2.1 Lifespan Results



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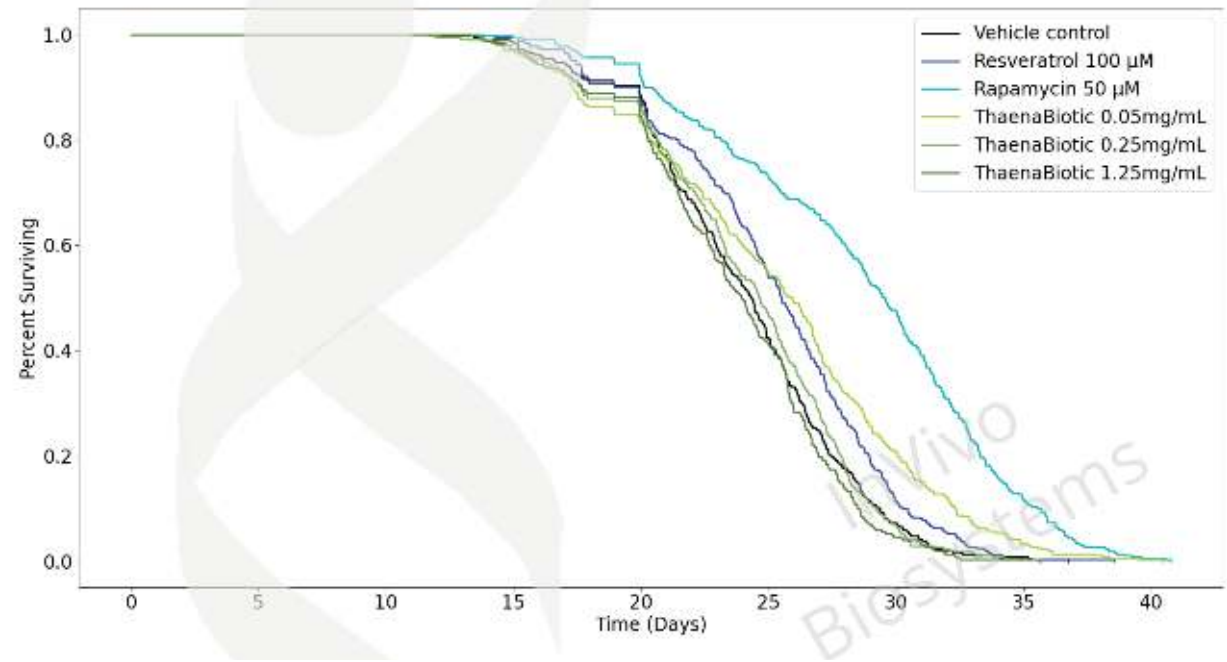

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Figure 1 Lifespan assay. Overall, the lifespan assay detected a significant positive increase in lifespan at the lowest concentration of ThaenaBiotic 0.05 mg/ml. Additionally, both the Resveratrol and Rapamycin treated animals showed an increase in lifespan. **A.** Kaplan-Meier Estimate of Survival function for ThaenaBiotic at

0.05 mg/mL and the vehicle control. **B.** Kaplan-Meier Estimate of Survival function for all conditions.

Animals were treated with three different doses of ThaenaBiotic (0.05, 0.25, or 1.25 mg/mL) as well as the reference treatments of 0.1mM Resveratrol or 0.05mM Rapamycin. **Treatment of animals with 0.05mg/mL of ThaenaBiotic had a significant increase in lifespan as compared to the untreated control (Figure 1A).**

Lifespan data are represented as the percent of animals surviving over time, known as the Kaplan-Meier Estimate of Survival function (Figure 1B). Treatments that increase lifespan, such as Rapamycin (light blue line), will shift the curve to the right relative to control. The survival curve resulting from 0.05mg/mL ThaenaBiotic treatment is not only right-shifted but has a different slope which diverges more from control over time after about day 20 (Figure 1A). This suggests that although there is not a salient early benefit, animals treated with ThaenaBiotic have a relatively lower mortality rate late in life.

Survival curves are holistically compared using a log-rank test to determine whether one treatment group lived longer than another (Table 1). This indicated the observed increase in lifespan with the 0.05mg/mL dose of ThaenaBiotic was statistically significant ($P < 0.0001$).

Table 1. Pairwise statistical analysis of survival curves.

Curve comparison	Mantel-Cox log-rank		Wilcoxon-Breslow-Gehan	
	Test statistic (X^2)	Log-rank test P-value	Test statistic (X^2)	Log-rank test P-value
ThaenaBiotic 0.05mg/mL vs. vehicle control	32.0	**<0.0001**	13.2	**<0.0001**
ThaenaBiotic 0.05mg/mL vs. Resveratrol 0.1mM	6.3	0.012	0.2	1.0
ThaenaBiotic 0.05mg/mL vs. Rapamycin 0.05mM	60.4	**<0.0001**	62.8	**<0.0001**
ThaenaBiotic 0.25mg/mL vs. vehicle control	0.2	0.70	0.6	1.0
ThaenaBiotic 1.25mg/mL vs. vehicle control	2.0	0.16	1.2	1.0
Resveratrol 0.1mM vs. vehicle control	13.9	0.00019	14.1	**<0.0001**
Rapamycin 0.05mM vs. vehicle control	195.7	**<0.0001**	138.2	**<0.0001**

The Mantel-Cox log-rank test is a non-parametric test that compares two survival functions across the duration of the lifespan. The Wilcoxon-Breslow-Gehan test weights each death time by the total number of

subjects at risk, thus assigning more weight to earlier death times. P-value is corrected for multiple comparisons (Bonferroni correction). Yellow highlights indicate significance at the p<0.05 level.

Survival curves can be compared descriptively with statistics such as mean, median, and maximum lifespan. The ThaenaBiotic had a mean survival of 25.3 days, whereas the vehicle-treated control group had a mean lifespan of 24.1 days (Figure 2). Rapamycin, a powerful lifespan prolonging compound, produced a mean lifespan of 28.6 days.

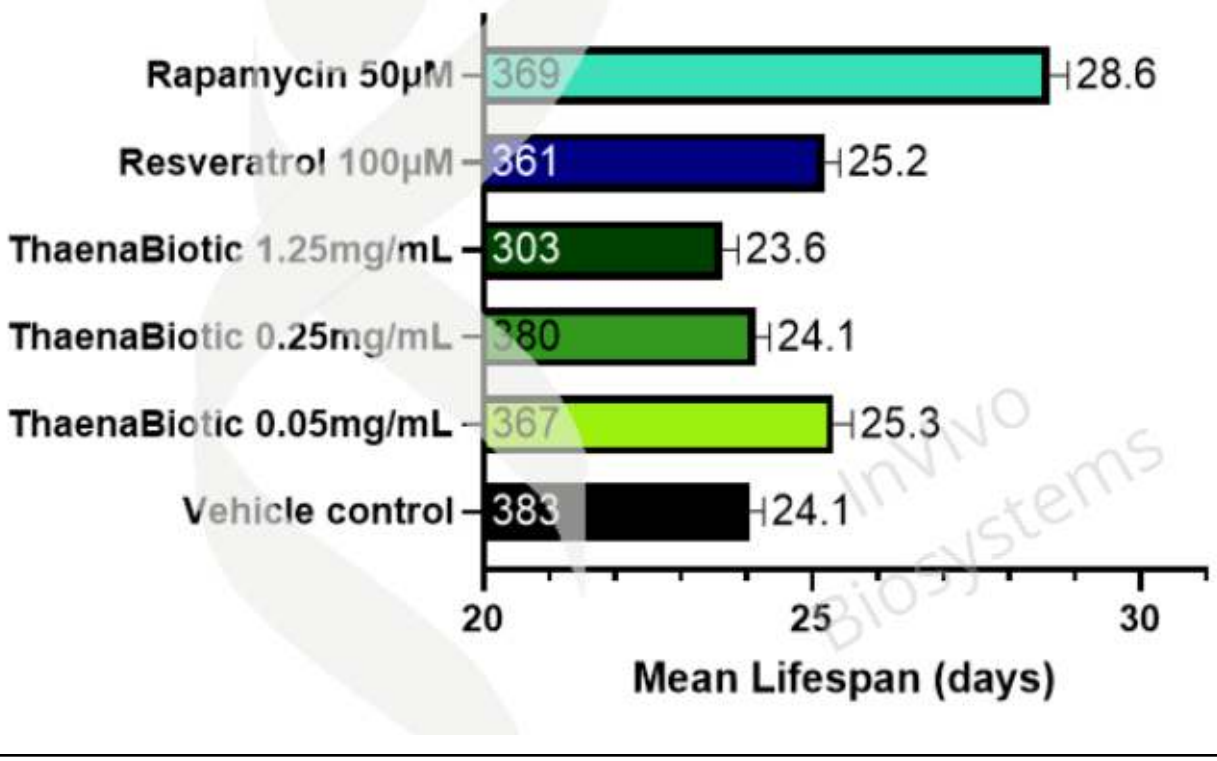

Figure 2. Mean lifespan for each condition along with the number of animals tested for each treatment.

The survival curves were also compared by examining the age of animals at a specific percent of population mortality (Table 2). Animals treated with the 0.05mg/mL of ThaenaBiotic reached the milestones of 50%, 75%, 90%, and 95% population mortality at a significantly later time point than control.

The median lifespan is the age at which 50% of the animals have died. **Treatment with 0.05mg/mL Thaenabiotic produced a significant increase in median lifespan over control** (Table 2, Table 3, P < 0.005). By convention, the "maximum lifespan" is typically the 95th percentile of lifespans recorded. **Treatment with 0.05mg/mL ThaenaBiotic increased the maximum lifespan from the control of 30.5 days to 34.3 days** (Table 2, far left column)**.** The Rapamycin group had a maximum lifespan of 36.1 days.

Table 2. Age in days at percent mortality

Treatment	Number of animals	25% mortality	50% mortality	75% mortality	90% mortality	95% mortality
Vehicle control	383	21.1	24.3	26.9	29.3	30.5
ThaenaBiotic 0.05mg/mL	367	21.5	25.9	29.1	32.3	34.3
ThaenaBiotic 0.25mg/mL	380	21.3	24.6	27.3	29.2	30.3
ThaenaBiotic 1.25mg/mL	303	20.9	23.9	26.5	28.5	29.8
Resveratrol 0.1mM	361	22.4	25.5	28.3	30.2	32.3
Rapamycin 0.05mM	369	24.6	29.5	32.8	35.7	36.1

Age at percent mortality is the age in days at which the given percentage of animals are dead. Age at 50% mortality is equal to the median lifespan. These analyses are useful for examining early or late life-specific effects or when the survival curves are not parallel. Yellow shading indicates a statistically significant difference from vehicle control at the p<0.05 level; Fisher's Exact Tests for differences at 25, 50, 75, 90, 95% mortality are shown in table 3.

Table 3. Fisher's Exact Test for survival differences at key time points.

Treatment vs. vehicle control	P-value at 25% mortality	P-value at 50%	P-value at 75%	P-value at 90%	P-value at 95%
ThaenaBiotic 0.05mg/mL	1	0.006	**<0.0001**	**<0.0001**	**<0.0001**
ThaenaBiotic 0.25mg/mL	1	0.96	1	1	1
ThaenaBiotic 1.25mg/mL	1	1	1	0.37	1
Resveratrol 0.1mM	0.044	0.013	0.00033	0.072	0.035
Rapamycin 0.05mM	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

Statistical analysis corresponding to the age at specific percent mortality shown in Table 2. Age at 50% mortality is equal to the median. Yellow highlights indicate significance at the p<0.05 level.

2.2. Healthspan Results

To measure healthspan, the animals' spatial location and movements were quantified throughout their lifespan. Animals treated with the ThaenaBiotic at 0.05 mg/ml showed increased activity relative to the vehicle control between days 20 and 28 (Figure 3A). In fact, while the Resveratrol and Rapamycin treated groups showed more activity early in life, the ThaenaBiotic treatment at 0.05 mg/mL had the highest activity of any compound at the later time period (Figure 3B). This result indicates that the animals treated with ThaenaBiotic retained higher levels of activity late in life–had a longer healthspan–than the control group.

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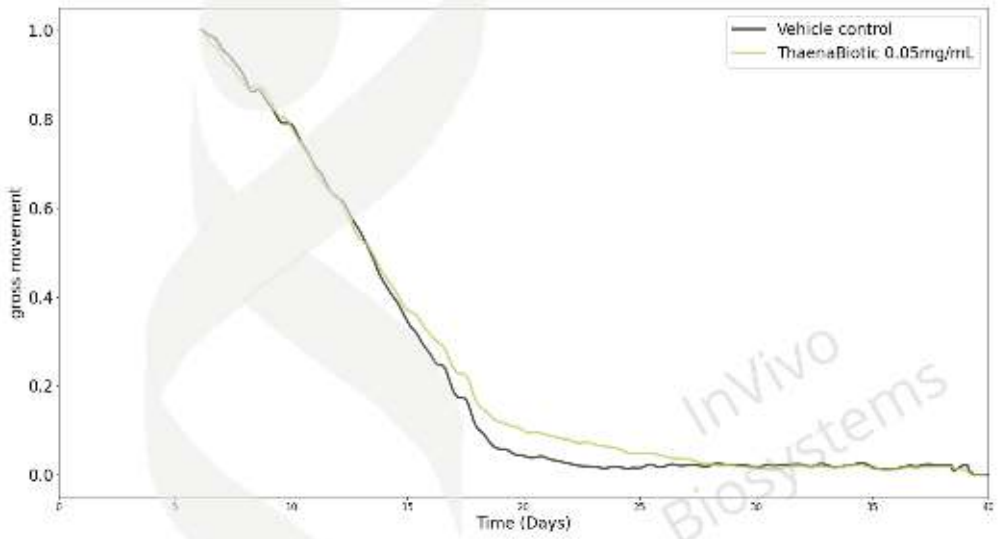

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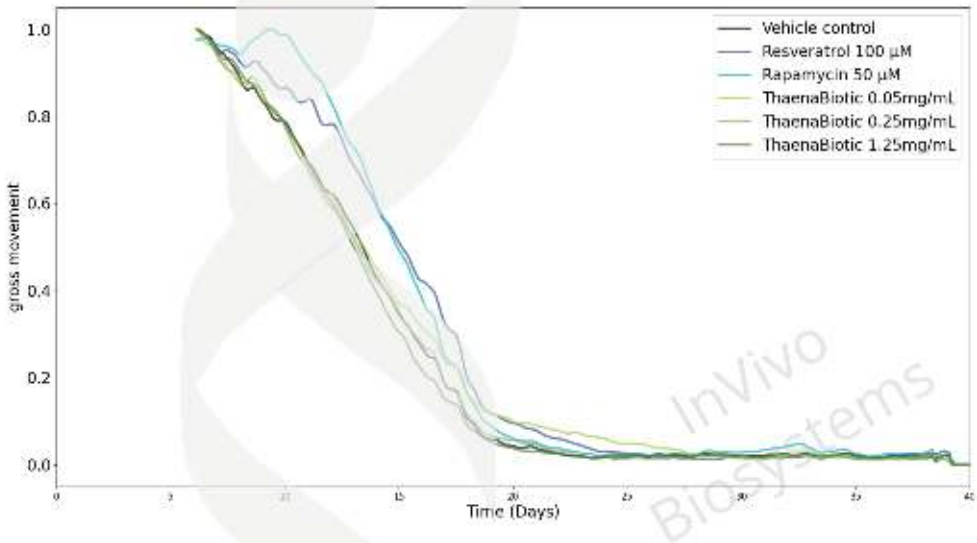

Figure 3. Activity analysis over the duration of lifespan. Gross movement as measured using Difference-Based Spatial-Temporal Entropy Image (DSTEI).[13] Worm activity was obtained by computationally analyzing the images from the automated lifespan instrument. **A.** ThaenaBiotic at 0.05 mg/mL and the vehicle control. **B.** All conditions.

Activity by animal was also measured (Figure 4). As animals age, they transition from vigorous movement to weak movement, to no movement, to death. Figure 4 shows the age at which the animals transition from vigorous movement to weak movement. **The animals treated with ThaenaBiotic at 0.05mg/mL show vigorous movement later than the vehicle control (Figure 4A) as well as later than any of the other treatments (Figure 4B).** This analysis also supports the result that the ThaenaBiotic treatment had a positive impact on healthspan.

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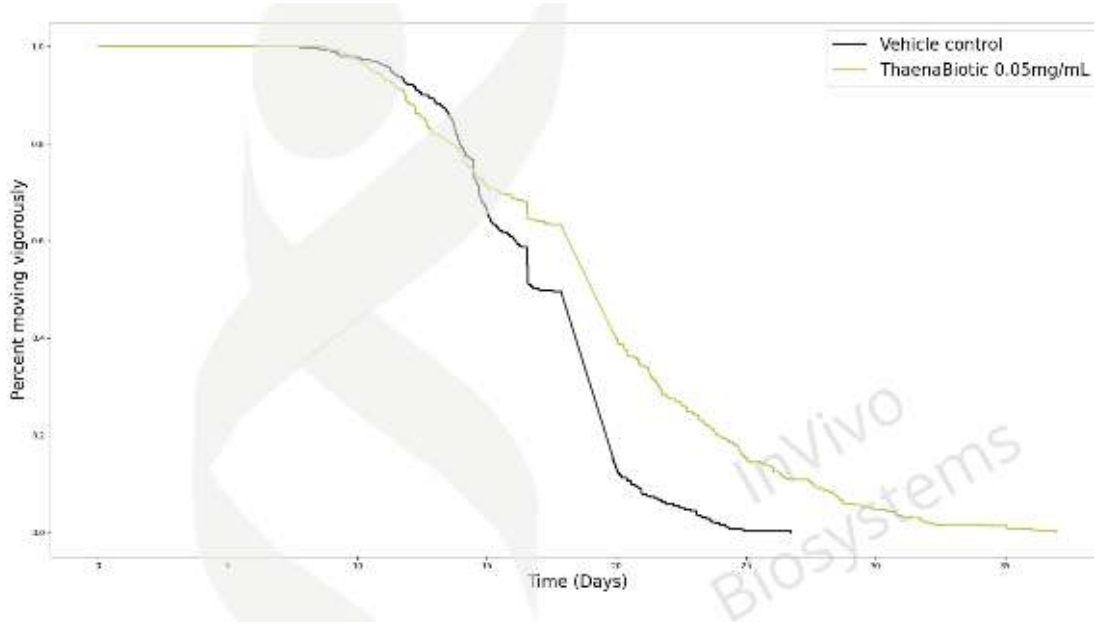

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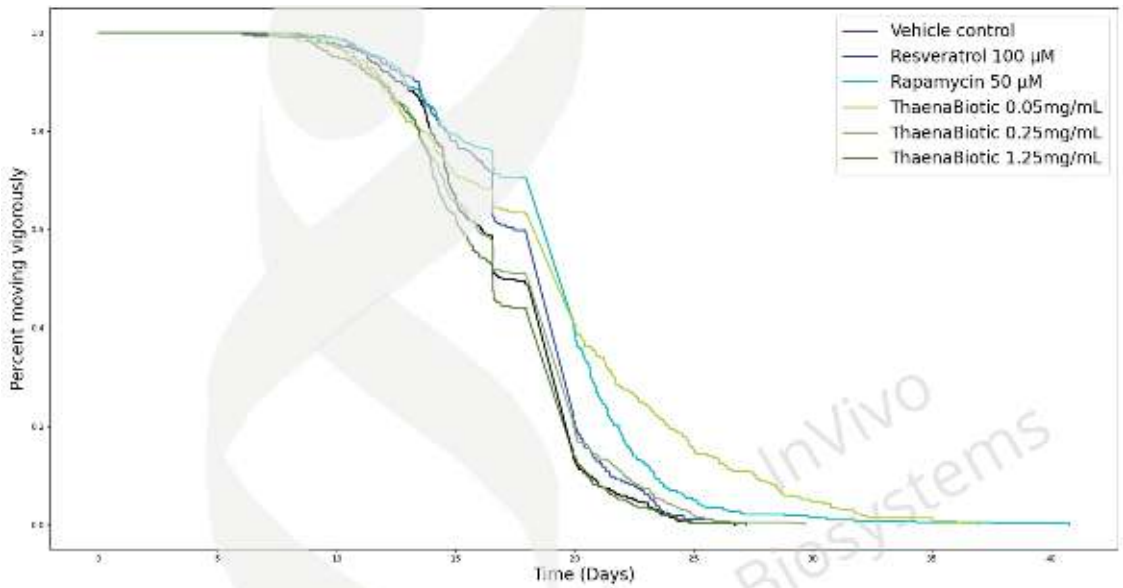

Figure 4. Age at last vigorous movement. Modeling was used to identify the time point when each animal transitioned from vigorous to weak movement. **A.** ThaenaBiotic at 0.05 mg/mL and the vehicle control. **B.** All conditions. These graphs show interpolated data for improved visualization. Original graphs can be found in Appendix 1.

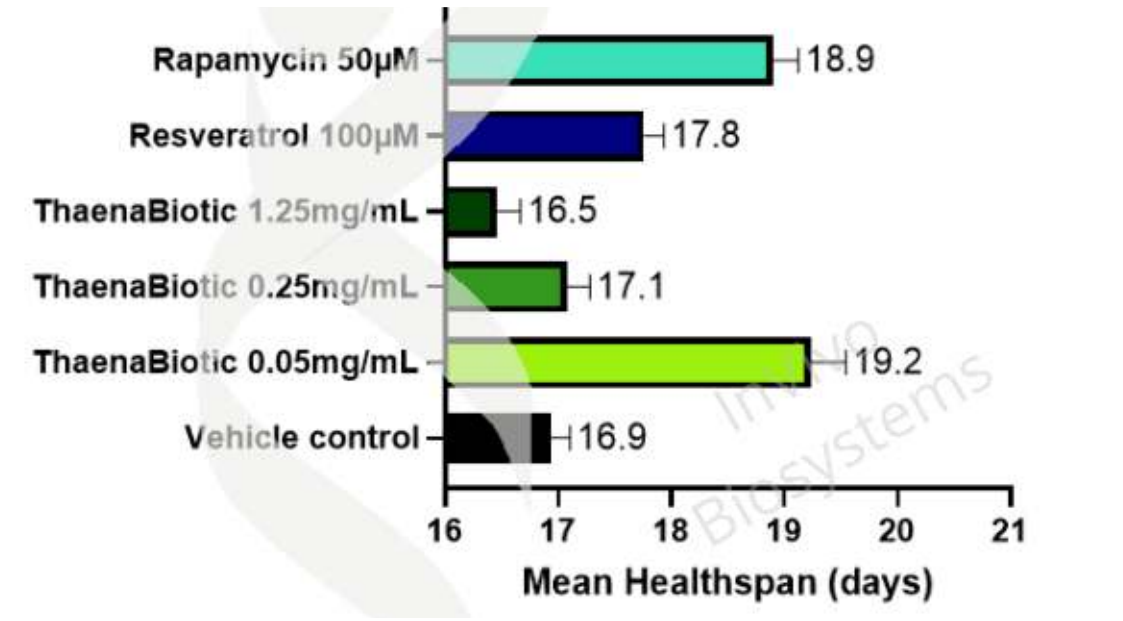

Figure 5. The mean healthspan for each condition as determined by the age at last vigorous movement.

Using the age at last vigorous movement we calculated a mean healthspan for each treatment (Figure 5). The animals treated with **ThaenaBiotic at 0.05 mg/mL maintained vigorous movement longer than any of the other treatments.** We used a Mantel-Cox log-rank test to compare the

healthspan curves from the age at last vigorous movement (Table 4). ThaenaBiotic treatment at 0.25mg/mL and 0.05mg/mL were significantly different from the vehicle control as were the reference treatments Resveratrol and Rapamycin.

Table 4. Pairwise statistical analysis of healthspan curves.

Curve comparison to vehicle control	Mantel-Cox log-rank	
	Test statistic (X^2)	Log-rank test P-value
ThaenaBiotic 0.05mg/mL	82.2	**<0.0001**
ThaenaBiotic 0.25mg/mL	6.7	0.0095
ThaenaBiotic 1.25mg/mL	0.010	0.92
Resveratrol 0.1mM	12.0	0.00052
Rapamycin 0.05mM	79.0	**<0.0001**

The Mantel-Cox log-rank test is a non-parametric test that compares two survival functions across the duration of the healthspan. P-value is corrected for multiple comparisons (Bonferroni correction). Yellow highlights indicate significance at the p<0.05 level.

The extension of activity to later in life can be seen looking at the age when 90% and 95% of the population has transitioned from vigorous to weak movement (Table 5). Both of these show a significant difference from vehicle control for the ThaenaBiotic treatment at 0.05mg/mL and Rapamycin treatment, indicating that animals from these populations remain active later in life.

Table 5. Age in days for % of population transitioning from vigorous to weak movement.

Treatment	**Number of animals**	**90%**	**P-value at 90% vs vehicle control**	**95%**	**P-value at 95% vs vehicle control**
Vehicle control	383	20.5		22.5	
ThaenaBiotic 0.05mg/mL	367	27.6	**<0.0001**	29.8	**<0.0001**
ThaenaBiotic 0.25mg/mL	380	22.1	0.038	23.7	0.49
ThaenaBiotic 1.25mg/mL	303	20.0	1	22.0	1
Resveratrol 0.1mM	361	21.5	0.25	23.2	0.9
Rapamycin	369	23.3	0.0014	25.1	0.0014

0.05mM					

This table captures the days at which the given percentage of animals have stopped vigorous movement. These analyses are useful for examining late life-specific effects or when the survival curves are not parallel. Yellow shading indicates a statistically significant difference from vehicle control at the p<0.05 level; Fisher's Exact Tests for differences at 90 and 95% are shown. This highlights the later stage population transition that is seen in Figure 4.

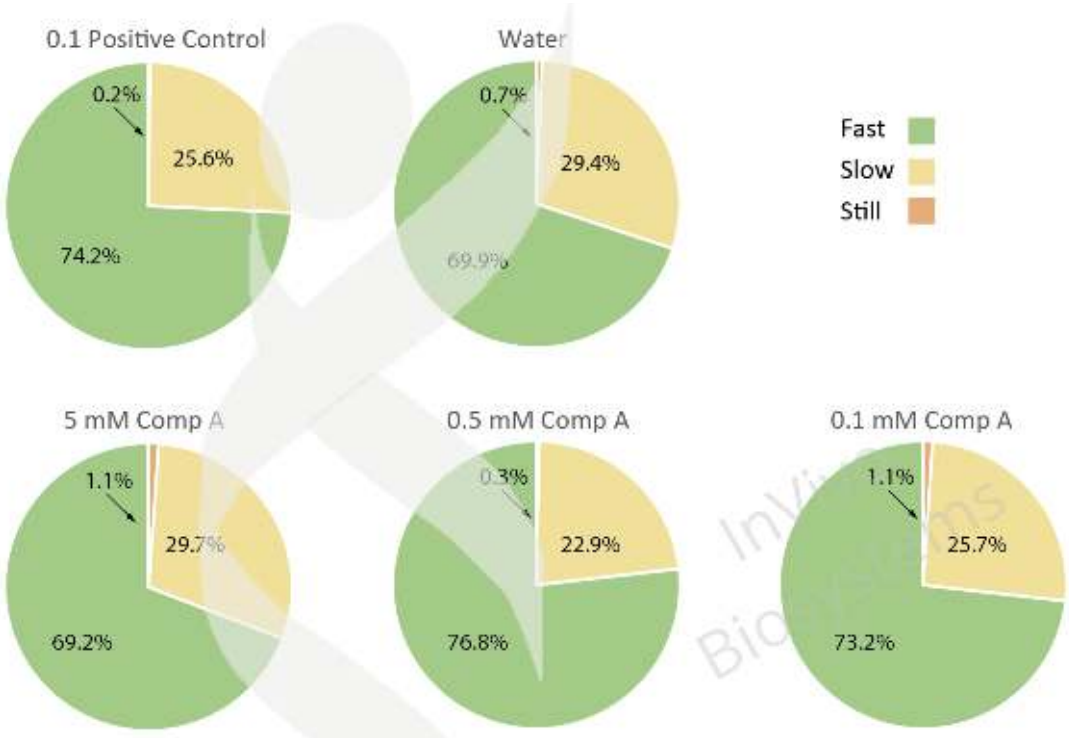

Figure 2.4 Activity phases over duration of lifespan. Animals were identified in the fast moving, slow moving, and still phases of their life. 0.25 mM and 0.05 mM Compound A increase the proportion of life spent in the healthy, fast-moving stage.

3. Reactive Oxygen Species (ROS) Study

- **Purpose:** Measure antioxidant effects of compounds for animals experiencing oxidative stress.
- **Approach:** Reactive Oxygen Species (ROS) lead to severe stress responses (decreased locomotion, altered morphology, and decreased brood size) and ultimately early death of the animal. Paraquat at 10 mM and higher concentration leads to a rapid stress response that is monitored as decreased locomotion.
- **Scope**: One concentration of test sample is assayed. Dosages are chosen at and below the NOAEL as determined in Step I. Includes one positive control and one negative control.
    1. **Negative control**: 0.05% DMSO.
    2. **Positive control**: Vitamin C at 10 mg/ml in 0.05% DMSO.
    3. **Thaenabiotic test**: NOAEL at concentrations of 1.25, 0.25, and 0.05 mg/mL.

3.1 ROS assay results

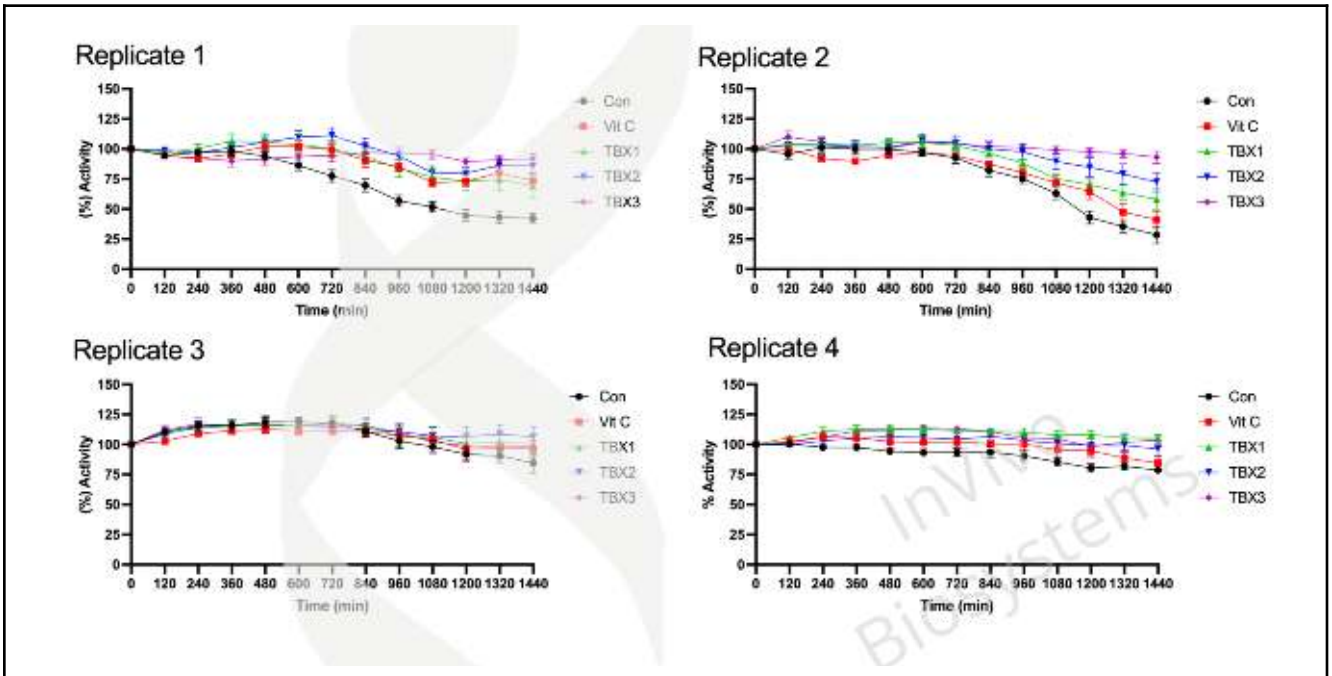

Figure 2a.1. Results of ROS assay using wMicroTracker. Worms were exposed to TheanaBiotic at 0.05, 0.25, or 1.25mg/mL (TBX1, 2, or 3), Vitamin C at 10mM, or water for 24 hours, then exposed to 10mM Paraquat at time 0 of recording. Four independent biological replicates are shown. Additional replicates in which Parquat effect was insufficient to interpret results are included in the data supplement. Paraquat was added to 10mM at time = 0. Positive control = 10mM Vitamin C. Error bars = standard error of the mean.

Pre-treatment of worms with ThaenaBiotic improved oxidative stress tolerance in a dose dependent manner. For all valid replicates, the ThaenaBiotic-treated worms showed greater activity over time under oxidative stress challenge than either vehicle control or the positive control, Vitamin C. Typically, the movement of antioxidant-treated worms will continue to decline over time, in some cases eventually returning to control levels, but the highest concentration of ThaenaBiotic remained protective for the duration of the assay. The molar concentration of Vitamin C was matched to Paraquat at 10mM as a point of reference, but it is possible that greater protective effect might be available with higher proportions of the positive control. In some trials, no effect was observed, but these were situations in which the Paraquat had failed to paralyze the worms, hence no effect could be observed. These trials are included in the data supplement for reference.

Conclusion

The impact of ThaenaBiotic treatment on the lifespan and healthspan of animals was measured using the Vitality Platform. Treatment with 0.05mg/mL of ThaenaBiotic had a positive impact on extending lifespan and healthspan. The median lifespan of the animals treated with 0.05mg/mL ThaenaBiotic was increased by more than a day compared to the untreated animals. This statistically significant increase in lifespan was matched by an increase in healthspan. The animals treated with ThaenaBiotic at 0.05mg/mL were active for longer in their life.

Because ThaenaBiotic is a complex mixture, the experimental design included a range of compound concentrations in order to capture the positive effects of treatment and minimize negative effects. Different concentrations may be therapeutic in different ways. In the case of ThaenaBiotic, preliminary growth assays showed that the highest concentrations tested resulted in rapid early growth which might counteract longevity benefits (Lee et al. 2016). While two of the final concentrations tested did not show a positive effect on vitality, the 0.05 mg/mL treatment did markedly increase both lifespan and healthspan. By contrast, higher concentrations of ThaenaBiotic demonstrated greater therapeutic benefits in the acute ROS assay in young adults. Testing a range of concentrations can reveal benefits of a treatment if the human equivalent dose has not been established or if the components of a mixture have different activities.

Next steps

Claims of vitality benefits can be bolstered by evidence of direct mechanistic action. To identify potential mechanisms through which ThaenaBiotic exerts vitality benefits, we can perform RNA-seq to reveal the global gene expression changes induced by treatment with the concentration used in the vitality assay. This data can be analyzed by mapping gene expression data to vitality-related pathways and identifying which pathways are activated or repressed by treatment with ThaenaBiotic.

Materials and Methods

Worm maintenance and media

Worm Feeding: To prevent chemical modification or metabolism of the test article by the food bacteria, animals were fed on a lawn of inactivated *E. coli*, strain OP50. Cultures of OP50 were inactivated via exposure to 0.5% paraformaldehyde for 1 hour followed by 5 washes in M9 (Beydoun et al. 2021). Bacteria were dispersed by passing through a 5µM filter during the wash steps. The quantity and distribution of food bacteria were calibrated to ensure adequate access to food for the duration of assay while maintaining visibility of the animals.

Solubilization: Thaenabiotic was a heterogeneous mixture that dissolved readily into water at 200mg/mL. After bath sonication and clearing by centrifugation, only a small amount of residue remained out of solution. The soluble supernatant was collected and filter sterilized before applying to medium.

Delivery strategy: The indicated compound dosage is based on the total volume of the plates with the assumption that the water-soluble compound diffuses throughout the agar. The compound is dissolved in a working solution and then combined directly with the food bacteria before seeding on agar plates. The food spots are dried slowly, allowing the compound to diffuse into the food bacteria and the agar for at least 24 hours before animals are introduced.

Vitality Platform

Instrumentation: To obtain high-resolution lifespan data and eliminate confounding factors such as worm handling and operator bias, lifespan data was collected using an Automated Lifespan Machine (ALM) (Stroustrup et al. 2013). The ALM used by InVivoBiosystems is based on the *Caenorhabditis elegans* lifespan machine published by Stroustrup et. al. (Stroustrup et al. 2013) with proprietary modifications to improve temperature stability and image acquisition. The unit consists of a modified EPSON V850 scanner and images are processed and analyzed using the ALM software (Stroustrup et al. 2013). The machine time-of-death calls are trained and validated using the "storyboarding" feature of the ALM software.

Vitality measurements were adapted from published protocols (Lucanic et al. 2017; Amrit et al. 2014). Three biological replicates, derived from synchronizing three independently-maintained lines of N2 animals, were distributed across instruments. Populations of all replicate groups were expanded to more than 1000 animals, then synchronized by bleaching and allowing larval animals to hatch and arrest in a food-deprived state. As development and aging are temperature-dependent, animals were kept strictly at 20° +/- 1°C throughout the experiment using dedicated incubators, a temperature-controlled room, and multiple temperature sensors. To eliminate the effect of bacterial metabolism and growth on lifespan, synchronized animals were only exposed to dead bacterial food. To suppress progeny, animals were transferred to media containing 5-Fluorodeoxyuridine (FUdR) within 54-60 hours post-plating. Animals were inspected 24 and 48 hours after this transfer to confirm infertility. Finally, the animals were inspected for general health and morphology before transferring to freshly prepared plates that they occupy for the remainder of the assay. The animals were incubated for an additional 2 days and inspected again before loading onto the ALM in a temperature-controlled room. Images of the animals were then collected for the next 35 days with

no planned interruption or manipulations; however, a data collection hardware failure interrupted recordings intermittently between days 17 and 19 of the lifespan leading to the step in the survival curve over that time interval.

Some plates were excluded after quality checks, but plate number and worm counts for all replicates exceeded the threshold for statistical significance. The total number of lifespans recorded per condition ranged from 303 to 383 (Table 2), surpassing the requirement of 150 required to eliminate subsampling errors and detect lifespan differences of 10% or more (Gruber et al. 2009). A Cox Proportional Hazards analysis was run to determine if factors other than the treatment could confound the data and no factors other than the incubator were detected. Qualitatively, the animals appeared morphologically and behaviorally normal suggesting that they were not impacted by confounding hazards such as contamination or toxicity.

Lifespan Analysis and Statistics
Time of death calls exported from the ALM software were analyzed and plotted using the Lifelines software package developed by Cam Davidson-Pilon *et. al* (Davidson-Pilon 2022). Additional analysis was performed using the OASIS2 analysis software (Han et al. 2016).

A standard Mantel-Cox log-rank test compares the curves globally over the course of the lifespan assigning equal weight to each timepoint, whereas the Wilcoxon-Breslow-Gehan weights each death by the number of subjects at risk, assigning greater weight to earlier deaths.

Healthspan analysis
Worm movement was tracked from the images acquired by the ALM during the lifespan assay. Worm Activity serves as a proxy for animal health. Changes in spatial distribution of the animals between time points is used to derive **gross movement** for the population over time. Foreground motion is calculated using an approach called difference based spatial temporal entropy image (DSTEI) (Ma and Zhang 2001).

State transitions were assessed using a Hidden Markov Model (HMM, (Oswal et al. 2021) trained on N2 wild-type *C. elegans* at 20°C. The HMM uses features such as movement and morphology to determine the life state of the worm. The transition from actively crawling on the plate (vigorous movement) to a stationary but moving phase (weak movement) is plotted as the age of last vigorous movement. Gross curation was performed by manual analysis of worm presence and worm death. A curve of the age of each worm at the time of transition from vigorous movement to weak movement is plotted.

Antioxidant Capacity Study
A large population of wild-type *C. elegans* was synchronized and grown to the L4 juvenile stage. Liquid culture medium was prepared fresh as follows: S medium, 50 uM FUdR, 200 ug/ml Streptomycin, and filtered OP50. The L4 worms were resuspended in liquid culture at about 2 worms/ul. 90ul of the worms in the liquid culture media were added to each well. 10ul of compound or vehicle were added to each well. Microplates were sealed and incubated at 20°C overnight on a shaker. The next day, activity was measured on the wMicrotracker (PhylumTech) for 2 hours. This is the basal measurement. Paraquat (100mM) is added to each well for a final concentration of 10mM. The plate is resealed and activity is recorded on the wMicrotracker for 48 hours. The activity is

grouped in blocks of 40 minutes and percent activity is calculated by normalizing to the basal activity measurements. Percent activity vs time is shown.

References

Amrit, Francis Raj Gandhi, Ramesh Ratnappan, Scott Alexander Keith, and Arjumand Ghazi. 2014. "The C. Elegans Lifespan Assay Toolkit." *Methods* 68 (3): 465–75.

Beydoun, Safa, Hyo Sub Choi, Gabrielle Dela-Cruz, Joseph Kruempel, Shijiao Huang, Daphne Bazopoulou, Hillary A. Miller, Megan L. Schaller, Charles R. Evans, and Scott F. Leiser. 2021. "An Alternative Food Source for Metabolism and Longevity Studies in Caenorhabditis Elegans." *Communications Biology* 4 (1): 258.

Davidson-Pilon, Cameron. 2022. *Lifelines, Survival Analysis in Python*. https://doi.org/10.5281/zenodo.6359609.

Gruber, Jan, Li Fang Ng, Suresh Kumar Poovathingal, and Barry Halliwell. 2009. "Deceptively Simple but Simply Deceptive--Caenorhabditis Elegans Lifespan Studies: Considerations for Aging and Antioxidant Effects." *FEBS Letters* 583 (21): 3377–87.

Han, Seong Kyu, Dongyeop Lee, Heetak Lee, Donghyo Kim, Heehwa G. Son, Jae-Seong Yang, Seung-Jae V. Lee, and Sanguk Kim. 2016. "OASIS 2: Online Application for Survival Analysis 2 with Features for the Analysis of Maximal Lifespan and Healthspan in Aging Research." *Oncotarget* 7 (35): 56147–52.

Lee, Yujin, Wooseon Hwang, Juyoung Jung, Sangsoon Park, Josephine Jill T. Cabatbat, Pan-Jun Kim, and Seung-Jae V. Lee. 2016. "Inverse Correlation between Longevity and Developmental Rate among Wild C. Elegans Strains." *Aging* 8 (5): 986–99.

Lucanic, Mark, W. Todd Plummer, Esteban Chen, Jailynn Harke, Anna C. Foulger, Brian Onken, Anna L. Coleman-Hulbert, et al. 2017. "Impact of Genetic Background and Experimental Reproducibility on Identifying Chemical Compounds with Robust Longevity Effects." *Nature Communications* 8 (February): 14256.

Ma, Yu-Fei, and Hong-Jiang Zhang. 2001. "Detecting Motion Object by Spatio-Temporal Entropy." In *IEEE International Conference on Multimedia and Expo, 2001. ICME 2001.*, 265–68.

Oswal, Natasha, Olivier M. F. Martin, Sofia Stroustrup, Monika Anna Matusiak Bruckner, and Nicholas Stroustrup. 2021. "A Hierarchical Process Model Links Behavioral Aging and Lifespan in C. Elegans." *bioRxiv*. https://doi.org/10.1101/2021.03.31.437415.

Stroustrup, Nicholas, Bryne E. Ulmschneider, Zachary M. Nash, Isaac F. López-Moyado, Javier Apfeld, and Walter Fontana. 2013. "The Caenorhabditis Elegans Lifespan Machine." *Nature Methods* 10 (7): 665–70.

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Appendix 1. Healthspan Curves

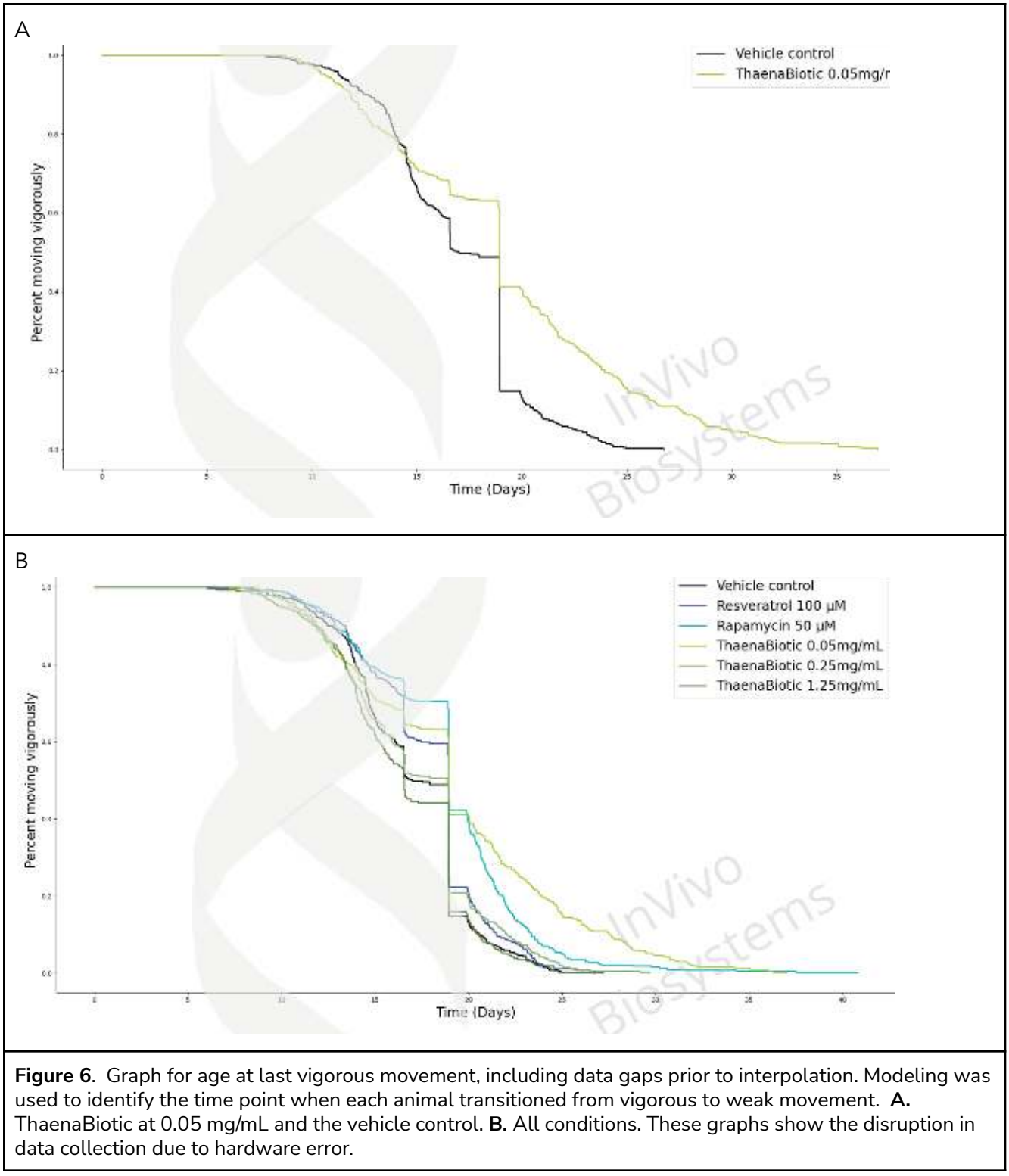

Figure 6. Graph for age at last vigorous movement, including data gaps prior to interpolation. Modeling was used to identify the time point when each animal transitioned from vigorous to weak movement. **A.** ThaenaBiotic at 0.05 mg/mL and the vehicle control. **B.** All conditions. These graphs show the disruption in data collection due to hardware error.