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RESEARCH REPORT

Influence of sex on intestinal microbiota of Pacific white shrimp *Penaeus vannamei*

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Abstract

Penaeus vannamei is a high-value aquaculture species. However, with the expansion in farming area and the rise in stocking density, the disease problems of *P. vannamei* have grown increasingly severe. Given the pivotal role of intestinal microbiota in regulating host health, including digestion, immune function, and metabolic homeostasis, understanding microbial dynamics is critical for disease control. Notably, although pronounced sexual dimorphism exists in *P. vannamei*, gender-specific microbiota variations remain uncharacterized. This study intends to evaluate the impact of gender variations on the intestinal microbiota of *P. vannamei*, using the 2bRAD-M technique. The results showed that the average growth rate of female shrimp was significantly higher than that of male shrimp. The Chao 1 index and Simpson index of female shrimp were greater than those of male shrimp. β-diversity research suggested that the female group samples might contain more microbial variety. At the phylum level, the microbial composition of the female and male shrimp groups is similar, with Pseudomonadota, Bacillota_A, Bacteroidota, Actinomycetota, and Planctomycetota being the dominating phyla. At the species level, the female group is predominantly formed of *Phaeobacter italicus*, *NSJ_50_sp014385105*, *Pseudoalteromonas spongiae*, and *Xanthomarina gelatinilytica*, while the male group contains a larger abundance of *Vibrio parahaemolyticus* and *Vibrio cholerae*. These data indicated that female *P. vannamei* not only display faster growth rates, but also possess a more complex and diverse intestinal microbiota, which may contribute to their higher disease resistance compared to male ones.

Key Words: *Penaeus vannamei*; intestinal microbiota; sex differences

Introduction

Penaeus vannamei, is one of the most important economic species in worldwide aquaculture (Wang *et al*., 2017). However, with the continuing expansion in farming density and scale, disease challenges have become increasingly prevalent, significantly harming its sustainable development. Crustaceans exhibit strong sexual dimorphism. For example, male *Macrobrachium rosenbergii* often have a faster growth rate than female ones (Liu *et al*., 2023). In contrast, in *Penaeus monodon*, *Fenneropenaeus chinensis*, and *P. vannamei*, females often grow larger than males after attaining sexual maturity(Browdy, 1998; Jones *et al*., 2020). Research achievements indicated that male *P. vannamei* have a competitive advantage in acquiring feed, implying that males are better at exploiting food resources than females (Moss and Moss, 2006). In addition, investigations have revealed that female *Procambarus clarkii* have a significant advantage in growth rate and abdominal meat content (Wang *et al*., 2014; Peng *et al*., 2021). Infection investigations on male and female *P. clarkii* demonstrated that female crayfish have greater disease resistance than male crayfish (Ren *et al*., 2022).

The intestinal microbiota has numerous critical roles in aquatic species, including controlling the host's digestive metabolism, improving immune protection, and preserving health (Louis *et al*., 2014). In recent years, research has found that the intestinal microbiota of aquatic animals is significantly different from that of terrestrial animals, primarily due to the specific living environment of aquatic animals, which makes them more susceptible to environmental

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microorganisms and results in lower intestinal microbiota stability. Intestinal microbiota plays a key role in host health, while their composition and structure are impacted by different factors, including food, developmental stage, individual size, and environmental conditions (Shen and May, 2015). The intestinal microbial diversity in shrimp can drastically vary under illness settings. For example, after infection with acute hepatopancreatic necrosis disease (AHPND), pathogenic *Vibrio* species grow significantly in the shrimp gut, resulting in a homogenized microbial community structure and a large reduction in microbial diversity (Quiroz-Guzmán *et al*., 2022). Gender factors are regarded as an essential component in the interaction between the host and its intestinal microbiota. Studies have demonstrated that in some species, sex can greatly impact the diversity, quantity, and function of intestinal microbiota. For example, gender has a substantial impact on the makeup and function of microbial communities in fish and crustaceans, such as the *Periophthalmus spp.* and the *Eriocheir sinensis* (Fang *et al*., 2024; Jiang *et al*., 2024). However, investigations on the gender variations in the intestinal microbiota of Pacific white shrimp are still rare.

This study applies the 2bRAD-M approach to investigate the variations in intestinal microbiota diversity and composition between normal male and female *P. vannamei*, studying the impact of gender on intestinal microbiota. These studies aim to expand the understanding of host-microbe interactions in invertebrates and provide scientific data for enhancing the health and illness resistance of *P. vannamei*.

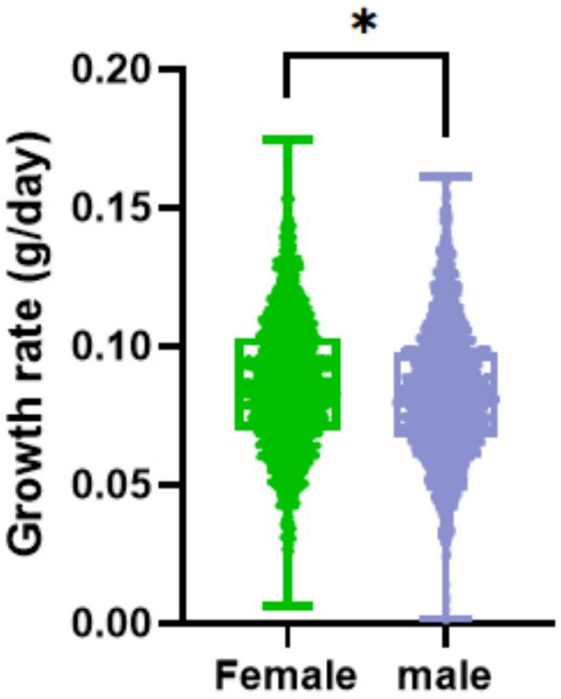

Fig. 1 Growth rate comparison between male and female *P. vannamei.* Boxplots show median growth rates (central line), interquartile range (box), and individual data points (dots)

Materials and Methods

Growth rate measurement

A total of 1,722 female and 1,670 male *P. vannamei*, provided by Hainan Zhongzheng Aquatic Science and Technology Co., Ltd, were selected for growth rate analysis. Shrimp with a wide range of initial weights were reared under controlled conditions: salinity of 20‰, water temperature of 25 ± 1 °C, and daily water exchange (1/3-1/2 total volume). The growth rate was calculated as:

$$\text{Growth rate (g/d)} = \frac{\text{Harvest body weight (g)-Initial body weight (g)}}{\text{Times from marking to harvest (d)}}$$

Initial and final weights were measured using a digital balance (precision: 0.01 g), and rearing duration was recorded. Data were analyzed with GraphPad Prism 8.0.2, and presented as boxplots with standard deviation and individual data points.

Experimental animals and sample collection

Healthy *P. vannamei* (average weight: 13 ± 1.2 g) were screened for common pathogens using the methods we previously developed (Xu *et al*., 2022; Li *et al*., 2023; Xu *et al*., 2023; Zhang *et al*., 2023a; Zhang *et al*., 2023b; Zhou *et al*., 2023; Li *et al*., 2024; Liu *et al*., 2024; Yang *et al*., 2024; Zhang *et al*., 2024a; Zhang *et al*., 2024b). For microbiome analysis, 30 males and 30 females were randomly divided into experimental and control groups (n = 5 per tank, triplicate tanks per group). Intestines from five size-matched shrimp per tank were aseptically dissected, pooled, flash-frozen in liquid nitrogen, and stored at -80 °C.

Extraction of intestinal microbial genome and 2bRAD-M sequencing

The library was generated and sequenced according to the approach of Wang (Wang *et al*., 2012). The technique includes the following steps: First, the DNA (1 pg-200 ng) was digested with 4 U of BcgI enzyme (R0545, NEB, USA) for 3 hours at 37 °C. Then, the junctions were connected to the DNA fragments, and the DNA was removed from the DNA fragments. Subsequently, the junctions are linked to the DNA fragments. The ligation process was carried out by combining 10 μL of digested DNA with 10 μL of a ligand master mixture containing 0.2 μM of each of the two junctions and 800 U of T4 DNA Ligase (M0202, NEB, USA). Ligation was conducted at 4 °C for 12 hours. Then, the ligation products were amplified, and the PCR products were submitted to 8% polyacrylamide gel electrophoresis. Approximately 100 bp bands were excised from the polyacrylamide gel, and the DNA was diffused in nuclease-free water for 12 hours at 4 °C. Sample-specific barcodes were introduced by PCR utilizing platform-specific primers containing barcodes. Each 20 μL PCR comprised 25 ng of gel-extracted PCR product, 0.2 μM of each primer, 0.3 mM dNTP, 1 × Phusion HF buffer, and 0.4 U of Phusion high-fidelity

Table 1 Sequencing data statistics

Sample	Raw_Reads	Enzyme_Reads	Clean_Reads	Percent
BM1	8220657	7839048	7372065	89.68%
BM2	7667991	7446725	7027769	91.65%
BM3	10247968	9583382	8958114	87.41%
BF1	7711522	7513815	7009005	90.89%
BF2	8196483	7809264	7288927	88.93%

DNA polymerase (M0530, NEB, USA). PCR products were purified using the QIAquick PCR purification kit (28106, Qiagen, Germany) and finally sequenced on the Illumina Nova PE150 platform.

Bioinformatics analysis

A total of 49,957,362 raw reads were obtained from sequencing. The data were subjected to quality control using Fastp, filtering out reads with an N-base proportion greater than 8% and those with more than 20% of their bases having a quality score below Q30. This resulted in 47,810,873 clean reads. The α-diversity (Chao 1, Shannon, and Simpson diversity indices) was analyzed using QIIME, and species and diversity index rarefaction curves were plotted to assess the adequacy of sequencing depth. Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) were used to calculate the β-diversity of each sample group, and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was employed to construct a clustering dendrogram. In order to further explore the differences in community structure between grouped samples, LEfSe analysis was used to identify the microbial species that differ between the groups.

Results

Growth rate comparison

The results showed that the average growth rate of female shrimp (0.087 ± 0.025 g/day) was significantly higher than that of male shrimp (0.083 ± 0.024 g/day) (Student's t-test, $p < 0.05$). The boxplot illustrates the distribution of growth rates for male and female shrimp (Figure 1).

Quality control and diversity assessment of sequencing data

After filtering low-quality reads (N-base proportion > 8%, Q30 < 20%), a total of 44,756,086 clean reads were obtained (Table 1). Rarefaction curves approached saturation (Figure 2A and 2B), indicating sufficient sequencing depth. The raw data has been uploaded to the Sequence Read Archive database with the accession number PRJNA1050063.

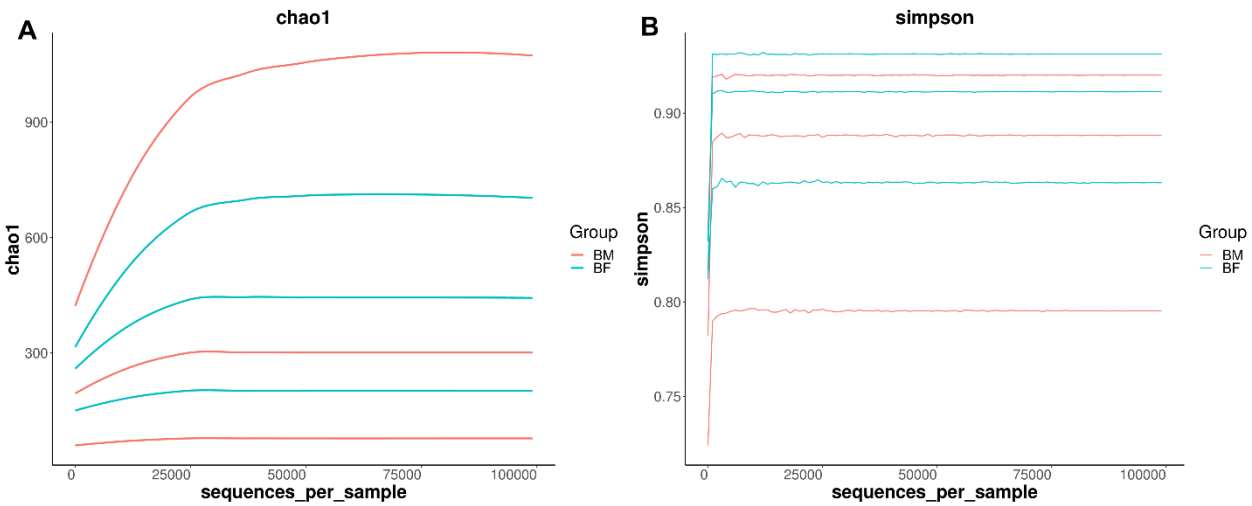

Fig. 2 Rarefaction curves of sequencing depth. (A) Chao1 index; (B) Shannon index. Curves approaching saturation indicate adequate sequencing depth

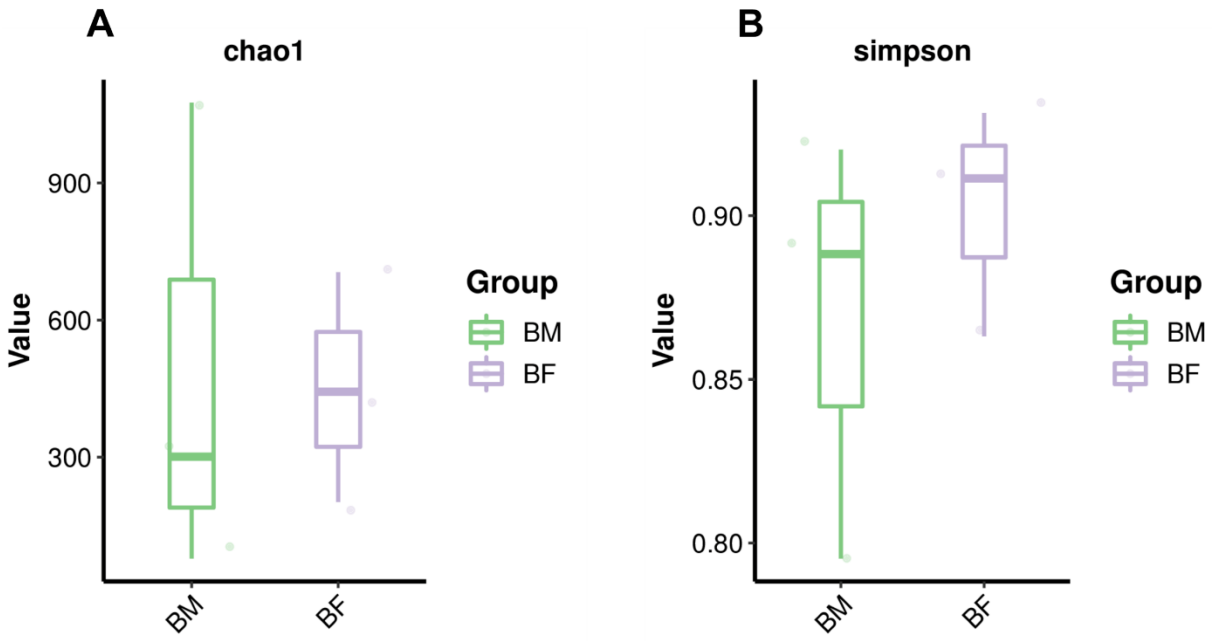

Fig. 3 α-Diversity indices of intestinal microbiota (A) Chao1 (species richness); (B) Simpson (diversity)

α-diversity analysis

The α-diversity analysis reveals the degree of species diversity in the biological environment. From the chao1 index, the quantity of intestinal microorganisms was higher in female shrimp *P. vannamei* (Figure 3A). The Simpson's index showed that the intestinal microbial diversity of female Pacific white shrimp was also higher (Figure 3B).

β-diversity analysis

Principal coordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) were employed to explore the β-diversity between the two sets of samples. The samples of female shrimp are more concentrated than those of male shrimp (Figure 4A and 4B).

Analysis of community structure

The findings of community composition studies of the intestinal samples of the two groups of shrimps are displayed in Figure 5. At the phylum level, both female and male shrimp were dominated by Pseudomonadota, Bacillota_A, Bacteroidota, and Actinomycetota. At the species level, female shrimp were dominated by *Phaeobacter_italicus*, *NSJ_50_sp014385105*, *Pseudoalteromonas_spongiae*, and *Xanthomarina_gelatinilytica*, while male shrimp were dominated by *Phaeobacter_italicus*, *NSJ_50_sp014385105*, *Spiribacter_roseus*, and *V. parahaemolyticus* (Figure 5A and 5B). The distribution of the top 20 most abundant intestinal flora in each sample at the phylum level represents the proportion of dominant microbial species in each group and the proportion of dominant microbial species among groups (Figure 5C).

Differential species analysis

Differential species analysis is a technique for examining variations in intestinal microbial composition among distinct people or groups. The outcomes of the differential species analysis are illustrated by box plots, with distinct colors denoting various sample groups, and the y-axis reflecting the relative abundance of species. As demonstrated in the figure, at the genus level, all differential taxa had higher relative abundance in the female shrimp (F group) compared to the male shrimp (M group) (Figure 6A). At the species level, most differential species likewise show higher relative abundance in the F group, except for *Vibrio cholerae* and *Aliiroseovarius*, which showed higher abundance in the M group (Figure 6B).

Discussion

Crustacean exhibit pronounced sexual dimorphism in both morphology and physiology, with emerging evidence suggesting sex-specific metabolic regulation of intestinal microbiota (Wang *et al*., 2024). As a crucial determinant of shrimp health, these microbial communities mediate nutrient assimilation and immune responses (Louis *et al*., 2014). While environmental modulators (diet, density) of *P. vannamei* microbiota have been well-documented, gender-driven variations remain uncharacterized (Zhang *et al*., 2014; Zeng *et al*., 2017; Gainza *et al*., 2018; Fan and Li, 2019; Quiroz-Guzmán *et al*., 2022). Here, we systematically characterized the gut microbiota of male and female *P. vannamei* using 2bRAD-M, revealing novel insights into the interplay between sexual dimorphism and intestinal microecology.

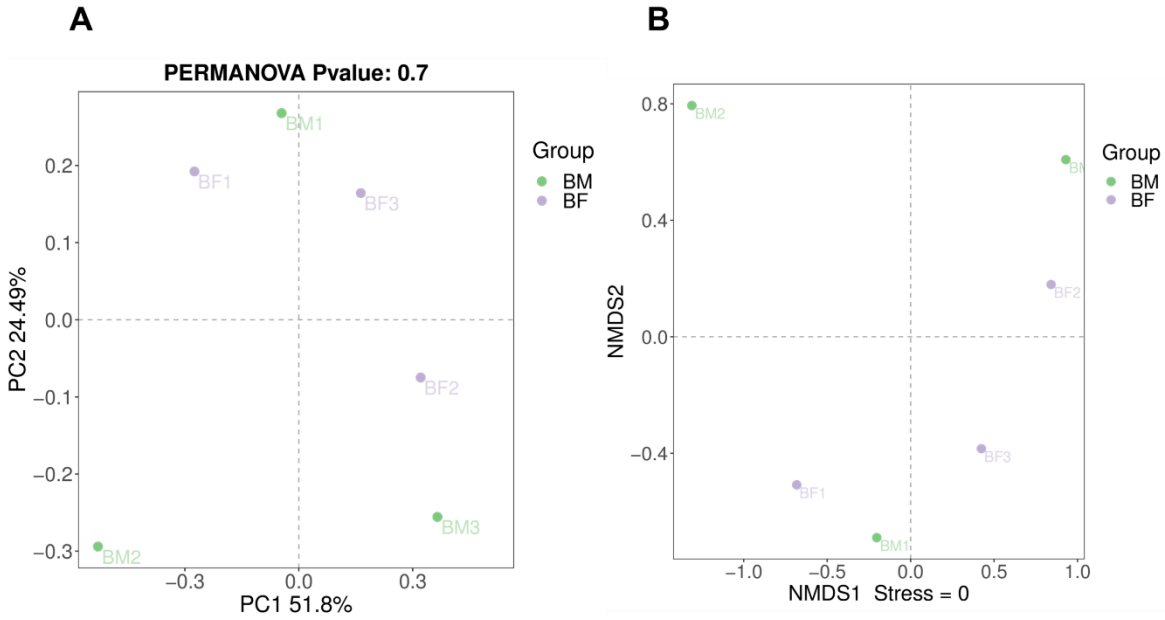

Fig. 4 β-Diversity analysis of intestinal microbiota (A) Principal coordinatesanalysis (PCoA); (B) Nonmetric multidimensional scaling (NMDS). Distinct clustering patterns between female (F) and male (M) groups

Sexual dimorphism in crustaceans is further exemplified by growth rate disparities. Female crayfish exhibit significantly higher growth rates and abdominal muscle mass than males (Wang *et al*., 2014), while female individuals of *P. monodon* and *F. chinensis* generally attain larger body sizes post-sexual maturation (Browdy, 1998). In this study, female Pacific white shrimp demonstrated significantly faster growth than male ones. These findings align with previous reports of female-biased growth in crustaceans, further supporting the role of sexual dimorphism in shaping growth performance across species.

The gut plays a crucial role in the absorption and metabolism of nutrients in animals. The unique immune mechanisms of shrimp make the intestinal microbiota an important factor in maintaining shrimp health (Sonnenburg and Backhed, 2016). The diversity of bacteria is considered an important indicator of community stability. Generally, the higher the bacterial diversity, the stronger the stability of the community, and the greater its resistance to pathogens (Shade *et al*., 2012; Sonnenburg *et al*., 2016). Dominant microbial phyla in *P. vannamei* intestines include Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, which are vital for maintaining intestinal health and disease resistance (Zheng *et al*., 2022). Studies have reported higher microbial diversity and stable community structures in healthy Pacific white shrimp, whereas AHPND infection leads to a significant reduction in diversity (Quiroz-Guzmán *et al*., 2022). Our findings on sex-specific differences in microbial communities are supported by the high sequencing depth (44,756,086 clean reads), which allowed for comprehensive and reliable analysis. Females exhibited higher α-diversity (Chao1, Simpson indices) and tighter β-diversity clustering (PCoA/NMDS), indicating a more stable and diverse microbiota. At the phylum level, both sexes shared similar microbial profiles dominated by Pseudomonadota, Firmicutes, Bacteroidetes, Actinobacteria, and Planctomycetota, aligning with prior findings. In summary, the results indicate that female shrimp exhibit a more stable and diverse intestinal microbiota compared to males, as evidenced by higher α-diversity and tighter β-diversity clustering. These results indicate that females may have a stronger immune capacity against external pathogens and better adaptability to environmental changes.

Firmicutes and Bacteroidetes are two key beneficial phyla in shrimp intestines, synergistically contributing to polysaccharide degradation, short-chain fatty acid (SCFA) production, and intestinal homeostasis (Zhang *et al*., 2015). Butyrate-producing Firmicutes improve intestinal mucosal health by metabolizing dietary fibers, while Bacteroidetes degrade fibers via glycolytic genes to support host energy metabolism. However, compared to terrestrial mammals, Bacteroidetes are less abundant in aquatic animals, possibly due to their lower carbohydrate metabolic efficiency (Duan *et al*., 2018). The Firmicutes/Bacteroidetes (F/B) ratio has emerged as a critical indicator of intestinal health (Fan and Li, 2019). In this study, male shrimp (M group) showed a higher F/B ratio than females (F group), indicating superior dietary fiber utilization. This finding aligns with previous reports that male shrimp are more efficient in resource exploitation.

Beyond beneficial phyla such as Firmicutes and Bacteroidetes, conditional pathogens including *Vibrio* also profoundly impact host health. The genus *Vibrio* (Proteobacteria), encompassing pathogens like *V. parahaemolyticus* and *V. cholerae*, could cause

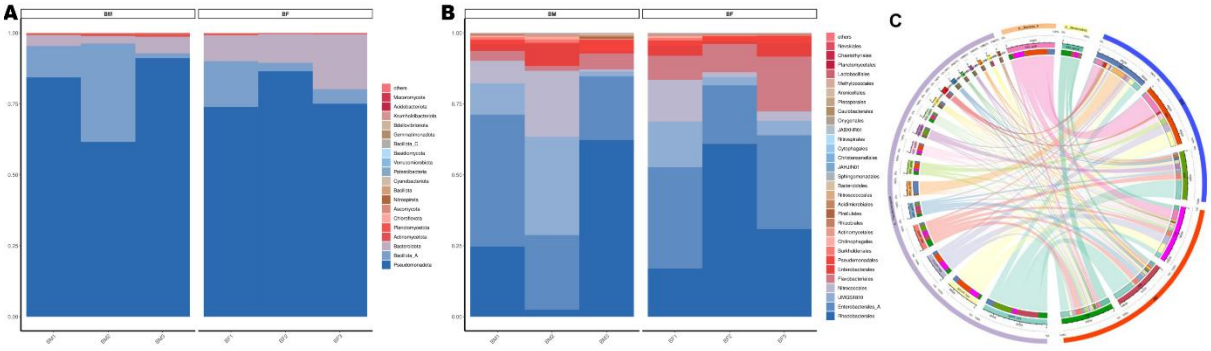

Fig. 5 The microbial community composition in the intestine. (A)The composition of the top 15 bacterial groups ranked in relative abundance at the phylum level. (B) The composition of the top 15 bacterial groups ranked in relative abundance at the species level. (C) The circus of top 20 relative abundant species in each group at phylum level

severe diseases including tail necrosis, red disease, and loose shell syndrome (Jayasree *et al*., 2006). *Vibrio* spp. thrive in crustacean intestines by metabolizing chitin (Holt *et al*., 2021). Previous studies have shown that white spot syndrome virus (WSSV) infection disrupts intestinal microbiota in *P. vannamei*, significantly increasing *Vibrio* abundance while reducing microbial diversity (Wang *et al*., 2019). Similarly, white feces syndrome (WFS) is associated with *Vibrio* dominance (Wang *et al*., 2020). Here, male shrimp (M group) harbored higher relative abundances of *V. parahaemolyticus* and *V. cholerae* than females (F group), implying that males may be more vulnerable to conditional pathogens under environmental stress.

Mesoflavibacter (Bacteroidetes), a genus of Gram-negative bacteria widely distributed in aquatic environments, plays a critical role in host immunological control and disease resistance (Lee *et al*., 2014; Zhang *et al*., 2018). It synthesizes zeaxanthin, a carotenoid with potent antioxidant and anti-inflammatory properties, which mitigates oxidative stress and enhances immune defenses (Duan *et al*., 2019). Previous studies have demonstrated that resistant starch (RS)-supplemented feeds significantly promote *Mesoflavibacter* proliferation in shrimp intestines, thereby improving immune function and disease resistance (Zhang *et al*., 2009). In this study, female *P. vannamei* (F group) exhibited significantly higher relative abundance of *Mesoflavibacter* compared to males (M group). These results indicate the elevated abundance of *Mesoflavibacter* in females may confer stronger immune defense capabilities through antioxidant protection.

Aliiroseovarius (Rhodobacteraceae, Proteobacteria), a marine bacterium with dual ecological functions, can act as both a pathogen (*Aliiroseovarius crassostreae* causing oyster septicemia syndrome) and a commensal involved in metabolic regulation (Duan *et al*., 2020). Dietary changes (yeast extract supplementation) significantly increase *Aliiroseovarius* abundance in *P. vannamei* intestines (Zheng *et al*., 2021). Consistent with findings in male *Panulirus argus*, male Pacific white shrimp (M group) in this study exhibited significantly

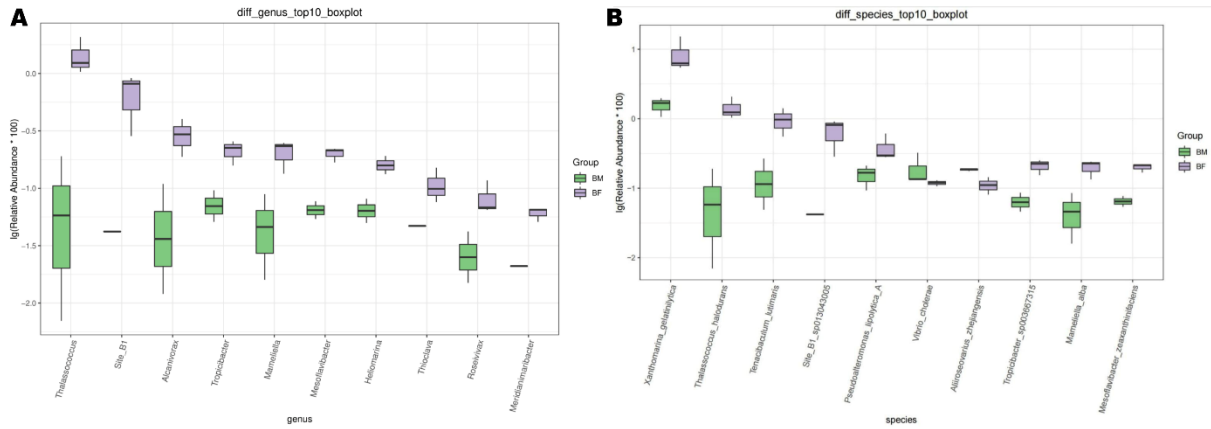

Fig. 6 Differential species analysis by LEfSe analysis. (A) Genus-level; (B) Species-level

higher Aliiroseovarius abundance than females (F group) (Zamora-Briseño *et al*., 2020). While high *Aliiroseovarius* abundance may enhance male metabolic efficiency (energy utilization), its pathogenic potential under environmental stress poses health risks. Although the precise role of *Aliiroseovarius* in crustaceans remains unclear, its male-biased abundance could influence host metabolism or pathogen competition, warranting further mechanistic studies.

Conclusion

This study revealed that the growth rate of female *P. vannamei* was significantly higher than that of males, indicating differences in energy utilization and metabolic efficiency. Additionally, the intestinal microbiota diversity and richness in females were significantly higher than in males, possibly due to physiological mechanisms that maintain microbial balance. The presence of opportunistic pathogens in males, such as *V. parahaemolyticus*, may compromise their health, while the richer microbiota in females likely enhances immune protection. These results demonstrate that female shrimp exhibit superior growth performance and immune resilience, supporting the development of sex-specific strategies for shrimp health management in aquaculture.

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