

**Characterizing Innate Immune Cell Trajectories and Microbial Community
Invasion Dynamics in Larval Zebrafish**

by

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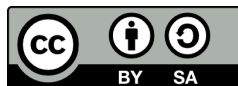
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DISSERTATION ABSTRACT

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Doctor of Philosophy in Physics

Title: Characterizing Innate Immune Cell Trajectories and Microbial Community Invasion Dynamics in Larval Zebrafish

The gastrointestinal tract harbors dense microbial communities that profoundly influence host physiology, yet our understanding of the spatial and temporal dynamics governing host-microbe interactions remains limited. This dissertation employs light sheet fluorescence microscopy and quantitative image analysis to characterize both innate immune cell behavior and bacterial population dynamics in the larval zebrafish gut.

The first study examines how neutrophils and macrophages perform immune surveillance around the intestine. Using dual-transgenic zebrafish and three-dimensional tracking over four-hour periods, I find that individual immune cells maintain remarkably stable motility states, remaining either predominantly motile or non-motile throughout observation rather than frequently transitioning between behaviors. Gut architecture exerts dominant influence over migration patterns: both cell types preferentially move along the intestinal anterior-posterior axis and maintain high probability of remaining gut-associated. Notably, motile macrophages display significantly reduced sphericity compared to non-motile macrophages, providing a morphological signature of motility state visible in static

snapshots. These surveillance patterns remain consistent across germ-free, conventionally reared, and pathobiont-colonized fish, indicating that tissue structure rather than microbial signals primarily determines baseline immune cell dynamics.

The second study investigates how a zebrafish-native *Vibrio* strain equipped with a Type VI Secretion System (T6SS) invades pre-established gut bacterial communities of varying complexity. Species identity emerges as a primary determinant of invasion outcome, with striking differences in susceptibility among founder species. Community complexity systematically attenuates invasion magnitude: three-species communities exhibit generally weaker suppression than single-species associations, revealing emergent resilience that cannot be predicted from pairwise interactions alone. Unexpectedly, a T6SS-deficient mutant produces suppression effects comparable to wild-type *Vibrio*, indicating that resource competition, motility, and other T6SS-independent mechanisms dominate invasion dynamics within the gut environment.

Together, these studies reveal that spatial organization and temporal dynamics in the gut are governed by biophysical principles including tissue architecture, cellular mechanics, and ecological interactions. The quantitative frameworks and open-source computational tools developed here enable translation to other model systems, contributing toward a general understanding of host-microbe interactions applicable from zebrafish to humans.

This dissertation includes previously published/unpublished co-authored material.

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DEDICATION

My Ma and Papa: नीलम और रमा रमण अमिताभ

*The curiosity you nurtured, the sacrifices you made, and the love you gave across the distance
have carried me further than I ever imagined.*

My Wife: हविषा खुराना

*You have been my anchor through frustrating setbacks, and the quiet triumphs that no one else
saw. Your belief in me never wavered, even when my own did. I could give my best as I knew you
were always there to support me.*

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(A) Experimental timeline. Germ-free zebrafish embryos were derived at 0 days post-fertilization (dpf) and inoculated with founder bacterial species at 4 dpf. After 24 hours of founder colonization, invader species were introduced at 5 dpf. Larvae were then euthanized at 6 dpf for gut dissection and bacterial quantification. **(B)** Experimental workflow and bacterial quantification methods. **Left:** A 6 dpf larval zebrafish, with dashed outline showing the gut. Scale bar = 500 μm (adapted from [2]). Larval guts were dissected and homogenized, then plated on HiChrome UTI agar for species-specific enumeration. **Middle:** Representative petri dish shows distinct colony morphologies for the five bacterial species studied: Acinetobacter (AC, milky

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Figure 3.2

Gut bacterial abundance across different founder and invader combinations **(A)** Total bacterial abundance (founders + invaders) across all experimental conditions. Each circle represents an individual larval zebrafish; black squares show mean $\pm$ 95% confidence interval. Total abundance remains consistent at $\sim 10^4$ CFU/gut regardless of species composition except for mono-association with AC which shows lower colonization levels. **(B)** Total founder abundance shows systematic suppression following *Vibrio* invasion. Founder-only controls (first seven conditions) maintain 10^4 – 10^5 CFU/gut with AC being the only exception with $\sim 10^{2.5}$ CFU/gut. Invaded communities (remaining conditions) show 1–3 orders of magnitude reduction in founder abundance with some specimen showing extinction events of founder species with the points corresponding to zero CFU/gut. **(C)** Total invader abundance is remarkably uniform across all founder variations. Founder-only controls (first seven conditions) correctly show no invading species abundance. In the invaded groups, both *Vib* WT and *Vib* dT6SS colonizes to $\sim 10^4$ CFU/gut independent of founder community composition. 89

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CHAPTER I

INTRODUCTION

1.1 Immune surveillance in vertebrates

Microorganisms are ubiquitous partners of multicellular life. Trillions of bacteria, archaea, fungi, and viruses inhabit the surfaces and interior niches of animals and plants, forming communities whose influence extends far beyond passive coexistence [3]. These microbial partners shape host development, metabolism, and physiology in ways that are only beginning to be understood [4,5]. Maintaining productive relationships with beneficial microbes while defending against pathogens requires sophisticated surveillance systems. In vertebrates, this balance is achieved through continuous monitoring by immune cells that patrol tissues, recognize microbial signals, and calibrate responses accordingly [6,7].

Innate immune cells, primarily neutrophils and macrophages, execute both defensive and homeostatic functions through continuous tissue surveillance [7,8]. These functions require precise spatial positioning and coordinated movement. Neutrophils provide rapid antimicrobial responses through phagocytosis, generation of reactive oxygen species, and formation of neutrophil extracellular traps [9,10]. Macrophages phagocytose bacteria and cellular debris while secreting numerous cytokines that modulate tissue inflammation [11,12]. However, the efficacy of these cellular responses depends critically on where

immune cells are positioned relative to epithelial barriers and microbes, how they move through tissue architecture, and when they arrive at sites requiring surveillance or intervention.

This gap raises fundamental questions about immune cell dynamics: How do immune cells perform surveillance in complex, three-dimensional tissue environments? Where do they position themselves relative to epithelial surfaces and resident microbes? What determines their movement patterns—do they patrol randomly, or follow stereotyped routes? When microbial populations change, whether through colonization by pathogenic species or shifts in resident community composition, how do immune cell behaviors respond spatially and temporally?

Answers to these questions have been elusive. *In vitro* studies have characterized fundamental properties of immune cell migration, including the transition between mesenchymal and amoeboid modes in response to mechanical constraints and the distinct mechanisms governing movement through two-dimensional versus three-dimensional environments [13,14]. Intravital microscopy has extended these observations to living mammals, visualizing immune cells within small tissue volumes and revealing migration dynamics, pathogen interactions, and coordination of immune responses across diverse inflammatory contexts [15,16]. However, comprehensive characterization of immune cell trajectories spanning entire organ segments over physiologically relevant timescales has remained lacking. Extracting kinetic parameters such as velocities and directional persistence [17,18], morphological states, and their relationships to tissue architecture and

microbial distributions across whole organs would address the surveillance questions posed above.

Understanding these immune cell dynamics is critical for several reasons. First, dysregulation of immune cell positioning and trafficking contributes to inflammatory diseases, and elucidating normal surveillance patterns could inform therapeutic strategies aimed at restoring immune homeostasis [19]. Second, the spatial distribution of immune cells determines which tissue regions are actively monitored, how rapidly cells can detect and respond to microbial perturbations, and whether surveillance is uniform or concentrated at vulnerable interfaces [20]. Third, the physical behaviors of immune cells—their speeds, directional biases, and positional preferences—represent integrated outputs of multiple biochemical signals and mechanical constraints. Quantifying these migration parameters may provide insights into cellular functional states and the local tissue microenvironments in which cells operate. Among the diverse tissues requiring immune surveillance, those with high microbial loads present particularly complex challenges, as immune cells must distinguish between beneficial residents and potential threats while operating in crowded, mechanically and chemically heterogeneous environments.

1.2 The gut as a central site of host-microbe interaction

The gastrointestinal tract (gut) exemplifies the challenge to the host immune system: maintaining tolerance to trillions of commensal bacteria while retaining the capacity to rapidly detect and eliminate pathogens [7,20]. In a healthy gut, immune cells maintain a

state of “restrained activation,” responding to bacterial signals without triggering destructive inflammation [21,22]. When this balance fails, either through excessive immune activation or insufficient responses, it results in diseases such as inflammatory bowel disease [19,23,24] or enteric infections [25].

The magnitude of this challenge reflects the gut’s exceptional microbial density. Just in the colon, proximal part of the gut, bacterial populations reach approximately 10^{11} cells per gram of luminal content [26,27], the highest densities found anywhere on or in the body. These resident microbes (collectively termed the gut microbiota) have attracted intense scientific and public interest in recent decades due to discoveries linking them to an unexpectedly broad range of host physiology and health outcomes [28]. Beyond intuitive roles in nutrient digestion and metabolism [4], gut bacteria have been shown to modulate immune system development and function [5,8], influence brain function and behavior [29–32], and contribute to susceptibility to diseases ranging from inflammatory bowel disease to metabolic disorders [33,34]. These connections demonstrate that the gut microbiota functions not merely as a passive collection of resident microbes, but as an active participant in host biology.

Similar host-microbe partnerships have been documented across the tree of life. Specific bacterial strains enable bobtail squid to produce bioluminescence for camouflage [35] and direct the formation of nitrogen-fixing root nodules in leguminous plants [36]. Understanding how these host-microbe systems assemble, persist, and function has thus become a central challenge in biology and medicine.

1.3 Spatial organization matters for gut biology

Most research on gut microbiota has focused on identifying *which* microbial species are present and *what* molecular pathways mediate host-microbe communication [37,38]. These approaches have revealed sophisticated biochemical dialogues: bacteria produce metabolites that signal to host cells, secrete factors that modulate immune responses, and compete through the deployment of antimicrobial compounds [39,40]. While these molecular studies have been enormously productive, they typically analyze homogenized tissue samples, fecal material, or cultured populations collected at single timepoints, losing information about *where* interactions occur within the gut and *how* they unfold over time. Yet spatial organization and temporal dynamics are likely to be critical: host tissues exhibit complex three-dimensional architecture, and both microbial communities and host cells are constantly in motion.

The importance of spatial structure in biological systems has long been recognized across scales, from millimeter-scale biofilms [41] to landscape-level ecosystems [42]. In the gut, bacteria are not uniformly distributed, exhibiting distinct localization patterns along the intestinal length and across the radial dimension from lumen to epithelium [24,43,44]. These patterns arise from multiple physical processes: fluid flow generated by peristaltic contractions transports motile and aggregated bacteria differently, mucus layers create chemical gradients and physical barriers [45], and epithelial crypts provide sheltered microenvironments [46]. The spatial distribution of bacteria, in turn, determines which host

cells encounter which microbial stimuli, potentially shaping immune responses and other host functions.

1.4 Larval zebrafish as a model system

Understanding spatial and temporal organization in the gut requires methods that can track both microbial and host cells throughout entire organs over physiologically relevant time-scales in living animals. The traditional compositional analysis approaches described above (analyzing biopsies, fecal samples, or homogenized tissues) fundamentally cannot capture spatial and temporal dynamics. Even repeated sampling provides only snapshots that cannot reveal continuous motion or transient interactions. *In vitro* models offer experimental control but lack the complex three-dimensional architecture, chemical milieu, and living tissue dynamics of actual intestines [47,48]. Larval zebrafish (*Danio rerio*) uniquely address these limitations through their optical transparency and genetic tractability. Light sheet fluorescence microscopy enables non-invasive, high-volume imaging of entire intestinal volumes with cellular resolution and minimal phototoxicity [49,50], allowing continuous observation over hours.

The larval gut, while simplified compared to mammalian intestines (lacking a stomach and consisting of a single epithelial tube), shares fundamental features including peristaltic motility, mucus secretion, and colonization by complex bacterial communities [51–53]. Critically, zebrafish also develop a vertebrate-typical immune system that reflects the basic organization found in other jawed vertebrates, including humans, which provides

a relevant framework for studying host-microbe interactions. This immune system appears early in development, and by 48 hours post-fertilization larvae already possess functional neutrophils and macrophages that can respond to microbial cues [54–56]. The availability of transgenic lines expressing fluorescent reporters in specific immune cell populations further supports gentle, real-time visualization of multiple cell types within the living intestine [57,58].

1.5 Overview of dissertation chapters

This dissertation examines the spatial dynamics of host-microbe interactions in the larval zebrafish gut, addressing both sides of this relationship: the movement patterns of host immune cells and the population dynamics of invading bacteria. Sections of this dissertation contain material previously published or prepared for publication. Chapter II is under review in PRX Life with co-author Raghuveer Parthasarathy. Chapter III describes ongoing collaborative research conducted with Vivian S. Winkler under the supervision of Principal Investigator Raghuveer Parthasarathy.

1.5.1 Chapter II: Immune cell surveillance dynamics

Chapter II focuses on characterizing how innate immune cells patrol the gut environment. Despite extensive knowledge of immune cell responses to purified stimuli *in vitro*, we lack quantitative understanding of how neutrophils and macrophages move within living gut tissue over extended periods. What are the typical movement patterns? Do cells

maintain consistent behavioral states or frequently switch between modes? How do tissue architecture and bacterial presence influence these dynamics?

Using dual-transgenic zebrafish expressing fluorescent markers for neutrophils (Tg(*mpx:eGFP*)) and macrophages (Tg(*mpeg1:mCherry*)), we tracked hundreds of individual immune cells across entire gut volumes for 4-hour durations at 3-minute intervals. We examined four experimental conditions: germ-free fish, conventionally reared fish (colonized by ambient microbes), and fish mono-associated with either wild-type *Vibrio* ZWU0020 or a mutant strain lacking the T6SS actin crosslinking domain (*Vibrio*ΔACD).

We developed computational methods to classify cellular motility states based on displacement thresholds and directional persistence measures, quantify morphological features including sphericity as an indicator of motility state, and characterize spatial relationships to gut architecture. Our analyses reveal several key findings. First, individual immune cells maintain remarkably stable motility phenotypes. They remain either predominantly motile or non-motile throughout multi-hour imaging periods rather than frequently transitioning between states. Second, gut architecture exerts a dominant influence on migration patterns. Immune cells show strong preferential movement along the intestinal anterior-posterior axis and exhibit high probability of remaining in gut-associated regions throughout the observation period. Third, macrophages display significantly higher sphericity during non-motile periods compared to motile periods. This provides a morphological signature of motility state that is visible in static snapshots. Notably, migration patterns remain consistent across diverse microbial conditions (germ-

free, conventionally reared, and mono-associated with either *Vibrio* strain), indicating that tissue structure, rather than microbial stimuli, primarily determines baseline immune surveillance dynamics. The only microbe-specific behavior observed was immune cell recruitment toward sites of tissue damage induced by wild-type *Vibrio*, consistent with prior observations [2].

These results establish that immune cell surveillance in the gut follows stereotyped patterns constrained by tissue geometry, with implications for understanding how innate immunity is spatially organized and how immune cells respond to perturbations.

1.5.2 Chapter III: Invasion of Established Gut Microbial Communities by a *Vibrio* Species Armed with a Type VI Secretion System

While Chapter II addresses the host immune response, Chapter III shifts focus to the role of gut-resident bacterial communities in mediating colonization resistance against an invading pathobiont. Building on prior work showing that *Vibrio* ZWU0020 employs T6SS-dependent mechanisms to damage tissue and recruit immune cells [2], we investigated *Vibrio* invasion dynamics within zebrafish hosts pre-colonized with simplified resident communities of one to three species. Specifically, we compared the invasive success of wild-type *Vibrio* (Vib WT) against a T6SS-deficient mutant (Vib dT6SS) to determine how this apparatus influences competition within diverse microbial landscapes.

We addressed the following questions: When *Vibrio* invades a gut already containing resident bacteria, how do bacterial abundances change over time? Does the T6SS confer an

advantage in overcoming colonization resistance? How does the composition and complexity of the resident community affect invasion outcomes?

Using high-throughput gut dissections and plating to measure total bacterial abundances, we characterized temporal changes in community composition during *Vibrio* invasion. Our work reveals how T6SS activity enables *Vibrio* to overcome colonization resistance provided by established residents. The results demonstrate that competitive interactions in the gut are mediated by direct T6SS-mediated killing as well as other factors, such as physical space limitation, resource competition, secretion of inhibitory metabolites, and host physiological responses.

These findings connect molecular virulence mechanisms to community-level ecological outcomes, showing that invasion success depends on the interplay between bacterial weapons and existing community structure.

1.6 Broader Significance

Together, these two projects provide complementary perspectives on gut biology. Chapter II characterizes the host immune surveillance landscape, the defensive framework within which bacterial populations must persist. Chapter III examines how invading bacteria navigate and disrupt this landscape through competitive mechanisms. The unifying theme is that spatial organization and temporal dynamics, governed by biophysical principles involving cellular mechanics, tissue architecture, and fluid flow, determine the outcomes of host-microbe interactions.

These findings carry implications for both inflammatory disorders and microbiome-targeted therapeutics. If immune surveillance patterns follow predictable rules determined by tissue architecture, and if community complexity confers resilience against invasion, then understanding these biophysical principles could inform more rational approaches to treating conditions such as inflammatory bowel disease and to engineering stable probiotic interventions. Chapter IV discusses these implications in detail.

CHAPTER II

CHARACTERIZING TRAJECTORIES OF INNATE IMMUNE CELLS IN LARVAL ZEBRAFISH

This chapter is adapted from co-authored work by P. Amitabh and R. Parthasarathy, “Characterizing trajectories of innate immune cells in larval zebrafish”, currently accepted for publication at PRX Life (Feb 9, 2026). I designed and performed all experiments, developed the image analysis and trajectory tracking pipeline, analyzed the data, and wrote the manuscript. R. Parthasarathy supervised the research and contributed to study design, interpretation, and manuscript editing.

2.1 Abstract

It is well established from in vitro studies of immune cells that stimulation by a wide range of potential signals leads to motility and morphology changes. How these physical behaviors manifest inside a living animal remains unclear due to limitations of conventional imaging and analysis approaches. Here, we establish a quantitative framework for imaging and tracking neutrophil and macrophage dynamics in larval zebrafish, spanning a large fraction of the animal for multi-hour timescales with few-minute temporal resolution. We focus especially on the gut, examining innate immune responses to different preparations of the intestinal microbiome. Using light sheet fluorescence microscopy and trajectory analysis

of hundreds of individual cells, we characterize speeds, directional persistence measures, and cellular morphology to reveal distinct population behaviors. Individual immune cells exhibit stable motility phenotypes, favoring predominantly motile or non-motile states rather than frequent transitions between them. Gut architecture constrains migration patterns as demonstrated by preferential anterior-posterior movement and a high probability of cells remaining in the vicinity of the gut throughout the imaging duration. Macrophages display significantly reduced sphericity during motile periods compared to non-motile periods, providing a morphological signature that may enable inference of dynamic behavior from static snapshots. Surprisingly, migration patterns remain consistent across diverse microbial conditions—germ-free, conventionally reared, and colonized by two strains of a zebrafish-native *Vibrio* species—indicating that tissue structure exerts a stronger influence than bacterial stimuli on immune surveillance dynamics. Previously observed tissue damage by the wild-type *Vibrio* strain, and the resulting recruitment of immune cells towards the damage site, provided the only microbe-specific cellular behavior. These findings reveal innate immune surveillance as a stereotyped process whose characteristics reflect both cellular decision-making and larger-scale anatomical structure.

2.2 Introduction

The innate immune system serves as the first line of defense against pathogens and supports tissue homeostasis throughout the body [6,59]. Both functions are especially important around the gastrointestinal tract, which serves as an interface between interior

and exterior spaces and between the cells of the host and vast numbers of microbes that include commensal as well as pathogenic organisms. Neutrophils and macrophages, the primary effector cells of innate immunity, communicate with each other and with other cell types through complex, multi-layered chemical networks involving numerous signaling molecules and regulatory pathways [60–63]. Immune cells are also highly motile and physically dynamic, and their strategies for navigating complex, three-dimensional, heterogeneous environments are an integral component of their overall efficacy. In vitro systems, for example collagen gels, have enabled insights into chemotaxis, cell polarity, activation, and force generation by neutrophils and macrophages [14,64,65]; while they afford experimental control and clarity, their geometry and homogeneity differ considerably from that encountered in live animals. Our understanding of how innate immune cells move in vivo, especially in and around gut-associated tissues, remains minimal, leaving open questions about what cellular features might characterize distinct motility states and whether gut microbes influence surveillance behavior.

Imaging immune cells in live animals is technically challenging but has progressed considerably in recent years. Studies using intravital multi-photon microscopy have revealed the dynamics of immune cells in mammals, but require extensive surgical preparation that can compromise physiological relevance [15,66–69]. Larval zebrafish (*Danio rerio*), due to their transparency and genetic tractability, provide an especially useful vertebrate model system for studies of the innate immune system [70,71]. Studies using zebrafish have shown, for example, specialized subsets of macrophages in close association with enteric neural

processes in the gut [72], fewer numbers of gut-associated neutrophils in germ-free animals than in animals colonized by microbes [73,74], different speeds and modes of migration (mesenchymal versus amoeboid) for neutrophils and macrophages [75], tissue-dependent T-cell motility modes likely reflecting mechanical constraints [76], and a two step search and run response of neutrophils to in-vivo chemical gradients [77]. Furthermore, cutting the caudal fin has become a well-established wound and regeneration assay, which has been used for example to show that neutrophils perform both forward and reverse migration in response to localized damage [18,78–81], and to test therapeutics for inflammation [82].

Despite the increasing number of investigations, there remains a lack of imaging-based approaches of innate immune cells in volumes large enough to span the entire larval zebrafish gut and over durations large enough to characterize motion types. We therefore used light sheet fluorescence microscopy to image fluorescent neutrophils (transgenic line *Tg(mpx:eGFP)*) and macrophages (*Tg(mpeg1:mCherry)*) [57,58] in live fish over length scales of millimeters and time scales of a few hours. Light sheet fluorescence microscopy provides rapid, high-volume, three-dimensional imaging with minimal phototoxicity [49,50,83], and its utility for imaging zebrafish intestinal bacteria and immune cells has been repeatedly demonstrated [2,84–87]. The resulting image datasets are large, roughly 24 GB per time-point and 1.92 TB per fish for typical dataset, necessitating efficient segmentation and tracking methods to yield immune cell trajectories.

We first validate this imaging and analysis approach with caudal fin cut experiments, providing a test of our analysis methods under conditions of known, directed immune cell

migration before applying them to the more complex gut-associated microenvironment. Then we describe neutrophil and macrophage trajectories in larval fish under various gut microbial preparations: germ-free, colonized by ambient microbes (“conventional”), or colonized solely by one of two variants of a zebrafish-isolate *Vibrio* strain, ZWU0020. In past work, we have shown that this strain induces inflammation and other physiological responses in the host animal, responses that require the presence of the actin crosslinking domain (ACD) of the bacterial Type VI Secretion System (T6SS). *Vibrio* with a complete T6SS causes tissue damage, induces macrophage redistribution towards the damage site, and leads to a roughly 100% increase in the amplitude of zebrafish intestinal contractions compared to *Vibrio* lacking the ACD or to non-*Vibrio* bacteria [2]. As described below, we find that under all bacterial conditions, both neutrophils and macrophages show stable motility states, i.e. remaining either motile or non-motile over roughly hour-long durations, maintain proximity to the gut, and migrate preferentially along the intestinal anterior-posterior axis, suggesting that gut architecture is the primary determinant of immune cell movement patterns. Notably, macrophages but not neutrophils show significantly higher sphericity during non-motile periods, providing a morphological indicator of motility state that reflects the greater pseudopodial extension of migrating macrophages.

2.3 Results

2.3.1 Validation of 3D immune cell tracking using the caudal fin cut wound model

We performed transverse amputations of the caudal fin in 5 days post-fertilization (dpf) zebrafish larvae, cutting midway between the posterior end of the notochord and the distal tip of the fin (Figure [2.1A](#)). Using the dual transgenic line *Tg(mpx:GFP;mpeg1:mCherry)*, we simultaneously imaged neutrophils (cells expressing the myeloperoxidase, *mpx*) and macrophages (cells expressing macrophage-expressed gene-1, *mpeg1*) during the wound response [57,58] (Figure [2.1B](#) and Supplemental Videos 1, 2). Time-lapse imaging was performed on a custom-built light sheet fluorescence microscope from 1 to 5 hours post-amputation, capturing a tissue volume of approximately $4.8 \times 10^7 \mu\text{m}^3$ (400 μm along the dorsal-ventral, 800 μm along the anterior-posterior, and 150 μm along the left-right axes), with images acquired every 3 minutes.

Following image pre-processing and drift correction, individual immune cells were segmented and tracked across time points to generate single cell trajectories (see Materials and Methods). Simple cell counts showed a greater number of both cell types in the field of view of the fin cut assay than the control (Supplemental Figure [A.1A](#)). The resulting trajectory data clearly demonstrated directed migration of both neutrophils and macrophages toward the amputation site (Figure [2.1C, D](#); Supplemental 3D Figures 1–4), consistent with the known recruitment of immune cells to sites of tissue damage [18,78–81]. All cell trajectories are provided as Supplemental Data.

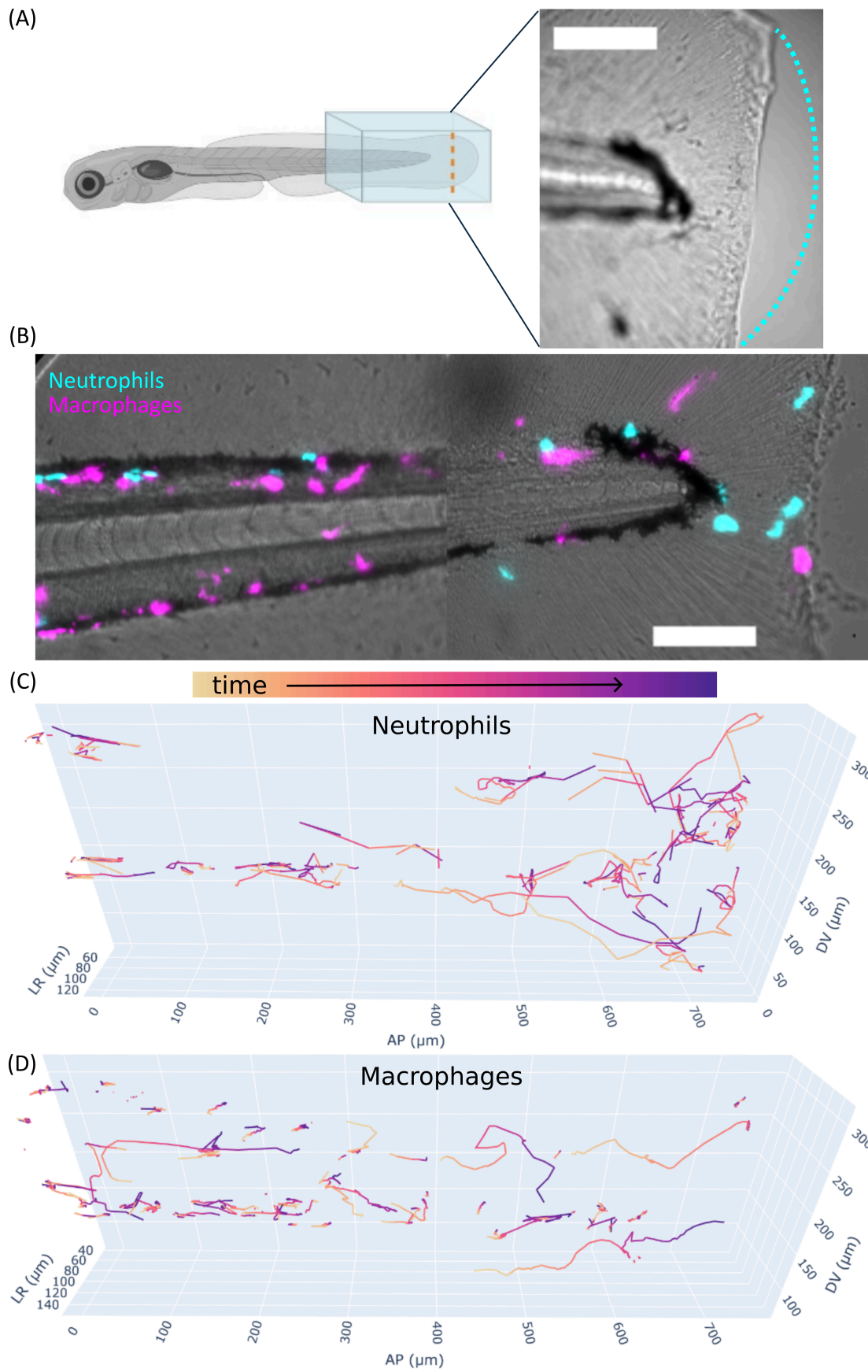


Figure 2.1. Fin cut assay validates immune cell tracking and demonstrates directed migration toward injury. (A) Left: Illustration of a larval zebrafish. Blue box indicates the imaging volume ($\approx 800\mu\text{m}$ along the anterior-posterior axis), with its posterior boundary aligned to the caudal fin tip; orange dashed line indicates the location of the caudal fin transection. **Right:** Representative brightfield image showing the fin clip, with the original fin shape approximated by the dotted cyan curve. **(B)** Representative composite image at 1 hour post-fin cut showing brightfield, macrophages (magenta), and neutrophils (cyan). **(C, D)** Cumulative tracks of neutrophils **(C)** and macrophages **(D)** over 1 to 5 hours post-fin cut from a representative specimen. Colors represent normalized time from track initiation (yellow) to termination (purple). Both cell types show migration towards the fin cut site (right). See Supplemental 3D Figures 1–4 for interactive 3D trajectories. AP: anterior-posterior; DV: dorsal-ventral; LR: left-right. All scale bars: $100\mu\text{m}$.

2.3.2 Establishment of motion classification criteria

To extract meaningful physical parameters from cellular trajectories, we developed a computational framework that accounts for the intermittent nature of immune cell migration. Individual cells exhibit alternating periods of active migration and relative quiescence, a characteristic feature of immune cell behavior in tissue environments [13]. To capture this temporal heterogeneity, we subdivided each complete trajectory into “subtracks” based on frame-to-frame displacement. Specifically, a new subtrack was initiated whenever the displacement transitioned above or below a threshold of half the mean cell diameter per 3 minute interval. We computed the spatial extent of each subtrack as the diameter of the smallest circumscribing sphere. We calculated the velocity cosine similarity of each subtrack, a measure of directional persistence defined as the cosine of the

angle between consecutive velocity vectors, averaged over the subtrack. Evaluating immune cells in the caudal fin area, pooling both cut and control fish, plots of spatial extent versus velocity cosine similarity for each subtrack show a positive correlation for both neutrophils and macrophages (Figure [2.2A](#)), indicating that more spatially extended tracks are also more directionally persistent.

To compare this correlation with what would arise from undirected random motion with the same track features and sampling as in the data, we generated simulated tracks by randomizing step directions while preserving each track's step-size distribution and track length, with detector noise matching experimental imaging conditions (see Materials and Methods). Simulated tracks showed no such correlation, with velocity cosine similarity clustered near zero regardless of spatial extent (Supplemental Figure [A.11A](#)), and both velocity cosine similarity and velocity anisotropy being significantly lower than experimental values (Supplemental Figure [A.11B, C](#)). These results confirm that the observed directional signatures reflect intrinsic directional bias rather than kinematic artifacts. To classify subtracks as motile or non-motile, we define a simple threshold of spatial extent being at least twice the mean cell diameter. To justify this beyond its intuitive validity, we consider for each cell type a linear fit of spatial extent versus velocity cosine similarity; the spatial extent corresponding to zero velocity cosine similarity is $47.2\mu\text{m}$ ($R^2 = 0.109$, $n = 13882$ datapoints) for neutrophils and $42.6\mu\text{m}$ ($R^2 = 0.115$, $n = 23765$) for macrophages, close to twice the mean cell diameter of $20.4\mu\text{m}$ and $19.5\mu\text{m}$ for neutrophils and macrophages respectively (Figure [2.2A](#)). Since zero velocity cosine similarity

corresponds to random or repetitive motion, including physiological noise from blood flow and small displacements from cell shape changes, this agreement implies that our motility threshold effectively separates persistent migration from non-directional motion.

Applying this motility classification scheme to the fin cut assay demonstrates its effectiveness in capturing differences in immune cell behavior. Motile subtracks from fin-cut fish exhibited significantly higher velocity cosine similarity compared to motile subtracks from the uninjured control group (Figure [2.2B](#)), confirming that our analysis pipeline successfully identified the expected increase in directional migration of both neutrophils and macrophages following tissue damage. In control fish, motile subtracks show velocity cosine similarity near zero, indicating largely random motion; small deviations from zero reflect residual noise not fully eliminated by the analysis pipeline. In injured fish, the innate immune cells show persistent motion, visually apparent as directed toward the wound site.

Having validated our motion analysis pipeline, we turn to investigations of immune cell behavior in response to different gut microbiota inoculations.

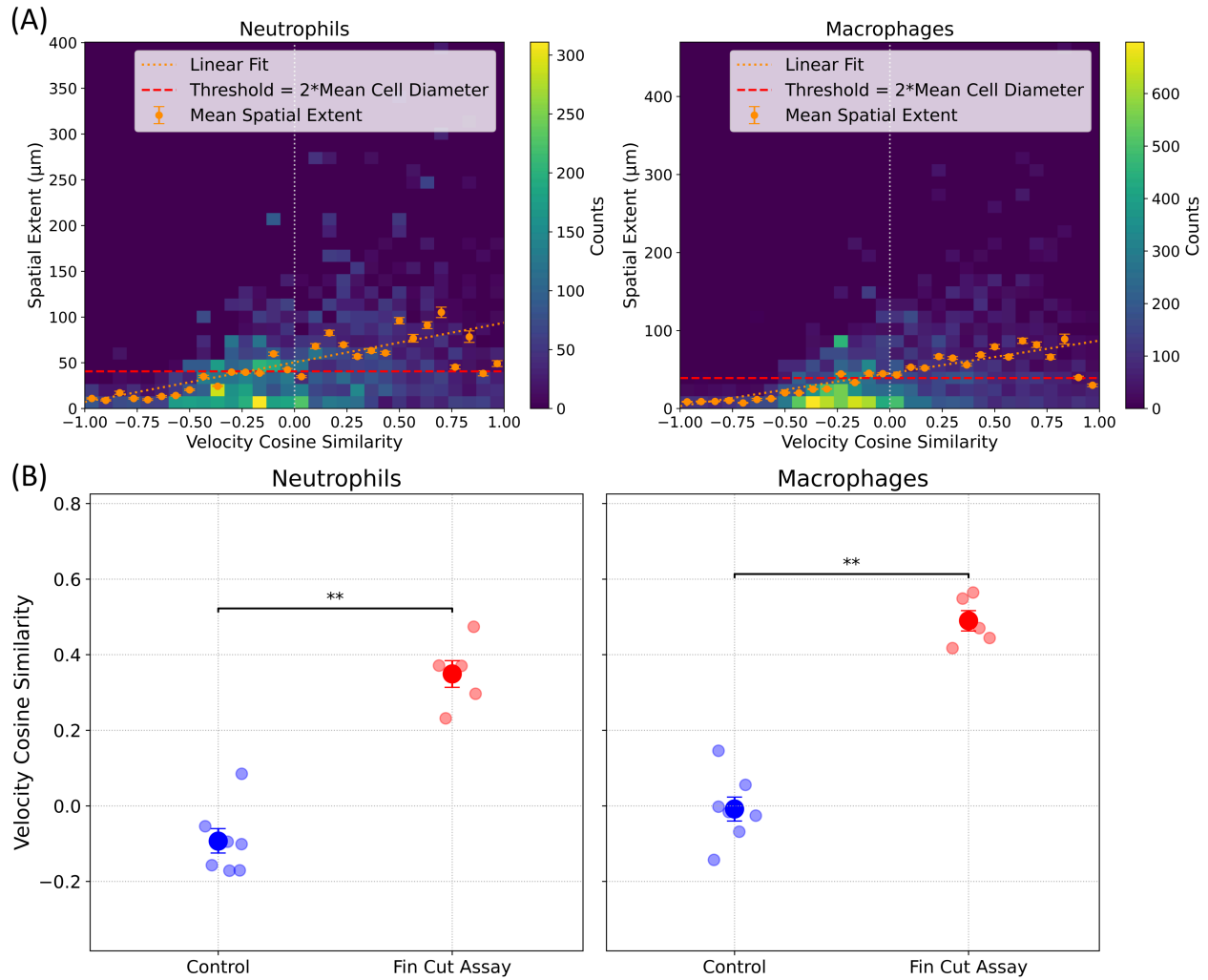


Figure 2.2. Motility classification reveals enhanced directional persistence during wound response. All panels show neutrophils (left) and macrophages (right). **(A)** 2D histograms of spatial extent versus mean velocity cosine similarity for subtracks in the caudal fin region, pooling control and fin-cut fish. The horizontal red line shows the motility classification threshold of twice the mean cell diameter. Orange points show mean spatial extent binned by velocity cosine similarity; the orange dashed line is a linear regression fit to all data points (see main text). **(B)** Velocity cosine similarity for motile subtracks in fin-cut and control fish. Both cell types show significantly higher velocity cosine similarity for the fin cut assay compared to the control, indicating more consistent directional movement.

$n_{\text{control}} = 5$, $n_{\text{fin cut assay}} = 7$; total $N = 12$. * : $p < 0.05$, ** : $p < 0.01$.

2.3.3 Three-dimensional immune cell tracking reveals gut-associated motility patterns

To investigate immune cell dynamics around the larval zebrafish gut in response to varied commensal bacterial groups, we assessed germ-free fish, conventionally reared fish with normal parental tank microbiota, and initially germ-free fish inoculated at 5 or 6 days post-fertilization (dpf) with one of two variants of the zebrafish-native *Vibrio* strain ZWU0020. As noted above, we showed in earlier work that colonization with wild-type *Vibrio* ZWU0020 (which we denote *Vibrio*^{WT}) causes tissue damage near the zebrafish vent (the posterior opening of the gut), which recruits macrophages to the vent from their normal midgut positions during a period of roughly 14 to 20 hours post inoculation (hpi) [2]. Vent damage and immune activation are minimal, with levels similar to germ-free fish, in larvae colonized with a *Vibrio* mutant lacking the actin crosslinking domain (ACD) of the Type VI Secretion System (T6SS), (denoted *Vibrio*^{ΔACD}) [2]. In prior work [2], macrophages positions were assessed at 20 minute intervals and neutrophils were not tracked.

In the studies described here, we began imaging at 9 hpi and tracked neutrophil and macrophage cells at 3-minute intervals through 13 hpi. This time window was chosen to investigate and capture immune cell behavior prior to the major macrophage redistribution to the vent previously observed at 14-20 hpi [2]. Gut dissection and plating at the end of the imaging period (13 hpi) confirmed successful *Vibrio*^{WT} colonization at 10³ to 10⁴ colony forming units (CFU) per gut (see Materials and Methods). Imaging of *Vibrio*^{WT}-dTomato fluorescence at representative timepoints (10, 11, and 13 hpi) showed bacterial localization

primarily at the gut bulb and the vent (see Materials and Methods; Supplemental Figure [A.2](#) and Supplemental Video 3). Germ-free, conventionally reared, or *Vibrio*-inoculated zebrafish were imaged with light sheet fluorescence microscopy at 3-minute intervals at 5 or 6 dpf from 9 to 13 hpi for inoculated fish or the same age for other non-inoculated fish. The imaging volume covered the entire gut: approximately 1600 μ m along the anterior-posterior (AP) axis, 100 μ m left-right (LR), and 400 μ m dorsal-ventral (DV) (Figure [2.3A](#)). Using the dual transgenic line *Tg(mpx:GFP; mpeg1:mCherry)*, we imaged and tracked individual neutrophils and macrophages throughout the 4-hour observation period (Figure [2.3B, C](#) and Supplemental Video 4, 5). All cell trajectories are provided as Supplemental Data. The mean track lengths were 24 and 32 frames (69 and 93 minutes) for neutrophils and macrophages, respectively, pooled across all groups. These cells showed a similar relationship between spatial extent and velocity cosine similarity as immune cells in the fin cut assay (Supplemental Figure [A.3](#)). As with the fin cut data, simulated random walks showed no such correlation, with velocity cosine similarity clustering around zero, while experimental data showed significantly higher directional persistence across nearly all conditions (Supplemental Figure [A.12A, B](#)). Vent width measurements confirmed the expected T6SS-dependent tissue damage at the end of the imaging window, with *Vibrio*^{WT}-associated fish showing the largest increase compared to other groups (Figure [2.3D](#), Supplemental Figure [A.4](#)).

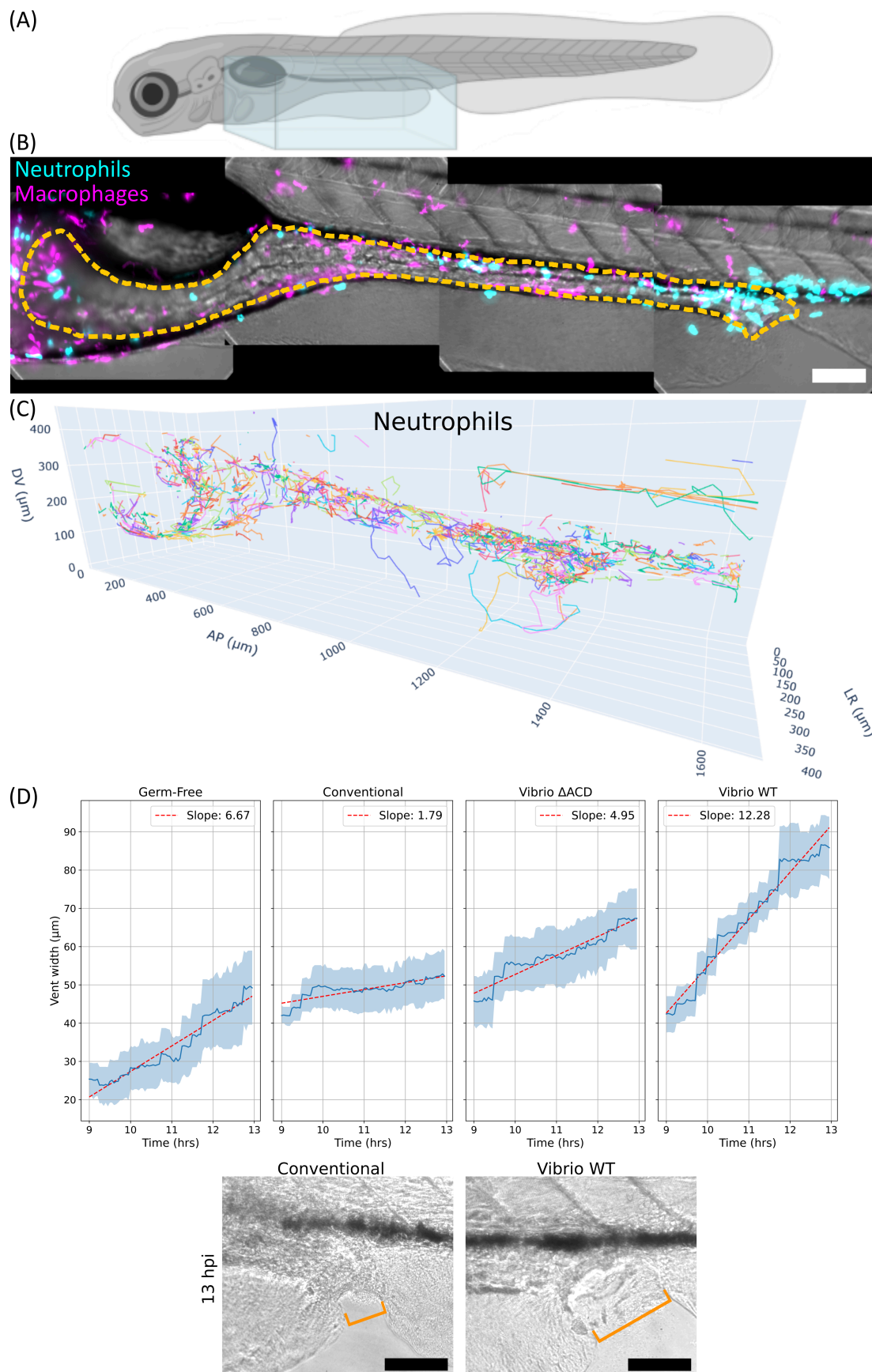


Figure 2.3. Gut imaging experimental design and *Vibrio*-induced tissue damage. (A)

Illustration of a larval zebrafish showing the imaging volume spanning the gut region as a blue box. (B) Composite maximum intensity projection image at a single time point (11 hpi, midpoint of imaging window) showing brightfield (gray), macrophages (magenta), and neutrophils (cyan). The orange dashed line outlines the gut. (C) Cumulative tracks of neutrophils over the 9 to 13 hpi imaging window. Different colors represent individual cell tracks, illustrating the dynamic movement of neutrophils throughout the gut region. (D) **Upper:** Zebrafish vent width over the course of imaging for the different experimental groups: Germ-free, conventionally reared (Conventional), *Vibrio*^{ΔACD}-inoculated, and *Vibrio*^{WT}-inoculated fish. Increased vent width is an indicator of tissue damage. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$. **Lower:** Brightfield images showing vent widths for conventionally raised and *Vibrio*^{WT}-inoculated fish at the end of the imaging window. Orange lines indicate the vent width. All scale bars: 100μm.

2.3.4 Immune cells maintain distinct motility states with robust motility patterns across microbial conditions

Using our framework for classifying the motility of subtracks, we found that both neutrophils and macrophages showed a tendency to remain either motile or non-motile for extended periods of time comparable to the mean track duration of over one hour rather than showing frequent transitions between motility states. Histograms of the motile subtrack fraction per track, grouped across all bacterial conditions, reveal bimodal distributions with peaks at 0 and 1 for both neutrophils and macrophages (Figure 2.4A; bin width 0.06̄). This bimodal pattern contrasts with the uniform distribution expected if there were frequent switching between motility states. While a subset of cells exhibits intermediate fractions of motile behavior, the observed distribution indicates that innate

immune cells preferentially adopt and maintain a consistent motile or non-motile phenotype over the four-hour observation period. Analysis included only tracks with minimum track lengths of 5 frames to ensure robust motility classification. This distribution pattern was consistent across all four experimental groups and in the fin cut assay (Supplemental Figures [A.5](#), [A.6](#)).

To more finely assess motility behaviors and possible dependence on microbial stimulation, we examined the number and fraction of motile and non-motile cells (subtracks) at each timepoint from 9 to 13 hpi. The numbers of motile neutrophils and macrophages remained fairly constant and similar across all four conditions (germ-free, conventional, *Vibrio*^{WT}, and *Vibrio*^{ΔACD}) (Figure [2.4B, C](#)). Non-motile neutrophils increased in number over time in *Vibrio*^{WT}-inoculated fish (Figure [2.4B](#)), which was mainly driven by the increase in the number of non-motile neutrophils near the vent region (Supplemental Figure [A.7](#)). Macrophages showed no significant temporal changes in non-motile subtrack numbers across any group (Figure [2.4C](#)). We note that for some experimental conditions there is an initial increase and a late decrease in motile cells (Figure [2.4B, C](#)). This is a likely consequence of the requirement of a minimum 5 frame (15 minute) track length, as motile cells have an appreciable likelihood to enter or leave the field of view during the peripheral 15 minutes, and so are not counted.

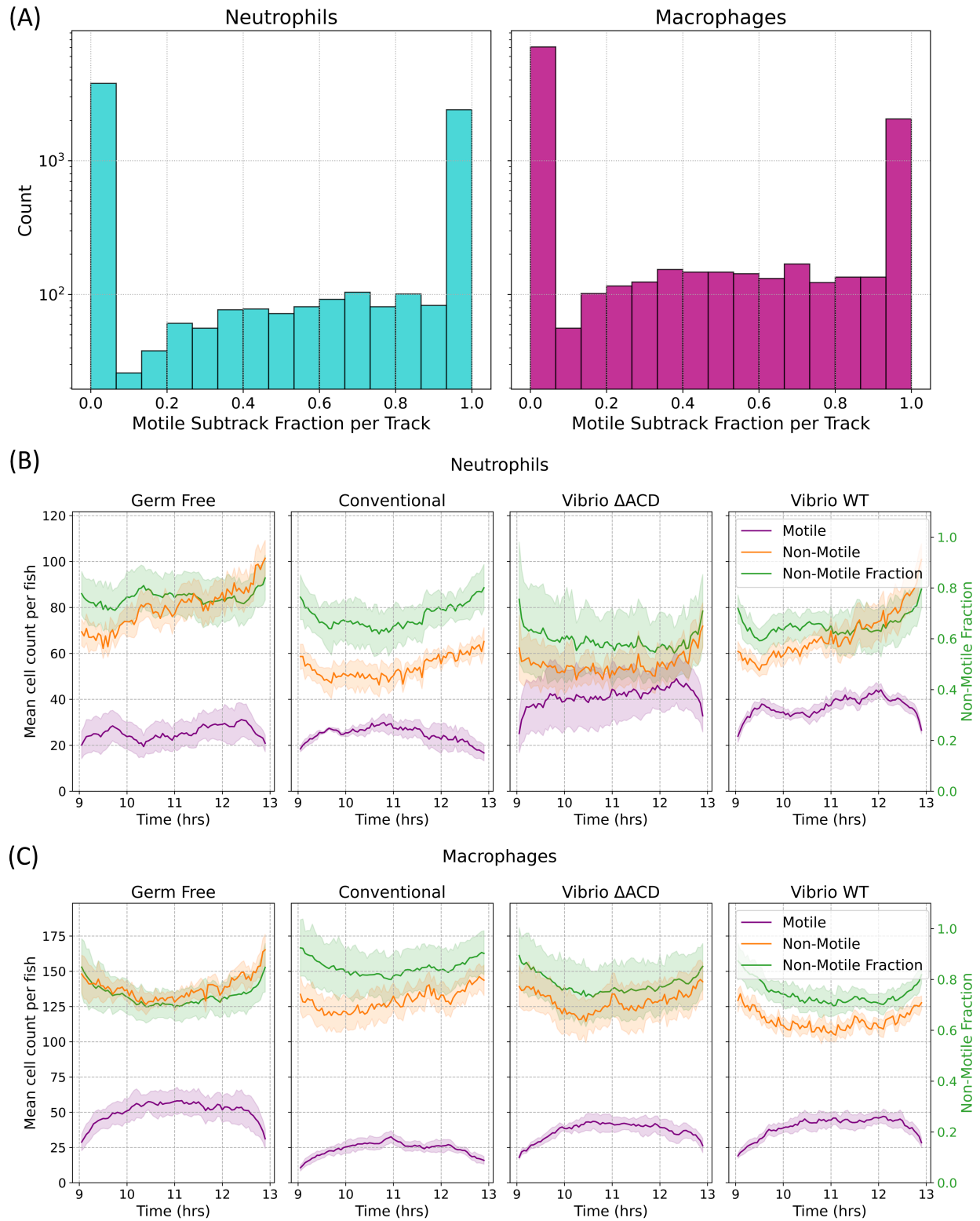


Figure 2.4. Immune cells maintain stable motility phenotypes over multi-hour timescales. (A) Histograms showing the motile subtrack fraction per track for neutrophils (left) and macrophages (right), pooled across all experimental groups. The distribution shows peaks at 0 and 1, indicating that immune cells generally adopt a consistent motile or non-motile phenotype over the 4-hour observation period, though a subset of cells exhibits intermediate behavior. Bin width = $0.0\bar{6}$. **(B, C)** Counts of motile and non-motile subtracks, and the ratio of non-motile to total subtracks from 9 to 13 hpi for **(B)** neutrophils and **(C)** macrophages across experimental groups: Germ-free, conventionally reared (Conventional), *Vibrio*^{ΔACD}-inoculated, and *Vibrio*^{WT}-inoculated fish. Motile cell numbers remain relatively constant across conditions. Error bars represent uncertainties from bootstrapping.

$$n_{\text{Germ Free}} = 5, n_{\text{Conventional}} = 5, n_{\text{Vibrio } \Delta\text{ACD}} = 5, n_{\text{Vibrio WT}} = 6; \text{ total } N = 21.$$

2.3.5 Macrophage morphology correlates with motility state

To investigate whether immune cell motility correlates with detectable morphological changes, we quantified cellular sphericity, defined as the ratio of the surface area of a sphere with the same volume as the measured cell to the measured surface area of the cell. Perfect spheres have a sphericity value of 1.0, whereas shape distortions reduce this value.

Neutrophils exhibited no significant differences in sphericity between motile and non-motile states across all experimental groups (Figure 2.5A). In striking contrast, macrophages demonstrated significantly higher sphericity during non-motile periods compared to motile periods, indicating increased cellular rounding when stationary. Representative microscopy images reveal that motile macrophages display prominent pseudopodial protrusions, whereas non-motile macrophages adopt a more rounded

morphology (Figure 2.5B and Supplemental Videos 6–9). This morphological distinction suggests that motile macrophages extend pseudopodia more extensively than their non-motile counterparts, providing a snapshot-based indicator of motility state that does not require temporal tracking to assess. This behavior is also observed in the response of these cells in the fin cut assay (Supplemental Figure A.1B). We examined numerous additional cellular parameters, including volume and normalized anterior-posterior position, but sphericity was the only metric that distinguished motile from non-motile behavior across all macrophage groups (Supplemental Figure A.8A, B and A.1C).

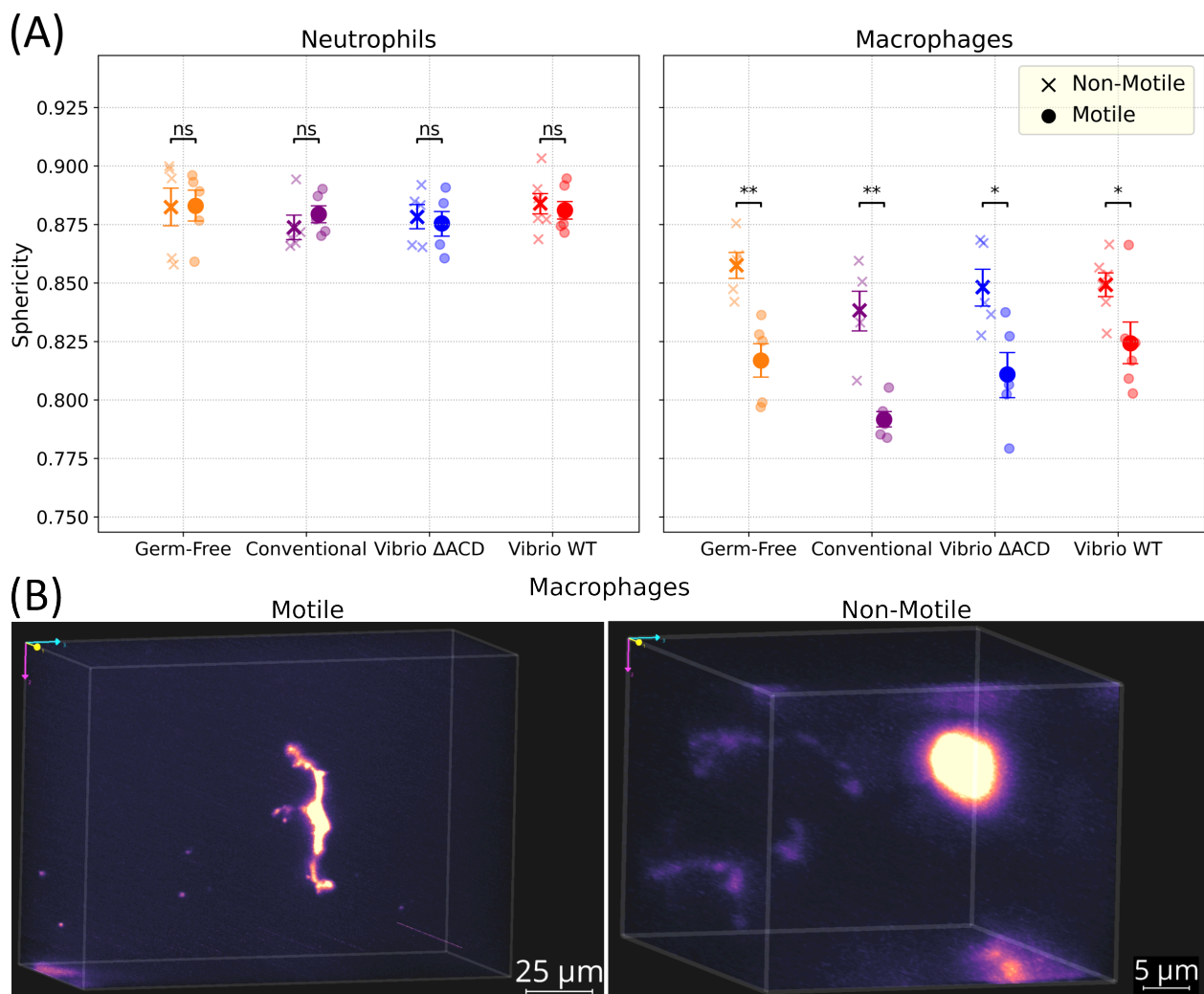


Figure 2.5. Macrophage morphology correlates with motility state. Macrophages exhibit reduced sphericity associated with pseudopodial extensions during motile phases. **(A)** Sphericity comparison between motile and non-motile subtracks for neutrophils (left) and macrophages (right) across all experimental groups. Macrophages exhibit significantly lower sphericity during motile periods compared to non-motile periods, providing a morphological signature of motility state that could enable inference from static snapshots. Neutrophils show no significant difference between motility states. **(B)** Representative images of macrophages during motile (left) and non-motile (right) phases. Motile macrophages display prominent pseudopodial protrusions, whereas non-motile macrophages adopt a more rounded morphology (Supplemental Videos 6–9). Error bars represent uncertainties from bootstrapping. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$. ns: not significant, * : $p < 0.05$, * * : $p < 0.01$.

2.3.6 Gut architecture constrains immune cell migration patterns

We manually segmented images to identify gut-associated regions, including the intestinal lumen and surrounding tissue (Figure 2.3B and Supplemental Video 10). We classify as “gut-associated” immune cells that are located within these boundaries at any point during imaging. To assess the persistence of gut association over extended periods, we considered subtracks with a duration of at least five frames and examined the fraction of time spent in the gut volume. Non-motile subtracks spent more than 80% of their time within the gut, considering averages across all experimental groups (Figure 2.6A). Surprisingly, motile subtracks also demonstrated high gut residence probability (>70% across all groups). Simulating random walks with the same average frame-to-frame displacement as immune cells and assessing their residence in a volume like that of the

larval gut gives a residence probability of 30-40% for walkers with the same median track lengths of the observed neutrophils and macrophages, indicating that gut-associated immune cells migrate predominantly along the boundary of the gut.

Analysis of motile subtrack characteristics revealed that most kinetic parameters, including mean speed and velocity components, showed no significant differences between experimental groups (Figure 2.6B and Supplemental Figure A.9). We also provide these parameters for the fin cut assay (Supplemental Figure A.10). However, we found that immune cell migration shows a strong directional bias. We calculated all cells' velocity anisotropy, given by:

$$\text{Velocity anisotropy} := \sqrt{\frac{2 \cdot V_{AP}^2}{V_{DV}^2 + V_{LR}^2}},$$

where V_{AP} , V_{DV} , and V_{LR} represent velocity components along the anterior-posterior, dorsal-ventral, and left-right axes, respectively. Velocity anisotropy consistently exceeded 1.0, the value for isotropic motion, across all experimental conditions (Figure 2.6C), and was significantly higher than the anisotropy of simulated random walks drawn from the experimental distribution of step sizes (see Materials and Methods; Supplemental Figure A.12C), indicating preferential movement along the anterior-posterior axis compared to dorsal-ventral or left-right directions.

Combined with the high gut residence probability of motile cells, this result demonstrates that gut-associated immune cells migrate primarily along the intestinal length (along anterior-posterior axes) rather than in left-right or dorsal-ventral directions,

suggesting that gut architecture fundamentally constrains immune cell movement patterns regardless of microbial stimulus.

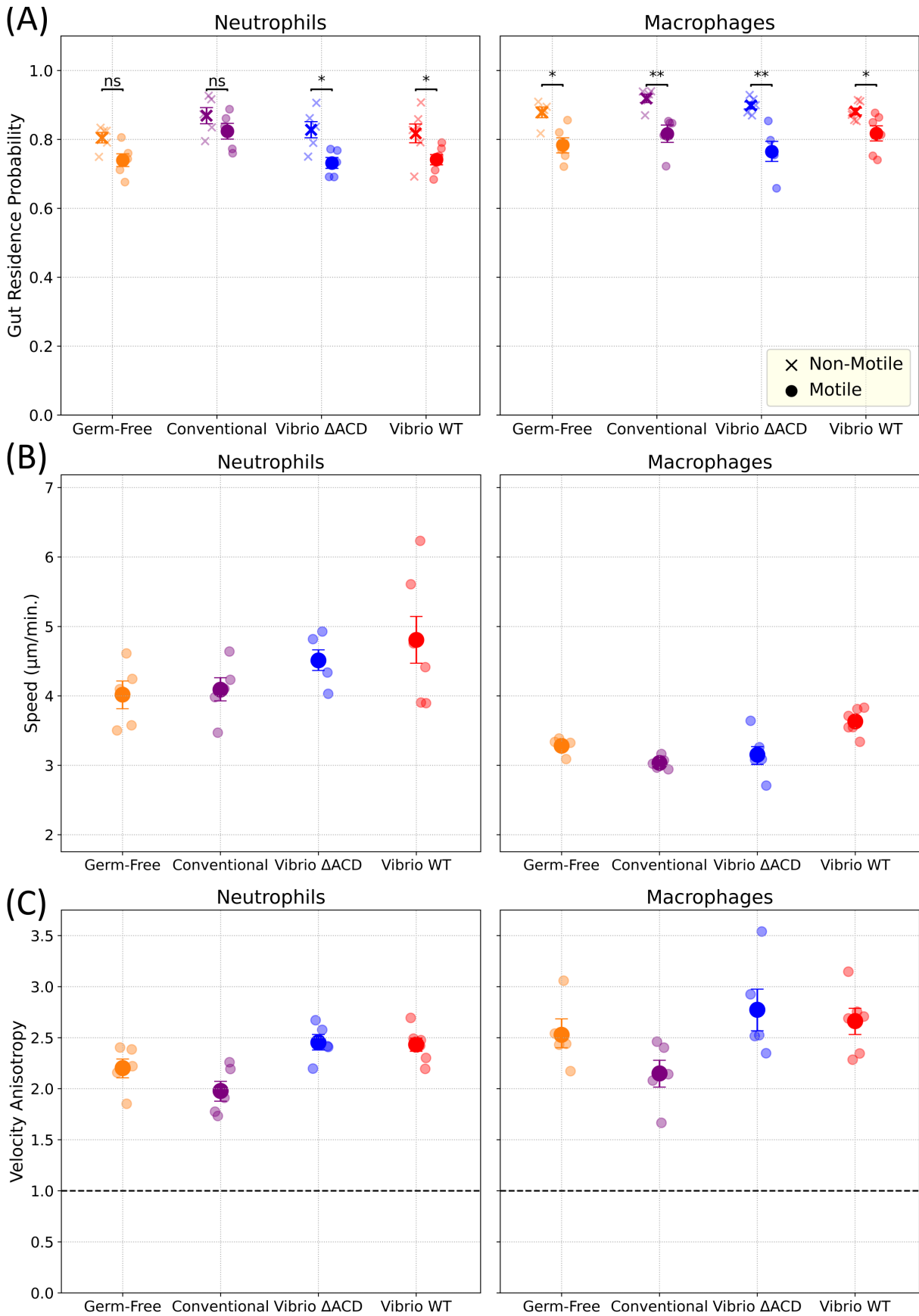


Figure 2.6. Gut architecture constrains immune cell migration patterns.

Characterization of immune cell residence and migration dynamics in gut-associated regions. All panels show neutrophils (left) and macrophages (right). **(A)** Gut residence probability, defined as the fraction of time a cell that is ever in the vicinity of the gut remains in the vicinity of the gut, for motile and non-motile subtracks. Both populations spend the majority of their time within gut-associated regions across all experimental conditions, indicating high gut affinity. **(B)** Average speed of motile subtracks, calculated from frame-to-frame displacements, shows no significant differences between experimental groups for either cell type. **(C)** Velocity anisotropy quantifies preferential movement along the anterior-posterior axis relative to perpendicular directions. Values consistently exceed 1.0 across all conditions, indicating that immune cells migrate primarily along the intestinal length rather than in dorsal-ventral or left-right directions. This pattern persists regardless of microbial colonization state. Error bars represent uncertainties from bootstrapping. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$. ns: not significant, * : $p < 0.05$, ** : $p < 0.01$.

2.4 Discussion

To date, there have been few quantitative analyses of immune cell dynamics in living organisms over volumes that span a large fraction of the animal, and to our knowledge, this study is the first that characterizes innate immune dynamics around the gastrointestinal tract. Given strong, multifaceted interactions between the intestinal microbiota and the host immune system [7,88–90], it is natural to expect that different microbial stimuli should lead to different immune cell motility patterns. Surprisingly, we find little difference in motility characteristics in germ-free larval zebrafish, conventionally reared fish, and fish inoculated with a pathobiont *Vibrio* species, other than spatial redistribution to the site of *Vibrio*-

induced tissue damage [2]. Immune cell motion is, however, strongly influenced by the anatomical context of the host. Neutrophils and macrophages that encounter the gut tend to spend a high fraction of their time near the gut, and migrate largely along the anterior-posterior axis. This strong axial preference was consistent across all microbial conditions, indicating that gut structure, not microbial signals, primarily dictates migration patterns.

The remarkable consistency of immune cell behavior across microbial conditions – germ-free, conventional, *Vibrio*^{WT}, and *Vibrio*^{ΔACD} – demonstrates robust migration patterns independent of bacterial stimuli. The bimodal distribution of motility states, constant numbers of motile cells, and invariant kinetic parameters across all conditions suggest that gut architecture exerts stronger influence than microbial signals during the 9-13 hpi window. Only the localized accumulation of non-motile neutrophils near *Vibrio*-induced tissue damage deviated from this pattern, indicating that while bacterial activity can create specific retention sites, it does not fundamentally alter migration dynamics. These findings suggest that therapeutic strategies targeting immune motion should consider the dominant role of tissue architecture over microbial influences.

On the timescale of a few hours, we found stable motility states (either motile or non-motile) for both neutrophils and macrophages, rather than frequent transitions in behavior. While our study made use of live imaging over several hours, it is interesting to ask whether motility states can be gleaned from single snapshots, potentially even from non-living animals or histological samples. We found no notable morphological differences between motile and non-motile neutrophils. Macrophages, in contrast, were much less

spherical when motile. This feature may allow the inference of macrophage dynamic behavior from static snapshots alone. Due to the lack of cell membrane and actin markers, we cannot definitively classify the cellular protrusions observed in the motile and less spherical subset of macrophages, though visual inspection suggests these protrusions are consistent with the general form of pseudopodia (see Supplementary Videos 6–9). Cellular protrusions can be classified into several types based on their morphology and cytoskeletal architecture, like lamellipodia (broad, sheet-like projections), filopodia (thin, finger-like extensions), and blebs (pressure-driven spherical extensions) [91]. Characterizing the protrusion types present in migrating gut macrophages, along with their mechanical characteristics and modes of migration (amoeboid versus mesenchymal), represents an interesting avenue for future investigation.

A key open question concerns the mechanistic basis of immune cell guidance *in vivo*. Neutrophils and macrophages are well known to migrate along chemokine gradients toward sites of infection or tissue damage [62], yet the striking anatomical constraint we observe, cells remaining in the vicinity of the gut and migrating along its axis, suggests that physical cues also play a role. The gut is a tubular structure, presenting curved surfaces that could engage curvotaxis, whereby cells preferentially migrate along regions of negative curvature through nucleus-mediated mechanosensing [92,93]. Cell migration can also be directed by durotaxis (stiffness gradients) and haptotaxis (substrate-bound signals) [94,95]. The extracellular matrix in zebrafish undergoes substantial remodeling during development, with region-specific changes in composition and mechanical properties [96,97]. Given that

different anatomical regions exhibit distinct ECM characteristics at different developmental stages, the relative contributions of chemotaxis, curvotaxis, and durotaxis to immune cell navigation likely vary across tissue contexts. Future work employing membrane and cytoskeletal markers, combined with ECM characterization, would help dissect these contributions.

Our study focused on neutrophils and macrophages in larval zebrafish at 5–6 days post-fertilization, a stage when innate immunity predominates and adaptive immune cells have not yet matured [98]. Notably, heterogeneous motility phenotypes within immune cell populations have been observed in other contexts. In lymph nodes, T cell populations contain both highly motile naive cells undergoing random walks and arrested cells forming stable conjugates with dendritic cells during antigen recognition, with cells maintaining their respective motility states for periods of 8–12 hours [15,69,99]. Similarly, heterogeneity in migration modes—mesenchymal versus amoeboid—has been described for neutrophils and macrophages in zebrafish tissues [75]. Whether the stable motile and non-motile phenotypes we observe in gut-associated immune cells correspond to distinct functional subtypes, analogous to the naive versus antigen-engaged distinction in T cells, or represent different stages of a behavioral program, remains an open question. More generally, motility patterns across the range of immune cell types remain under-explored. The immune systems of zebrafish and mammals are highly conserved but differ in key aspects, including, in zebrafish, the absence of lymph nodes and reliance on the kidney as the primary hematopoietic organ [55,56]. Extending our framework to mammals with more complex

organ structure and tissue compartmentalization, to later developmental stages when adaptive immunity is established, and to other immune cell types would be important next steps and would reveal whether the behaviors uncovered in innate immune cells in larval zebrafish are reflected in other organisms and other stages of development. The ability to track hundreds of cells in 3D across multi-hour timescales, combined with emerging single-cell transcriptomic atlases [100], could ultimately link motility phenotypes to transcriptional states.

The combination of stable motility states, architectural constraints, and cell-type-specific morphological signatures reveals immune surveillance as a stereotyped process whose characteristics reflect both cellular decision-making and larger-scale structure. The quantitative framework established here and the open-source computational pipeline we provide should allow translation to other model systems and cell types, identifying conserved versus context-specific features of immune surveillance mechanisms during development and disease.

2.5 Materials and Methods

2.5.1 Zebrafish husbandry and transgenic lines

All experiments with zebrafish followed standard procedures [101] and were performed in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee (protocol numbers AUP-22-02 and AUP-20-16). Fish from

which the larvae used in these studies were derived were maintained in the University of Oregon Zebrafish Facility at 28°C with a 14-h/10-h light/dark cycle.

The transgenic line Tg(mpeg1:mCherry), allele number: gl23, is described in reference [57]. The transgenic line Tg(tnf α :GFP);Tg(mpeg1:mCherry), allele number pd1028Tg, is described in reference [102].

2.5.2 Gnotobiotic derivation and bacterial colonization

Embryos were derived germ-free following established protocols [103]. Fertilized eggs were incubated for 6 hours in sterile embryo medium (EM) containing antibiotics (100 μ g/mL ampicillin, 10 μ g/mL gentamycin, 1 μ g/mL tetracycline, 1 μ g/mL chloramphenicol, 250 ng/mL amphotericin B). After surface sterilization with 0.003% sodium hypochlorite and 0.1% polyvinylpyrrolidone-iodine, embryos were distributed into T25 flasks at one embryo per mL in 15 mL sterile EM.

Vibrio ZWU0020 (PRJNA205585) was previously isolated from the zebrafish intestinal tract. From freezer stocks kept at -80°C , cultures were grown overnight in Luria Broth at 30°C with agitation. Then, bacterial cultures were pelleted through centrifugation for 3 min at $7,000 \times g$, washed once in sterile EM, and inoculated at 10^6 CFU/mL directly into flask water. Larvae were colonized at 5 or 6 dpf, with time-lapse imaging from 9 to 13 hpi.

2.5.3 Construction of Vibrio ZWU0020 T6SS mutants

The actin crosslinking domain deletion mutant (*Vibrio*^{ΔACD}) was constructed using markerless allelic exchange with the pAX2 vector as previously described. In brief, splice-by-overlap extension generated deletion cassettes with the following primers:

5' HR: 5'-CCCAATGATAGCCACGGTTG-3' + 5'-

CCATTCCATTTTCCACTAGGCTAAAGGACACACCTT-3'; 3' HR: 5'-

AAGGTGTGTCCTTTAGCCTAGTGGAAAATGGAATGG-3' + 5'-

GGCGCAAGATTTTCAATCA-3'. After conjugation into *Vibrio* using *E. coli* SM10,

merodiploids were selected on TSA-gentamicin at 37°C. Deletion was confirmed by PCR

using flanking primers: 5'-CGGAGCTTTGGTCAATCTCA-3' + 5'-

AGGTCTCTCCGTGGAAAACA-3'.

2.5.4 Caudal fin amputation

For wound response assays, 5 dpf larvae were anesthetized in 120 µg/mL MS-222 before mounting on glass slides using 4% methyl-cellulose. The caudal fin was transected midway between the notochord end and fin tip using a sterile scalpel. Larvae recovered in fresh EM at 28°C before imaging.

2.5.5 Quantification of intestinal bacterial abundance

To quantify intestinal bacterial abundance, larval zebrafish were euthanized by hypothermic shock at the indicated timepoints (hours post-inoculation, hpi). Intestines were removed under a dissection microscope, placed in 1 mL sterile embryo medium, and

homogenized with zirconium oxide beads using a Bullet Blender® tissue homogenizer (Next Advance, Troy, NY). Homogenates were serially diluted 10^{-1} and 10^{-2} , and 100 μL volumes were spread onto LB-agar plates. Plates were incubated at 30°C overnight, and colony-forming units (CFU) were enumerated to calculate bacterial load per intestine.

2.5.6 Light sheet fluorescence microscopy

Imaging was performed using a custom-built light sheet fluorescence microscope described in detail elsewhere [104,105]. A galvanometer-scanned light sheet of excitation illumination from 448 or 561 nm lasers (Coherent Sapphire, operating at 5 mW) was oriented perpendicular to the optical axis of a 40x, 1.0 NA water-immersion detection objective. Images were captured by a 5.5 MP sCMOS camera (Excelitas pco.edge). Three-dimensional volumes were acquired by scanning the sample along the detection axis in 1 μm steps with 33 ms exposures per plane. The resulting image resolution is approximately 0.4 μm in the dimensions parallel to the sheet and 5 μm perpendicular to the sheet. All hardware components were controlled using $\mu\text{Manager}$ software [106].

For gut imaging, four overlapping sub-regions covering the entire intestine (approximately $1,200 \times 300 \times 150 \mu\text{m}^3$) were sequentially acquired and computationally registered. Time series were collected at 3-minute intervals for 4 hours (9-13 hpi). For fin wound assays, single volumes were acquired at 3-minute intervals for 4 hours (1-5 hours post-amputation). Original voxel dimensions were 1 μm (z-axis) $\times$ 0.1625 μm (y-axis) $\times$ 0.1625 μm (x-axis).

2.5.7 Sample mounting

Larvae were anesthetized in 120 $\mu\text{g}/\text{mL}$ MS-222 and embedded in 0.7–1.0% low-melt agarose within glass capillaries. Specimens were partially extruded to avoid optical interfaces and oriented with ventral illumination. Up to four samples were mounted simultaneously in sterile EM at 28°C.

2.5.8 Bacterial distribution analysis

To quantify bacterial distribution along the gut during the imaging period, we analyzed *Vibrio*^{WT}-dTomato fluorescence. To reduce host cell and background autofluorescence interference, we created 3D gut lumen masks to restrict analysis to regions where bacteria are present in the zebrafish gut. Using bright-field and dTomato fluorescence images, 2D gut lumen boundaries were manually drawn at every 10th z-plane for each field of view. These 2D masks were extruded across the intermediate planes to generate complete 3D volumes, then stitched across all imaging positions to cover the entire gut (Supplemental Video 3). The gut was masked using this 3D mask and the resulting image was median background subtracted. Pixel intensities were binned along the anterior-posterior axis to generate line profiles of bacterial distribution. Anterior-posterior position is normalized such that 0 and 1 represent the start and end of the gut, respectively.

2.5.9 Gut segmentation

Binary masks that identified the larval zebrafish gut region in images were manually segmented using a Wacom Cintiq[®] graphics tablet on brightfield images acquired at the median sagittal plane. To optimize efficiency while maintaining accuracy, masks were initially drawn at every 10th timepoint and subsequently interpolated across all 80 imaging timepoints (Supplemental Video 10). The resulting 2D masks were extruded along the left-right axis to encompass the complete gut volume within the z-stack imaging boundaries.

2.5.10 Image analysis

All image processing and data analysis were performed with code written in Python using the NumPy [107], SciPy [108], scikit-image [109], and pandas [110] libraries. Code is available at https://github.com/rplab/Immune_Cell_Motion_Analysis_2025.

2.5.10.1 Preprocessing

Each 2D slice of a 3D image volume (z-stack) was downsampled by a factor of 4 in both x and y directions to reduce computation time. Raw image stacks were then drift-corrected using phase correlation between bright field images from consecutive time points. For gut imaging, 4-5 overlapping fields of view along the anterior-posterior axis were acquired. Stage positions saved in the acquisition metadata were extracted from configuration files and represent global coordinates of the images which were then transformed to local pixel coordinates which was used to stitch different fields of view to generate complete gut volumes.

2.5.10.2 Cell segmentation

Immune cells were segmented using a two-stage approach combining iterative thresholding with morphological Active Contour Without Edges (ACWE) [111,112].

In brief, we applied iterative simple thresholding to get loose boundaries around objects. Initial thresholding used median + 2σ , where σ is the standard deviation of pixel intensities. Objects exceeding $10^5 \mu\text{m}^3$, considerably larger than the possible volume of a single cell, were iteratively re-thresholded at their local median + $n\sigma$, incrementing n until all objects fell below the volume limit or n reached 7. Objects smaller than a sub-cellular threshold of $10^3 \mu\text{m}^3$ were removed.

For each remaining object, we tightened/refined boundaries around the object using morphological ACWE with the thresholded region as the initial level set. The algorithm ran for 10 iterations with parameters: smoothing=1, $\lambda_1=1$, $\lambda_2=4$, with early stopping after 3 iterations of no change.

2.5.10.3 Cell property analysis

Cell properties including volume, centroid, and mean intensity were extracted for each segmented object.

Surface area was calculated via a marching cubes algorithm on Gaussian-smoothed segmented objects with 5-pixel padding to prevent edge artifacts. Supplemental Video 11 shows the generated surfaces on the segmented macrophages. Sphericity quantified morphological complexity and was computed as:

$$\text{Sphericity} = \frac{(36\pi V^2)^{1/3}}{A}$$

where V is cell volume and A is surface area. This metric equals 1 for perfect spheres and decreases with surface complexity, providing a quantitative measure of pseudopodial extension. As this metric is meaningful only between 0 and 1, the resulting values were clipped to this range.

Gut association was determined by spatial overlap with manually defined gut boundaries.

2.5.10.4 Tracking and trajectory analysis

Segmented cells were tracked across time points using a bidirectional nearest-neighbor algorithm. For each consecutive time point pair, we computed Euclidean distances between all cell centroids in 3D space. A cell at time t was linked to its nearest neighbor at time $t + 1$ only if the reverse mapping (from $t + 1$ to t) also identified the original cell as the nearest neighbor. This bidirectional validation is a stringent criterion, such that tracks terminate when cells exit the imaging volume or when local crowding prevents unambiguous assignment; however, it also prevents spurious connections. The total number of tracked cells at each timepoint shows gradual changes without large fluctuations (Supplemental Figures [A.13](#) and [A.14](#)), consistent with stable tracking over time.

Trajectories were subdivided into “subtracks” at transitions above/below a displacement threshold of $0.5 \times$ mean cell diameter, separating motile from non-motile phases. A patience

parameter (n=3 timepoints) was applied to ensure subdivision only occurred after cells maintained the new motility state for 3 consecutive timepoints to prevent oversegmentation from transient behavior.

For each subtrack, we calculated:

- **Velocity components:** Frame-to-frame velocity components V_{AP} , V_{DV} , and V_{LR} along anterior-posterior (AP), dorsal-ventral (DV), and left-right (LR) axes
- **Velocity cosine similarity:** $\cos(\theta)$ between consecutive velocity vectors
- **Spatial extent:** Diameter of smallest circumscribing sphere of the subtrack
- **Velocity anisotropy:** Normalized ratio of velocity components along the anterior-posterior direction (V_{AP}) versus the other two directions (V_{DV} and V_{LR}):

$$\text{Velocity Anisotropy} = \sqrt{2V_{AP}^2 / (V_{DV}^2 + V_{LR}^2)}$$

2.5.11 Simulated random walk

To compare the observed spatial extent and velocity cosine similarity relations with what would arise from randomly directed motion, we generated simulated trajectories for each experimental track. For each track, we generated random walks with the same starting position, number of time points, and set of frame-to-frame step magnitudes, drawing step sizes at random and assigning directions drawn uniformly from the unit sphere. Positions were computed by cumulative summation from the original starting position. To replicate imaging, we applied simulated detection effects: Gaussian localization noise ($\sigma_{xy} = 0.21\mu\text{m}$,

$\sigma_z = 0.43\mu\text{m}$) followed by quantization to the imaging pixel resolution ($0.5\mu\text{m}$ in xy , $1.0\mu\text{m}$ in z). Simulated tracks were analyzed using the same pipeline applied to experimental data.

2.5.12 Statistical analysis

Custom code written in Python was used for data analysis and plotting. All p-values reported are from Mann–Whitney U tests, which are nonparametric and so do not assume normally distributed data. Unless otherwise noted, uncertainties are the 16th and 84th percentile of the distribution calculated from bootstrap subsampling.

2.6 Data Availability Statement

Analyzed neutrophil and macrophage trajectories are available as Supplemental Information. Analysis code is publicly available at https://github.com/rplab/Immune_Cell_Motion_Analysis_2025.

2.7 Bridge to Chapter III

The analyses presented in this chapter establish that innate immune cells patrol the larval zebrafish gut in stereotyped patterns constrained primarily by tissue architecture rather than by the bacterial species present. Neutrophils and macrophages maintain stable motility states, preferentially migrate along the intestinal anterior-posterior axis, and remain gut-associated regardless of whether fish are germ-free, conventionally reared, or colonized by a pathobiont *Vibrio* species. The only bacterial-dependent deviation from this baseline

surveillance pattern was the localized accumulation of non-motile neutrophils near sites of *Vibrio*-induced tissue damage.

These findings raise a complementary question: if the host immune surveillance landscape is relatively invariant to bacterial stimuli, how do bacterial populations themselves respond when new species attempt to colonize an already-occupied gut? Prior work has demonstrated that *Vibrio* ZWU0020 employs a Type VI Secretion System (T6SS) capable of both inter-bacterial killing and host cell targeting [2]. The T6SS-dependent tissue damage we observed as vent widening in this chapter represents one mechanism by which *Vibrio* perturbs the gut environment. However, the ecological consequences of T6SS activity for competitive dynamics within established bacterial communities remain unexplored.

The following chapter addresses this question by investigating how *Vibrio* invades gut communities comprising one to three founder bacterial species. Where Chapter II characterized the host's defensive framework, Chapter III examines how an invading pathobiont navigates and disrupts resident microbial communities through competitive interactions. Together, these perspectives illuminate complementary aspects of the host-resident microbe-invading microbe tripartite relationship that shapes gut ecology and host health.

CHAPTER III

INVASION OF ESTABLISHED GUT MICROBIAL COMMUNITIES BY A VIBRIO SPECIES ARMED WITH A TYPE VI SECRETION SYSTEM

This chapter describes ongoing collaborative research conducted under the supervision of Principal Investigator R. Parthasarathy. My contributions included the conceptualization and design of the study, as well as overarching project management and experimental scheduling. I performed bacterial cultures, derived germ-free larvae, and conducted *in vitro* competition experiments. Additionally, I developed the computational framework and code for all data processing, formal analysis of bacterial abundances, and visualization. I wrote the entirety of this chapter.

V.S. Winkler contributed to the study design and led the experimental execution. Her work included bacterial cultures, performing *in vitro* killing assays, and conducting zebrafish bacterial inoculations. Furthermore, she executed the primary *in vivo* work, comprising hundreds of zebrafish gut dissections and subsequent plating for colony-forming unit (CFU) quantification.

R. Parthasarathy served as the senior advisor, providing institutional resources, overarching study guidance, and critical interpretation of the research findings.

3.1 Abstract

Intestinal microbial communities profoundly influence host health, yet the mechanisms governing community composition and stability remain incompletely understood. Competition between bacterial species and perturbation of the host environment contribute to shaping these communities, but the factors determining resistance to invasion are only beginning to be characterized. Here we investigate how a zebrafish-native *Vibrio* strain (ZWU0020), referred as Vib WT, possessing a Type VI Secretion System (T6SS) capable of targeting both bacterial and host cells, invades pre-established gut bacterial communities of varying complexity. Using larval zebrafish colonized with defined communities comprising one to three founder bacterial species, we quantify invasion outcomes through gut dissection and colony enumeration. We use invasion and correlation coefficients framework to systematically compare suppression magnitudes across species and community configurations. All founder species examined show reduced abundance following Vib WT inoculation, but with striking species-specific differences: *Acinetobacter calcoaceticus* (ZOR0008) exhibits remarkable resistance while *Aeromonas veronii* (ZOR0001) and *Enterobacter cloacae* (ZOR0014) show strong susceptibility. Critically, community complexity systematically attenuates invasion magnitude, with three-species communities showing substantially weaker suppression than mono-associations. Unexpectedly, a T6SS-deficient Vib WT mutant produces suppression effects comparable to or exceeding wild-type, indicating that T6SS-independent mechanisms—likely including physical space limitation, resource competition, and secretion of inhibitory metabolites—dominate invasion dynamics in this system. These observations

reveal that species identity and community diversity, rather than T6SS-mediated killing alone, are primary determinants of gut microbiome resistance to invasion.

3.2 Introduction

3.2.1 The gut microbiome: composition, stability, and colonization resistance

The gastrointestinal tract of vertebrates harbors dense and diverse communities of microorganisms that influence a remarkably broad range of host processes, including nutrient metabolism, immune system development, and neurological function [113,114]. Human intestines contain an estimated 10^{13} bacterial cells comprising thousands of species, achieving densities approximately 100-fold greater than overnight laboratory cultures [26]. Despite this complexity, the gut microbiome exhibits structured patterns of variation: composition is highly body-site specific, relatively stable within individuals over time, yet distinct across individuals [115]. Understanding the rules governing this organization and particularly the factors that determine community stability and resistance to perturbation represents a fundamental challenge in host-microbiome studies.

A key concept in microbiome ecology is colonization resistance: the ability of an established community to resist invasion by exogenous species [116]. This phenomenon has clinical relevance, as disruption of the resident microbiota by antibiotics can enable colonization by pathogens such as *Clostridioides difficile* [116,117]. Mechanisms underlying colonization resistance include competition for nutrients and physical space, production of

antimicrobial compounds, modulation of host immune responses, and direct inter-bacterial antagonism [25,118,119]. The relative contributions of these mechanisms, and how they depend on community composition, remain active areas of investigation.

3.2.2 The Type VI Secretion System: molecular machinery for inter-bacterial warfare

Among the mechanisms bacteria employ for inter-species competition, the Type VI Secretion System (T6SS) has emerged as a particularly potent molecular apparatus. The T6SS is a contact-dependent secretion machine found in approximately 25% of Gram-negative bacteria, functioning as a molecular syringe that punctures adjacent cells and delivers toxic effector proteins [120,121]. Structurally, the T6SS comprises a membrane-spanning baseplate complex and a contractile sheath that propels a needle-like structure, formed by stacked Hcp hexamers tipped with trimeric VgrG spike proteins, into target cells [122].

The effector repertoire delivered by T6SS varies across species and strains, enabling targeting of both prokaryotic competitors and eukaryotic host cells. Anti-bacterial effectors include peptidoglycan-degrading enzymes, phospholipases, and DNases [123]. Notably, both the human-pathogen and zebrafish-native *Vibrio cholerae* strains possess a T6SS spike protein (VgrG-1) with a C-terminal actin crosslinking domain (ACD) capable of disrupting the cytoskeleton of eukaryotic cells [2,120,124]. This dual functionality—attacking both competing bacteria and modulating host cells—makes T6SS-armed bacteria particularly interesting agents of community disruption.

3.2.3 *Vibrio* T6SS modulates host physiology and bacterial competition

Previous studies from our laboratory have demonstrated that the human-pathogen *Vibrio* strain (El Tor C6706) T6SS activity can influence both host physiology and inter-bacterial competition in the larval zebrafish gut [86]. More recently, Ngo et al. investigated the mechanism connecting T6SS activity to intestinal motility using a zebrafish-native *Vibrio* strain ZWU0020 (hereafter Vib WT) [2]. This strain, which lacks cholera toxin and toxin-coregulated pili, colonizes larval zebrafish at high abundance and has been characterized as a “pathobiont”—a member of the normal microbiota with potential for pathogenicity—due to its pro-inflammatory characteristics [125,126]. Using a mutant deficient in the entire T6SS gene cluster (hereafter Vib dT6SS), Ngo et al. demonstrated that enhanced intestinal contractions require the T6SS and are associated with T6SS-dependent tissue damage and redistribution of intestinal macrophages.

3.2.4 *Spatial organization and motility in gut bacterial competition*

The physical organization of bacteria within the intestine plays a crucial role in population dynamics and inter-species interactions. Schlomann et al. discovered a quantitative relationship between bacterial aggregation behavior and spatial distribution along the intestinal axis [85]. More cohesive populations localize posteriorly, while planktonic populations concentrate anteriorly. This relationship has implications for competitive dynamics: aggregated bacteria are susceptible to peristaltic expulsion, whereas motile, planktonic bacteria resist displacement [84,88].

Bacterial swimming motility confers substantial advantages for intestinal persistence. Wiles et al. demonstrated that motile Vib WT can counter intestinal flow and maintain anterior localization, while non-motile mutants are progressively expelled [88]. Importantly, both Vib WT and Vib dT6SS possess functional flagella and are highly motile and planktonic, characteristics that may contribute to competitive success independent of T6SS activity.

3.2.5 Larval zebrafish and native gut bacteria as a model system

The larval zebrafish (*Danio rerio*) offers unique advantages for studying gut microbiome dynamics that are inaccessible in other vertebrate models [51,53]. Established protocols enable derivation of germ-free animals and subsequent colonization with defined microbial communities [103]. Light sheet fluorescence microscopy provides rapid, three-dimensional imaging with single-bacterium resolution [104].

A collection of zebrafish intestinal bacterial isolates has been developed and engineered to constitutively express fluorescent proteins (GFP and dTomato), enabling direct observation and species discrimination [89,127]. Among these, three species serve as “founder” populations in this study: *Aeromonas veronii* ZOR0001 (hereafter AE), *Acinetobacter calcoaceticus* ZOR0008 (hereafter AC), and *Enterobacter cloacae* ZOR0014 (hereafter EN). These Proteobacteria represent common members of the zebrafish gut microbiota and exhibit distinct colonization behaviors—AE forms mixtures of planktonic cells and aggregates, while EN forms highly cohesive aggregates localized to the midgut [85].

3.2.6 Higher-order interactions in gut microbial communities

Ecological theory predicts that pairwise species interactions may not fully predict community dynamics when higher-order interactions are present [128]. In the zebrafish gut microbiome, Sundarraman et al. demonstrated that higher-order interactions can dampen pairwise competition, with communities showing weaker competitive effects than predicted from two-species experiments [129]. This observation suggests that community complexity itself may confer emergent properties relevant to invasion resistance.

3.2.7 Aims of this study

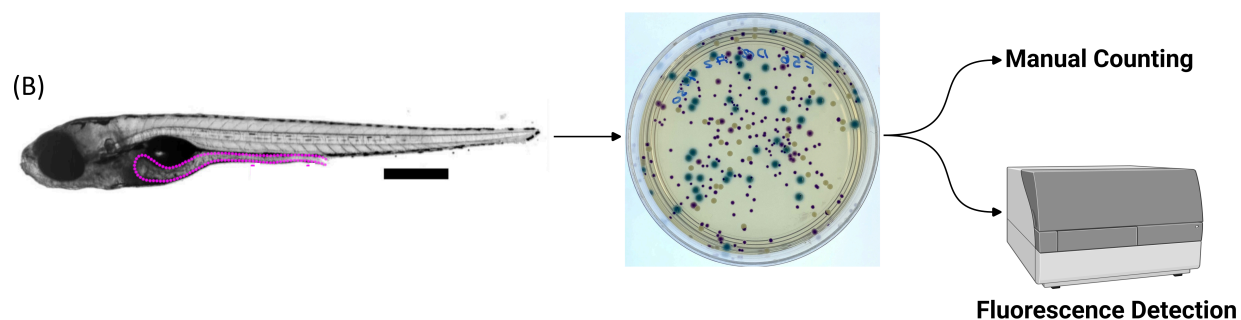
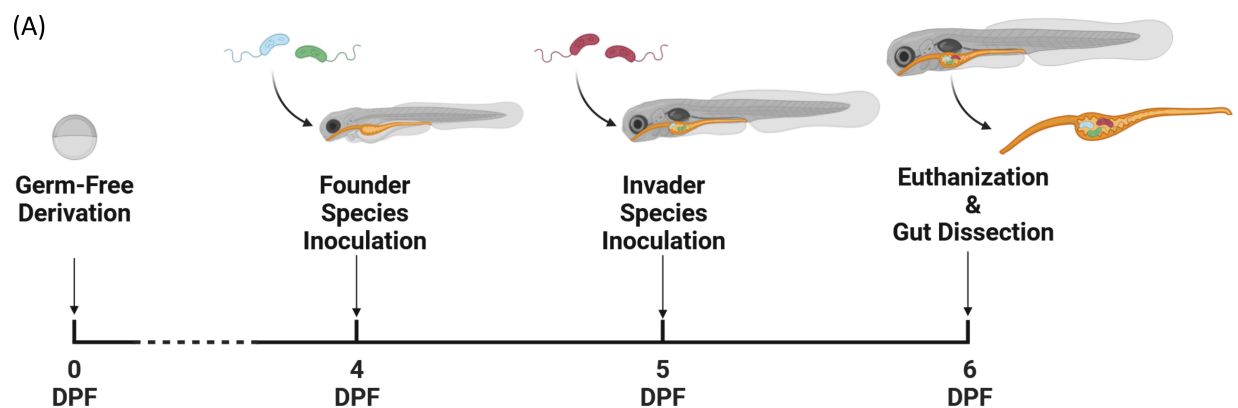
Building on the foundation established by prior T6SS studies in zebrafish, we investigate how *Vibrio* invades and disrupts pre-established gut bacterial communities of varying complexity. Specifically, we ask: (1) Does *Vibrio* invasion reduce the abundance of resident founder species, and does this depend on species identity? (2) Does community complexity—the number of co-colonizing founder species—influence invasion outcomes? (3) What is the role of T6SS in competitive displacement within complex communities?

We address these questions using a systematic experimental design in which germ-free larval zebrafish are first colonized with communities of one, two, or three founder species (AC, AE, EN), then challenged with *Vibrio* invaders (Vib WT or Vib dT6SS). Culture-based quantification of bacterial populations, combined with an invasion coefficient framework, enables rigorous comparison across conditions.

3.3 Results

To systematically examine how *Vibrio* invasion affects established gut bacterial communities, we colonized germ-free larval zebrafish with founder communities of varying complexity (one, two, or three species) at 4 days post-fertilization (dpf), then introduced Vib WT or Vib dT6SS at 5 dpf (Figure [3.1](#)). Gut bacterial abundances were quantified 24 hours post-invasion at 6 dpf by dissection and plating.

This design generates 21 experimental conditions: 7 founder configurations $\times$ 3 invasion treatments (Control-no invader, Vib WT, Vib dT6SS). Each condition includes $N = 7$ –42 individual larvae. See Figure [3.1C](#) for all conditions and sample sizes.



(C)

Founder × Invader Matrix

		Invader			
		Control (No Invader)	Vib WT	Vib dT6SS	
Founder Community	Mono-association	AC	$n = 32$	$n = 7$	$n = 12$
		AE	$n = 35$	$n = 11$	$n = 11$
		EN	$n = 35$	$n = 10$	$n = 12$
	Di-association	AC + AE	$n = 31$	$n = 8$	$n = 10$
		AC + EN	$n = 31$	$n = 7$	$n = 12$
		AE + EN	$n = 32$	$n = 11$	$n = 11$
	Tri-association	AC + AE + EN	$n = 42$	$n = 17$	$n = 12$

21 total experimental groups

Figure 3.1. Experimental design for studying *Vibrio* invasion in established gut communities. **(A)** Experimental timeline. Germ-free zebrafish embryos were derived at 0 days post-fertilization (dpf) and inoculated with founder bacterial species at 4 dpf. After 24 hours of founder colonization, invader species were introduced at 5 dpf. Larvae were then euthanized at 6 dpf for gut dissection and bacterial quantification. **(B)** Experimental workflow and bacterial quantification methods. **Left:** A 6 dpf larval zebrafish, with dashed outline showing the gut. Scale bar = 500 μm (adapted from [2]). Larval guts were dissected and homogenized, then plated on HiChrome UTI agar for species-specific enumeration. **Middle:** Representative petri dish shows distinct colony morphologies for the five bacterial species studied: *Acinetobacter* (AC, milky white), *Aeromonas* (AE, dark purple/red), *Enterobacter* (EN, blue/teal), *Vibrio* WT (dark purple/red), and *Vibrio* dT6SS (dark purple/red). All founder species (AC, AE, EN) carry constitutive GFP fluorescent markers, while *Vibrio* strains lack fluorescent tags. **Right:** Colony counts were performed manually using chromogenic colony morphology, with fluorescent plate reader measurements used to differentiate GFP-positive founders from non-fluorescent *Vibrio* invaders in mixed samples. **(C)** Experimental condition matrix. Seven founder community configurations (rows) were tested: three mono-associations (AC, AE, EN), three di-associations (AC+AE, AC+EN, AE+EN), and one tri-association (AC+AE+EN). Each founder configuration was combined with three invader treatments (columns): control (no invader), *Vib* WT invasion, or *Vib* dT6SS invasion, generating 21 experimental groups. Sample sizes (n) for each condition are shown within cells. Background colors distinguish mono-association (blue), di-association (yellow), and tri-association (pink) founder communities.

3.3.1 Total bacterial abundance is maintained despite community perturbation

We first examined whether *Vibrio* invasion alters total bacterial load in the gut. Remarkably, total bacterial abundance (founders + invaders) remained consistent at $\sim 10^4$ CFU/gut across nearly all experimental conditions (Figure 3.2A). The sole exception was mono-association with AC, which showed lower colonization levels ($\sim 10^{2.5}$ CFU/gut) even

in the absence of invasion. This consistency suggests that the larval zebrafish gut has a characteristic carrying capacity that is largely independent of species composition.

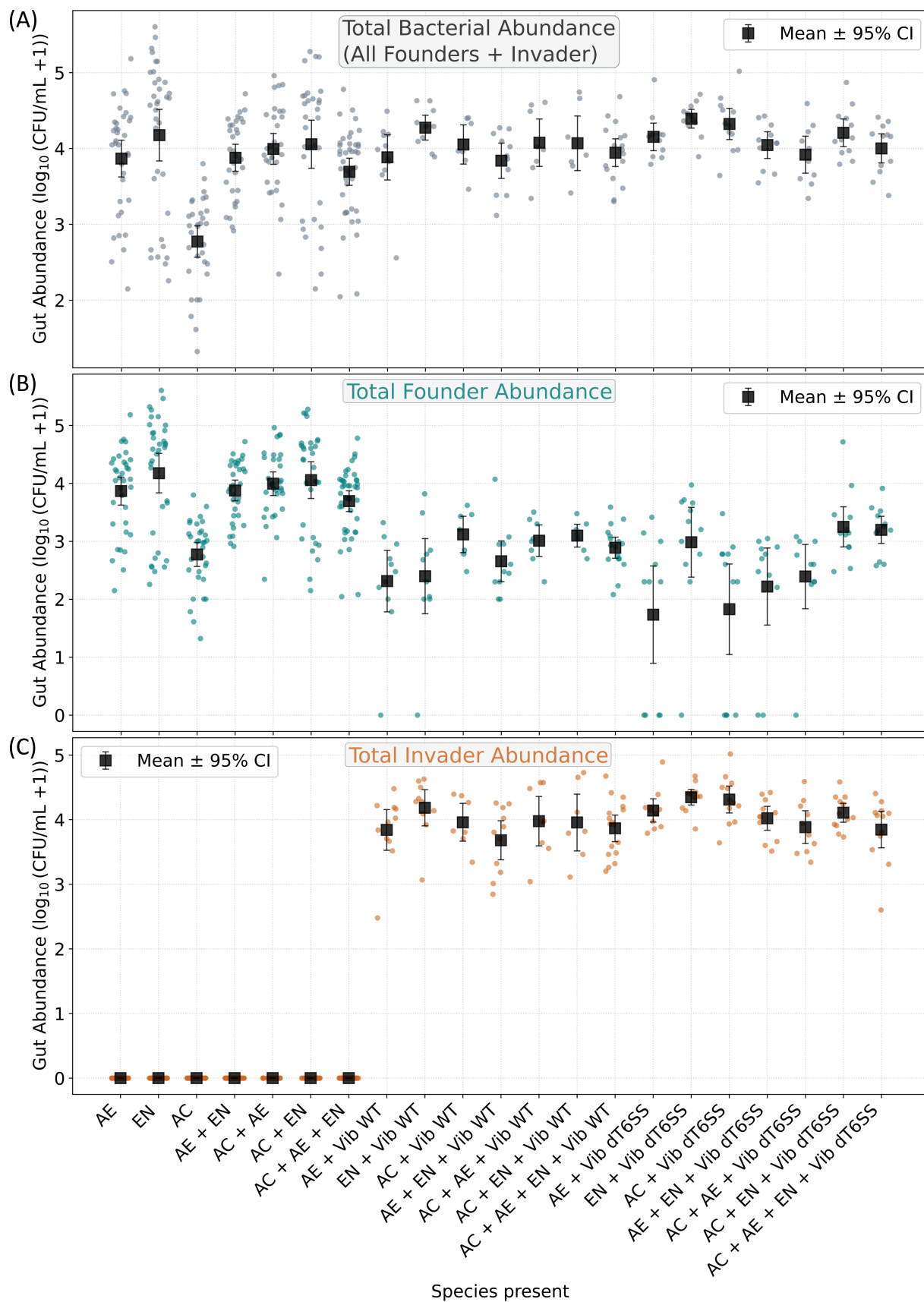


Figure 3.2. Gut bacterial abundance across different founder and invader combinations

(A) Total bacterial abundance (founders + invaders) across all experimental conditions. Each circle represents an individual larval zebrafish; black squares show mean $\pm$ 95% confidence interval. Total abundance remains consistent at $\sim 10^4$ CFU/gut regardless of species composition except for mono-association with AC which shows lower colonization levels. **(B)** Total founder abundance shows systematic suppression following *Vibrio* invasion. Founder-only controls (first seven conditions) maintain 10^4 – 10^5 CFU/gut with AC being the only exception with $\sim 10^{2.5}$ CFU/gut. Invaded communities (remaining conditions) show 1–3 orders of magnitude reduction in founder abundance with some specimen showing extinction events of founder species with the points corresponding to zero CFU/gut. **(C)** Total invader abundance is remarkably uniform across all founder variations. Founder-only controls (first seven conditions) correctly show no invading species abundance. In the invaded groups, both *Vib* WT and *Vib* dT6SS colonizes to $\sim 10^4$ CFU/gut independent of founder community composition.

3.3.2 *Vibrio* invasion suppresses founder populations

While total bacterial abundance remained constant, the distribution between founders and invaders shifted dramatically following invasion. In founder-only controls (no invader), founder species maintained abundances of 10^4 – 10^5 CFU/gut, with AC again being the exception at $\sim 10^{2.5}$ CFU/gut (Figure 3.2B). Following *Vibrio* invasion, founder abundances decreased by 1–3 orders of magnitude across all conditions tested. Notably, some individual larvae showed complete extinction of founder species (zero CFU/gut), indicating that invasion can drive local founder elimination.

Both *Vib* WT and *Vib* dT6SS colonized to $\sim 10^4$ CFU/gut regardless of founder community composition (Figure 3.2C). This uniformity demonstrates that *Vibrio*

colonization success is robust to variation in the resident community, consistent with the invader's motility and growth characteristics enabling rapid establishment. Species-level abundance data across all experimental groups are provided in Supplemental Figure [B.1](#).

3.3.3 Species-specific susceptibility to invasion

Normalizing founder abundances to their respective controls revealed striking species-specific responses to invasion (Figure [3.3](#); Supplemental Figure [B.3](#)). AC exhibited remarkable resistance: following invasion, AC abundance remained near or even slightly above control levels (normalized abundance $\sim 10^0$; Figure [3.3A](#)). This resistance was observed regardless of whether the invader was Vib WT or Vib dT6SS.

In contrast, AE showed strong susceptibility, with 1–2 orders of magnitude reduction in abundance following invasion (Figure [3.3B](#)). EN exhibited similar vulnerability, with normalized abundances dropping to 10^{-2} – 10^{-1} relative to controls (Figure [3.3C](#)). Two-species founder communities showed intermediate suppression patterns (Figure [3.3D](#); Supplemental Figure [B.2](#)).

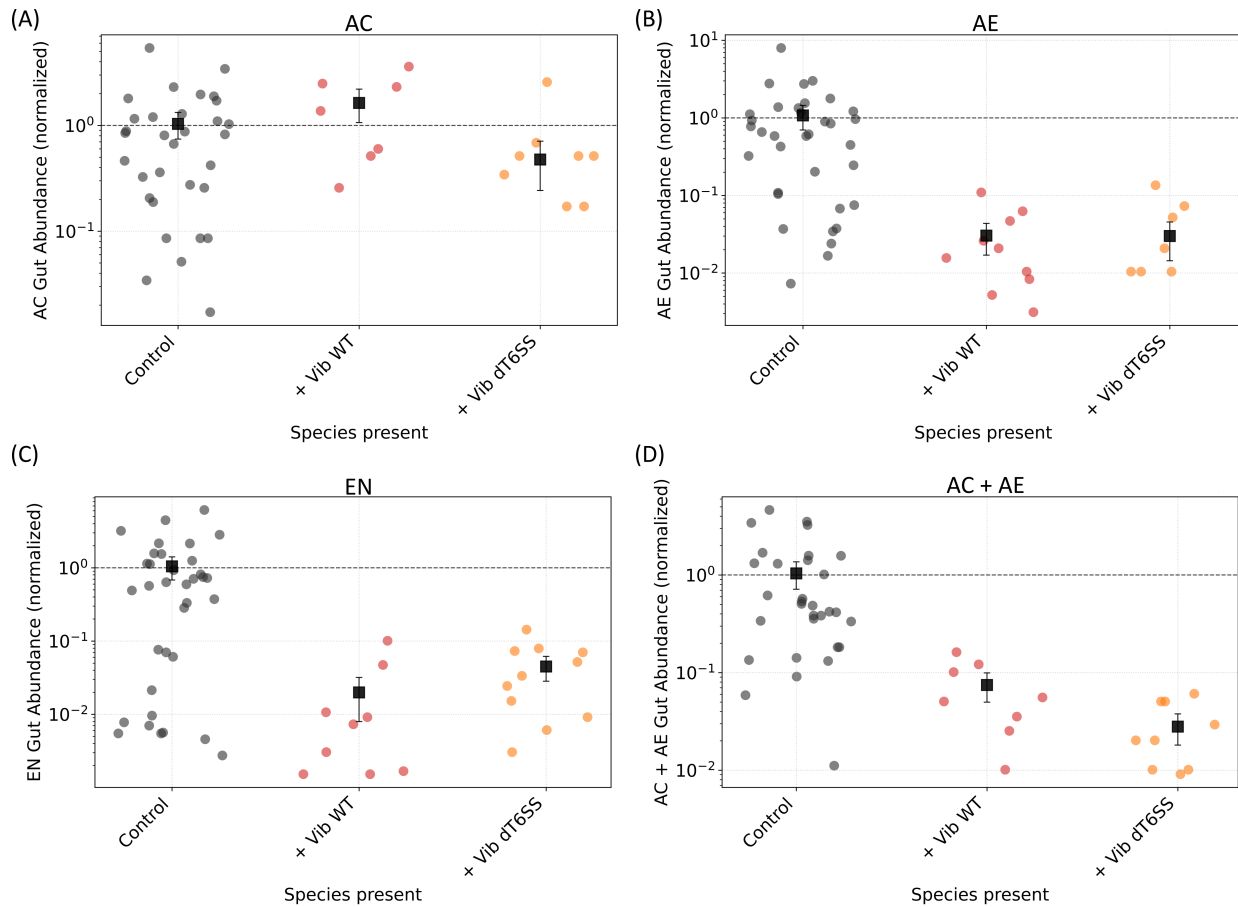


Figure 3.3. Founder-specific responses to *Vibrio* invasion Founder abundance was normalized to founder-only controls (dashed line at 10^0) to quantify invasion effects. Each circle represents an individual larval zebrafish; black squares show mean $\pm$ bootstrapped uncertainty. **(A)** AC abundance remains near control levels or slightly elevated during invasion, indicating resistance. **(B)** AE shows strong suppression, with 1-2 orders of magnitude reduction in invaded conditions compared to control. **(C)** EN exhibits similar susceptibility to AE, with normalized abundance dropping between 10^{-2} – 10^{-1} in most invaded conditions. **(D)** Two-species community (AC+AE) demonstrates intermediate suppression patterns. See supplemental for other community combinations. Colors distinguish invasion by Vib WT (red) and Vib dT6SS (orange) invasion conditions from controls (gray).

3.3.4 Invasion coefficients reveal systematic patterns

To quantify suppression magnitude across all founder-invader combinations, we computed invasion coefficients (β) representing $\log_{10}$ fold-change in founder abundance *with* versus *without* invader. More specifically, for the effect of invader i on founder f , β_{fi} is defined as:

$$\beta_{fi} = \log_{10} P_f^{+i} - \log_{10} P_f^{-i}$$

where P_f^{+i} is founder abundance in invaded conditions and P_f^{-i} is founder abundance in non-invaded controls. For brevity, we write β without subscripts where founders and invaders are clear from context. Values of β_{fi} indicate the interaction type: negative for suppression, zero for no effect, and positive for facilitation. The resulting heatmap reveals several key patterns (Figure 3.4A).

First, species identity is a primary determinant of invasion outcome. AC consistently showed β values near zero or slightly positive ($\beta \approx 0$ to $+0.35$), confirming its resistance to invasion. AE and EN showed strongly negative coefficients ($\beta \approx -1.5$ to -2.1), indicating susceptibility.

Second, community complexity systematically attenuates invasion magnitude. Organizing invasion coefficients by founder complexity (Figure 3.4B) reveals a clear trend: mono-associations show the strongest suppression (most negative β), di-associations show intermediate effects, and the three-species community (AC+AE+EN) shows the weakest

suppression ($\beta \approx -0.5$ to -0.8). This pattern suggests emergent community-level resilience that cannot be predicted from single-species responses alone (Supplemental Figure [B.2C](#)).

Third, and unexpectedly, Vib dT6SS produced suppression effects comparable to or exceeding those of Vib WT in many conditions. For some founder species (particularly AE), Vib dT6SS invasion resulted in even stronger suppression than Vib WT invasion.

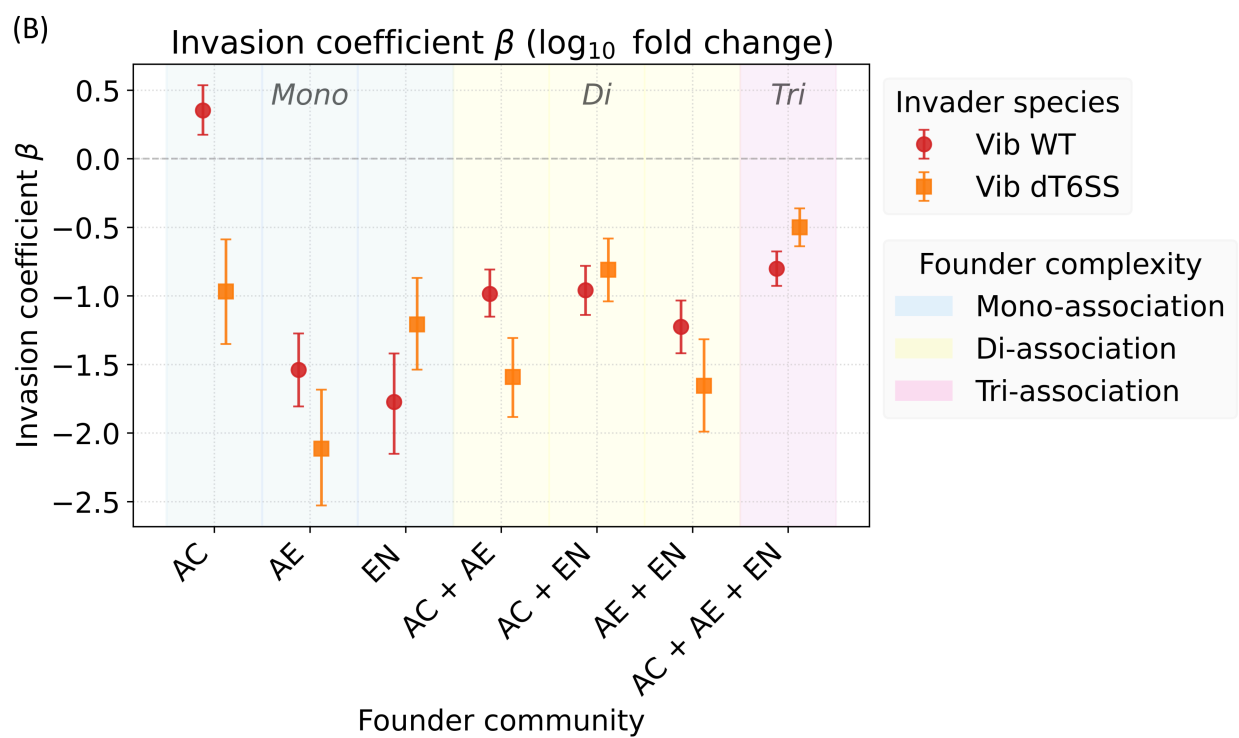
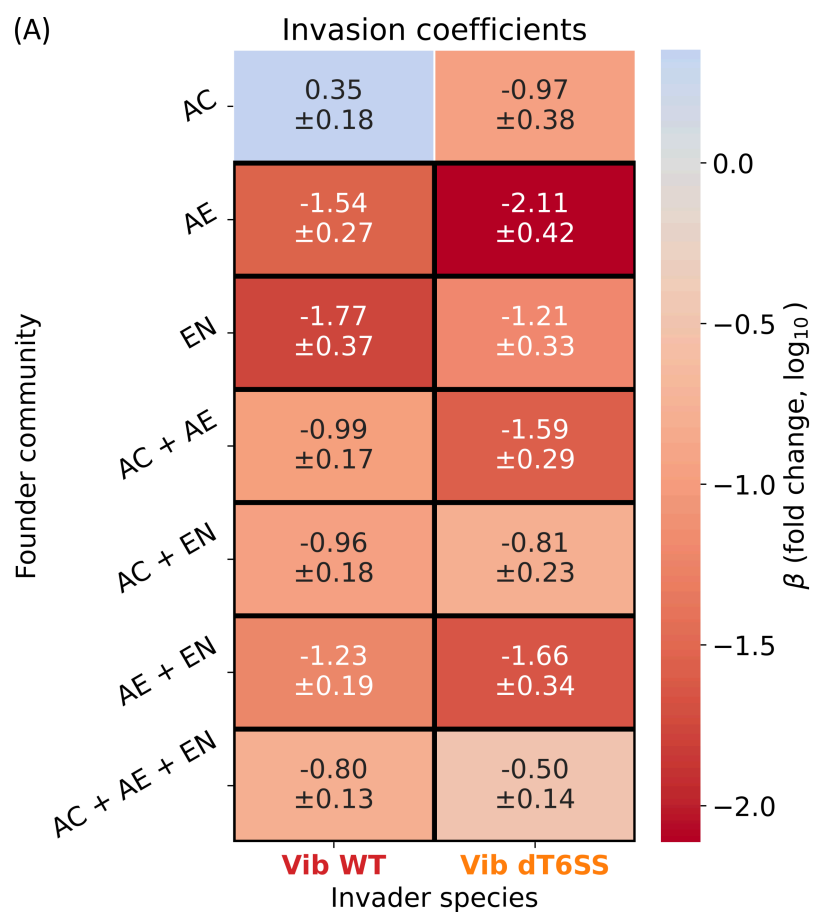


Figure 3.4. Invasion coefficients quantify founder suppression and community complexity effects. Invasion coefficient β represents $\log_{10}$ fold change in founder abundance with versus without invader. **(A)** Heatmap of invasion coefficients across all founder-invader combinations. Each cell shows β value (above) and bootstrapped uncertainty (below). Bold black borders indicate coefficients differing from zero by more than three standard deviations, representing statistically robust invasion effects. Color scale ranges from light blue showing resistance or facilitation ($\beta > 0$) to dark red representing strong suppression ($\beta < -2$). AC exhibits resistance ($\beta \approx 0$ to $+0.35$), while AE and EN show strong susceptibility ($\beta \approx -1.5$ to -2.1). Community complexity systematically attenuates invasion magnitude, with three-species communities showing weakest effects ($\beta \approx -0.5$ to -0.8). **(B)** Invasion coefficients organized by founder complexity. Background colors distinguish mono-association (blue), di-association (yellow), and tri-association (pink) founder communities. Circles (Vib WT, red) and squares (Vib dT6SS, orange) show mean $\beta \pm$ bootstrapped uncertainty for each founder-invader combination. Horizontal dashed line at $\beta = 0$ indicates no invasion effect. An approximate trend from more negative β values in simple communities to less negative values in complex communities demonstrates emergent community-level resilience.

3.3.5 Pairwise interaction coefficients reveal asymmetric relationships

To understand the mechanistic basis of invasion outcomes, we computed pairwise interaction coefficients (C_{ij}) quantifying how each species affects the abundance of others in di-association experiments (Figure 3.5; Supplemental Figure B.4). Specifically, C_{ij} is defined by fitting the relationship:

$$\log_{10} P_i^{\text{II}} = \langle \log_{10} P_i^{\text{I}} \rangle + C_{ij}^{\text{II}} \cdot \log_{10} P_j^{\text{II}}$$

where P_i^{I} is the abundance of species i in mono-association, P_i^{II} is its abundance in di-association with species j , and $\langle \cdot \rangle$ indicates the mean across replicates. The coefficient C_{ij}

thus captures the effect of species j abundance on species i in log-space (see Methods for higher order interactions formula).

Founder-founder interactions were generally weak. Among AC, AE, and EN, most pairwise coefficients were small ($|C_{ij}| < 0.15$), indicating that these species coexist without strong mutual inhibition. One notable exception was the asymmetric AE-EN interaction: AE inhibited EN ($C_{ij} = -0.47 \pm 0.08$), but EN had negligible effect on AE ($C_{ij} = -0.03 \pm 0.04$).

Invader-founder interactions were substantially stronger and species-specific. Vib WT exerted strong inhibition on AE ($C_{ij} = -0.69 \pm 0.15$) and EN ($C_{ij} = -0.76 \pm 0.18$), but weak, non-significant effects on AC. Strikingly, Vib dT6SS showed even stronger inhibition of AE ($C_{ij} = -1.30 \pm 0.40$) than Vib WT, while showing somewhat weaker effects on EN ($C_{ij} = -0.41 \pm 0.12$). Both invader strains showed minimal effects on AC.

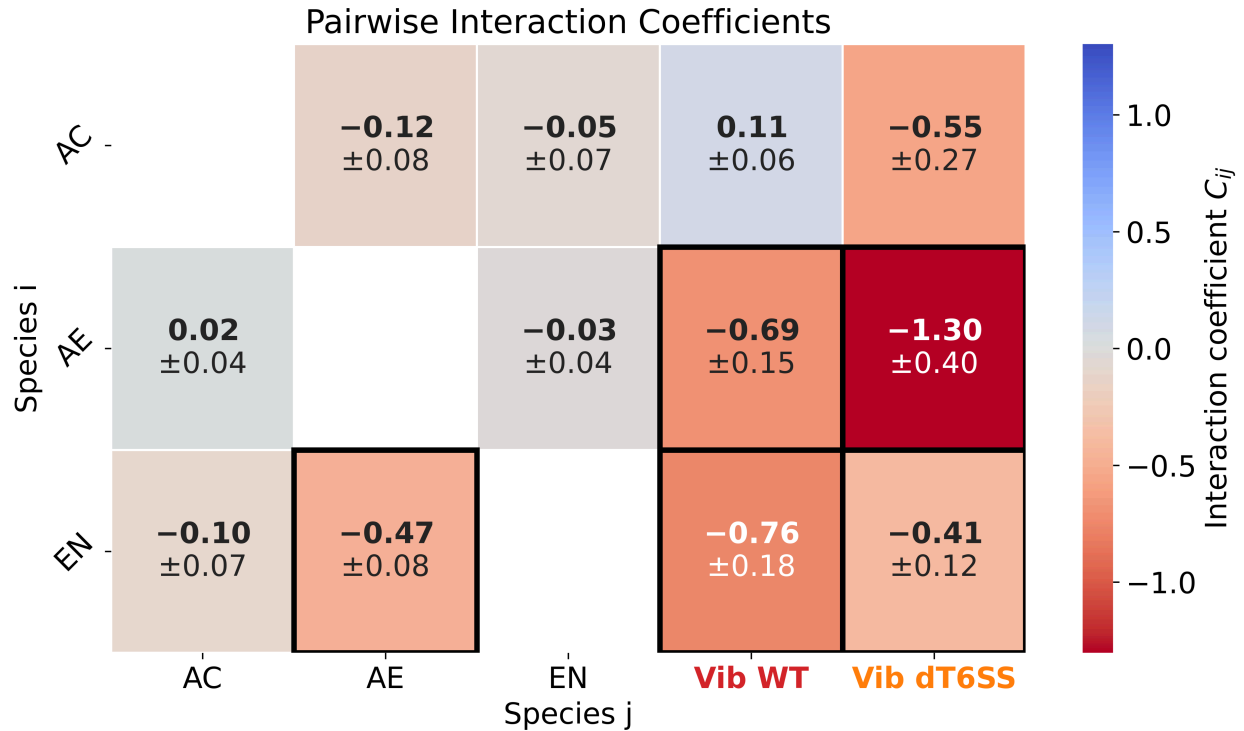


Figure 3.5. Pairwise interaction coefficients reveal asymmetric invader-founder relationships Interaction coefficients (C_{ij}) represent the effect of species j (columns) on species i (rows) in di-association experiments. Each cell displays the coefficient value (top) and bootstrapped uncertainty (bottom). Bold black borders indicate interactions differing from zero by more than three standard deviations. Color scale ranges from strong inhibition (dark red, $C_{ij} \approx -1.3$) through neutral (gray, $C_{ij} \approx 0$) to facilitation (dark blue, $C_{ij} > 0$).

Founder-founder interactions (Left 3×3 region): Weak interactions among AC, AE, and EN ($|C_{ij}| < 0.15$ for most pairs), with moderate asymmetric AE-EN antagonism with AE inhibiting EN ($C_{ij} = -0.47 \pm 0.08$) but EN not having any appreciable effect on AE ($C_{ij} = -0.03 \pm 0.04$).

Invader-founder interactions (Right 3×2 region): Vib WT exerts strong inhibition on AE ($C_{ij} = -0.69 \pm 0.15$) and EN ($C_{ij} = -0.76 \pm 0.18$), but weak non-significant effects on AC. Vib dT6SS shows even stronger inhibition of AE ($C_{ij} = -1.30 \pm 0.40$) but lesser effect on EN ($C_{ij} = -0.41 \pm 0.12$). Vib dT6SS shows slightly negative correlation with AC. See supplemental for correlation coefficients between all experimental groups.

3.3.6 T6SS is not required for competitive displacement in vivo

Perhaps the most surprising finding of this study is that T6SS is not required for *Vibrio* to suppress founder populations *in vivo*. In nearly all conditions tested, Vib dT6SS achieved suppression magnitudes comparable to or exceeding Vib WT.

This result contrasts with *in vitro* killing assays (Supplemental Figure [B.5](#)). On agar plates, Vib WT substantially reduced AE abundance compared to controls, while Vib dT6SS showed only intermediate killing—a clear T6SS-dependent phenotype. Yet *in vivo*, Vib dT6SS produces *stronger* suppression of AE than Vib WT. EN presents the opposite discordance: resistant to both *Vibrio* strains *in vitro*, yet among the most susceptible founders *in vivo* ($\beta \approx -1.5$ to -2.1).

3.4 Discussion

3.4.1 Species identity as a primary determinant of invasion outcome

Our results reveal that species identity is a dominant factor in determining susceptibility to *Vibrio* invasion. AC showed consistent resistance across all experimental conditions, while AE and EN were strongly suppressed. What distinguishes AC from the susceptible species?

One possibility relates to spatial organization and growth mode. Prior work established that bacterial cohesion—the tendency to form aggregates—correlates with spatial distribution in the zebrafish gut [85]. AC may occupy spatial niches or adopt growth

modes that reduce contact with invading *Vibrio*. Alternatively, AC may possess intrinsic resistance mechanisms, such as immunity proteins or cell surface modifications that prevent effector delivery.

The asymmetric AE-EN interaction (AE inhibits EN but not vice versa) suggests that founder species are not equivalent competitors. These pre-existing interaction hierarchies may influence how communities respond to invasion, with dominant species potentially buffering subordinate species from invader effects.

3.4.2 In vitro killing assays fail to predict in vivo outcomes

A striking finding of this study is the species-specific discordance between *in vitro* killing assays and *in vivo* invasion outcomes. On agar plates, T6SS function determines competitive success: Vib WT kills AE effectively while Vib dT6SS shows only intermediate killing (Supplemental Figure [B.5](#)). Yet within the gut, this relationship inverts—Vib dT6SS suppresses AE *more strongly* than Vib WT.

EN presents the mirror-image discordance. *In vitro*, EN exhibits remarkable resistance to both *Vibrio* strains, with survival approaching control levels. *In vivo*, EN is among the most susceptible founders, showing $\beta \approx -1.5$ to -2.1 regardless of invader genotype.

These reversals have important implications for interpreting bacterial competition studies. The gut environment introduces factors absent from agar plates: intestinal flow and peristalsis, spatial structure along the gut axis, host immune surveillance, mucus layers, and

dynamic nutrient availability. Our results suggest that these environmental factors can override or even reverse the competitive hierarchies established by direct bacterial antagonism. Studies relying solely on *in vitro* co-culture or killing assays may therefore misidentify the mechanisms governing community assembly *in vivo*.

3.4.3 Community complexity confers emergent resilience

A central finding of this study is that community complexity systematically attenuates invasion magnitude. Three-species communities showed substantially weaker suppression than mono-associations, with di-associations showing intermediate effects. This pattern is consistent with ecological theory predicting that diverse communities exhibit greater stability and invasion resistance [130].

Several mechanisms could underlie this emergent resilience. First, statistical averaging: if species differ in susceptibility (as demonstrated here), community-level abundance reflects a mixture of resistant and susceptible components. Second, functional redundancy: multiple species may occupy overlapping niches, such that suppression of one species creates opportunity for others to expand. Third, higher-order interactions: the presence of multiple species may modify pairwise interactions in ways that dampen invasion effects [129].

Our observation that tri-associated founders maintain higher relative abundances than di-associations ([B.2C](#)) supports the hypothesis that complexity itself provides protection beyond simple averaging effects. The nonlinear relationship between founder

number and invasion resistance suggests genuine higher-order interactions rather than mere dilution of susceptible species.

3.4.4 Mechanistic interpretations of T6SS-independent suppression

The finding that Vib dT6SS produces suppression effects comparable to or exceeding Vib WT *in vivo* represents a significant departure from the expectation that T6SS-mediated killing drives competitive displacement. What mechanisms might explain T6SS-independent suppression?

First, resource competition: *Vibrio* is a fast-growing species that may outcompete founders for limiting nutrients regardless of direct killing. The gut represents a nutrient-limited environment where rapid growth and efficient resource acquisition confer substantial advantages [88].

Second, motility-driven displacement: both Vib WT and Vib dT6SS possess functional flagella and are highly motile and planktonic. These characteristics enable resistance to intestinal expulsion and maintenance of anterior gut localization [88]. Intestinal peristalsis—even without T6SS-induced enhancement—could disproportionately expel aggregated founder species while sparing planktonic *Vibrio*.

Third, indirect effects via host physiology: *Vibrio* colonization induces immune responses, including neutrophil and macrophage recruitment, that may disadvantage founders independently of T6SS activity [2]. Even Vib dT6SS elicits host responses that could alter the competitive landscape.

The observation that Vib dT6SS shows stronger effects on AE than Vib WT is particularly intriguing. One possibility is that T6SS activity imposes a metabolic cost on Vib WT, diverting resources from growth and motility toward effector production and secretion apparatus assembly. Alternatively, T6SS-mediated host tissue damage by Vib WT may paradoxically benefit certain founder species by releasing nutrients or creating spatial refugia.

3.4.5 Implications for microbiome ecology and engineering

Our findings have implications for understanding and manipulating gut microbial communities. The observation that community diversity confers invasion resistance suggests that maintaining or restoring microbiome diversity could enhance protection against pathogens—consistent with clinical observations that antibiotic-depleted microbiota are vulnerable to pathogen colonization [116].

The species-specific nature of invasion susceptibility indicates that community composition, not just diversity, matters for colonization resistance. AC's remarkable resistance suggests that certain species may serve as keystone members that stabilize communities against perturbation. Identifying the mechanisms of AC resistance could inform strategies for engineering invasion-resistant communities or selecting probiotic strains.

The disconnect between *in vitro* and *in vivo* outcomes cautions against using plate-based killing assays to predict ecological dynamics. Screening for T6SS activity or *in vitro*

antagonism may identify strains with competitive potential, but the gut environment introduces additional selective pressures that can fundamentally alter competitive outcomes. Future efforts to engineer protective communities should prioritize *in vivo* validation over *in vitro* characterization.

Finally, the finding that T6SS is not required for competitive success challenges the assumption that bacterial warfare machinery is the primary determinant of community dynamics. While T6SS clearly contributes to inter-bacterial competition in some contexts [86], our results suggest that ecological factors—resource competition, motility, spatial organization—may be equally or more important in determining invasion outcomes within complex, host-associated communities.

3.5 Conclusion

We investigated how a T6SS-armed *Vibrio* strain invades pre-established gut bacterial communities of varying complexity in larval zebrafish. Three principal findings emerge from this work.

First, species identity is a primary determinant of invasion susceptibility. AC exhibited remarkable resistance to *Vibrio* invasion across all conditions, while AE and EN were consistently suppressed. This species-specificity was not predictable from *in vitro* killing assays, which showed different—and in some cases opposite—patterns of susceptibility.

Second, community complexity confers emergent resilience. Invasion magnitude decreased systematically from mono-associations to di-associations to the three-species community, suggesting that diversity itself provides protection beyond simple averaging of species-specific responses.

Third, T6SS is not required for competitive displacement *in vivo*. Vib dT6SS achieved suppression comparable to or exceeding Vib WT, indicating that T6SS-independent mechanisms—likely including resource competition, motility-driven displacement, and host-mediated effects—dominate invasion dynamics within the gut environment.

These results highlight the limitations of *in vitro* approaches for predicting microbial community dynamics and underscore the importance of host context in shaping competitive outcomes. Future work will examine the temporal dynamics of invasion, the spatial organization of founders and invaders within the gut, and the molecular basis of species-specific resistance.

3.6 Materials and Methods

3.6.1 Zebrafish husbandry

All experiments with zebrafish followed standard procedures [101] and were performed in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee (protocol numbers AUP-22-02 and AUP-20-16). Fish from which the larvae used in these studies were derived were maintained in the University of

Oregon Zebrafish Facility at 28°C with a 14-h/10-h light/dark cycle. Wild-type (AB × TU) zebrafish (*Danio rerio*) were used for all experiments.

3.6.2 Gnotobiotic derivation

Embryos were derived germ-free following established protocols [103]. Fertilized eggs were incubated for 6 hours in sterile embryo medium (EM) containing antibiotics (100 µg/mL ampicillin, 10 µg/mL gentamycin, 1 µg/mL tetracycline, 1 µg/mL chloramphenicol, 250 ng/mL amphotericin B). After surface sterilization with 0.003% sodium hypochlorite and 0.1% polyvinylpyrrolidone-iodine, embryos were distributed into T25 tissue culture flasks at one embryo per mL in 15 mL sterile EM. Germ-free status was verified by plating and microscopic examination.

3.6.3 Experimental design

The experimental scheme (Figure [3.1](#)) proceeds as follows: (1) germ-free larval zebrafish are colonized at 4 dpf with founder communities comprising one species (AC, AE, or EN alone), two species (AC+AE, AC+EN, or AE+EN), or three species (AC+AE+EN); (2) at 5 dpf, fish are inoculated with invaders (*Vib* WT or *Vib* dT6SS), or left uninoculated as controls; (3) at 6 dpf (24 hours post-invasion), larvae are euthanized for gut dissection and plating.

This design generates 21 experimental conditions: 7 founder configurations $\times$ 3 invasion treatments (no invader, Vib WT, Vib dT6SS). Each condition includes $N = 7$ –42 individual larvae. See Figure [3.1C](#) for all conditions and sample sizes.

3.6.4 Bacterial strains

Founder species comprise zebrafish intestinal isolates: *Aeromonas veronii* ZOR0001 (AE), *Acinetobacter calcoaceticus* ZOR0008 (AC), and *Enterobacter cloacae* ZOR0014 (EN). These species were previously engineered to constitutively express fluorescent proteins (GFP or dTomato) enabling species discrimination [89,127].

Invader strains include *Vibrio* sp. ZWU0020 (Vib WT; NCBI BioProject PRJNA205585), previously isolated from the zebrafish intestinal tract [126], and the T6SS-deficient mutant Vib dT6SS, constructed as previously described [2]. All bacterial stocks were maintained in 25% glycerol at -80°C .

3.6.5 Bacterial inoculation

One day prior to inoculation, bacteria from frozen glycerol stocks were grown overnight in Lysogeny Broth (LB: 10 g/L NaCl, 5 g/L yeast extract, 12 g/L tryptone, 1 g/L glucose) at 30°C for 16 h with shaking. Samples of 1 mL from each overnight culture were washed once by centrifugation at $7000 \times g$ for 2 min, removal of supernatant, resuspension in 1 mL sterile embryo media, and inoculated directly into flask water.

At 4 dpf, germ-free larvae in tissue culture flasks were inoculated with founder species (AC, AE, EN, or combinations thereof) at 10^6 CFU/mL. For multi-species founder communities, equal concentrations of each species were added. After 24 h of founder colonization, invader species (Vib WT or Vib dT6SS) were introduced at 5 dpf at 10^6 CFU/mL. Control groups received no invader. Flasks were maintained at 28°C until gut dissection at 6 dpf (24 h post-invasion).

3.6.6 Culture-based quantification

Larval guts were dissected following established procedures [104]. Dissected guts were placed in sterile saline (0.7%) with zirconium oxide beads and homogenized using a bullet blender (30 s, power level 4). Homogenates were serially diluted and plated on HiCrome UTI agar, which enables species discrimination based on colony morphology and color: AC produces milky opaque colonies, AE produces reddish-purple colonies, EN produces blue/teal colonies, and both *Vibrio* strains produce same color colonies as AE. Vib WT and Vib dT6SS were distinguished from AE by fluorescent markers using a commercial plate reader.

3.6.7 Invasion coefficient framework

To quantify invasion effects, we define the invasion coefficient β_{fi} for the effect of invader i on founder f as:

$$\beta_{fi} = \log_{10} P_f^{+i} - \log_{10} P_f^{-i}$$

where P_f^{+i} is founder abundance in invaded conditions and P_f^{-i} is founder abundance in non-invaded controls. The sign of β_{fi} reflects the ecological interaction: $\beta_{fi} < 0$ indicates suppression, $\beta_{fi} = 0$ indicates no effect, and $\beta_{fi} > 0$ indicates facilitation. Bootstrapped confidence intervals (10,000 resamples) were computed for all coefficients.

3.6.8 Pairwise and higher-order interaction coefficients

Following Sundarraman et al. [129], we computed interaction coefficients C_{ij} representing the effect of species j on species i . In di-association experiments:

$$\log_{10} P_i^{\text{II}} = \langle \log_{10} P_i^{\text{I}} \rangle + C_{ij}^{\text{II}} \cdot \log_{10} P_j^{\text{II}}$$

where P_i is abundance of species i (CFU/gut), superscript I denotes mono-association, superscript II denotes di-association, and $\langle \cdot \rangle$ indicates the mean across replicates. This formulation generalizes to multi-species communities:

$$\log_{10} P_i^N = \langle \log_{10} P_i^{\text{I}} \rangle + \sum_{j \neq i} C_{ij}^N \cdot \log_{10} P_j^N$$

where superscript N denotes the number of co-colonizing species. We computed C_{ij}^{II} for all pairwise founder and invader combinations (Figure 3.5), and C_{ij}^N for tri-association and four-species (three founders + invader) communities (Supplemental Figure B.4).

This formulation is motivated by the approximately log-normal distribution of gut bacterial abundances, with species interactions capable of inducing orders-of-magnitude changes [129]. Qualitatively, positive C_{ij} indicates that abundance of species i increases in the presence of j ; negative C_{ij} indicates decline. The coefficient C_{ij} can be derived as the

interaction parameter in a competitive Lotka-Volterra model modified to act on log-abundances (see [129] Supplemental Text). Mean and standard deviation of interaction parameters were estimated by bootstrap subsampling.

3.6.9 Pseudocount transformation

All log-transformations of gut bacterial abundances, including calculations of different coefficients, were computed with a pseudocount of 1 (i.e., $\log_{10}(P + 1)$) to accommodate zero values arising from extinction events. This approach retains information from complete founder elimination, which represents a biologically meaningful invasion outcome that would otherwise be excluded from analysis.

3.6.10 Quantifying T6SS-mediated bacterial killing in vitro

Overnight cultures of fluorescently labeled founder species (AC, AE, or EN) and either Vib WT or Vib dT6SS were mixed at a 1:1 ratio. Mixtures were washed once in 0.7% saline, serially diluted, and spotted onto tryptic soy agar (TSA; 5 μ L/spot). Spots were incubated overnight at 30°C and imaged using an Amersham™ Typhoon™ biomolecular imager. Spot boundaries were manually masked and total fluorescence intensity within each spot was quantified as a proxy for founder abundance. Four biological replicates were performed per dilution factor per bacterial combination.

3.6.11 Statistical analysis

All analyses were performed using Python with NumPy, SciPy, and Matplotlib. Unless otherwise noted, uncertainties represent the 16th and 84th percentiles of the distribution calculated from bootstrap subsampling. Coefficients were considered statistically robust when differing from zero by more than three standard deviations.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

This dissertation has examined the spatial dynamics of host-microbe interactions in the larval zebrafish gut through two complementary studies. Chapter II characterized the surveillance patterns of innate immune cells, while Chapter III investigated how an invading bacterial species displaces established gut microbial communities. Together, these studies reveal that physical constraints, whether imposed by tissue architecture on immune cell migration or by ecological context on bacterial competition, play unexpectedly dominant roles in shaping the outcomes of host-microbe interactions. This chapter synthesizes the principal findings, discusses their theoretical and practical implications, acknowledges limitations, and outlines directions for future research.

4.1 Summary of Findings

The first study (Chapter II) established a quantitative framework for imaging and tracking neutrophils and macrophages throughout the larval zebrafish gut over multi-hour timescales [131]. Using light sheet fluorescence microscopy and three-dimensional trajectory analysis of hundreds of individual cells, I characterized speeds, directional persistence, and cellular morphology across diverse microbial conditions: germ-free, conventionally reared, and colonized by wild-type or mutant *Vibrio* strains. Three principal

findings emerged. First, individual immune cells exhibit stable motility phenotypes over four-hour imaging periods, favoring predominantly motile or non-motile states rather than frequent transitions between them. This bimodal distribution was consistent across all microbial conditions and both cell types. Second, gut architecture fundamentally constrains immune cell migration patterns. Both neutrophils and macrophages showed preferential movement along the anterior-posterior intestinal axis and maintained high gut residence probability (greater than 70% of imaging time), consistent with migration along the gut boundary rather than random exploration. Third, macrophages but not neutrophils displayed significantly reduced sphericity during motile periods compared to non-motile periods, providing a morphological signature that may enable inference of dynamic behavior from static snapshots. Remarkably, migration patterns remained consistent across all microbial conditions tested. The only microbe-specific response observed was the localized accumulation of non-motile neutrophils near *Vibrio*-induced tissue damage [2], indicating that while bacterial activity can create specific retention sites, it does not fundamentally alter surveillance dynamics.

The second study (Chapter III) examined how *Vibrio* species armed with a Type VI Secretion System (T6SS) invades pre-established bacterial communities in the zebrafish gut. Using gnotobiotic techniques to construct communities of defined composition (mono-, di-, and tri-associations of zebrafish-native bacterial species), we quantified invasion outcomes through gut dissection and colony enumeration. Three principal findings emerged. First, species identity is the primary determinant of invasion susceptibility. *Acinetobacter* (AC)

exhibited resistance to *Vibrio* invasion, maintaining or even slightly increasing its abundance following challenge, while *Aeromonas* (AE) and *Enterobacter* (EN) showed strong susceptibility, with 1–2 orders of magnitude reduction in abundance. Second, community complexity systematically attenuates invasion magnitude. Founder communities with three species showed weaker invasion effects than di-associations, which in turn generally showed weaker effects than mono-associations, demonstrating emergent community-level resilience that cannot be predicted from pairwise interactions alone [129]. Third, and most unexpectedly, the T6SS was not required for competitive displacement *in vivo*. Wild-type *Vibrio* and T6SS-deficient mutants produced comparable suppression of founder species, despite the established role of T6SS in mediating inter-bacterial killing *in vitro* [2]. This finding indicates that resource competition, motility advantages, or host-mediated mechanisms dominate over direct T6SS-mediated killing in the complex gut environment.

4.2 Theoretical Contributions

These studies contribute to understanding host-microbe interactions in several ways. First, they highlight the role of tissue architecture in shaping immune surveillance. Extensive *in vitro* work has demonstrated that immune cells respond to local chemical signals [6,7], and this is undoubtedly true. However, the present findings suggest that at the scale of whole-organ surveillance in larval zebrafish, anatomical constraints also play a substantial role. Immune cells that encounter the gut tend to remain there and migrate

along its length, and this pattern persists regardless of whether the gut harbors conventional microbiota, a pathobiont, or no bacteria at all. Whether this architectural influence generalizes to other tissues and organisms remains to be tested.

Second, the finding that community complexity confers resilience against invasion extends ecological theory to the vertebrate gut microbiome. Recent work has shown that microbiome diversity protects against pathogens through nutrient blocking [132], and reviews have synthesized the mechanisms underlying colonization resistance [133,134]. The present results provide direct experimental evidence that, in a vertebrate gut, increasing species richness from one to three generally reduces invasion by a competitive species. Importantly, this emergent resilience operates through mechanisms that cannot be captured by pairwise interaction coefficients, as the damping of competitive effects in multi-species communities exceeds predictions from simple additive models [129].

Third, the discordance between *in vitro* killing assays and *in vivo* invasion outcomes highlights the limitations of reductionist approaches to understanding host-microbe interactions. The T6SS is a well-characterized molecular weapon that demonstrably kills competitor bacteria in co-spotting assays [135]. Yet in the complex environment of the living gut, T6SS-mediated killing does not appear to be the defining contributor to competitive outcomes. This finding does not diminish the importance of understanding T6SS biology but rather emphasizes that molecular mechanisms must be evaluated in their physiological context. Resource competition, host immune responses, spatial structure, and other factors may overwhelm the effects of direct killing in determining community composition.

4.3 Practical Implications

The findings from this dissertation carry implications for therapeutic strategies targeting the gut microbiome and intestinal immunity. Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis involve dysregulated immune responses to gut microbiota [19,23]. Current treatments often target systemic immune pathways without considering the spatial organization of immune responses within the gut. If immune surveillance patterns follow predictable rules determined by tissue architecture, as the present findings suggest, then spatially targeted interventions may offer more precise control over inflammation than systemic approaches. For example, strategies that modulate immune cell retention at specific gut locations, rather than globally suppressing immune activity, might provide therapeutic benefit with fewer side effects.

Similarly, interventions aimed at manipulating the microbiota through probiotics, prebiotics, or fecal microbiota transplantation increasingly seek to improve gut health [136,137], yet predicting which interventions will successfully establish in a given individual's gut remains challenging. The present findings suggest that the complexity of the resident community is a critical factor: simpler communities are more vulnerable to invasion, while more diverse communities exhibit emergent resilience. This principle could inform strategies for probiotic design, as engineered strains may have higher establishment rates in individuals with depleted microbiota, for example following antibiotic treatment [116,117].

The observation that T6SS-mediated killing does not dominate competitive outcomes *in vivo* also carries practical implications. Considerable effort has been devoted to understanding and potentially exploiting bacterial secretion systems as targets for antimicrobial intervention, including the design of peptides that inhibit T6SS assembly [138]. The present results suggest caution in assuming that blocking a killing mechanism *in vitro* will translate to altered community dynamics *in vivo*. Effective microbiome manipulation may require attention to ecological context, including resource availability, spatial structure, and host-mediated effects, rather than individual molecular mechanisms alone.

4.4 Limitations and Scope

The studies presented here represent methodological advances that also define the boundaries of current investigation. Chapter II provides, to our knowledge, the first comprehensive three-dimensional characterization of gut-associated immune cell dynamics in a living vertebrate, spanning germ-free, conventionally colonized, and pathobiont-associated conditions over multi-hour timescales. The imaging and analysis framework enables quantification of speeds, directional persistence, morphology, and spatial distributions for hundreds of individual cells. However, the biophysical approach that makes this characterization possible does not access molecular mechanisms. The signaling pathways that maintain stable motility phenotypes, the receptors that mediate gut residence, and the transcriptional states distinguishing motile from non-motile cells remain unknown.

Integrating the present framework with emerging techniques for *in vivo* biosensors or single-cell transcriptomics could bridge this gap.

The four-hour imaging window captures a snapshot of immune surveillance during a specific developmental and colonization phase (9-13 hours post-inoculation in 6 dpf larvae). Whether the architectural constraints and stable motility phenotypes observed here persist across longer timescales, during active infection, or through resolution of inflammation remains to be tested. Similarly, larval zebrafish possess only innate immunity; the interplay between the patterns described here and adaptive immune responses in adult vertebrates represents an important area for future investigation.

Chapter III presents one of the first *in vivo* studies of bacterial invasion into pre-established gut communities of defined composition in a vertebrate host. The gnotobiotic approach enables precise manipulation of community membership and absolute quantification of species abundances, revealing species-specific invasion susceptibility and complexity-dependent resilience. However, the reliance on gut dissection and colony enumeration sacrifices spatial information. Work from our laboratory has demonstrated that bacterial spatial organization within the gut, including aggregation patterns, localization along the anterior-posterior axis, and proximity to the epithelium, strongly influences population dynamics and host interactions [85]. Whether the invasion coefficients measured here reflect uniform displacement throughout the gut or spatially heterogeneous competitive outcomes remains unknown. Live imaging of fluorescently labeled communities during invasion could address this gap.

Finally, the focus on a single *Vibrio* strain and three founder species constrains generality. The principles identified here, including the dominance of resource competition over T6SS-mediated killing and the attenuation of invasion in complex communities, await testing with additional invaders and more diverse founder communities.

4.5 Future Research Directions

4.5.1 Short-term Research Opportunities

Several immediate extensions of this work would strengthen and expand the findings presented here. First, longer-duration imaging studies on innate immune cells spanning 12-24 hours would test whether the stable motility phenotypes observed over four hours persist across extended timescales, or whether cells eventually transition between states. Such studies would also reveal whether immune cell behaviors change as bacterial populations grow and community dynamics unfold.

Second, and most directly, experiments combining the immune cell imaging of Chapter II with the defined bacterial communities of Chapter III would bridge these complementary perspectives. Simultaneous tracking of fluorescently labeled immune cells and bacteria in the same animal would address several open questions. Do immune cells redistribute when *Vibrio* displaces founder species, or does surveillance proceed independently of competitive outcomes? Does the complexity-dependent resilience of bacterial communities correlate with differences in immune engagement? A combined

imaging approach could reveal whether immune cells preferentially associate with invading or resident species, and whether such associations influence competitive outcomes.

Third, live imaging of bacterial populations during invasion would reveal the spatial dynamics of competitive displacement. Where within the gut does *Vibrio* first establish? Do founder species retreat to location specific niches or undergo uniform depletion? Combining bacterial imaging with this invasion model could test whether spatial structure predicts invasion susceptibility.

Fourth, extending the invasion experiments to additional time points (6, 12, 24, 48 hours post-invasion) would characterize the temporal dynamics of community restructuring and test whether initial invasion patterns predict long-term outcomes.

4.5.2 Long-term Research Opportunities

Several broader research directions emerge from this work. A central question is whether the principles identified here generalize across host-microbe systems. The architectural constraint of immune surveillance, the complexity-dependent resilience of microbial communities, and the context-dependence of bacterial killing mechanisms may reflect general features of gut biology or may be specific to larval zebrafish. Testing these principles in other tissues (skin, lung, oral cavity), other model organisms (mouse, *Drosophila*, *C. elegans*), and in adult animals with mature adaptive immunity would establish the scope of their applicability.

The morphological signature linking macrophage sphericity to motility state raises the possibility of inferring dynamic behavior from static snapshots. Developing this approach for application to fixed tissue samples, including clinical biopsies, could enable retrospective analysis of immune surveillance patterns in health and disease states where live imaging is impossible.

The mechanisms underlying complexity-dependent resilience of bacterial communities remain unclear. Higher-order interactions, resource partitioning, spatial structure, and host-mediated effects could all contribute. Systematic manipulation of community composition combined with metabolomics and transcriptomics could identify the mechanistic basis of emergent resilience and suggest strategies for engineering robust probiotic communities.

Finally, the observation that immune surveillance patterns are largely independent of microbial context, while bacterial competitive outcomes depend strongly on community composition, raises questions about how these two systems interact over longer timescales. Does sustained immune activity at sites of bacterial competition alter invasion outcomes? Do shifts in bacterial community composition eventually reshape immune surveillance patterns? The tools developed here, applied longitudinally and in combination, could begin to address how immune and microbial dynamics co-evolve within a single host.

4.6 Concluding Remarks

The gut represents a dynamic interface where host tissues continuously negotiate with microbial communities. This dissertation has approached the interface from two complementary perspectives: the host immune system surveilling for threats, and bacterial populations competing for residence. Both studies converge on a common theme: the importance of physical and ecological context in determining the outcomes of host-microbe interactions.

Immune cells do not simply respond to chemical signals; they move through tissues whose architecture constrains and channels their motion. Bacteria do not compete solely through molecular weapons; they navigate ecosystems where community composition and resource availability modulate the effects of any single mechanism. These findings argue for integrating biophysical and ecological perspectives into our understanding of host-microbe relationships. The reductionist approach of isolating molecular mechanisms in controlled conditions has been enormously productive, but the present work suggests that such mechanisms must be evaluated in their physiological context. Tissue geometry, community complexity, and spatial organization are not background noise to be controlled away; they are determinants of outcome.

The larval zebrafish gut, transparent and tractable, has enabled visualization of dynamics that remain hidden in other systems. The quantitative frameworks established here, for tracking immune cell trajectories in three dimensions and for quantifying invasion across communities of varying complexity, provide tools applicable to new questions. As imaging technologies improve and computational methods mature, the principles identified

in this small vertebrate may illuminate the larger challenge of understanding and manipulating the human gut microbiome.

The gut microbiome affects virtually every aspect of human health, from nutrition and metabolism to immunity and even behavior. Therapeutic manipulation of this system holds enormous promise but remains largely empirical. Probiotics often fail to engraft; fecal transplants succeed unpredictably and rarely persist; inflammatory bowel diseases resist durable treatment. Progress toward rational intervention will require understanding of not just what species are present, but how they interact with each other and with the host, in space and over time. This dissertation represents a step toward that understanding.

APPENDIX A

SUPPLEMENTAL INFORMATION FOR CHAPTER 2

This appendix contains supplemental information that support the main findings presented in chapter 2 of the dissertation.

A.1 Supplemental Information: Contents

1. Supplemental Figures and Captions
2. Supplemental 3D Figure Captions
3. Supplemental Video Captions
4. Supplemental Dataset Descriptions

A.2 Supplemental Figures and Captions

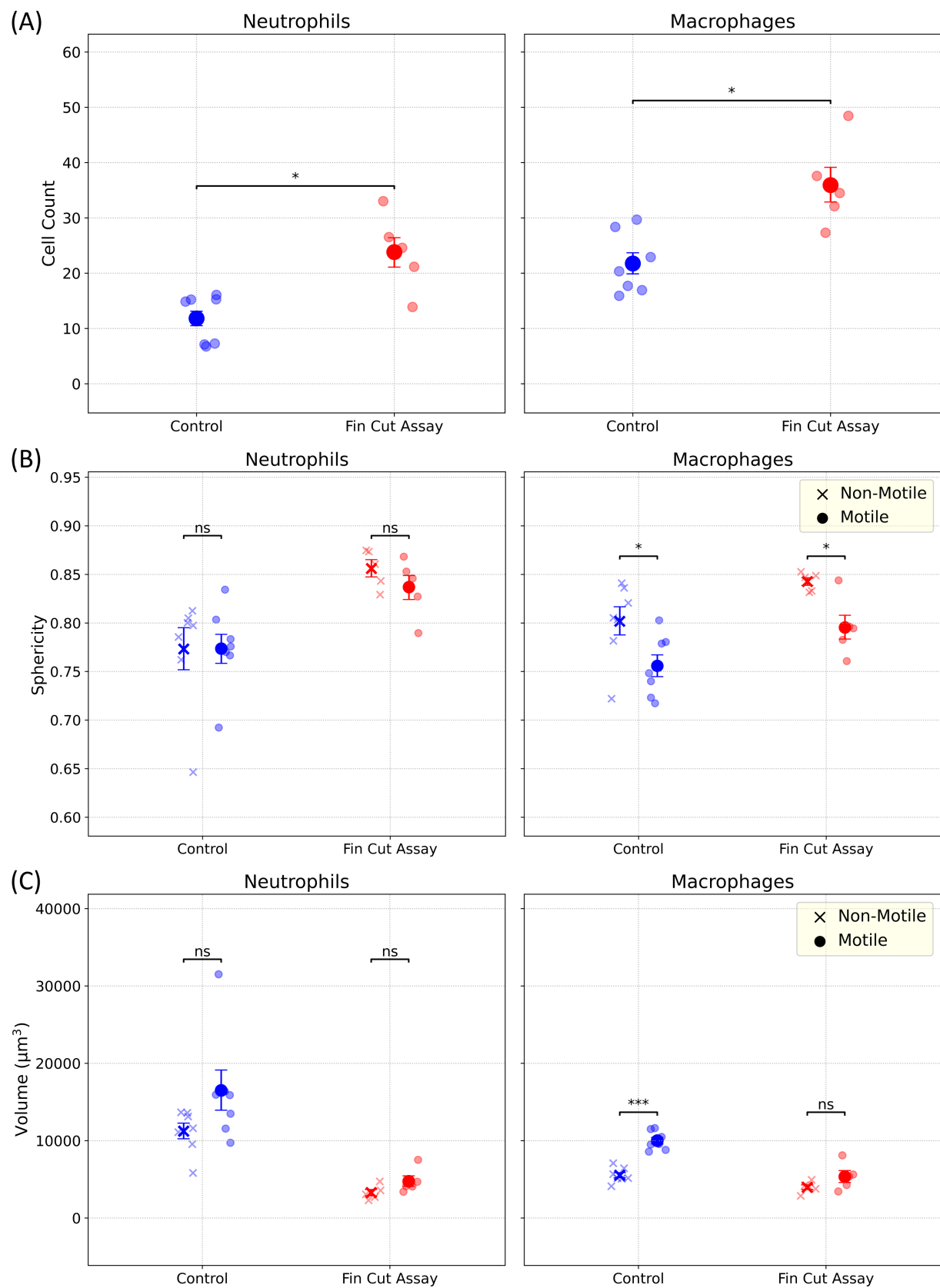


Figure A.1. Supplemental Fin Cut Assay: All panels show data for neutrophils (left) and macrophages (right). (A) Mean cell count in the field of view of imaging (approximately 800 μm from the posterior end of the caudal fin) over 1-5 hours post-amputation in control and fin cut conditions. Neutrophil numbers increased following fin amputation compared to controls, while macrophage counts remained unchanged between conditions. (B) Sphericity comparison between motile and non-motile subtracks across both experimental groups. Macrophages exhibit significantly higher sphericity during non-motile periods for both control and fin cut assay, indicating increased cellular rounding when stationary, while neutrophils show no morphological distinction between motility states. (C) Volume comparison between motile and non-motile subtracks in both experimental groups. Error bars represent uncertainties from bootstrapping. $n_{\text{Control}} = 5$, $n_{\text{Fin Cut Assay}} = 7$; total $N = 12$. ns: not significant, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

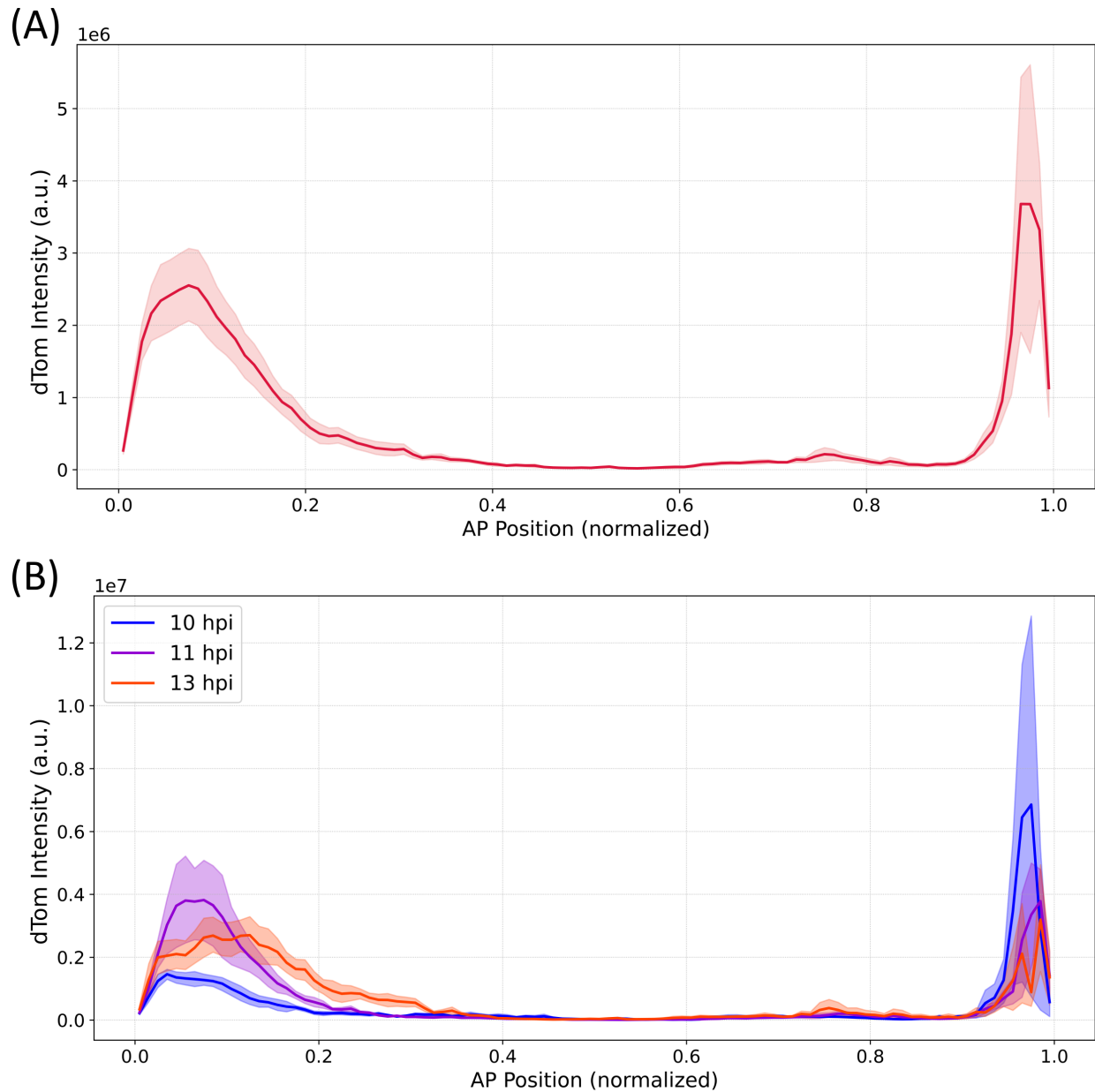


Figure A.2. Bacterial distribution and colonization during the imaging period. (A) Pooled median background-subtracted line profile of dTomato intensity along the anterior-posterior axis for *Vibrio*^{WT}-dTomato inoculated fish from 10-13 hpi. (B) Line profiles of dTomato intensity showing bacterial distribution at 10, 11, and 13 hpi. Bacterial fluorescence localizes primarily at the gut bulb (anterior) and the vent (posterior) at representative timepoints throughout the 9-13 hpi imaging window. Anterior-posterior position is normalized such that 0 and 1 represent the start and end of the gut, respectively. dTomato intensity is shown

in arbitrary units (a.u.). Error bars represent uncertainties from bootstrapping. $n = 4$ per timepoint, total $N = 12$.

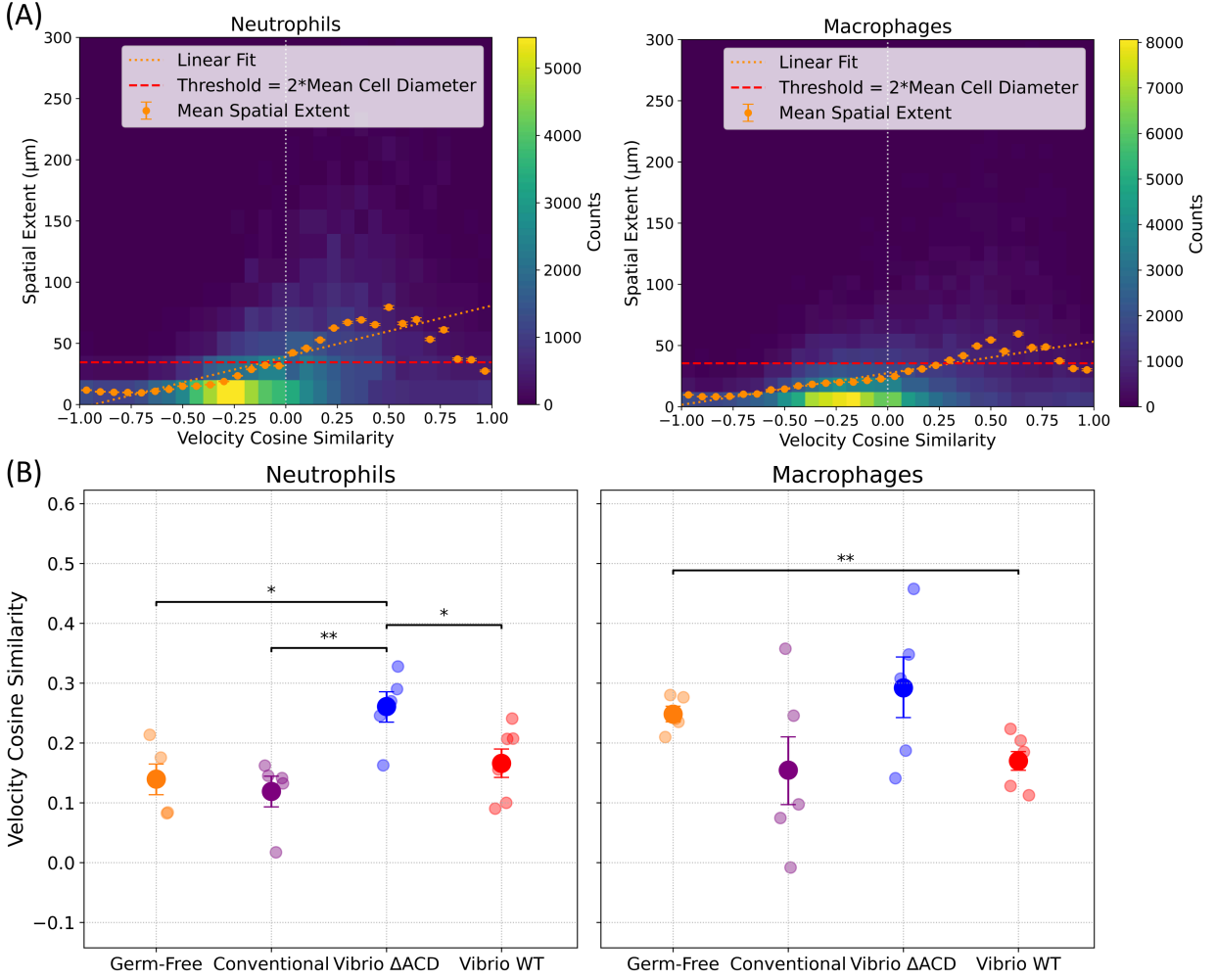


Figure A.3. Supplemental Gut Bacterial Association: All panels show data for neutrophils (left) and macrophages (right). (A) 2D histograms of spatial extent versus mean velocity cosine similarity for subtracks in the imaging region around the gut, pooled across control, conventional, and all *Vibrio*-associated fish. The horizontal red line shows the motility classification threshold of twice the mean cell diameter. Orange points show mean spatial extent binned by velocity cosine similarity; the orange dashed line is a linear regression fit to all individual data points. The y-intercept of the linear fit, i.e., spatial extent corresponding to zero velocity cosine similarity, is $38.98 \pm 0.23 \mu\text{m}$ ($R^2 = 0.134$, $n = 1.40 \times 10^5$ datapoints) for neutrophils and $27.38 \pm 0.12 \mu\text{m}$ ($R^2 = 0.120$, $n = 2.47 \times 10^5$) for macrophages. (B) Velocity Cosine Similarity for motile subtracks in control, conventional and *Vibrio*-associated fishes. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$. * : $p < 0.05$, ** : $p < 0.01$.

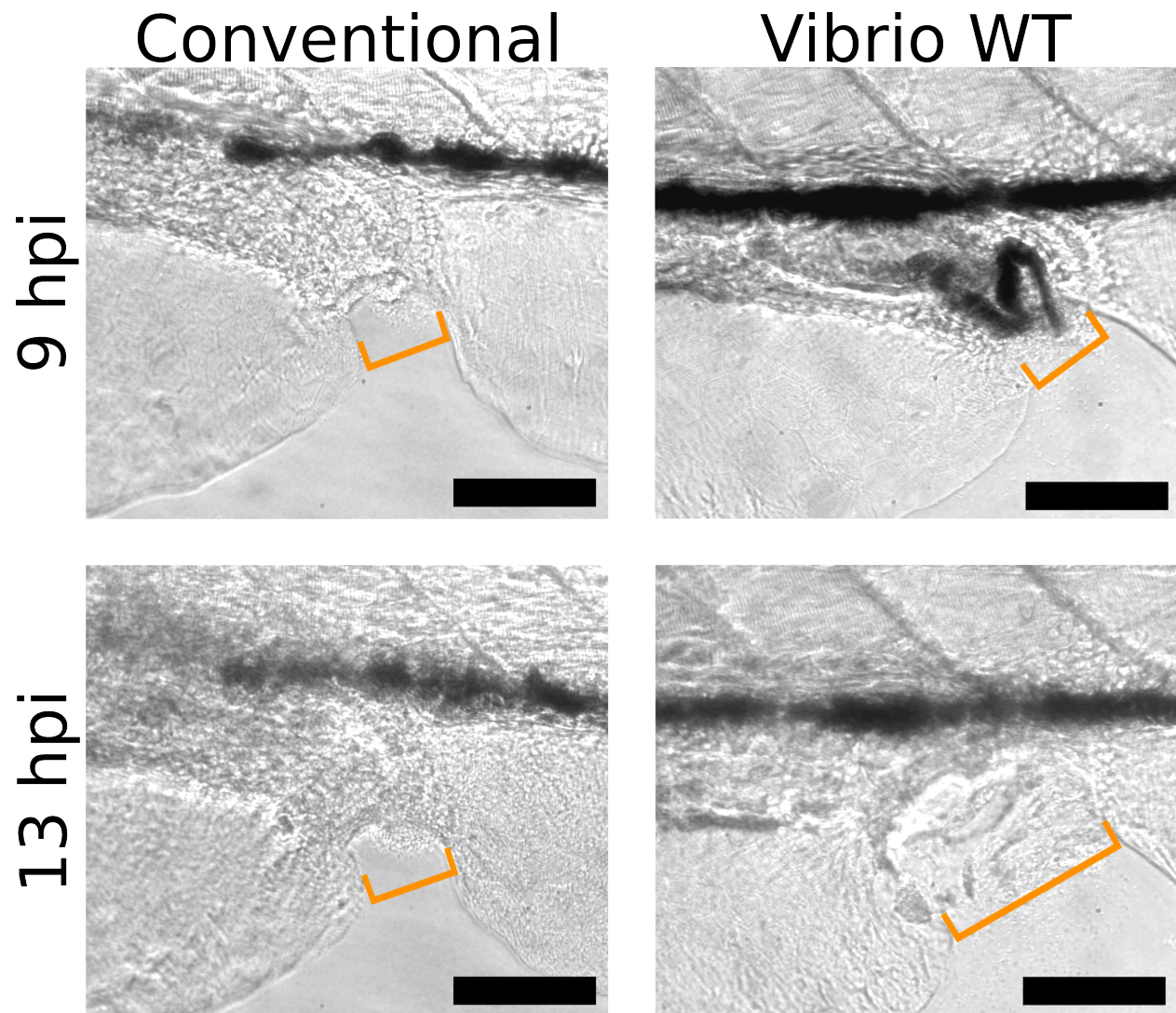


Figure A.4. Supplemental Gut Bacterial Association: Brightfield images showing vent widths for conventionally raised (left) and *Vibrio*^{WT}-inoculated fish (right) at the start and end of the imaging window. Orange lines indicate the vent width. All scale bars: 100µm.

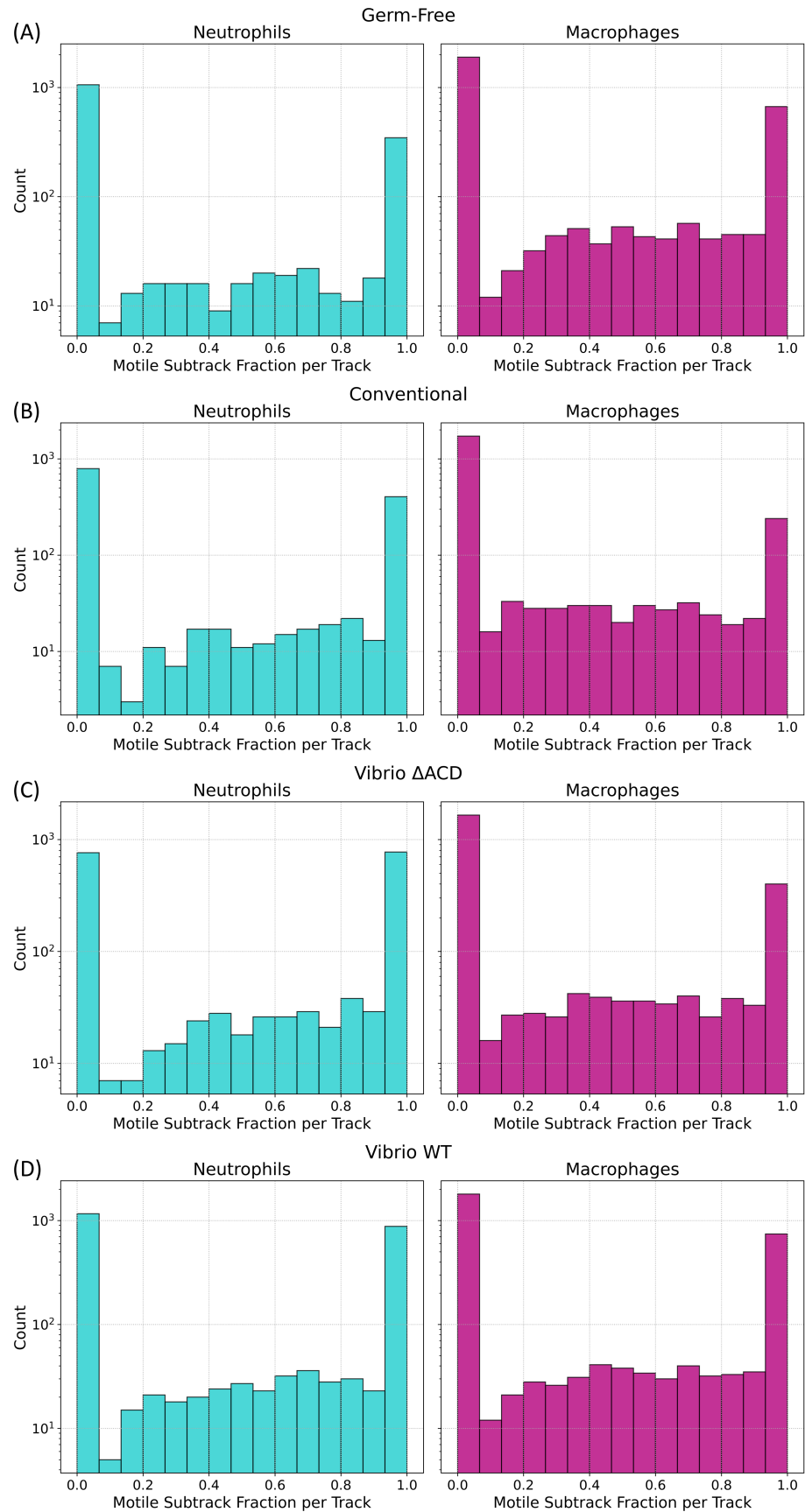


Figure A.5. Supplemental Gut Bacterial Association: Histograms showing the fraction of each complete cell track exhibiting motile behavior for neutrophils (left) and macrophages (right), for (A) Germ-free (GF), (B) conventionally reared (Conventional), (C) *Vibrio*^{ΔACD}-inoculated, and (D) *Vibrio*^{WT}-inoculated fish. The distribution shows peaks at 0 and 1, indicating that immune cells generally adopt a consistent motile or non-motile phenotype over the 4-hour observation period, though a subset of cells exhibits intermediate behavior. Analysis included only tracks with minimum track lengths of 5 frames. Bin width = 0.06̄.

$n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$.

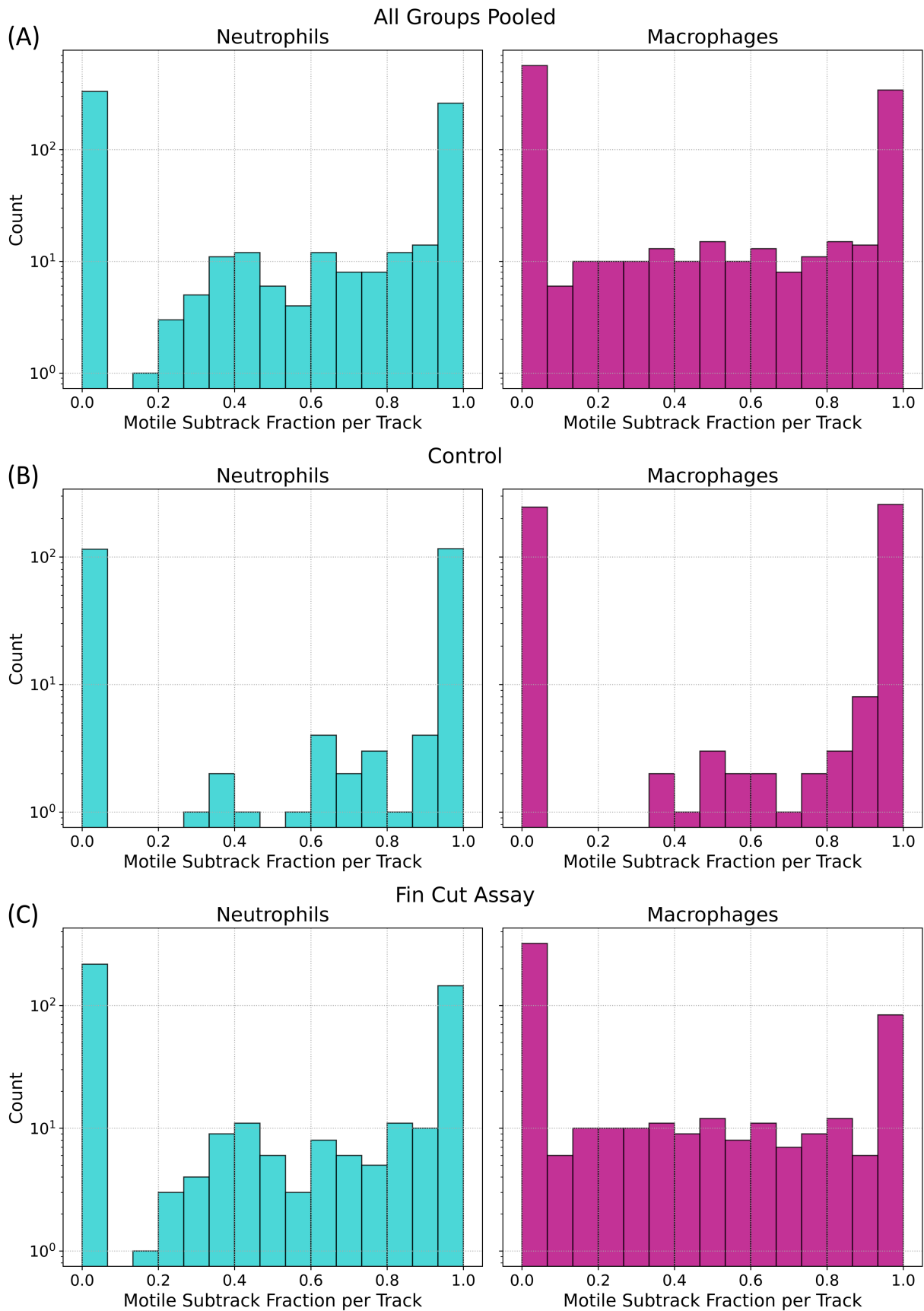


Figure A.6. Supplemental Fin Cut Assay: Histograms showing the fraction of each complete cell track exhibiting motile behavior for neutrophils (left) and macrophages (right), (A) pooled across all experimental groups, (B) only fin cut assay, and (C) only control (uninjured) group. The distribution shows peaks at 0 and 1, indicating that immune cells generally adopt a consistent motile or non-motile phenotype over the 4-hour observation period, though a subset of cells exhibits intermediate behavior. Analysis included only tracks with minimum track lengths of 5 frames. Bin width = $0.0\bar{6}$. $n_{\text{Control}} = 5$, $n_{\text{Fin Cut Assay}} = 7$; total $N = 12$.

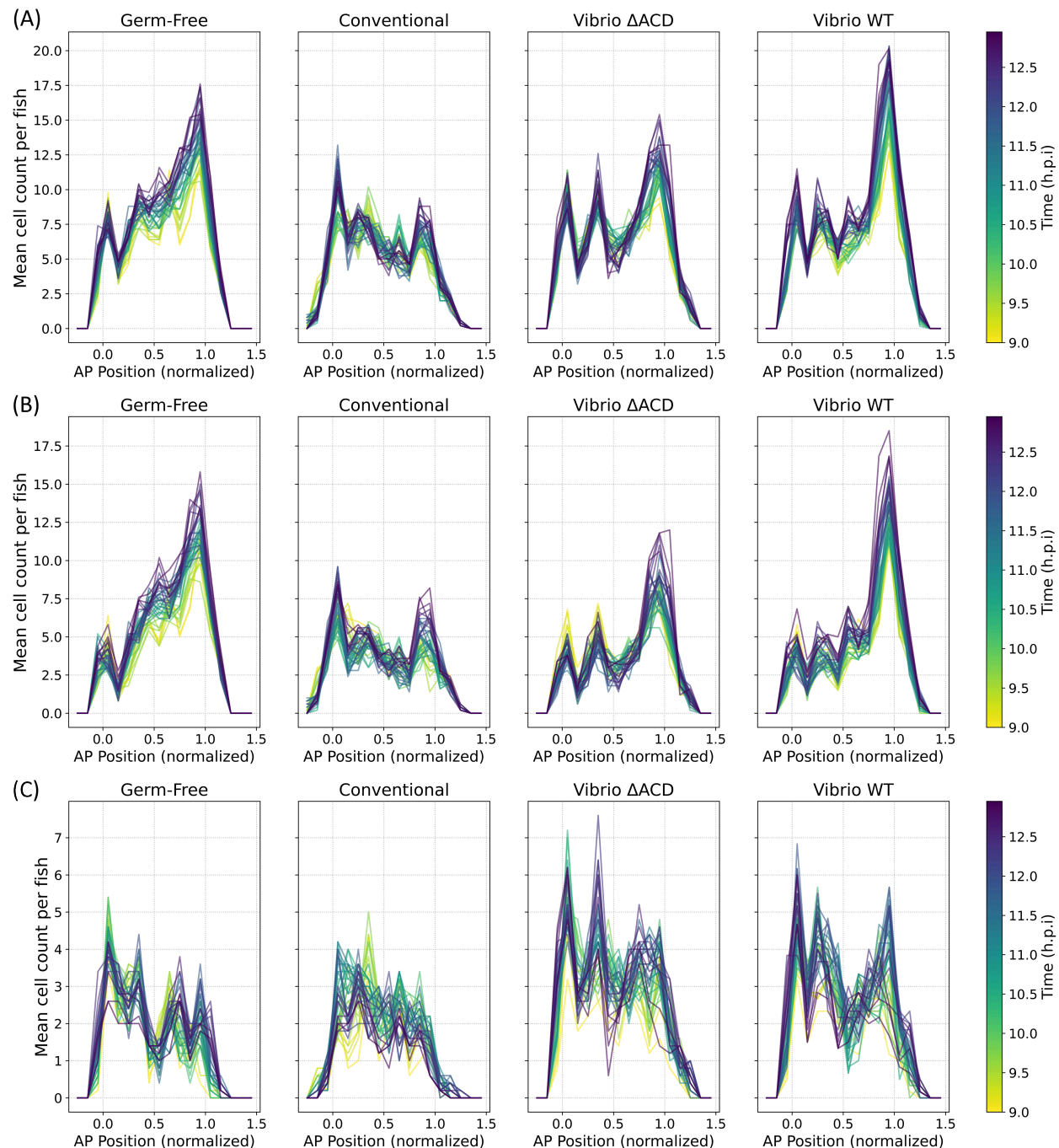


Figure A.7. Supplemental Gut Bacterial Association: Cell distribution along the anterior-posterior gut axis over time. Anterior-posterior position is normalized such that 0 and 1 represent the start and end of the gut, respectively. Color scale indicates time progression in hours post inoculation (h.p.i), where yellow represents early timepoints and dark purple represents late timepoints. (A) Total cell counts along the gut axis regardless of motility state. (B) Distribution of non-motile subtracks, showing increased cell accumulation near

the vent region over time. (C) Distribution of motile subtracks. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$.

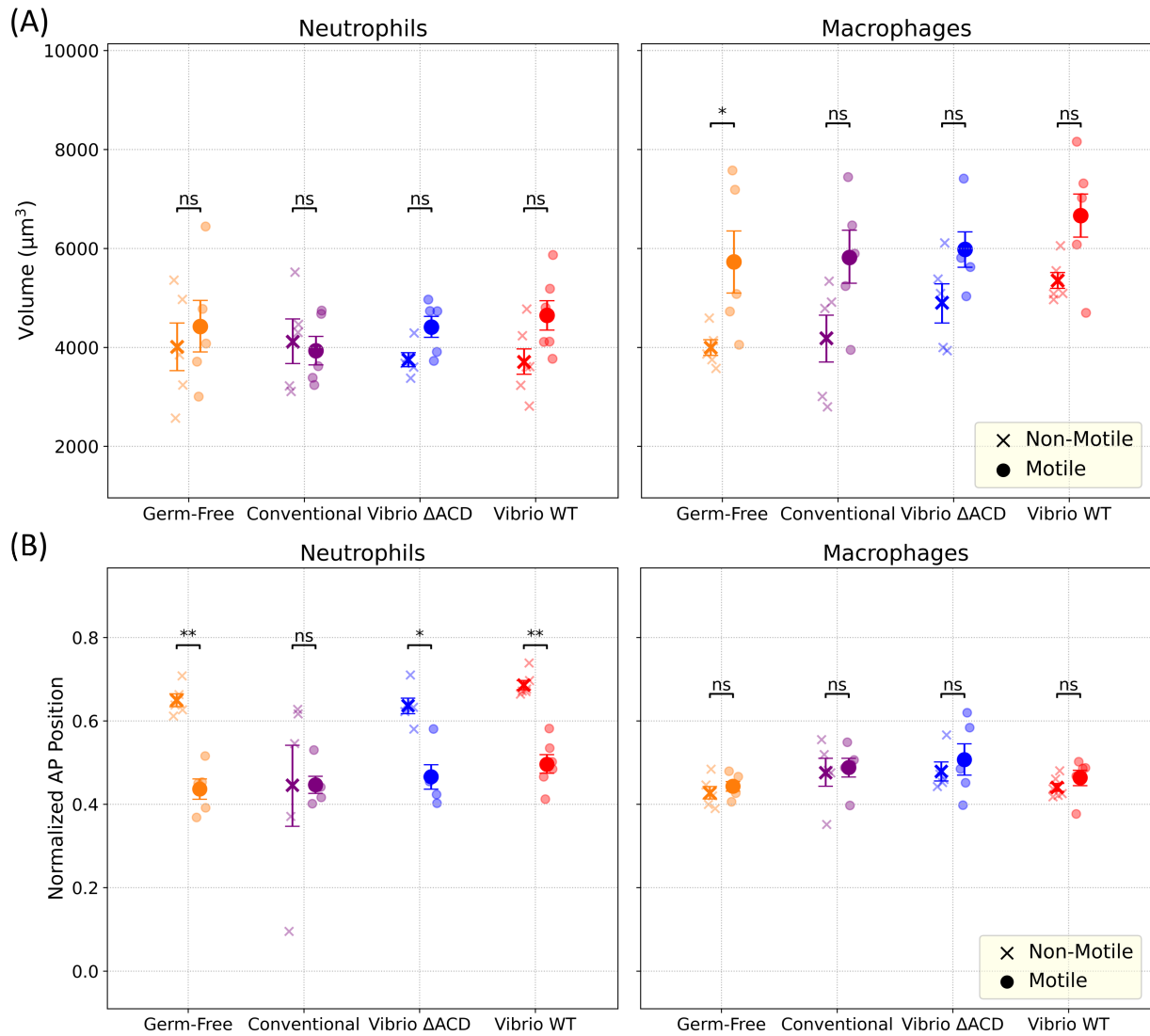


Figure A.8. Supplemental Gut Bacterial Association: (A) Volume comparison between motile and non-motile subtracks for neutrophils (left) and macrophages (right) across all experimental groups. Neither cell type shows significant differences between the cellular volumes. (B) Anterior-posterior position is normalized such that 0 and 1 represent the start and end of the gut, respectively. Non-motile neutrophils are generally more posteriorly located than motile neutrophils for all groups except the conventional. Macrophages show no significant difference in localization among the two motion types across all the groups, with average values around 0.5 implying they are equally distributed along the anterior-posterior axis. Error bars represent uncertainties from bootstrapping. $n_{\text{Germ Free}} = 5$,

$n_{\text{Conventional}} = 5, n_{\text{Vibrio } \Delta\text{ACD}} = 5, n_{\text{Vibrio WT}} = 6$; total $N = 21$. ns: not significant, $*$: $p < 0.05$, $**$: $p < 0.01$, $***$: $p < 0.001$.

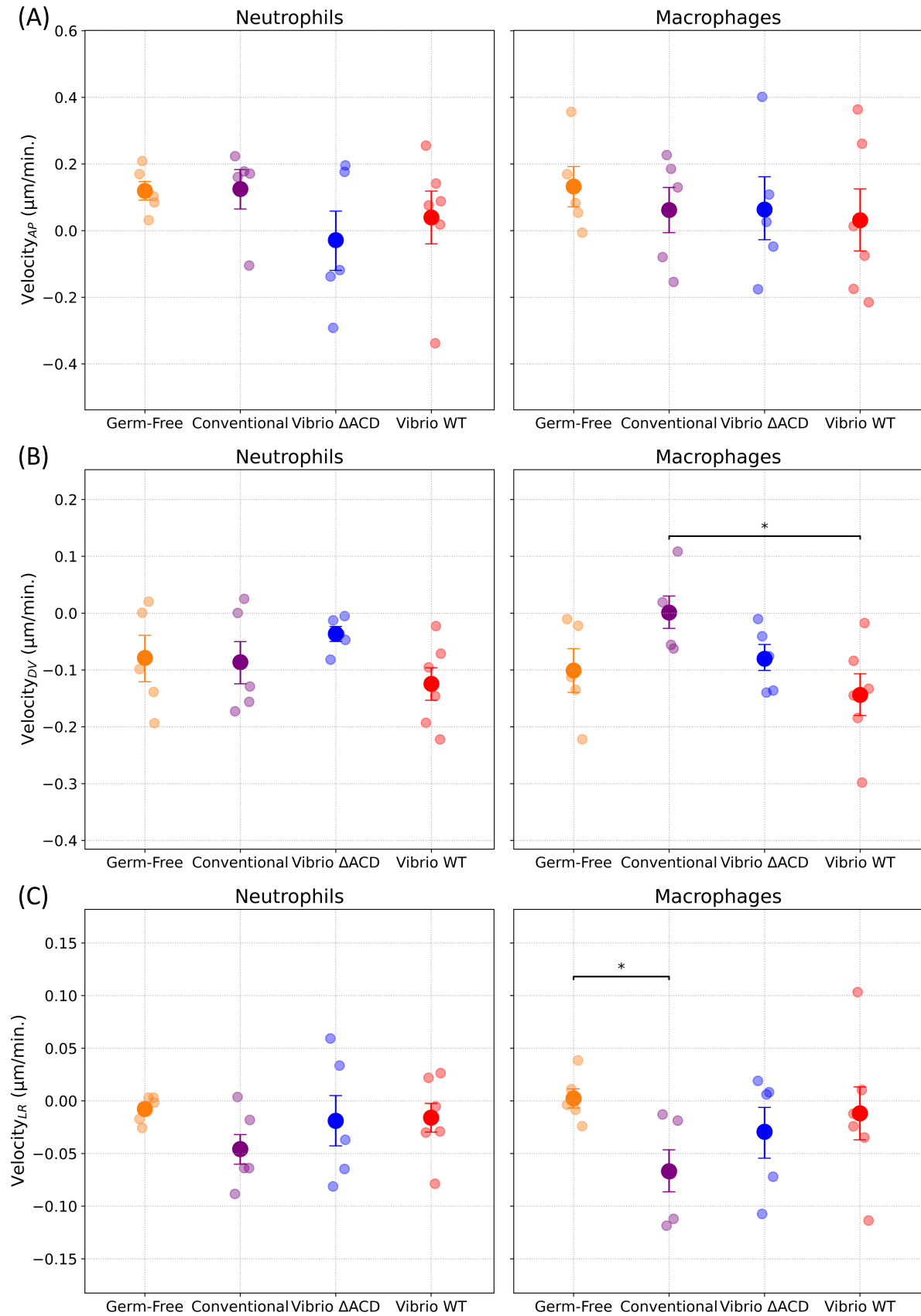


Figure A.9. Supplemental Gut Bacterial Association: The velocity component along the (A) anterior-posterior, (B) dorsal-ventral, and (C) left-right directions, calculated from frame-to-frame displacements, shows no significant differences between most experimental groups for either cell type. Error bars represent uncertainties from bootstrapping. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$. * : $p < 0.05$.

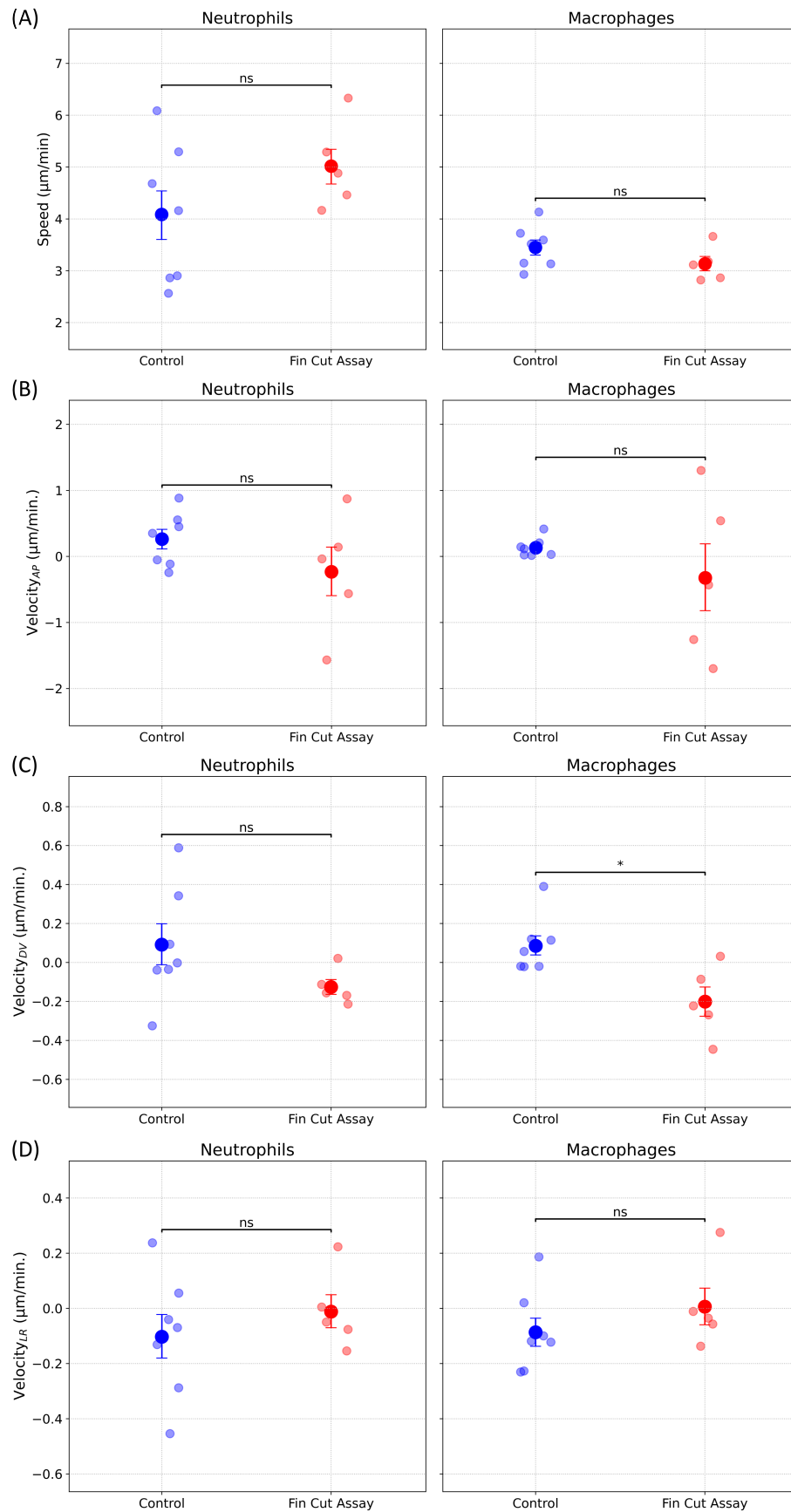


Figure A.10. Supplemental Fin Cut Assay: The average speed (A) and the velocity component of motile subtracks along the (B) anterior-posterior, (C) dorsal-ventral, and (D) left-right directions, calculated from frame-to-frame displacements, shows no significant differences between experimental groups for either cell type. Error bars represent uncertainties from bootstrapping. $n_{\text{Control}} = 5$, $n_{\text{Fin Cut Assay}} = 7$; total $N = 12$. ns: not significant, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

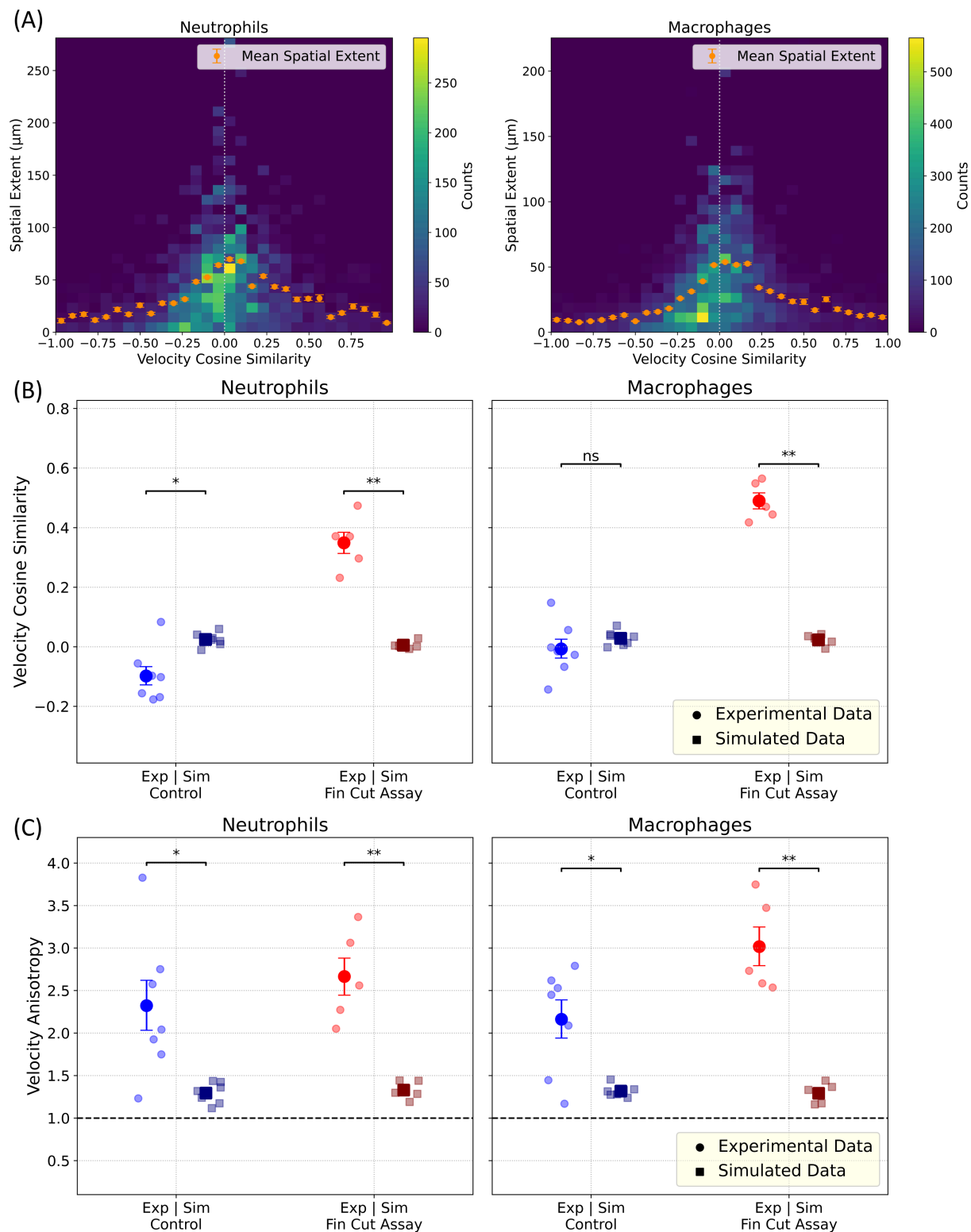


Figure A.11. Supplemental Simulated Fin Cut Assay: All panels show data for neutrophils (left) and macrophages (right). Simulated tracks were generated by randomizing step

directions while preserving each track's step-size distribution and track length, with detector noise applied to match experimental imaging conditions (see Materials and Methods). (A) 2D histograms of spatial extent versus velocity cosine similarity for simulated subtracks, pooling control and fin-cut conditions. Orange points show mean spatial extent binned by velocity cosine similarity. Unlike experimental data (Figure 2A), simulated tracks show no linear correlation; velocity cosine similarity clusters almost symmetrically around zero regardless of spatial extent. (B) Velocity cosine similarity for simulated data clusters around zero, confirming random directionality. Experimental data show significantly higher directional persistence than simulated data for both cell types in the fin-cut condition. (C) Velocity anisotropy for motile subtracks. Dashed line indicates isotropy (anisotropy = 1). Experimental tracks exhibit significantly higher anisotropy than simulated tracks across all conditions. Error bars represent uncertainties from bootstrapping. ns: not significant, * : $p < 0.05$, ** : $p < 0.01$.

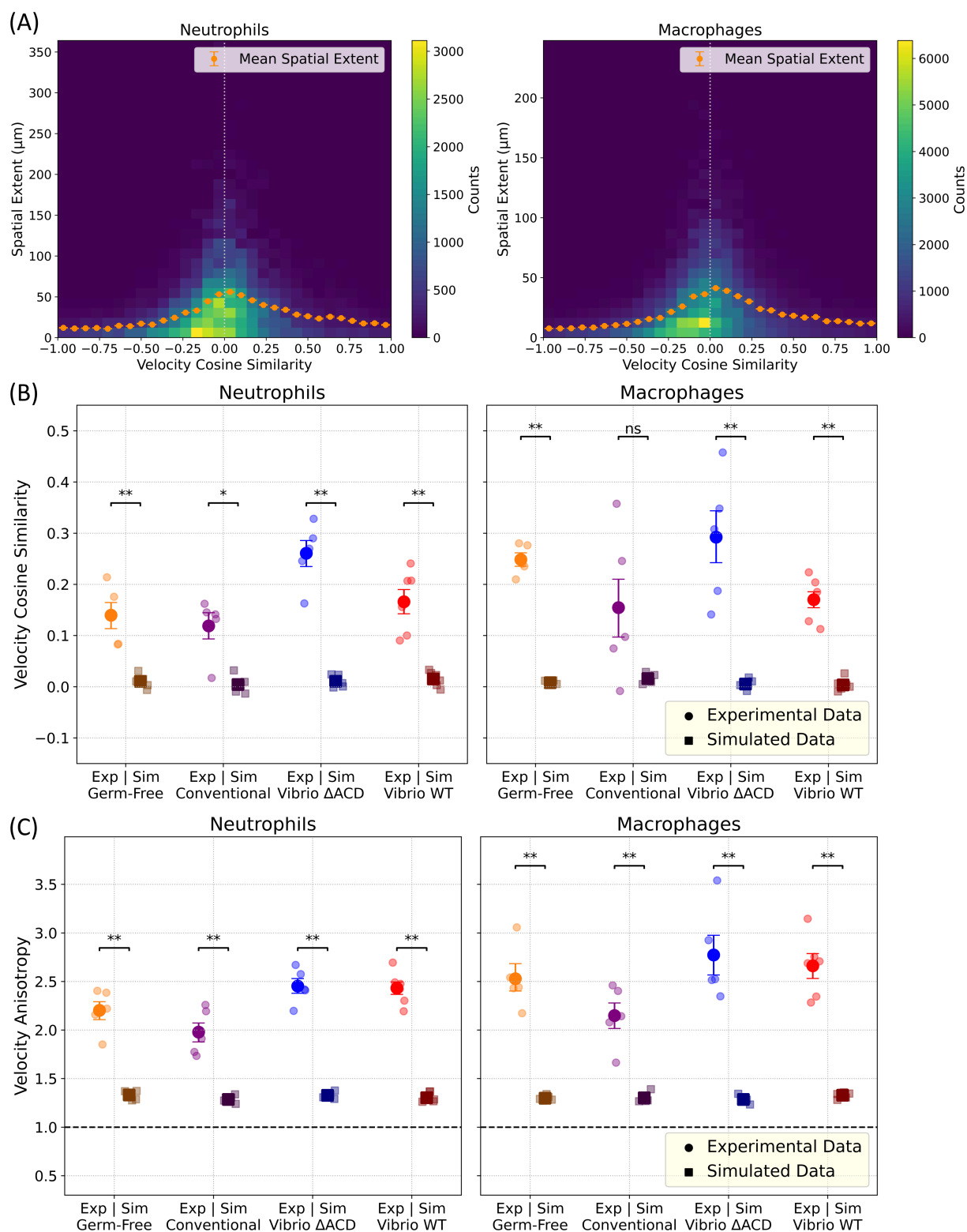


Figure A.12. Supplemental Simulated Gut Bacterial Association: All panels show data for neutrophils (left) and macrophages (right). Simulated tracks were generated by randomizing

step directions while preserving each track's step-size distribution and track length, with detector noise applied to match experimental imaging conditions (see Materials and Methods). (A) 2D histograms of spatial extent versus velocity cosine similarity for simulated subtracks, pooling all conditions. Orange points show mean spatial extent binned by velocity cosine similarity. Unlike experimental data (Supplemental Figure 3A), simulated tracks show no linear correlation; velocity cosine similarity clusters symmetrically around zero regardless of spatial extent. (B) Velocity cosine similarity for simulated data clusters around zero, confirming random directionality. Experimental data show significantly higher directional persistence than simulated data for both cell types across nearly all conditions. (C) Velocity anisotropy for motile subtracks. Dashed line indicates isotropy (anisotropy = 1). Experimental tracks exhibit significantly higher anisotropy than simulated tracks across all conditions for both cell types. Error bars represent uncertainties from bootstrapping. ns: not significant, * : $p < 0.05$, ** : $p < 0.01$.

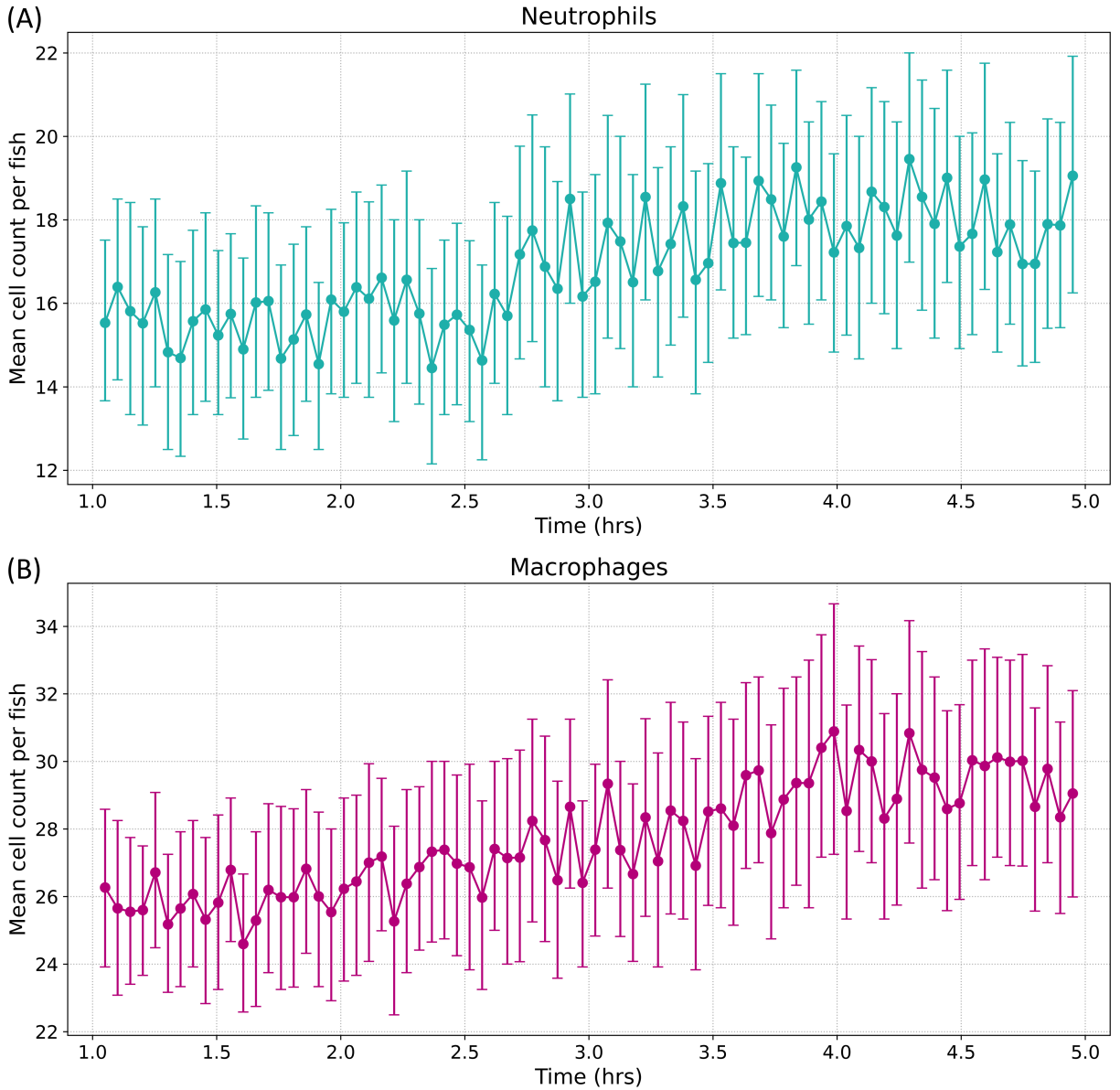


Figure A.13. Supplemental Fin Cut Assay: Mean cell count over time during fin cut assay experiments. (A) Neutrophils and (B) macrophages tracked in the caudal fin region from 1 to 5 hours post-amputation. Cell counts show gradual increases without large sudden fluctuations, indicating stable tracking of tissue-resident cells throughout the imaging period. Error bars represent standard error of the mean. Data pooled across all fin cut assay fish. $n_{\text{Control}} = 5$, $n_{\text{Fin Cut Assay}} = 7$; total $N = 12$.

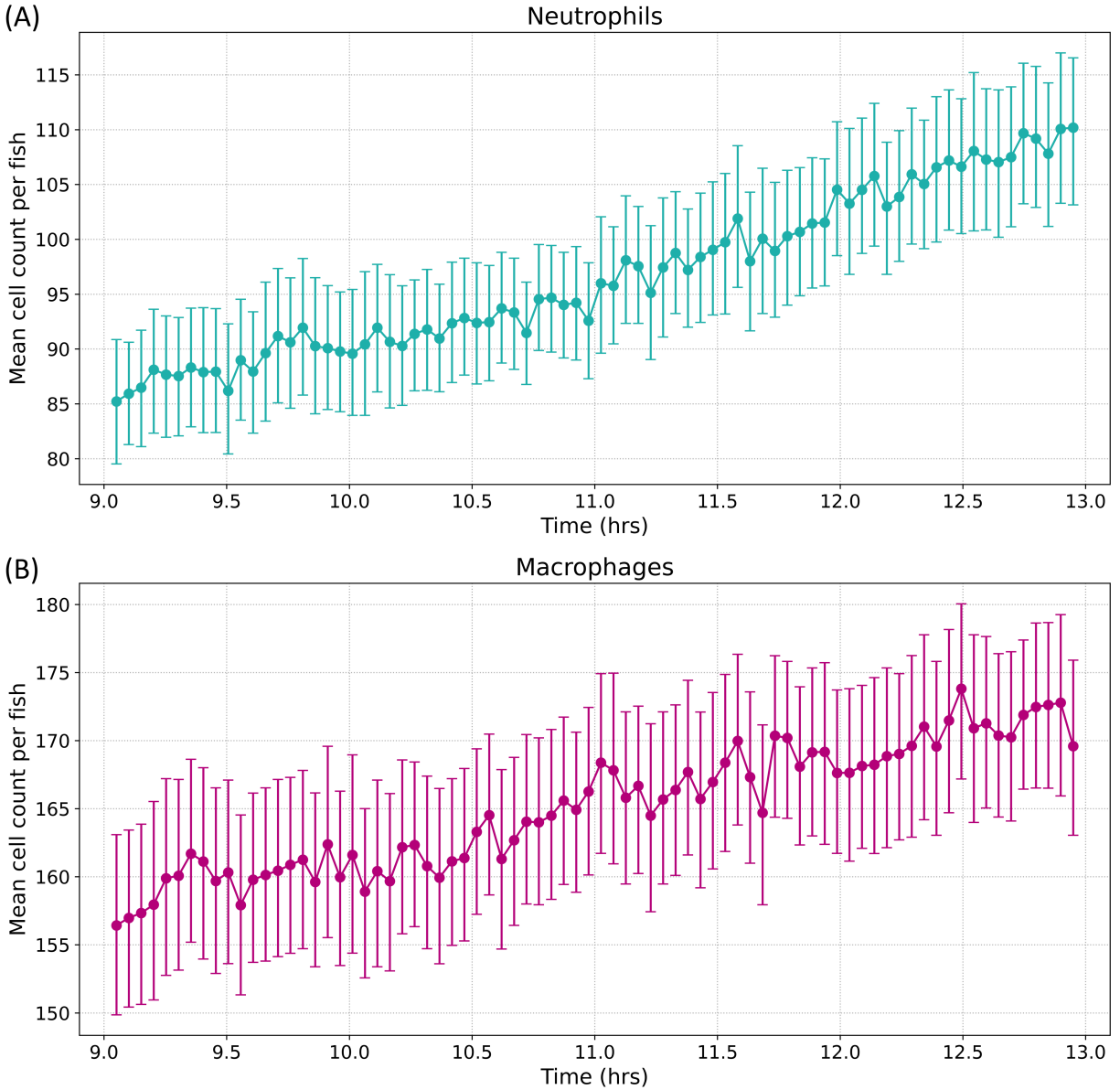


Figure A.14. Supplemental Gut Bacterial Association: Mean cell count over time during gut imaging experiments. (A) Neutrophils and (B) macrophages tracked in the gut region from 9 to 13 hours post-inoculation. Cell counts increase gradually without large sudden fluctuations, indicating stable tracking of tissue-resident cells throughout the imaging period. Error bars represent standard error of the mean. Data pooled across all experimental groups. $n_{\text{Germ Free}} = 5$, $n_{\text{Conventional}} = 5$, $n_{\text{Vibrio } \Delta\text{ACD}} = 5$, $n_{\text{Vibrio WT}} = 6$; total $N = 21$.

A.3 Supplemental 3D Figure Captions

Supplemental 3D figures are available at [Parthasarathy Lab Dropbox folder](#).

1. Supplemental 3D Figure 1. 3D neutrophil trajectories colored by normalized

time: Interactive 3D visualization of neutrophil migration trajectories during fin cut assay from 1 to 5 hours post amputation. Track colors represent normalized temporal progression from track initiation (yellow) to termination (purple), with intermediate timepoints shown in gradient. AP: anterior-posterior; DV: dorsal-ventral; LR: left-right. Data corresponds to 3D representation of Figure 1C.

2. Supplemental 3D Figure 2. 3D macrophage trajectories colored by normalized

time: Interactive 3D visualization of macrophage migration trajectories during fin cut assay from 1 to 5 hours post amputation. Track colors represent normalized temporal progression from track initiation (yellow) to termination (purple), with intermediate timepoints shown in gradient. AP: anterior-posterior; DV: dorsal-ventral; LR: left-right. Data corresponds to 3D representation of Figure 1D.

3. Supplemental 3D Figure 3. 3D neutrophil trajectories colored by track identity:

Interactive 3D visualization of neutrophil migration trajectories during fin cut assay from 1 to 5 hours post amputation. Each unique track is assigned a distinct color based on track ID. Hover information displays timepoint and additional parameters for individual trajectories. AP: anterior-posterior; DV: dorsal-ventral; LR: left-right.

4. **Supplemental 3D Figure 4. 3D macrophage trajectories colored by track identity:**

Interactive 3D visualization of macrophage migration trajectories during fin cut assay from 1 to 5 hours post amputation. Each unique track is assigned a distinct color based on track ID. Hover information displays timepoint and additional parameters for individual trajectories. AP: anterior-posterior; DV: dorsal-ventral; LR: left-right.

A.4 Supplemental Video Captions

Supplemental videos are available at [Parthasarathy Lab Dropbox folder](#).

1. **Video 1. Fin cut assay composite movie:** Composite image showing fin cut in gray, neutrophils in cyan and macrophages in magenta. Both immune cell types can be seen migrating to the fin amputation to the right of the figure from 1 to 5 hours post amputation. Scale bar = 100 μm .
2. **Video 2. Segmented neutrophils in maximum intensity projection responding to caudal fin transection:** Neutrophil response in fin cut assay showing 3D image stacks overlaid with segmented cells, unique colors show different segmented objects from 1 to 5 hours post amputation. Scale bar = 100 μm .
3. **Video 3. Z-scan through zebrafish gut showing bacterial distribution:** Volumetric z-scan along the left-right axis of the zebrafish gut at 13 hours post-inoculation with *Vibrio*^{WT}-dTomato. Image orientation: anterior-posterior (left to right), dorsal-ventral (top to bottom). Orange lines show the outline of the manually segmented gut lumen boundary at every 10th z-slice (see Methods and Materials). Scale bar = 100 μm .

4. **Video 4. Neutrophil trajectories on 4D image stacks:** Neutrophil dynamics across the entire gut field of view showing 3D image stacks with cells overlaid with pink dots showing centroids and unique colors showing different trajectories over 4 hours of imaging. Scale bar = 100 μm .
5. **Video 5. Macrophage trajectories on 4D image stacks:** Macrophage dynamics across the entire gut field of view showing 3D image stacks with cells overlaid with pink dots showing centroids and unique colors showing different trajectories over 4 hours of imaging. Scale bar = 100 μm .
6. **Video 6. 3D visualization of motile macrophage morphology:** Representative motile macrophage with rotating field of view demonstrating prominent pseudopodial extensions and reduced sphericity characteristic of the motile state. Scale bar = 20 μm .
7. **Video 7. Temporal dynamics of motile macrophage:** Representative motile macrophage in static view showing dynamic pseudopodial protrusions over time. Scale bar = 25 μm .
8. **Video 8. 3D visualization of non-motile macrophage morphology:** Representative non-motile macrophage with rotating field of view demonstrating rounded morphology and increased sphericity characteristic of the non-motile state. Scale bar = 10 μm .
9. **Video 9. Temporal dynamics of non-motile macrophage:** Representative non-motile macrophage in static view showing minimal morphological changes and absence of pseudopodial activity over time. Scale bar = 5 μm .

10. **Video 10. Neutrophil trajectories on maximum intensity projection with gut**

mask: Maximum intensity projection of 3D image stack demonstrating manual gut segmentation and neutrophil dynamics across the entire gut field of view. Neutrophils are shown in cyan; the brightfield image is gray, overlaid with pink dots showing centroids and unique colors showing different trajectories over 4 hours of imaging. Scale bar = 100 μm .

11. **Video 11. 3D surface mesh visualization of gut bulb macrophages:** 3D surface

mesh visualization of segmented macrophages in the gut bulb region, demonstrating the conversion from segmented objects to three-dimensional surface representations for morphological analysis. Scale bar = 50 μm .

A.5 Supplemental Dataset Descriptions

CSV files containing all tracked cell positions and other information are available at [Parthasarathy Lab Dropbox folder](#). The experimental datasets are:

1. **gut_neutrophil_track_data_simplified.csv:** Trajectory and subtrack data for neutrophils from gut bacterial association experiments.
2. **gut_macrophage_track_data_simplified.csv:** Trajectory and subtrack data for macrophages from gut bacterial associations experiments.
3. **fin_cut_assay_neutrophil_track_data_simplified.csv:** Trajectory and subtrack data for neutrophils from fin cut assay experiments.

4. **fin_cut_assay_macrophage_track_data_simplified.csv**: Trajectory and subtrack data for macrophages from fin cut assay experiments.

The corresponding simulated datasets are available at [Parthasarathy Lab Dropbox folder](#).

The simulated datasets are:

1. **simulated_gut_neutrophil_track_data.csv**: Trajectory and subtrack data for neutrophils from gut bacterial association experiments.
2. **simulated_gut_macrophage_track_data.csv**: Trajectory and subtrack data for macrophages from gut bacterial associations experiments.
3. **simulated_fin_cut_assay_neutrophil_track_data.csv**: Trajectory and subtrack data for neutrophils from fin cut assay experiments.
4. **simulated_fin_cut_assay_macrophage_track_data.csv**: Trajectory and subtrack data for macrophages from fin cut assay experiments.

The columns in each CSV file are:

1. **Identification and Temporal Columns**

- **track_id**: Unique identifier for each complete cell trajectory
- **subtrack_id**: Unique identifier for trajectory subdivisions based on motility state transitions
- **time_point**: Frame number in the time series
- **slice**: Z, Y, X-slice position in the image stack according to numpy array slicing convention

- **fish_id**: Unique identifier for each individual zebrafish containing information about the experimental group.
- **dpf**: Days post fertilization at time of imaging
- **time_post_treatment_mins**: Minutes elapsed since treatment
- **time_post_treatment_hours**: Hours elapsed since treatment

2. Morphological Measurement Columns

- **volume**: Cell volume in pixels³.
- **volume_um**: Cell volume in μm^3 .
- **user_surface_area_um**: Cell surface area in μm^2
- **sphericity**: Measure of how closely the cell shape approximates a sphere (0-1 scale).
- **holes**: Number of holes detected in the cell mesh produced by marching cubes. Any object with non-zero holes is not a closed object and discarded from further computations.
- **intensity_mean**: Average fluorescence intensity in the cell.

3. Spatial Position Columns

- **pos**: the relative position of the image stack during acquisition. Final Image stacks are produced by stitching images from multiple positions.
- **x-dv-px, y-ap-px, z-lr-px**: Intensity-weighted cell centroid coordinates in pixels (dorsal-ventral, anterior-posterior, left-right directions defined as x, y, z axes respectively). These are global coordinates for the final stitched 3D image stack.

- **x-dv-um, y-ap-um, z-lr-um:** Intensity-weighted cell centroid coordinates in μm (dorsal-ventral, anterior-posterior, left-right directions defined as x, y, z axes respectively). These are global coordinates for the final stitched 3D image stack.
- **mask_synced_y_ap_px_normalized:** Normalized anterior-posterior position in pixels using hand-drawn masks.

4. **Motion Parameters Columns** All parameters are computed per subtrack unless otherwise noted.

- **speed_um-per-min:** Frame-to-frame cell speed in $\mu\text{m}/\text{min}$.
- **velocity_x_um-per-min, velocity_y_um-per-min, velocity_z_um-per-min:** Frame-to-frame velocity components along each axis in $\mu\text{m}/\text{min}$.
- **distance_um:** Frame-to-frame distance traveled in μm . Computed per track.
- **distance_px:** Frame-to-frame distance traveled in pixels. Computed per track.
- **velocity_cosine_similarity:** Measure of directional persistence computed as cosine of angle between consecutive velocity vectors.
- **spatial_extent_um:** Diameter of smallest circumscribing sphere of the subtrack in μm .
- **anisotropy_index_normalized:** Measure of trajectory directional bias along the anterior-posterior axis relative to the other two axes. Normalized such that isotropic motion yields a value of 1.

5. **Motility Classification Columns**

- **motility:** Classification of motility states, either “motile” or “non-motile”.

- **distance_px > mean_radius/2:** Boolean indicating if displacement exceeds half the mean cell radius.
- **spatial_extent > 2*mean_diameter:** Boolean indicating if spatial extent exceeds twice the mean cell diameter.

6. Track Characteristics Columns

- **subtrack_length:** Number of timepoints in each subtrack.
- **track_length:** Total number of timepoints in the complete trajectory.
- **label:** Segmentation-derived identifier for individual cells within each image stack.
Together with `timepoint` and `pos`, this forms a unique composite key that unambiguously identifies each segmented cell event in the entire dataset.

7. Columns with Anatomical Context for Gut Bacterial Association Experiments

- **in_gut:** Boolean indicating whether cell is located within the gut region.
- **gut_associated:** Binary classification of gut association. True if the cell trajectory intersects with the manually defined gut boundary at any timepoint within the subtrack.
- **vent_width_um:** Width of the vent in μm . Manually measured from bright-field images.

8. Scale Information Columns

- **Pixel to micron conversion:** Anisotropic voxel size with 1 pixel = $0.65 \mu\text{m}$ in x-y directions and 1 pixel = $1.0 \mu\text{m}$ in the z direction.
- **Temporal resolution:** 3 minutes/frame.

9. Analysis Parameters Columns

- **Motility threshold:** $0.5 \times$ mean cell diameter
- **Patience parameter:** 3 timepoints for subtrack subdivision

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER 3

This appendix contains supplemental information that support the main findings presented in chapter 3 of the dissertation.

B.1 Supplemental Information: Contents

1. Supplemental Figures and Captions
2. Supplemental Dataset Descriptions

B.2 Supplemental Figures and Captions

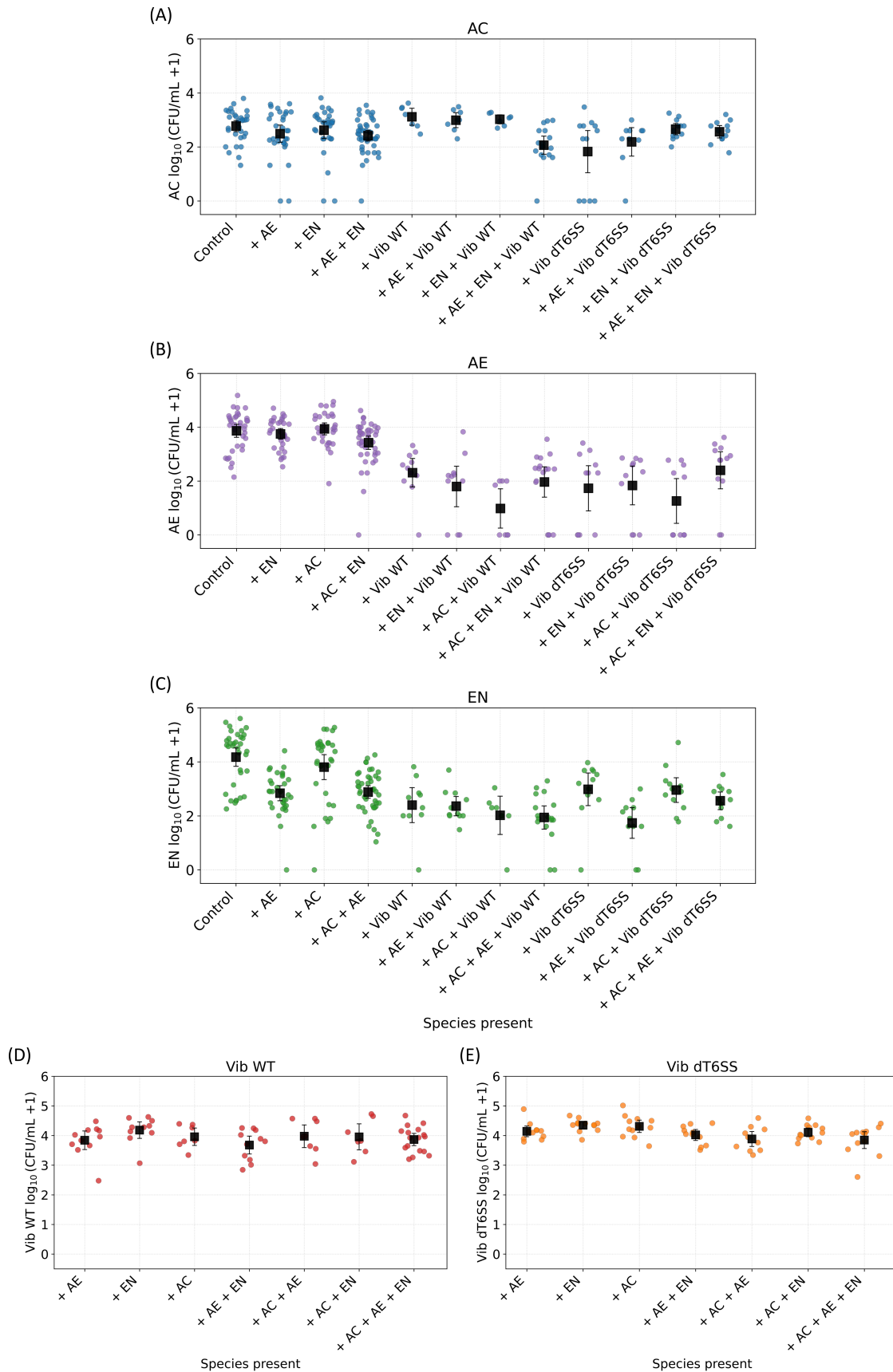


Figure B.1. Gut Bacterial Abundance across all experimental groups for (A) AC, (B) AE, (C) EN, (D) Vib WT, and (E) Vib dT6SS. Each circle represents an individual larval zebrafish; black squares show mean $\pm$ 95% confidence interval.

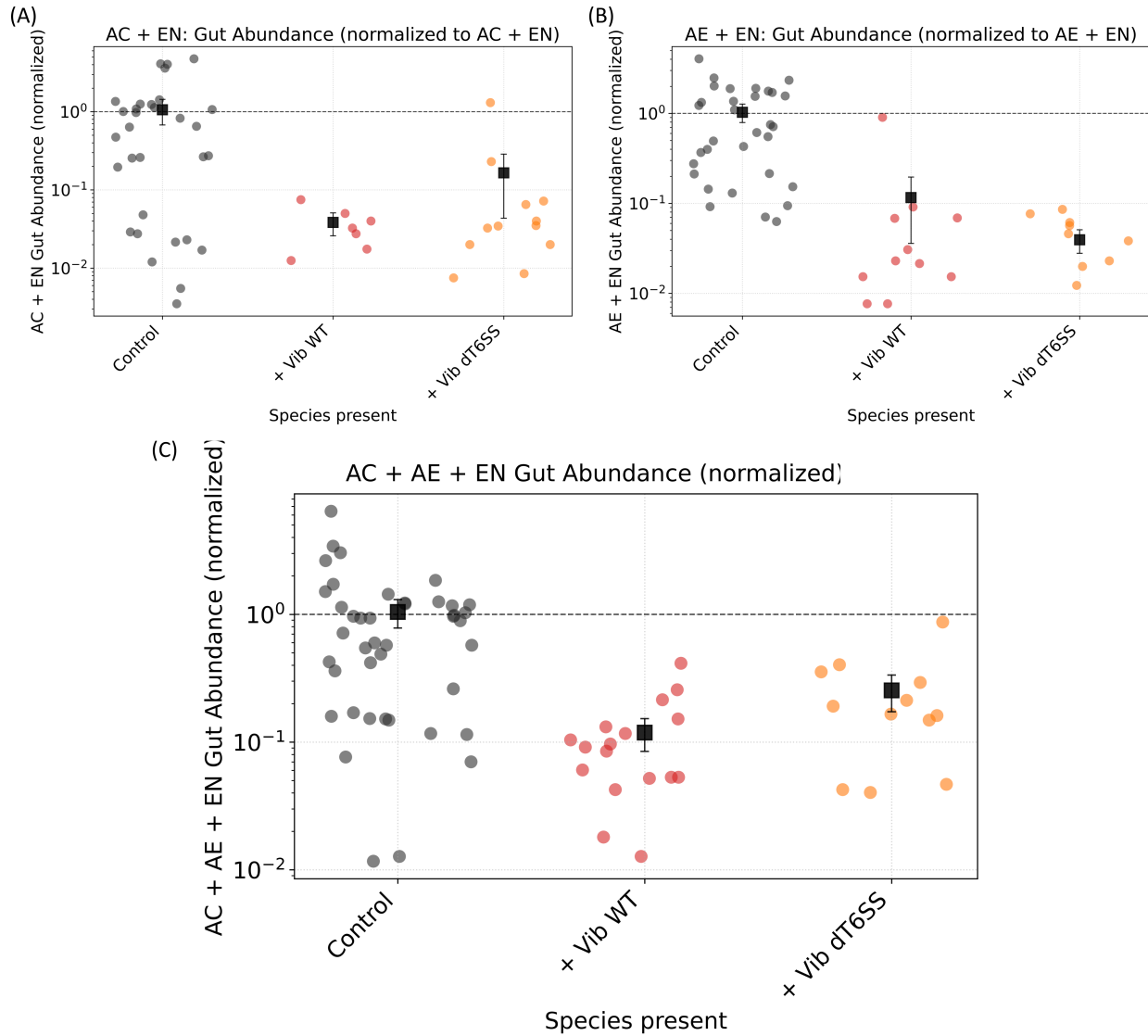


Figure B.2. Combinations of 2 and 3 founder abundances were normalized to founder-only controls (dashed line at 10^0) to quantify invasion effects. Each circle represents an individual larval zebrafish; black squares show mean $\pm$ bootstrapped uncertainty. Di-associated founder combinations are shown in (A) AC + EN and (B) AE + EN. (C) Shows tri-association of founder species AC + AE + EN. Tri-associated founders show higher relative abundances than di-associations. Colors distinguish invasion by Vib WT (red) and Vib dT6SS (orange) invasion conditions from controls (gray).

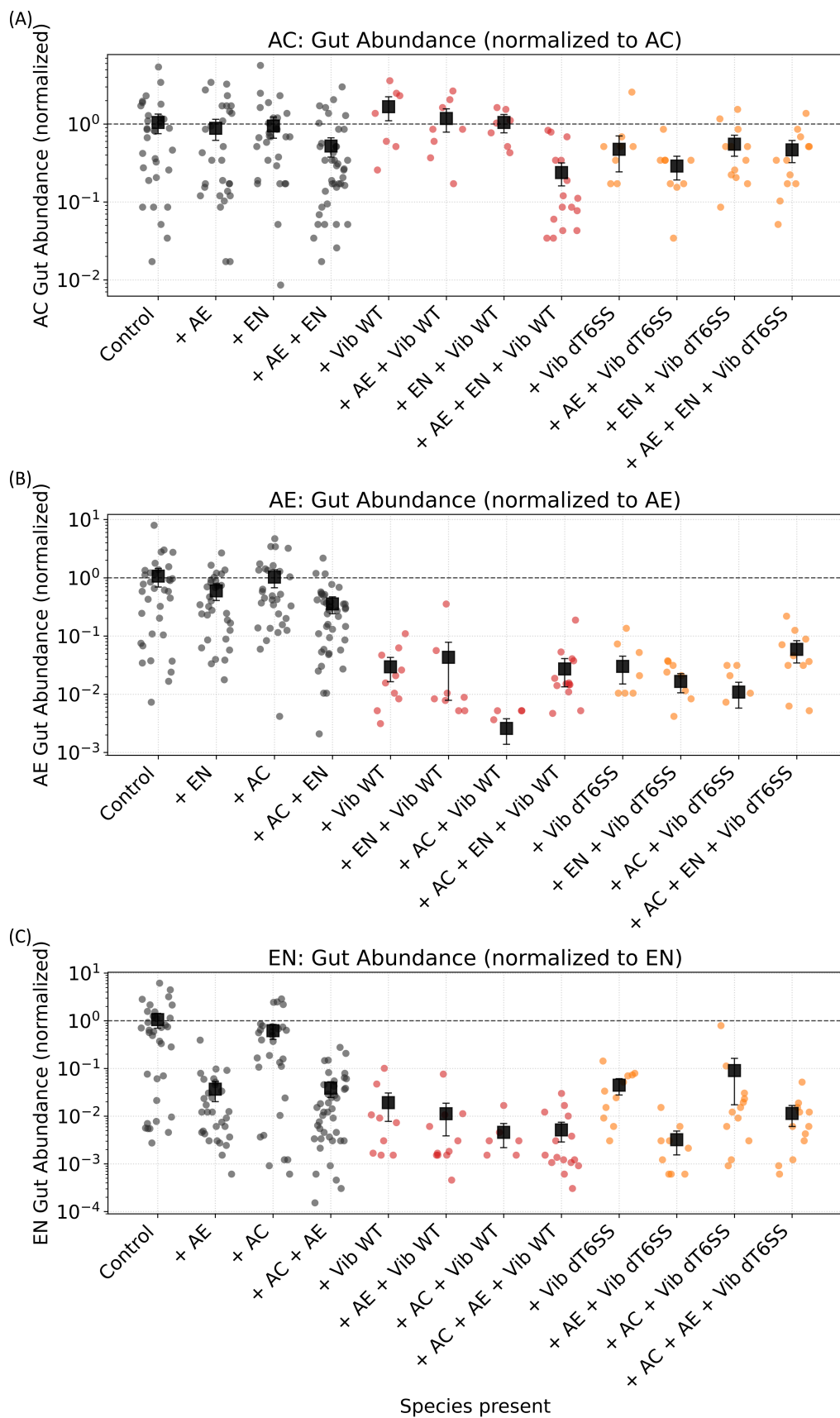


Figure B.3. Normalized founder abundances for individual bacterial species was found by normalizing to founder-only controls (dashed line at 10^0). Each circle represents an individual larval zebrafish; black squares show mean $\pm$ bootstrapped uncertainty. Founder relative abundances across all experimental groups are shown for (A) AC, (B) AE, and (C) EN. Colors distinguish invasion by Vib WT (red) and Vib dT6SS (orange) from no-invasion conditions (gray).

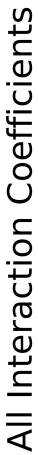


Figure B.4. Comprehensive matrix showing the interaction coefficients (C_{ij}) indicating the effect of species j (columns) on species i (rows) across all experimental groups. Bold black borders indicate interaction coefficients differing from zero by more than three standard deviations. Color scale ranges from strong inhibition (dark red, $C_{ij} < -0.6$) through neutral (gray, $C_{ij} \approx 0$) to facilitation (dark blue, $C_{ij} > 0.6$).

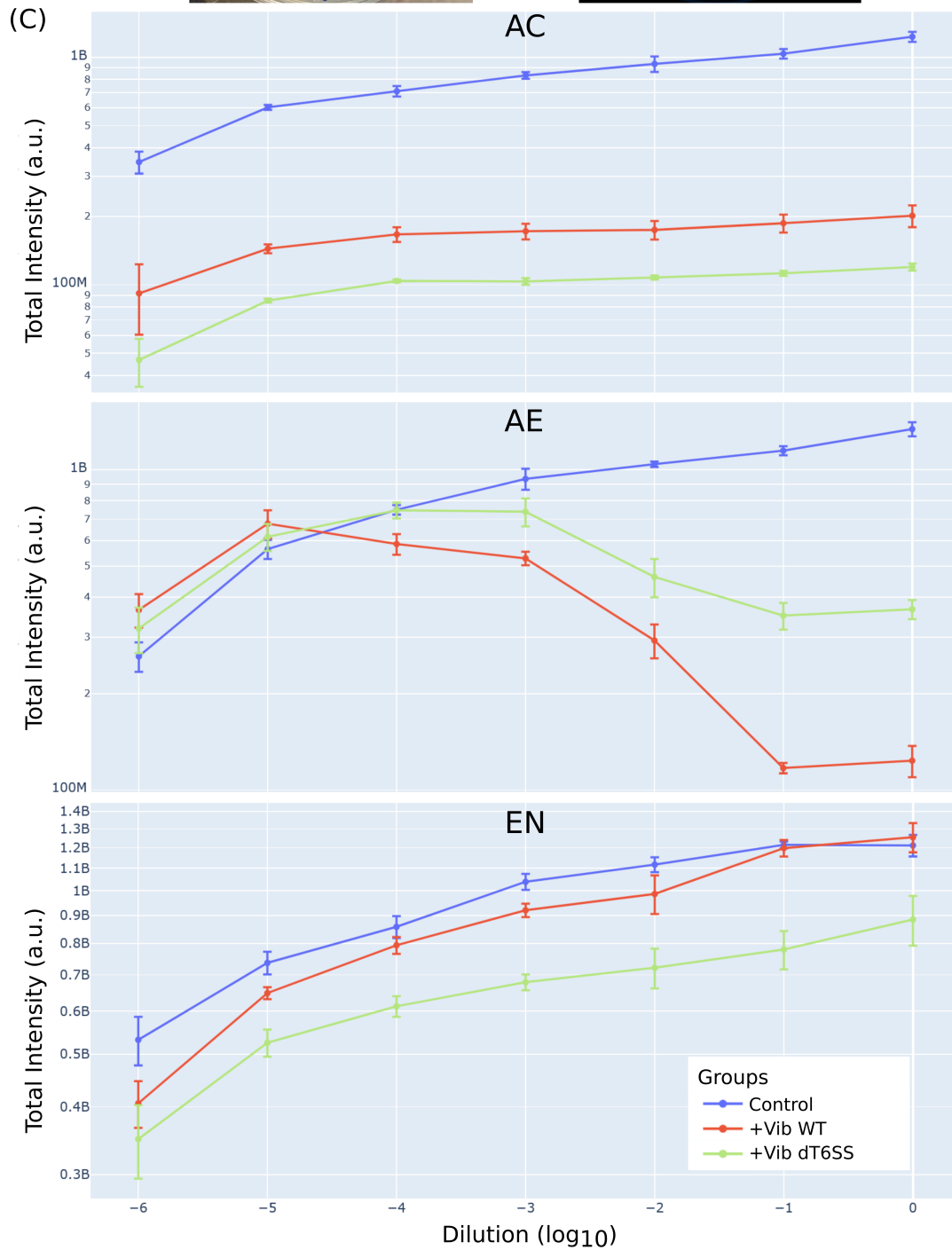
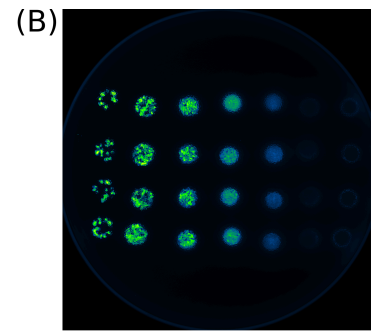
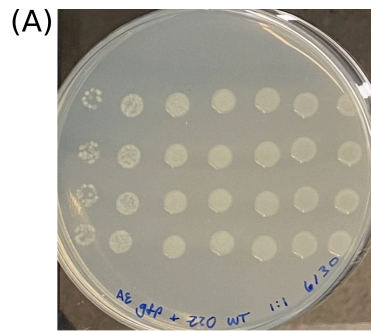


Figure B.5. In vitro assays of T6SS-mediated inter-bacterial killing. (A) Representative image of GFP-expressing target bacteria spotted onto LB agar either alone (Control) or together with Vib WT or Vib dT6SS, photographed under standard illumination. Rows correspond to different replicates; columns represent serial tenfold-increasing initial bacterial concentrations (left to right). (B) The same plate as in (A) imaged under GFP fluorescence excitation, with four biological replicates shown. GFP fluorescence intensity serves as a proxy for target bacterial abundance. (C) Quantification of total GFP fluorescence intensity (arbitrary units, a.u.) as a function of dilution factor ($\log_{10}$) for three zebrafish-native bacterial species that were used as founder species in *in vivo* experiments: AC, AE, and EN. Data points represent the mean and error bars indicate the standard deviation across $N = 4$ replicates. For AE, co-spotting with Vib WT results in markedly reduced fluorescence intensity compared to controls across all dilution factors, consistent with T6SS-mediated inter-bacterial killing. Co-spotting with Vib dT6SS yields an intermediate phenotype, indicating partial killing in the absence of a functional T6SS, potentially attributable to other competitive mechanisms. AC shows minimal susceptibility to *Vibrio*-mediated killing, with fluorescence remaining relatively stable across conditions. EN exhibits the greatest resistance to killing by both *Vibrio* strains, with fluorescence intensity trajectories nearly converging with control values at higher bacterial concentrations, suggesting species-specific resistance mechanisms to T6SS-mediated antagonism that manifest differently *in vitro* compared to *in vivo*.

B.3 Supplemental Dataset Descriptions

CSV files containing all computed coefficients is available at [Parthasarathy Lab Dropbox folder](#). The experimental datasets are:

1. **Higher order interaction coefficients.csv**: Interaction coefficient data for all the combinations. It has the following columns:
 - sp_i: species (or combination) i
 - sp_j: species (or combination) j
 - mean_c_ij: mean interaction coefficient C_{ij}
 - unc_lower: bootstrapped lower uncertainty
 - unc_upper: bootstrapped upper uncertainty
 - std: bootstrapped standard deviation
2. **Invasion coefficients.csv**: Invasion coefficient data for all the combinations. It has the following columns:
 - founder: founder single or a combination of species
 - invader: invader species, either Vib WT or Vib dT6SS
 - beta: invasion coefficient β
 - beta_unc_lower: bootstrapped lower uncertainty
 - beta_unc_upper: bootstrapped lower uncertainty

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