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Pathogenic mechanisms of *Enterocytozoon hepatopenaei* through the parasite-gut microbiome-shrimp (*Litopenaeus vannamei*) physiology axis

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ABSTRACT

The progressive impact of *Enterocytozoon hepatopenaei* (EHP) infection on gut microbial function in *Litopenaeus vannamei* remains poorly understood beyond static comparisons between healthy and infected individuals. To close this knowledge gap, metagenomic sequencing was used to characterize the gut microbiomes of normal, long, medium, and short-sized adult shrimp categorized by increasing severity of infection. EHP infection suppressed digestive activity while inducing immune responses compared with healthy shrimp. Increasing infection severity was associated with a gradual decline in gut α-diversity and an expansion of potential pathogens and virulence factors (VFs). In addition, dysbiosis in gut microbiota composition and function, as well as reduced network stability among differential species, intensified with infection severity. Accordingly, we identified 24 EHP-discriminatory species that contributed an overall 83.3% accuracy in diagnosing infection severity without false negatives. Functional pathway analysis revealed significant suppression of metabolic, degradative, and biosynthetic processes in EHP-infected shrimp compared with healthy controls. Among them, map00630 glyoxylate and dicarboxylate metabolism and map00280 valine, leucine and isoleucine degradation were consistently depleted in infected individuals, thereby impairing their digestive function and anti-inflammatory responses. Additionally, EHP infection diversified VFs directly affecting shrimp gut microbiome. These findings support a conceptual model linking EHP pathogenesis to the parasite-gut microbiome-shrimp physiology axis.

Keywords: Increasing *Enterocytozoon hepatopenaei* infection; Gut microbiome; Microbiota dysbiosis; EHP-discriminatory species; Virulence factors

INTRODUCTION

Litopenaeus vannamei is the most extensively farmed shrimp species globally, contributing significantly to the global aquaculture industry. However, various infectious diseases pose major challenges to shrimp production (Thitamadee et al., 2016; Xiong et al., 2024). Among emerging pathogens, *Enterocytozoon hepatopenaei* (EHP) has become a widespread and economically detrimental threat in recent years (López-Carvallo et al., 2022; Xu et al., 2025). EHP is an intracellular microsporidian parasite that colonizes hepatopancreatic epithelial cells, leading to hepatopancreatic microsporidiosis (Biju et al., 2016). During infection, hepatopancreatic tissue fragments and EHP spores are released into the gut, facilitating parasite transmission and further compromising host physiology (López-Carvallo et al., 2022). Although EHP infection is generally nonlethal, it substantially retards shrimp growth. Consequently, research on EHP has lagged behind studies on acute shrimp diseases, such as acute hepatopancreatic necrosis disease (AHPND). Furthermore, EHP-infected individuals are highly susceptible to secondary infections, including co-infection with AHPND (Aranguren et al., 2017), white feces syndrome (WFS) (Caro et al., 2020), and white spot syndrome virus (WSSV) (Suryakodi et al., 2022). Overlooking EHP infection is therefore unwise, as it not only stunts growth but also exacerbates susceptibility to opportunistic pathogens.

The hepatopancreas and gut play central roles in shrimp

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digestion and immunity, and disruption of hepatopancreatic function by EHP infection further compromises host physiology and health (Aranguren et al., 2017; Shen et al., 2022a). Additionally, the gut microbiota is essential for maintaining shrimp health (Sha et al., 2022; Xiong et al., 2024), and dysbiosis has been implicated in various shrimp diseases (Mao et al., 2023; Sha et al., 2022). For example, *Arcobacter*, *Propionigenium*, and *Photobacterium* spp. are enriched in the gut microbiota of WSSV-infected shrimp (Wang et al., 2019), whereas *Photobacterium*, *Pseudoalteromonas*, and *Vibrio* commensals are recruited in response to *Vibrio parahaemolyticus* exposure (Zhang et al., 2021). These findings suggest that gut microbiota responses are pathogen-specific, paralleling differential immune responses to bacterial and viral infections (Lu et al., 2022). The robust interplay between the gut microbiota and innate immune system of shrimp is well established (Zhou et al., 2025). Understanding this synergistic mechanism is crucial for a deeper understanding of how EHP infection alters this tripartite relationship among infection severity, gut microbiome, and host physiology.

Available studies have primarily focused on the morphology of EHP (Gao et al., 2020), quantitative detection of EHP spores (Karthikeyan et al., 2017), and the relationship between EHP spore burden and hepatopancreatic microsporidiosis (Ning et al., 2019). However, the extent to which EHP infection alters the functional architecture of the shrimp gut microbiota remains incompletely understood, despite its relevance for elucidating EHP pathogenesis. Current research indicates significant compositional differences in gut microbiota between EHP-infected and healthy cohorts (Babu et al., 2023; Xu et al., 2025). Nevertheless, these studies typically compare a single infected cohort with matched healthy controls, leaving uncertainty as to whether gut microbiota alterations follow a linear trajectory with increasing infection severity. Given that distinct bacterial communities can perform comparable metabolic functions through functional redundancy (Yang, 2021), a deeper functional characterization of the gut microbiota is needed. Metagenome-assembled genomes provide insights into the functional potential of microbial communities (Lu et al., 2022; Zhang et al., 2019). Functional annotation using multiple databases enables the construction of comprehensive gut microbiome profiles, facilitating the identification of shifts in functional pathways under EHP infection. However, data on how escalating EHP infection severity influences gut microbial function remain scarce. In particular, the identification of gut microbial signatures characterizing EHP infection could diagnose EHP incidence, and inform probiotic-based interventions to improve shrimp health and aquaculture productivity.

Moving beyond static comparisons of paired diseased and healthy cohorts, metagenomic sequencing was employed to assess the extent to which the shrimp gut microbiome is altered with increasing EHP infection severity across three infection levels. This study aimed to: (i) evaluate how the gut microbiome responds to progressive EHP infection; (ii) identify gut bacterial biomarkers indicative of infection severity; and (iii) explore the interactions among EHP infection severity, gut microbiome, and shrimp physiology. In addition, a conceptual model was proposed to integrate these findings and provide mechanistic insights into EHP pathogenesis.

MATERIALS AND METHODS

Experimental design

Adult *L. vannamei* shrimp were collected from six independent ponds at an aquafarm in Xiangshan, Ningbo, China. The ponds were uniformly managed under standardized conditions, including identical rearing water quality, stocking density (200 larval shrimp per m^2), and diet. The shrimp were cultured for 70 days before collection. Given that growth retardation is a hallmark symptom of EHP infection, the shrimp were categorized into four groups based on body length: normal-size (NS, maximum body size among those sampled), long-size (LS), medium-size (MS), and short-size (SS) (Supplementary Figure S1). Since all shrimp cohabited within the same ponds, environmental influences on gut microbiome composition were minimized. Gut and hepatopancreatic tissues were dissected from each individual for analysis. In total, 24 samples (four size groups × six replicates) were included. Hepatopancreatic digestive and immune activities were analyzed using commercial kits (Jiancheng Bioengineering Institute, China), following previously described protocols (Shen et al., 2022a).

Metagenomic sequencing and data processing

Genomic DNA of gut microbes was extracted using a NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs, USA), following the manufacturer's protocols. Sequencing libraries were prepared as described in our previous work (Lu et al., 2021). In short, 5 μg of DNA from each sample was fragmented into approximately 250 bp using a sonicator. The fragmented DNA was end-repaired, ligated with adaptors and index codes for sample attribution. The desired fragments were cut from agarose gel, and purified using kit (Qiagen, France). The resulting libraries were sequenced on the Illumina HiSeq 2500 platform at Guangdong MagiGene Technology Co., Ltd, China.

Raw sequencing reads were assessed for quality using FastQC (v.0.11.6) (Brown et al., 2017) and further processed using Trimmomatic (Bolger et al., 2014) to remove non-biological sequences (e.g., adaptors and index codes) and to filter reads shorter than 36 bp or with an average quality score below Q20. Host-associated genetic contamination from *L. vannamei* was removed using Bowtie 2 (v.2.5.1) (Langdon, 2015; Zhang et al., 2019). High-quality reads were assembled using MEGAHIT (v.1.2.9) with the "meta-sensitive" mode and default settings (Li et al., 2015). Taxonomic annotation of bacterial and eukaryotic sequences was performed using Kraken 2 (v.0.38) (Wood et al., 2019). Open reading frames (ORFs) were predicted with MetaProdigal (v.2.6.3) (Hyatt et al., 2010), and their abundances were quantified using Salmon (v.0.11.3) (Patro et al., 2017). Finally, to obtain functional profiles and virulence factors (VFs), ORFs were blasted against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and virulence factor database (VFDB) (https://www.mgc.ac.cn/VFs/) using DIAMOND (v.0.9.18) (Buchfink et al., 2015).

Gut microbiota dysbiosis index (MDI) in EHP-infected shrimp

The MDI was applied to quantify dysbiosis of the gut microbiota in the LS, MS, and SS cohorts compared with NS shrimp, calculated using the equation:

$$MDI = \log_{10} \frac{\sum ES(X)}{\sum DS(X)} \quad (1)$$

where $\sum ES(X)$ and $\sum DS(X)$ represent the summed abundances of enriched taxa and depleted taxa in LS, MS, or SS shrimp compared with NS controls, respectively. A higher MDI indicates more severe dysbiosis in the gut microbiota (Toto et al., 2024).

Construction of a diagnostic model for EHP infection

A Random Forest model was developed to identify key predictors of EHP infection severity using the "randomForest" package in R (Strobl et al., 2007). To assess whether taxonomic resolution influences diagnostic accuracy, model performance was compared across EHP-discriminatory lineages at bacterial phylum, class, order, family, genus, and species levels. Relative abundances of all taxonomic lineages were stratified according to shrimp size categories using default parameters, rather than a binary classification of healthy and EHP-infected cohorts. A 10-fold cross-validation algorithm was employed to ascertain the minimum number of EHP-discriminatory lineages that achieved the best diagnostic accuracy, using a 50% threshold to distinguish between predicted and observed size categories (Strobl et al., 2007). To evaluate the generalizability of the diagnostic model, gut microbiota profiles from EHP-infected shrimp collected from six geographically distant farms were included for validation (Xu et al., 2025).

Statistical analysis

All statistical analyses were performed in R v.4.1.3 (R Core Team, 2018). Gut microbiota stability was quantified using average variation degree (AVD) (Xun et al., 2021). Differences in α-diversity and AVD across shrimp size categories were assessed using one-way analysis of variance (ANOVA). Non-metric multidimensional scaling (NMDS) was performed to visualize the effects of experimental factors on gut microbiota variance based on Bray-Curtis distance. Permutational multivariate analysis of variance (PERMANOVA, permutation= 999) was further applied to quantify their contributions to community dissimilarity (Anderson, 2001). Differential species were identified using a negative binomial generalized linear model ($|\log_2$ Fold Change (FC)$|>2$ and $-\log_{10} P_{adj}>1.3$) using the "DESeq2" package (Love et al., 2014). Co-occurrence networks among differential species were constructed using correlation coefficients of $|r|>0.75$ and adjusted $P<0.05$ (Deng et al., 2012). Functional differences between paired groups were identified using STAMP software (Parks et al., 2014). Pathway diagrams of interest were retrieved from the KEGG database (https://www.genome.jp/kegg/). A partial least squares path model (PLS-PM) was adopted to infer interrelations among EHP abundance, shrimp growth traits, hepatopancreatic enzyme activity, gut bacterial community structure, VFs, and functional composition (Sanchez et al., 2017). The model was based on the following *a priori* theoretical framework: (a) EHP infection directly alters VFs, shrimp enzyme activities, and gut microbiota composition, and (b) these alterations subsequently modulate shrimp physiology.

RESULTS

Growth traits and enzymatic activities in relation to increasing EHP infection severity

Shrimp were classified into four severity categories based on body size (Supplementary Figure S1A), with infection severity further validated by the relative abundance of EHP within gut eukaryotic communities (Supplementary Figure S1B). Progressive EHP infection led to a linear decline in shrimp body length and weight (Supplementary Figure S1C, D). In addition, EHP abundance exhibited significant negative correlations with both body length (Pearson $r=-0.757$, $P=0.018$) and body weight ($r=-0.897$, $P<0.001$).

Enzyme activity profiles revealed that superoxide dismutase activity increased linearly with infection severity, while the digestive enzymes α-amylase, lipase, and pepsin showed a progressive decline. In contrast, acid phosphatase activity peaked in the SS cohort, whereas lysozyme activity was highest in the LS cohort (Supplementary Figure S2).

Effects of EHP infection on shrimp gut microbiota

After quality control, metagenomic sequencing of 24 gut samples yielded a total of 324.1 GB of high-quality reads, with 94.0% of sequences surpassing the Q30 threshold. Following the removal of host-derived sequences, microbial reads accounted for approximately one-third of the total data, averaging 4.43±0.65 GB per sample and 18.6 million reads per sample (Supplementary Table S1). Previous studies have established that a sequencing depth exceeding 15 million reads is sufficient to achieve stable species composition in metagenomic analyses (Liu et al., 2022). Thus, sequencing depth in this study was sufficient for capturing gut microbial diversity. Notably, α-diversity analysis revealed a significant reduction in both Shannon index and richness ($P<0.05$) across all EHP-infected cohorts compared with NS shrimp, with a consistent decrease with increasing infection severity (Figure 1A). The most abundant bacterial class was Gammaproteobacteria (NS: 31.0%; LS: 37.2%; MS: 40.3%; SS: 54.1%), whose relative abundance increased proportionally with EHP severity. In contrast, Alphaproteobacteria was more prevalent in NS shrimp (25.4%), but exhibited reduced abundance in infected groups (LS: 17.3%; MS: 23.4%; SS: 18.2%). Additionally, Bacilli and Actinobacteria were enriched in LS shrimp but declined in abundance as infection severity increased (Figure 1B). NMDS biplot showed a clear separation of gut microbial communities among the four shrimp size categories (Figure 1C). PERMANOVA confirmed significant structural differences across cohorts (Global $R^2=0.25$, $P=0.04$). Microbiota stability was highest in NS shrimp, as evidenced by the lowest AVD, whereas MS shrimp exhibited the most unstable gut bacterial community, surpassing even the SS cohort (Figure 1D).

Differential species and network dynamics along increasing EHP infection severity

Gut microbiota composition varied with EHP infection severity, with LS and NS shrimp sharing the highest number of commensal species (n=1 324), while SS and NS shrimp exhibited the lowest overlap (n=1 173) (Supplementary Table S2; Figure 2A). Several species were consistently enriched or depleted across all infected groups compared with NS controls. Notably, opportunistic pathogens, such as *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Lysinibacillus sphaericus*, were significantly enriched in all EHP-infected groups. Conversely, beneficial taxa, such as *Limosilactobacillus fermentum*, *Lactococcus formosensis*, *Brevibacterium intestinavium*, and *Virgibacillus* spp., were predominantly enriched in the NS group (Supplementary Table S3). To characterize the degree of gut dysbiosis, the MDI was calculated based on differential species (Figure 2A). MDI values were significantly elevated in

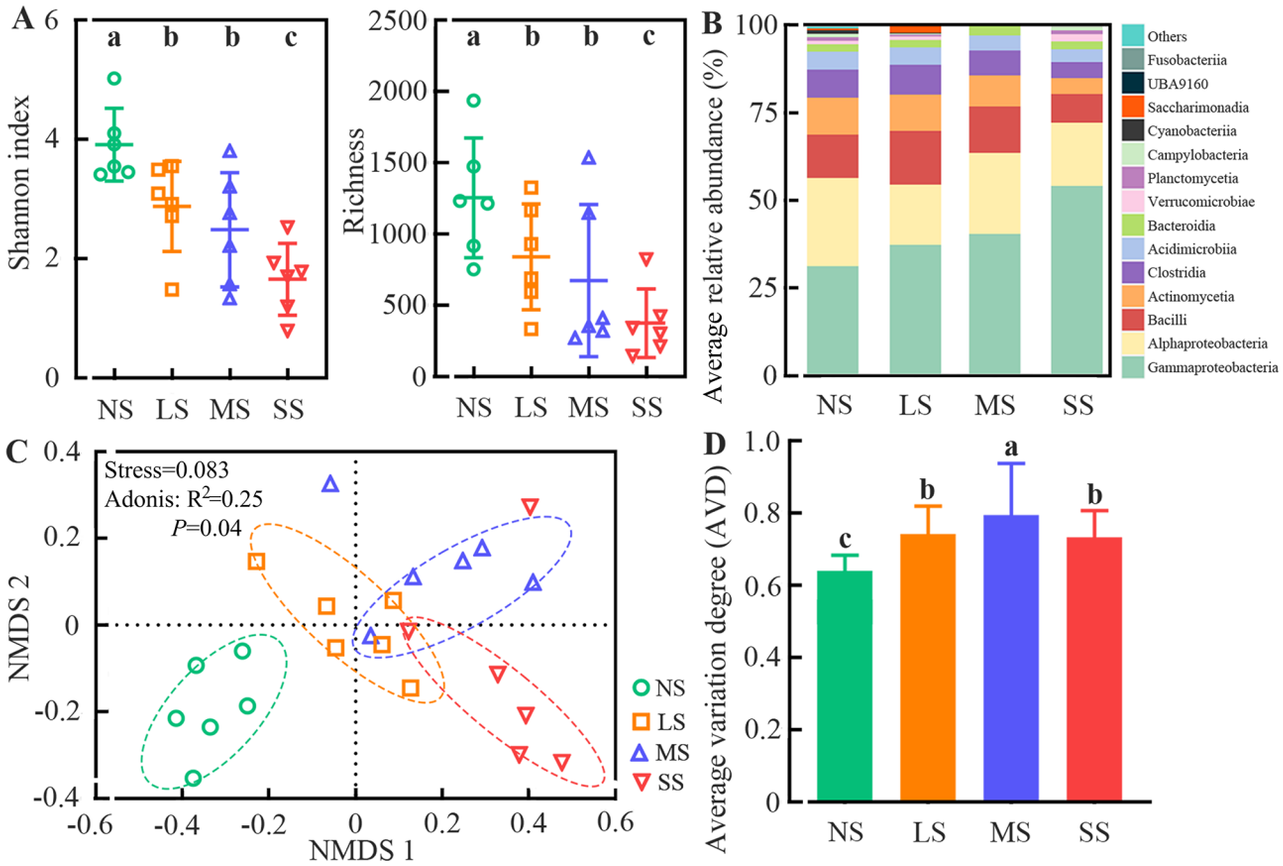

Figure 1 Overall characteristics of gut microbial communities with increasing *Enterocytozoon hepatopenaei* (EHP) infection severity

A, B, D: Comparison of gut microbial diversity (A), relative abundance of dominant bacterial phyla (B), and average variation degree (D) among four different-sized shrimp groups. C: Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance matrices depicting variances in gut microbiota with increasing EHP infection severity. Different letters indicate significant differences among groups using one-way analysis of variance (ANOVA) at $P<0.05$ level. NS: Normal-size; LS: Long-size; MS: Medium-size; SS: Short-size.

all infected groups relative to NS shrimp, with a progressive increase as infection severity intensified (LS: $P=0.035$; MS: $P=0.0065$; SS: $P=0.00021$) (Figure 2B). Network analysis was performed to assess microbial interactions between differential species in each EHP-infected cohort compared with NS shrimp (Figure 2C). The proportion of negative correlations between enriched and depleted species progressively increased with infection severity, indicating intensified mutual exclusion (positive/negative: LS: 59.4/40.6; MS: 35.7/64.3; SS: 13.6/86.4, Supplementary Table S4) and a shift toward a more destabilized microbial network under severe infection conditions. The average clustering coefficient and average degree also exhibited an upward trend with disease severity, reflecting greater microbial interconnectivity in highly infected shrimp (Figure 2C). Key microbial hubs were identified as species with degree>15 and eigenvector centrality (EC)>0.20 (Supplementary Figure S3). In the LS vs. NS network, *Phaeobacter italicus* (degree=18, EC=0.234), *Clostridium sporogenes* (degree=17, EC=0.217), and *Lactococcus garvieae* (degree=16, EC=0.222) emerged as central hubs, all significantly enriched in LS shrimp. In the MS vs. NS network, *V. parahaemolyticus* (degree=37, EC=0.241) was identified as an enriched species, while *Ruegeria lacuscaerulensis* (degree=38, EC=0.209) was identified as a depleted species. In the SS vs. NS network, only *V. campbellii* (degree=24, EC=0.232) functioned as a network hub, showing significant enrichment in the SS cohort (Supplementary Figure S3).

Diagnostic model for assessing EHP infection severity

Given the significant differences in gut microbiota with increasing EHP infection severity (Figure 1), a diagnostic model was developed to identify biomarkers that were indicative of infection severity. Among taxonomic resolutions tested, species-level biomarkers achieved the best diagnostic accuracy (83.3%) (Supplementary Table S5; Figure 3), and were therefore selected for further analysis. A 10-fold cross-validation algorithm ascertained that the top 24 EHP-discriminatory species yielded the lowest classification error (Figure 3A). The model demonstrated 100% classification accuracy for NS and severely infected SS cohorts. However, classification performance was lower for LS individuals, with only 50% correctly assigned to their observed category (Figure 3B). Notably, all misclassified LS samples were assigned to other infected groups rather than the NS cohort (Supplementary Table S6), ensuring no false negatives were present in the model. The 24 diagnostic biomarkers were ranked based on their importance in the diagnostic model (Figure 3C). The abundance patterns of these biomarkers corroborated with their known functional roles. For instance, pathogenic species such as *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *V. campbellii*, *V. parahaemolyticus*, *V. owensii*, and *V. fluvialis* exhibited a gradual increase with disease severity. Conversely, beneficial taxa such as *Brevibacterium intestinavium* and *V. hepatarius* showed a declining trend (Figure 3D). To assess the generalizability of the diagnostic model, its performance was

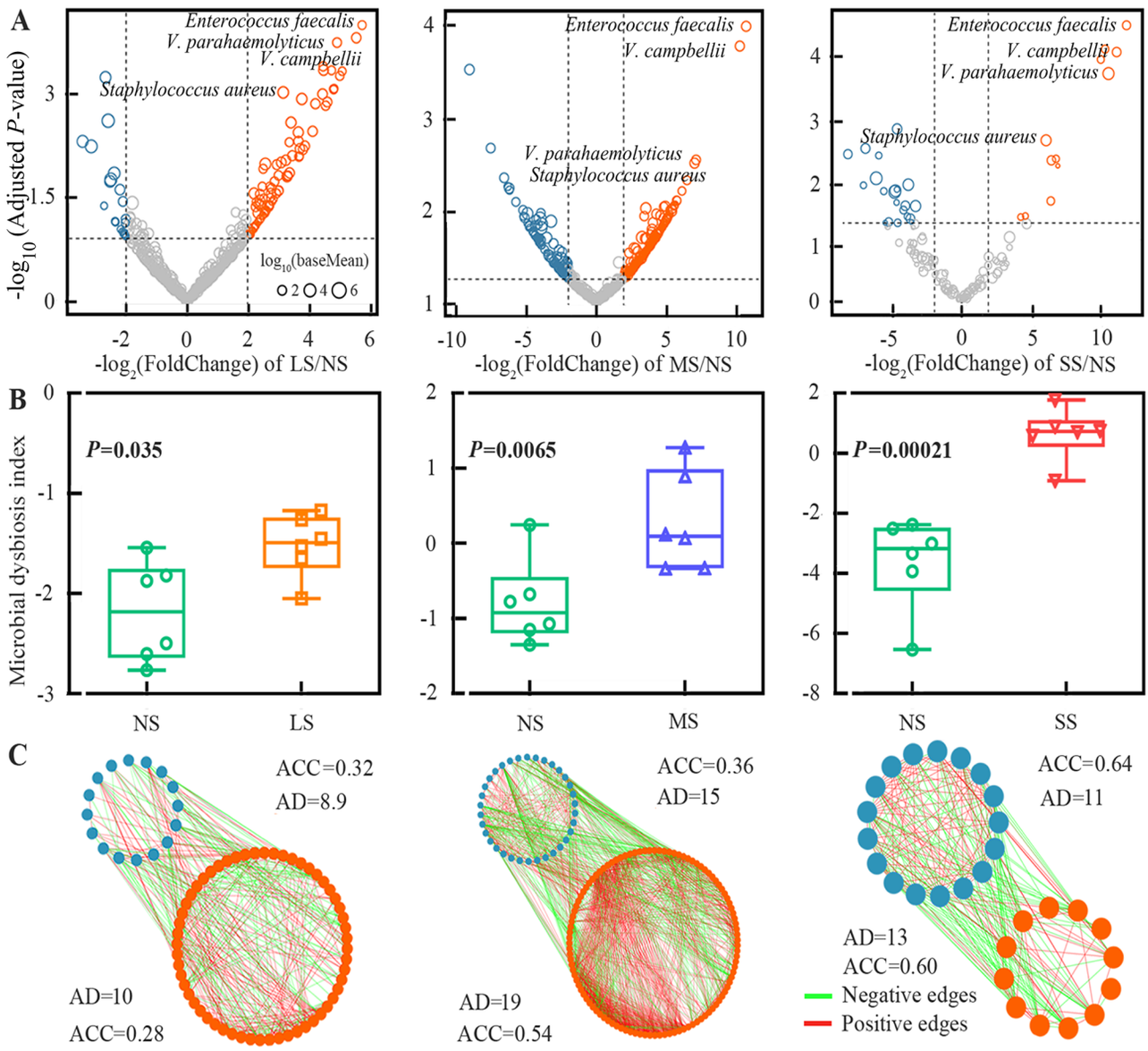

Figure 2 Differential species and networks between healthy and each infected cohort

A: Volcano plots depicting distribution of differential species between LS vs. NS, MS vs. NS, and SS vs. NS. Circle diameter is proportional to mean abundance of a given bacterial species. B: Comparison of microbial dysbiosis index between healthy and infected cohorts using Wilcoxon rank-sum test. C: The networks of differential species between healthy and infected cohorts. Above and below nodes are depleted and enriched species in EHP-infected shrimp compared with healthy controls, respectively. ACC: Average clustering coefficient; AD: Average degree. NS: Normal-size; LS: Long-size; MS: Medium-size; SS: Short-size.

evaluated using gut microbiota data from EHP-infected shrimp that were not included in the model construction. The model achieved an overall accuracy of 88.1% (Supplementary Table S7), confirming its robustness and applicability across independent datasets (Xu et al., 2025).

Functional differences in the gut microbiome with progressive EHP infection

Significant shifts in the gut microbial functional profiles were observed among the four shrimp groups (R^2=0.30, P=0.01) using the profiles of KEGG Orthology (KO) annotations (Figure 4A). Functional richness in the gut microbiome progressively declined with decreasing shrimp body length (Figure 4B). Most KOs were shared among the four cohorts (n=7 925, 80% of all obtained KOs). However, the overlap between NS and severely infected SS shrimp was minimal (n=35, 0.32%), suggesting the greatest functional divergence between these two groups. Moreover, the number of shared KOs between NS shrimp and each infected group gradually decreased with disease severity (NS vs. LS: n=650, 5.9%; NS vs. MS: n=71, 0.64%, Figure 4C). Key functional pathways related to protein, amino acid, lipid, and carbohydrate metabolism were significantly depressed in all EHP-infected cohorts compared with NS shrimp (Figure 4D–F). Among these differential pathways, map00630 glyoxylate and dicarboxylate metabolism and map00280 valine, leucine and isoleucine degradation were consistently depleted across all infected cohorts. A total of 77 and 39 KOs were affiliated with map00630 and map00280 pathways, respectively (Supplementary Figure S4). The relative abundance of these KOs was highest in NS shrimp and decreased linearly with infection severity (Supplementary Figures S5, S6). Notably, the primary end-products of map00630 and map00280 were

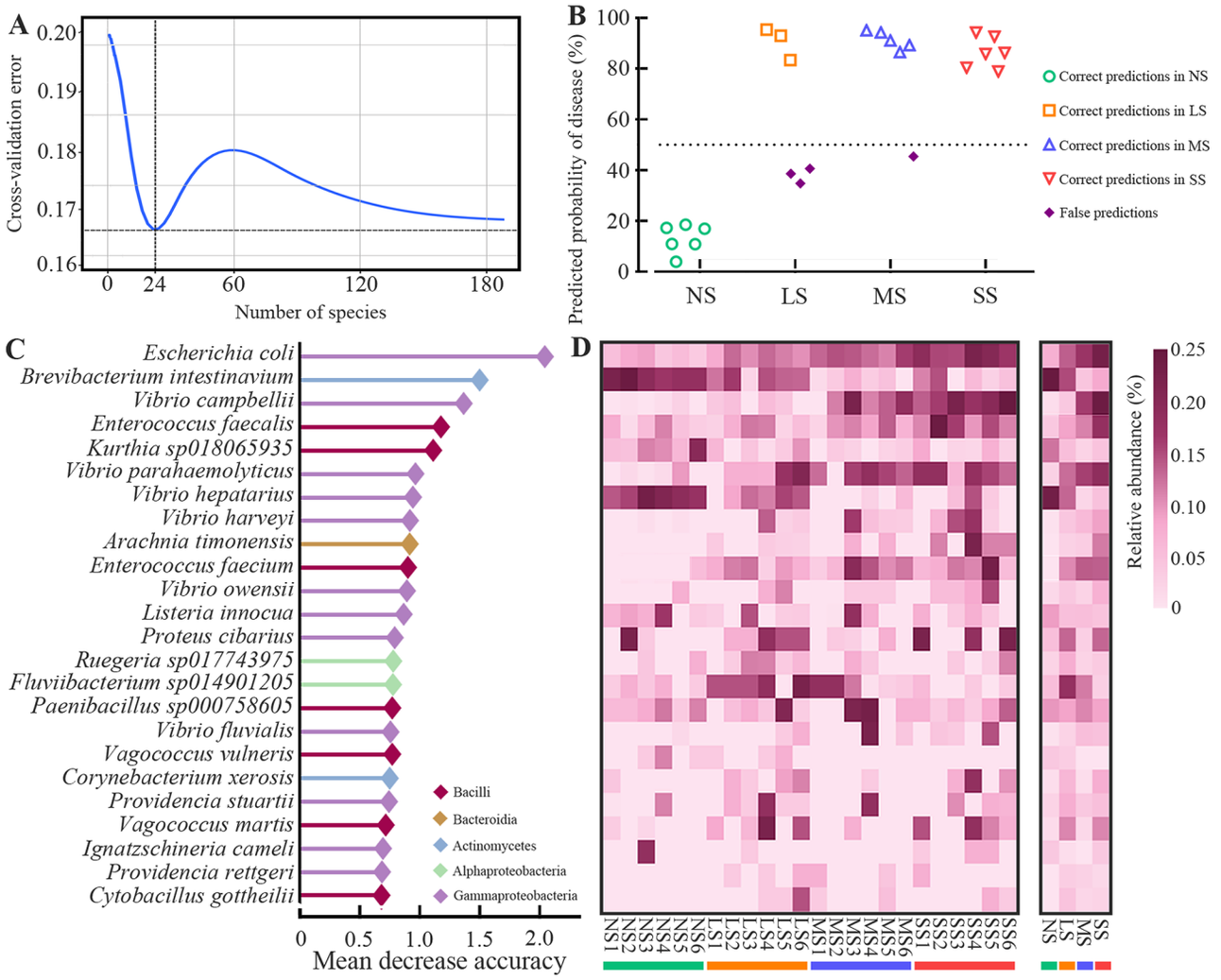

Figure 3 Identification of biomarkers for diagnosing EHP infection severity using a random forest model

A: 10-fold cross-validation was used to determine minimum number of species that achieved the lowest error rate. B: Probability of EHP infection severity diagnosed using 24 EHP-discriminatory species. A diagnosis was considered correct when the predicted category matched the observed category with a probability threshold of >50%. C: Top 24 biomarkers ranked in descending order based on mean decrease accuracy (MDA, degree of reduced prediction accuracy in diagnostic model, with a larger value indicating greater biomarker importance). D: Heatmaps depicting relative abundances of biomarkers across samples (left) and averaged within each group (right). Color gradient is proportional to relative abundance of biomarkers. NS: Normal-size; LS: Long-size; MS: Medium-size; SS: Short-size.

formate (Supplementary Figure S5) and branched chain fatty acids (BCFAs, Supplementary Figure S6), respectively.

Diversification of VFs in response to EHP infection

Across the 24 shrimp samples, 146 VFs were identified, with significant diversification in all three infected groups (Figure 4G). Differentially abundant VFs were consistently enriched in the three infected cohorts compared with healthy individuals. The SS group exhibited the greatest divergence in VFs relative to NS shrimp, with an increased abundance of virulence-associated elements such as mobile flagella, Type IV pili, and Type III and VI secretion systems (T3SS1 and T6SS) (Figure 4H).

Interplay among EHP, VFs, gut microbiome and shrimp growth traits

PLS-PM revealed that increasing EHP abundance exerted significant and negative effects on shrimp enzyme activity (–0.412, P<0.05) and growth traits (–0.840, P<0.01), but positive effects on VFs (0.427, P<0.05) and microbial community (0.506, P<0.05). Enzyme activity (0.878, P<0.01) and functional composition (0.404, P<0.05) were the primary drivers of shrimp growth traits, whereas gut microbiota composition imposed an indirect effect (0.427, P<0.05). Additionally, EHP-induced VF diversification had a direct impact on gut microbial community (0.397, P<0.05) and functional structure (0.753, P<0.01) (Figure 5).

DISCUSSION

Shrimp growth is influenced by diverse abiotic and biotic factors, including dietary composition, rearing conditions, and developmental stage (Cornejo-Granados et al., 2018; Mao et al., 2023; Xiong et al., 2017). However, these confounding factors were controlled in this study, as all shrimp were sourced from biological batches. By utilizing metagenomic sequencing, progressive shifts in gut microbiota composition and function were examined across varying degrees of disease severity, leading to the identification of diagnostic biomarkers.

Despite cohabitation under identical environmental conditions, the shrimp exhibited substantial variation in body

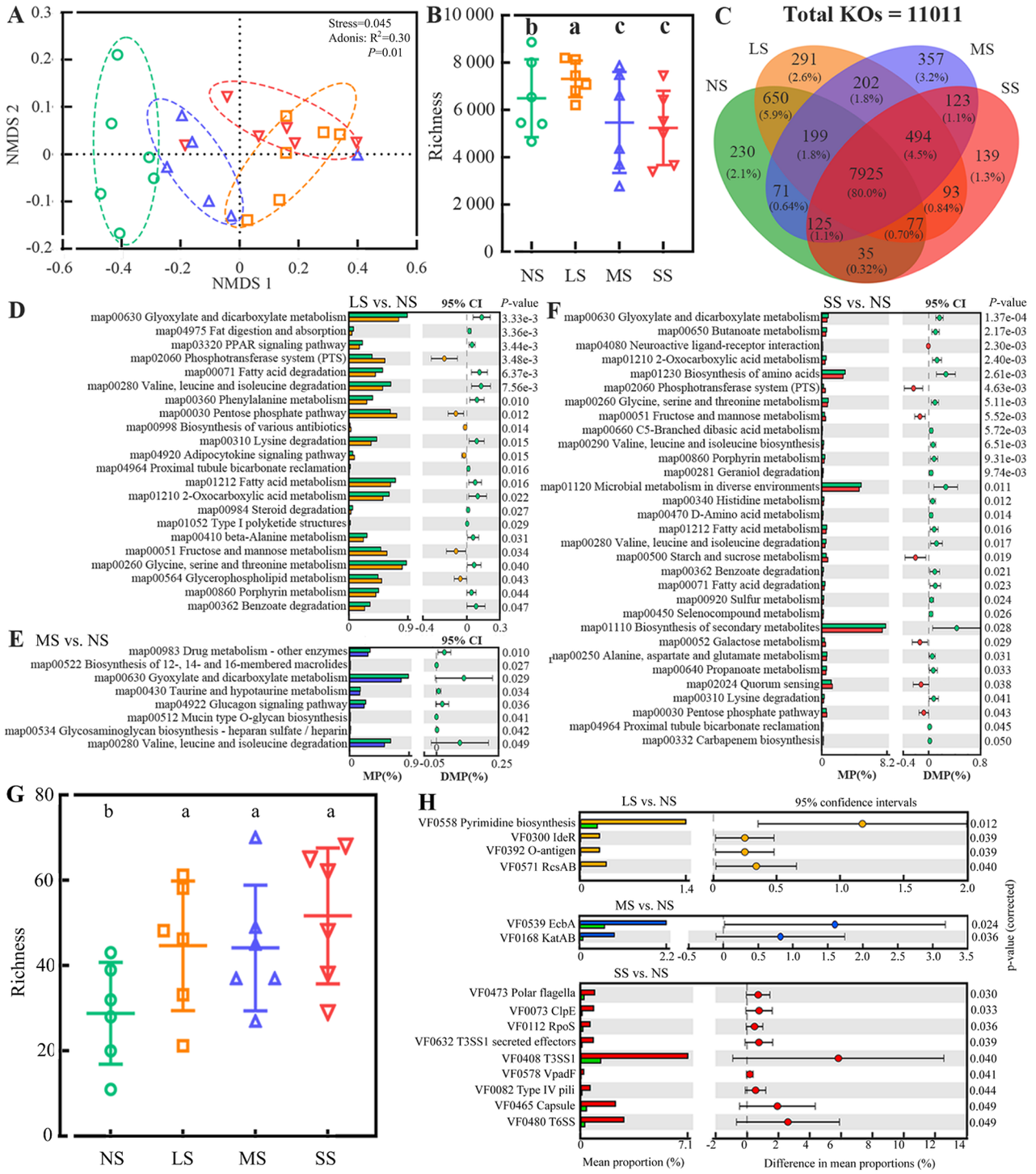

Figure 4 Functional structures and virulence factors (VFs) of the gut microbiome across increasing EHP infection severity

A: NMDS ordination based on Bray-Curtis distance matrices of KEGG Orthology (KO) showing shifts in gut microbial functional composition structures with increasing EHP infection severity. B: Comparison of functional diversity across infection severity groups. C: Venn diagram showing distribution of KOs in each group. D–F: Differentially enriched functional pathways between LS vs. NS (D), MS vs. NS (E), and SS vs. NS (F). CI: Confidence intervals. G: Comparison of VFs diversity across increasing EHP infection severity. H: Differential VFs between LS vs. NS, MS vs. NS, and SS vs. NS. Different letters indicate significant differences among groups using one-way ANOVA at $P<0.05$ level. MP: Mean proportion. DMP: Difference in mean proportion. NS: Normal-size; LS: Long-size; MS: Medium-size; SS: Short-size.

weight and length (Supplementary Figure S1A), which were supposed to be infected by EHP. This assertion was supported by the elevated relative abundance of EHP in infected cohorts (Supplementary Figure S1B). Notably, EHP abundance in SS shrimp was lower than in MS shrimp (Supplementary Figure S1B). However, previous studies have reported that EHP proliferation can be suppressed or halted under nutrient deprivation, particularly during the late stages of infection, with parasite replication resuming only when conditions become favorable (López-Carvallo et al., 2022). This cyclic infection pattern may partially explain why EHP infection is nonlethal. Hepatopancreatic immune activity, as

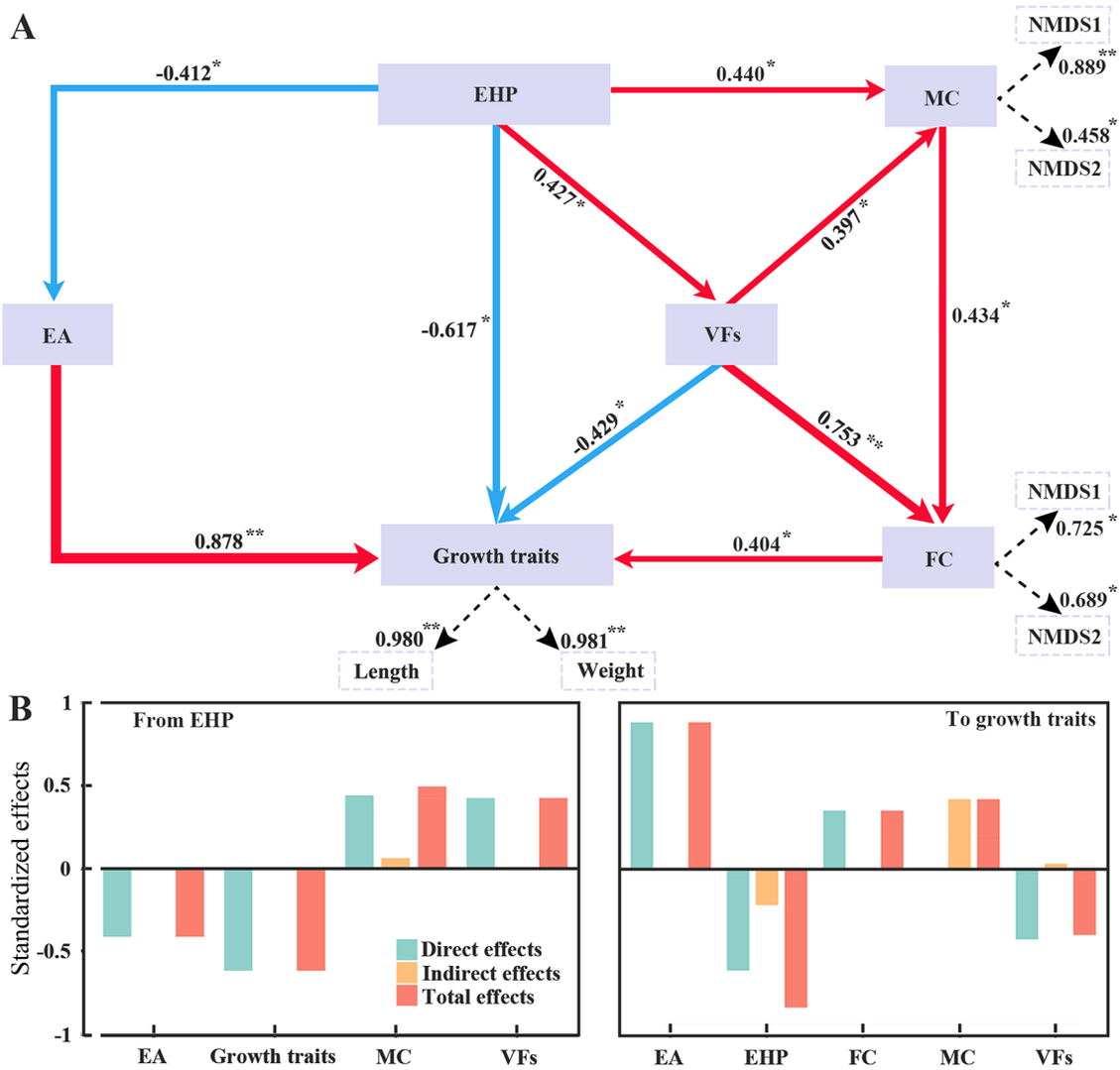

Figure 5 Effects of EHP infection on shrimp growth traits based on partial least squares path modeling (PLS-PM)

A: PLS-PM depicting interrelationships among EHP abundance, enzyme activity (EA), microbial community (MC), virulence factors (VFs), function composition (FC), and growth traits. Path coefficients are proportional to arrow width. Red and blue stands indicate positive and negative relationships, respectively. *: $P<0.05$ and **: $P<0.01$ after 1 000 bootstrap replicates. B: Standardized effects of each factor on growth traits in PLS-PM. Direct and indirect effects were summed for total effects.

indicated by elevated superoxide dismutase and acid phosphatase levels, was highest in the SS cohort (Supplementary Figure S2A), aligning with prior reports that EHP invasion triggers immune responses in shrimp (Cao et al., 2023; Yang et al., 2021). However, digestive enzyme activity declined progressively with increasing EHP infection severity (Supplementary Figure S2B), consistent with the well-established link between digestive function and overall shrimp health (Dai et al., 2018; Shen et al., 2022a). This suppression may result from reduced food intake due to EHP infection, leading to an energy-conserving mechanism that down-regulates digestive activity (Kumar et al., 2022; Subash et al., 2023). Alternatively, the intracellular parasitism of EHP may cause hepatopancreatic tissue damage, resulting in the loss of normal digestive function (Zhu et al., 2022).

Gut microbial diversity plays a critical role in maintaining community stability. A more diverse microbiome provides functional redundancy, mitigating the impact of species loss and enhancing resistance to external disturbances, including pathogen invasion (Xiong et al., 2024). By this logic, reduced gut microbial diversity may increase host disease susceptibility. Consistently, a progressive decline in gut bacterial diversity was observed with increasing EHP infection severity (Figure 1A). Considering the rapid progression of shrimp diseases and the inherent sensitivity (non-resilience) of gut microbiota to shrimp diseases (Xiong et al., 2024), we proposed the supplementation with keystone probiotics may offer a strategy for restoring microbial homeostasis during the initial stages of infection. A notable shift in microbial composition was observed as EHP infection severity increased, particularly with the enrichment of Gammaproteobacteria (Figure 1B). Diseased shrimp often harbor a higher relative abundance of Gammaproteobacteria compared to healthy counterparts, a trend largely attributed to the proliferation of *Vibrio* species (Sha et al., 2022; Xiong et al., 2018). EHP infection frequently occurs alongside secondary infections, including co-infection with AHPND or WFS (Aranguren et al., 2017; Caro et al., 2020; Tang et al., 2016). Pathogenic *V. campbellii*, *V. parahaemolyticus*, *V. owensii*, and *V. fluvialis* have been identified as causative

agents of AHPND and WFS (Kumar et al., 2020; Sha et al., 2024). Intriguingly, these pathogens were also detected in this study, with their abundance increasing linearly with increasing EHP severity (Figure 3C). These findings further reinforce the role of EHP infection in predisposing shrimp to secondary infections.

Progressive EHP infection significantly altered the composition and structure of the shrimp gut microbiota (Figure 1C). Distinct microbial communities have been consistently observed between healthy and diseased shrimp, independent of the causing pathogens (Mao et al., 2023; Rungrassamee et al., 2016; Sha et al., 2022), supporting the notion that a balanced gut microbiota is essential for maintaining shrimp health (Huang et al., 2020; Xiong et al., 2024). Notably, our results showed that microbial divergence increased proportionally with infection severity (Figure 1D), a pattern also reported in shrimp suffering from advanced WFS, AHPND, and WSSV infections (Lu et al., 2023; Sha et al., 2024; Wang et al., 2019). Evidence suggests that the extent of gut bacterial recovery is positively correlated with shrimp survival rate during *V. harveyi* infection (Rungrassamee et al., 2016). By extension, dysbiosis is expected to escalate with increasing EHP infection severity.

Differential abundance analysis identified species that were consistently enriched or depleted between healthy shrimp and each infected cohort (Figure 2A). Intriguingly, 13 species exhibited significant shifts across infected cohorts (Supplementary Table S3), including *V. parahaemolyticus*, *V. campbellii*, *V. harveyi*, *Enterococcus faecalis*, and *Brevibacterium* sp., which were also identified as diagnostic biomarkers (Supplementary Table S3; Figure 3). Among species enriched in EHP-infected cohorts, *Clostridium perfringens* is a well-documented pathogen responsible for necrotic enteritis (García-Vela et al., 2023), while *Staphylococcus aureus* is a globally recognized opportunistic pathogen affecting a wide range of hosts, including humans (Mrochen et al., 2020). Among the depleted species, *Limosilactobacillus fermentum* is a safe probiotic used in shrimp aquaculture to mitigate vibriosis and AHPND incidence (Fernandes et al., 2023). *Lactococcus formosensis* plays a role in host immunomodulation and cholesterol assimilation (Kingkaew et al., 2022), whereas *Virgibacillus* sp. contributes to amino acid metabolism (Zhu et al., 2023). The suppression of these beneficial microbes could be the targets for microbial-based interventions aimed at improving shrimp disease resistance.

To quantitatively assess dysbiosis, the MDI was calculated using differential species (Toto et al., 2024). Similar to the increasing instability observed in gut microbiota AVD, EHP infection severity was associated with a progressive increase in gut MDI (Figure 2B). In addition, the proportion of negative interactions between enriched and depleted species declined substantially with increasing infection severity (Supplementary Table S4; Figure 2C), accompanied by a reduction in network hubs (Supplementary Figure S3). Ecological theory suggests that a higher prevalence of competitive interactions potentiates network stability (Coyte et al., 2015). This can be attributed to the mutual interdependence that underpins cooperative microbial interactions —when sensitive species are lost, cascading effects destabilize the entire community (Dai et al., 2019; Mackenzie et al., 2017). By this logic, a reduced proportion of competitive interactions could compromise colonization resistance, thereby facilitating the expansion of alien species. Accordingly, increasing EHP severity was accompanied by a linear increase in the abundance of diverse potential pathogens (Figure 3C).

Given the progressive dysbiosis observed in the gut microbiotas with increasing EHP infection (Figure 1), microbial biomarkers were identified to classify infection severity (Figure 3). Several *Vibrio* strains, including *V. harveyi*, *V. campbellii*, *V. parahaemolyticus*, *V. owensii*, and *V. fluvialis*, exhibited a stepwise increase in abundance with escalating EHP infection severity (Figure 3D). *V. harveyi* and its closely related species, e.g., *V. alginolyticus* and *V. parahaemolyticus*, are well-known shrimp pathogens (Thompson et al., 2004; Xiong et al., 2024). For instance, *V. campbellii* is a causative agent of luminescent vibriosis in shrimp hatcheries, resulting in 100% larval mortality (Kumar et al., 2021). Similarly, *V. parahaemolyticus* and *V. owensii* have been identified as primary pathogens responsible for AHPND in shrimp (Kumar et al., 2020; Liu et al., 2018). In addition to its impact on aquaculture, *V. fluvialis* is a notorious seafood- and waterborne pathogen capable of causing severe and sometimes lethal diarrhea in humans (Igbinosa & Okoh, 2010). Beyond *Vibrio* species, other opportunistic pathogens, including *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium* (Almohamad et al., 2014; Yadav et al., 2022), also showed gradual increase in abundance with increasing EHP infection severity (Figure 3D). In contrast, *V. hepatarius* exhibited a declining trend (Figure 3D). Recent findings suggest that *V. hepatarius* functions as a probiotic strain capable of preventing *V. parahaemolyticus* colonization in the shrimp gut (Ramirez et al., 2022). Indeed, not all *Vibrio* strains are pathogenic; some, such as *V. diabolicus* and *V. fischeri*, play beneficial roles as symbionts or probiotics (Restrepo et al., 2021). In addition, gut-associated *Brevibacterium* sp. may contribute to host cellulose digestion (Bai et al., 2022). Collectively, the enrichment or depletion patterns of these biomarkers were largely consistent with their known functions, reinforcing their diagnostic utility. The overall classification accuracy of the diagnostic model reached 83.3%, although accuracy was lower for the LS shrimp (Figure 3B). This limitation may stem from the relatively small sample size, suggesting that increased sample representation is required to improve model robustness in future studies. Notably, no LS samples were misdiagnosed as healthy, ensuring that the model produced no false negatives (Supplementary Table S6). Although conventional polymerase chain reaction (PCR)-based assays can detect EHP within hours, these methods provide only qualitative results, lacking a defined threshold for predicting disease severity. In contrast, the identified biomarkers may serve as quantitative indicators, allowing for a more precise diagnosis of EHP infection severity. While further validation and optimization are required to develop this approach into a practical diagnostic tool, the model remains a valuable framework for advancing EHP monitoring and management in shrimp aquaculture.

Increasing EHP infection severity substantially altered the functional architecture of the gut microbiota (Figure 4A), leading to a marked decline in functional diversity (Figure 4B). These findings indicate that the shrimp gut microbiome lacks functional redundancy, reinforcing the essential role of a balanced microbial community in maintaining digestive efficiency and immunomodulatory activities (Xiong et al., 2019; Zhou et al. 2025). Consistently, the proportion of shared functional genes between the NS and infected cohorts

declined progressively with worsening EHP infection (Figure 4C). In addition, diverse functional pathways, particularly those involved in amino acid, carbohydrate, and energy metabolism, were significantly suppressed in EHP-infected shrimp (Figure 4D–F). This disruption may contribute to the characteristic growth retardation observed in affected individuals. The map00630 glyoxylate and dicarboxylate metabolism pathway produces formate (Supplementary Figure S5), an essential short-chain fatty acid that plays crucial roles in gut energy metabolism, mucosal barrier protection, and anti-inflammatory responses (Tsukuda et al., 2021). Similarly, BCFAs, end-products of the map00280 valine, leucine and isoleucine degradation pathway (Supplementary Figure S6), strengthen epithelial integrity and mitigate pro-inflammatory cytokine responses by suppressing the NF-κB signaling pathway (Ezzine et al., 2022). A reduction in gut BCFAs and SCFAs may relieve the expression of negatively regulated histone deacetylase, thereby up-regulating pro-inflammatory cytokines such as IL-1β and TNF-α, which facilitate pathogen clearance (Nie et al., 2017). In line with these observations, the linear suppression of map00630 and map00280 pathways correlated with dampened digestive activity and impaired anti-inflammatory responses as EHP infection severity increased (Figure 4; Supplementary Figures S5, S6). These alterations may further predispose shrimp to secondary infections. Concurrently, the inflammatory response induced by EHP infection also triggers reactive oxygen species (ROS), resulting in the expansion of facultative anaerobic bacteria (e.g., *Vibrio* spp.) at the expense of obligate anaerobic bacteria (e.g., *Lactococcus formosensis*, Supplementary Table S3). Notably, the observed decline in functional diversity was accompanied by a significant enrichment of VFs (Figure 4). Among these, VF0558, a pyrimidine biosynthesis-related factor primarily derived from *Enterococcus faecalis* and *Staphylococcus aureus*, facilitates immune evasion (Cao et al., 2016; Thurlow et al., 2009). VF0539 EcbA, exclusively produced by *Enterococcus faecium*, confers bacterial adhesion to host tissues (Hendrickx et al., 2009). Additionally, VF0408 T3SS1, encoded by *V. parahaemolyticus*, exerts cytotoxic effects on host cells by inducing autophagy and cell lysis (Zhou et al., 2008). Intriguingly, these pathogens exhibited consistent enrichment in the three EHP-infected cohorts (Supplementary Table S3; Figure 3). The sequential increase in VFs abundance suggests a stepwise process in which pathogens evade host immune clearance, establish adherence, and exert cytotoxic effects as infection severity progresses (Figure 4G, H). Further validation of VFs expression is warranted to elucidate their mechanistic roles in EHP pathogenesis and their potential as therapeutic targets in shrimp disease management.

Increasing EHP infection significantly altered the compositional and functional structures of the gut microbiota (Figures 1, 4), leading to enrichment of potential pathogens and a diversification of VFs (Supplementary Table S3; Figures 3, 5). To elucidate these complex interrelationships, *a priori* PLS-PM was employed (Figure 5). Analysis revealed that EHP infection led to a marked decline in digestive enzyme activity, which strongly influenced shrimp growth traits. Notably, as infection severity increased, VFs encoded by the gut microbiota diversified, further exacerbating the negative impact on shrimp growth (Figure 5). Intensive studies have reported the frequent co-occurrence of EHP and bacterial infections (Babu et al., 2023; Shen et al., 2022a; Xu et al., 2025). Consistently, an increased abundance of various pathogens was observed in EHP-infected cohorts (Supplementary Table S3), with some of these species also identified as diagnostic biomarkers for EHP infection severity (Figure 3). The direct disruption of gut microbiota composition by EHP infection (Figure 5) aligns with the observed patterns of dysbiosis and increasing disease severity (Shen et al., 2022b). Furthermore, shifts in gut microbial composition indirectly affected shrimp growth traits via functional alterations (Figure 5). This finding supports the notion that microbial taxonomy alone is a poor predictor of ecological function, whereas functional traits more directly determine microbial contributions to host physiology (Yang, 2021). Collectively, these findings deepen our understanding of the pathogenic mechanisms of EHP through the lens of the parasite-gut microbiome-shrimp physiology axis.

This study comprehensively characterized gut microbiome responses to increasing EHP infection severity, rather than limiting comparisons to paired healthy and infected shrimp. Specifically, a conceptual model was proposed to summarize these findings (Figure 6). In healthy shrimp, the diverse and stable gut microbiome was enriched in beneficial symbionts, such as *Lactococcus formosensis* and *Limosilactobacillus fermentum*, which contributed key metabolites, including formate and BCFAs. These metabolites play essential roles in maintaining digestive function and immune homeostasis. However, EHP invasion disrupted this homeostasis, leading to a decline in compositional and functional diversity, as well as widespread dysbiosis, creating ecological niches that favor pathogen colonization. As these pathogens proliferated, their associated VFs sequentially increased in abundance and diversity, further destabilizing the gut microbiota. The cumulative effects of these disruptions intensified as infection progressed, resulting in compromised digestive activity and growth retardation in shrimp (Figures 1, 6). Given the linear alteration in gut microbiota with increasing EHP infection, microbial biomarkers were identified for diagnosing infection severity. Although the model exhibited limited accuracy in detecting early-stage infections, it effectively distinguished between EHP-infected and healthy shrimp without false negatives. Further refinement and validation are required to enhance its diagnostic utility and translate these findings into practical applications for shrimp health management.

DATA AVAILABILITY

All sequences reported in this study were deposited in the Genome Sequence Archive database (http://gsa.big.ac.cn/) under accession number CRA019394, Science Data Bank (https://www.scidb.cn/) under doi: 10.57760/sciencedb.j00139.00171, and NCBI database under BioProjectID PRJNA1225847.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.C. and J.B.X. designed the research. Y.M.L. and Q.Z. performed the experiments. Y.M.L. and J.Q.L. performed data and laboratory analyses. Y.M.L. wrote the manuscript. J.B.X. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

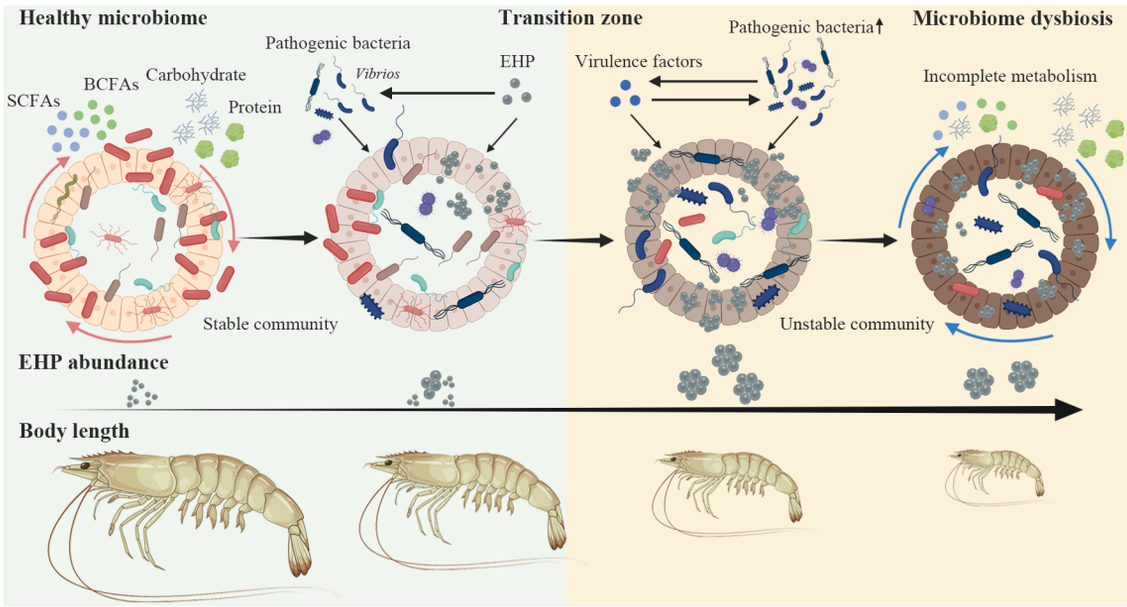

Figure 6 Proposed mechanisms of EHP infection impacting shrimp growth via the parasite-gut microbiome-shrimp physiology axis

In a healthy state, a diverse and stable gut microbiome is enriched with beneficial symbionts that produce short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs), sustaining normal digestive function and immune homeostasis. However, as EHP infection progresses, this microbial balance is progressively disrupted, creating ecological niches that facilitate pathogen proliferation and the enrichment of diverse virulence factors, ultimately compromising shrimp growth and physiology.

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