TITLE:

Using Single-Worm Data to Quantify Heterogeneity in *Caenorhabditis elegans*-Bacterial Interactions

AUTHORS AND AFFILIATIONS:

Megan N. Taylor[1], Satya Spandana Boddu[2], Nic M. Vega[1,2]

[1]Department of Biology, Emory University, Atlanta, GA

[2] Department of Physics, Emory University, Atlanta, GA

Corresponding author:

Nic M. Vega (nvega@emory.edu)

Co-authors:

Megan N. Taylor (megan.nicole.taylor@emory.edu)

Satya Spandana Boddu (satya.spandana.boddu@emory.edu)

KEYWORDS:

Caenorhabditis elegans, microbiome, heterogeneity, host-microbe, bacteria, transmission

SUMMARY:

This protocol describes a 96-well disruption of individual bacterially colonized *Caenorhabditis elegans* following cold paralysis and surface bleaching to remove external bacteria. The resulting suspension is plated on agar plates to allow accurate, medium-throughput quantification of bacterial load in large numbers of individual worms.

ABSTRACT:

The nematode *Caenorhabditis elegans* is a model system for host-microbe and host-microbiome interactions. Many studies to date use batch digests rather than individual worm samples to quantify bacterial load in this organism. Here it is argued that the large inter-individual variability seen in bacterial colonization of the *C. elegans* intestine is informative, and that batch digest methods discard information that is important for accurate comparison across conditions. As describing the variation inherent to these samples requires large numbers of individuals, a convenient 96-well plate protocol for disruption and colony plating of individual worms is established.

INTRODUCTION:

Heterogeneity in host-microbe associations is observed ubiquitously, and variation between individuals is increasingly recognized as a contributing factor in population-level processes from competition and coexistence[1] to disease transmission[2–4]. In *C. elegans,* "hidden heterogeneity" within isogenic populations has been observed repeatedly, with sub-populations of individuals showing distinct phenotypes in heat shock response[5,6], ageing, and lifespan[7–11], and many other aspects of physiology and development[12]. Most analyses that attempt to identify sub-population structure provide evidence for two sub-populations in experimental populations of isogenic,

synchronized worms[5,7,8], though other data suggest the possibility of within-population distributions of traits rather than distinct groups[7,12,13]. Of relevance here, substantial heterogeneity in intestinal populations is observed even within isogenic populations of worms colonized from a shared source of microbes[13–16], and this heterogeneity can be concealed by the batch digest measurements that are widely used[17–20] for bacterial quantification in the worm.

This work presents data suggesting a need for greater reliance on single-worm measurements in host-microbe association, as well as protocols for increasing accuracy and throughput in single-worm disruption. These protocols are designed to facilitate mechanical disruption of large numbers of individual *C. elegans* for quantification of viable bacterial load, while providing better repeatability and lower effort per sample than pestle-based disruption of individual worms. A recommended gut-purging step, where worms are permitted to feed on heat-killed *E. coli* prior to the preparation for disruption, is included to minimize contributions from recently ingested and other transient (non-adhered) bacteria. These protocols include a cold-paralysis method for cleaning the cuticle with a low-concentration surface bleach treatment; surface bleaching can be used as a preparatory step in single-worm disruption or as a method for preparing live, externally germ-free worms. This surface-bleaching method is sufficient to remove a wide range of external microbes, and cold treatment provides an alternative to conventional levamisole-based paralysis; while levamisole will be preferred for cold-sensitive experiments, cold paralysis minimizes contributions to hazardous waste streams and allows rapid resumption of normal activity. While the full protocol describes a laboratory experiment where worms are colonized with known bacteria, the procedures for cleaning worms and single-worm disruption can readily be applied to worms isolated from wild samples or colonized in microcosm experiments. The protocols described here will produce live bacteria extracted from the worm intestine, suitable for plating and quantification of colony forming units (CFUs) in individual worms; for sequencing-based intestinal community analysis, subsequent cell lysis and nucleic acid extraction steps should be added to these protocols.

PROTOCOL:

Worms used in these experiments were obtained from the *Caenorhabditis* Genetic Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Bristol N2 is the wild-type. DAF-2/IGF mutants *daf-16(mu86)* I (CGC CF1038) and *daf-2(e1370)* III (CGC CB1370) are used to illustrate differences in intestinal bacterial load.

HT115(DE3) *E. coli* carrying the *pos-1* RNAi vector is from the Ahringer library[21]. The MYb collection of *C. elegans* native gut bacteria[22] was obtained from the Schulenburg lab. *Salmonella enterica* LT2 (ATCC 700720) *attB*:GFP-KmR is from this lab[23]. *Pseudomonas mosselii* was isolated in this lab. *Staphylococcus aureus* MSSA Newman pTRKH3-mGFP was obtained from the LaRock lab at Emory University.

All worm buffers and media are prepared according to previously published literature[24] with minor modifications (see **Supplementary File 1**).

1. Preparation of synchronized sterile *C. elegans*

NOTE: In this section, step-by-step procedures are described for generating a synchronized population of reproductively sterile adult worms. Feeding on *pos-1* RNAi plates is used here to prevent production of progeny because this interference is embryonic lethal; L1 larvae raised to adulthood on *pos-1* RNAi develop into egg-laying hermaphrodites, but these eggs are inviable[25]. The RNAi feeding protocol is as in the "Reverse genetics" chapter of Wormbook[26].

1.1. Before synchronizing worms, ensure that fresh 10 cm NGM + *pos-1* RNAi plates are available. Plates can be prepared fresh from concentrated induced liquid culture (+Amp +IPTG) or inoculated as lawns on NGM + 100 µg/mL ampicillin + 1 mM IPTG and allowed to grow at 25 °C in the dark for 1 day[27].

NOTE: Carbenicillin (25 µg/mL) is often used instead of ampicillin on RNAi plates. Ampicillin is less expensive but less stable; if using ampicillin, plates should be seeded immediately once dry and used as soon as possible (can be stored for <1 week at 4 °C)[27]. The high antibiotic concentration recommended here will help to ensure adequate selection.

1.2. Start with several (typically two to four) NGM plates with large populations of gravid hermaphrodites. Isolate eggs using bleach-NaOH synchronization[24].

1.2.1. Wash worms off agar plates using 2 mL of sterile ddH_2O per plate. Distribute the liquid evenly into 1.5 mL microcentrifuge tubes (one tube per plate or hermaphrodites).

1.2.2. Spin down for ~5 s in a benchtop minicentrifuge (2,000 x *g*) to pull adults to the bottom of the tubes. Pipette off the supernatant and discard.

1.2.3. Wash with 1 mL of sterile ddH_2O; spin down as before and discard the supernatant.

1.2.4. Repeat the previous step to reduce remaining bacterial debris.

1.2.5. Resuspend the contents of each tube in 1 mL of sterile ddH_2O. Add to each tube 130 µL of commercial bleach (8.25% sodium hypochlorite) and 130 µL of 5 N sodium hydroxide (NaOH, final concentration 0.5 N).

1.2.6. Vortex tubes vigorously for at least 10–15 s every 2 min until adult bodies have broken up. Do not allow bleach-NaOH treatment to go longer than 5 min to avoid killing eggs.

1.2.7. Spin in a minicentrifuge for 30–60 s at 2,000 x *g* to pellet the eggs. Pipette off the supernatant and discard. There may or may not be a visible pellet, this is normal.

1.2.8. Add 1 mL of M9 worm buffer and spin for 30–60 s at 2000 x *g*. Discard the supernatant.

1.2.9. Repeat the rinse step (1.2.8) 5x to thoroughly remove bleach-NaOH mixture, removing as much of the supernatant as possible without disturbing the egg pellet.

1.2.10. Transfer eggs to 10 mL of M9 worm buffer in a 50 mL conical tube or 30 mL culture tube with cap. If using conical tubes, leave the lid unscrewed slightly and use a bit of tape to keep it secure. Incubate with shaking overnight (16 h) at 25 °C and 200 RPM to allow the larvae to hatch.

1.3. Transfer synchronized L1 larvae to RNAi plates to grow to adulthood.

1.3.1. Add 2 mL of sterile M9 buffer + 0.01% Triton X-100 (henceforth M9TX-01) to each L1 tube and transfer the entire volume (12 mL) to a 15 mL screw-top conical tube.

1.3.2. Place 15 mL tubes with L1 worms at 4 °C for 10 min to slow the larval movement.

1.3.3. Spin down 15 mL conical tubes in a large tabletop centrifuge (1,500 x *g* at 4 °C for 3 min; acceleration and deceleration should be no higher than 80% of maximum).

1.3.4. Carefully pipette off the supernatant without disturbing the L1 pellet. Discard the supernatant.

1.3.5. Add 12 mL of cold M9TX-01 to each tube. Repeat the centrifugation. Carefully pipette off and discard the supernatant. Each tube should have ~200 µL remaining.

1.3.6. Rinse a 200 µL pipette tip in M9TX-01 to keep worms from sticking to the plastic, then use this tip to resuspend the worm pellet. Transfer resuspended worms to prepared *pos-1* plates by pipetting drops of liquid onto the bacterial lawn.

1.3.7. Incubate the plates at 25 °C until the first day of adulthood.

NOTE: If growing worms on *pos-1* RNAi plates, worms MUST feed *ad libitum* on the RNAi bacteria until they have fully transitioned to adulthood to ensure high penetrance of the embryonic-lethal phenotype. Check the plates at 24 and 48 h. If the plates appear starved or nearly starved, the worms will need to be moved to fresh plates to finish growing into full-sized adults. To avoid depleting plates before worms are grown, aim to add 250–500 L1 larvae to each 10 cm RNAi plate.

1.4. Harvest adults and clear intestinal *E. coli* to create germ-free worms.

1.4.1. Rinse adult worms from plates using 5 mL of M9TX-01 per plate. Transfer buffer + worms to a 15 mL conical tube and allow adults to settle to the bottom of the tube.

1.4.2. Rinse adults in changes of 10 mL of fresh M9TX-01 buffer until no visible bacterial turbidity remains (typically 1–2x). Tubes can be centrifuged at 700 x *g* for 30 s to pellet worms, or adults can be allowed to settle by gravity.

1.4.3. Perform one additional wash with 10 mL of M9TX-01 to reduce external bacteria.

1.4.4. Transfer worms to 50 mL conical tubes or 30 mL culture tubes containing 5 mL S Medium + 2x heat-killed *E. coli* OP50 (~5 x 10^9 killed cells/mL) + 200 µg/mL of gentamycin + 50 µg/mL of chloramphenicol. If using conical tubes, leave the lid unscrewed slightly and use a bit of tape to keep it secure. Use glass pipettes or rinse plastic pipettes in M9TX-01 to keep the worms from sticking.

1.4.5. Incubate adults at 25 °C with shaking at 200 RPM for 24–48 h to produce germ-free adults.

NOTE: If the worms are to remain in antibiotics for >24 h, more heat-killed OP50 may have to be added to ensure that the worms have an adequate food source. Check tubes at 24 h and supplement with heat-killed OP50 if turbidity is visibly reduced.

1.5. Sucrose wash adults according to the Wormbook protocols[24] to obtain clean, reproductively sterile, synchronized adult-only stocks for bacterial colonization.

1.5.1. Ensure that cold volumes of 60% sucrose, M9 worm buffer, and M9TX-01 are ready for the use. For simplicity, these can be left at 4 °C the night before.

1.5.2. For each sample to be washed, create a labeled 15 mL conical tube containing 8 mL of M9TX-01 and set aside on ice. These will be needed in 1.5.10.

1.5.3. Add 5 mL of M9TX-01 to each 50 mL tube containing L1 larvae. Transfer the entire volume (now 10 mL) to an empty 15 mL screw-top conical tube and allow adults to settle to the bottom of the tube.

1.5.4. Carefully pipette off the supernatant and discard.

1.5.5. Add 10 mL M9TX-01 to each tube and move tubes to an ice bucket for 5–10 min.

NOTE: Starting at this point, worms and all buffers should be kept on ice.

1.5.6. Use the "fast temp" setting to cool a large tabletop centrifuge to 4 °C.

1.5.7. Add 10 mL of cold M9TX-01 to each tube to rinse off any remaining debris. Let worms settle on ice; remove the supernatant and discard.

1.5.8. Sucrose float: Add 5 mL of cold M9 buffer and 5 mL of cold 60% sucrose solution to each tube, mixing thoroughly. Then, carefully float 1 mL of cold M9 buffer on top of the sucrose-buffer mixture in each tube. Do not mix after the float has been added.

CAUTION: Move quickly for the next few steps—worms can desiccate if exposed to high concentrations of sucrose for too long!

1.5.9. Centrifuge at 1500 x *g* for 3 minutes at 4 °C. Live adult worms will be at the interface of the M9 and the sucrose, approximately 1 mL from the top of the tube.

1.5.10. Use a glass 5 mL serological pipette to transfer the worm layer to prepared 15 mL conical tubes with cold M9TX-01 (from step 1.5.2). Be very careful to get the layer of live worms without pipetting up too much of the sucrose.

1.5.11. If necessary, add M9TX-01 to get equal volumes of 10–12 mL/tube. Centrifuge at 1500 x *g* at 4 °C for 1 min, then pipette off the supernatant. Worms can be returned to room temperature at this point.

1.5.12. Repeat the wash step 1.5.11 twice, reducing the speed to 700 x *g* at 4 °C and time to 30 s.

2. Feeding worms on live bacteria in liquid culture

NOTE: This protocol is used to colonize worms with laboratory-grown bacteria in well-mixed conditions in liquid culture (**Supplementary Figure 1**). Worms can be colonized with individual isolates from pure culture (e.g., pathogens such as *Enterococcus faecium*[28,29]) or mixtures of isolates (e.g., minimal microbiome communities[14]).

2.1. Start with sucrose washed synchronized adult worms from protocol step 1.5 in a 15 mL conical tube. Wash the worms once in 12 mL of S buffer and discard the supernatant.

2.2. Resuspend the washed worms in the volume of S medium needed for the experiment. Consider the volume of experimental conditions, the number of conditions over which worms will be split, and the final concentrations of worms and bacteria.

NOTE: Feeding in worms varies with bacterial availability[30] and worms can be stressed by crowding[31]. For colonization in liquid culture, <1000 worms/mL and >10^7 CFU/mL are recommended; 10^{11} CFU/mL is considered "*ad libitum*" feeding density on *E. coli*[32].

2.3. Spin down bacterial cultures. Pour off the supernatant; aspiration or pipetting can be used to remove the supernatant for bacteria that form loose pellets.

NOTE: For cultures >5 mL, transfer to 15 mL tubes and spin at ~2800 x *g* in a large tabletop centrifuge for 8–10 min. Cultures <5 mL can be transferred to 1.5 mL tubes and centrifuged at 9000 x *g* for 1–2 min in a small tabletop centrifuge. Highly motile bacteria (e.g., many species of *Pseudomonas*) may need to be chilled at 4 °C for 10–15 min to facilitate formation of a stable pellet, and it may be better to centrifuge at 4 °C.

2.4. Resuspend bacterial cultures in one volume of S buffer and centrifuge again to pellet. Remove and discard the supernatant as before.

2.5. Resuspend bacterial cultures in S medium at the desired density for the experiment, plus any antibiotics for selection. The antibiotics to be used, if any, will depend on the resistance profile of the bacteria used for colonization.

2.6. Using a pipette tip coated in M9TX-01, pipette worms gently up and down until worms are thoroughly resuspended in S medium, then transfer to tubes or plate wells for bacterial colonization.

2.7. Add bacterial suspension to each worm culture to reach the desired bacterial concentration and final volume.

2.8. If using a multi-well plate for colonization, cover the plate with a sterile 96-well gas-permeable sealing membrane.

2.9. Incubate with shaking at 200 RPM to prevent bacteria from settling during incubation.

3. Mechanical disruption of individual worms in a 96-well format

NOTE: This section describes a 96-well plate format protocol for mechanical disruption of individual bacterially colonized *C. elegans*. The first steps in the protocol (3.1–3.8) describe a method for purging non-adhered bacteria from the worm intestine and cleaning the exterior of the worms using cold paralysis and surface-bleaching. These steps will produce clean, live adult worms that can be mechanically disrupted for quantification of bacterial contents (3.8–end) or used for further experiments (**Supplementary Figure 1**). This protocol can be adapted to quantify bacteria in worms colonized in liquid culture (Section 2), on agar plates, or from natural or microcosm soil.

3.1. Place an aliquot of M9TX-01 on ice to chill (4–5x the number of samples in mL).

3.2. Prepare an aliquot of M9TX-01 + bleach (6% sodium hypochlorite, 1:1000 or 1:2000 v/v, 1 mL per sample + 1 mL extra) and place on ice to chill. This aliquot will be used in step 3.8.

3.3. Prepare 96-well plates for serial dilution of disrupted worm samples.

3.3.1. Obtain sterile 300 µL capacity 96-well plates with lids; this protocol uses one dilution plate per 12 worms digested.

3.3.2. Use a 96-well multichannel pipettor to fill rows B–D of each 96 well plate (300 µL capacity) with 180 µL of 1x PBS buffer. Leave the top row empty. Rows B–D will become 10x serial dilutions of the worm digests [0.1x, 0.01x, 0.01x].

3.3.3. Set plates aside. Dilution plates will be used in step 3.13.

3.4. Resuspend each worm sample in 1 mL of M9TX-01 in a 1.5 mL microcentrifuge tube.

3.5. Spin tubes briefly (2–3 s) in a low speed minicentrifuge (2,000 x *g*) at 25 °C to pellet adults. Pipette off the supernatant and discard, being sure not to disturb the worm pellet.

3.6. Using the centrifugation settings in step 3.5, rinse worms twice with 1 mL of M9TX-01, then once with 1 mL of M9 worm buffer, to reduce external bacteria.

3.7. Purge non-adhered bacteria from the worm intestine.

3.7.1. Resuspend each sample of worms in 1 mL of S medium + 2x heat-killed OP50 in a culture tube.

3.7.2. Incubate at 25 °C for 20–30 min to allow passage of any non-adhered bacteria from the gut.

NOTE: This will also purge any extracellular fluorescent protein from the lumen and allow clearer visualization of labeled bacteria adhered to the intestinal epithelium, particularly when acid-fast fluorophores (e.g., mCherry, dsRed) are used.

3.8. Surface bleach worms to clear external bacteria.

3.8.1. Rinse purged worms twice with 1 mL of cold M9TX-01 and discard the supernatant.

3.8.2. Allow tubes to chill for 10 min on ice (preferred) or at 4 °C. This will paralyze worms and prevent ingestion of bleach.

NOTE: Other protocols use a chemical paralysis agent such as levamisole; this is an established approach[33] which requires addition of a hazardous waste stream.

3.8.3. Add 1 mL of ice-cold M9 worm buffer + unscented bleach (8.25% sodium hydroxide, 1:1000 or 1:2000 v/v) to each tube. Allow tubes to sit on ice (preferred) or at 4 °C for at least 10 min to kill external bacteria.

NOTE: Do not exceed 1:1000 concentration of bleach. Even in paralyzed worms, mortality can result.

3.8.4. Pipette off bleach buffer and discard; return tubes to ice to ensure worms do not resume pumping until bleach is cleared.

3.8.5. Add 1 mL of cold M9TX-01 to each tube. Spin for ~5 s in a minicentrifuge (2,000 x *g* at 25 °C); return tubes to ice. Remove the supernatant and discard.

3.8.6. Repeat this rinse step with another 1 mL of cold M9TX-01, discarding as much of the supernatant as possible.

NOTE: If using worms for further experiments, skip the permeabilization step (Protocol 3.9) and instead transfer freshly surface-bleached adults to ice-cold buffer in a 6 cm Petri dish and separate worms into experimental conditions as in Protocol 3.10. Keep worms cold to prevent motility from resuming but work quickly — keeping worms for >30 min on ice can potentially result in <100% resumption of normal activity[34].

3.9. Chemical permeabilization of worm cuticle with sodium dodecyl sulfate and dithiothrietol (0.25% SDS + 300 mM DTT) (based on[35])

CAUTION: DTT is a reducing agent and irritant. Wear PPE and work in a fume hood when handling dry stocks or solutions. A hazardous waste stream is required.

3.9.1. In the fume hood, prepare enough SDS/DTT solution to allow 100 µL for each sample. For 1 mL, to 965 µL of M9 worm buffer or M9TX-01 in a 1.5 mL microcentrifuge tube, add 5 µL of 5% (w/v) SDS and 30 µL of 1M DTT.

NOTE: 1 M DTT solution (aqueous) should be prepared fresh or stored in aliquots at -20 °C to ensure potency. Aliquots should be sized to be used up in two to three experiments to avoid excessive freeze-thaw cycling.

3.9.2. Move microcentrifuge tubes containing surface-bleached worms to a room-temperature tube rack. Each tube should contain worms in ~20 µL of buffer.

3.9.3. Add 100 µL of SDS/DTT solution to each worm sample. Dispose of any remaining SDS/DTT solution in the appropriate hazardous waste stream.

3.9.4. Allow the treatment to proceed for up to 8 min on the bench to partially break down the resistant cuticle of the adult worms. Worms will die and settle to the bottom of the tube during this time.

3.9.5. After permeabilization time is up, carefully pipette off the SDS/DTT supernatant and dispose of it in an appropriate SDS/DTT hazardous waste stream.

3.9.6. Add 1 mL of M9TX-01 to each tube. Spin briefly in a table-top centrifuge to pellet the worms or allow worms to settle by gravity to the bottom of the tubes, then draw off the supernatant and dispose of it in an SDS/DTT hazardous waste stream.

3.9.7. Resuspend worms in 1 mL M9 worm buffer + 0.1% Triton X-100 until ready to use.

3.10. Separate worms into a deep 96-well plate with silicon carbide grit for mechanical disruption. Prepare the 96-well disruption plate as under.

3.10.1. Obtain a sterile 2 mL deep-well 96-well plate and a matching silicon 96-well plate cover.

NOTE: It is important to use plates that are compatible with the 96-well adaptors for the tissue disruptor. Tiny differences in external dimensions make the difference between a plate that can be removed from the adaptors and one that cannot.

3.10.2. Using a sterile scoop spatula, add a small amount of sterile 36-grit silicon carbide to each well of the plate that will receive a worm. Use enough grit to barely cover the bottom of the well (about 0.2 g per well). Excessive material will make it difficult to get a pipette tip to the bottom of the well when retrieving the contents.

3.10.3. Add 180 µL of M9 worm buffer to each well.

3.10.4. Label the columns or rows to indicate where each sample will go, then cover the plate loosely with the silicon 96-well plate cover.

3.11. Transfer individual worms to the 96-well plate for disruption.

3.11.1. Move permeabilized worms carefully to a small (35 or 60 mm) Petri dish containing sufficient M9TX-01 to fill the dish to a depth of ~1 cm.

NOTE: If a large number of worms are present, it may not be feasible to transfer the entire sample as the liquid will become crowded and it will be difficult to pipette individual worms.

3.11.2. Using a dissecting microscope or other low-magnification device, pipette off individual worms in 20 µL volumes, and transfer these worms to individual wells of the 96-well plate.

NOTE: It is best to harvest only freshly killed worms. Avoid worms with a rigid linear shape, as these worms may have been dead for some time. Try to take worms that are curved or S-shaped, with normal gross physiology and an intact gut.

3.11.3. After transferring each volume, make sure that the selected worm was actually ejected into the well. To do this, pipette up 20 µL of M9TX-01 from a clear area of the Petri dish and release the full volume back into the dish; this will normally eject the worm if it is stuck to the pipet. If the worm was stuck, remove 20 µL from the well and try the transfer again.

3.11.4. Once all worms have been transferred, cover the 96-well plate with a sheet of commercially available flexible paper-backed sealing film (2 x 2 squares), making sure that the paper-backed side of the sealing film is facing down onto the sample wells. Be careful not to stretch the sealing film too thin, or it will be very difficult to remove later.

3.11.5. Place the silicon sealing mat lightly on top of the flexible sealing film; do not press the cover down into the wells at this time.

3.11.6. Move the plate to 4 °C to chill for 30–60 min. This will prevent over-heating during disruption, which can damage the samples.

NOTE: This is a break point in the protocol. In most cases, the plate can be left at 4°C for up to 4 h before grinding. Do not leave the worms overnight, as this will change the bacterial counts.

3.12. Load 96-well plates onto a tissue disruptor to break up worm tissues and release intestinal bacteria.

NOTE: (Optional) If using an odd number of 96-well plates for digests, it is necessary to prepare a counterweight before proceeding. Use an empty deep 96-well plate and fill wells with water until it weighs the same as the first plate.

3.12.1. Press the silicon sealing mat down firmly into the wells to create a seal, making sure the lid lies flat across the entire surface of the plate.

NOTE: If the flexible sealing film is too thick after stretching, it will be difficult to secure the silicon lid such that it is lying flat in all wells. This will result in an insufficient seal and well-to-well contamination during shaking.

3.12.2. Secure plates in the tissue disruptor using the 96-well plate adaptors. Shake plates for 1 min at 30 Hz, then rotate plates 180° and shake again for 1 min. This will help ensure even disruption in all wells of the plate.

3.12.3. Tap plates firmly on the bench two or three times to dislodge any grit from the flexible sealing film.

3.12.4. Using a large centrifuge with two 96-well plate adaptors, spin the plates down at 2400 x *g* for 2 min to gather all material to the bottom of the wells.

3.12.5. Remove the silicon lid and carefully pull off the flexible sealing film.

NOTE: If the flexible sealing film sticks in any of the wells, use a 200 µL pipette tip to remove it. This is common when the flexible sealing film was stretched too thin.

3.13. Serially dilute worm digest samples in 300 µL in 96-well plates.

3.13.1. Using a multi-well pipettor set to 200 µL, pipette up and down several times slowly and carefully to re-mix the contents of the wells, then draw off as much of the liquid as possible. Transfer this liquid to the top rows of the 96-well plates prepared in step 3.3.

3.13.2. Using a 96-well pipettor set to 20 µL, remove this volume of liquid from the top row and dispense into row B. Pipette up and down 8–10x to mix. Discard tips.

3.13.3. Repeat step 3.13.2, starting from the 0.1x samples in row B to create 0.01x dilution samples in row C.

3.13.4. Repeat step 3.13.2 again, going from row C to row D.

3.13.5. Plate onto solid agar for bacterial quantification. For mono-colonized worms, it is generally sufficient to plate 10–20 µL drops of each dilution [1x–0.001x] on agar plates. For multi-species colonization, plate each dilution separately by pipetting 100 µL onto a 10 cm agar plate; spread immediately using glass plating beads.

4. Cleaning silicon carbide grit for re-use

NOTE: This procedure is used to clean and sterilize the grinding material, silicon carbide grit, for re-use after experiments. This protocol should be followed in its entirety before first use, as silicon carbide grit is an industrial product and does not come pre-sterilized. Si-carbide grit (3.2 g/cc) is a dense, rough-edged material that works efficiently to disrupt tough samples. However, the particles can wear down over repeated use and should be replaced when wear becomes apparent. Fortunately, the material is inexpensive, and the sizes typically sold (~1 lb) are sufficient for many experiments.

4.1. After removing samples for plating, add 10% bleach solution to all wells of the 96-well plate and let sit for at least 10 min.

4.2. Remove the bulk of the grit by rapidly inverting the 96-well plate over a small high-sided tray or empty P1000 pipette tip container large enough to catch all the contents. The grit will sink immediately to the bottom of the tray. Pour off the bleach solution into a sink.

4.3. Re-fill the 96-well plate with tap water and invert into the same tray to rinse out remaining grit. Pour the water off into the sink.

4.4. Repeat one to three more times with tap water until plate is completely clear of grit.

4.5. Rinse grit 2x in tap water, filling the tray each time.

NOTE: The 96-well deep-well plate can be washed in a laboratory dishwasher, covered securely with foil, and autoclaved with other reusable plastics. Grit does not need to be washed immediately and can be set aside at this point. Used grit is usually accumulated from multiple experiments before washing and autoclaving.

4.6. Wash grit in a solution of laboratory detergent for 30 min, agitating occasionally by swirling or mixing with a metal spatula.

4.7. Rinse away all traces of detergent in several (8–10) changes of tap water, then rinse 2x with distilled water.

4.8. Spread grit in an open tray, such as a shallow polypropylene autoclave tray, and dry at 40–70 °C for several hours.

NOTE: If the grit is clumpy when dry, it was not cleaned or rinsed sufficiently. Repeat the cleaning protocol starting at step 4.6, adding additional rinses in step 4.7.

4.9. Distribute clean, dry grit into screw-top autoclavable glass bottles to a maximum depth of 5–6 cm. Autoclave on pre-vacuum cycle for 30 min to sterilize.

REPRESENTATIVE RESULTS:

Bleach sterilization of live worms

Surface-bleached worms are effectively free of external bacteria until motility returns and excretion resumes. Under the conditions used here, rapid extinction of bacteria in buffer is observed (**Figure 1A–C, Supplementary Figure 2, Video 1**) without disturbing the gut-associated bacteria in cold-paralyzed worms (**Figure 1D–F, Video 2**). These data indicate that surface bleaching can be used effectively to sanitize worms externally without compromising the intestinal contents (comparisons of surface-bleached vs. no-bleach worm-associated CFU counts are non-significant, Wilcoxon rank-sum test $p > 0.05$).

Variations on multi-sample mechanical disruption

The 96-well technique for mechanical disruption of worms is robust to the specific materials used, and practical considerations dictate the choice of grinding material. Similar to a previous report[33], manual disruption (**Figure 2A**) resulted in more heterogeneity than the standard 96-well protocol (silicon carbide grit, **Figure 2B**) ($var(\log_{10}CFU) = 0.499$) across all buffer conditions, as compared with 0.229 for Si-carbide, 0.243 for large glass beads (**Figure 2C**), and 0.227 for small glass beads (**Figure 2D**). Nonetheless, most differences in CFU/worm distributions were not significant (Kruskal-Wallace, $p = 0.017$ with $df = 3$; significant post-hoc Wilcoxon tests for large beads vs. small beads, $p = 0.021$, and large beads vs. silicon carbide grit, $p = 0.02$). The use of Triton X-100 as a surfactant was not associated with any significant difference in yield when considered as an individual factor (Kruskal-Wallace, $p = 0.94$, $df = 3$), although there is an apparent increase in yield in no-Triton vs. Triton-containing samples when large beads (2.7 mm) were used (**Figure 2C**), possibly attributable to the excessive “foaming” observed in these wells when Triton was present. These results indicate that large glass beads, while ideal for use in homogenization tubes[33], are not suitable for the 96-well technique. While small glass beads produced reasonable results (**Figure 2D**), they consistently clogged 200 µL pipette tips during mixing and plating. The standard material in this assay, silicon carbide grit, is inexpensive, too large to clog standard tips, and like glass beads can be washed and reused after autoclaving. The

grit does release a small amount of "dust" into the buffer, which does not interfere with plating but needs to be filtered off if the products of disruption are to be used for flow cytometry.

Heterogeneity in bacterial colonization in adult worms

Successful disruption of individual worms reveals heterogeneity in bacterial colonization. Individuals from isogenic synchronized populations of worms, colonized at the same time on the same pool of bacteria, consistently show 100-fold or greater range in intestinal bacterial load. This is observed for different bacterial colonists (**Figure 3A**) and during colonization on multi-species bacterial communities (**Figure 3B**). This heterogeneity is also evident in individual-worm measurements of fluorescence when worms are colonized with bacteria expressing a fluorescent protein (GFP) (**Figure 3C–D**). The properties of the host play a role in shaping this heterogeneity, as can be seen by comparing colonization of wild-type Bristol N2 worms to colonization by the same bacteria in DAF-2/IGF mutants; this *daf-16* mutant supports larger populations of many bacteria as compared with N2, while *daf-2* is resistant to colonization by a range of bacteria[36] (**Figure 3B,D**). This heterogeneity is characteristic, showing variation across different combinations of host and colonist(s), while retaining a consistent structure over different runs of the same experiment (**Figure 3E–F**).

Importance of individual heterogeneity for accurate comparison of groups

The importance of individual heterogeneity can be easily seen by considering how batch digests could alter the distributions of data. Colonization by native microbiome bacteria MYb53 (*Rhodococcus erythropolis*) and MYb120 (*Chryseobacteria* spp.) (**Figure 3A, 4A**) in N2 adults are used as examples. The individual worm data are clearly similar in distribution (two-tailed t-test, $p = 0.9$, Wilcoxon rank sum, $p = 0.59$). When resampling these data to simulate the effects of batch digests, the batch extrapolated CFU/worm pulls toward the upper quantiles of the data due to the positive skew in these distributions (mean > median). As batching effectively averages over the individuals within a batch, batch-extrapolated CFU/worm will center around the arithmetic mean of the individual data, with decreasing distance to this mean as batches become large according to the central limit theorem (**Figure 4B–D**). Accordingly, signal from biological variation is quickly lost; batch-inferred CFU/worm measurements converge toward the average, which is not a representative metric of these log-scale-distributed data. Differences in inferred colonization by MYb53 vs. MYb120 quickly become significant in simulated batch digests (t-test batch 5, $p = 0.049$; batch 10, $p = 2.27e\text{-}4$; batch 20, $p = 1.19e\text{-}15$; Wilcoxon rank sum test batch 5, $p = 2.27e\text{-}4$; batch 10, $p = 2.70e\text{-}06$; batch 20, $p = 1.80e\text{-}09$) as the original signal is obscured.

Effects of individual heterogeneity on microbial transmission

As individual worms show substantial heterogeneity in bacterial colonization, it is reasonable to ask whether this heterogeneity has downstream effects. For example, it is reasonable to expect that transmission might be a function of intestinal bacterial load. By transferring individual surface-bleached worms to a clean environment, it is possible to observe inoculation of the environment with excreted live bacteria. In these experiments, surface-bleached pre-colonized adults, carrying generally substantial populations (10^3-10^5 CFU/worm, **Figure 5**) of commensal *Ochrobactrum* MYb14-GFP or pathogenic *S. aureus*-GFP, were allowed to roam on heat-killed

OP50 lawns on NGM agar for 1.5 h. When these worms are re-harvested from excretion plates and disrupted for bacterial quantification, there is no significant relationship between bacterial load and excretion rate of live bacteria (Pearson correlations between log-transformed colonies/hr and CFU/worm: MYb14 rho = 0.19, p = 0.45; *S. aureus* rho = 0.02, p = 0.9) (**Figure 5**). Nor is there a significant relationship between the presence/absence of colonies on a plate and intestinal bacterial load (binomial logistic regression with log-transformed CFU/worm as factor: p = 0.15 with df = 53). A substantial fraction of plates remained free of new growth (9/18 plates for MYb14, 10/36 plates for *S. aureus*), indicating low overall excretion rates.

When worms are allowed to excrete onto agar plates, the actual number of live excreted bacteria per worm is confounded by "farming", where worms pass through colonies and create trails of new growth (**Figure 6**)[37]. A plate with *n* colonies represents at least one, and at most *n*, events where live colony-forming bacteria were excreted. From this observation, it is not possible to know how many excretion events in (1,*n*) actually occurred, nor is it possible to know how many bacteria were excreted in each event. It is therefore not possible to precisely estimate excretion rates of live bacteria from the gut using these data. However, it is possible to infer some bounds. Although the number of colonies per plate is not very informative, presence/absence data can be used for rough inference of excretion rates. For simplicity, if it is assumed that excretion rate of live bacteria is not a function of bacterial load and that excretion is a Poisson process, there is a ~50% chance of observing at least nine events in 18 trials when $\lambda \approx 0.33$ worm^{-1} hr^{-1} in MYb14. For *S. aureus*, similar plausible rates of $\lambda \approx 0.2$ worm^{-1} hr^{-1} are obtained. While these rough calculations suggest low rates of excretion of live bacteria, more precise quantification of this process over larger numbers of individual worms will be necessary to obtain reliable estimates.

Data Availability:

Data shown here are available on Dryad (https://doi.org/10.5061/dryad.7wm37pvw2).

FIGURE AND TABLE LEGENDS:

Figure 1. Low-concentration surface-bleaching treatment rapidly kills bacteria in buffer but does not disturb intestinal communities in cold-paralyzed worms. (**A–C**) Bacterial CFU/mL in M9 worm buffer during surface bleaching at three different concentrations (1:1000, 1:2000, 1:5000 v/v; unbleached control for comparison), targeting (**A**) *S. aureus* Newman, (**B**) *S. enterica* LT2, or (**C**) *E. coli* OP50. Samples were taken at indicated time points up to 20 min post-exposure and washed twice with sterile buffer to prevent bleach from killing colonies on plates. Data for the 1:1000 condition are offset slightly so that these data are visible on the plot. (**D–F**) Intestinal bacteria in individual N2 worms (n = 24 worms per experiment, two or three independent runs on separate days). All comparisons of surface-bleached and no-bleach worm-associated CFU counts are non-significant (Wilcoxon rank-sum test p > 0.05). Grey horizontal lines represent threshold of detection, defined as the density (40 CFU/worm) at which probability of observing at least one colony is ~60% when plating 10 µL aliquots from 200 µL volumes.

Figure 2. The 96-well disruption protocol produces consistent results and is robust to the choice of materials. N2 adult worms colonized with a single bacterial species for 48 h (*P.*

mosselii) were surface bleached and permeabilized according to standard protocols, then individual worms (n = 24 per condition) were mechanically disrupted for CFU plating using (**A**) manual disruption in individual 0.5 mL tubes, using a motorized pestle or (**B–D**) variations on the 96-well disruption protocol described in detail in the Protocol. Disruption was carried out in M9 worm buffer containing varying concentrations of Triton X-100 (x-axis, 0–0.1%, v/v) and one of (**B**) 36-grit silicon carbide, (**C**) small (425-600 µm) glass beads, or (**D**) large (2.7 mm) glass beads. For all plots, data shown are $\log_{10}$(CFU/worm), and each point is one individual worm.

Figure 3. Heterogeneous bacterial colonization of the *C. elegans* intestine. (**A**) Single-species colonization of N2 adult hermaphrodites prepared as in Methods. Bacteria are four species from the MYb native worm microbiome collection (Dirksen et al. 2016) (n = 24 worms, one experiment each) and two pathogens, *Staphylococcus aureus* MSSA Newman (SA) and *Salmonella enterica* LT2 (SE) (n = 96–144 worms over two/three independent experiments). Colonization by native microbiome species was assessed after a 48 h incubation at 25°C in liquid S medium + 10^8 CFU/mL bacteria; colonization by pathogens was assessed after incubation on lawns on NGM worm agar for 24 (SA) or 48 (SE) h at 25°C. (At 48 h, worms on *S. aureus* have mostly died.) (**B**) Total CFU/worm in N2, *daf-16(mu86)*, and *daf-2(e1370)* adults colonized for 4 days in liquid media on an eight-species minimal native microbiome (data from Taylor and Vega, 2021)[14]. (**C–D**) Green fluorescence in individual worms colonized with GFP-expressing bacteria, observed by large object flow cytometry. In (**C**), synchronized populations of N2 adults were colonized with OP50 (non-fluorescent, n = 1908 individual adult worms), *S. aureus* (GFP, n = 968), or *S. enterica* (GFP, n = 1153) as described in (**A**); the OP50 control indicates typical levels of green-channel autofluorescence in day-3 adult N2 worms. In (**D**), synchronized populations of N2 (n = 1165), *daf-16(mu86)* (n = 1180), and *daf-2(e1370)* (n = 2267) adults were colonized with commensal *Ochrobactrum* MYb14-GFP for 2 days on plates as described in (**A**). (**E–F**) Day-to-day variation in colonization by *S. aureus* (**E**) and *S. enterica* (**F**) (same data as in **panel A** and **Figure 1**, n = 48 worms per experiment). The x-axis indicates the day of sampling. Grey horizontal lines represent threshold of detection, defined as the density at which probability of observing at least one colony is ~60% (40 CFU/worm for single-species colonization and four CFU/worm for multi-species colonization, due to different plating volumes of 10 µL and 100 µL respectively out of 200 µL).

Figure 4. Batching erases biological variation in skewed log-scale data. CFU/worm data from **Figure 3** were resampled with replacement to create n = 25 replicate sets of simulated data for each batch size, where size is the number of individual worms per batch. CFU/worm is the total CFU in each simulated batch divided over the number of worms per batch. In the raw data (**panel A),** average CFU/worm for MYb53 is 4450.8 ($10^{3.6}$), and for MYb120, 1398.3 ($10^{3.1}$); the batch-inferred numbers converge to these values as batch size increases (**B**, five worms/batch; **C**, 10 worms/batch; **D**, 20 worms/batch), consistent with expectations from central limit theorem.

Figure 5. Excretion of live bacteria is poorly correlated with CFU load in the intestine of individual worms. Here, N2 adults were colonized by feeding for 1 or 2 days respectively on lawns of *S. aureus*-GFP or MYb14-GFP. Worms with detectable GFP fluorescence (total GFP > 1.8

logs on large object flow cytometer) were sorted from the bulk population, surface bleached as described in Methods, and transferred individually to NGM + heat-killed OP50 plates as described for **Figure 5**. Pearson correlations between log-transformed colonies/h and CFU/worm are non-significant (MYb14 rho = 0.19, p = 0.45; *S. aureus* rho = 0.02, p = 0.9).

Figure 6. Bacterial "farming" obscures the number of excretion events on agar plates. Here are two plates with MYb14-GFP colonies from worm excreta. The first plate (**A**) has clear evidence of "farming" along worm paths and appears to represent at least two separate excretion events based on differences in GFP expression (visible as yellowish pigmentation) across colonies. While the second plate (**B**) is more ambiguous, farming cannot be ruled out based on the positions of the colonies. In these experiments, N2 adult worms were pre-colonized for 48 h by feeding on agar plates containing lawns of MYb14-GFP. After colonization, worms were prepared and surface bleached according to Methods, then transferred in 5 µL aliquots of M9 worm buffer + 0.1% Triton X-100 to 6 cm NGM + heat-killed OP50 plates (prepared by allowing 50 µL spots of 5x concentrated heat-killed OP50 to dry on the surface). Worms were permitted to roam for 1.5 h at 25°C, then picked from plates and disrupted for CFU/worm plating (manual disruption in 20 µL buffer in individual 0.5 mL tubes, using a motorized pestle). Plates were incubated at 25°C for 2 days before counting.

Video 1. Visualization of N2 worms colonized with GFP fluorescent *S. aureus* without surface bleaching. A small number of fluorescent cells on the cuticle move into and out of focus as the image passes through the body of the worm, and spatially heterogeneous colonization of the gut becomes visible as the field of view moves from the body surface into the intestine. Z-stack image was taken at 20x magnification on an inverted fluorescent microscope. Bright-field and GFP filtered fluorescent images were overlaid, and images across the Z-stack stitched together, using the vendor software. Image is from the same slide as in **Supplementary Figure 2**.

Video 2. Visualization of a N2 worm colonized with GFP fluorescent *S. aureus* with surface bleaching (1:1000 v/v for 20 min). Spatially-heterogeneous colonization by fluorescent bacteria is visible in the intestine of this individual, and bacteria have infiltrated the body tissues, indicating advanced infection. No bacteria are visible on the cuticle. Z-stack image was taken at 20x magnification on an inverted fluorescent microscope. Bright-field and GFP filtered fluorescent images were overlaid, and images across the Z-stack stitched together, using the vendor software. Image is from the same slide as in **Supplementary Figure 2B**.

Supplementary Figure 1. Overview of the Protocol. Here, synchronized adult worms are mono-colonized with red bacteria, surface-bleached, and permeabilized before mechanical disruption of individual worms in a 96-well format. Bacteria released from the intestine are dilution plated in 10x series for CFU/worm quantification; plates shown are typical for observed heterogeneity.

Supplementary Figure 2. Visualization of N2 worms colonized with GFP fluorescent *S. aureus* with and without surface bleaching (1:1000 v/v for 20 min). (**A**) In the unbleached sample, external bacteria are visible at low magnification as areas of green fluorescence not associated with worms or worm body fragments. (**B**) In the surface-bleached sample, GFP fluorescence is

restricted to the interior of worm bodies (one worm body fragment is visible mid-image). All images were taken at 4x magnification on an inverted fluorescent microscope. Bright-field and GFP filtered fluorescent images were overlaid, and images from adjacent fields of view stitched together, using the vendor software.

Supplementary File 1: Buffer and solution recipes.

DISCUSSION:

Here data are presented on the advantages of single-worm quantification of bacterial load in *C. elegans*, along with a 96-well disruption protocol to allow the rapid and consistent acquisition of large data sets of this type. As compared with existing methods[33], these protocols allow higher-throughput measurement of intestinal microbial communities in the worm.

This approach has plating as a rate-limiting step and is not truly "high-throughput". Large-object flow cytometry (**Figure 3B,C**) is a useful high-throughput method for quantifying fluorescently labeled bacteria in individual worms[16], although the number of simultaneous fluorophores is a limitation in multi-species communities. Linking multi-well plate disruption with community sequencing is another way to increase throughput; however, the 96-well disruption procedure described here was optimized specifically to leave bacterial cells intact. Sequencing-based analysis, where thorough lysis of cells is desirable, will require addition of a nucleic acid extraction step or modification of the beating protocol (Protocol 3.10–3.11) to extract cell contents instead of live bacteria. Protocols for single-worm disruption and extraction of nucleic acids have been published elsewhere[38,39].

Bacterial total abundance in the worm intestine is heterogeneous, and the data shown here suggest that batch-based measurement can produce erroneous results in comparisons between groups. However, other measures of bacterial communities in the worm may be less sensitive to the effects of batching. Of note, relative abundances in worm-associated communities seem to vary very little if at all with total intestinal population size, regardless of whether interactions among microbes are neutral[40] or not[14]. It is plausible that, compared to count data, relative abundance measures will be less susceptible to the false-positive rate issue described. Sequencing-based community analysis, which generates relative abundance data for community composition, may therefore not require measurement of single worms. Further investigation is needed on this point.

Here, we use cold treatment to paralyze worms for surface bleaching. Other work has found that worms resume normal activity rapidly (<15 min) if time on ice is kept under 30 min, allowing immediate use in further assays, in contrast with chemical paralysis agents which can require extended periods before full recovery[34]. If worms are to be disrupted immediately for bacterial quantification, this feature is dispensable, and the main advantage of chilling vs. chemical paralysis is avoiding the need for a controlled waste stream. Extended cold treatment should be used with caution when investigating stress responses, particularly if there is a known connection to temperature. The cold paralysis protocols described here entail shorter acute cold exposure than used in experiments for cold stress (20–30 min vs 2+ h at 2–4°C)[41–43], and a 1 h

cold shock produces no apparent phenotype in wild-type worms[43]. Short-term (90 min) incubation at 4 °C induces changes in cold-stress gene expression (measured by expression of a TMEM-135::GFP reporter), but expression returns to unstressed levels within minutes once worms are returned to room temperature[34]. However, the effects on stress-sensitive worm genotypes may be more severe than in wild-type. This procedure should be validated under the experimental conditions to be used.

The surface bleaching protocol described here can be used as a way to limit or eliminate passaging of external microbes in experiments. This method has additionally been used to clear fungal contaminants by surface bleaching and transferring only L1/L2 larvae to fresh plates (transfer of surface-bleached adults resulted in failure to clear the contaminant, presumably due to carriage in the intestines of the larger animals). It is critically important to ensure that bleach concentration does not exceed 1:1000 v/v, as damage to the worms and mortality will result. This procedure may be useful in experimental host-microbe evolution and host-pathogen interactions. For example, the low excretion rate of live bacteria observed here can help to explain the highly variable rates observed for bacterial transmission from hermaphrodites to offspring[15]. The lack of correlation between intestinal bacterial load and excretion rate observed here is interesting, but requires further investigation; a larger number of data points across a range of conditions will be needed to determine where (or whether) this observation will hold.

It may not always be necessary to clean worms to the extent provided by surface bleaching. Multiple washes in sterile buffer are likely sufficient when worms are internally colonized with a single microbe if the minimum expected CFU/worm is much higher (10–100-fold) than the concentration of bacteria in buffer supernatant, as this carryover will minimally affect counts (see **Figure 1**). Additionally, if the microbe(s) of interest primarily colonize(s) the cuticle, surface bleaching should clearly be avoided. Thorough cleaning is more important to ensure accuracy when dealing with mixed microbial communities (to ensure that all colonies/reads in a sample are from worm-associated bacteria and not from the environment), when bacteria adhered to the cuticle interfere with reading the internalized population, when expected minimum CFU/worm is low, etc.

Acknowledgments:
The authors would like to acknowledge H. Schulenberg and C. LaRock for their generous sharing of bacterial strains used in these experiments. This work was supported by funding from Emory University and NSF (PHY2014173).

Disclosures:
The authors have no conflicts of interest.

References:
1. Armitage, D. W., Jones, S. E. How sample heterogeneity can obscure the signal of microbial interactions. *The ISME Journal*. **13** (11), 2639–2646 (2019).

2. Stephenson, J. et al. Host heterogeneity affects both parasite transmission to and fitness on subsequent hosts. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **372** (1719), 20160093 (2017).
3. VanderWaal, K. L., Ezenwa, V. O. Heterogeneity in pathogen transmission: mechanisms and methodology. *Functional Ecology*. **30** (10), 1606–1622 (2016).
4. Dwyer, G., Elkinton, J. S., Buonaccorsi, J. P. Host heterogeneity in susceptibility and disease dynamics: tests of a mathematical model. *The American Naturalist*. **150** (6), 685–707 (1997).
5. Wu, D., Rea, S. L., Yashin, A. I., Johnson, T. E. Visualizing hidden heterogeneity in isogenic populations of C. elegans. *Experimental Gerontology*. **41** (3), 261–270 (2006).
6. Yashin, A. I. et al. Heat shock changes the heterogeneity distribution in populations of Caenorhabditis elegans does it tell us anything about the biological mechanism of stress response? *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*. **57** (3), B83–B92 (2002).
7. Zhao, Y. et al. Two forms of death in ageing Caenorhabditis elegans. *Nature Communications*. **8** (1), 1–8 (2017).
8. Eckley, D. M. et al. Molecular characterization of the transition to mid-life in Caenorhabditis elegans. *AGE*. **35** (3), 689–703 (2012).
9. Rea, S. L., Wu, D., Cypser, J. R., Vaupel, J. W., Johnson, T. E. A stress-sensitive reporter predicts longevity in isogenic populations of Caenorhabditis elegans. *Nature Genetics*. **37** (8), 894–898 (2005).
10. Kinser, H. E., Mosley, M. C., Plutzer, I. B., Pincus, Z. Global, cell non-autonomous gene regulation drives individual lifespan among isogenic C. elegans. *eLife*. **10**, e65026, doi: 10.7554/eLife.65026 (2021).
11. Churgin, M. A. et al. Longitudinal imaging of Caenorhabditis elegans in a microfabricated device reveals variation in behavioral decline during aging. *eLife*. **6**, e26652, doi: 10.7554/eLife.26652 (2017).
12. Perez, M. F., Francesconi, M., Hidalgo-Carcedo, C., Lehner, B. Maternal age generates phenotypic variation in Caenorhabditis elegans. *Nature*. **552** (7683), 106–109 (2017).
13. Baeriswyl, S. et al. Modulation of aging profiles in isogenic populations of Caenorhabditis elegans by bacteria causing different extrinsic mortality rates. *Biogerontology*. **11** (1), 53 (2009).
14. Taylor, M., Vega, N. M. Host immunity alters community ecology and stability of the microbiome in a Caenorhabditis elegans model. *mSystems*. **6** (2), 00608-20 (2021).
15. Diaz, S. A., Restif, O. Spread and transmission of bacterial pathogens in experimental populations of the nematode Caenorhabditis elegans. *Applied and Environmental Microbiology*. **80** (17), 5411–5418 (2014).
16. Twumasi-Boateng, K., Berg, M., Shapira, M. Automated separation of C. elegans variably colonized by a bacterial pathogen. *Journal of Visualized Experiments: JoVE*. (85), e51090 (2014).
17. Ortiz, A., Vega, N. M., Ratzke, C., Gore, J. Interspecies bacterial competition regulates community assembly in the C. elegans intestine. *The ISME Journal*. **15** (7), 2131–2145 (2021).
18. Berg, M. et al. TGFβ/BMP immune signaling affects abundance and function of C. elegans gut commensals. *Nature Communications*. **10** (1), 604 (2019).

19. Portal-Celhay, C., Blaser, M. J. Competition and resilience between founder and introduced bacteria in the Caenorhabditis elegans gut. *Infection and Immunity*. **80** (3), 1288–1299 (2012).
20. Scott, E., Holden-Dye, L., O'Connor, V., Wand, M. E. Intra strain variation of the effects of gram-negative ESKAPE pathogens on intestinal colonization, host viability, and host response in the model organism Caenorhabditis elegans. *Frontiers in Microbiology*. **10**, 3113 (2020).
21. Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., Ahringer, J. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. *Genome Biology*. **2** (1), research0002.1-research0002.10 (2001).
22. Dirksen, P. et al. The native microbiome of the nematode Caenorhabditis elegans: gateway to a new host-microbiome model. *BMC Biology*. **14**, 38, doi: 10.1186/s12915-016-0258-1 (2016).
23. Vega, N. M., Allison, K. R., Samuels, A. N., Klempner, M. S., Collins, J. J. Salmonella typhimurium intercepts Escherichia coli signaling to enhance antibiotic tolerance. *Proceedings of the National Academy of Sciences*. **110** (35), 14420–14425 (2013).
24. Stiernagle, T. Maintenance of C. elegans. *WormBook*. doi: 10.1895/wormbook.1.101.1 (2006).
25. Tabara, H. et al. The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell*. **99** (2), 123–132 (1999).
26. Ahringer, J. Reverse genetics. *WormBook*. doi: 10.1895/wormbook.1.47.1 (2006).
27. Rual, J.-F. et al. Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. *Genome Research*. **14** (10b), 2162–2168 (2004).
28. Revtovich, A. V. et al. Development and characterization of high-throughput Caenorhabditis elegans – Enterococcus faecium infection model. *Frontiers in Cellular and Infection Microbiology*. **11**, 667327, doi: 10.3389/fcimb.2021.667327 (2021).
29. Anderson, Q. L., Revtovich, A. V., Kirienko, N. V. A high-throughput, high-content, liquid-based C. elegans pathosystem. *JoVE (Journal of Visualized Experiments)*. (137), e58068, doi: 10.3791/58068 (2018).
30. Scholz, M., Dinner, A. R., Levine, E., Biron, D. Stochastic feeding dynamics arise from the need for information and energy. *Proceedings of the National Academy of Sciences*. **114** (35), 9261–9266 (2017).
31. Wu, T. et al. Pheromones modulate learning by regulating the balanced signals of two insulin-like peptides. *Neuron*. **104** (6), 1095-1109.e5 (2019).
32. Ching, T.-T., Hsu, A.-L. Solid plate-based dietary restriction in Caenorhabditis elegans. *Journal of Visualized Experiments: JoVE*. (51), e2701 (2011).
33. Walker, A. C., Bhargava, R., Vaziriyan-Sani, A. S., Brust, A. S., Czyz, D. M. Quantification of bacterial loads in Caenorhabditis elegans. *Bio-protocol*. **12** (2), e4291–e4291 (2022).
34. Manjarrez, J. R., Mailler, R. Stress and timing associated with Caenorhabditis elegans immobilization methods. *Heliyon*. **6** (7), e04263, doi: 10.1016/j.heliyon.2020.e04263 (2020).
35. Zhang, S., Banerjee, D., Kuhn, J. R. Isolation and culture of larval cells from C. elegans. *PLoS ONE*. **6** (4), e0019505 (2011).
36. Garsin, D. A. et al. Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. *Science (New York, N.Y.)*. **300** (5627), 1921 (2003).

37. Thutupalli, S. et al. Farming and public goods production in Caenorhabditis elegans populations. *Proceedings of the National Academy of Sciences*. **114** (9), 2289–2294 (2017).
38. Ly, K., Reid, S. J., Snell, R. G. Rapid RNA analysis of individual Caenorhabditis elegans. *MethodsX*. **2**, 59–63 (2015).
39. Johnke, J., Dirksen, P., Schulenburg, H. Community assembly of the native C. elegans microbiome is influenced by time, substrate, and individual bacterial taxa. *Environmental Microbiology*. **22** (4), 1265–1279 (2020).
40. Vega, N. M., Gore, J. Stochastic assembly produces heterogeneous communities in the Caenorhabditis elegans intestine. *PLOS Biology*. **15** (3), e2000633 (2017).
41. Gulyas, L., Powell, J. R. Cold shock induces a terminal investment reproductive response in C. elegans. *Scientific Reports*. **12** (1), 1338 (2022).
42. Jiang, W. et al. A genetic program mediates cold-warming response and promotes stress-induced phenoptosis in C. elegans. *eLife*. **7**, e35037 (2018).
43. Robinson, J. D., Powell, J. R. Long-term recovery from acute cold shock in Caenorhabditis elegans. *BMC Cell Biology*. **17** (1), 2 (2016).