

# Contrasting Ecological Processes and Functional Compositions Between Intestinal Bacterial Community in Healthy and Diseased Shrimp

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**Abstract** Intestinal bacterial communities play a pivotal role in promoting host health; therefore, the disruption of intestinal bacterial homeostasis could result in disease. However, the effect of the occurrences of disease on intestinal bacterial community assembly remains unclear. To address this gap, we compared the multifaceted ecological differences in maintaining intestinal bacterial community assembly between healthy and diseased shrimps. The neutral model analysis shows that the relative importance of neutral processes decreases when disease occurs. This pattern is further corroborated by the ecosphere null model, revealing that the bacterial community assembly of diseased samples is dominated by stochastic processes. In addition, the occurrence of shrimp disease reduces the complexity and cooperative activities of species-to-species interactions. The keystone taxa affiliated with Alphaproteobacteria and Actinobacteria in healthy shrimp gut shift to Gammaproteobacteria species in diseased shrimp. Changes in intestinal bacterial communities significantly alter biological functions in shrimp. Within a given metabolic pathway, the pattern of enrichment or decrease between healthy and diseased shrimp is correlated with its functional effects. We propose that stressed shrimp are more prone to invasion by alien strains (evidenced by more stochastic assembly and higher migration rate

in diseased shrimp), which, in turn, disrupts the cooperative activity among resident species. These findings greatly aid our understanding of the underlying mechanisms that govern shrimp intestinal community assembly between health statuses.

**Keywords** Shrimp intestinal community · Health status · Ecological process · Species-to-species interactions · Functional composition

## Introduction

In an aquaculture ecosystem, shrimp and microbes share a water environment; therefore, the intestinal microbial community directly interacts with the planktonic microbiota [1, 2]. Indeed, the rearing microbiota furnishes a source of infectious agents and also provides probiotics that prevent diseases due to the competition with each other for space, available nutrients, and ecological niches [2–4]. Thus, the association between the intestinal microbiota and the surrounding microbial community is of great interest to the field of aquaculture, particularly in locations where shrimp diseases are widespread [5]. There is ample evidence that domesticated and wild shrimp share similar intestinal bacterial communities, though their respective ambient bacterioplankton communities are remarkably distinct [6, 7]. This pattern suggests that shrimp exert a strong selection over which bacterial species colonize the gut, regardless of the regional species pool [8, 9]. Some recent studies however indicate that rearing water microbiota substantially alters the gut bacterial composition of adult shrimp [2] and larval tilapia [10]. Consistently, it has been shown that pathogen exposure significantly disrupts shrimp intestinal bacterial community, which in turn results in the occurrences of shrimp disease [11]. This discrepancy addresses the question which factors affect the colonization of

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rearing water bacteria (e.g., pathogens) into shrimp gut. A relevant study delicately shows that the presence of closely related species can increase the chance of alien colonizers into gut for both enteropathogen and beneficial bacteria [4]. However, it appears that more phylogenetically related species are less easily able to coexist according to the Darwin-based niche theory, e.g., competitive exclusion [12]. Therefore, understanding the ecological processes that determine intestinal assembly is of essential interest from both a commercial and scientific perspective.

It is now recognized that the colonization of microorganisms into the gut is controlled by both host- and non-host-related factors [8, 13]. Such factors include the already present intestinal assemblage [4, 14], host physiological condition [15], growth stage of the host [16, 17], and environmental conditions [2]. While the list of potential factors is long, they can be categorized into two major processes: deterministic and stochastic processes [18, 19]. Determinism refers to the processes (e.g., host selection and species-to-species interactions) that favor one species over the other due to differences in specific ecological traits among individuals [20, 21]. Alternatively, stochastic processes assume that species are ecologically equivalent, thus community assembly is structured by the random dispersal and drift [16, 22]. In an ecological context, deterministic processes assemble defined community composition trajectories over space and in time [23]. Stochastic processes, in contrast, lead to unpredictable communities [21, 24]. It has been shown that deterministic processes contribute reproducible long-term changes in bacterial community composition and functional performance in engineered systems [23]. In this perspective, quantifying the relative importance of these two processes could provide ecological rationale on efficient gut microbial management [1, 25].

Recently, studies have designed a framework to discern the importance of stochastic processes in generating microbial community assembly using neutral model approaches [21, 22]. The neutral model assumes that species that deviate from the model expectations are the candidates for undergoing selection, thereby facilitating a quantitative estimation on the taxa that are present as a result of selective rather than neutral processes [22]. Indeed, this approach has been applied to quantify the relative importance of deterministic and stochastic processes in maintaining vertebrate fishes' intestinal bacterial communities, which reveals that the relative importance of deterministic processes increase as hosts mature and are independent of the bacterial community in surrounding water [16, 17]. Thus, a healthy adult host exerts deterministic filters on intestinal assemblages. Our recent study has found that the magnitude of changes in intestinal bacterial communities is parallel to the disease severity of adult shrimp, with increased abundances of potential pathogens [2]. Indeed, there is accumulating evidence that normal intestinal microbiota is crucial for preventing pathogen invasion [4, 13], whereas stressed

hosts suffer from increased invasibility [14, 26]. For these reasons, we hypothesized that the ability of shrimp to select intestinal microbiota (e.g., host filtering) would decrease when disease was present, thereby weakening the relative importance of deterministic processes. Pathogen invasion significantly alters shrimp intestinal bacterial community, resulting in mortality—in other words, destabilizing the functional stability of the intestinal community [11]. Indeed, the invasibility of a pathogen has been served as an indicator for both compositional and functional stabilities of bacterial community [14, 18]. Given the functional importance of intestinal microbiota in stimulating host nutrient acquisition and preventing pathogen colonization [4, 13], we hypothesized that the disruption of a normal microbiota would alter functional composition, although it is assumed that microbial communities have functional redundancy; that is, the ability of one microbial community contributes a functional process at the same rate as another [27]. In addition, increasing evidence shows that the responses of a community to disturbance (e.g., disease) depend on species-to-species interactions, rather than the sum of the traits of individual species [28, 29]. Recently, the species-to-species interactions approach has been applied to evaluate the robustness of community stability to disturbance [20, 30]. For example, an increased species-to-species complexity is positively associated with microbial function potentials [30, 31]. These findings led us to determine the extent of the species-to-species interactions that are affected by the occurrence of shrimp disease.

## Materials and Methods

### Experimental Design and Molecular and Statistical Methods

For the present study, we used a paired-end 16S rRNA gene Illumina sequencing data (the open accession number was DRA002865 in DDBJ, <http://www.ddbj.nig.ac.jp/>) from our previous study, in which we demonstrated that the magnitude of deviation in Pacific white shrimp (*Litopenaeus vannamei*) intestinal bacterial community are positively associated with disease severity [2]. A brief description of the study design is as follows: after 70 days of inoculation, shrimp disease emerged in 3 out of 30 ponds, resulting in a massive mortality. The diseased shrimp stopped eating with white fecal strings, gradually ulcerated, and died within a few days, referred to as white feces syndrome [5]. We collected healthy (in 4 ponds) and diseased (in 3 ponds) shrimp and water samples from the corresponding ponds. To improve statistical power, 2–3 fishing nets were used to collect shrimp in each pond, resulting in 9 pseudo-biological shrimp replicates for each health status (e.g., healthy and diseased). Readers are referred to Xiong et al. for additional details [2].

Given the limited number of sub-healthy shrimp, these samples were removed in this study.

To obtain bacterial metabolic functional traits for the functional redundancy assay, bacterial sequences were binned into operational taxonomic units (OTUs, 97 % similarity) and then were classified taxonomically against a closed reference (Greengenes database 13.8) and aligned using PyNAST [32]. After taxonomies had been assigned, OTUs that were affiliated with Archaea, chloroplasts, unclassified (not affiliated with bacteria), and singleton OTUs were removed from the dataset prior to subsequent analysis. To correct for unequal sequencing depth, normalization was completed across samples through a randomly selected subset of 7770 reads from each sample. Subsequently, the OTU table was normalized by dividing the abundance of each OTU by its predicted 16S copy number, producing the Kyoto Encyclopedia of Genes and Genomes (KEGG) classified functions of the community by the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [33]. The non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) were used to evaluate the overall differences in predicted functional composition using Bray–Curtis distance [34]. A Pearson correlation was performed to test the association between changes in bacterial phylogenetic composition and predicted functional composition [35]. The significances in the pathways between healthy and diseased shrimp were tested by computing the response ratio [36]. Briefly, the 99 % confidence interval =  $rr_i \pm 2.58 \times \sqrt{v_i}$ , where  $rr_i = \ln(\bar{x}_i/\bar{y}_i)$  ( $i = 1 \cdots n$ ),  $\bar{x}$  and  $\bar{y}$  are the mean abundance of pathway  $i$  in diseased and healthy shrimps, respectively; the variance ( $v_i$ ) is  $v_i = \frac{s_{x_i}^2}{m_{x_i}\bar{x}_i} + \frac{s_{y_i}^2}{m_{y_i}\bar{y}_i}$  ( $i = 1 \cdots n$ ), where  $s_{x_i}$  and  $s_{y_i}$  are the standard deviation of pathway  $i$  in diseased and healthy shrimp, and  $m_{x_i}$  and  $m_{y_i}$  are the number of pathway  $i$  across healthy and diseased shrimp, respectively [36]. A permutational analysis of multivariate dispersion (PERMDISP) was used to test whether the dispersion among biological replicates for each group is indistinguishable from the null expectation [19].  $P < 0.05$  was considered significant.

### Neutral Model and Ecosphere Null Model

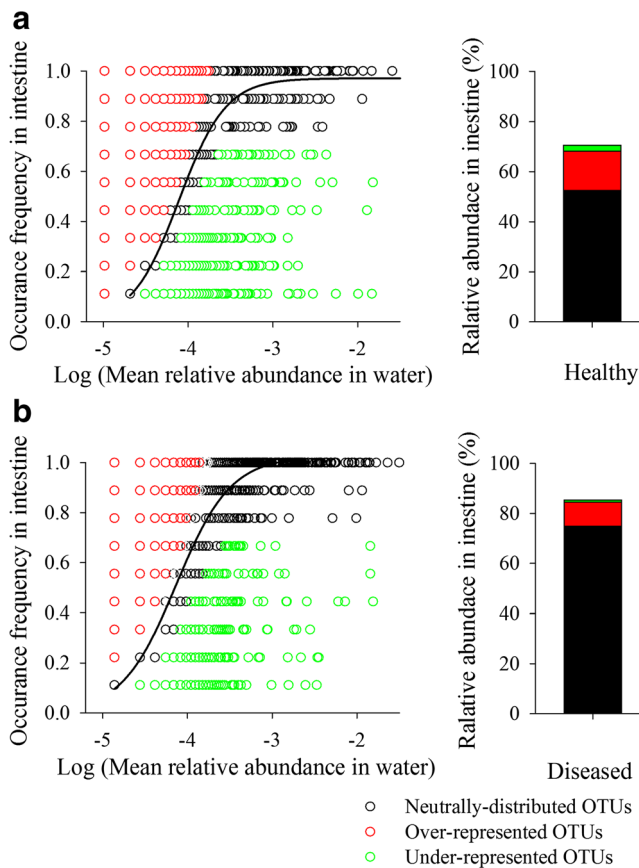
We used the Sloan Neutral Community Model to determine the relative importance of neutral processes (random dispersal from water and ecological drift) in maintaining the shrimp intestinal bacterial community [37]. For this purpose, we considered the bacterial community in water as the source of the bacteria in shrimp intestine. To compare the relative importance of neutral processes between health statuses, the neutral model was applied independently for healthy and diseased samples. This neutral model predicts that the probability of detecting an OTU in shrimp intestine due to random dispersal is directly proportional to the abundance of that OTU in the

water community. The migration rate was estimated by fitting the observed frequency of OTUs in the shrimp intestine and their abundance in ambient water, using a non-linear least-squares fitting with the `minpack.lm` package in R [38], which provides an index of dispersal limitation [16]. Binomial proportion 95 % confidence intervals (Wilson score) around the model predictions were calculated using the `HMisc` package in R [39, 40]. The overall fit of the neutral model to the observed data were accessed by comparing the sum of squares of residuals to the total sum of squares [41]. In addition, we assessed the fit of a binomial distribution model to evaluate whether incorporating drift and dispersal limitations would improve the fit of the neutral model beyond just random sampling of the species pool [42]. The Akaike information criterion (AIC) was calculated to compare the fit of the neutral and binomial model. OTUs were sorted into three categories depending on whether they occurred more frequently than the other OTUs (over-represented), less frequently than the other OTUs (under-represented), or within (neutrally distributed) the 95 % confidence interval of the neutral model predictions (Fig. 1). The cumulative relative abundance of sequences in these three categories of OTUs in shrimp intestine served as a proxy to evaluate the importance of neutral processes ( $\sum$  relative abundance of sequences within neutrally distributed OTUs) and determinism ( $\sum$  relative abundance of sequences in overrepresented and underrepresented OTUs) in shaping the intestinal bacterial community [22].

It should be noted that the above neutral model only focused on the shared OTUs between intestine and ambient water while ignoring the unique OTUs for each habitat. For this reason, we applied the ecosphere null model to quantify the role of deterministic selection processes in shaping the variations of the entire bacterial community [19, 20]. The proportional determinacy was calculated as the proportion of the difference between the observed similarity and the similarity expected under the null hypothesis divided by the observed similarity [43] while the complement of proportional determinacy provides a quantitative estimation of the importance of stochastic processes in structuring community composition [44].

### Bacterial Species-to-Species Interactions

To evaluate the effects of a disease outbreak on intestinal bacterial species-to-species interactions, molecular ecological network analysis was performed using an open-accessible pipeline (<http://ieg2.ou.edu/MENA>) [45]. This approach automatically identifies a threshold for a molecular network using a random matrix theory [45]. The OTU table was split into two datasets: healthy and diseased shrimp intestinal samples. To quantitatively compare the differences in intestinal bacterial interactions between healthy and diseased shrimp, a set of topology properties was calculated, including



**Fig. 1** Neutral model applied to the shrimp intestinal microbiota with ambient water as the source. *Stacked bar chart* depicts the relative abundances of sequences in the neutrally distributed, over-represented, and under-represented OTUs in shrimp intestine. The model was applied independently for healthy (a) and diseased (b) shrimp. The neutral model can be applied only to shared OTUs between the intestine and ambient water, thus the cumulative abundances do not add up to 100 % in the bar chart

average path length, clustering coefficient, modularity and positive co-occurrences [45, 46]. The network was visualized in Cytoscape 3.3.0 [47].

## Results

### Relative Importance of Neutral Processes Decreases When Disease Occurs

The abundance weighted null model test (PERMDISP) show that bacterial communities within each group significantly ( $P < 0.05$  in all cases) differ from the null random expectation (Table 1), suggesting that differences among biological replicates for each group was not overdispersion. Thus, the true variance and certainty are unlikely overestimated in subsequent analysis [48]. Further, the neutral model was respectively applied to data derived from healthy and diseased shrimp, as well as the corresponding water samples (Fig. 1). We found

that up to 52.4 % of the sequences recovered from healthy shrimp intestine sorted into OTUs consistent with neutral distribution (black points), up to 15.8 % of the sequences sorted into OTUs were overrepresented in the shrimp intestine (red points), and up to 2.5 % of the sequences were sorted as underrepresented OTUs (green points) (Fig. 1a). Alternatively, the OTUs categorized into neutral distribution accounted for 74.9 % of the sequences, while the overrepresented and underrepresented OTUs accounted for 9.6 and 0.9 % sequences in diseased shrimp intestine, respectively (Fig. 1b). In both cases, the neutral model offered a better fit than a binomial distribution model did (Table 2). Therefore, the effects of passive dispersal and ecological drift exceed random sampling effects of the source community. Additionally, the estimated migration rate is lower in healthy shrimp than that in diseased shrimp (Table 2), revealing that bacterial communities tend to be less physically inhibited (e.g., host filtering) in diseased shrimp.

To integrate the unique OTUs in each habitat (intestine or water) in the analysis, the ecosphere null model was further applied to quantify the determinacy in structuring the bacterial community assembly of intestine or water. The results showed that the intestinal bacterial communities were principally shaped by deterministic processes in healthy shrimp, whereas the occurrence of disease resulted in stochasticity dominated assembly. For example, the deterministic processes of the healthy shrimp increased 55.4 % in relation to the diseased shrimp (Fig. 2). A similar trend was detected between ponds with healthy and ponds with diseased shrimp, with an increase of 30.5 % determinism (Fig. 2).

### The Occurrence of Shrimp Disease Reduces the Complexity of Network Interaction

The co-occurrence patterns of bacterial taxa depend on the Pearson correlation among them, while low present OTUs could reduce the statistic power [29, 45]. Therefore, the OTUs detected in five or more replicates were selected for correlation calculation, which resulted in 681 and 823 OTUs from healthy and diseased shrimp intestine, respectively (Table 3). In both cases, the network connectivity distribution curves had comparable similarity thresholds when plotted and fitted with the power law model ( $R^2 > 0.89$ , Table 2), suggesting that the constructed networks were scale free. The network plots revealed that bacterial network interactions were more complex and better connected in the healthy shrimp than those in the diseased shrimp (Fig. 3); however, more OTUs for diseased shrimp were retained in the analysis (Table 2). This pattern was confirmed by the key topological properties, that is, the average path of healthy shrimp was lower than that in diseased shrimp, while the average clustering coefficient and average degree exhibited opposite trends (see the notes of Table 2). The values of these parameters in empirical networks

**Table 1** Bray–Curtis distance-based significance test of centroid differences between the observed communities and the null model simulations for each group

Group	Actual centroid	Null centroid	<i>F</i>	<i>P</i> value
Healthy shrimp intestine	0.469	0.570	6.3	0.0232
Diseased shrimp intestine	0.364	0.568	54.9	<0.0001
WH	0.209	0.472	61.3	0.0002
WD	0.360	0.477	2.9	0.0370

WH water sample from ponds with healthy shrimp, WD water sample from ponds with diseased shrimp

were out of the range in random networks (Table 2); therefore, the co-occurrence relationships within both cases are non-random, e.g., correlated microbial pairs [49]. In addition, the percentage of positive co-occurrence is much higher in healthy shrimp than that in diseased shrimp (73.0 versus 51.9 %, respectively; Table 2), which suggests a greater overlap of preferred environmental conditions or a higher degree of cooperative activities in healthy shrimp [50]. Notably, the keystone species affiliated with Alphaproteobacteria (dominated by Rhodobacterales, data not shown) and Actino bacteria (dominated by Actinomycetales) in healthy shrimp shifted to Gammaproteobacteria (dominated by Vibrionales and Alteromonadales) species in diseased shrimp (Fig. 3).

**Association Between Shifts in Bacterial Phylogenetic and Functional Structure**

Based on the predicted bacterial KEGG pathways across the samples, an NMDS ordination analysis showed that the functional compositions were substantially distinct between healthy and diseased shrimp intestines, primarily separated by the first axis (Fig. 4a). This is also the case for the bacterioplankton communities between the ponds with healthy and diseased shrimp (Fig. 4a). The pattern was further confirmed by the ANOSIM, revealing the significant differences in the bacterial functional compositions between healthy and diseased shrimp ( $r = 0.63$ ,  $P = 0.023$ ) and water ( $r = 0.44$ ,  $P = 0.029$ ). In addition, there was a significant and positive (Pearson correlation,  $r = 0.516$ ,  $P < 0.001$ ) association between the phylogenetic and functional compositions (Fig. 4b). Notably, the abundance of the KEGG pathways

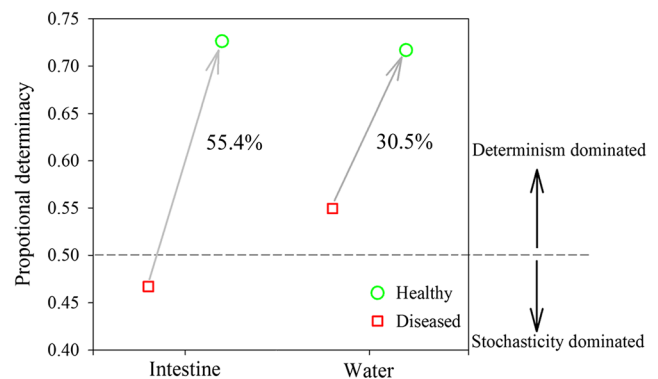
generally decreased in the diseased shrimp compared to the abundance of pathways in the healthy shrimp. In contrast, some KEGG pathways involved in pathogen infection exhibit an opposite pattern (Fig. 5). For example, compared with the healthy shrimp, the pathways of focal adhesion and disease infection significantly increased, while the antibacterial pathways, such as endocytosis, lysosome, and peroxisome pathways, decreased in the diseased shrimp (Fig. 5).

**Discussion**

Disruption of intestinal microbial communities has been linked to numerous host diseases [2, 11]; therefore, unraveling the mechanisms that govern bacterial community assembly could guide the establishment of new strategies against pathogenic infections [1, 25]. For this purpose, we quantified the relative contributions of neutral processes (random dispersal from water and ecological drift) and selective processes (species-to-species interaction and selection by the occurrence of disease) in shaping the community assembly of the shrimp intestinal bacteria. Recently, studies have focused on the processes that drive fish intestinal bacterial community assembly in growth stages [16, 17]. However, to our knowledge, how and to what extent the bacterial intestinal community assembly is altered by the occurrence of disease remains unknown.

**Table 2** Neutral model fit increases when shrimp disease occurs. These parameters include goodness of fit of the Sloan neutral model, comparison of the maximum likelihood fit of the neutral and binomial models, and the estimated migration rate in shrimp intestine bacterial communities

Parameter	Healthy	Diseased
Model fit ( $R^2$ )	0.293	0.314
Akaike information criterion for neutral model	1332.5	750.2
Akaike information criterion for binomial model	1127.8	614.5
Estimated migration rate ( $m$ )	0.241	0.292



**Fig. 2** Changes in the determinacy of bacterial communities in the healthy (green circles) and diseased (red squares) shrimp and water. The dashed line denotes equal roles for both. Below the line, the stochastic process is dominant, and above it, the determinant process is dominant. The numbers associated with each line indicate a percentage increase in determinacy. (Color figure online)

**Table 3** Topological properties of the empirical molecular ecological networks (MENs) of bacterial communities and their associated random MENs

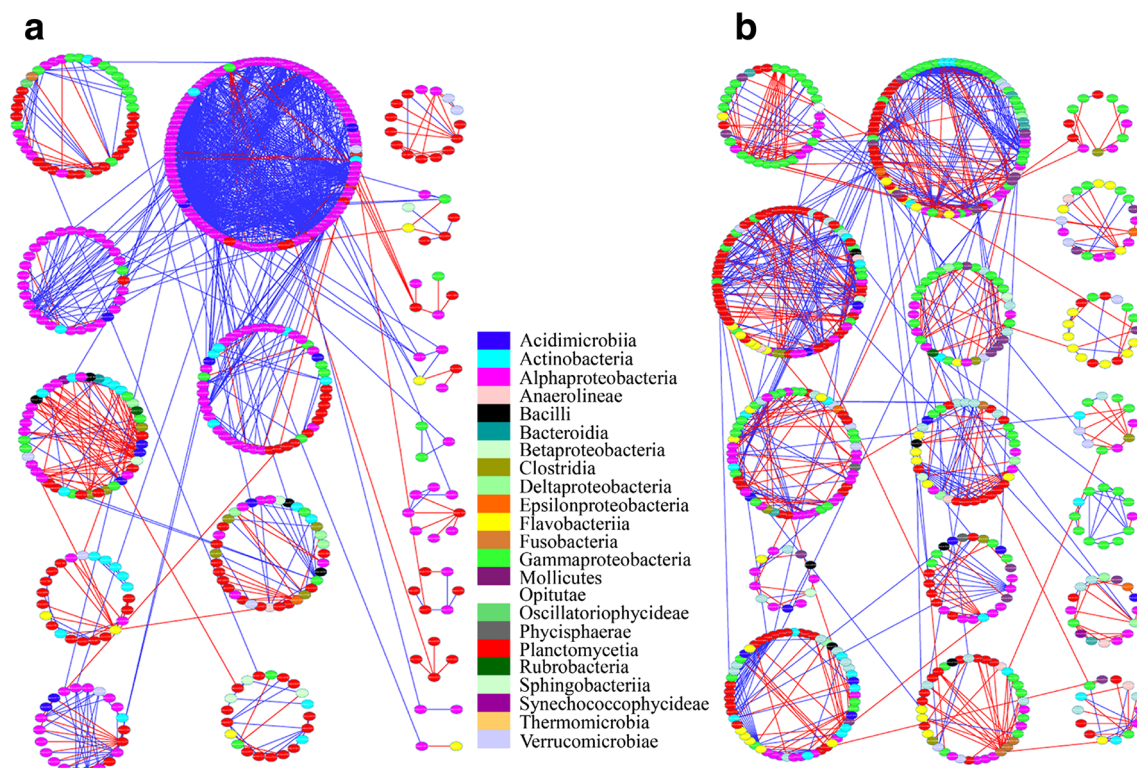
	Topological properties	Health status	
		Healthy	Diseased
Empirical networks	Similarity threshold (st)	0.91	0.90
	Network size ( $n$ )	681	823
	Links ( $n$ )	1469	1349
	Links per node	2.157	1.639
	R <sup>2</sup> of power law	0.890	0.914
	Average path <sup>a</sup>	2.611	3.587
	Average clustering coefficient <sup>b</sup>	0.160	0.143
	Average degree <sup>c</sup>	4.314	3.278
	Positive co-occurrence	73.0 %	51.9 %
	Modularity <sup>d</sup>	0.657	0.857
Random networks	Average path	3.428 ± 0.091	4.501 ± 0.113
	Average clustering coefficient	0.038 ± 0.005	0.007 ± 0.002
	Modularity	0.458 ± 0.004	0.599 ± 0.004

<sup>a</sup> Average network distance between all pairs of nodes. A smaller average path indicates all the nodes in the network are closer [46]

<sup>b</sup> The extent of module structure present in a network. It describes how well a node is connected with its neighbors [69]

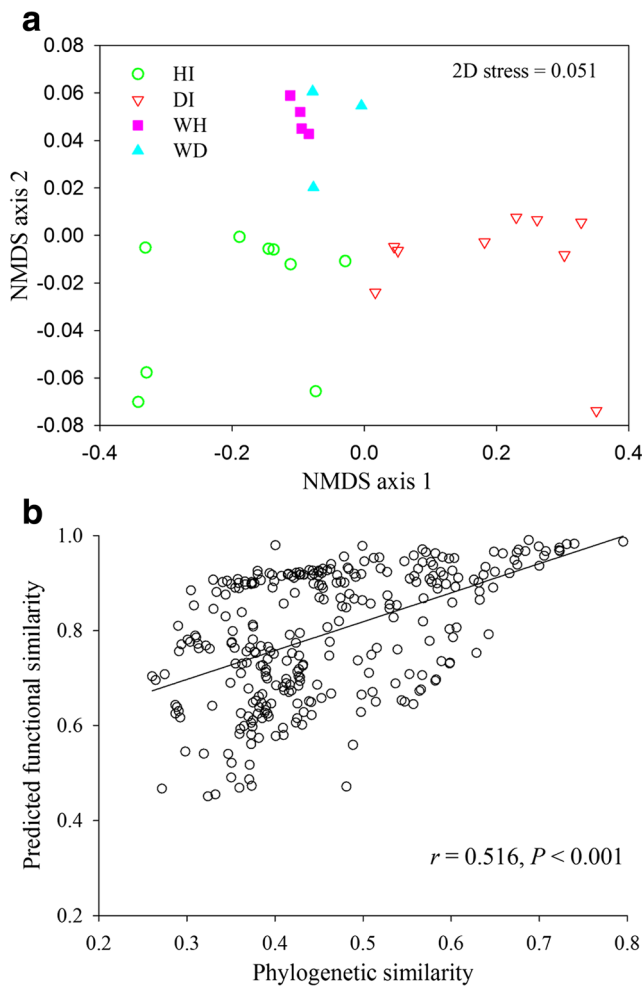
<sup>c</sup> Node connectivity. It is the connection strength between nodes, a higher avgK signifies a more complex network [45]

<sup>d</sup> Modularity >0.4 indicates that the partition produced by the modularity algorithm can be used to detect distinct communities within the network. It is used to measure how well a network is able to be separated into modules [70]



**Fig. 3** The network interaction graph for healthy (a) and diseased (b) shrimp intestinal bacterial communities. Each *node* represents a bacterial OTU. *Colors* of the nodes indicate OTUs affiliated to different major

classes. A *blue edge* indicates a positive interaction, whereas a *red edge* indicates a negative interaction between two individual nodes. (Color figure online)

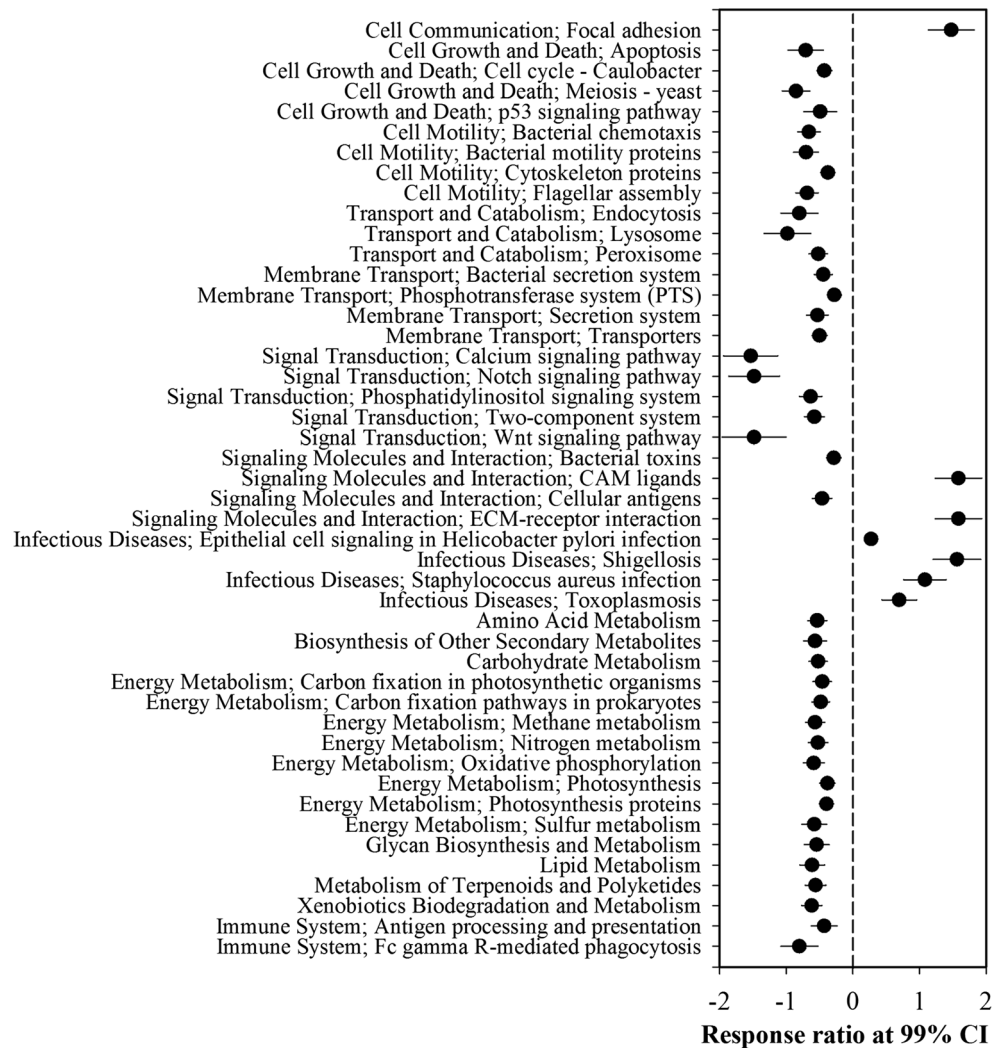


**Fig. 4** Non-metric multidimensional scaling (NMDS) plot visualizing bacterial functional community dissimilarities using Bray–Curtis distance (a), correlation between phylogenetic similarities (1-weighted UniFrac distance) and functional similarities (b). *HI* healthy shrimp intestine, *DI* diseased shrimp intestine, *WD* water sample from ponds with diseased shrimp, *WH* water sample from ponds with healthy shrimp

Our recent work has shown that some dominant bacterial assemblages exhibit a similar change pattern between healthy and diseased samples, regardless of the habitats (water and intestine) [2]. Thus, the intestinal bacterial community interacts with the surrounding water to a certain extent, as studies have shown shrimp reared under distinct environments share similar intestinal assemblages [6, 7]. Over the past decade, numerous studies have documented the parameters that define alien species invasion, such as a positive diversity–invasibility relationship [51], negative evenness–invasibility [52], and the resident bacterial assemblages [4]. However, we found that the intestinal bacterial community compositions dramatically changed between the healthy and diseased shrimp, rather than a significant change in the bacterial diversity [2]. Indeed, there is ample evidence that a disruption of intestinal microbiota dramatically increases the risk of infection [53, 54]. These

findings lead us to investigate the ecological processes generating this pattern. Within each health status, there were numerous bacterial taxa distributions deviated from the neutral predictions (Fig. 1), which are ecologically important. For example, the over-represented OTUs are specifically adapted to and have a competitive advantage in host filtration [22]. From a practical point, these OTUs are good candidates for probiotic development because they have a better chance of colonizing the shrimp intestine [25, 55]. Alternatively, shrimp hosts may select against the under-represented OTUs, which are potential pathogens [16]. If true, this would explain why some under-represented OTUs in healthy shrimp are neutrally distributed in diseased shrimp (data not shown). In addition, a remedy for the pathogen proliferation could be established, e.g., application of narrow-spectrum antibiotics to target a given pathogen, if the virulence of the potential pathogens is confirmed by pure culture work in the future. Consistent with our hypotheses, the neutral processes become relatively more important when disease occurs (Fig. 1). This pattern is further substantiated by the ecosphere null model; instead of only focusing on the shared taxa between intestine and water pool, the model suggests that the bacterial community assembly of the diseased samples was controlled by stochastic dominated processes (Fig. 2). One possible explanation is that the host selective pressures on alien bacterial species colonization is reduced under environmental stresses, e.g., significant increases in  $\text{NO}_2^-$  and  $\text{PO}_4^{3-}$  concentrations in ponds with diseased shrimp [2], thereby increasing their susceptibility to enteric infection [53, 56]. Consistently, it has been reported that environmental stress leads to the decline of shrimp innate immunity [26]. It is also probable that changes in intestinal bacterial community provide an open niche (a consequent lack of competition) for transient bacterial species. Stressed shrimp are subject to stochastic colonization by dispersed microorganisms from ambient water, evidenced by a higher estimated bacterial immigration rate in diseased shrimp (Table 2). Indeed, it has been shown that stressor exposure increases the permeability of the host intestine [53]. However, this is not to say that the neutrally distributed OTUs are functionally trivial. Rather, the selection of beneficial taxa may occur without host filtering if they maintain intestinal conditions that are favorable to defensive microbes [57], for example, some OTUs affiliated with *Bdellovibrionales*, which are neutrally distributed and known predators on *Vibrio* pathogens [58]. Currently, it is difficult to disentangle the causal relationship between the changes in intestinal community and the occurrences of disease. On the other hand, we note that the water quality deteriorated in ponds with diseased shrimp [2]; thus, the disruption of intestinal community tended to be the causal factor. The ultimate goal of studying host-associated

**Fig. 5** Significant changes in intestinal bacterial Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways between healthy and diseased shrimp using the response ratio method at a 99 % confidence interval (CI). *Circles* at the right column indicate the abundance of the corresponding pathway significantly increased, while those at the left column indicate decreased in diseased shrimp. *Error bars* represent the deviation of that pathway across the samples



communities is to manipulate the host microbiota for disease prevention and therapy. As such, our results demonstrate the ecological differences in the bacterial distribution between healthy and diseased shrimp and provide updated insights for the candidate sources of probiotics and identifying potential pathogens.

Species-to-species interactions reflect niche processes that drive co-occurrence within biological communities, which can be used to infer stability and ecological traits in assembling communities [18, 49]. For example, studies, albeit limited, have shown that the complexity of species-to-species interactions is positively associated with functional potentials [30, 31]. In species-to-species interactions, the correlation and distribution of modules could serve as proxies for niche partitioning and synergistic relationships [20, 59]. Thus, the emergence of disease results in a disruption of proper intestinal functions and is expected to decrease syntrophic interactions among species [29], which should be translated into networks with lower connectivity [45, 46]. Consistently, we found that the network complexity increased when disease

occurred, as evidence by increased average path and decreased average clustering coefficient and average degree of network connectivity in diseased shrimp (Table 3). Notably, the percentage of positive co-occurrence of bacterial taxa in healthy shrimp was substantially higher than that in diseased shrimp (Fig. 3 and Table 3), revealing that the bacterial taxa are more commonly preferred environmental conditions or cooperative activities in healthy shrimp [59]. On the basis of these results, we propose that the occurrence of disease reduces the host-filtering effects on alien strains, resulting in enhanced competitions between probiotics and/or commensal bacteria with new detrimental colonizers. This assertion is based on the observations that increases in estimated migration rate (Table 2), reduced positive co-occurrence (Table 3) and shifts in the modules occur from Alphaproteobacteria and Actinobacteria species in healthy shrimp to Gammaproteobacteria species in diseased shrimp (Fig. 3). This pattern is consistent with the notion that Actinobacteria species, e.g., *Streptomyces* sp., are producers of antibacterial and growth-promoting substances [60]. In contrast, taxa affiliated with



Gammaproteobacteria, such as Vibrionaceae and Pseudoalteromonadaceae species, are well-known pathogens in coastal aquaculture environments [20, 58]. Accordingly, we infer that strong environmental changes exert stresses on shrimp, thus opening the niche for the detrimental colonizers, which in turn disrupts the cooperative syntrophy among taxa. Thus, the emergence of disease could be a result of changes in intestinal bacterial communities, combined with the disruptions in bacterial co-occurrence pattern.

It is now broadly recognized that the disruption of intestinal microbial homeostasis can result in disease [2, 61]. However, the interplay between phylogenetic and functional similarities is generally obscured by community functional redundancy [62]. For example, it has been reported that different shrimp species harbor distinct gut bacterial compositions, but these bacteria communities have similar biological functions [7]. It appears that the strength of composition and function association depends on the type of function examined and the stochasticity of a community [63]. Nevertheless, we used the same approach as Tzeng et al. [7] to predict metagenome function and found that disease-induced changes in intestinal bacterial community significantly altered biological functions in shrimp (Fig. 4a). In addition, there was a significant correlation between the phylogenetic and the functional structures of the bacterial communities (Fig. 4b). Similarly, substantial functional differences in gut microbiota have been identified between healthy and diseased infants by using metagenomic sequencing [64]. There are several hypotheses as to why such a pattern was observed. First, a host exhibits strong selective pressures on exogenous bacterial colonizers, which leads to the recruitment of phylogenetically coherent and adaptive specialists [22]. This assertion is supported by the notion that when bacterial communities are shaped by niche processes (e.g., host filters), it results in intensified linkages between function and composition [23]. Alternatively, the functional redundancy might be overstated [27, 65]; however, synchronized changes in microbial composition and function, e.g., respiration and enzyme activities, have been frequently detected [35, 36, 65]. Indeed, subtle fluctuations in intestinal bacterial composition between individual hosts contribute substantial differences in host susceptibility to gut invasions or probiotic therapy [4]. Similarly, there is evidence that the magnitude of changes in an intestinal bacterial community is associated with disease severity [2, 66]. These findings suggest that the degree of functional redundancy of intestinal microbiota is relatively low. Consistent with this notion, we found that a large number of bacterial KEGG pathways significantly changed between healthy and diseased shrimp (Fig. 5). Of particular interest, the patterns of pathway increase or decrease are correlated with their known functions. For example, focal adhesion is a virulence factor for pathogens to establish infection [67]; therefore, an increase in this pathway would be expected to promote invasibility. As a consequence, pathways

for diseases caused by infections, such as those used by *Helicobacter pylori* and Shigellosis infection, significantly increased in diseased shrimp compared to pathways in healthy shrimp (Fig. 5). In contrast, endocytosis and lysosome enzymes are known as control centers for cellular clearance and energy metabolism [68]. Accordingly, decreases in abundances of these pathways attenuate the capability of the immune system and metabolic processes needed for proper function (Fig. 5). Collectively, shifts in shrimp intestinal bacterial community could translate to a similar change in functions, which in turn results in the occurrence of disease.

To our knowledge, this is the first attempt to explore the effects of disease on shrimp intestinal bacterial community assembly. It is likely that stressed shrimp are more prone to be invaded by alien bacterial species (e.g., more stochastic assembly and higher migration rate), which in turn disrupts the cooperative activity among resident bacterial strains. In practice, knowing which bacterial strains have the competitive advantage (e.g., over-represented OTUs) to persist in gut is vital for selection of probiotic candidates. Notably, changes in intestinal assemblages significantly alter the biological functions in shrimp; therefore, the functional redundancy was low in shrimp gut community. For this reason, examining the bloom of under-represented OTUs (potential pathogens) could be a helpful approach in assessing the risk of disease. These findings of the present study provide valuable and complementary information to the ecological processes that govern intestinal bacterial community assembly in invertebrates between health statuses, rather than well-studied vertebrate gut communities. However, our data comes from a snapshot sampling, while shrimp intestinal communities are temporal dynamic over growth stages. Thus, a time-series design is needed to verify these ecological patterns in future work.

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