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TITLE:

Using the Protozoan *Paramecium Caudatum* as a Vehicle for Food-Borne Infections in Zebrafish Larvae

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Paramecia, *Paramecium caudatum*, zebrafish, *Danio rerio*, food-borne infection, enteric pathogens

SUMMARY:

Zebrafish (*Danio rerio*) are becoming a widely-used vertebrate animal model for microbial colonization and pathogenesis. This protocol describes the use of the protozoan *Paramecium caudatum* as a vehicle for food-borne infection in zebrafish larvae. *P. caudatum* readily internalizes bacteria and get taken up by larval zebrafish through natural preying behavior.

ABSTRACT:

Due to their transparency, genetic tractability, and ease of maintenance, zebrafish (*Danio rerio*) have become a widely-used vertebrate model for infectious diseases. Larval zebrafish naturally prey on the unicellular protozoan *Paramecium caudatum*. This protocol describes the use of *P. caudatum* as a vehicle for food-borne infection in larval zebrafish. *P. caudatum* internalize a wide range of bacteria and bacterial cells remain viable for several hours. Zebrafish then prey on *P. caudatum*, the bacterial load is released in the foregut upon digestion of the paramecium vehicle, and the bacteria colonize the intestinal tract. The protocol includes a detailed description of paramecia maintenance, loading with bacteria, determination of bacterial degradation and dose, as well as infection of zebrafish by feeding with paramecia. The advantage of using this method of food-borne infection is that it closely mimics the mode of infection observed in human disease,

leads to more robust colonization compared to immersion protocols, and allows the study of a wide range of pathogens. Food-borne infection in the zebrafish model can be used to investigate bacterial gene expression within the host, host-pathogen interactions, and hallmarks of pathogenicity including bacterial burden, localization, dissemination and morbidity.

INTRODUCTION:

Zebrafish share morphologically and functionally conserved features with mammals, including granulocytic lineages (*e.g.*, neutrophils), monocyte/macrophage-like cells, Toll-like receptors, pro-inflammatory cytokines, and antimicrobial peptides[1]. The intestinal tract in zebrafish is fully developed at 6 days post fertilization (dpf) and shows morphological and functional conservation with the mammalian gastrointestinal tract, such as conserved transcriptional regulation in intestinal epithelial cells[2]. This makes zebrafish an excellent model for intestinal microbial colonization and pathogenesis. A wide range of enteric microbes has been studied in the zebrafish model, including enterohemorrhagic *Escherichia coli*[3], *Vibrio cholerae*[4,5], *Salmonella enterica*[6], the zebrafish microbiota[7,8], and the role of probiotics in intestinal immunity[9]. A distinct advantage of the zebrafish model is that it is colonized by many microbes without disrupting the endogenous microbiota, which allows the investigation of microbial behavior in the context of mixed microbial populations[3,6]. Currently, most zebrafish models of gastrointestinal colonization and disease rely on the administration of microbes by bath immersion, where zebrafish are incubated in a bacterial suspension for a specific amount of time[10]. However, this makes it difficult to determine the exact dose of bacteria administered, and leads to limited colonization with some microbes, particularly with non-pathogenic bacteria. Alternatively, a bacterial suspension is administered to fish *via* oral gavage[11], but this is technically challenging and limited to older larvae and adult fish.

This protocol describes the use of the unicellular protozoan *Paramecium caudatum* as a vehicle for food-borne delivery of microbes to the gastrointestinal tract of zebrafish larvae. Paramecia are easy and cheap to maintain and are capable of feeding on a wide variety of microbes, including algae, fungi, and bacteria, which they internalize through a ciliated oral groove[12-14]. Once internalized, bacteria are held in vacuoles, which eventually acidify and contents are degraded over a time frame of several hours[15]. Larval zebrafish capture paramecia as natural prey soon after hatching, around 3 - 4 dpf depending on temperature[16], and take them up with high efficiency. The process of prey capture takes on average 1.2 s from detection to capture[17], and captured paramecia are quickly digested in the zebrafish foregut, such that internalized viable bacteria are released into the intestinal tract[3]. As a result, paramecia can be used as a quick and easy method to deliver a high and consistent dose of bacteria into the gastrointestinal tract of zebrafish. The delivered bacteria can either be transformed to express a fluorescent protein, such as mCherry as described here, or, in the case of genetically intractable bacteria, they can be pre-stained with a fluorescent dye to allow visualization within the gastrointestinal tract.

This protocol describes the food-borne delivery of enteropathogenic *E. coli* (enterohemorrhagic *E. coli* [EHEC] and adherent invasive *E. coli* [AIEC]), and *Salmonella enterica* ssp. Typhimurium. Both pathogenic *E. coli* and *S. typhimurium* are transmitted *via* the fecal-oral route[18,19], and can be acquired *via* contaminated food, such as meat, vegetables, and dairy. Using *P. caudatum* as a

vehicle, *E. coli* and *S. typhimurium* successfully colonize the zebrafish larvae within 30 - 60 min of co-incubation with the paramecium vehicle. The achieved bacterial burden is robust enough to visualize colonization and determine burden by plating tissue homogenates.

PROTOCOL:

Zebrafish care, breeding, and experiments described here are in accordance with the Guide for the Care and Use of Laboratory Animals, and have been approved by the Institutional Animal Welfare Committee of the University of Texas Health Science Center, protocol number AWC-16-0127.

1. Growth and Maintenance of Paramecia

1.1. Obtain live paramecia cultures from sources such as the Zebrafish International Resource Center (ZIRC).

1.2. From a live growing culture, add 1 mL of the paramecia culture, 1 mL of an *E. coli* MG1655 culture (optical density OD_{600} 1.0 - 2.0) resuspended in 1x E3 (0.29 g/L NaCl, 13 mg/L KCl, 44 mg/L $CaCl_2$, 81 mg/L $MgSO_4$, 0.48 g/L HEPES, pH 7.0, sterile), and 8 mL of 1x E3 into a 10 mL tissue culture flask. Swirl lightly and then store at 22 °C.

1.3. To maintain a culture, every two weeks, passage 1 mL of a paramecia culture into a 10 mL tissue culture flask with 9 mL of fresh 1x E3 medium containing 10^8 CFU/mL of *E. coli* MG1655 resuspended in 1x E3.

2. Determination of Bacterial Dose Administered to Zebrafish

2.1. Determine bacterial half-life within paramecium

NOTE: The half-life of bacteria within paramecia is determined by plating viable *E. coli* recovered from paramecium, as described below.

2.1.1. Following a 2 h incubation of bacteria (OD_{600} of 1.0) with paramecia at 22 °C, combine the paramecia and bacterial co-culture into a 50 mL conical tube, wash the paramecia with 1x E3, and count the number of paramecia per milliliter (as described below in steps 3.2.7 and 3.2.8).

2.1.2. After counting the number of paramecia, remove 50 µL of the paramecia and bacterial co-culture every hour (for 6 h post-exposure) and add each sample into a fresh 1.5 mL tube.

2.1.3. Place 950 µL of 1% nonionic surfactant (see **Table of Materials**) in phosphate buffered saline (PBS) solution into each of the 1.5 mL tubes and vortex for 1 min to lyse the paramecia. Perform 1:10 dilutions of each sample using sterile PBS as a diluent (*i.e.*, 100 µL in 900 µL).

2.1.4. Plate 100 µL of each dilution onto selective plates (LB tet medium: 1 g/L tryptone, 0.5 g/L yeast extract, 1 g/L NaCl, 30 µg/mL tetracycline) and incubate at 37 °C for 16 h. The next day,

count and record the number of bacterial colonies on the plate (colony forming units, CFUs) by counting only the isolated and distinct individual colonies. Identify a plate with a dilution that gives 30 - 300 CFU.

2.1.5. Determine the dilution factor used. For example, if 1 µL of the bacterial culture is mixed with 99 mL of sterile PBS, this is a 1:100 (0.01) dilution. This number will be the number of CFU in the dilution. Perform the calculation below, for every time point, to determine the CFU in the original sample:

$$\frac{CFU\ from\ dilution}{Dilution\ factor\ x\ volume\ plated} = CFU\ in\ orginal\ sample$$

2.1.6. Calculate and graph the number of viable *E. coli*/paramecium corresponding to the hours post exposure using the paramecia concentration calculated in step 3.3.1 and determine the half-life within the paramecia (**Figure 1**).

2.1.7. Using the CFU number calculated for each time point, calculate and graph the number of viable *E. coli* per paramecium on the y-axis *versus* the hours post incubation on the x-axis, using the paramecia concentration calculated in step 3.3.1. Determine the half-life within the paramecia.

2.2. Determine bacterial dosing from bacterial half-life and preying rate.

NOTE: To determine the bacterial dosage, the half-life of the bacteria inside the paramecia and the preying rate of zebrafish on paramecia (see step 3.4) have to be taken into account.

2.2.1. Use the following formula to determine bacterial decay within paramecia:

(1) $Nt = N_0 e^{-k\tau}$

Where N_0 is the initial quantity of bacteria per paramecia after the 2 h incubation, Nt is the remaining quantity after time t, τ is the time after which the number of viable bacteria has halved, and k is the decay constant.

2.2.2. From the degradation experiment, determine the decay constant k using the bacterial half-life (*i.e.*, the time after which the amount of viable bacteria/paramecium has halved).

(2) $ln\left(\frac{Nt}{No}\right) = -k * \tau$

For the determination of half-life, the term $\frac{Nt}{No} = \frac{1}{2}$ and

(3) $ln\left(\frac{1}{2}\right) = -k * \tau$ or

(4) $k = -\frac{\ln\frac{1}{2}}{\tau}$

NOTE: Based on **Figure 1**, the half-life of *E. coli* in paramecia is approximately 2.3 h. Thus, using formula (4), the decay rate, k, for *E. coli* is:

(5) $k = -\frac{\ln\frac{1}{2}}{\tau} = -\frac{\ln\frac{1}{2}}{2.3} = 0.30 \cdot h^{-1}$

2.2.3. Determine the decay rate (k) from the half-life experiment to find the dose of viable bacteria (Nt) taken up by the zebrafish larvae after preying time (t), where (P) is the preying rate or the number of paramecia eaten by one fish per hour:

(6) $ln\ \frac{Nt}{No \cdot P} = -k * t$ or

(7) $Dosage\ Nt = \frac{P \cdot N_0}{e^{k \cdot t}}$

NOTE: Per **Figure 1**, the initial quantity of bacteria per paramecia (N_0) after a 2 h incubation (t) is 790 CFU. Per **Movie 1** and **Figure 2**, the preying rate (P) is 1539.

2.2.4. Using these values to substitute into equation (7), calculate the bacterial dosage consumed by a zebrafish following a 2 h incubation as:

(8) $Dosage\ Nt = \frac{P \cdot N_0}{e^{k \cdot t}} = \frac{1539 \cdot 790}{e^{0.30 \cdot 2}} = 6.7 \cdot 10^{5} CFU$

3. Food-borne Infection of Zebrafish

3.1. Incubate bacteria with paramecia.

3.1.1. Prepare a co-culture of paramecia and *E. coli* MG1655 the night prior to infection. Combine 8 mL of E3 media, 1 mL of an ongoing paramecia culture, and 1 mL of an *E. coli* MG1655 culture (OD_{600} = 1.0) resuspended in 1x E3 in T25 tissue culture flasks. Incubate the flasks at room temperature (RT) overnight. For each treatment condition, prepare two flasks of paramecia.

3.1.2. Inoculate bacterial growth media (LB: 1 g/L tryptone, 0.5 g/L yeast extract, 1 g/L NaCl) with infectious strain of bacteria, by picking an individual bacterial colony from a plate using a sterile inoculation loop. Incubate the liquid culture at 37 °C and leave shaking at 110 rotations per minute (rpm) overnight.

NOTE: Personal protective equipment (a laboratory coat and gloves) should be worn and biosafety level 2 facilities should be used when handling infectious agents.

3.1.3. On the next day, measure the OD_{600} of the overnight culture. Calculate the volume of culture required to achieve an OD_{600} of 1 when resuspended in 11 mL of media.

3.1.4. Harvest the volume of bacteria from step 3.1.2 *via* centrifugation at 6000 x g for 5 min, one volume for each flask of paramecia. Discard the supernatant and resuspend the bacterial pellet in 1 mL of E3 media.

3.1.5. Optionally, pre-stain the bacteria with a fluorescent dye.

3.1.5.1. Add 1 µL of FM 4-64FX bacterial stain (5 mg/mL stock solution). Cover tube with foil to protect from photobleaching and incubate rotating end-over-end at RT for 15 min.

3.1.5.2. Remove excess dye by washing with 1x E3: Pellet bacteria *via* centrifugation at 6000 x g for 1.5 min, then resuspend pellet in 1 mL of E3 media. Repeat wash step two times.

3.1.5.3. Harvest stained bacteria *via* centrifugation at 6000 x g for 5 min. Discard the supernatant and resuspend the bacterial pellet in 1 mL of E3 media.

3.1.6. Add 1 mL of the bacterial suspensions to each of the two flasks of fresh paramecia. Incubate at RT for 2 h.

NOTE: If working with stained bacteria, incubate in the dark at RT for 2 h.

3.2. Wash bacteria/paramecia co-culture.

3.2.1. Combine the contents of both flasks of paramecia/bacteria co-culture into a 50 mL conical tube. Centrifuge samples at 300 x g at 15 °C for 10 min. Make sure that the centrifuge is pre-cooled prior to this step.

3.2.2. Remove approximately 10 mL of the E3 supernatant using a serological pipette and add approximately 10 mL of fresh 1x E3 to the conical tube.

NOTE: During all wash steps, it is essential to be very quick when removing the supernatant, as the paramecia will begin to swim out of the pellet. Spin and remove supernatant from one tube at a time to ensure quick enough handling at this step, and avoid loss of paramecia in the supernatant.

3.2.3. Spin samples *via* centrifugation at 300 x g at 15 °C for 5 min. Remove approximately 10 mL of the E3 supernatant using a serological pipette, and add approximately 10 mL of fresh 1x E3 to the conical tube. Repeat this step twice.

3.2.4. Centrifuge samples at 300 x g at 15 °C for 5 min. Remove approximately 10 mL of the E3 supernatant, taking care not to disrupt the pellet.

3.2.5. Resuspend pellet into the remaining 10 mL of E3 media and transfer 500 µL of the suspension into a new 1.5 mL microcentrifuge tube. Pellet the 500 µL of paramecia by centrifuging at 300 x g for 5 min to count the number of paramecia.

3.2.6. Remove 400 µL of the E3 supernatant from the 500 µL sample. Add 20 µL of 36.5% formaldehyde solution to the remaining 100 µL of paramecia and gently resuspend, and incubate for 5 min at 22 °C.

NOTE: This step kills the paramecia to allow for counting.

3.2.7. Measure actual total volume using the pipette and record. Dilute the paramecia suspension 1:1 v/v with 0.4% trypan blue solution.

3.2.8. Use a cell counter or hemocytometer to count the number of dead paramecia/mL.

NOTE: Because of the prior fixation step, most paramecia will be dead at this point, but this number reflect the number of live paramecia for the co-incubation experiment. The authors have not found significant paramecia death due to bacterial co-incubation, so this can be disregarded as a factor here.

3.3. Co-incubate Paramecia and zebrafish larvae.

3.3.1. Calculate the concentration of paramecia:

$$\frac{500\ \mu L}{\text{measured volume (from step 3.2.7)}} = \text{dilution factor}$$

$$\frac{\text{measured paramecia concentration (step 3.2.8)}}{\text{dilution factor}} = \text{original paramecia concentration}$$

NOTE: This calculation gives the concentration of paramecia in the 50 mL conical tube from step 3.2.5.

3.3.2. Calculate the volume of washed paramecia required for a concentration of 2 x 10^5 paramecia/mL in a final volume of 3 mL of E3.

NOTE: The concentration of paramecia can be adjusted based on the desired bacterial dosage, which is subject to optimization.

3.3.3. Anesthetize zebrafish by adding tricaine in 100 mM Tris pH 8.0 to a final concentration of 100 mg/L. Transfer 10 zebrafish into each well of a 6-well plate into a total volume of 3 mL of fresh E3 containing the appropriate concentration of paramecia (calculated in step 3.3.2). Ensure to transfer larvae in a minimal amount of liquid, to ensure they recover from anesthesia in the recipient well.

3.3.4. Incubate at 30 °C for 2 h in a diurnal incubator under day-light conditions, to ensure optimal lightning conditions for preying.

3.3.5. Wash zebrafish at least 5 times by transferring fish into a new well containing 3 mL of fresh E3 containing 100 mg/L tricaine each time.

NOTE: Do not attempt to omit the tricaine during the washing step. Transferring mobile larvae without anesthesia increases the risk of damage and distress to the animal.

3.3.6. Optionally, prepare zebrafish for imaging by embedding zebrafish in 3 mL of 1% low-melt agarose in a black-walled 6-well plate: Low-melt agarose is made up in 1xE3 and heated in a microwave. Once molten, add tricaine to a final concentration of 160 mg/mL. Position fish under a stereomicroscope, using a clipped gel loading tip, making sure that the head is on the left and the tail is on the right (**Figure 3**). Wait for 5 minutes for the agarose to set, then overlay the embedded fish with 1xE3 containing 160 mg/mL tricaine for imaging.

3.4. Determine the preying rate.

NOTE: No separate experiment needs to be set up to determine the preying rate. Rather, this can be done during step 3.3.4., as described below.

3.4.1. During step 3.3.4, view preying zebrafish on a stereomicroscope and capture video footage of the prey capture.

3.4.2. Score the video footage. Prey capture is characterized by striking of zebrafish toward the prey. Count each strike as one prey capture event, although this is only an approximation (see **Discussion**).

3.4.3. Calculate the average number of prey capture events per hour from multiple video clips, each representing a different zebrafish larva (**Figure 2**).

REPRESENTATIVE RESULTS:

Paramecium caudatum readily internalizes a wide range of bacteria into storage vacuoles. The intracellular bacterial density depends on the densities of bacteria and paramecia in the co-culture, as well as the bacterial species used. Over time, the vacuoles acidify and bacterial degradation ensues. The rate of degradation has to be individually determined for all strains used. For pathogenic *E. coli*, the initial bacterial density is 790 bacteria/paramecium, and bacteria are degraded with a half-life of approximately 2.3 h (**Figure 1**).

[Place Figure 1 here.]

Further, the zebrafish preying rate, that is, the rate at which zebrafish internalize bacteria-loaded paramecia upon co-incubation, was studied. Larval zebrafish start to hunt and capture live prey

from 5 dpf [20], although it was found that, when raised at 30 °C, larval development is accelerated and animals display preying behavior from 4 dpf. Preying is accompanied by a characteristic striking behavior[20] (**Figure 2A**), and the determination of the preying rate is based on the assumption that each strike leads to internalization of one paramecium, although this can only be regarded an approximation (see **Discussion**). Based on the herein described observations of preying zebrafish larvae, the rate of paramecia uptake is approximately 1539 per hour (**Figure 2B**).

[Place Figure 2 here.]

Following internalization of paramecia, the zebrafish efficiently degrades the prey in the foregut, releasing infectious bacteria into the digestive system. As described herein, paramecia degradation proceeds quickly, and free bacteria can be detected in the intestinal tract within 30 minutes of preying. Free bacteria then move from the foregut to the mid- and posterior intestine, where they are detected approximately 1 - 2 h after the beginning of preying (**Figure 3**). Bacterial persistence in the intestine depends on species and dose but ranges from several hours to several days in the case of *E. coli* and *S. enterica*. *S. enterica* localizes primarily in the intestinal mucosae, with some epithelial invasion (**Figure 3D**), leading to infiltration of neutrophils into the epithelium (**Figure 3C**).

[Place Figure 3 here.]

FIGURE AND TABLE LEGENDS:

Figure 1: Determination of bacterial half-life in paramecia. (**A**) Following 2 hours of co-incubation with infectious *E. coli*, *P. caudatum* was washed and transferred to medium without bacteria. At the indicated time points, numbers of viable *E. coli* cells were determined by dilution plating on selective agar. Results are means ± standard error of the mean (SEM; n = 3). (**B**) Typical image of paramecium carrying internalized bacteria, with bright field (**Bi**), fluorescent bacteria (**Bii**), and merged channels (**Biii**). Scale bar, 20 µm.

Figure 2: Determination of zebrafish preying rate. (**A**) Still images from a preying video, showing a zebrafish larvae (5 dpf) preying on paramecia carrying fluorescent bacteria. Time in [seconds]. Arrow indicates the main axis of movement during striking. (**B**) Quantification of preying rate (paramecia intake per hour), based on n = 10 videos taken over the full 2-hour exposure time.

Figure 3: Colonization of zebrafish with bacteria. Zebrafish at 5 dpf were left uninfected (**A**) or colonized with mCherry expressing (**B**) *E. coli* or (**C**) *S. enterica*. Infection experiments may be performed in wild type (**A** and **B**) fish or transgenic lines (*e.g.*, the line Tg(MPO::EGFP)^{i114} expressing green fluorescent neutrophils shown in (**C**). The rectal opening is marked by an arrow. (**D**) Higher magnification of intestinal section from whole-mount embedded larvae infected with *Salmonella enterica* infection. (**Di**) Blue – Hoechst marking nuclei, (**Dii**) Purple – phalloidin marking F-actin, (**Diii**) Red – *Salmonella*, (**Div**) merge. Scale bar, 5 µm.

Movie 1: Video footage of the prey capture.

DISCUSSION:

The basic protocol described here has been optimized for pathogenic *E. coli*, and has been successfully adapted for other bacterial species, including *Salmonella enterica* and *Vibrio cholerae*. For some species that do not colonize the zebrafish gut following bath immersion, including some *Salmonella enterica* strains and some anaerobes, food-borne infection as described here can be used to successfully establish colonization. Compared to microgavage, which is also used to establish high bacterial burdens in the larval intestinal tract, food-borne infection is technically less challenging and requires less specialized equipment. However, critical parameters should be optimized for the bacterial species and strains to be used. Such factors include bacterial and paramecium density for the bacteria-paramecium co-culture step: If bacterial numbers within paramecia are low, this could be improved by increasing the bacterial density in the co-culture step. Some bacterial species may cause damage to the paramecium host, and this should be assessed by microscopy.

Another important factor in this protocol is prey capture by zebrafish. The preying rate as described here is based on the assumption that every prey capture strike results in the ingestion of one paramecium. High densities of paramecia per fish are used in the protocol to ensure high preying rates. However, prey capture is dependent on the density of paramecia in the system, and in very dilute paramecium cultures, preying rates may be as low as 13 - 15 paramecia per hour[21,22]. A limitation is that prey capture rates are also strongly dependent on lighting conditions and in the dark, capture rates are 80% lower than in light conditions[21] and this should be taken into account when setting up experiments. If exposure times to prey have to be expanded to optimize colonization, consideration has to be given to secondary exposure to bacteria through feces. Under the conditions described above – 2 hours of prey exposure – this exposure is negligible, since gut passage time of bacteria is more than 1 h and the concentration of bacteria in the vehicle is much higher than in feces. However, if prey exposure time is significantly increased, this may become a significant factor.

Appropriate controls should be included in this protocol, including colonization of zebrafish following feeding with paramecia containing non-pathogenic *E. coli* MG1655. If multiple bacterial strains are compared for their ability to colonize the zebrafish host, it is important to test whether their half-life within paramecia is comparable. Bacterial mutations, including those compromising cell wall integrity or acid sensing, may compromise bacterial stability within paramecia. In such cases, zebrafish feeding has to be adjusted to account for the differences in dosage.

The protocol described here can be used to investigate bacterial colonization and its consequences, including by imaging bacterial colonization of zebrafish as described above, as well as by determining CFU per zebrafish from tissue homogenate[3], or investigating infection-associated morbidity and mortality. Ideally, for bacterial visualization, bacterial strains expressing fluorescent proteins such as mCherry or red fluorescent protein (RFP) should be used. This will allow the visualization of growing bacterial populations. If the bacterial strain is not genetically tractable or the use of fluorescent protein expression is precluded for other reasons, bacteria

may be stained with a fluorescent dye, such as FM 4-64FX, prior to co-culture with paramecia. When using the protocol described here, co-culture with paramecia does not decrease the brightness of the dye and stained bacteria are clearly visible in the zebrafish intestine. However, the dye will be diluted over time should significant bacterial proliferation occur within the zebrafish host. In either case, red-fluorescent bacteria are preferable over green-fluorescent bacteria, since tissue autofluorescence can be higher in the green than in the red channel.

It has been found that this protocol can be adapted for aerobic and microaerophilic bacterial species. It may be possible to adapt it for the feeding of spores and fungal species, although this remains to be tested experimentally.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

Click here to access/download;Figure;Figure 1.tif

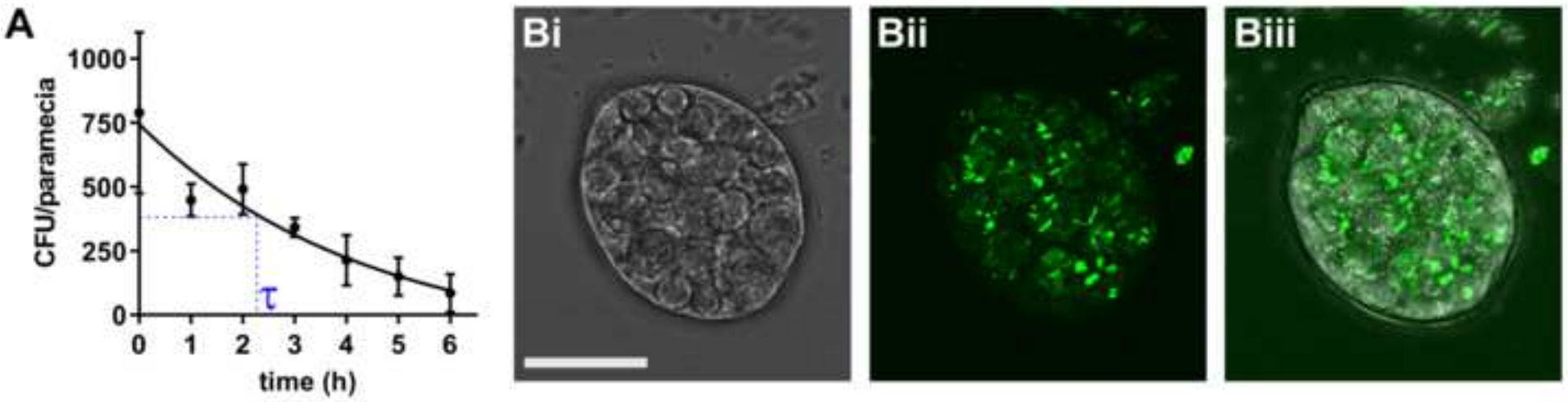

Figure2

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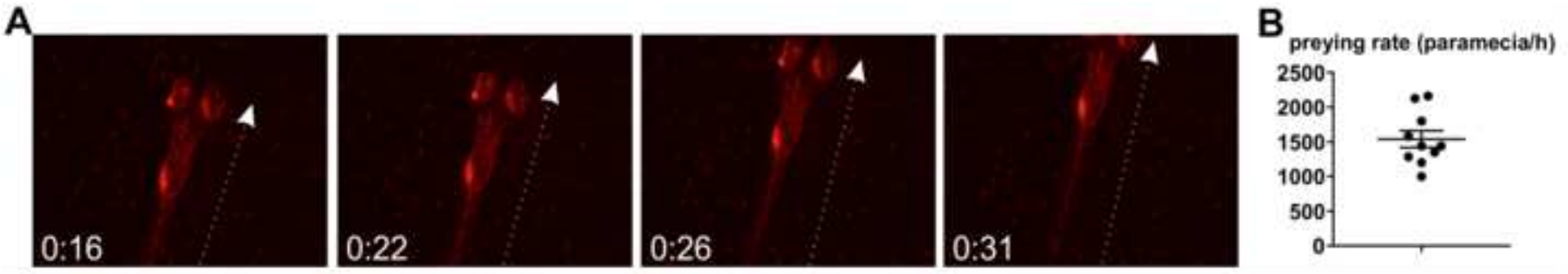
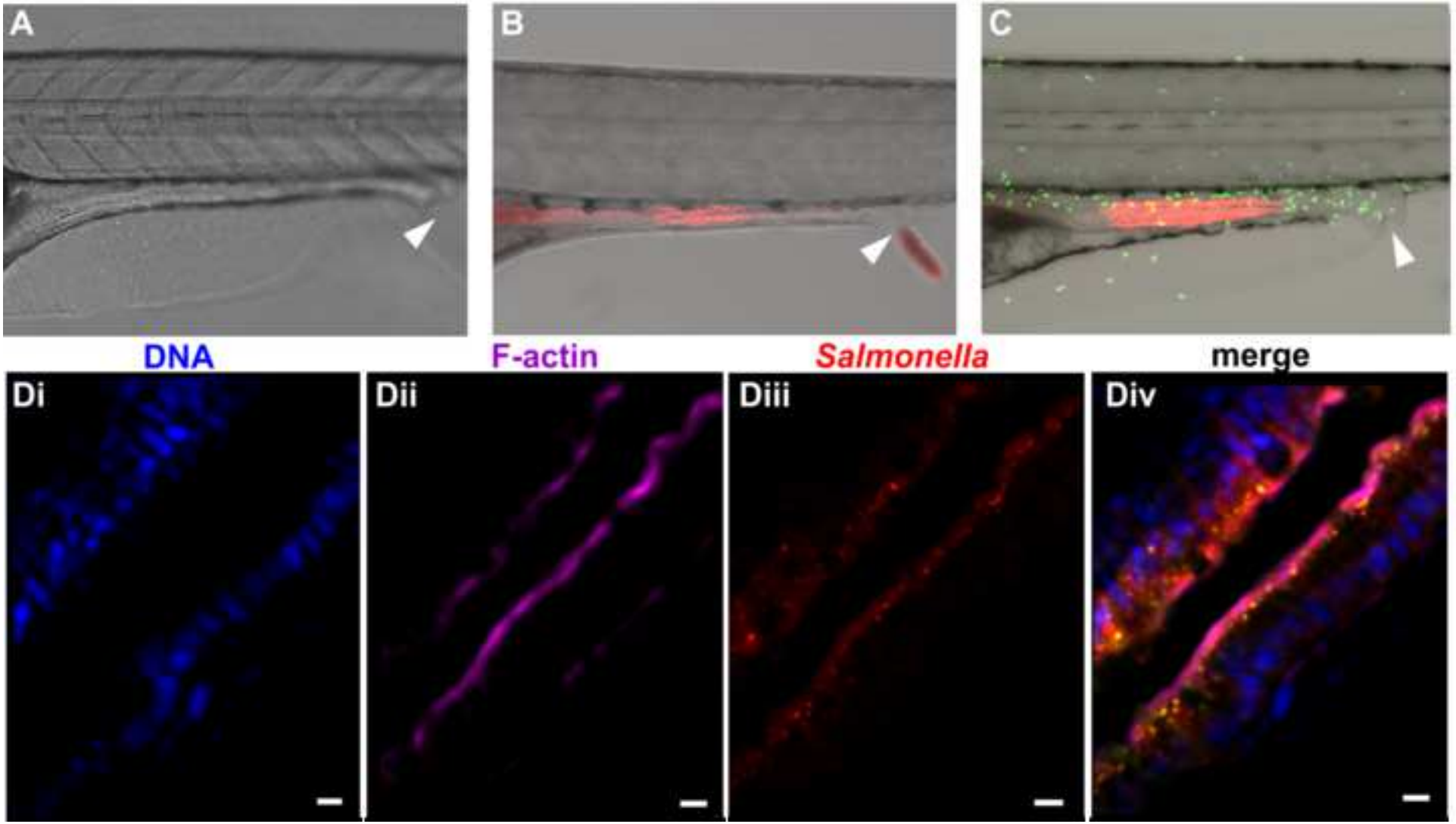


Figure3

Click here to access/download;Figure;Figure 3.tif



Movie1

Click here to access/download

Video or Animated Figure

PreyCapture.mp4

Name of Material/ Equipment	Company	Catalog Number
Paramecium caudatum , live	Carolina	131554
0.4% Trypan Blue Solution	Sigma	T8154-20ML
Dimethyl sulfoxide (DMSO)	Sigma	276855-100ML
Escherichia coli , MG1655	ATCC	ATCC 700926
FM 4-64FX stain	Thermo Fisher	F34653
Formaldehyde	Sigma	F8775-4X25ML
LB Broth	Sigma	L3397-1KG
Phosphate buffered saline tablets	Thermo Fisher	18912014
Tetracycline	Sigma	87128-25G
Tricaine (Ethyl 3-aminobenzoate methanesulfonate)	Sigma	E10521-10G
Triton X-100	Sigma	X100-100ML
Trypan Blue Solution, 0.4%	Sigma	93595-50ML
UltraPure Low Melting Point Agarose	Thermo Fisher	16520050
hemocytometer or cell counter	any	
stereomicroscope	any	
table-top centrifuge		
microwave		
rotator wheel		
heated shaking incubator		
aquatics facilities		
breeding tanks		

Comments/Description

no not store growing cultures below room temperature

liquid, sterile-filtered, suitable for cell culture; prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic

store in a solvent safety cabinet

can be replaced by any other non-pathogenic *E. coli* strain

aliquot and store frozen

toxic, irritant

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Author(s): Flores, Thompson, Sirisaengtaksin, Nguyen, Ballard Krachler.

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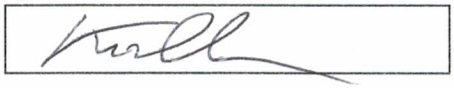
Signature:	
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Microbiology and Molecular Genetics

Anne-Marie Krachler, Ph.D.
Associate Professor

September 17, 2018

Re: Manuscript JoVE58949

Dear Editor,

Please find enclosed our revised manuscript entitled **'Using the protozoan *Paramecium caudatum* as a vehicle for food-borne infections in zebrafish larvae'**. We would like to thank you and the reviewers for your positive responses and really appreciate your suggestions on how to improve our work. We have updated the manuscript to reflect our response to your comments. Please find attached below a detailed breakdown of our response to the editorial and the reviewers' comments and resulting changes to the manuscript. We hope you will now find our work suitable for publication.

Sincerely,

Anne-Marie Krachler, Ph.D.
Associate Professor of Microbiology and Molecular Genetics

_____

Response to Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. – *We proofread the manuscript.*
2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. – All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: falcon, Triton, FM 4-64FX, etc. – *We replaced Falcon tube with conical tube. Triton and FM 4-64 are specific reagents, which cannot be replaced by other, generic reagents.*
3. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). – *We have edited the protocol to avoid these phrases.*
4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. – *We have amended this, except in the Discussion section where we feel these phrases are appropriate.*
Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
1.2: What is E3? Please provide its composition. What is taken from a live growing culture? It is unclear. – *We have added the composition of E3 upon its first mention in section 1.2*.
1.3: How to measure density and what is the peak density? – *We do not actually measure the culture density, and have removed the corresponding phrasing.*
2.1.1: What is the incubation temperature? What is used to wash the paramecia? How to count the number? Steps 4.1 and 4.2 do not exist.- *We have added the incubation temperature and amended the reference to the correct steps.*
2.1.5: What are the selective plates and how to count CFUs? – *We have added a description of the media composition and a more detailed description of the CFU counting.*
2.1.6: Please provide an equation if possible. – *We have now provided an equation.*
3.1.3: Please describe how to inoculate bacterial growth media with infectious strain of bacteria. Please provide composition of bacterial growth media. – *We have added the medium composition and procedure.*
3.1.6: Step 4.1.5 does not exist. Should it be 3.1.5? *Yes, corrected.*
3.1.8.3: What is used to wash? Please specify the wash step repeated here. – *We added that the wash is performed with 1xE3.*
3.2.3: What is used to remove the supernatant? – *Added that a serological pipette is used.*
3.2.9: What is the incubation temperature? – *Added the incubation temperature.*
3.3.1: Steps 4.2.10 and 4.2.6 mentioned in this step do not exist. Please revise. – *Amended.*
3.3.3: Step 4.3.2 does not exist. Please revise.- *Amended to 3.3.2.*
6. 2.2 and sub-steps: Can these steps be moved after step 3.3.4. As currently organized, the readers/viewers have to refer back and forth in order to complete the protocol.- *We have moved this section to the end of the protocol.*

7. 2.3.1 and 2.3.2: Please break up into sub-steps.- *We broke it up into sub-steps.*
8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. – *We have combined steps wherever we thought possible.*
9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia. Please note that calculation steps are not appropriate for filming.
11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
12. Figure 2: Please state the unit of time in panel A in the figure legend. – *Included in figure legend.*
13. Discussion: Please discuss any limitations of the technique and the significance with respect to existing methods. – *We have included some more discussion of limitations and comparison with existing techniques.*
14. References: Please do not abbreviate journal titles.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
good, detailed procedure to produce a useful experimental tool. Only minor concerns that can be addressed in text and possibly with addition of already existing data

Minor Concerns:
Line 249 reference to 4.1.5 should be 3.1.5 – *Thank you, this has been corrected.*
274 insight into gravitational tolerance of paramecium would be nice. Does harder for shorter kill the paramecium or can they tolerate a shorter spin at higher g? – *We use the minimal force required to pellet them efficiently, and see no benefit in increasing the spin force.*
277 recommend pipette or decanting to rapidly remove supernatant without disturbing motile pellet? – *We added to add a serological pipette for this step.*
296 I assume a standard 4% PFA? PFA is missing from materials section – *We have specified the formaldehyde concentration and added the reagent to the materials list.*
320 why is it necessary for the embryos to be knocked out prior to transfer*? – Anesthetizing the embryos facilitates fast and efficient transfer and minimizes distress and risk of causing damage to the animals.*
324 please add necessity of lighting to achieve high/reproducible prey rates. Is lab bench lighting sufficient or is more direct light required? – *We have added the note: 'Incubation should be carried out in a diurnal incubator under day-light conditions, to ensure optimal lightning conditions for preying.'*
333 add homogenisation and plating steps and indicative results – *The halflife experiment does not as such contain a homogenization step. The plating step is described in step 2.1.5, and representative results in Fig. 1.*

Reviewer #2:

Manuscript Summary:
In this manuscript, Flores and colleagues describe using paramecia as a delivery system for pathogenic bacteria into the intestines of larval zebrafish. Zebrafish is increasingly being used as a model for investigating gnotobiology and bacterial pathogenesis, so this approach is timely and should be very useful to the research community. However, as described below, there are issues that need to be resolved before this manuscript can be published and used as the basis for a video.

Major Concerns:
1) At the beginning of the protocol, the experiments are described as being in accordance with the Animal Scientific Procedures Act 1986 which is from the UK. Since the work is covered by a University of Texas Health Science Center Institutional Animal Welfare Committee protocol number, the authors should change this to the Guide for the Care and Use of Laboratory Animals, or something else specific to the US. – *The section has been amended accordingly.*

2) A list of abbreviations and solutions would be useful. – *we have added the composition of E3, and formaldehyde, which are the only relevant solutions.*

3) In step 2.1.2, are the samples from different time points added to the same 1.5mL tube? Or is each sample added to a different tube? It doesn't make sense to me why all of the samples would be added together. But reading the protocol it isn't clear that they aren't. Assuming there are different tubes for each time point, then 2.1.3 should say tubes plural, rather than tube singular, which reinforces the idea that all the samples go into a single tube.- *They all go into separate tubes, and we have edited the section to improve clarity.*

4) In step 2.2.2 and lines 352-359 the authors define the number of prey capture events as the number of strikes. They acknowledge, in the discussion, that the number of strikes and the number of prey captures aren't necessarily the same. I think it would be useful to include that information here, or at least include a reference to the discussion. – *We have added references to the Discussion in step 2.2.2. and in the representative results section, as suggested.*

5) It's not at all clear what is shown in Figure 2. The authors should have pictures that include both a larva and the fluorescent bacteria. And why is the entire larva, including the eyes, fluorescing in red? – *As explained in the legend, Figure 2 shows a larva preying on paramecia that carry internalized bacteria (small round objects in the frames). Single bacteria would be too small to visualize at this magnification. To visualize the larva movements, we have to use both the red fluorescent channel, as well as a minimal amount of bright field backlighting. The latter leads to the red appearance of the larvae, which is the only way to track both larva and paramecia for these videos.*

6) How do the authors know that the bacteria in Figure 3 are alive? *Dead bacteria, when visualized under the microscope, rapidly lose membrane integrity and thus, fail to contain fluorescence.*

7) Most of the fluorescence in Figure 3 is in intestinal epithelial cells, not in bacteria. It would be useful to include a blow up that shows both the autofluorescent intestinal epithelial cells and the fluorescent bacteria. – *We have added an uninfected control fish to Figure 3, which highlights that there is little to no autofluorescence in the red channel coming from the epithelial cells. The reason the epithelium appears red is because the bacteria are tightly associated with the*

mucosae and, to some extent, invade the epithelium. To highlight this, we have added a series of higher magnification images (Fig. 3D), which show bacterial localization in respect to the intestinal epithelium.

8) The equations in 3.3.1 have several errors. First, there is no step 4.2.10. Second, I don't see how paramecia concentration/dilution factor can equal the dilution factor. Third, there is no step 4.2.6. – *We have amended the formula, to reflect both the correct step numbers as well as the correct algebra.*

9) What is the dosing experiment of step 3.3 that is referred to in 3.3.2? – *We have edited the text to read ' The concentration of paramecia can be adjusted based on the desired bacterial dosage, which is subject to optimization.'*

10) 3.3.3 would be easier to follow if broken up into two steps - first anesthetize the larvae and place them in a well with fresh E3 - and second add the paramecia. Alternatively, first prepare the well with fresh E3 and paramecia - and second add anesthetized larvae. It also needs to be made clear whether the larvae are still anesthetized when they are in the well with the paramecia. I wouldn't think so, as they wouldn't be able to hunt, but this isn't clear from the manuscsript. If the larvae are not anesthetized when they are in the well, why are they anesthetized first? Is this just for handling purposes? And when is the anesthesia removed? This should all be clarified. – *We have edited this step for clarity. The larvae are anaesthetized for ease of handling and to reduce the risk of damage and distress. The target well does not contain tricaine, and care has to be taken to transfer the larvae with a minimal amount of liquid to ensure they recover from anesthesia once they reach the recipient well.*

11) In 3.3.5 are the fish transferred from well to well for the five washes? Or is the tricaine E3 sucked out of the well? Does this matter? Does it matter whether the larvae are anesthetized? – *Yes, and each well has E3 plus tricaine. This minimizes chances to do damage and cause distress to the animal, which we have now added to the text.*

12) More information should be given about the embedding step in 3.3.6. – *We have added more detail regarding the embedding step.*

13) Line 222-223 refer to video 1 which was not made available to review. – *We have added the video to the uploaded files.*

Minor Concerns:
1) Unclear why some of the protocol has yellow highlighting.- *This is a requirement of the journal for video purposes.*

2) What are the units of preying rate? *[s^{-1}]*

3) In 3.1.3, or elsewhere, it would be prudent to say something about appropriate PPE to wear while working with infectious bacteria. – *We have added a note to that effect to the inoculation step.*

4) 3.1.6 refers to 4.1.5. But there is no 4.1.5. – *Amended to 3.1.5.*

5) There is an extra with in 3.1.8.2. – *Thank you, has been removed.*

6) More detail on the washing would be useful in 3.1.8.3. – *We have specified number, volume,*

and media used for washes, as well as centrifugation speed to be used.

7) 3.1.9 has an extra the. – *Thanks, this has been removed.*

8) Why are two flasks used in 3.1.9 and then the contents combined in 3.2.1? – *We use 2 T-25 flasks containing 10 ml each, since this guarantees optimal aeration of the cultures.*

9) In 3.2.6 is the remaining E3 what is left in the tube after removing as much as possible in the previous step? *We edited this and the prior steps to enhance clarity.*

10) What is the final concentration of tricaine used for anesthesia? *For transfer steps, we use 0.1 mg/L of tricaine, and for embedding and long-term procedures 160 mg/L tricaine, as noted in the manuscript.*

Reviewer #3:

Manuscript Summary:
This protocol describes the use of the protozoan Paramecium caudatum as a vehicle for food-borne infection in zebrafish larvae.

Major Concerns:
1. Is there a stable phase of bacteria dose in the paramecia after co-incubation? – *There might be a short (few minute-long) plateau prior to acidification which might be considered a stable phase. However, since this timeframe is very short in comparison to co-incubation and subsequent gut transit time, we found it more practical to consider bacterial half-life using the formulas presented in the manuscript to work out the dose of viable bacteria at any given point during incubation.*
2. Is it considered that the bacteria in the feces could be prey again? – *Over the timeframe of prey exposure (2hrs – gut passage time is more than 1 hr), and considering the relative concentration of bacteria in paramecium vs in feces, this constitutes a negligible contribution.* We have added this consideration to the discussion.
3. Line 304. Counting the dead paramecia. In co-incubation of Paramecia and zebrafish larvae protocol, whether the dead paramecia should been removed? –*The paramecia will be dead because of the formaldehyde fixation at this point, but this number will reflect the concentration of live paramecia for the co-incubation experiment. We have determined that co-incubation with bacteria does not result in significant death of paramecia, but rather in proliferation, so this can be disregarded and no removal is necessary. We have added a note to explain this.*

Minor Concerns:
Explain the E3 medium.- *We have added a description of contents upon first mention.*