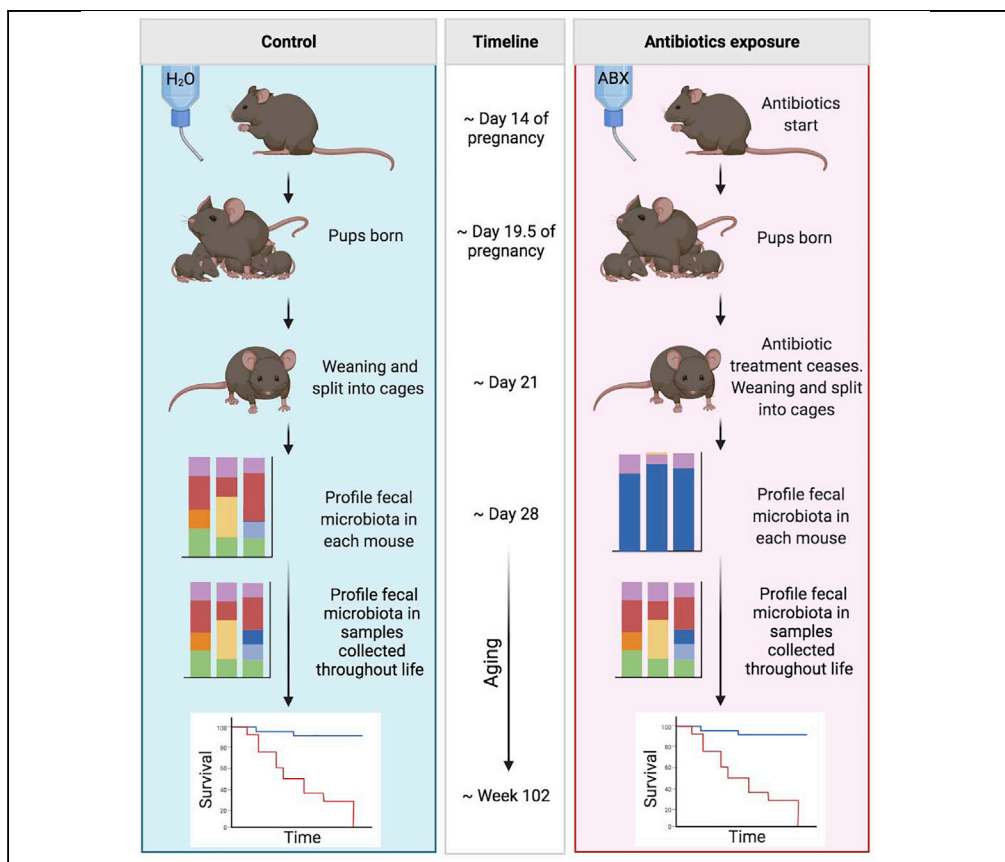


Protocol

Protocol to assess the impact of early-life antibiotic exposure on murine longevity



We present this protocol using a mouse model to assess the impact of early-life antibiotic exposure on mammalian lifespan and the composition of the gut microbiota over time. We describe longitudinal fecal sampling and health monitoring following early-life antibiotic exposure. We detail DNA extraction and 16S rRNA gene sequencing to longitudinally profile the composition of the fecal microbiota. Finally, we discuss how to address potential confounders such as the stochastic recolonization of the gut microbiota following antibiotic exposure.

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Highlights

We describe a mouse model of early-life antibiotic exposure

Bacterial load rapidly depleted following antibiotic exposure but recovers quickly

Low diversity, highly variable microbiota colonizes after antibiotic exposure

We describe criteria for health monitoring as the mice age

Lynn et al., STAR Protocols 3,
101220
March 18, 2022 © 2022 The
Author(s).
[https://doi.org/10.1016/
j.xpro.2022.101220](https://doi.org/10.1016/j.xpro.2022.101220)



Protocol

Protocol to assess the impact of early-life antibiotic exposure on murine longevity

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SUMMARY

We present this protocol using a mouse model to assess the impact of early-life antibiotic exposure on mammalian lifespan and the composition of the gut microbiota over time. We describe longitudinal fecal sampling and health monitoring following early-life antibiotic exposure. We detail DNA extraction and 16S rRNA gene sequencing to longitudinally profile the composition of the fecal microbiota. Finally, we discuss how to address potential confounders such as the stochastic recolonization of the gut microbiota following antibiotic exposure. For complete details on the use and execution of this protocol, please refer to Lynn et al. (2021).

BEFORE YOU BEGIN

The intestinal microbiota consists of a diverse ecosystem of microorganisms, predominantly bacteria, which have wide-ranging roles in supporting homeostasis, metabolism, and immunity in the gut and systemically. Disruption to the normal homeostasis of the gut microbiota, via antibiotic exposure for example, has been associated with a range of metabolic and immune-mediated diseases including metabolic disease, obesity and allergy (Cox and Blaser, 2015). Accumulating evidence suggests that increased diversity of the gut microbiota is associated with healthy aging in humans (Kong et al., 2016; Biagi et al., 2016). In our recent study (Lynn et al., 2021), we investigated the impact of early life antibiotic exposure on otherwise healthy, normal chow fed, wildtype mice, monitoring these mice for >700 days in comparison to untreated control mice. We found that differences in the composition of the gut microbiota following antibiotic exposure differentially affected host health and longevity in later life.

In this protocol, we describe our mouse model of early life antibiotic exposure and how to assess the impact of this exposure on the composition of the gut microbiota over the life of the mice. This model is not intended to be an exact preclinical model of any specific clinical situation where infants are prescribed antibiotics, however, this model most closely resembles the antibiotics that are frequently prescribed to neonates with sepsis (or suspected sepsis). The WHO recommends that “ampicillin (or penicillin; cloxacillin if staphylococcal infection is suspected) plus gentamicin” (an aminoglycoside like neomycin) for empiric treatment of neonates with suspected clinical sepsis (Fuchs et al., 2016). In our study, we chose to use a cocktail of two antibiotics, ampicillin and neomycin. These classes of antibiotic are frequently used to treat neonates with sepsis, though they would not usually be administered orally and our model uses a supra-clinical dose of neomycin. Neomycin has the added advantage in that it is poorly absorbed outside the gut and therefore oral administration targets the gut microbiota (Taylor, 2005).



Before you begin, it is necessary to consider which cocktail of antibiotics you will use to deplete the gut microbiota. Although we and others routinely use a combination of two different antibiotics to deplete the gut microbiota (Josefsdottir et al., 2017; Brandl et al., 2008; Lynn et al., 2018), cocktails of three (Candon et al., 2015; Kim et al., 2017; Brandl et al., 2008), four (Ganal et al., 2012), or five different antibiotics have been used in some studies (Deshmukh et al., 2014; O'Connor et al., 2021; Hertz et al., 2020).

Preparation of antibiotic-supplemented drinking water

⌚ Timing: 1 h

This model of early life antibiotic exposure involves administration of antibiotics in the drinking water to dams prior to the birth of their litters and during the pre-weaning period. This approach overcomes the challenges of directly administering antibiotics to murine neonates. Once the pups are old enough (around day 14 post birth) they will also be directly exposed to antibiotics as they begin to consume the drinking water.

1. Prepare 100× stocks of ampicillin and neomycin (or other desired cocktail of antibiotics) in advance.
 - a. Dissolve 5 g of ampicillin in 50 mL of sterile drinking water.
 - b. Filter sterilize solution through a 0.22 μm filter using a 50 mL syringe.
 - c. Aliquot in volumes of 3.2 mL into 5 mL tubes.
 - d. Dissolve 2.5 g of neomycin in 50 mL of sterile drinking water.
 - e. Filter sterilize solution through a 0.22 μm filter using a 50 mL syringe.
 - f. Aliquot in volumes of 3.2 mL into 5 mL tubes.

🛑 Pause point: 100× ampicillin and neomycin stocks may be stored at −20°C for up to 3 months.

Escherichia coli for 16S rRNA qRT-PCR standard curve

⌚ Timing: 3 days

16S rRNA gene real-time quantitative PCR (qRT-PCR) is used to estimate bacterial load in fecal samples to demonstrate that the gut microbiota has been depleted in antibiotic treated mice. The 16S rRNA gene primers detect the bacterial 16S rRNA gene. Bacterial DNA of a known concentration is required to generate a standard curve for this analysis. In this protocol, DNA from *E. coli* DH5α has been used to generate the standard curve.

2. Culture DH5α *E. coli* competent cells.
 - a. Prepare LB agar plates.
 - i. Prepare and autoclave the LB agar media according to manufacturer's instructions.
 - ii. Pour approximately 20 mL of LB agar into petri dishes and cool. Store at 4°C until use. (Maximum storage time is 1–2 months).
 - b. Prepare LB broth media.
 - i. Prepare and autoclave the LB broth media according to manufacturer's instructions.
 - ii. Store at 4°C until use. (Maximum storage time is 3–4 months).

Note: LB broth and agar can be purchased from most suppliers including Merck Millipore, Oxoid and Thermo Fisher Scientific.

Note: LB broth and agar should be prepared a day ahead, so that media can cool/set before use.

- c. Obtain a pure culture of *E. coli* DH5 α competent cells.
 - i. Purchase *E. coli* DH5 α competent cells from a laboratory reagent vendor such as Thermo Fisher Scientific.
 - ii. Streak out *E. coli* on an LB agar plate using a disposable inoculating loop.
 - iii. Incubate at 37°C overnight (12–18 h).
- d. Culture *E. coli* in LB broth.
 - i. Dispense 5 mL of LB broth into a 10 mL falcon tube.
 - ii. Pick a single colony from the agar plate using a disposable inoculating loop and inoculate culture in LB broth.
 - iii. Incubate for 2 h or more in an orbital shaker at 37°C.
 - iv. Read OD at 600 nm on spectrophotometer every hour until an OD reading of 1 is achieved.
- e. Calculate the colony forming units (CFU) for the *E. coli* DH5 α culture.
 - i. Prepare serial dilutions of the culture in a 96 well plate.
 - ii. Dispense 180 μ L of LB in wells into 8 well in a single column of 96 well plate.
 - iii. Add 20 μ L of the *E. coli* culture to the first well to make the first dilution and pipette up and down.
 - iv. Using a new tip and transfer 20 μ L of the first dilution to the well directly below to make the second serial dilution.
 - v. Repeat until 8 or more successive dilutions have been created.
 - vi. Plate 10 μ L of each serial dilution onto LB agar plates in triplicate and incubate overnight (12–18 h) at 37°C.
 - vii. Count colonies for each serial dilution.
 - viii. Calculate the colony forming units using the following equation:

$$\text{no. colonies} \times \text{dilution factor/volume plated in ml} = \text{cfu/ml}$$
- f. Extract DNA from *E. coli* culture using a DNeasy Powerlyzer Powersoil kit (Qiagen) in accordance with manufacturer's instructions as outlined in **Part 2: DNA extraction**.
 - i. Briefly, Spin down the *E. coli* culture at 3,500 \times g for 10 min at 4°C.
 - ii. Discard supernatant.
 - iii. Resuspend the bacterial pellet with 750 μ L of the DNeasy Powerlyzer "bead solution" using a wide bore barrier tip and add the solution to the provided glass "bead tube" containing 60 μ L of the C1 solution.
- g. Continue with the protocol as described in DNA extraction **Part 2: DNA extraction**.
 - i. Elute in 100 μ L of nuclease-free H₂O.

III Pause point: Extracted DNA may be stored at –20°C until use.

- h. Calculate the concentration of *E. coli* DNA using a Qubit™ dsDNA BR Assay kit (Thermo-Fisher).
- i. Prepare the Qubit™ dsDNA BR Assay kit standard 1 and 2 as per manufacturer's instructions: <https://www.fishersci.ca/shop/products/invitrogen-quant-it-qubit-dsdna-br-assay-kit-2/q32853>.
 - i. Prepare an adequate volume of the Qubit™ "working solution" by diluting the Qubit™ dsDNA BR reagent 1 in 200 in the provided buffer for each sample.
 - ii. Load 2 μ L of sample DNA and 198 μ L of "working solution" into a Qubit™ 0.5 mL PCR tube.
 - iii. Incubate at room temperature (RT; 20°C–25°C) for 2 min.
 - iv. Vortex and spin down.
 - v. Read using Qubit™ 3.0 Fluorometer.

Mouse model of early life antibiotic exposure

© Timing: ~4 weeks

This early life antibiotic exposure model involves the administration of antibiotics to dams prior to birth and in the pre-weaning period via the drinking water. The suitability of mice for the model should be considered before commencing these studies. Factors including the strain and age of the mice should be considered. Our study used C57BL/6J mice that were 8–12 weeks old as dams.

Note: Our mice were housed in individually ventilated cages (Techniplast) with access *ad libitum* to commercial food pellets (2018 Teklad global 18% protein, Envigo) and autoclaved water. Standardized housing conditions were maintained with 12 h day/night cycle as well as regulated temperature and humidity.

- All animal procedures need to be approved by the appropriate Institutional Animal Care and Use Committee. All experiments in this protocol were approved by the South Australia Health and Medical Research Institute Animal Ethics Committee (SAM#112).
- Consider experimental group sizes needed to achieve experimental aims. For survival experiments consider at least $n=20$ per group as a rough guide.
- Consider number and appropriateness of female mice for the study. We usually use virgin adult female mice aged 8–12 weeks for these studies.

Note: Consider using multiple cages with 5 mice per cage for each experimental group. Randomize littermates across different cages but don't mix mice from different experimental groups. Five pregnant dams are needed per experimental group (i.e., no treatment and antibiotics treated) to account for failed pregnancies and neonatal mortality which is commonly observed.

Note: We usually use virgin adult female mice as dams in these and similar experiments, however there may be advantages to using multiparous dams (e.g., larger litter size, reduced cannibalization of litters). In either case, prior co-housing of dams prior to mating is highly recommended to ensure a similar microbiota among dams in each experimental group prior to mating and treatment.

- Consider choice of male mice for studs. Practiced studs, housed individually for 1–2 weeks prior, should be utilized for timed-mating.
- Co-house female mice for use in both control (unexposed) and antibiotic exposed groups prior to mating and following plugging to limit differences in the gut microbiota in the dams prior to antibiotic exposure.
- Randomly allocate pregnant dams to the treatment groups (antibiotics or no antibiotics).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|--------------------------|---------------|
| Chemicals, peptides, and recombinant proteins | | |
| Ampicillin sodium salt | Merck | Cat#A0166 |
| Neomycin trisulfate salt hydrate | Merck | Cat#N1876 |
| LB broth base | Invitrogen | Cat#12780-052 |
| Agar bacteriological | Oxoid | Cat#LP0011 |
| 1× PBS | Merck | Cat#D8537 |
| Bacterial and virus strains | | |
| DH5 α competent cells | Thermo Fisher Scientific | Cat#18265017 |
| Biological samples | | |
| Mouse fecal samples | (Lynn et al., 2021) | N/A |

(Continued on next page)

| <i>Continued</i> | | |
|---|----------------------------------|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| <i>Critical commercial assays</i> | | |
| Qubit™ ds DNA BR kit | Invitrogen | Cat#Q32850 |
| SYBR™ Green PCR Master Mix | ABI via Thermo Fisher Scientific | Cat# 4309155 |
| DNeasy Powerlyzer Powersoil kit | QIAGEN | Cat#12855-50 |
| <i>Deposited data</i> | | |
| 16S rRNA gene sequence data | SRA Bioproject | PRJNA645716 |
| <i>Experimental models: Organisms/strains</i> | | |
| C57BL/6J | The Jackson Laboratory | Cat#000664 |
| <i>Oligonucleotides</i> | | |
| 16S f 5«-TCCTACGGGAGGCAGCAGT-3« | (Nadkarni et al., 2002) | N/A |
| 16S r 5«GGACTACCAGGTATCTAATCCTGTT-3« | (Nadkarni et al., 2002) | N/A |
| 515F 5'TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGGTGCCAGCMGCCGCGTAA-3' | Illumina | N/A |
| 806R 5'GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTWTCTAAT-3' | Illumina | N/A |
| <i>Software and algorithms</i> | | |
| QuantStudio 7 Real-Time PCR System Software | Applied BioSystem | version 1.7.1 |
| GraphPad Prism 7.01 | GraphPad Software Inc | version 7.01 https://www.graphpad.com/scientific-software/prism/ |
| QIIME2 | (Bolyen et al., 2019) | version 2019.10 https://qiime2.org/ |
| DADA2 | (Callahan et al., 2016) | version 1.8 https://benjjneb.github.io/dada2/ |
| FastTree | (Price et al., 2010) | version 2 http://www.microbesonline.org/fasttree/ |
| GreenGenes | (DeSantis et al., 2006) | version 13.8 https://greengenes.secondgenome.com/ |
| PICRUSt2 | (Douglas et al., 2020) | version 2.3 https://huttenhower.sph.harvard.edu/picrust |
| PhyloSeq | (McMurdie and Holmes, 2013) | version 1.3 https://joey711.github.io/phyloseq/ |
| R | R Core Team | version 3.6.3 https://www.r-project.org/ |
| ggplot2 | (Wickham, 2016) | version 3.3 https://ggplot2.tidyverse.org/ |
| R code for analysis and plots | (Lynn et al., 2021) | https://bitbucket.org/lynnlab/longevity |
| <i>Other</i> | | |
| QuantStudio 7 Real-Time PCR | Applied BioSystem | Cat# 4485701 |
| Precellys® 24 Tissue Homogenizer | Bertin Instruments | Cat#P000669-PR240-A |
| Qubit Fluorometer | Invitrogen | Version 3.0 |
| Millex-GP Syringe Filter Unit, 0.22 µm | Merck | Cat# SLGP033RS |
| MiSeq system | Illumina | Cat# SY-410-1003 |
| Nextera XT Index Kit v2 | Illumina | Cat# FC-131-1001 |

MATERIALS AND EQUIPMENT

Consumables including barrier tips, microcentrifuge tubes, 96-well plates, etc. are not included in the [key resources table](#) as these items should be acquired from local laboratory suppliers. Basic laboratory items are also needed including a conventional benchtop microcentrifuge, a vortex, heating block, homogenizer, a bacterial culture incubator, an orbital shaker, an autoclave, and glassware for media preparations.

Alternatives: This protocol describes the steps for the manual processing of fecal samples using the DNeasy Powerlyzer Powersoil kit (Qiagen). This kit is also compatible with the QIAcube instrument and may be adapted in a semi-automated fashion for this system.

Alternatives: This protocol uses the Precellys 24 homogenizer to homogenize the fecal samples. Alternatives include Fisherbrand™ Bead Mill 24 Homogenizer (Fisher Scientific) and the Bullet Blender Storm Pro™ (Next Advance).

Alternatives: For qRT-PCR, this protocol uses the SYBR™ Green PCR Master Mix and the QuantStudio 7 Real Time PCR system. The SYBR™ Green PCR Master Mix is also compatible with other PCR detection systems including the LightCycler® 480 (Roche) and other QuantStudio systems including QuantStudio 1, 3, 5, and 6 Pro.

STEP-BY-STEP METHOD DETAILS

Part 1: Generation of mice exposed to antibiotics in early life

⌚ Timing: ~6 weeks

This mouse model of early life antibiotic exposure involves administration of two antibiotics, ampicillin and neomycin, via the drinking water to dams in late pregnancy and during the pre-weaning period. A comparable group of control mice not exposed to antibiotics should also be maintained.

1. Set up timed-mating.
 - a. Co-house females (aged 8–12 weeks), 5 females to a cage for 2 weeks in advance of mating.
 - b. Place dirty bedding from studs' cages into the female cages ~3 days in advance of mating to induce a synchronized estrous cycle. We suggest adding 75–100 mL of urine soaked bedding into each of the female cages.
 - c. Weigh each mouse individually prior to timed mating.
 - d. Place 1–2 females in each studs' cage.
 - e. Check females for vaginal plugs every morning for the duration of the timed mating. Remove plugged females from the stud cages upon observation of plug, randomly co-house 5 plugged females to a cage.
 - f. Record date of plugging for each female.

Note: The “plug” is comprised of coagulated secretions from the male. It tends to fill the female's vagina and may persist for 8–24 h after breeding. Lift the base of the female's tail and inspect for a whitish mass in her vaginal opening.

Note: We usually add the dirty bedding before the weekend and place the females into the studs' cages on a Tuesday afternoon and check for vaginal plugs every morning for 3 consecutive days; Wednesday, Thursday and Friday morning. We find the majority of C57BL/6J females are plugged on day 1 or day 3 of timed-mating, with only a small portion plugging on day 2 of the timed-mating.

Note: Any unplugged females should be separated from the males after 3 days of attempted mating and placed into cages of 5 females/cage. Mice should be rested prior to reuse for 4–6 weeks. It is possible that a small proportion of these females may become pregnant, though they may be unsuitable for use as the date of plugging will not be known.

2. Co-house plugged dams 5 mice to a cage until day 14 of pregnancy (or 14 days post-plugging).
3. Collect fecal samples.
 - a. Collect fecal samples from dams in both groups at day 14 of pregnancy (prior to antibiotic exposure) and at day 2 and day 21 post birth of pups.
 - b. To do this, place each pregnant dam individually onto a clean cage base where she will spontaneously defecate.

- c. Pick up the fecal sample using a sterile toothpick/tip and place into a pre-labeled 2 mL sterile microcentrifuge tube. Place collected sample directly onto dry ice.
- d. Replace pregnant dam into cage. Clean the cage base and gloved hands with an appropriate reagent (i.e., 70% ethanol/F10) to sterilize.

Note: A light scruff will encourage any mouse not spontaneously defecating to do so.

Note: Two or more fecal samples should be collected for each individual mouse.

△ **CRITICAL:** Fecal samples must be placed on dry ice immediately to preserve the integrity of the sample.

4. Re-weigh plugged dams at day 14 of pregnancy.
 - a. Assess pregnancy based on increase of 3–5 g of weight and whether mice are visibly pregnant.

Note: The authors have only carried out this protocol using virgin females, 8–12 weeks of age. Within this window, the authors have found that pregnant dams put on 3–5 g of weight by day 14 of pregnancy, independent of starting weight.

Note: A visibly pregnant dam will develop a “pear-like” appearance with a bulge in their center and enlarged nipples. The size of the bulge will be dependent on the number of fetuses that are being carried.

- b. Re-house pregnant females, 1 dam per cage, at day 14 of pregnancy based on the plugging date recorded for each dam.
5. Randomly assign pregnant dams to antibiotic exposure or control groups.
 - a. Prepare a 1× dilution of ampicillin and neomycin from the pre-made stock solutions.
 - i. Thaw out an adequate number of aliquots of the 100× frozen stock solutions **before you begin: step 1** (3 mL of 100× ampicillin and 3 mL of 100× neomycin are needed for each treated cage).
 - ii. Prepare a 1× stock in a sterile water bottle by mixing 3 mL of 100× ampicillin stock and 3 mL of 100× neomycin stock in 300 mL of sterile drinking water (Figure 1) (enough for a single cage for 3 days). Replace lid and shake to mix.
 - iii. Place laminated “Antibiotic treatment” label on water bottle.
 - iv. Place antibiotic treated water into position on mouse’s cage.
 - v. Untreated mice receive untreated water.

△ **CRITICAL:** Use sterilized drinking water for stock and diluent.

Note: Drinking water supplemented with certain antibiotics may taste bitter to mice, and they may be reluctant to drink it, although this was not the case with the antibiotics used in this protocol. If mice are reluctant to drink antibiotic supplemented water, we recommend supplementing the water with sucrose. Importantly, if this is done, sucrose should also be added to the drinking water of the control mice to avoid this being a confounder.

Note: Monitor drinking water levels to ensure that both antibiotic-treated and untreated mice are drinking adequate volumes of water. Consider marking water level on outside of drinking bottle or weighing water bottles on a daily basis.

Note: Antibiotic supplemented drinking water bottles may be tinted, laminated or wrapped in aluminum foil or equivalent to prevent light damage if using light sensitive antibiotics.

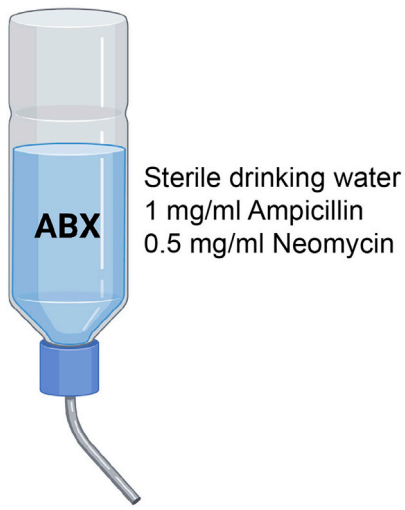


Figure 1. Schematic of sterile antibiotic drinking preparation

Note: Replace antibiotic supplemented drinking water every 3 days for the duration of exposure.

Alternatives: Even in the presence of a sweetener mice may be reluctant to drink water supplemented with certain antibiotics. If this is the case a possible alternative strategy would be to administer antibiotics via oral gavage on a daily basis to achieve similar results (Reikvam et al., 2011). However, daily oral gavage may cause unnecessary stress to pregnant and lactating dams.

6. Record date of birth for pups.
 - a. Record date of birth and number of pups born to each dam.

Note: C57BL/6J pups are born to dams approximately 19.5 days post plugging. Time from plugging to birth may vary from mouse strain to strain.

Note: It is critical to disturb the mice as little as possible during this time (birth to day 7). We expect some loss of pups in the time from birth to weaning, however, limiting any disruption during this time will help to reduce any loss of pups.

7. Wean pups at day 21 post birth.

Note: Cease antibiotic treatment at day 21 +/- 1 day post birth and replace antibiotic-supplemented drinking water with sterile drinking water. Wean all pups at this timepoint also.

Note: Depending on your ethics approval, unexposed dams may be reused for other purposes.

Note: It is important to wean antibiotic exposed pups separately from unexposed pups, as exposed pups have a depleted microbiome. It is recommended to wean the antibiotic treated pups first and wean the unexposed pups secondly.

- a. Humanely kill dams (as antibiotic exposed dams cannot usually be re-used for breeding or other experiments).
- b. Wean antibiotic exposed and unexposed pups separately.
- c. Separate pups in each litter into males and females.

- d. Ear notch pups for identification.
- e. Place one pup from each litter in a new cage with 4 other mice, each from different litters (same sex and within the same treatment group).
- f. Collect a fecal sample (consisting of 2–3 pellets) at this timepoint as described [Collect fecal samples: step 3](#).

△ **CRITICAL:** One littermate from each dam should be randomly assigned to each cage. Antibiotic exposed and control pups need to be caged separately. Co-housing would likely result in a more rapid recovery in the microbiota of antibiotic exposed mice due to coprophagy.

Note: We only used male pups for this experiment, as metabolic differences such as weight gain, hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hyperleptinemia are often easier to observe in male mice ([Hwang et al., 2010](#)). However, both sexes may be used.

Note: Although some fecal samples can be collected from mice from day 15 post birth, its availability is unreliable. Mice must be humanely culled to collect cecal contents prior to this timepoint.

Longitudinal fecal sampling

⌚ **Timing:** 1 h for duration of experiment

Collect fecal samples longitudinally throughout the experiment to assess changes to the composition of the gut microbiota in antibiotic exposed mice compared to control mice and recovery following antibiotic exposure. In our study, we collected fecal samples at 3, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, and 102 weeks post birth and at the same timepoints from control mice.

8. Collect fecal samples from antibiotic exposed and control mice throughout the duration of the experiment.
 - a. Collect fecal pellets using the methods described in [Collect fecal samples: step 3](#).
 - b. Collect 1–2 fecal samples from each individual mouse 7 days following cessation of antibiotic exposure, at day 28 of life.
 - c. Collect fecal samples every 7 days for the first 4 weeks following cessation of antibiotics, and at least every 3 months subsequently for the duration of the experiment.
 - d. Monitor body condition and record weight monthly for each individual mouse for the duration of the project weekly as per ethics requirements.

Note: By 7 days following the cessation of antibiotic exposure we consistently observe that the gut microbiota is recolonized by a low diversity microbiota, typically dominated by blooms of taxa that are present at a low relative abundance in untreated mice such as the *Enterobacteriaceae*, *Enterococcus*, *Lachnospiraceae* and/or *Akkermansiaceae*.

△ **CRITICAL:** While there are some common features, recolonization of the gut microbiota following antibiotic exposures appears to be somewhat stochastic in nature. It is therefore essential not to assume that the composition of the microbiota following antibiotic exposure will be the same/similar between different experiments, cages or even individual mice. To assess this, it is essential to collect fecal samples individually from all mice in each independent experiment. One should also note that the time required for the gut microbiota to recover to a state similar to unexposed mice is also likely to be variable between different individuals/cages/experiments.

Longitudinal health monitoring

⌚ Timing: 1–2 h weekly for duration of experiment

To assess whether antibiotic exposure in early life is associated with impaired healthspan or lifespan, mice in both the antibiotic exposed and unexposed groups should be monitored for at least 100 weeks. This is considered old age as conventionally housed SPF mice typically live for up to 2 years (Flurkey et al., 2007). As the mice age, weekly in-depth health monitoring is recommended. Body weight and body condition monitoring should be increased from monthly monitoring (from 4 weeks to 20 weeks) to weekly monitoring (from >20 weeks of age) as per ethics requirements. Humane endpoints should be determined in advance of the experiment starting. Consider the expected decline in aged mice when defining humane endpoints. Deaths should be recorded as soon as they are observed, and necropsies should be carried out wherever possible.

Note: In many cases mice will reach humane endpoints rather than dying, but in our experience, mice will also be found dead in cages without prior overt evidence of a decline in health.

9. Conduct health monitoring on aged mice on a weekly basis from 20 weeks of age.
 - a. Weigh each mouse individually on a weekly basis and record weights.
 - i. Monitor for 15% weight loss within a week.
 - ii. Monitor for long-term weight loss of 15% below the 50th percentile as defined by the growth weight curve for the colony at 12 weeks of age, accounting for sex.
 - iii. If either of these weight loss criteria are met, the mouse should be humanely culled.
 - iv. Record the date and reason for humane culling in an appropriate database.

Note: The rationale for using body weight at 12 weeks of age as a benchmark for a humane endpoint is that it is assumed that mice have reached an adult lean body mass by this time.

- b. Score body condition for each mouse on a weekly basis based on criteria outlined by Ullman and Foltz (Ullman-Cullere and Foltz, 1999).
 - i. Monitor for hunching.
 - ii. Monitor for coat condition.
 - iii. Monitor for movement upon stimulation.
 - iv. Monitor gait.
 - v. Monitor for orbital tightening.
 - vi. Score each mouse weekly from 0 to 5 based on these criteria.
- c. Consult the Animal Welfare Officer or equivalent if a body score of 3 out of 5 is reached and consider humane culling to avoid any unnecessary suffering.
- d. Humanely cull any mouse with a body condition score of 4 out of 5.
- e. Request or preform a necropsy to investigate possible reasons for decline in body condition.
- f. If a mouse is found dead, notify the Animal Welfare Officer or equivalent immediately and request/perform a necropsy.
- g. Record date of death and any details available including possible cause of death, and appearance of age related conditions, for example seminal vesicles appearance aged male mice etc.

Note: As the mice age, it may be necessary to terminate the experiment to avoid any unnecessary suffering. In the case of our experiment, we ended the experiment at 102 weeks, when one of the experimental groups had reached 40% survival, to ensure that were enough mice left at the designated termination date for organ collection.

Part 2: DNA extraction

⌚ Timing: 4–6 h

DNA is extracted from individual fecal samples using the DNeasy Powerlyzer Powersoil kit (Qiagen) to assess bacterial load via a 16S rRNA gene qRT-PCR assay and to assess the composition of the fecal microbiota by 16S rRNA gene sequencing.

10. Extract DNA from fecal samples as per manufacturer's instructions

<https://www.qiagen.com/us/resources/resourcedetail?id=329362e4-03e6-4ae1-9e4e-bbce41abe4b7&lang=en>

Note: A reagent control consisting of 1 × PBS instead of a fecal sample should be used with every extraction to ensure that reagents are not contaminated.

Note: Recommend processing up to 24 samples at once (including a reagent control) for this protocol.

▲ **CRITICAL:** Weight of the starting fecal sample is necessary for subsequent analysis.

- a. Weigh fecal sample.
 - i. Label and weigh microcentrifuge tube to be used for each sample DNA extraction.
 - ii. Place 1–2 fecal pellets into the pre-weighed tube.
 - iii. Re-weigh the microcentrifuge tube containing the fecal sample(s).
 - iv. Record the weight of fecal sample minus the weight of the tube.
- b. Resuspend fecal samples in 1 × Phosphate Buffered Saline (PBS) solution.
 - i. Add 1 mL of 1 × PBS to fecal samples collected from an adult mouse and 500 µL to fecal samples collected from a 3–4 week old mouse. Vortex vigorously.
 - ii. Spin solution at 13,000 × g for 5 min at RT and remove supernatant.

▮▮ **Pause point:** The fecal pellet may be safely stored at –80°C at this step.

- c. Continue with extraction procedure as directed by the manufacturer.
 - i. Label a glass bead tube (provided by the manufacturer) and 1.5 mL microcentrifuge tubes (not provided) for each step in the protocol; C2, C3, C5 and a spin column (provided by the manufacturer) for each sample to be processed.
 - ii. Label a 2 mL microcentrifuge tube for the C4 step for each sample.
 - iii. Add 60 µL of C1 solution to the glass bead tube.
- d. Resuspend fecal pellet in 750 µL of the “bead solution” and add it to the glass bead tube using a wide bore barrier tip.
 - i. Briefly vortex the glass bead tubes and incubate at 65°C for 10 min on a heating block.
 - ii. Transfer glass bead tubes containing heated solution to a 24 Tissue Homogenizer (Precellys) and pulse at 6.5 setting for 2 cycles of 60 s.
 - iii. Spin down the homogenized sample at 10,000 × g at RT for 3 min.
- e. Add 250 µL of solution C2 to a new microcentrifuge tube (labeled C2) and up to 500 µL of the homogenized supernatant.
 - i. Incubate the C2 tube on ice for 10 min.
 - ii. Spin down at 10,000 × g for 3 min.
- f. Add 200 µL C3 solution to the tube labeled C3, and up to 600 µL of C2 supernatant.
 - i. Vortex briefly.
 - ii. Incubate on ice for 10 min.
 - iii. Spin at 10,000 × g for 1 min at RT.
- g. Transfer 1,200 µL of C4 solution to the 2 mL C4 labeled tube.
 - i. Add up to 750 µL of C3 supernatant.
 - ii. Vortex contents briefly.
 - iii. Load 650 µL of C4 solution to the provided spin column.

- iv. Spin at 10,000×g for 1 min, discard flow-through and replace collection tube (provided by manufacturer).
- v. Load remaining C4 solution to the provided spin column.
- vi. Spin at 10,000×g for 1 min, discard flow-through. Replace collection tube.
- vii. Add 500 μL of C5 solution to spin column, spin at 10,000×g for 1 min, discard flow-through. Spin again at 10,000×g for 3 min.
- viii. Prepare a new collection tube (not provided by manufacturer); remove lid from a 2 mL microcentrifuge tube, and carefully place the spin column inside.
- ix. Add DNA with 50 μL of C6 or nuclease-free H₂O to spin column filter and incubate for 1 min. Spin at 10,000×g for 1 min.
- x. Repeat elution with 50 μL C6 or nuclease-free H₂O, incubate for 1 min and spin again.
- xi. Collect eluant in a new microcentrifuge tube (not provided).

▮▮▮ **Pause point:** Eluted DNA may be stored at -80 °C.

11. Quantify DNA concentration in each sample using a Qubit™ dsDNA BR Assay kit (ThermoFisher). https://www.thermofisher.com/documentconnect/documentconnect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFAssets%2FSLG%2Fmanuals%2FQubit_dsDNA_BR_Assay_UG.pdf

Note: The Qubit™ dsDNA BR Assay kit may be swapped for the high-sensitivity Qubit™ dsDNA HS Assay kit if bacterial DNA concentration is very low.

- a. Prepare standard 1 and 2 as per manufacturer's instructions.
- b. Prepare an adequate volume of working solution by diluting the Qubit™ stock solution 1 in 200 in provided buffer for each sample.
- c. Load 2 μL of sample DNA and 198 μL of working solution into a Qubit™ 0.5 mL PCR tube.
- d. Vortex briefly
- e. Read using a Qubit™ 3.0 Fluorometer.

▮▮ **Pause point:** DNA can be stored at -80°C.

Note: In our experience, samples collected from antibiotic treated dams at day 2 post birth and pups at day 21 post birth have a fecal DNA concentration <3 ng/μl. However, these values will depend on the antibiotics used.

Part 3: 16S rRNA gene qRT-PCR

⌚ **Timing:** 3–4 h

12. Prepare 16S rRNA gene qRT-PCR

To assess whether the antibiotic treatment successfully depleted the gut microbiota, a qRT-PCR assay assessing the number of 16S rRNA gene copies in extracted fecal samples is recommended. All reactions should be carried out in duplicate.

Note: Master mix, primers and nuclease-free H₂O are combined and added in a single solution to plate.

⚠ **CRITICAL:** Use separate barrier tips to ensure no mixing of DNA.

- a. Calculate the amount of master mix components needed by multiplying the volumes in [Table 1](#) by the total number of reactions to be performed including samples, controls, and standards in duplicate, and a few extra for pipetting errors, etc.

- b. Prepare the master mix solution consisting of SYBR green PCR master mix and the forward and reverse primer, in a sterile 1.5 mL microcentrifuge tube and keep on ice (in a template-free room).
- c. Dilute the template DNA 1 in 100 in sterile nuclease-free H₂O for untreated mice, 1 in 10 for antibiotic-treated mice.
- d. Add 3 μ L diluted DNA onto a PCR plate (96/384 depending on setup) to the bottom of each well (equivalent to 1–3 ng of DNA/reaction).
- e. Add 7 μ L master mix solution to each well.

Note: Use lab texter to inscribe sections onto plate to assist with plating out. Load DNA first. Add master mix second.

- f. Prepare a no template DNA control (NTC), where DNA is substituted for 3 μ L nuclease-free H₂O and include in every analysis.
- g. Prepare a negative reagent control (NRC), where 1 \times PBS is used instead of fecal samples during DNA extraction.
- h. Prepare a standard curve using DNA extracted from DH5 α competent cells [Culture DH5 \$\alpha\$ Escherichia coli: step 2](#).
 - i. Prepare 6 successive serial dilutions 1 in 10 with nuclease-free H₂O using DNA extracted from DH5 α competent cells
 - ii. Load 3 μ L of standards in place of template DNA.

Note: The starting concentration of our *E. coli* stock is usually 2.8 ng/ μ l following the protocol as outlined, [before you begin: step 2](#).

- i. Briefly spin the PCR plate at 200 \times g for 10 s to remove any air bubbles and ensure liquid is at the bottom of the well.
- j. Load plate using the settings outlined in [Table 2](#).

Note: Calculations for obtaining copy number per mg of feces are detailed in Part 5: 16S rRNA gene standard curve calculations.

Part 4: 16S rRNA gene library preparation and sequencing

⌚ Timing: 1–2 weeks

Use 16S rRNA gene sequencing to longitudinally profile the composition of the fecal microbiota following early life antibiotic exposure. Extracted fecal DNA is used to generate amplicons of the V4 hypervariable region of the 16S rRNA gene using the 515F and 806R primers (Illumina). For an in-depth protocol for 16S rRNA gene sequencing library preparation, please refer to the Nextera XT Index kit.

13. Generate 16S rRNA gene sequencing reads
 - a. Multiplex samples using a dual-index approach with the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions (https://sapac.support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf).
 - b. Generate, clean, index and sequence amplicons according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html).
 - c. Sequence amplicon libraries on the Illumina MiSeq system using 2 \times 300 bp runs.

Table 1. Preparation of the 16S rRNA gene qRT-PCR to assess bacterial load

| Component | Volume |
|---|-----------------------------|
| SYBR™ Green PCR Master Mix | 5 μ L |
| 2 μ M of 16S rRNA gene forward primer | 1 μ L |
| 2 μ M of 16S rRNA gene reverse primer | 1 μ L |
| DNA template | 3 μ L |
| Total | 10 μL |

Note: Library generation and sequencing is routinely offered by genomics core facilities. We recommend the use of these facilities for library preparation and sequencing.

Note: Sequencing analyses are detailed in [Part 6: 16S rRNA gene sequence data normalization and taxonomic assignment](#).

Survival analysis

14. Generate a Kaplan Meier curve to assess differences in survival between control and antibiotic exposed mice.
 - a. Use the Gehan-Breslow-Wilcoxon or log-rank tests to assess whether differences between experimental groups are statistically significant.

Note: We used the Gehan-Breslow-Wilcoxon test in our study as this test gives more weight to deaths at early time points, which is important since all mice are expected to begin to reach end-of-life towards the end of the 102 week experiment.

Part 5: 16S rRNA gene standard curve calculations

The *E. coli* DNA standard curve is used to estimate the number of copies of the 16S rRNA gene in each sample.

15. Calculate bacterial load using the *E. coli* DH5 α DNA standard curve.
 - a. Input the starting concentration of the standard curve into the qRT-PCR software program. Insert number of dilutions and dilution factor into the software to enable the software to generate the standard curve.

Note: The qRT-PCR programs, (including the Applied Biosystems software), uses the standard curve (mean C_q – y-intercept) / slope) to generate a corresponding quantity for each sample.

Note: We use *E. coli* DNA at 2.8 ng/ μ l as our highest standard and create successive 1 in 10 serial dilutions to create our standard curve.

△ CRITICAL: The cfu/ml of the standard curve is known (before you begin: step 2). *E. coli* has 7 16S copies per genome (Nadkarni et al., 2002), thus cfu/ml multiplied by 7 is equal to the number of copies/ml.

- b. Account for any dilution that has been made to your sample.
- c. Multiply the cfu/mL by 7 to calculate the average of 16S rRNA gene copies for the *E. coli* standard curve.
- d. Multiply copies/mL by 100 to get total copies for the DNA extraction, as the fecal DNA was eluted in 100 μ L.
- e. Divide the copies/extraction by the weight of the fecal samples (in mg) to give an approximate bacterial load per mg of feces.

Table 2. The PCR cycling conditions for each 16S rRNA gene qRT-PCR assay

| PCR cycling conditions | | | |
|------------------------|-------------|---------|--------------|
| Steps | Temperature | Time | Cycles |
| Initial denaturation | 95°C | 10 min | 1 |
| Denaturation | 95°C | 15 s | 35–40 cycles |
| Annealing & extension | 60°C | 60 s | |
| Final extension | 72°C | 10 min | |
| Hold | 4°C | forever | 1 |

Note: This calculation differs for different bacterial species. If the colonizing species is known, a more accurate number for the 16S rRNA copies may be generated using this method. However, in the case of a mix of different bacterial species, this method works well to detect 10-fold increases/decreases in bacterial load between groups.

Note: We find that estimation of bacterial loads by qRT-PCR is possible even with as little as 10 mg of feces is extracted. However, the dilution used for the qRT-PCR will need to be adjusted as low concentrations of DNA may lead to overestimation/underestimation of bacterial load, (Nadkarni et al., 2002) for more information.

Part 6: 16S rRNA gene sequence data normalization and taxonomic assignment

In brief, the analysis pipeline for the 16S rRNA amplicon data consists of quality control assessments of the raw reads, generating exact sequence variants (ESVs), assigning taxonomy, and analysis of alpha and beta-diversity to characterize the composition of the gut microbiota.

16. Normalize and assign taxonomy to generated 16S rRNA gene sequence data.
 - a. Demultiplex paired end 16S rRNA gene sequences and import into QIIME2 for processing (Bolyen et al., 2019).
 - b. Correct sequence errors and generate counts of error-corrected reads per sample using DADA2 (Callahan et al., 2016).
 - c. Construct a phylogenetic tree of error-corrected sequences using FastTree (Price et al., 2010).
 - d. Assign taxonomy to sequences with the sklearn plugin for QIIME2 with an 80% confidence threshold, using a recent version of the GreenGenes database (DeSantis et al., 2006).
 - e. Predict relative abundance using PICRUSt2 (Douglas et al., 2020).
 - f. Generate alpha and beta diversity statistics using the R package PhyloSeq version 1.3 (McMurdie and Holmes, 2013).
 - g. Perform further statistical analysis using R version 3.6.3, graphing using ggplot2 v3.3.0 and normalize all count data for library size prior to visualization or statistical testing.

Note: As an example of the statistical analysis, please refer to the original article (Lynn et al., 2021) and (Lynn et al., 2018). The 16S rRNA gene sequence data has been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA645716. All original code has been deposited at the Lynn Laboratory Bitbucket repository (<https://bitbucket.org/lynnlab/longevity>).

EXPECTED OUTCOMES

Antibiotic treatment (ABX) results in significantly reduced bacterial load in antibiotic exposed dams

For dams treated with the ampicillin and neomycin cocktail of antibiotics described in this protocol, it is expected that the number of 16S rRNA gene copies in fecal DNA samples collected during antibiotic exposure will be reduced from $\sim 10^8$ copies to $\sim 10^4$ copies (i.e., a 4 log fold reduction in bacterial load) (Figure 2). It is expected that the 16S rRNA gene copy number will be reduced at least 3–4 log

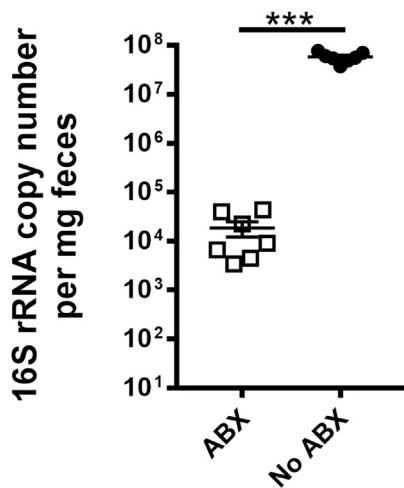


Figure 2. Antibiotic treatment (ABX) results in significantly reduced bacterial load in antibiotics exposed dams

Bacterial load as determined by copies of the 16S rRNA genes in fecal samples collected from antibiotic treated (ABX) and untreated dams (NO ABX) at day 21 post-birth. Data are represented as mean \pm SEM. A Mann Whitney test was used to assess statistical significance, where *** $p < 0.001$.

fold at the day 2 post birth and remain low until day 21 post birth of the pups. Significantly higher bacterial load levels in antibiotic exposed mice may indicate colonization by antibiotic resistant bacteria.

Significantly reduced bacterial load rapidly recovers after antibiotic exposure

Fecal samples are also collected from the pups upon weaning. Fecal samples are extracted and 16S rRNA gene copy number is assessed. At this timepoint, a significantly reduced copy number is also expected in the antibiotic exposed pups ($\sim 10^3$ – $\sim 10^4$ copies per mg of feces) compared to controls ($\sim 10^8$ – $\sim 10^9$ copies per mg of feces). Following cessation of antibiotic treatment and rehousing of the pups, bacterial load is expected to recover rapidly. By 7 days post the cessation of antibiotics 16S rRNA gene copy number is expected to increase to approximately one log fold lower than untreated mice (Figure 3).

Following antibiotic exposure, it is expected that the gut will be rapidly recolonized. We have observed that a low diversity microbiota typically recolonizes within a week following the cessation of antibiotic exposure. 16S rRNA gene sequencing reveals that this recolonization is frequently highly variable between different experiments, cages and within-cage differences are sometimes even evident. For instance, in our original study (Lynn et al., 2021) two different low-diversity microbiota community types, which we referred to as post-antibiotic microbiota I (PAM I) and PAM II, were evident following the cessation of antibiotic exposure (Figure 4). The composition of the fecal microbiota post antibiotic exposure is typically dominated by a small number of bacterial families including the *Enterobacteriaceae*, *Enterococcus*, *Lachnospiraceae* and/or *Akkermansiaceae*, which are usually at a low relative abundance in untreated control mice. This variation in the composition of the microbiota following antibiotic exposure may explain difficulties in reproducing the effects of antibiotic exposure on different phenotypes across studies. It is imperative that future studies carefully assess differences in the composition of the microbiota following antibiotic exposure by longitudinally profiling the composition in individual mice and cages in each independent experiment.

Following antibiotic exposure, the composition of the fecal microbiota will slowly recover over many weeks. For example, in our study, differences in the composition of the fecal microbiota between antibiotic exposed and control mice started to resolve around 48 weeks (Figure 4). Co-housing antibiotic exposed mice with control mice at weaning would likely accelerate this recovery in the gut microbiota due to coprophagy.

LIMITATIONS

The choice of antibiotics will determine the types of bacteria that are depleted. In the (Lynn et al., 2021) study, antibiotic exposure using a cocktail of ampicillin and neomycin resulted in a four

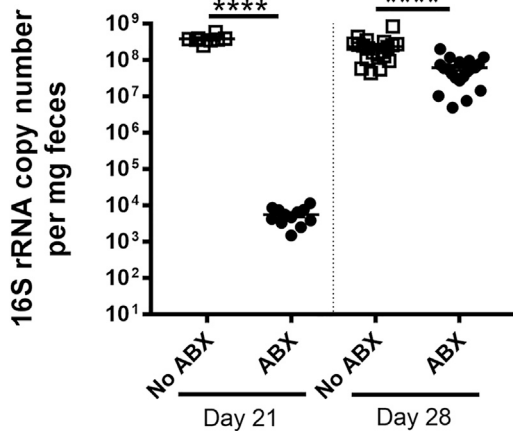


Figure 3. Antibiotic treatment (ABX) results in significantly reduced bacterial in antibiotics pups

Bacterial load as determined by copies of the 16S rRNA genes in fecal samples collected from antibiotic treated (ABX) and untreated dams (NO ABX) at day 21 post-birth. Data are represented as mean \pm SEM. A Mann Whitney test utilized to assess statistical significance, where **** $p < 0.0001$.

log-fold reduction in 16S rRNA gene copies detectable in the antibiotic exposed mice. Use of more antibiotics in the cocktail may reduce the residual bacterial load even further. Similarly, exposing mice to a single antibiotic or a lower dose of antibiotics may result in higher bacterial load during antibiotic exposure.

Our antibiotic exposure model is a long antibiotics exposure model (approximately 3.5 weeks), starting at day 14 of pregnancy and ending at weaning (day 21 of life). A shorter antibiotic exposure period, as described in (Lynn et al., 2018) has also been shown to induce similar dysbiosis in early life though its long-term impact on longevity has not been investigated to date. Moreover, the antibiotics exposure in this mouse model begins at day 14 of pregnancy, and thus there may be some *in utero* exposure, which may influence responses in the offspring.

Following the cessation of antibiotics there is a stochastic recolonization of the gut microbiota. This stochastic recolonization is also evident in antibiotic treated humanized germ free mice (Lavelle et al., 2019) and may vary over time and between facilities. We find that following antibiotic exposure the gut microbiota is dominated by a small number of dominant bacterial families (Lynn et al., 2018, 2021) and that the composition of the microbiota that recolonizes may vary from cage to cage and even from mouse to mouse within a single cage. In our study, even randomizing antibiotic-treated littermates across 4 cages resulted in two very distinct post-antibiotics microbiota (PAM) community types (Lynn et al., 2021). Is it difficult to predict or guide the stochastic nature of the post-antibiotic recolonization (Shaw et al., 2019). Co-housing with control mice would likely accelerate recovery in the composition of the gut microbiota but this may make it more difficult to assess effects of dysbiosis on phenotypes of interest.

Fecal sample collection is a non-invasive procedure that acts as a proxy for the composition of the microbiota in the gut and can be collected longitudinally. However, the microbiota may vary in different parts of the gastro-intestinal tract (Donaldson et al., 2016). Similarly, the fecal microbiota does not necessarily reflect the mucosal adherent microbiota, which can be significantly distinct from the fecal microbiota, especially in disease status such as cirrhosis (Shen et al., 2021). An alternative would be to collect ceca or intestinal contents, but this would require humane killing at each sampling timepoint, significantly increasing the number of mice needed.

A pure bacterial culture, such as the *E. coli* standard used in our 16S rRNA gene qRT-PCR assay, is commonly used to estimate bacterial load (Nadkarni et al., 2002). However, this method does have limitations as 16S rRNA gene copy numbers are variable between different bacterial families. Nonetheless the level of accuracy required to measure the difference in bacterial load between an

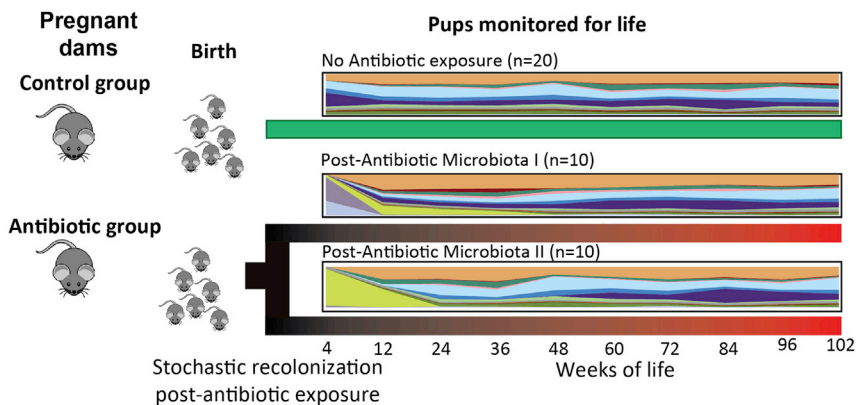


Figure 4. Composition of the fecal microbiota in antibiotic exposed and unexposed mice throughout life

The composition of microbiota in unexposed mice (no ABX, $n=20$) and mice exposed to antibiotics in early life ($n=20$) was profiled using 16S rRNA gene sequencing of DNA extracted from pooled fecal samples collected at specific intervals from 1 week post-antibiotic exposure (week 4 of age) to week 102 of life. Two distinct microbiota community types were evident: post-antibiotic exposure (PAM I and PAM II, $n=10/\text{group}$). Note: Reprinted from “The composition of the gut microbiota following early life antibiotic exposure affects host health and longevity in later life,” by (Lynn et al., 2021). Reprinted with permission.

untreated and an antibiotics treated samples is so great, (4 log fold difference), that this assay can still function adequately here. Other antibiotic cocktails may induce more subtle effects that may require more accurate bacterial load measurements. Other solutions including the use of commercially available synthetic standards may be utilized to more accurately estimate the bacterial load using digital droplet PCR (Barlow et al., 2020).

Although, a routine technology in studying the composition of the microbiota, 16S rRNA sequencing is limited in its taxonomic resolution and does not detect viruses, fungi or microbial eukaryotes. Similarly the taxonomic resolution of the gene varies between different bacterial taxa (Yarza et al., 2014) and databases may be limited in their coverage and have a bias towards human associated bacteria.

TROUBLESHOOTING

Problem 1

Ineffective antibiotic treatment.

Light damage rendering antibiotic cocktail ineffective in reducing bacterial load.

Potential solution

Antibiotic supplement may be administered in tinted or laminated water bottles. Alternatively wrap bottles in aluminum foil to prevent light damage. Change antibiotic supplement every 2–3 days. [Randomly assign pregnant dams to antibiotic exposure or control groups: step 5.](#)

Problem 2

Higher mortality rates in pups born to antibiotic treated dams.

Often unavoidable, higher mortality rates may be associated with pups exposed to antibiotics due to stress associated with antibiotics exposure.

Potential solution

Allow for excess dams in the antibiotic treated group, to account for loss of pups. [Before you begin: step 4.](#)

Problem 3

Stochastic recolonization of the gut microbiota following antibiotic exposure.

Following the cessation of the antibiotic treatment there is a stochastic recolonization of the gut microbiota. Within an individual cage, it is likely that the mice will have a similar gut microbiota as mice routinely ingest feces from within the cage. Mice housed in separate cages may have a distinct gut microbiota.

Potential solution

It is necessary to sample every mouse in each cage and not to assume that mice in different cages have the same microbiota. 16S rRNA gene sequencing should be performed approximately 7 days following antibiotic exposure to assess differences between individual mice and cages and ideally this should be continued longitudinally. If this is not possible, colonization of germ-free mice may be utilized to ensure no cage to cage variation. [Collect fecal samples from antibiotic exposed and control mice throughout the duration of the experiment: step 8.](#)

Problem 4

Insufficient fecal material.

In the first 18 days of life, it is not possible to reliably collect fecal samples from pups.

Potential solution

We use the dam as a proxy for antibiotic driven depletion of the gut microbiota at day 2 of life and at weaning. A fecal sample is collected and extracted from dams at these time points and 16S rRNA qRT-PCR is performed to assess the impact of antibiotic exposure on bacterial load. We recommend collecting 2 or more fecal pellets at day 21 and day 28 of life to offset these issues in the younger mice. Intestinal or cecal contents of pups following antibiotic exposure could also be collected though this will substantially increase the number of mice required as mice will need to be humanely killed at each timepoint. This approach would also prohibit longitudinal profiling in the same individuals. [Collect fecal samples: step 3.](#)

Problem 5

Failure to reproducibly obtain standard curve as part of 16S rRNA gene assay.

Inconsistent adherence to 16S rRNA qRT-PCR protocols may lead to failure to obtain accurate standard curves.

Potential solution

Make multiple aliquots of the DNA stock and follow protocols carefully, using a fresh aliquot of DNA standard on each occasion. [Prepare 16S rRNA gene qRT-PCR: step 12.](#)

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Lynn (David.lynn@sahmri.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets/code generated or analyzed during this study.

ACKNOWLEDGMENTS

The authors acknowledge the facilities and technical assistance of staff in the South Australian Health and Medical Research Institute (SAHMRI) bioresources and histology facilities. Flow cytometry analysis was performed at the ACRF Cellular Imaging and Cytometry Core Facility in SAHMRI. The ACRF Facility is generously supported by the Detmold Hoopman Group, Australian Cancer Research Foundation, and Australian Government through the Zero Childhood Cancer Program. The authors acknowledge the facilities and scientific and technical assistance of the National Imaging Facility, a National Collaborative Research Infrastructure Strategy (NCRIS) capability, at SAHMRI. We also acknowledge the SAHMRI genomics core for their assistance with RNA-seq and 16S rRNA library preparation and sequencing. This study was financially supported by an EMBL Australia Group Leader award to D.J.L.

AUTHOR CONTRIBUTIONS

Conceptualization, D.J.L.; investigation, M.A.L.; writing – original draft, M.A.L. and F.J.R.; writing – review & editing, M.A.L., C.Y.T., F.J.R., S.C.B., and D.J.L.; funding acquisition, D.J.L.; supervision, D.J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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