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Research Article

Keywords: Zebrafish, Microbiome, Host-genetics, Pseudocapillaria tomentosa

Posted Date: November 18th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10385040/v1

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Additional Declarations: No competing interests reported.

Host genetics influence parasite infection severity and gut-microbiome response to parasite exposure in zebrafish

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Abstract

Background: The composition of the gut microbiome is shaped by a variety of external factors, including environmental and host-related influences. However, the extent to which fixed factors, such as host genetics, drive differences in microbiome composition, particularly in the presence of external stressors like parasitic infection, remains poorly understood.

Results: Using 16S rRNA gene sequencing, we analyzed the gut-microbiomes of four genetic zebrafish strains after exposure to *Pseudocapillaria tomentosa* at two timepoints previously identified as peak infection severity days. We built linear regressions to identify significant drivers of alpha diversity (Shannon Entropy and richness), beta diversity (Bray-Curtis distance) and the abundances of significantly differentially abundant taxa. Host strain showed minimal influence on baseline gut microbiomes, with strain significantly associated with alpha diversity but not beta diversity in control fish. Parasite exposure and infection burden emerged as stronger drivers of microbiome variation than host genetics. However, significant strain by exposure interactions revealed that strains exhibited different microbiome trajectories following infection, despite similar parasite burdens at sampling timepoints. Five taxa showed consistent associations with infection across all strains: *Barnesiellaceae*, *Cetobacterium*, *Paraclostridium*, and *ZOR0006* (depleted) and *Pseudomonas* (enriched), suggesting conserved microbiome responses that override genetic variation.

Conclusions: Host genetic background has a minimal effect on baseline zebrafish gut microbiomes. Instead of fundamentally different microbial communities, strains harbor similar baseline microbiomes that diverge along strain-specific trajectories during infection. The identification of conserved microbial signatures across genetically diverse strains indicates that *P. tomentosa* triggers core microbiome responses independent of host genetic background. Taken together, these findings suggest that microbiome resilience may be modestly shaped by host genetics. Implications include understanding host-parasite-microbiome dynamics and developing robust microbiome-based biomarkers.

Keywords: Zebrafish, Microbiome, Host-genetics, *Pseudocapillaria tomentosa*

Background

Deciphering the biological factors that shape health outcomes is essential for advancing our understanding and control of disease. It is well established that the gut microbiome plays a critical role in maintaining host health and mediating disease through processes such as infection protection [1], nutrient metabolism [2], and biochemical signaling [3]. In the case of infection protection, gut-microbiome derived metabolites have been associated with decreased gastrointestinal infections [4], emphasizing the importance of understanding gut microbiome structure and function in the context of health maintenance. This microbial ecosystem is shaped by exogenous factors such as diet [5], environment [6], and heavy metal exposure [7], which influence its ability to maintain homeostasis. Host genetics independently select for microbial taxa across host species due to differing niches [8–10], but the impact of genetic variation within a species remains largely unexplored. While a bidirectional relationship between microbiome composition and parasitic infections has been observed [4, 11], and some evidence suggests

that host genetic variation is associated with infection development [12–14], the response of the gut microbiome to parasitic infection across different host genetic backgrounds remains poorly understood. This gap limits our understanding of how interstrain genetic variation influences gut microbiome composition and infection responses. Such insights have far-reaching implications for revealing the unique contribution of biological influences on microbiome structure and for clarifying the causal pathways through which microbiome-mediated responses to environmental stressors affect host health.

of interstrain genetic variation's influence on gut microbiome composition and infection responses.

Zebrafish (*Danio rerio*) have emerged as a powerful model for studying the vertebrate gut microbiome and its responses to external stressors due to their genetic tractability, optical transparency, large sample sizes, and well-characterized microbial communities [15, 16]. Previous studies have identified a core zebrafish microbiome [17, 18], and documented compositional shifts across developmental stages [19]. Our prior work has demonstrated environmental factors such as diet [5] and temperature [6], contribute to the variation in zebrafish gut microbiome composition. However, the role that host genetics play in this variation is unclear. While it has been shown that different host species select for distinct microbial communities [10], the potential selectivity of genetic variation within species is not well understood. A complicating factor in zebrafish microbiome research is the use of multiple wildtype genetic strains across studies, notably the 5D, AB, TU, and Casper genetic lines, wherein 5D is an outbred pet-store derived fish, AB and TU are established inbred laboratory lines, and Casper is a genetic line derived from AB that carries specific pigmentation mutations [20]. This strain variability may introduce uncontrolled variation in microbiome studies that confounds cross-study comparisons and limits reproducibility.

Pseudocapillaria tomentosa is an intestinal nematode that commonly infects laboratory zebrafish. Due to its high prevalence in zebrafish research facilities [11, 21, 22], it poses a significant health burden, often leading to chronic inflammation, epithelial damage, and altered nutrient absorption. These outcomes,

along with tumor promotion [23], can introduce undesirable variation in zebrafish studies conducted in facilities known to have *P. tomentosa* [24]. Its consistent presence and well-defined pathology make *P. tomentosa* a valuable model for studying host–parasite interactions and the role of the gut microbiome in mediating infection outcomes. Moreover, with zebrafish becoming a widespread biomedical model, it is vital to understand how *P. tomentosa* infections alter experimental results and how *P. tomentosa* may interact with other experimental components, such as fish strain, to influence study endpoints. Importantly, *P. tomentosa* early infection dynamics differ across fish strains [25]. Moreover, *P. tomentosa* infection has been linked to shifts in the gut microbial community composition [4, 11], though these studies only utilized a single zebrafish strain (5D) leaving a critical gap in knowledge of whether these shifts are strain-specific. This gap offers a unique opportunity to investigate how host genetic background influences microbiome susceptibility to perturbation and subsequent divergence patterns under parasitic stress.

Building on Schuster et al.'s [25] finding that *Pseudocapillaria tomentosa* infection progression varies across zebrafish strains, we hypothesized that these strain-driven differences in infection dynamics would manifest as distinct gut-microbiome response patterns. If zebrafish strains harbor distinctly different gut microbiomes, then differences in zebrafish parasite infections outcomes may be, at least in part, attributable to strain-specific gut microbiome variations. Understanding how host genetics shape microbiome-mediated responses to perturbations will enhance the reproducibility and relevance of microbiome research, leading to more reliable and impactful findings. Addressing how host genetics influence these interactions could also inform strategies to enhance immunity and reduce inflammation, ultimately contributing to more effective medical treatments and improved health outcomes.

Results

The zebrafish used for microbiome analysis represent a subset of a larger exposure study described by Schuster et al, 2023 [25]. From the original 720 fish (180 each of 5D, AB, Casper, and TU strains) examined across multiple timepoints (8, 15, 22, 31, and 44 dpe), we selected 127 fish from the 31 and 44 days-post-exposure (dpe) timepoints for microbiome analysis (Figure 1). These fish showed peak infection prevalence: a biologically meaningful observation since many worms are mature and associated with severe intestinal pathologic changes after about 3 weeks [11]. The subset included 36 5D fish (18 exposed, 18 control), 26 AB fish (13 exposed, 13 control), 34 Casper fish (17 exposed, 17 control), and 31 TU fish (18 exposed, 13 control). This subset exhibited representative infection patterns compared to the full dataset (Table S1). Notably, a supersaturation event occurred at 3-6 dpe that differentially affected AB (46 mortalities of 180 fish) and 5D (5 mortalities of 180 fish) strains in the exposed groups only, though control fish in the same water system remained unaffected.

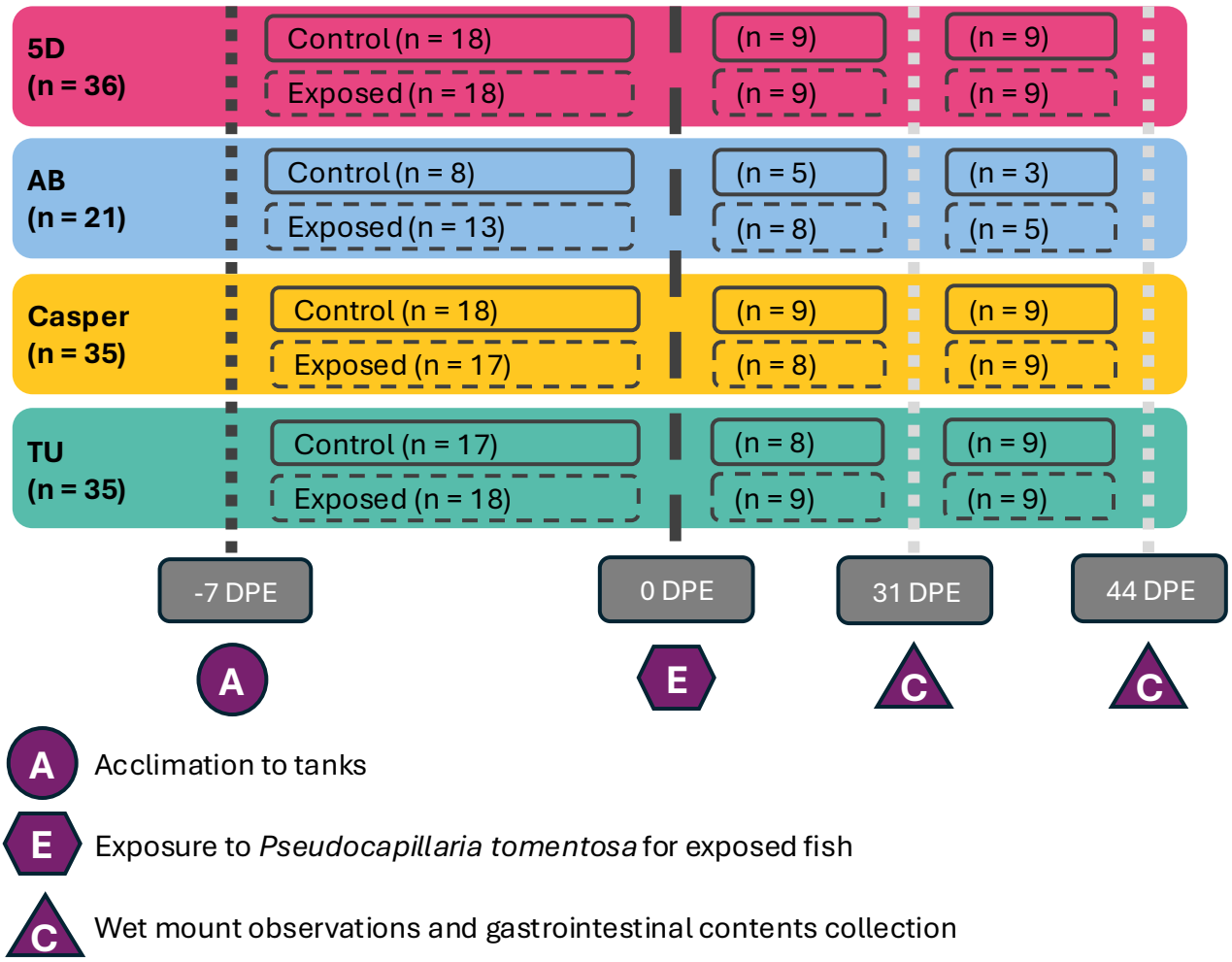


Figure 1. Experimental design showing treatment and husbandry events. Dashed lines indicates when an experimental event occurred at each timepoint. (P) 127 fish of 4 different zebrafish strains were assigned to exposure groups and reared from 0 to 124-126 days post fertilization (dpf). (E) At 124-126 dpf (or 0 days post exposure; dpe) fish in the exposure groups were exposed to *Psuedocapillaria tomentosa*. (C) At 31 and 44 dpe a subset of each strain and exposure group were euthanized, assessed for weight, sex and worm burden, and samples were collected for microbiome analysis.

Zebrafish strains exhibit similar susceptibility and weight differences in response to parasite infection

We first examined whether zebrafish strain or sex influenced susceptibility to parasite infection in our subset. Susceptibility was defined as a positive infection status (detectable worms at sampling by wet mounts), with prevalence ranging from 65% in Casper to 100% in AB fish (Table S1). These same fish were all positive by a specific qPCR test for *P. tomentosa* [25] indicating the presence of parasite DNA; detection only by qPCR is consistent with early-stage infection, whereas concurrent positivity by qPCR and wet mount indicates fully developed infections detectable by microscopy. A binomial GLM showed a modest but non-significant strain effect ($P=0.06$) and no effect of sex ($P > 0.05$; Table S2), indicating that infection susceptibility did not differ meaningfully by genetic background or sex at these time points when the infection had fully developed.

We next examined infection severity, measured as total worm count. We built a negative binomial GLM to determine if infection severity differed by zebrafish strain or dpe. We found that infection burden does not vary significantly as a function of strain ($P>0.05$, Figure 2A, Table S3), but that infection severity was significantly higher at 44 dpe than 31 dpe ($P<0.05$, Figure 2B, Table S3). Notably, while our subset showed no strain differences in worm burden at these later timepoints, Schuster et al. [25] found that AB strains developed significantly heavier infections than other strains at earlier timepoints (8-22 dpe), suggesting infection dynamics equilibrate by 31 dpe. These findings suggest that infection severity does not differ significantly between zebrafish strains, indicating that susceptible individuals exhibit similar infection severity regardless of genetic background after the infection had fully progressed. Overall, infection severity did not differ among strains, but increased with time, underscoring the importance of temporal dynamics in host–parasite interactions.

To determine whether parasite exposure or infection status impact zebrafish health, we next considered how these factors associate with fish weight using Wilcoxon ranked-sum tests. Exposed versus unexposed fish showed a non-significant trend ($P = 0.086$), whereas infected fish were significantly lighter than non-

infected fish (P = 0.027; Figure 2C), though post-hoc estimated marginal means with Tukey adjustments for multiple comparisons revealed no significant differences between infection status groups within strains (P > 0.05, Table S4). Thus, infection status was more strongly associated with weight than exposure alone across strains, although this effect was not significant within individual strains after accounting for the strain × infection interaction indicating that the observed association at the population level does not uniformly extend to all strain backgrounds.

Finally, we used stepwise regression to identify factors associated with weight among exposed fish. The optimized GLM included strain, sex, infection result, and the interaction between strain and sex. Weight differed significantly by infection result, by at least one strain, and between sexes (all P < 0.05; Table S5). Tukey tests showed that AB fish were significantly lighter than all other strains, and that Casper fish were lighter than TU and marginally lighter than 5D (Table S6). Notably, while our subset showed no strain differences in worm burden at these later timepoints, Schuster et al. (2023) found that AB strains developed more severe infections than other strains at earlier timepoints (8-22 dpe), suggesting infection dynamics equilibrate by 31 dpe. The significant interaction effect we observed is likely attributable to a larger ratio of males in our AB population, given that female fish are significantly heavier that male fish (Wilcoxon Ranked-Sum test, P < 0.05).

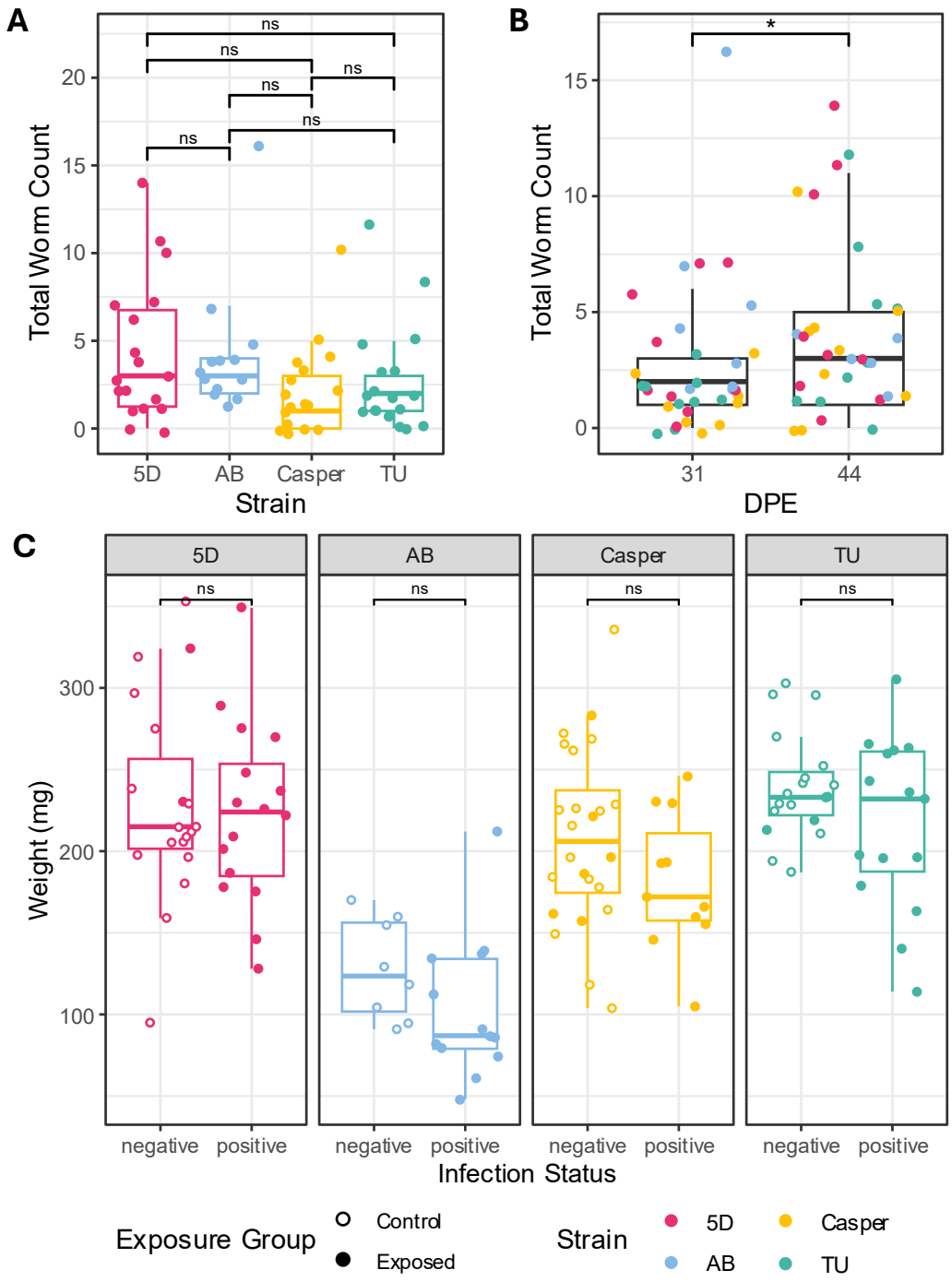


Figure 2. Zebrafish strains exhibit similar physiological trends in response to parasite infection.

(A) Boxplots of zebrafish from each strain separated by infection status show minor, but non-significant differences in weight. Boxplots of the total number of worms detected in each infected zebrafish show no significant difference in worm burden when separated by (B) strain but do show a significant difference when separated by (C) days post exposure (DPE). A "*" indicates statistical significance

below a “0.05” threshold and “ns” represents a comparison of non-significance (above a “0.05” threshold).

Zebrafish harbor similar gut microbiomes across strains with differences limited to a few taxa

Next, we evaluated the extent to which zebrafish gut microbiomes associate with host genetic background. To determine whether gut microbial diversity varies across zebrafish strains we used stepwise regression to build optimized GLMs for alpha diversity as measured by Shannon Entropy and richness (observed genus counts) (Figure S1A). We found that alpha diversity significantly varies as a function of zebrafish strain across both diversity metrics ($P<0.05$; Table S7 & Table S8). A post-hoc Tukey test clarified that gut microbial alpha diversity significantly differed between AB and Casper fish strains for both alpha diversity metrics, and that richness significantly differed between Casper and 5D zebrafish ($P<0.05$; Table S9). To assess whether the effect of strain was consistent across exposure groups, we built separate GLMs, one for each diversity metric, using strain as the sole predictor. This was done separately for the parasite-exposed and unexposed groups because the optimal models did not include a strain-by-exposure interaction (Figures 3A & 2B). Shannon Entropy did not differ significantly by strain in either the unexposed or exposed zebrafish ($P>0.05$, Tables S10 & S11, Figures 3A & 3B). Richness did not differ significantly by strain in the unexposed zebrafish ($P>0.05$, Table S12, Figure 3A) but did differ significantly by strain in the exposed zebrafish ($P<0.05$, Table S13, Figure 3B). These findings point to only modest strain-level differences in alpha diversity, with much of the apparent variation emerging when exposed and control fish are analyzed together. Analyzing each exposure group separately reveals weaker strain effects, suggesting that exposure-driven shifts, rather than inherent baseline differences, account for much of the observed pattern.

To evaluate how zebrafish strain associates with community composition, we quantified the Bray-Curtis dissimilarity between all samples and generated distance matrices (Figure 3C & S3). We used stepwise regression to select an optimal model which included strain, exposure, dpe, total worm count, and three two-way interactions between strain, exposure and dpe. There was significant clustering of samples by zebrafish strain, exposure, dpe, total worm count and the interaction between strain and exposure ($P<0.05$, Table S14). A homogeneity of dispersion test found significant differences in dispersion by strain; a post-hoc Tukey's test revealed a significant difference in the dispersions of AB and 5D fish strains, meaning that the gut microbial community compositions of AB fish exhibit elevated levels of dissimilarity compared to those of 5D fish ($P<0.05$, Figure S3). To test the dissimilarity of control zebrafish gut microbiomes, we constructed a Bray-Curtis dissimilarity matrix including only the unexposed fish and factors that did not include exposure or total worm count (Figure 3D) and found no significant difference in the beta diversity of different zebrafish strains ($P>0.05$, Table S15). These results suggest that while different zebrafish strains harbor broadly similar gut microbiome communities under control conditions, parasitic infection impacts these communities in strain-dependent ways.

Finally, we sought to determine whether specific genera differentially associated with different zebrafish strains. To do so, we built GLMs for the comparison of microbial taxonomic abundances between the zebrafish strains in only unexposed zebrafish. We found nine genera (genera in the Chitinibacteraceae, Gammaproteobacteria, Gemmataceae, and Rhizobailes Incertae Sedia families, and the genera *Reyranella*, *Candidatus Alysiosphaera*, *Crenobacter*, *Paucibacter*, and *Brevibacterium*) were significantly enriched and one (the genus *Paraclostridium*) was significantly depleted in Casper fish compared to AB fish. Two genera (a genus in the Obscuribacterales family, and the genus *Phreatobacter*) that were significantly enriched and two (genera in the Chitinibacteraceae and X67.14 families) that were significantly depleted in TU fish compared to AB fish. One genus (*ZOR0006*) that was significantly enriched

in 5D fish compared to AB fish (Figure S1B). These results indicate that each zebrafish strain gut environment may selectively modulate the abundances of a small number of taxa that differ from other zebrafish strains.

Collectively, these findings show that zebrafish strains are broadly similar in their baseline gut microbiome composition when they are reared in identical conditions, with only modest differences in alpha diversity and in the abundances of a small number of taxa. In contrast, parasite infection elicits strain-dependent shifts, indicating that host genetic background plays a more prominent role in shaping microbiome responses to environmental perturbation than in determining baseline community structure.

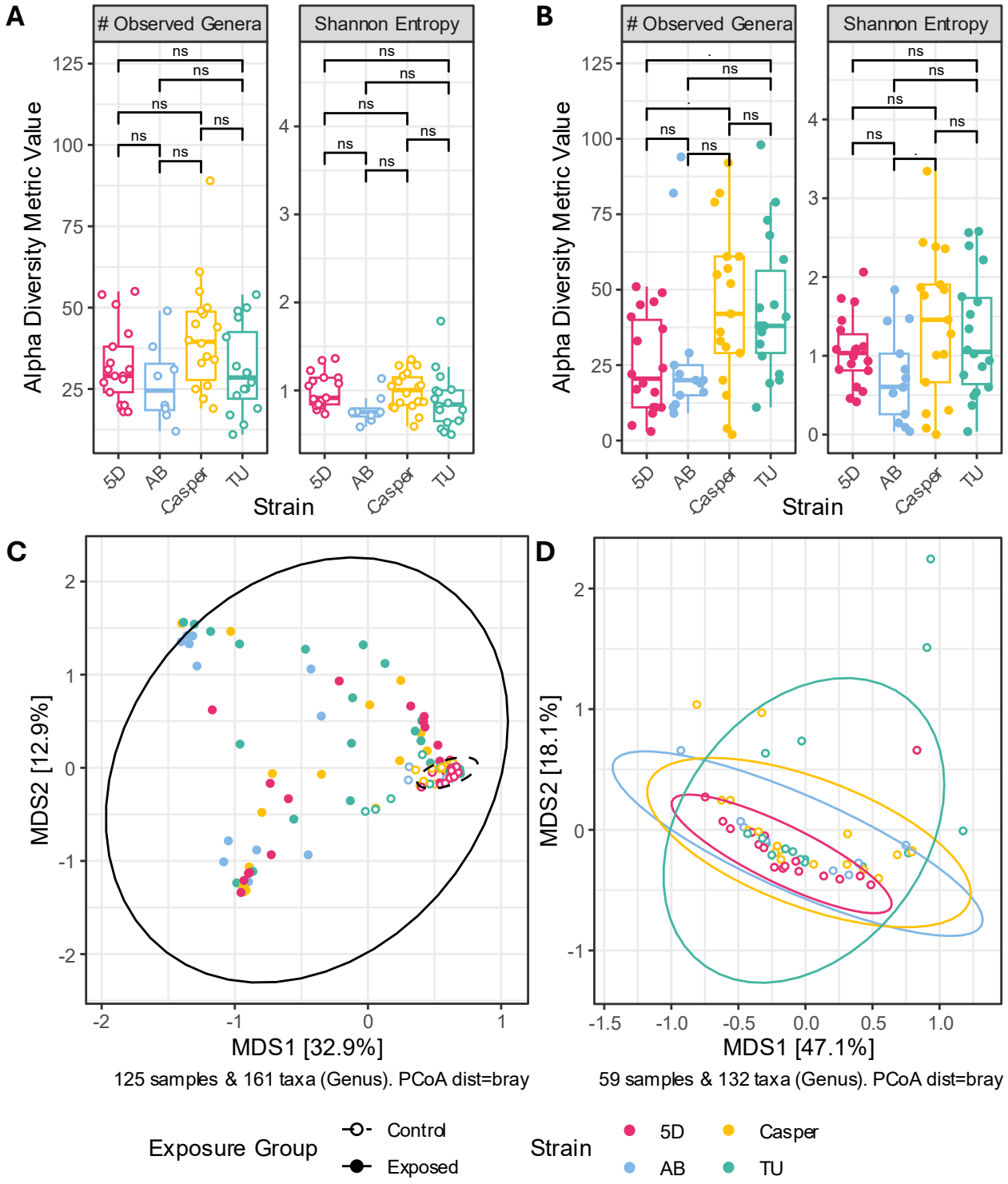


Figure 3. The gut microbiomes of zebrafish strains exhibit modest differences in diversity and composition. Boxplots of alpha diversity metrics, Shannon Entropy and the number of Observed Genera for Control zebrafish (A) and zebrafish exposed to *Pseudocapillaria tomentosa* (B) show

increased variation in alpha diversity of exposed zebrafish gut microbiomes (Tables S10 & S11). Significance determined using post-hoc tukey-tests (Tables S16 & S17). A "." indicates statistical significance between "0.05" and "0.1". (C) Principle Coordinates Analysis (PCoA) plot based on the Bray-Curtis dissimilarity of gut microbiome composition of zebrafish by strain shows significant differences in dispersion between control and exposed groups as indicated by the ellipses. (D) PCoA plot based on the Bray-Curtis dissimilarity of gut microbiome composition of just control zebrafish shows significant overlap in the compositions and dispersions between zebrafish strains. Ellipses in panels C and D indicate 95% confidence intervals.

Zebrafish gut microbiomes of different strains respond uniquely to parasite infection

To explore the influences of host genetics, parasite exposure and infection severity on the gut microbiomes of zebrafish, we used the previously created GLMs for alpha diversity as measured by Shannon Entropy and observed richness (Figure 4A) incorporating both exposure group and worm burden as predictors. Shannon Entropy significantly differs as a function of parasite exposure ($P<0.05$, Table S7) in addition to strain ($P<0.05$, Table S7), with exposure resulting in a higher Shannon Entropy. To test the relationship between Shannon Entropy and worm burden we ran a linear model and found that Shannon Entropy does not differ significantly as a function of worm burden ($P>0.05$, Figure S2A). Worm burden emerged as a significant predictor of observed genus richness ($P<0.05$, Table S8, Figure S2B), whereas parasite exposure as well as the interaction between strain and parasite exposure did not (P.0.05, Table S8). Taken together, these results indicate that *P. tomentosa* infection can alter the zebrafish gut microbiomes in a severity-dependent manner, suggesting that infection influences not only host pathologic changes and physiology directly, but may indirectly influence host physiology through shifts in microbial community structure.

To evaluate how parasite exposure associates with community composition, we quantified Bray–Curtis dissimilarities among samples (Figures 4B & S3). We detected significant clustering based on strain, exposure, dpe, and worm burden, as well as the interaction between strain and exposure ($P<0.05$, Table S14). The strong contribution of worm burden indicates that variation in infection intensity is a major driver of microbiome structure, in some cases comparable to or exceeding strain effects (Figure 4C). To determine whether these patterns persisted within infected fish, we repeated the analysis using only exposed samples. Here, worm burden again explained significant variation in community composition, alongside strain, dpe, and the interaction between strain and dpe ($P<0.05$, Table S18). These results show that microbiome responses to infection are shaped not only by host genetic background but also by infection dynamics, with worm burden emerging as a significant factor in community divergence over the course of exposure.

To assess differences in microbiome variability, we tested homogeneity of dispersion (HOD) across exposure groups and zebrafish strains using a composite group variable (details in methods section). A Tukey post-hoc test revealed two patterns. First, strains did not differ in dispersion within either exposure group ($P > 0.05$, Table S19), indicating similar within-strain variability under both conditions. Second, all strains showed significantly greater dispersion in exposed fish compared to controls ($P < 0.05$, Table S20). Taken together, these findings show that parasite exposure leads to a consistent increase in microbiome variability across all strains.

Given the significant clustering we found based on dpe, we next examined dispersion across infection timepoints. HOD tests revealed significant strain-dependent differences between 31 and 44 dpe ($P < 0.05$, Table S14). Dispersion increased over time in 5D and Casper fish but remained stable in AB and TU. At 31 dpe, several comparisons between zebrafish strains differed significantly from one another, whereas by 44 dpe these differences were no longer detectable. These patterns suggest that the microbiomes of different

zebrafish strains vary in their early susceptibility to perturbation, with greater divergence in community composition at earlier stages of infection that diminishes as infection progresses. Notably, this pattern aligns with prior observations that zebrafish strains differ most in their susceptibility to worm burden at earlier stages of infection, even though those studies did not examine microbiome composition [25]. Together, these results indicate that while parasite exposure broadly increases microbiome variability, the trajectory and magnitude of this variability over time are shaped by host strain and infection dynamics.

Finally, we sought to identify genera that are significantly associated with parasite exposure and compared associations between strains. To do so, we built GLMs for the comparison of taxonomic abundances between exposure groups for each of the zebrafish strains. There were 47 taxa that were significantly differentially abundant between exposure groups in at least one of the four zebrafish strains (Figure 5). Of the significantly different taxa, 42 of these were significantly differentially abundant between exposure groups in at least one, but not all, strains. Five of the identified taxa were consistently associated with infection across all strains; four were depleted in infected zebrafish (a genus in the Barnesiellaceae family, and the genera *Cetobacterium*, *Paraclostridium*, and *ZOR0006*), whereas the genus *Pseudomonas* was significantly enriched in infected zebrafish (GLM, FDR < 0.25), although the genus in the *Barnesiellaceae* family, and the genera *Paraclostridium*, and *ZOR0006* pass a stricter filtering threshold across all 4 zebrafish strains (FDR <0.1). The genus *Pseudomonas* has previously been associated with *Pseudocapillaria tomentosa* infection in 5D zebrafish [4, 11]. We found that the positive association between abundance of the genus *Pseudomonas* and *P. tomentosa* worm burden varied in strength across zebrafish strains with the correlation being stronger in the AB, Casper and TU zebrafish than in the 5D zebrafish (Figure S4). These results indicate that while many microbial taxa are affected by parasite infection, a select few exhibit consistent responses across all four zebrafish strains regardless of host genetics or early environmental stressors, suggesting the presence of a shared microbial signature of *P.*

tomentosa infection. Taken together, these findings reveal that infection severity is associated with progressively greater disruption of gut microbiome structure relative to controls, as well as the prominence of specific taxa that are correlated with severity.

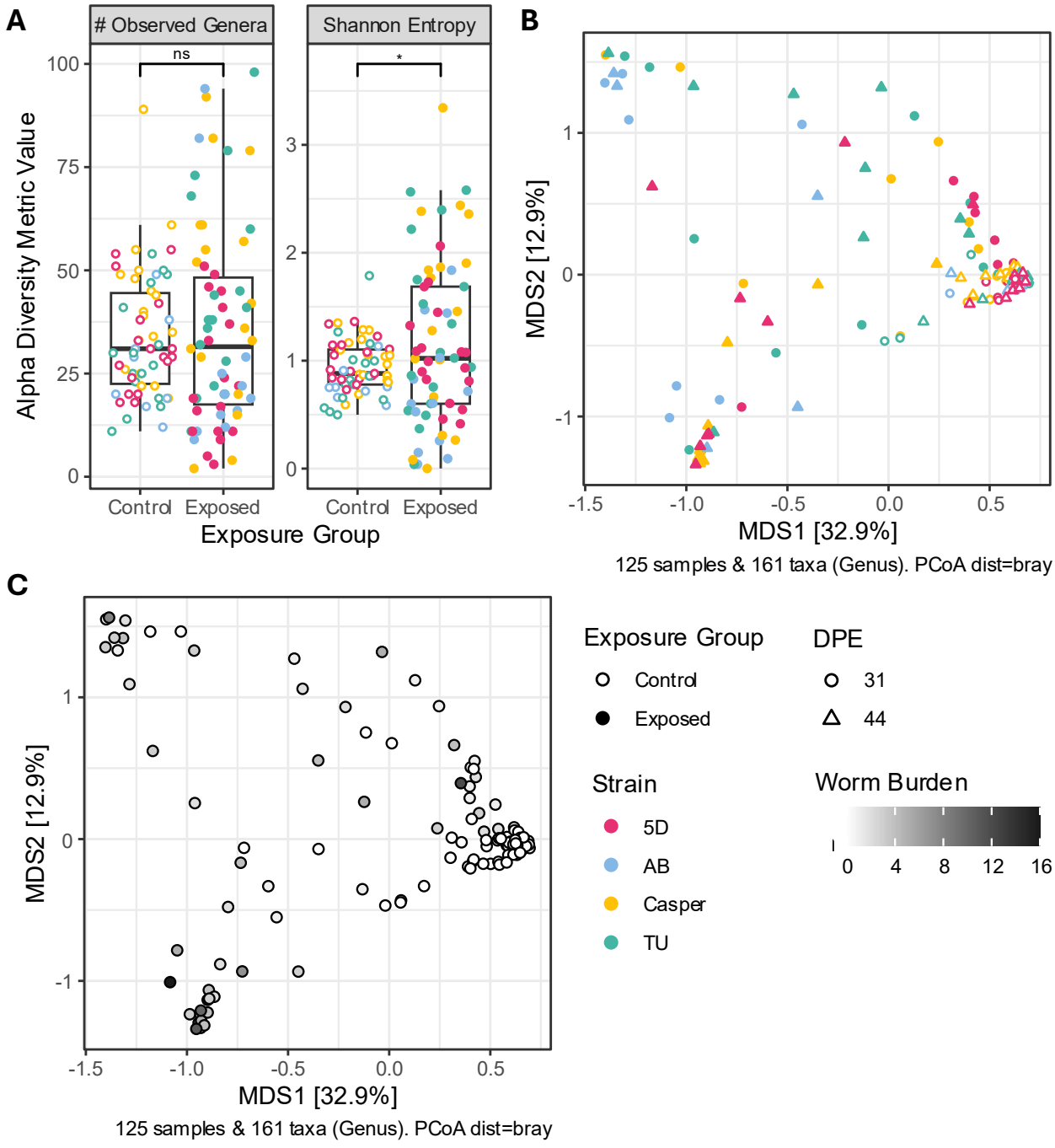


Figure 4. Exposure to *Pseudocapillaria tomentosa* induces strain-specific shifts in zebrafish gut microbiomes. (A) Boxplots show increased variance in Shannon Entropy and Observed Genus in

zebrafish exposed to the parasite as compared to control zebrafish. A "*" indicates statistical significance below the "0.05" level. (B) A PCoA of Bray–Curtis dissimilarities shows that *P. tomentosa* exposure increases microbiome dispersion relative to controls, with exposure-driven clustering patterns that vary across host strains. (C) A PCoA of Bray-Curtis dissimilarities shows differences in worm burden between clusters.

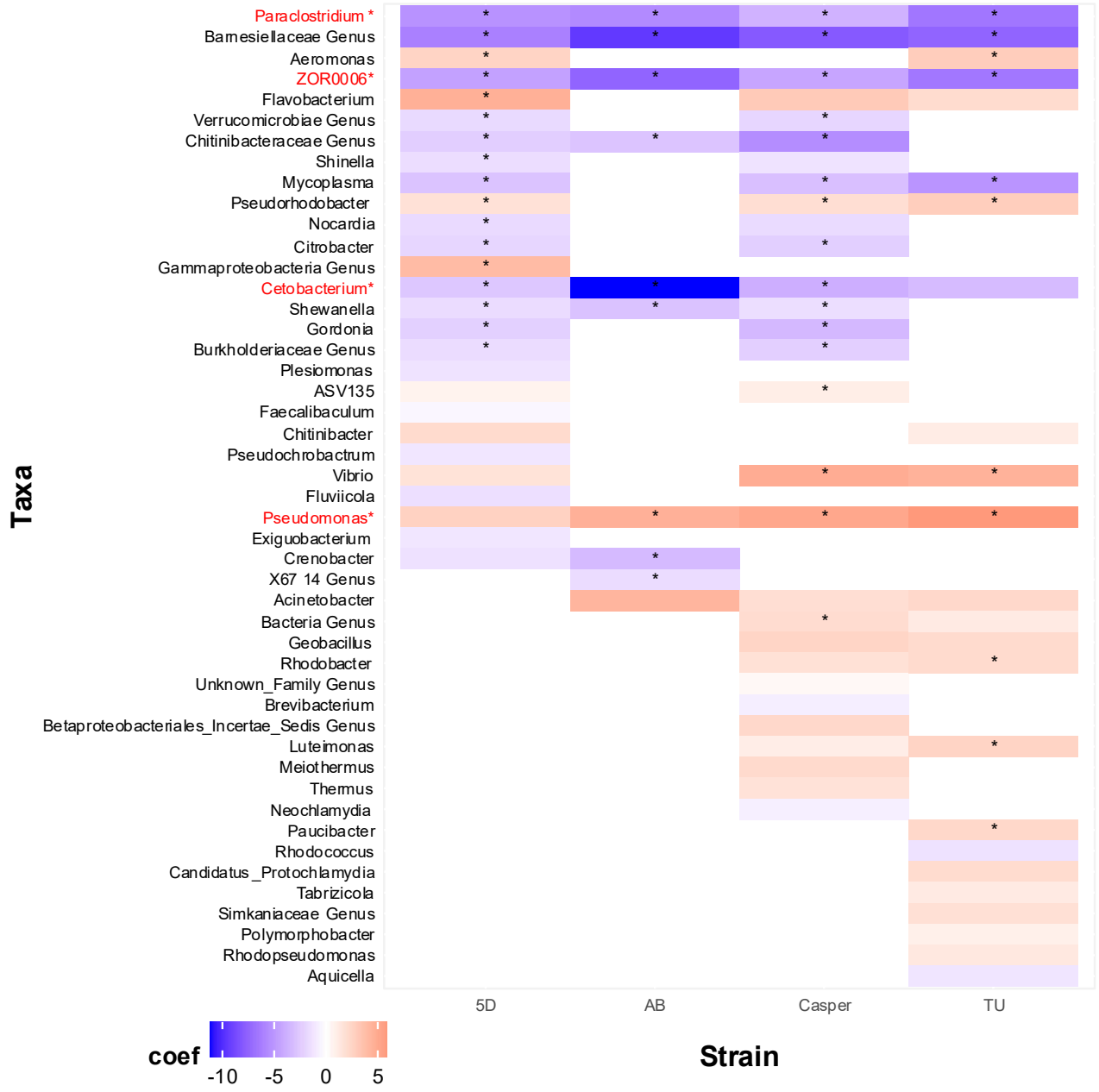


Figure 5. Evidence for taxonomic signatures of *Pseudocapillaria tomentosa* infection. A heatmap shows strain-specific taxonomic responses to parasite exposure (FDR < 0.25). Coeffecient values correspond to significantly differentially abundant taxa that are either depleted (negative coefficient) or enriched (positive coefficient) in the gut microbiomes of exposed zebrafish as compared to control zebrafish of the same strain. Comparisons with an FDR <0.1 are denoted by a "*" in the corresponding cell. Taxa that are significantly differentially abundant in all four strains are denoted with red text and a "*" along the y-axis.

Discussion

The gut microbiome is increasingly recognized as a modulator of host health, yet the relative contributions of host genetics versus environmental factors remain poorly understood. In this study, we analyzed gut microbiomes from a subset of zebrafish from Schuster et al. [25] to examine how host genetic background influences both baseline microbiome composition and responses to parasitic infection. Our findings reveal that zebrafish strain has a surprisingly modest effect on baseline gut microbiome structure, with more pronounced strain-specific differences emerging only under parasitic challenge. This pattern suggests that host genetics may primarily influence microbiome resilience and response to perturbation rather than fundamental community assembly.

Under control conditions, we found minimal evidence for strong genetic determination of microbiome composition. While alpha diversity metrics showed some strain differences, beta diversity did not differ significantly between strains in unexposed fish. These results differ from a previous zebrafish study that reported substantial genetic effects on the gut microbiome [26], though this work employed genetic manipulation techniques to determine the effect of specific genes rather than our effort to assess the effect of broad genotypic variation on the microbiome. Our results suggest that within the range of natural

292 genetic variation in laboratory zebrafish (outbred 5D, inbred AB and TU, and pigmentation-mutant Casper),
293 strain identity plays a minor role in shaping the gut microbiome under stable conditions.

294 The role of host genetics in shaping microbiome composition became more apparent following parasite
295 exposure. We observed significant strain by exposure interactions for beta diversity, indicating that the
296 gut microbiomes of different zebrafish strains respond differently to *P. tomentosa* infection. However,
297 even these effects were moderate. Notably, infection intensity (worm burden) emerged as a stronger
298 predictor of microbiome changes than either strain or exposure status alone. The identification of five
299 taxa consistently associated with infection across all strains – including depletion of *Barnesiellaceae*,
300 *Cetobacterium*, *Paraclostridium*, and ZOR0006, and enrichment of *Pseudomonas* – suggests that *P.*
301 *tomentosa* infection triggers conserved microbiome responses that largely override host genetic
302 variation.

303 An important caveat to our findings is the supersaturation event that occurred early in the experiment (3-
304 6 dpe), causing differential mortality in AB (46 deaths) and 5D (5 deaths) strains among exposed fish only.
305 While this event preceded our sampling by several weeks, it may have selected for more resilient
306 individuals in these strains. Interestingly, the elevated microbiome dispersion we observed in AB and 5D
307 strains could reflect lasting effects of this early stress. However, the microbiome clustering pattern
308 (AB/TU vs 5D/Casper) did not align with supersaturation impact, suggesting that any lasting effects were
309 subtle.

310 Our temporal analysis provides additional insights into infection-microbiome dynamics. Building on
311 Schuster et al [25], who found that AB strains developed heavier infections at early timepoints (8-22 dpe),
312 our analysis at 31 and 44 dpe showed no significant strain differences in worm burden. This temporal
313 pattern indicates that initial strain-specific differences in infection susceptibility equilibrate over time.
314 Despite this equilibration in parasite load, microbiome differences persisted and even increased

between timepoints in some strains (5D and Casper), suggesting that microbiome responses reflect cumulative infection history rather than current parasite burden alone.

The modest genetic effects observed here have important implications for zebrafish as a model organism. First, they suggest that microbiome variability between studies using different strains may be less problematic than previously assumed, at least under standard laboratory conditions when husbandry conditions are the same amongst the different strains. Second, they indicate that strain selection may be more critical for infection or stress studies where genetic background modulates response dynamics. Third, the strong conservation of infection-associated taxa across strains suggests that microbiome-based biomarkers for *P. tomentosa* infection may be broadly applicable regardless of host genetics.

Conclusions

In conclusion, our study demonstrates that while zebrafish strains harbor broadly similar baseline gut microbiomes, parasitic infection reveals strain-dependent differences in microbiome responses. Across strains, parasite exposure consistently increased microbiome variability. Downstream infection-related factors such as worm burden and time post-exposure further shaped the magnitude and trajectory of these shifts. Strain-dependent microbiome responses have important implications for experimental design, cross-study comparisons, and the development of microbiome-based interventions in both model organisms and translational contexts. These findings refine our understanding of host genetic influences on the microbiome, suggesting that genetics may be more important for determining microbiome resilience and response capacity than for establishing baseline community structure. Additionally, our work suggests the presence of microbial signatures of *P. tomentosa* infection across zebrafish strains. By clarifying how host genetics and infection dynamics interact to shape gut microbial communities, this work

contributes to a growing effort to understand when and how the microbiome reflects host resilience, susceptibility, and disease progression. Future studies will be essential for disentangling the endogenous factors that modulate host responses to environmental stressors and for defining the microbiome's role in these processes.

Methods

Animal husbandry

All experiments were conducted under the approval of Institutional Animal Care and Use Committee at Oregon State University (protocol 5151). This study used 4-5 month old 5Ds, ABs, TUs, and Caspers to evaluate microbiome differences across different zebrafish strains. 5D, AB, and Casper fish lines were acquired from the Sinnhuber Aquatic Research Laboratory, Oregon State University (Corvallis, OR), a facility free from *Pseudoloma* and *Pseudocapillaria tomentosa*. TUs were obtained from the Zebrafish International Resource Center (Eugene OR), where only *Mycobacterium chelonae* and *Pseudoloma neurophila* have been detected.

Experiments were conducted in the Nash Hall Vivarium (OSU) on a flow through system supplied by dechlorinated city water. Water parameters were maintained as follows: Temperature 23.6-26.2°C, ammonia 0-0.25 ppm, pH 7.6, hardness 0-25 ppm, and conductivity 90-110 uS/cm. Fish were fed Gemma Micro 300 (Skretting; Fontaine-les-Vervins, France) once a day 5 days per week. To minimize the likelihood of supersaturation events, tanks received moderate waterflow and supplemental aeration with air stones. Symptoms of supersaturation were monitored by looking for fish rapidly breathing at the bottom of the tank, small bubbles on the side of the tank, and bubbles on the fishes fins/eyes [27]. Each zebrafish strain was housed individually, in 9L tanks, which were randomly stocked with 30 fish per tank (180 fish/strain).

Each strain had 3 unexposed and 3 *P. tomentosa* exposed tanks. Tanks were placed on separate fish racks based on their exposure group and their position on each rack was randomized. Lastly, fish were allowed to acclimate in the tanks for one week prior to the start of the experiment.

Supersaturation events

It must be noted that supersaturation events occurred between 3-6 dpe [25]. Across all tanks 5 5D, 46 AB, and 1 TU mortalities were attributed to supersaturation. Fish which exhibited symptoms of supersaturation included presence of bubbles in eyes, on the fins, body, and rapid breathing on the bottom of the tank.

Parasite exposure

Acclimated zebrafish in the exposure group were infected with *P. tomentosa* by exposing them to effluent water containing *P. tomentosa eggs* from 0-24 dpe. Above the uninfected control fish, 40 infected zebrafish (> 21 dpe) were housed in 2 9L Aquaneering (San Marcos, CA, USA) tanks (20 fish per tank). A manifold was used to distribute *P. tomentosa* water effluent to the uninfected tanks (n=12). To further enhance the infections, 200mL of tank water from *P. tomentosa* infected fish was distributed to each recipient tank at 5, 10, 15, 20, and 25 dpe. To minimize tank effects, each tank's rack position was rotated every 2 days (each tank occupied every rack position twice). The control fish had an identical manifold infection setup and tank rotation but did not use *P. tomentosa* infected fish.

Weight, sex determination and worm count

Fish weight, sex*, P. tomentosa* infection severity, and microbiome compositions were evaluated at 31 and 44 dpe (days post exposure). Due to the large sample sizes (120 fish per timepoint), evaluations were conducted over two days (*P. tomentosa* exposed day one, and controls day two). For each sampling day, fish were first euthanized with 500ppm of MS-222 (Syndel, WA) and then weighed. Fish weights were

recorded by weighing each fish in a tared petri dish filled with fish water. Before the fish was added to the petri dish, excess water was removed by dabbing the transfer net with paper towels.

Sex was determined by a gross examination of external and internal phenotypes (testes or ovaries with eggs). *P. tomentosa* infections were evaluated using wet mounts of whole zebrafish intestines. Ethanol sterilized forceps and micro snips were used to isolate the intestine from other visceral organs using a dissecting microscope. A Leica (Wetzlar, Germany) DMLB microscope at 200x magnification was then used to evaluate the total number of worms, mature females, and the number of parasite eggs in each wet mount slide. *P. tomentosa* infection outcomes were based on the prevalence (infection status) and severity (number of worms) of infections for all detected worms.

Microbiome sample selection

Sample selection for the microbiome analysis was conducted using a systematic approach to ensure balanced representation across experimental conditions. The original study [25] included 270 exposed and 269 control fish, which were sampled at 8, 22, 31 and 44 dpe across 3 replicate tanks/strains for each exposure regime. The present study represents a subset of these fish for microbiome analysis, using fish from 31 and 44 dpe as at this time > 70% of the fish were positive by wet mounts and 100% by PCR [25]. These timepoints were selected because wet mount examinations at these time points provided many infected fish across the strains. The dataset was first cleaned by removing samples with missing timepoint or worm count data, as well as samples marked as lost during processing. Additionally, tank "IS02" was excluded due to documented strain mixing issues in the experimental records. The final dataset included two timepoints (31 and 44 dpe) across 25 experimental tanks, with samples collected from both control and exposed groups. To achieve balanced sampling for downstream analyses, a random sampling strategy was implemented where two individuals were selected per tank per exposure group per timepoint, resulting in an average of six individuals per strain across the experimental design. This sampling approach

was repeated 10 times to generate multiple sample sets, each maintaining the same sampling structure while accounting for potential variation in individual selection. The selected samples were stratified by strain, exposure group, tank, and timepoint to ensure comprehensive coverage of the experimental design space, with particular attention to maintaining balanced sex ratios within each sampling group. Due to various sample losses (freezer issues, moribund individuals), the final sample sizes were 61 and 66 total samples for the control and exposed groups, respectively. The tenth randomly generated sample set was selected for final analysis based on having the most even sex ratio distribution across sample populations and the narrowest spread between the highest and lowest number of each sex per timepoint, strain, and exposure group combination (Figure 1). The resulting sample sets were saved as R data objects for subsequent analyses, providing a robust foundation for microbiome comparisons while maintaining statistical power and experimental balance.

Microbiome sampling

Following infection analysis, the gastrointestinal contents were flash frozen in liquid nitrogen and stored at -80˚C, until DNA was extracted using the DNeasy Powersoil Pro Kit (Qiagen, Hilden Germany) as previously described [25]. PCR amplification was done on the V4 region of the 16S rRNA gene using Earth Microbiome Project (EMP) primers and protocols [28, 29]. Samples were prepared using 1 µl of purified DNA, 2µl of a 2.5µM barcoded 515F primers and 0.5µl the 806R primer, 5µl of Platinum Hot-Start PCR Master Mix (Thermo Fisher, Carlsbad, CA), and 2µl water with the following PCR conditions, 94°C, 3m; (94°C, 30s; 50°C, 30s; 70°C, 1m)x 35; 70°C 10m. PCR products were visualized on a 1.5% agarose gel and quantified using Quant-iT 1X dsDNA HS kit (Qubit, Carlsbad, CA) on the BioTek Synergy H1 Hybrid Multi-Mode Plate Reader (Winooski, Vermont). A 100ng aliquot of DNA was selected from each of the 127 samples, the pooled DNA was cleaned using the QIAGEN QIAquick PCR purification kit (Hilden, Germany), and quantified using Qubit dsDNA HS kit (Carlsbad, CA) on the Qubit 2.0. The quality of the pooled library

was verified on the Agilent TapeStation 4200 (Santa Clara, CA). The prepared library was submitted to the Center for Quantitative Life Sciences (CQLS) at OSU for sequencing on the Illumina (San Diego, CA) MiSeq platform using a paired-end 2 x 300 bp configuration.

Sequence processing

All subsequent DNA sequence processing and analyses were conducted in R (v 4.3.1) [30]. Two samples (ISF542 and ISF545) were removed due to low input read numbers. Fastq files were trimmed to 210 bp for forward read files, and 190 bp for reverse read files, and merged into contigs using DADA2 [31]. Amplicon sequence variants (ASVs) were then matched for identification using the SILVA database (v 138.1) [32]. ASVs identified as Eukaryota, Mitochondria, and Chloroplast were removed along with any bacterial reads that were unidentified at the Phylum level. All remaining ASVs were filtered at a minimum prevalence of 2, resulting in 447 identified ASVs among all samples. Observed richness was calculated using the ps_calc_richness function in the phyloseq package (v 1.46.0) [33]. Shannon Entropy was calculated using the ps_calc_diversity function in the phyloseq package (v 1.46.0). The OTU table from the Phyloseq object was used to create a distance matrix. The beta diversity distance matrix was created using the vegdist() function in the vegan package (v 2.6-4) using Bray-Curtis measurements of dissimilarity [34, 35]. Two samples were omitted from subsequent analysis due to low reads (samples 542 and 545).

Data transformations and substitutions

One value was missing for weight (sample 516), the weight value was replaced with the average weight of all fish in the same exposure group of the same strain and sex (Control, TU, male). Weight, observed richness, and Shannon Entropy were checked for normality using Shapiro tests for normality. If results were not normally distributed, a transformation method was determined using the bestNormalize() function in the bestNormalize package (v 1.9.1) [36].

Model selection

Kruskal-Wallis tests were used for the determination of significant differences in weights between groups with more than two factors. Wilcox Ranked-Sum tests were used for the determination of significance between groups with only 2 factors.

To identify variables that may be associated with weight, Shannon Entropy, and Observed Genus counts, full models were built using the lm() function, step-wise regression selections were performed using the step() function in the stats base R package (v 4.3.1) [30] with direction set as "both", and best models were determined by lowest AIC value using the following base formulae:

Weight (mg) ~ Strain + Sex + Infection.Status + Strain*Sex + Strain* Infection.Status + Sex*Infection.Status

Observed Genus Counts ~ Strain + Exposure + DPE + Sex + Weight + Infection.Status + Total.Worm.Count + Strain*Exposure + Strain*DPE + Exposure* DPE + Strain*Exposure*DPE

Shannon Entropy ~ Strain + Exposure + DPE + Sex + Weight + Infection.Status + Total.Worm.Count + Strain*Exposure + Strain*DPE + Exposure* DPE + Strain*Exposure*DPE

Strain was treated as a biologically fixed factor due to its relevancy to the central objectives of this study and was retained in all models.

The optimal model for the prediction of zebrafish weight was a significant predictor of zebrafish weight ($F_{8,116} = 18.37$, adj. $R^2 = 0.5285$, $P < 2.2e\text{-}16$).

Weight(mg) ~ Strain + Sex + Infection.Status + Strain:Sex

The optimal model for the prediction of the number of Observed Genera was significant ($F_{14,110} = 2.471$, adj. $R^2 = 0.1424$, $P = 4.47e\text{-}03$):

Observed Genera ~ Strain + Exposure + DPE + Weight_mg + Total.Worm.Count + Strain:Exposure + Strain:DPE + Exposure:DPE.

The optimal model for the prediction of Shannon Entropy was a significant predictor ($F_{7,117} = 3.346$, adj. $R^2 = 0.117$, $P = 2.75e\text{-}03$).

Shannon Entropy ~ Strain + Exposure + DPE + Weight_mg + Exposure:DPE

To determine the significance of variables associated with parasite infection, we built a binomial generalized linear model. We used the AB fish strain as the reference group because it had the highest rate of infection (100%) and included only samples that were exposed to the parasite. The model used was the following:

Infection.Status ~ Strain + Sex

To determine the significance of variables associated with infection severity (as measured by total worm count), we built a negative binomial generalized linear model. The model used was the following:

Total.Worm.Count ~ Strain + DPE

To identify variables that may be associated with Beta we built a full model using the capscale() function in the vegan package (v 2.6-4).

Beta Diversity Matrix ~ Strain + Exposure + DPE + Sex + Weight + Infection.Status + Total.Worm.Count + Strain*Exposure + Strain*DPE + Exposure* DPE + Strain*Exposure*DPE

Step-wise model selection for beta diversity was done using the ordistep() function in the vegan package (v 2.6-4) with direction set as “both”. The resulting optimal model for the prediction of the betadiversity matrix captured 36.5% of the total inertia in the constrained axes:

Beta Diversity Matrix ~ Strain + Exposure + DPE + Total.Worm.Count + Strain:Exposure + Strain:DPE + Exposure:DPE.

Analysis of Variance (ANOVA) and Permutational Analysis of Variance (PERMANOVA) tests were used for the determination of significant associations in the Generalized Linear Models (GLMs) and distance-based models, respectively.

Taxonomic modeling

To identify potential taxa at the genus level that differentially associate with various factors, we built GLMs using MaAsLin2 to explore the following comparisons: between exposed and control fish within strains and between dpe within strains and exposure groups [37]. To do this, we ran separate GLMs for each of the "within" groups with the "between" factor as the factor included in the model. All significantly (FDR < 0.25) differentially abundant taxa from each "within" analysis were compiled and used to assemble a heatmap.

Abbreviations

dpe = days post exposure

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC; Protocol No. 5151) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Consent for publication

Not applicable

Availability of data and material

All code generated during this analysis is available in the Interstrain Study repository at the following URL: https://github.com/emileemlance/Interstrain_Zebrafish_Gut_Microbiome. All sequencing data generated and analyzed during this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1415777. All other data supporting the findings of this study are provided within the article and its supplementary materials.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Science Foundation Grant 2025457.

Author contributions

TJS and MLK conceived the project. TJS, MLK and CJS designed the experiments. CJS, MLK, and CL performed experiments. KDK, MLK, CJS, and CL assisted in the generation of data. EML and MJS conducted data and statistical analyses. EML generated all visualizations. TJS, MJS, and EML wrote the paper, and all authors edited the paper.

Acknowledgements

We would like to thank Corbin J. Schuster for laying the groundwork for this study including conceptualization and conduction of the original experiment. We also thank Coleen E. Al-Samarrie for assisting in the rearing of the fish and data collection. We appreciate Sinnhuber Aquatic Research Laboratory and the Zebrafish International Resource Center for providing the zebrafish. All work was conducted at Oregon State University, a land grant university, located on the traditional homelands of the Kalapuya people, who are now absorbed into the Grande Ronde and Siletz Nations.

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Supplemental material

Sample metadata and all supplemental tables are available in the Supplementary Table file.

Supplemental Table 1. Sample metadata for the present study.

Supplemental Table 2. Infection status for Schuster et al. (2023) and the present study.

Supplemental Table 3. ANOVA results for the pathology outcome model.

Supplemental Table 4. Post-hoc tukey test results for the physiology differences within strains between pathology outcomes.

Supplemental Table 5. ANOVA results for the optimal physiology model.

Supplemental Table 6. Post-hoc tukey test results for the physiology model.

Supplemental Table 7. ANOVA results for the Shannon Entropy model.

Supplemental Table 8. ANOVA results for the observed genus model.

Supplemental Table 9. Post-hoc Tukey test for all samples – Alpha diversity metrics.

Supplemental Table 10. ANOVA results for Shannon Entropy model with just control samples.

Supplemental Table 11. ANOVA results for Shannon Entropy model with just exposed samples.

Supplemental Table 12. ANOVA results for # observed genera model with just control samples.

Supplemental Table 13. ANOVA results for the # of observed genera model with just exposed samples.

Supplemental Table 14. PERMANOVA results for the beta diversity model.

Supplemental Table 15. PERMANOVA results of the beta diversity model with only the control samples.

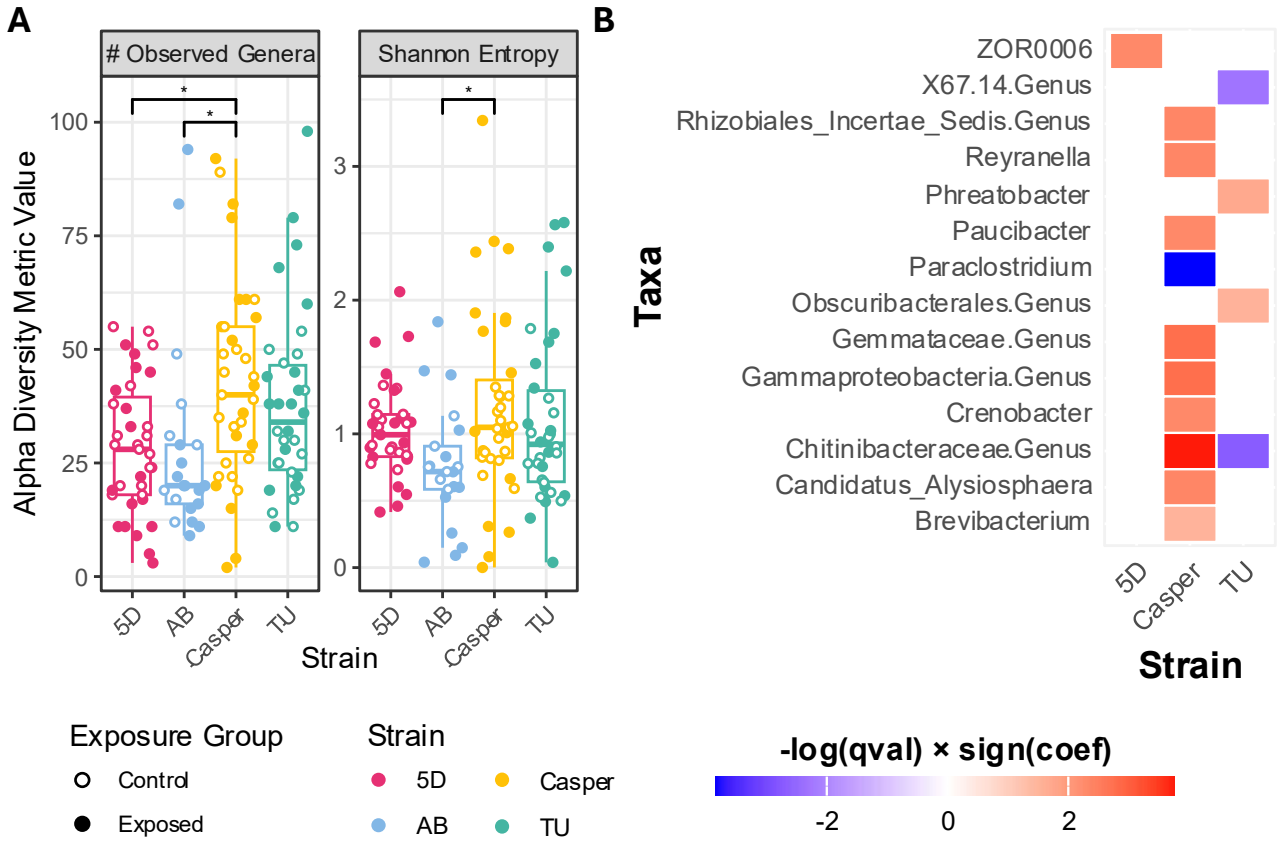
Supplemental Table 16. Post-hoc Tukey's test results for just control samples for both alpha diversity metrics.

Supplemental Table 17. Post-hoc Tukey's test results for just exposed samples for both alpha diversity metrics.

Supplemental Table 18. PERMANOVA results for the beta diversity model with only exposed samples.

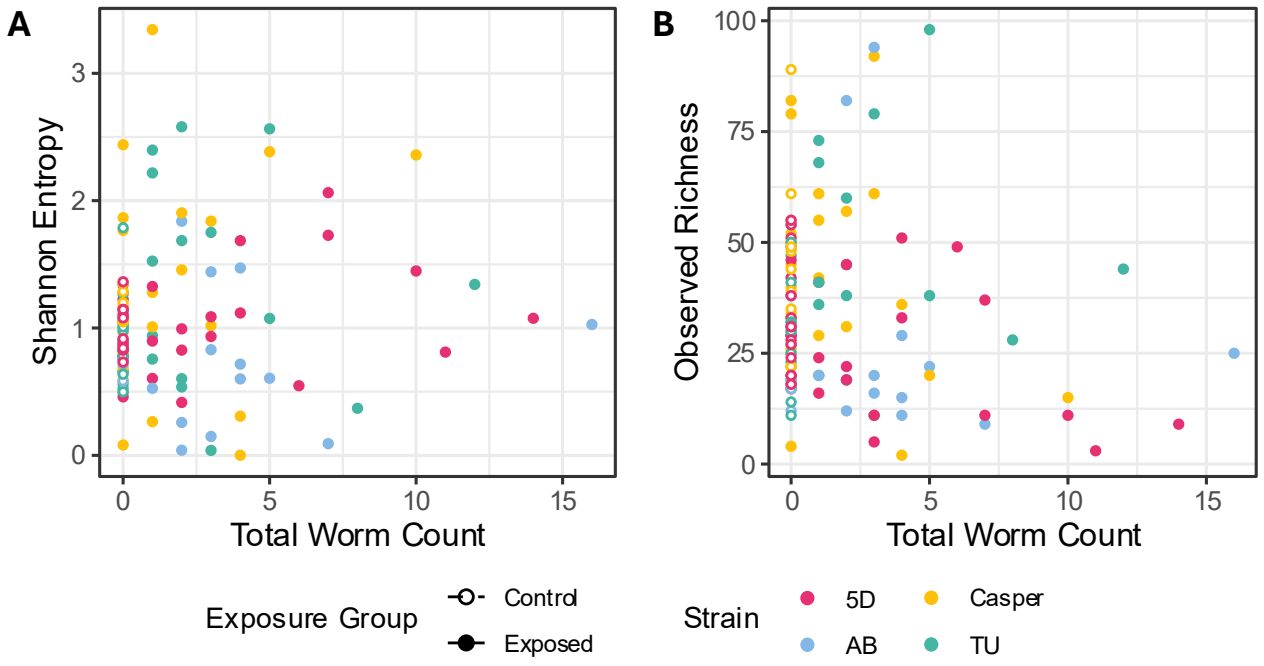
Supplemental Table 19. Post-hoc Tukey's test results comparing beta dispersion between strains in the same exposure groups.

Supplemental Table 20. Post-hoc Tukey's test results comparing beta dispersion between exposure groups of the same strain.



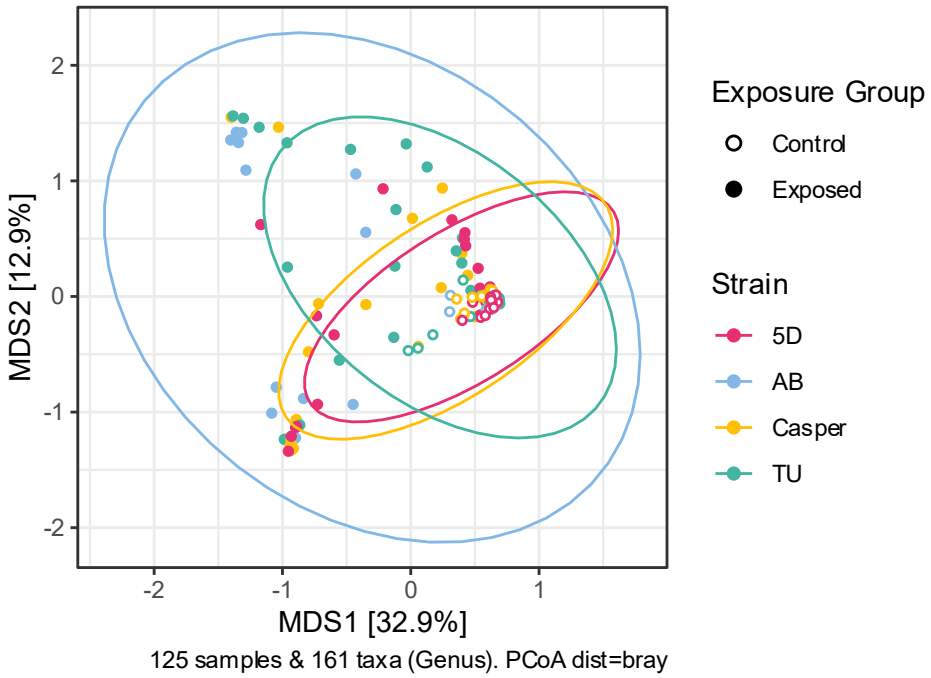
Supplemental Figure 1. Gut microbiomes of zebrafish strains exhibit similar diversity. (A) Boxplots of alpha diversity metrics, the number of Observed Genera and Shannon Entropy, of all zebrafish show modest differences in alpha diversity of zebrafish gut microbiomes when both control and exposed zebrafish are assessed together. Only statistically significant comparisons are shown. A "*" indicates statistical significance below the "0.05" level. Heatmap (B) showing differentially abundant genera associated with Casper, TU and 5D zebrafish compared to AB zebrafish in only the control group.

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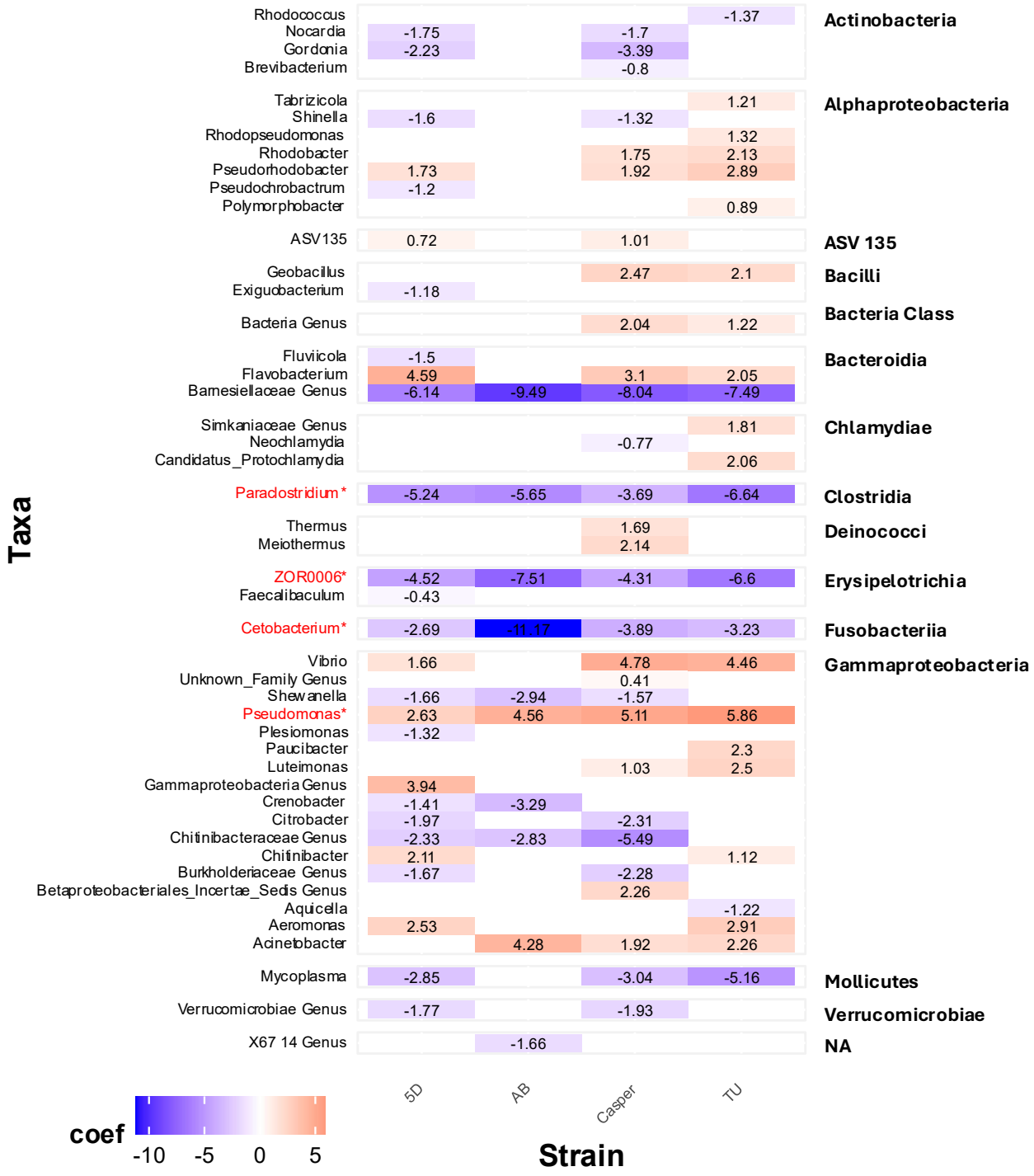
Supplemental Figure 2. Total worm count modestly associates with alpha diversity. Scatterplots of total worm count and alpha diversity metrics show a non-significant association with Shannon Entropy (A) and a significant association with the observed number of genera (B). Points are colored by zebrafish strain and filled conditionally by exposure group.

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Supplemental Figure 3. Zebrafish gut microbiomes exhibit non-homogenous dispersions by strain. Principle Coordinates Analysis based on the Bray-Curtis dissimilarity of gut microbiome composition of fish by strain. Ellipses indicate 95% confidence interval.

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Supplemental Figure 4. Taxa differ in their association with exposure across host strains. Heatmap showing MaAsLin2 result coefficients for taxa from that differ between exposure groups by strain. Taxa

are grouped by class, shown on the right side of the plot. Positive coefficient values indicate taxa that are positively associated with *Pseudomonas tomentosa* exposure within the zebrafish strain.

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.xlsx