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Supplementary Information for

Random encounters and amoeba locomotion drive the predation of *Listeria monocytogenes* by *Acanthamoeba castellanii*

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Materials and Methods

Microfabrication of chemotaxis assay. This section provides details concerning the fabrication steps to facilitate the fabrication of LGG microfluidic chip. Microfabrication began with the preparation of two layers of PDMS (10:1 ratio of base-elastomer to curing agent; cured for 4 h at 70 °C; Sylgard 184): a flat basal layer (25 mm × 25 mm × 1 mm) and a feature layer, molded from an Su-8 resin template on a silicon wafer (25 mm × 20 mm × 5 mm). An inlet and an outlet to each channel were made using a punch (Miltex® Biopsy Punch, 1.5-mm diameter). The layers were plasma-bonded on a glass slide using a plasma oven (Diener electronic, Zepto B model 1 plasma oven), first bonding the base layer to the glass slide and then the feature layer to the base layer. To complete the bonding process, the microfluidic chip was placed for 15 min on an 80 °C hotplate.

Before fabricating the hydrogel walls, the chip was chemically treated to allow the hydrogel to bond to the PDMS. The chip was: 1) rinsed with 1 mL methanol; 2) rinsed with 1 mL deionized water; 3) blow-dried within the channels with low-pressure N_2 gas; 4) filled with 10 wt% benzophenone in ethanol for 10 min; 5) rinsed with 1 mL ethanol; 6) rinsed with 1 mL deionized water, and finally 7) blow-dried within the channels with N_2 gas. Steps 5 and 6 were repeated until there was no white deposit visible that resulted from the recrystallization of benzophenone residues upon exposure to water.

Using a 1000 µL pipette, the microfluidic chip was then filled with the hydrogel solution [10 mL 20% (w/v) acrylamide (Sigma-Aldrich A9099) and 1% (w/v) N,N′-methylenebis(acrylamide) (Sigma-Aldrich 146072) in deionized water with 20 mg/mL 4-(2-hydroxyethoxy)phenyl 2-hydroxy-2-propyl ketone (Irgacure D-2959; Sigma-Aldrich 410896)]. A base solution of 20% (w/v) acrylamide and 1% (w/v) N,N′-methylenebis-(acrylamide) can be prepared well in advance and stored in a 4°C fridge. With the addition of 4-(2-hydroxyethoxy)phenyl 2-hydroxy-2-propyl ketone, the hydrogel polymerization can occur also at daylight. It is therefore recommended to make small batches of the hydrogel solution prior to polymerization. Filling the microfluidic chip using a 1000 µl pipette should be done in a careful and controlled manner, slowly pressing in the plunger of the pipette to avoid the creation of air pockets. Air-pockets will reduce the effectiveness of the polymerization as the initiator 4-(2-hydroxyethoxy)phenyl 2-hydroxy-2-propyl ketone will consume oxygen when being exposed with UV-light.

To stop flow within the chip in order to facilitate hydrogel polymerization, the inlets and outlets to all three channels were plugged using PDMS-filled tubing (Cole-Parmer Tygon® Tubing PG-06419-01). This step can also help in removing air pockets by pressing the plugs a little deeper into the chip. Optimally the plugs should not be pushed into the in- and outlets to deep as they will be harder to remove and sudden pressure changes at the end of the polymerization process might damage the hydrogel wall.

The hydrogel walls were then polymerized within the microfluidic chip by exposing the required regions to UV light by the use of a 20× objective (Nikon CFI S Plan Fluor ELWD 20XC) on a Nikon T*i* microscope with a DAPI cube (EX: 350/50 nm; Chroma 49025) connected to a metal halide fluorescent light source (Prior Lumen 200) at 100% power. The fluorescent pathway diaphragm (octagon) was adjusted to have a width of 500 µm (the intended width of the hydrogel walls) using a fluorescein sodium salt solution (10 mM in DI water; Sigma Aldrich F6377) to visualize the illuminated area. The area to be exposed was marked using the lasso tool from the 'Area and Annotations' toolset in NIS elements. The 'ND acquisition' tool was used to automatically move the stage (Prior Scientific Instruments, H117P222/F stage connected to HH31XYZE stage controller) during polymerization the length of each wall at a speed of 15 µm/s for the first run and 90 µm/s for the second run. To make the stage travel between the two-hydrogel wall anchor points, we saved the locations of each anchor point within the 'XYZ'-positions tab of the 'ND acquisition' tool. An optimization point that can be implemented here is to alter the physical position of the chip on the microscope stage to reduce the difference in x-values or y-values (depending on the orientation of the polymerization pathway) between the two anchor points to a minimum. This optimization step is useful as the microscope stage we used was not very effective making lateral changes along its path of travel. The speed of the stage can be changed within NIS elements by going to the Devices >> My devices >> XY-drive. In our case the stage operated at a speed of 12000 µm/s by default. For our purpose, the stage speed was changed to 15 µm/s for the first polymerization run and 90 µm/s for the second run. When polymerizing multiple walls within one device, it is highly recommended to change the stage speed to default between each runs, in order to avoid excessive waiting times when altering stage positions.

Prior to storage, the PDMS-filled plugs blocking inlets and outlets were removed. The remaining unpolymerized hydrogel solution was extracted carefully using a 1000 µL pipette. The microfluidic chip was then filled with deionized water and stored at 4 °C until use.

Data availability.
The data used to create all plots can be found under the following link: (TBA)

Python scripts. This section will provide more details and links to the scripts used in the analysis of chemotaxis and capture rate experiments.

1) The script themselves are available at the following GitHub repository: https://github.com/FdeSchae/Acanthamoeba-Listeria
   Each script file has extra annotations behind each coding line explaining the function of the code in detail.
2) **TrackPy parameters chemotaxis assays**
   dia = 9 # diameter: approximate diameter of the particles to be located
   MinMass = 0 *# Minimum mass: the minimum integrated brightness/darkness (filter to remove* spurious objects)
   Nsize = 1 *# noise size: width of the Gaussian blur filter to remove noise*
   Msize = 17 *# maximum diameter size (in pixels) of the particles to be located*
   Pmem = 15 *# particle memory: the maximum number of frames during which a feature can vanish, then reappear nearby, and be considered the same particle.*
   TrackTresh = 25 *# minimum number of points frames for a trajectory to survive*
3) **TrackPy parameters capture rate experiments**
   dia = 11 *# diameter: approximate diameter of the particles to be located*
   MinMass = 600 *# The minimum integrated brightness*
   MaxMass = 2000 *# The maximum integrated brightness*
   Nsize = 1.5 *# Gaussian blur kernel*
   MaxDia = 21 *#maximum diameter: approximate maximum diameter of the particles to be located*
   Pmem = 17 *# Particle memory: the maximum number of frames during which a feature can vanish, then reappear nearby, and be considered the same particle.*
   TrackTresh = 17 *# Minimum number of points (video frames) to survive*
   MaxT = 10 *# Maximum amount of pixels a bacterium can move in between two consecutive frames*

Manual tracking of individual GFP-expressing *L. monocytogenes* trapped on the outer surface of *A. castellanii*. This section provides a detailed tutorial on how to track GFP expressing *L. monocytogenes* within ImageJ.

1) Open the recorded raw video file (Trapped_Lmo_tracking_2020.lif) in ImageJ by go in to File >> Open and select the required file. Bio-Formats Import Options will open after selecting the file, make sure to select Hyperstack under Stack viewing >> View Stack with; Composite under Color options >> Color mode and activate the Autoscale option under Color options. Press OK.
2) A new window opens with a list of recordings, select one of the recorded time-lapses for tracking purposes. Press OK.
3) The selected recorded opens in a window. Save the video as an uncompressed .avi file with a frame rate of 20 fps, using File >> Save as. The
4) Reopen the .avi file in ImageJ, and make sure no options (such as virtual stack) are activated.
5) Select an *Acanthamoeba* that you would like to track. Find the first frame where a GFP labelled *L. monocytogenes* (bright green dots) is trapped on the outer surface of the *selected Acanthamoeba.*
6) Select the Oval tool and draw a circle on the trapped bacterium. Use the color picker tool to change the color. Finally press D on your keyboard to flatten the label on the frame.
7) Go to the following frame, you can use the arrows left and right of the slider bar underneath open frame to change frames. The circle you drew should still be in the same place. If the position of the trapped bacterium changes, you can use the arrow keys on your keyboard to move the circle. Press D on your keyboard to flatten the label on the frame. Repeat this step until the targeted trapped bacterium ended up in a backpack.
8) In each recording, one *Acanthamoeba* was selected and up to 5 bacteria were labelled.

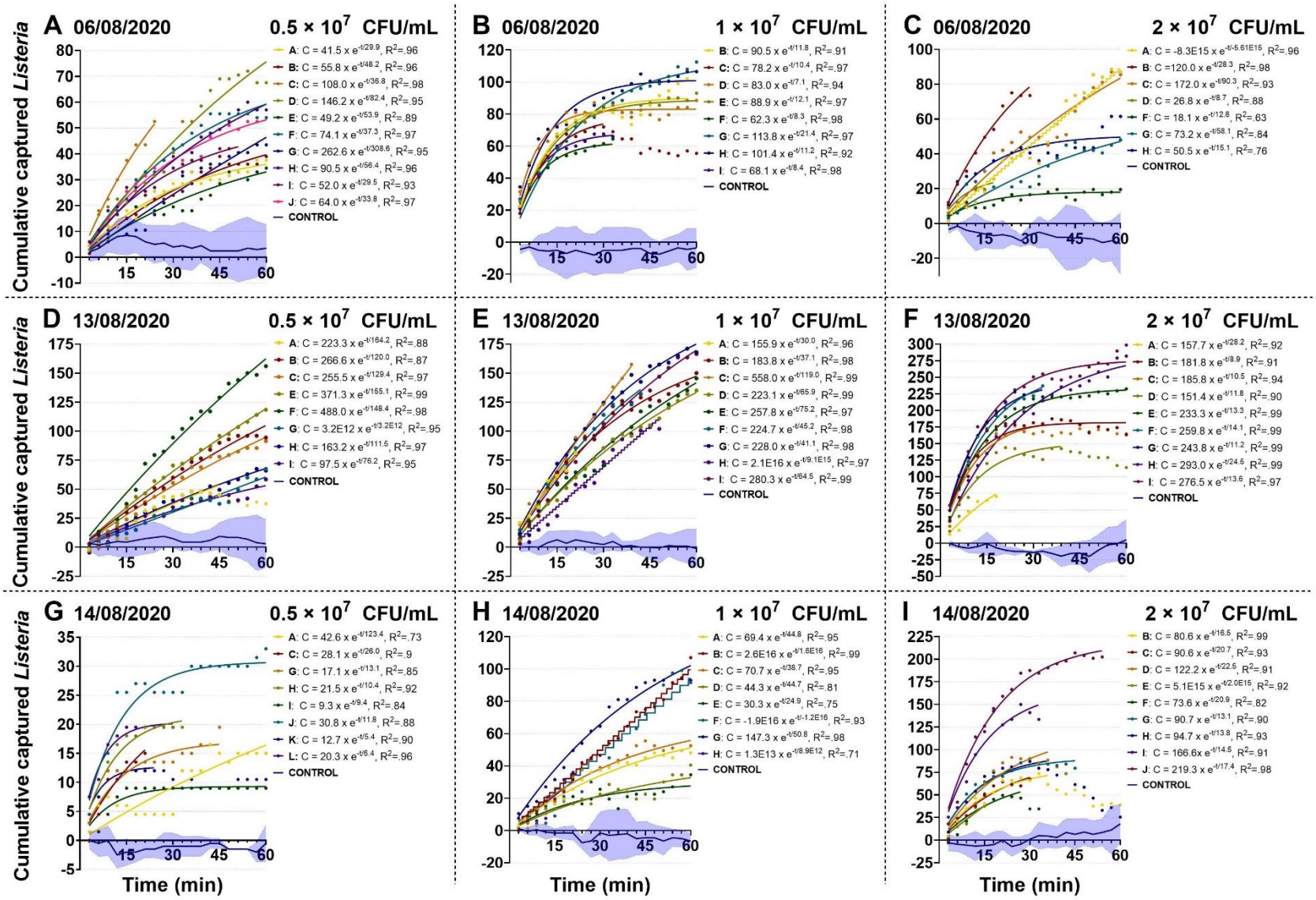
Visualization of backpacks.

A. castellanii cells (see culture conditions) were gently scraped from the bottom of the flask with one-directional movement using a cell scraper (Bioswisstec) and diluted with PYG broth to a concentration of approximately 5×10^4 cells/mL.

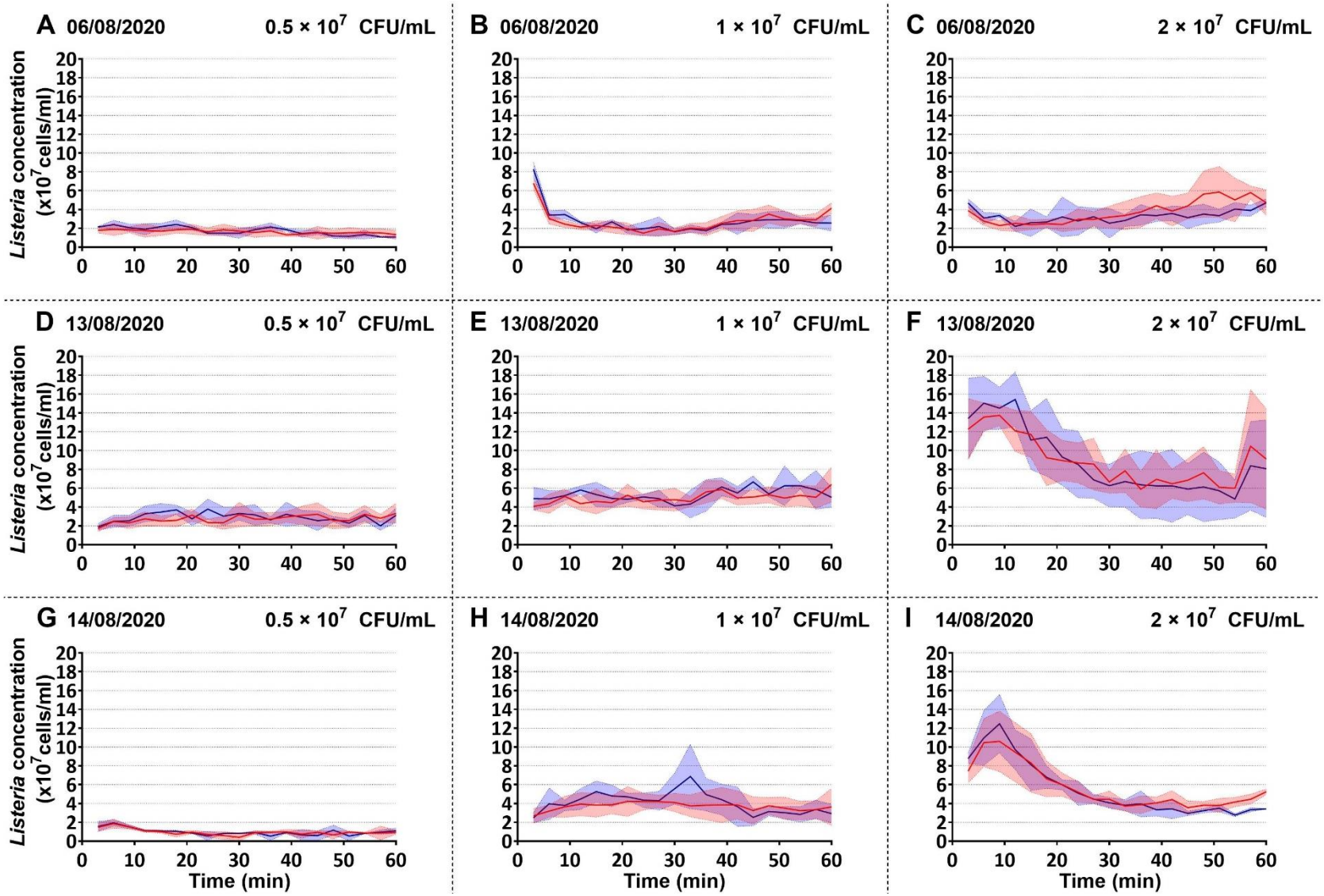
For confocal laser scanning microscopy (TCS-SPE, Leica Microsystems), 200 µL of *A. castellanii* suspension was added into a well of an 8-well IbiTreat µ-Slide (Ibidi, Germany) and left for 20–30 min to allow cells to attach to the lower surface. In case of the backpack visualization in PAS buffer broth (Fig. 1*A,B,C*), the settled amoebae were washed three times in total with 200 µL PAS buffer. The amoebae were then covered with 200 µL suspension of pre-washed *L. monocytogenes* Scott A::pPL2P$_{hyp}$*gfp* in PYG buffer, at a concentration of 2×10^7 CFU/mL (see culture conditions). Images of *A. castellanii trophozoites* were captured at 15 min, 30 min and 1 h after the start of co-incubation. In case of the backpack visualization in PYG broth (*SI appendix*, Fig. S8*A,B,C,D*), settled amoebae were washed three times with 200 µL PYG broth instead. The amoebae were then covered with 200 µL suspension of pre-washed *L. monocytogenes* Scott A::pPL2P$_{hyp}$*gfp* in PYG buffer, at a concentration of 2×10^7 CFU/mL (see culture conditions). Images of *A. castellanii trophozoites* were captured at 30 min and 1 h after the start of co-incubation. In case of the backpack visualization using starved *A. castellanii* trophozoites, settled amoebae were washed ten times with 200 µL PAS buffer. After the final wash, the amoebae were starved for 24 h at room temperature in 200 µL PAS buffer. After starvation, the PAS buffer was replaced with a 200 µL suspension of pre-washed *L. monocytogenes* Scott A::pPL2P$_{hyp}$*gfp* in PAS buffer at a concentration of 2×10^7 CFU/mL (see culture conditions). Images of *A. castellanii trophozoites* were captured at 1 h after the start of co-incubation (*SI appendix*, Fig. S8*E,F,G*). All images were taken with a confocal laser scanning microscopy (TCS-SPE, Leica Microsystems) using a 100× objective (Leica HCX PL FLUOTAR Oil 100×) and an excitation wavelength of 488 nm and an emission range of 500–570 nm.

For scanning electron microscopy (SEM), a droplet of *A. castellanii* suspension was placed on a carbon-coated glass disk (12-mm diameter, Menzel Gläser, VWR), and left for 20–30 min to allow cells to attach to the surface. Attached amoebae were washed three times in total with PAS buffer. A droplet of pre-washed *L. monocytogenes* Scott A wild type in PAS buffer, with a concentration of 2×10^7 CFU/mL, was added and left for 1 h to allow backpacks to form. The co-culture was fixed for 1 h with 2.5% glutaraldehyde (diluted from 50% glutaraldehyde, EM Grade, Chemie Brunschwig, Switzerland) in phosphate buffered saline (PBS, pH 7.4, ThermoFisher). After washing with PBS buffer three times, the sample was post-fixed with 1% OsO_4, dehydrated with increasing concentrations of ethanol (50%, 75%, 90%, 98%, 100% in milliQ water) and critical-point dried with liquid CO_2 (CPD 931, Tousimis, USA). The specimen was then painted with silver glue for good contact and conductivity, followed by sputter coating with 7 nm Pt–Pd using a CCU-010 Metal Sputter Coater (Safematic, Switzerland). Images were obtained using a Zeiss Merlin scanning electron microscope (Carl Zeiss Meditec AG, Germany).

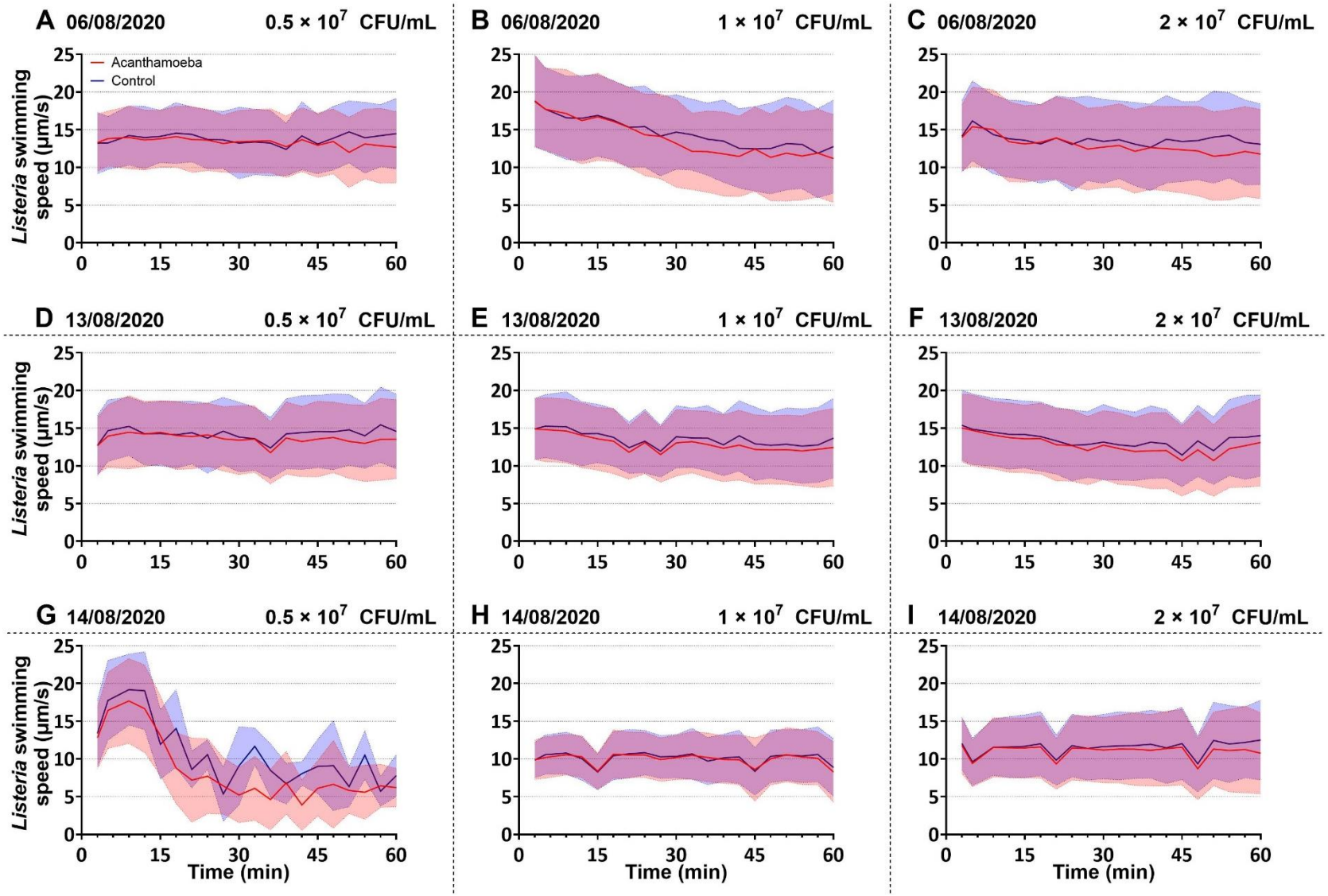
Supplementary Figures



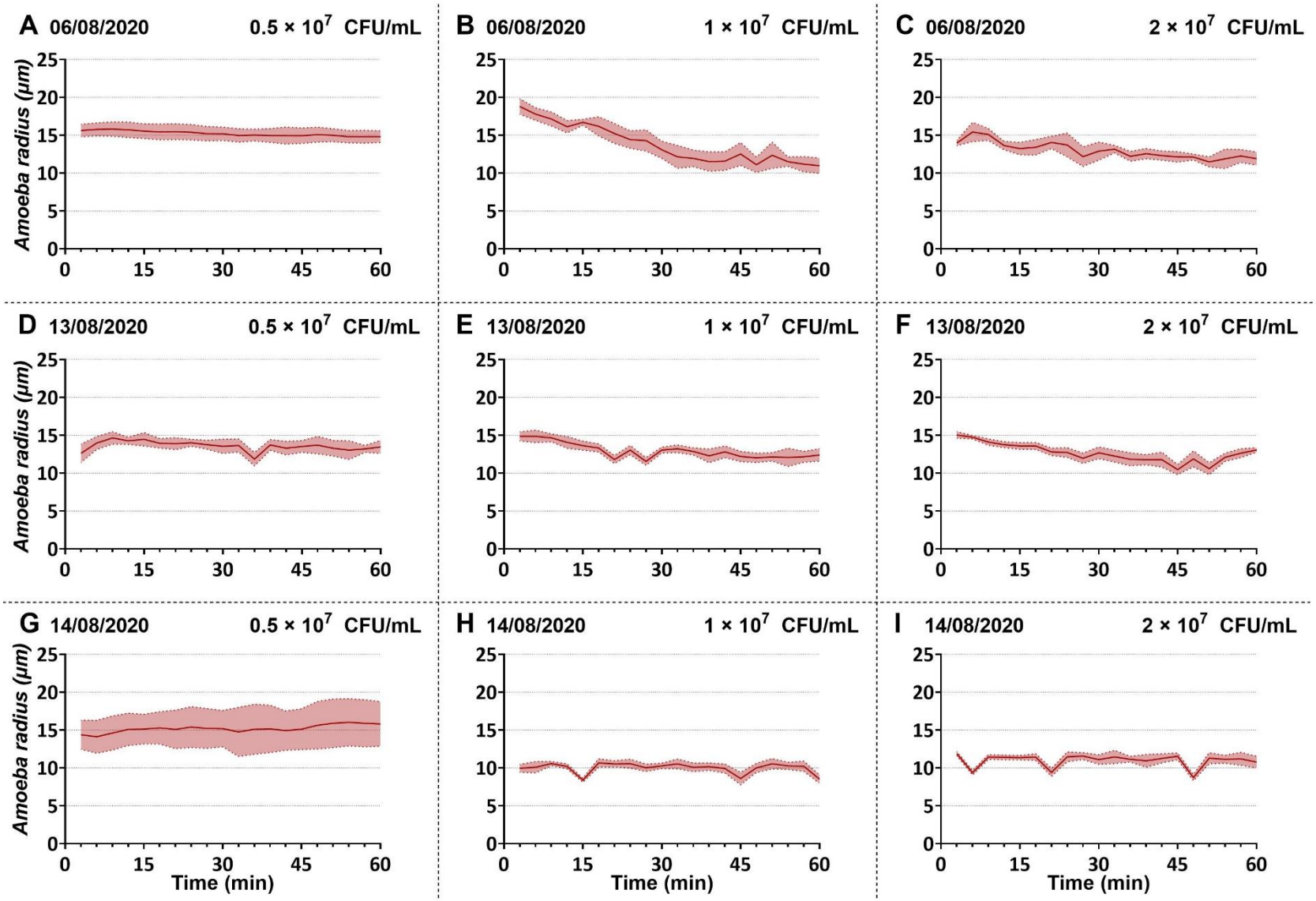
Supplementary Figure 1. Cumulative capture plots of *L. monocytogenes* by *A. castellanii*. Each panel (*A*–*H*) represents one experiment, labelled with the initial *L. monocytogenes* concentration and date. Within each panel, experimentally derived cumulative capture curves for each *A. castellanii* trophozoite (color codes given in legend) are plotted as a function of time (dots) and are accompanied by their saturating exponential regressions (lines of same color). The legend also contains the equation of the best fitting regression for each *A. castellanii* trophozoite, together with the R^2 value of the fit. Average cumulative capture curves for the digital controls $n = 3$ ("Control" in the legend) are displayed as a dark blue line, with shading indicating the standard deviation.



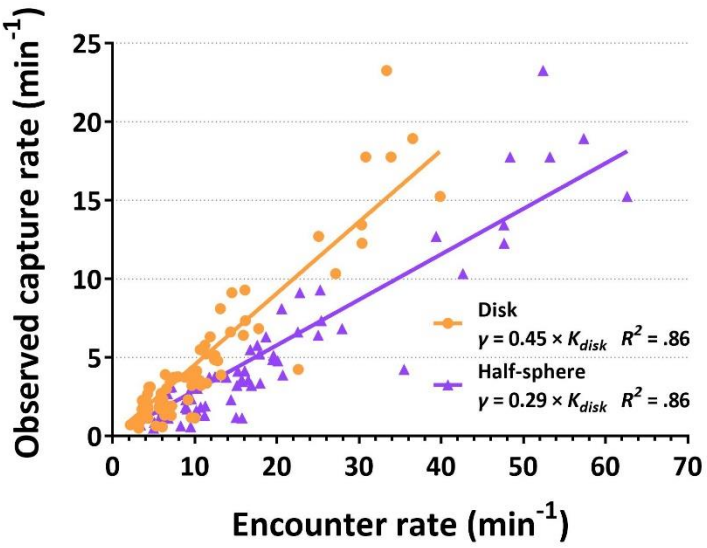
Supplementary Figure 2. Concentration of *L. monocytogenes* over time determined in capture rate experiments. Each panel (*A*–*H*) represents one experiment, labelled with the initial *L. monocytogenes* concentration and date. Curves show the mean concentration of *L. monocytogenes* as a function of time, within the regions of interest (350 × 350 pixels or 113.75 × 113.75 µm) surrounding tracked *A. castellanii* trophozoite (red) and surrounding digital control areas containing no *A. castellanii* (blue). Shading indicates the standard deviation, in each case for $n = 3$ control regions and $n = 7$ to 10 for *Acanthamoeba* regions. No difference was observed between regions of interest surrounding individual *Acanthamoeba* cells and digital control regions.



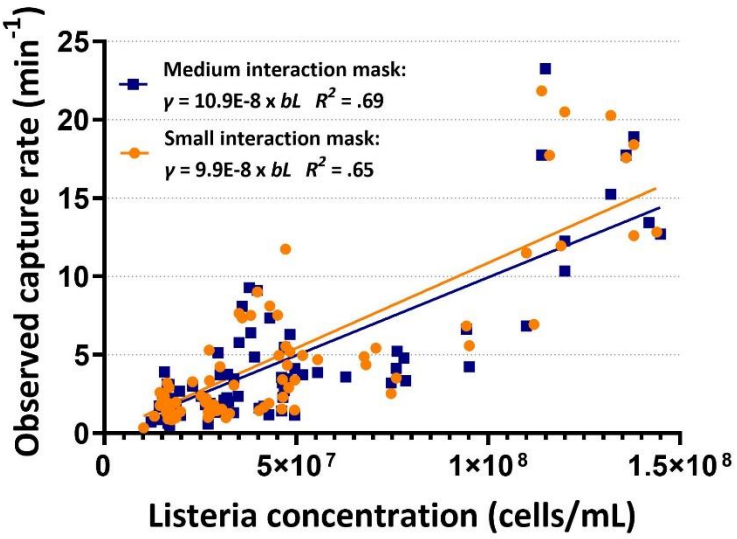
Supplementary Figure 3. Swimming speed of *L. monocytogenes* cells over time in the capture rate experiments**.** Each panel (*A*–*H*) represents one experiment, labelled with the initial *L. monocytogenes* concentration and date. Curves show the average swimming speed of *L. monocytogenes* as a function of time, within the regions of interest (350 × 350 pixels or 113.75 × 113.75 µm) surrounding an *A. castellanii* trophozoite (red) and surrounding the digital control areas containing no *A. castellanii* cell (blue). Shading indicates the standard deviation, in each case for $n = 3$ control regions and $n =$ 7 to 10 for *Acanthamoeba* regions. No difference was observed between regions of interest surrounding individual *Acanthamoeba* trophozoites and digital control regions.



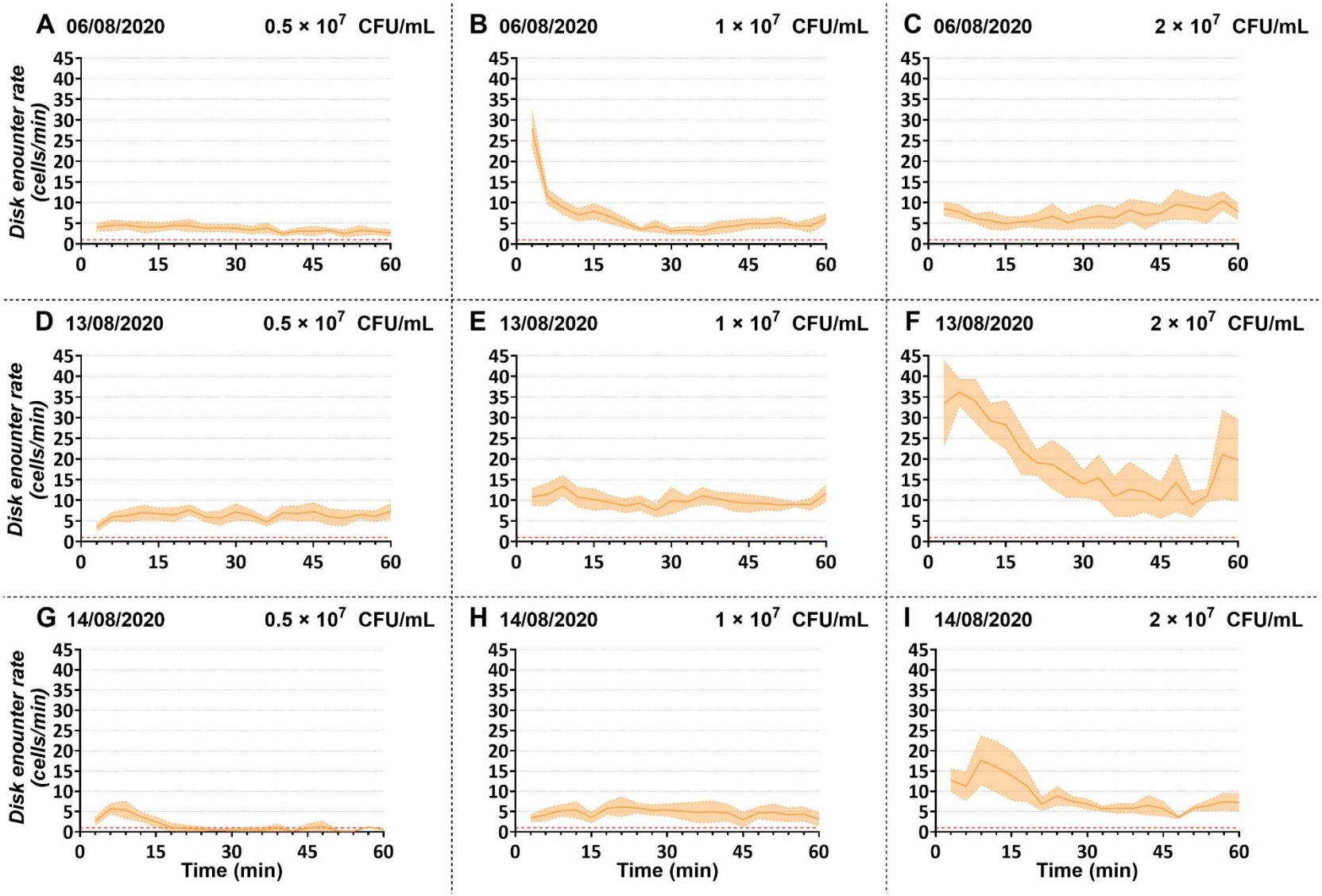
Supplementary Figure 4. Radius of *A. castellanii* trophozoites over time in the capture rate experiment. Each panel (*A*–*H*) represents one experiment, labelled with the initial *L. monocytogenes* concentration and date. Curves show the average radius of tracked *A. castellanii* (red line) as a function of time, with the standard deviation shown by shading. Sample size corresponds to the number of labelled trophozoites in Fig. S1.



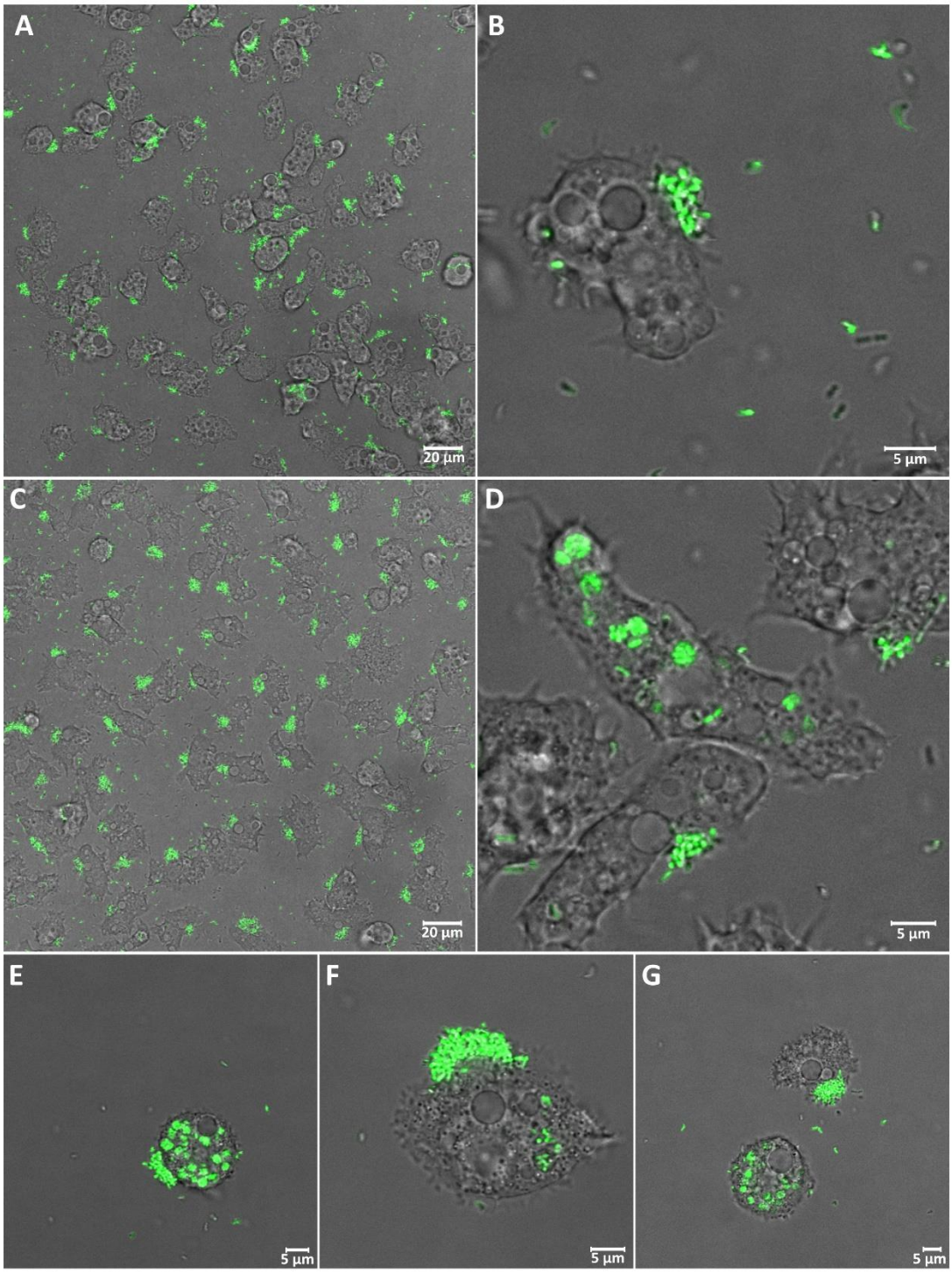
Supplementary Figure 5. Observed capture rates, γ (equation [2] using a small interaction mask), of individual *A. castellanii* trophozoites plotted as a function of the theoretical encounter rates calculated using the model for a disk (orange circles) or for a half-sphere (purple triangles) — that assume random bacterial encounter and perfect adsorption (see Results). The two models were parameterized for each *A. castellanii* trophozoite using values for *Listeria* concentration, *Listeria* swimming speed, and *A. castellanii* radius quantified from individual experiments. Solid lines represent linear regressions for each model in the respective color (Disk encounter model (orange): γ = 0.50 x b_L with R^2 = 0.90; medium interaction mask data (blue): γ = 0.32 x b_L with R^2 = 0.90).



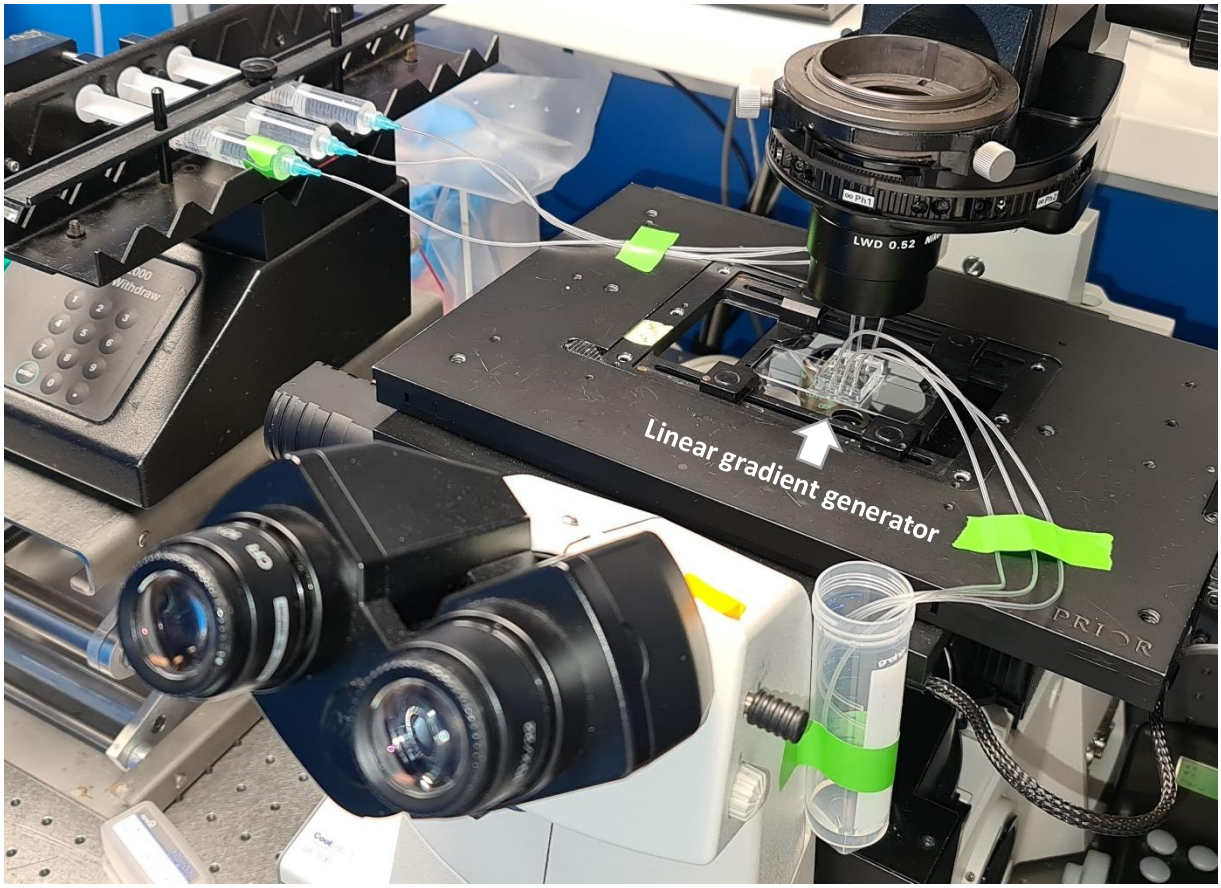
Supplementary Figure 6. Observed capture rates, γ (equation [2]), of individual *A. castellanii* trophozoites plotted as a function of the average *L. monocytogenes* concentration in the region of interest surrounding each trophozoite (dots and squares). Orange dots represent data for the small interaction mask, while blue squares represent data for the medium interaction mask. The solid lines represent linear regression fits (small interaction mask data (orange): C = 1.1E-7 x b_L with R^2 = 0.65; medium interaction mask data (blue): C 9.9E-8 x b_L with R^2 = 0.69).



Supplementary Figure 7. Disk encounter rates K_{disk} based over time in the capture rate experiment (based on the medium interaction mask). Each panel (*A*–*H*) represents one experiment, labelled with the initial *L. monocytogenes* concentration and date. Curves show the average disk encounter rate (orange) as a function of time, with the standard deviation shown by shading. The red dotted line represents an encounter rate of 1 cells/min. Sample size corresponds to the number of labelled trophozoites in Fig. S1.



Supplementary Figure 8. Confocal laser scanning micrographs of *A. castellanii* trophozoites co-incubated with GFP-expressing *L. monocytogenes* (strain Scott A::pPL2P_{hyp} *gfp*) for two scenarios: (A-D) co-incubation in PYG broth, images were taken after 30 min (A,B) and 60 min (C,D); (E-G) co-incubation in PAS buffer using starved *A. castellanii* trophozoites, images were taken after 1 h. (*SI appendix*: Materials and Methods: Visualization of backpacks)



Supplementary Figure 9. The experimental setup used in the chemotaxis assays. More specifically, this image depicts the preloading step of the hydrogel wall with the respective sample prior to image acquisition. Three syringes were mounted on a syringe pump (upper left corner): one syringe was loaded with the respective sample (marked with green tape) and connected to one side channel of the microfluidic linear gradient generator (marked with a white arrow); the other two syringes were loaded with PAS buffer and connected to the other side channel and to the central channel (Fig. 2A). Outlets were connected via tubing to an empty 50 mL Falcon tube to collect waste liquid.

Supplementary Movies

Supplementary Movie 1. Quantification of capture dynamics. An *A. castellanii* trophozoite imaged for 150 s to track captures of motile *L. monocytogenes* (visible as small black dots). Directly on top of the *Acanthamoeba* is a white circle representing the *Acanthamoeba* mask that was used to remove detected particles that impaired the calculation of trajectories by the image analysis script. *Acanthamoeba castellanii* is surrounded by a light grey area representing the interaction mask that was used to count the entry and exit of *L. monocytogenes* cells, and thereby estimate the cumulative number of captured cells.

Supplementary Movie 2. Trapped *L. monocytogenes* are aggregated in a backpack on a moving *Acanthamoeba*. The movie shows the capture and aggregation of motile *L. monocytogenes* cells into a backpack by a moving *Acanthamoeba* trophozoite. The same example is presented as a time series of images in Fig. 4*A* ("moving"). *Listeria monocytogenes* cells are visible as bright green rods. Trapped *L. monocytogenes* that were tracked over time are marked with colored circles.

Supplementary Movie 3. Backpack formation was not observed on non-moving *Acanthamoeba*. The movie shows the capture of motile *L. monocytogenes* cells by a non-moving *Acanthamoeba* trophozoite, but these bacteria are not then aggregated into a backpack. The same example is presented as a time series of images in Fig. 4*A* ("non-moving"). *Listeria monocytogenes* cells are visible as bright green rods. Trapped *L. monocytogenes* that were tracked over time are marked with colored circles