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Structure, dynamics and predicted functional ecology of the gut microbiota of the blue (*Haliotis fulgens*) and yellow (*H. corrugata*) abalone from Baja California Sur, Mexico

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The gastro-intestinal (GI) microbiota of abalone contains a highly complex bacterial assemblage playing an essential role in the overall health of these gastropods. The gut bacterial communities characterized so far reveal considerable interspecific variability, likely resulting from bacterial interactions and constrained by the ecology of their host species; however, they remain poorly investigated. Additionally, the extent to which structural changes in the microbiota entail functional shifts in metabolic pathways of bacterial communities remains unexplored. In order to address these questions, we characterized the gut microbiota of the northeast Pacific blue (*Haliotis fulgens* or HF) and yellow (*Haliotis corrugata* or HC) abalone by *16S rRNA* 454 pyrosequencing to shed light on: (*i*) their gut microbiota structure; (*ii*) how bacteria may interact among them; and (*iii*) predicted shifts in bacterial metabolic functions associated with the observed structural changes. Our findings revealed that *Mycoplasma* dominated the GI microbiome in both species. However, the structure of the bacterial communities differed significantly in spite of considerable intra-specific variation. This resulted from differences of the species with most reads in each GI metagenome, suggesting host-specific adaptation of bacterial lineages to these sympatric abalone. We hypothesize that the presence of exclusive OTUs in each microbiota may relate to host-specific differences in competitive pressure. Significant differences in bacterial diversity were found for the explored metabolic pathways between species despite their functional overlap. A more diverse array of bacteria contributed to each function in HC, whereas a single or much fewer OTUs were generally observed in HF. The structural and functional analyses allowed us to describe a deep taxonomic and functional split between the microbiota of HF and HC abalone.

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ABSTRACT

The GI microbiota of abalone contains a highly complex bacterial assemblage playing an essential role in the overall health of these gastropods. The gut bacterial communities characterized so far reveal considerable interspecific variability, likely resulting from bacterial interactions and constrained by the ecology of their abalone host species; however, they remain poorly investigated. Additionally, the extent to which structural changes in the microbiota entail functional shifts in metabolic pathways of bacterial communities remains unexplored. In order to address these questions, we characterized the gut microbiota of the northeast Pacific blue (*Haliotis fulgens* or HF) and yellow (*Haliotis corrugata* or HC) abalone by *16S rRNA* 454 pyrosequencing to shed light on: (*i*) their gut microbiota structure; (*ii*) how bacteria may interact among them; and (*iii*) predicted shifts in bacterial metabolic functions associated with the observed structural changes. Our findings revealed that *Mycoplasma* dominated the GI microbiome in both species. However, the structure of the bacterial communities differed significantly in spite of considerable intra-specific variation. This resulted from changes in predominant species composition in each GI metagenome, suggesting host-specific adaptation of bacterial lineages to these sympatric abalone. We hypothesize that the presence of exclusive OTUs in each microbiota may relate to host-specific differences in competitive pressure. Significant differences in bacterial diversity were found for the explored metabolic pathways between species despite their functional overlap. A more diverse array of bacteria contributed to each function in HC, whereas a single or much fewer OTUs were generally observed in HF. The structural and functional analyses allowed us to describe a deep taxonomic and functional split between the microbiota of HF and HC abalone.

Background

The gastro-intestinal (GI) tract of metazoans may be considered a highly complex ecosystem inhabited by a large number of bacteria [1]. For instance, the commensal microbiota harbored by the human GI tract far exceeds the total number of cells in the entire human body, and their collective genome (microbiome) is orders of magnitude larger than our own [1,2]. Moreover, the GI microbiome has been associated with essential physiological activities such as food digestion, nutrient assimilation, and defense against invasion of foreign bacterial species; which in turn may prevent epidemiologic outbreaks [3–5]. Also, functional studies have revealed that the relationship between the gut microbiome and its host may be so close that bacteria may be directly involved in the maturation of the GI tract of the hosts species [2,6,7].

As documented by cultured and uncultured approaches, the composition of the abalone gut microflora may be influenced by a great variety of factors such as diet, environmental conditions and ontogenetic stages [3,4,8–11]. Also, the use of probiotics has revealed that interspecific bacterial relationships may also shape the final gut microbiome composition of several marine invertebrates, including abalone [12–14]. Overall, these factors may explain the consistent differences in the gut microbiome of abalone species studied so far. In this context, the most abundant bacteria in homogenate samples of the entire GI of *H. discus hannai* were fermenter γ- *proteobacteria*, such as *Vibrio halioticoli* as well as other *Vibrio* species, α- *proteobacteria*, *Mollicutes* and *Fusobacteria* [10,16]. Moreover, the intestinal microflora (from stomach to anus) of *Haliotis diversicolo*r was dominated by *Mollicutes*, *Flammeovirga*, as well as β and α-, γ- and δ- *proteobacteria* [17]. In contrast, the bacterial composition of *H. gigantea* (from homogenate samples of the entire GI) appears less complex with a preponderance of γ- *proteobacteria* and *Mollicutes* [14].

The peninsula of Baja California harbors seven exploitable abalone species [18], two of which, the blue abalone *Haliotis fulgens* (HF, henceforth) and the yellow abalone *Haliotis corrugata* (HC, henceforth), sustain a high-valued fishery in the NW Mexican Pacific [18,19]. Despite the importance of the GI microbiomes for the survival of these abalone species, no efforts have been made to characterize them. Furthermore, it is equally uncertain which factors may shape their final composition as well as the functional roles played by the most representative bacterial groups.

Here, with the aim of addressing these questions, we analyze the structure of the GI microbiota of wild- caught specimens of HC and HF by means of *16S rRNA* amplification and 454 pyrosequencing (Roche). Additionally, we analyze the functional shifts involved in structural changes using a predictive metagenomic analysis to focus on 86 genes involved in several metabolic pathways.

Materials and Methods

Sample collection and genetic analyses.

Wild abalone (n = 31 HF, n = 35 HC) were sampled from the commercial harvested along the Pacific coast of central Baja California, Mexico. Approximately 30 mg of post esophageal tissue were dissected from visually healthy animals bearing no signs of the withering syndrome [24], and immediately transferred to sterile 1.5 ml microcentrifuge tubes containing molecular grade ethanol, until further analysis. Abalone and bacterial DNA was extracted and purified from preserved tissues using DNeasy blood & tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's protocols.

A fragment of the bacterial ribosomal *16S rRNA* spanning V1-V3 regions was PCR amplified using universal eubacterial primers 28F: 5' - GAGTTTGATCNTGGCTCAG - 3' [45] and 519R: 5' - GTNTTACNGCGGCKGCTG - 3' [46]. PCR reactions (20 µl) contained: 1X PCR Buffer (Kapa Biosystems, Woburn, MA, USA), 1.5 mM magnesium chloride (Kapa Biosystems, Woburn, MA, USA), 0.2 mM dNTPs (New England Biolabs), 0.5 µM each primer, 0.4 mM bovine serum albumin (New England Biolabs, Beverly, MA, USA), 1U *Taq* polymerase (Kapa Biosystems, Woburn, MA, USA), and 100 ng purified DNA. Thermal cycling consisted of an initial incubation at 94 °C for 4 min, followed by 40 cycles of: 94 °C for 1 min; 62°C for 30 sec. and 72 °C for 30 sec., and a final incubation of 8 min at 72 °C. Confirmation of amplification was carried out by 1.5% agarose gel electrophoresis. Amplicons were subsequently tagged using Roche 454 adaptors and multiplex identifier (MID) tags for each organism, following the bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) approach of Dowd *et al.* [47]. Following normalization, Roche 454 pyrosequencing was carried out in a GS FLX Titanium platform by Research and Testing Laboratory (Lubbock, TX).

Bioinformatic analyses

The *16S rRNA* reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software version 1.9.1 [48]. The first step consisted in demultiplexing and primer removal. Subsequently, reads were filtered according to Phred quality scores obtained from the

454 pyrosequencing. Acceptance quality criteria consisted of: (*i*) minimum and maximum lengths of 250 and 550 bp, respectively; (*ii*) default minimum quality Phred score of 25; (*iii*) no mismatch in primer sequences and MID-tag; (*iv*) maximum homopolymer length of 8 bp.

Sequences that met quality criteria were clustered in operational taxonomic units (OTUs) at 97% sequence similarity using the UCLUST algorithm [49]. The longest sequence from each OTU was selected as representative and these were subsequently aligned using Python Nearest Alignment Space Termination (also PyNAST) algorithm [50]. ChimeraSlayer [51] was used to detect and remove chimeras/singleton reads as implemented in QIIME. An additional chimera control test and taxonomic assignment were carried out by a BLAST search against the SILVA database (http://www.arb-silva.de/).

Ecological analysis

The number of reads of each OTU was used as an abundance proxy to estimate metagenomic diversity and structure of the abalone gut microbiota. Species richness (S), Shannon-Wiener (H) and Equitability (J) indices were calculated using Past V. 2.17c [52]. Taxonomic differences between abalone species, were evaluate by both Student t-tests using R [53] and by a linear discriminant analysis (LDA) effect size (LEfSe) [54]. To assess how exhaustively bacterial communities of both abalone species were sampled, rarefaction curves of discovered OTUs were generated for increasing numbers of sampled abalone. Also, OTU abundance was used to compute the non-parametric species richness estimator Chao 1 [55]. Rarefaction curves were obtained using the EstimateS V.9.0.1 program [56]. Microbiome community structure was evaluated using non-paramteric multidimensional scaling (MDS) analyses using Bray-Curtis and Sorensen similarity indices based on read abundance and on presence/absence, respectively, as implemented in PRIMER V.6 [57]. The statistical comparison of MDS results was performed with ANOSIM as implemented in Past V. 2.17c [52]. To determine which OTUs were primarily responsible for the similarities within each species and dissimilarity between HF and HC, a SIMPER analysis with square root transformed data was performed using PRIMER V.6 [57].

Bacterial interactions in the microbiomes of both abalone species were estimated using Jaccard distance (J_d) as implemented in PRIMER V.6 [57]. J_d is a measure of dissimilarity for all pairwise combinations of a data set [25] and was calculated using OTUs presence/absence. J_d

values close to 0 (from 0 to 0.33) were interpreted as co-occurrence (or putative mutualistic relationships) and values close to 1 (from 0.68 to 1) as interactions leading to exclusion (or putative competitive) [59]; whereas intermediate values were considered neutral relationships. We followed two approaches in the estimation of bacterial interactions. First, we computed J_d distances between all pairs of OTUs obtained from individual reads (putative species level). However, these mathematical results may not necessarily reflect a biological interaction. In order to improve the analysis, we posited that phylogenetically-related species share ecological functional attributes [23]. Thus, in an attempt to cluster bacterial species with similar functions and explore ecological interactions within those groups, we grouped OTUs at the ≥ 90% DNA sequence similarity and recomputed mean Jaccard distances within those groups only (i.e., referred to as "phylogenetic groups" henceforth). Distance matrices were computed with Geneious R9 [60]. Finally, in order to test the hypothesis that abundant bacteria in the microbiomes are subject to less competition and more neutral or positive interactions, we correlated the mean Jaccard distance (or $\bar{J}_d$) of each OTU with its abundance (i.e., number of reads).

Functional prediction of metagenomes

A phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) [61] was carried out to predict the functional attributes of metabolic genes from HF and HC GI microbiomes. Briefly, PICRUSt is a bioinformatic approach that uses information from a number of genetic markers, including the *16S rRNA*, to predict the metagenome functional content [61,62]. These predictions were obtained by matching our *16S rRNA* gene sequences against the prearranged genomic KEGG database [61,62]. The central result of PICRUSt consists of a table reporting the functional gene frequencies known as KEGG Orthologs (or KOs). KOs are hierarchically organized in sets of homologous sequences with known molecular function and assigned to biological pathways. We analyzed the data using the raw KOs counts as well as categorizing them by biological pathway. PICRUSt analyses use Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) as taxonomic and functional reference database. To implement quality control, we computed weighted nearest sequenced taxon index (NSTI) values for each individual metagenome. NSTI was developed to evaluate the prediction accuracy

of PICRUSt, since it reflects the average genetic distance (measured as number of substitutions per site) between an OTU against a reference genome [61,62]. Following suggested guidelines [61], we eliminated observations with a NSTI higher than 0.17.

In order to assess the contribution of individual OTUs to predicted KO functions, first we focused our analyses on genes involved in metabolic pathways (KEGG IDs from EC:1.1.1 to EC:6.5.1). Next, we categorized the relative importance of KO genes by ranking them according to their raw counts. These counts were obtained with PICRUSt v.1.1.0 [61] and were log normalized [63]. Finally, we focused our attention on a random subset (n =10) of KOs with the highest counts, as a first order analyses to characterize functional differences between these microbiomes. These analyses were carried out with the script *metagenome contributions.py*.

Finally, in order to compare the predicted ecological functions and the KOs abundance between microbiomes of both species of abalone, non-parametric MDS analysis based on Bray-Curtis similarity using log(x+1) transformation of data were performed in PRIMER V6 [57].

Results

Pyrosequencing and metagenome structure

Pyrosequencing yielded 451,095 raw *16S rRNA* reads of which 245,779 (54.5%) met quality criteria and were assigned to 281 OTUs, of which 87 had no match to databases (i.e., "No Hit"). The most abundant phyla (number of OTUs in parenthesis), included *Bacteroidetes* (13), *Fusobacteria* (14), *Proteobacteria* (56) and *Tenericutes* (96). Additionally, classified OTUs were assigned to 25 families and 47 genera (Table S1). The five most abundant OTUs (n > 15876 reads) were assigned to class *Mollicutes* (order *Mycoplasmatales*). Notably, LEfSe analysis revealed that 63 of 96 *Mollicutes* OTUs were exclusive to either in HC or HF (Fig. S1). Similar results were observed for other predominant bacterial families such as *Fusobacteriaceae* and *Vibrionaceae* (Fig. S1).

The classes *Fusobacteria*, *Mollicutes*, α- and γ-*protobacteira* comprised 99% of the identifiable reads. Rarefaction curves suggest that the bacterial communities were sufficiently sampled in both abalone species, given their asymptotic shape and the proximity of the observed number of taxa in each species to CHAO 1 estimates (Fig. 1). Species richness was significantly higher ($t = 6.07$; $p < 0.01$) in HF (mean $\pm$ S.D. HF: 56.45 ± 12.49; HC: 36.66 ± 13.16).

Despite their similar composition at the class level (Fig. 2), MDS analyses at the highest taxonomic resolution revealed a clear-cut structural difference between the metagenomes of both species, in the presence of considerable intraspecific variation, using read numbers as proxy for abundance (Fig. 3) or solely on the basis of presence/absence data (Fig. S2). Significant interspecific differentiation was corroborated by ANOSIM analyses in both cases ($R_{abundance} = 0.72$; $p < 0.001$; $R_{presence/absence} = 0.73$; $p < 0.001$).

Jaccard distances revealed that interspecific relationships among OTUs changed by an order magnitude with most involving competition (HC: 24,242 and HF: 24,118), followed by neutral (HC: 1321 and HF: 2123) and a smaller number of mutualistic interactions (HC: 315 and HF: 323). Furthermore, the $\bar{J}_d$ of the majority of OTUs decreased significantly with increasing read number in both species ($p < 0.001$; Fig. 4). The same trend was observed within phylogenetic groups (Fig. S3).

Functional profiling

Most abalone possessed mean NSTI values from 0.07 to 0.17, except for seven HCs with higher values that made them unreliable; hence they were excluded from further analyses. PICRUSt identified 4,201 KOs genes (Table S2) involved in 262 metabolic functions (Table S3). A one order of magnitude drop in log-normalized abundance was observed in the ranking of KOs (Fig S4); hence, *metagenome contribution*s analysis was carried out on a random set of 10 of the 86 most abundant Kos (i.e. Log (KOs counts > 4). According to PICRUSt, the metabolic functions in the HF microbiomes were generally enriched by one primary OTU, whereas many more OTUs contributed to the same function in HC (Fig.5). MDS analyses performed using both KOs genes and ecological function counts revealed no clear functional separation of the GI microbiota of both species, even though the scatter of individual microbiomes is much larger in HC, which is consistent with its higher diversity (Fig. 6).

Discussion

Abalone microbiome composition and dynamics

Our data revealed that the post esophageal microflora of HC and HF are dominated by the same bacterial classes. *Mollicutes*, mostly represented by *Mycoplasma* spp., was by far the most abundant class, followed by *Fusobacteria*, α-*proteobacteria* and γ- *proteobacteria*, with the latter represented by the genera *Vibrio* and *Francisella*. These bacteria have also been found dominating in the GI microbiota of other abalone species (*H. discus hannai*, *H. diversicolo*r and *H. gigantean* [10,14,16,17]). Even though the taxonomic composition of HC and HF GI microbiotas bears resemblance at high taxonomic levels, the species level composition showed significant differences. For instance, several *Mycoplasma* species predominant in one abalone were either absent or at low abundance in the other. Furthermore, all *Vibrio* spp. presented a higher prevalence in HC (Fig. S1). Notably, *Vibrio halioticoli* was absent in both HF and HC whereas it has been found at prevalence ranging from 40 to 65% in the GI of other marine invertebrates, including several abalone species [9]. The discrepancy may in part be related to the anatomical source of the microbiomes, our samples come from post esophageal tissue only whereas other studies have analyzed the entire GI tracts [14,16]. Consequently, the presence of *V. halioticoli* in the rest of GI tissues of HC and HF should be explored.

Our findings suggest that for most bacterial species their abundance decreased with increasing competition (lower $\bar{J}_d$ values), as observed for *Mycoplasma*. Furthermore, $\bar{J}_d$ for a single bacterium changed according to its host, which suggests that the same bacterial species faces different competitive pressures depending on the microbiota composition (Table S4). In other worlds, bacteria that may avoid competition in one microbiome, may be out competed in the other. However, this interpretation assumes untested hypothetical interspecific interactions [22]. Consequently, the same analysis was conducted focusing on genetically similar (≥ 90% similarity) OTUs, assuming that genetically related bacteria may share similar ecological roles [23]. This second approach produced similar results (Fig.S1), which may support the analysis carried out using all pairs of OTUs.

Some pathogenic bacteria, such as *Candidatus* Xenohaliotis californiensis and *Francisella*, were observed in the microbiomes of healthy abalone. The former has been recognized as the

etiological agent of the withering syndrome, a chronic wasting disease that possibly affects all north American abalone species [24]. This bacterium is a pleomorphic, gram-negative coccobacillus that inhabits abalone gastrointestinal epithelia and is considered an obligate endoparasite, like other *Rickettsiales* [25–27]. We observed the presence of *Candidatus* Xenohaliotis californiensis in healthy blue and yellow abalone, which supports that the presence of this pathogen is not sufficient to trigger withering syndrome as previously proposed [28]. Moreover, its absence and/or low intensity in abalone with morphological and histological signs of withering syndrome has already been reported [28–30]. Accordingly, we posit that further investigations are needed to reveal all the factors involved in withering syndrome outbreaks.

Francisella is a γ- proteobacterium and has previously been found in abalone microbiota [31]. It is a non-motile, pleomorphic gram-negative coccobacillus mainly known to be a facultative intracellular parasite of a wide range of hosts, including humans [31,32]. At the moment, this genus consists of five validated species [31]. Additionally, a novel species named *F. halioticida* [31] has been isolated from a die-off of farmed *H. gigantea* in Japan, for which it was identified as the etiological agent [33]. The *Francisella* ribotype detected in *H. corrugata* from Mexico was only 89% similar to *F. halioticida 16S rRNA* sequence (Genbank accession number NR_118116), which suggests a different species. Moreover, the presence of this bacterium in only HC organisms and its low prevalence (n = 2), suggests that this strain should not be considered an established member of the HC abalone bacterial core. Also, our limited data do not support the pathogenicity of the detected strain.

Finally, many bacterial genera were detected in low read frequencies and prevalences. Some of these (e.gr., *Acinetobacter*, *Alteromonas*, *Bacillus*, *Flavobacterium*) bear strong relationships with macroalgae [34] and may have been ingested while grazing.

Ecological functions of the abalone microbiome

According to the MDS of KOs gene counts and ecological functions, the inferred functionality of the GI microbiota of HF appears less variable than that of HC, which may reflect a higher degree of diet specialization. The diet diversity of wild HF in Baja California has been shown to be more limited and dominated by *Phyllospadix torreyi* (47%) and algae in the order *Gelidiales* (13%). On

the other hand, the diet of sympatric wild HC is more diverse consisting of different species of Phaeophyceae (10-20%), Rhodophyta (20%) and *Gelidiales* (20%) among others [35]. Possibly, those dietary preferences may be the result of bathymetric adaptations; indeed, HC are generally found in relative deep waters (between 10-20m), whereas HF generally inhabit shallow regions (between 3-10m) [35].

In the microbiota of both HC and HF, *Mycoplasma* contributed to all predicted KEGG. However, a given KO was generally enriched by a single predominant *Mycoplasma* OTU in HF, whereas in HC it was generally enriched by two or more *Mycoplasma* as well as other bacterial-OTUs (Fig.5). In other words, our results not only support that different *Mycoplasma* species may be highly host-specific [36], but also that they may also be highly specific to ecological tasks and/or to specific steps along a metabolic route. This may also be reflected by the relative low J_d (interpreted as absence of competitive interactions) of *Mycoplasma* OTUs. Indeed, ecological specialization should translate into niche partitioning and a reduction of inter-specific competition (Table S4).

To our knowledge, this is the first time that the ecological functions of *Mycoplasma* have been explored by a predictive analysis. *Mycoplasma* possess the smallest genome size among bacteria [7]; because of their small genome and number of genes, *Mycoplasma* species have been considered as obligate parasites and/or commensals [7,37]. Furthermore, our results suggest that these bacteria should be considered members of the bacterial core in the GI microflora of abalone.

Vibrio was the second most common genus observed enriching the majority of predicted KEGG. These bacteria are Gram-negative γ- *proteobacteria* that include both pathogenic and non-pathogenic species [38–40]. As it is the case in Asian abalone [9,14,17], our results confirm that *Vibrio* may be considered one of the main components of abalone GI microbiota in the eastern Pacific, at least in HC. Also, *Vibrio* has been found to play a pivotal role converting alginate to acetic acid [9,10]. In *Vibrio cholera*, possibly the best studied *Vibrio* species, the genome has been found to be dynamic, continuously acquiring and losing genes over time [38,41]. Consequently, as suggested by the PICRUSt analysis, *Vibrio* may be involved in a large number of ecological functions in the GI of abalone. We also speculate that the dominance of *Vibrio* in HC may contribute to their ability to process a wider spectrum of food sources.

PICRUSt analysis also revealed that several other bacteria may play major roles in several metabolic and/or enzyme production pathways. *Fusobacteriaceae* (mainly represented by

Psychrilyobacter) are obligate anaerobic gram-negative bacilli and are generally found in anoxic marine sediments. Species in this bacterial family have been detected in several marine invertebrate hosts such as shrimp [42], muricid snail [43], and abalone [16] and have been shown to be involved in the degradation of organic compounds [16,42,44]. Our findings suggest that these bacteria may be involved in several metabolic and/or enzyme production pathways such as glycero-phospholipid metabolism (K00057), starch and sucrose metabolism (K07024) and seleno-compound metabolism (K117170), among others, where they may take part in the production of dehydrogenases, lyases and other hydrolytic enzymes.

Conclusion

Using bTEFAP we characterized the microbiota of two commercially important and sympatric abalone in Mexico. Our results revealed novel microbiomes with significant shifts in bacterial species composition between them and with other species of abalone in the world. Given that these structural differences in microbiome composition do not necessarily result in distinct functional signatures, we posit that interspecific bacterial competition and the ecological differences of their host (i.e., diet and bathymetric distribution) may be responsible for these differences. These results may provide baseline references for future temporal and spatial sampling, and to assess microbiome changes related to ontogeny as well as physiological/health conditions. Additional efforts should also be directed towards understanding the roles of environment variables or other factors that may alter the GI microbiome of abalone.

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REFERENCES

1. Backhed F. Host-Bacterial Mutualism in the Human Intestine. Science 2005;307:1915–20. doi:10.1126/science.1104816

2. Bates JM, Mittge E, Kuhlman J, Baden KN, Cheesman SE, Guillemin K. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev. Biol. 2006;297:374–86. doi: 10.1016/j.ydbio.2006.05.006

3. ten Doeschate KI, Coyne VE. Improved growth rate in farmed *Haliotis midae* through probiotic treatment. Aquaculture; 2008;284:174–9. doi: 10.1016/j.aquaculture.2008.07.018

4. Zhao J, Shi B, Jiang QR, Ke CH. Changes in gut-associated flora and bacterial digestive enzymes during the development stages of abalone (*Haliotis diversicolor*). Aquaculture. 2012;338–341:147–53. doi: 10.1016/j.aquaculture.2012.01.016

5. Blaut M, Clavel T. Metabolic diversity of the intestinal microbiota: implications for health and disease. J. Nutr. 2007;137:751S–5S.

6. Bry L, Falk PG, Midtvedt T, Gordon JI. A model of host-microbial interactions in an open mammalian ecosystem. Science. 1996. p. 1380–3.

7. Bano N, DeRae Smith A, Bennett W, Vasquez L, Hollibaugh JT. Dominance of *Mycoplasma* in the guts of the Long-Jawed Mudsucker, *Gillichthys mirabilis*, from five California salt marshes. Environ. Microbiol. 2007;9:2636–41. doi:10.1111/j.1462-2920.2007.01381.x

8. Meryandini A, Junior MZ, Rusmana I. The Role of Agarolytic Bacteria in Enhancing Physiological Function for Digestive System of Abalone (*Haliotis asinina*). J. Appl. Environ.Biol. Sci. 2015;5:49–56.

9. Sawabe T, Setoguchi N, Inoue S, Tanaka R, Ootsubo M, Yoshimizu M, et al. Acetic acid production of *Vibrio halioticoli* from alginate: A possible role for establishment of abalone *V. halioticoli* association. Aquaculture. 2003;219:671–9. doi: 10.1016/S0044-8486(02)00618-X

10. Tanaka R, Sugimura I, Sawabe T, Yoshimizu M, Ezura Y. Gut microflora of abalone *Haliotis discus hannai* in. Fish. Sci. 2003;69:951–8. doi: 10.1046/j.1444-2906.2003.00712

11. Pang SJ, Xiao T, Bao Y. Dynamic changes of total bacteria and *Vibrio* in an integrated seaweed–abalone culture system. Aquaculture. 2006;252:289–97. doi: 10.1016/j.aquaculture.2005.06.050

12. Macery B., Coyne VE. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. Aquaculture. 2004;245:249–61. doi: 10.1016/j.aquaculture.2004.11.031

13. Rungrassamee W, Klanchui A, Maibunkaew S, Chaiyapechara S, Jiravanichpaisal P, Karoonuthaisiri N. Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). PLoS One. 2014;9:e91853.

14. Iehata S, Nakano M, Tanaka R, Maeda H. Modulation of gut microbiota associated with abalone *Haliotis gigantea* by dietary administration of host-derived *Pediococcus* sp. Ab1. Fish. Sci. 2014;80:323–31. doi: 10.1007/s12562-013-0691-9

15. Iehata S, Inagaki T, Okunishi S, Nakano M, Tanaka R, Maeda H. Improved gut environment of abalone *Haliotis gigantea* through *Pediococcus* sp. Ab1 treatment. Aquaculture. 2010;305:59–65. doi: /10.1016/j.aquaculture.2010.04.012

16. Tanaka R, Ootsubo M, Sawabe T, Ezura Y, Tajima K. Biodiversity and in situ abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. Aquaculture. 2004;241:453–63. doi: 10.1016/j.aquaculture.2004.08.032

17. Huang Z-B, Guo F, Zhao J, Li W-D, Ke C-H. Molecular analysis of the intestinal bacterial flora in cage-cultured adult small abalone, *Haliotis diversicolor*. Aquac. Res. 2010;41:e760–9. doi: /10.1111/j.1365-2109.2010.02577.x

18. Morales-Bojórquez E, Muciño-Díaz MO, Vélez-Barajas JA. Analysis of the Decline of the Abalone Fishery (*Haliotis fulgens* and *H. corrugata*) along the Westcentral Coast of the Baja California Peninsula, Mexico. J. Shellfish Res. 2008;27:865–70. doi: 10.2983/0730-8000(2008)27[865:AOTDOT]2.0.CO;2

19. SAGARPA. Sustentabilidad y Pesca Responsable en México. Evaluación y Manejo. 2009.

20. Thompson FL, Gevers D, Thompson CC, Dawyndt P, Hoste B, Munn CB, et al. Phylogeny and Molecular Identification of Vibrios on the Basis of Multilocus Sequence Analysis. Appl. Environ. Microbiol. 2005;71:5107–15. doi: 10.1128/AEM.71.9.5107-5115.2005

21. Hyun DW, Kim MS, Shin NR, Kim JY, Kim PS, Whon TW, et al. Shimia haliotis sp. nov., a bacterium isolated from the gut of an abalone, *Haliotis discus hannai*. Int. J. Syst. Evol. Microbiol. 2013;63:4248–53. doi: 10.1099/ijs.0.053140-0#tab2

22. Faust K, Raes J. Microbial interactions: from networks to models. Nat. Rev. Microbiol. Nature Publishing Group; 2012;10:538–50. doi: 10.1038/nrmicro2832

23. Chaffron S, Rehrauer H, Pernthaler J, Mering C. A global network of coexisting microbes from environmental and whole-genome sequence data. Genome Res. 2010;2010:947–59. doi: 10.1101/gr.104521.109

24. Friedman CS. Infection with *Xenohaliotis californiensis*. Man. Diagnostic Tests Aquat. Anim. 2012;511–23.

443 25. Friedman CS, Andree KB, Beauchamp K a, Moore JD, Robbins TT, Shields JD, et al.
444 "*Candidatus* Xenohaliotis californiensis", a newly described pathogen of abalone, *Haliotis* spp.,
445 along the west coast of North America. Int. J. Syst. Evol. Microbiol. 2000;50:847–55. doi:
446 10.1099/00207713-50-2-847#tab2
447
448 26. Moore JD, Finley C a., Friedman CS, Robbins TT. Withering syndrome and restoration of
449 southern California abalone populations. Calif. Coop. Ocean. Fish. Investig. Reports.
450 2002;43:112–7.
451
452 27. Crosson LM, Wight N, VanBlaricom GR, Kiryu I, Moore JD, Friedman CS. Abalone
453 withering syndrome: Distribution, impacts, current diagnostic methods and new findings. Dis.
454 Aquat. Organ. 2014;108:261–70. doi: 10.3354/dao02713.
455
456 28. Cáceres-Martínez J, Vásquez-Yeomans R, Flores-Saaib RD. Intracellular prokaryote
457 *Xenohaliotis californiensis* in abalone *Haliotis* spp. from Baja California, México. Cienc. Pesq.
458 2011;19:5–11. doi: 10.1099/00207713-50-2-847
459
460 29. Balseiro P, Aranguren R, Gestal C, Novoa B, Figueras A. *Candidatus* Xenohaliotis
461 californiensis and *Haplosporidium montforti* associated with mortalities of abalone *Haliotis*
462 *tuberculata* cultured in Europe. Aquaculture. 2006;258:63–72. doi:
463 10.1016/j.aquaculture.2006.03.046
464
465 30. Horwitz R, Mouton A, Coyne VE. Characterization of an intracellular bacterium infecting
466 the digestive gland of the South African abalone *Haliotis midae*. Aquaculture. 2016;451:24–32.
467 doi: 10.1016/j.aquaculture.2015.08.024
468
469 31. Brevik OJ, Ottem KF, Kamaishi T, Watanabe K, Nylund A. *Francisella halioticida* sp. nov.,
470 a pathogen of farmed giant abalone (*Haliotis gigantea*) in Japan. J. Appl. Microbiol.
471 2011;111:1044–56. doi: 10.1111/j.1365-2672.2011.05133.x.
472
473 32. Sjödin A, Svensson K, Ohrman C, Ahlinder J, Lindgren P, Duodu S, et al. Genome
474 characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in
475 pathogens of mammals and fish. BMC Genomics. 2012;13:268. doi: 10.1186/1471-2164-13-268
476
477 33. Kamaishi T, Miwa S, Goto E, Matsuyama T, Oseko N. Mass mortality of giant abalone
478 *Haliotis gigantea* caused by a *Francisella* sp. bacterium. Dis. Aquat. Organ. 2010;89:145–54.
479 doi: 10.3354/dao02188
480
481 34. Goecke F, Labes A, Wiese J, Imhoff J. Chemical interactions between marine macroalgae
482 and bacteria. Mar. Ecol. Prog. Ser. 2010;409:267–99. doi: 10.3354/meps08607
483
484 35. Guzman del Próo S a, Serviere-Zaragoza, Siqueiros-Beltrones D. Natural diet of juvenile
485 abalone *Haliotis fulgens* and *H. corrugata* (Mollusca: Gastropoda) in Bahia Tortugas, Mexico.
486 Pacific Sci. 2003;57:319–24.
487
488

489 36. Register KB, Thole L, Rosenbush RF, Minion FC. Multilocus sequence typing of
490 *Mycoplasma bovis* reveals host-specific genotypes in cattle versus bison. Vet. Microbiol.
491 2015;175:92–8. doi: 10.1016/j.vetmic.2014.11.002
492
493 37. Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, et al. The
494 Minimal Gene Complement of *Mycoplasma genitalium*. Science. 1995;270:397–403. doi:
495 10.1126/science.270.5235.397.
496
497 38. Heidelberg JF, Eisen J a, Nelson WC, Clayton R a, Gwinn ML, Dodson RJ, et al. DNA
498 sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature. 2000;406:477–
499 83. doi: 10.1038/35020000
500
501 39. Colwell RR. Global climate and infectious disease: the cholera paradigm. Science.
502 1996;274:2025–31. doi: 10.1126/science.274.5295.2025
503
504 40. Sawabe T, Inoue S, Fukui Y, Yoshie K, Nishihara Y, Miura H. Mass mortalities of Japanese
505 abalone *Haliotis discus hannai* caused by *Vibrio harveyi* infection. Microbes Environ.
506 2007;22:300–8. doi: 10.1264/jsme2.22.300
507
508 41. Okada K, Iida T, Kita-Tsukamoto K, Honda T. Vibrios commonly possess two
509 chromosomes. J. Bacteriol. 2005;187:752–7. doi: 10.1128/JB.187.2.752-757.2005
510
511 42. Chaiyapechara S, Rungrassamee W, Suriyachay I, Kuncharin Y, Klanchui A, Karoonuthaisiri
512 N, et al. Bacterial Community Associated with the Intestinal Tract of *P. monodon* in Commercial
513 Farms. Microb. Ecol. 2012;63:938–53. doi: 10.1007/s00248-011-9936-2
514
515 43. Ngangbam AK, Baten A, Waters DLE, Whalan S, Benkendorff K. Characterization of
516 Bacterial Communities Associated with the Tyrian Purple Producing Gland in a Marine
517 Gastropod. PLoS One. 2015;10:e0140725. doi: 10.1371/journal.pone.0140725
518
519 44. Hassenruck C, Hofmann LC, Bischof K, Ramette A. Seagrass biofilm communities at a
520 naturally CO2-rich vent. Environ. Microbiol. Rep. 2015;7:516–25. doi: 10.1111/1758-
521 2229.12282
522
523 45. Ludwig W, Mittenhuber G, Friedrich CG. Transfer of Thiosphaera pantotropha to
524 *Paracoccus denitrificans*. Int. J. Syst. Bacteriol. 1993;43:363–7. doi: 10.1099/00207713-43-2-
525 363
526
527 46. Ruff-Roberts AL, Kuenen JG, Ward DM. Distribution of cultivated and uncultivated
528 cyanobacteria and Chloroflexus-like bacteria in hot spring microbial mats. Appl. Environ.
529 Microbiol. 1994;60:697–704.
530
531 47. Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeehan T, Hagevoort RG, et al. Evaluation
532 of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX
533 amplicon pyrosequencing (bTEFAP). BMC Microbiol. 2008;8:125. doi: 10.1186/1471-2180-8-
534 125

48. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–6. doi: 10.1038/nmeth.f.303

49. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460–1. doi: 10.1093/bioinformatics/btq461

50. Caporaso JG, Bittinger K, Bushman FD, Desantis TZ, Andersen GL, Knight R. PyNAST: A flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010;26:266–7. doi: 10.1093/bioinformatics/btp636

51. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward D V., Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 2011;21:494–504. doi:10.1101/gr.112730.110

52. Hammer Ø, Harper D a. T, Ryan PD. Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 2001;4:9–18.

53. Team RC. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/. Http://www.R-Project.Org/. 2015. p. 2013.

54. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WSW, et al. Metagenomic biomarker discovery and explanation. Genome Biol. [Internet]. 2011;12:R60. doi: 10.1186/gb-2011-12-6-r60

55. Chao A. Nonparametric Estimation of the Number of Classes in a Population. Scand J Stat. 1984;11:265–70. URL: http://www.jstor.org/stable/4615964

56. Colwell RK. EstimateS Statistical estimation of species richness and shared species from samples. Version 9. User's Guide and application published at httppurl.oclc.orgestimates.pdf. 2013.

57. Clarke KR, Warwick RM. A further biodiversity index applicable to species lists: Variation in taxonomic distinctness. Mar. Ecol. Prog. Ser. 2001;216:265–78. doi: 10.3354/meps216265

58. Jaccard P. Nouvelles Recherches Sur la Distribution Florale. Bull. Soc. Vaudoise Sci. Nat. 1908;44:223–270.

59. Rahel FJ. Homogenization of Fish Faunas Across the United States. Science. 2000;854–6. doi: 10.1126/science.288.5467.854

60. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9. doi: 10.1093/bioinformatics/bts199

581
582 61. Langille M, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes J, et al. Predictive
583 functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat.
584 Biotechnol. 2013;31:814–21. doi: 10.1038/nbt.2676
585
586 62. de Voogd NJ, Cleary DFR, Polónia ARM, Gomes NCM. Bacterial community composition
587 and predicted functional ecology of sponges, sediment and seawater from the thousand islands
588 reef complex, West Java, Indonesia. FEMS Microbiol. Ecol. 2015;91:1–12. doi:
589 10.1093/femsec/fiv019
590
591 63. Urbanová Z, Bárta J. Microbial community composition and in silico predicted metabolic
592 potential reflect biogeochemical gradients between distinct peatland types. FEMS Microbiol.
593 Ecol. 2014;90:633–46. doi: 10.1111/1574-6941.12422

Figure 1

Sampling taxa control

Rarefaction curves of the cumulative number of observed OTUs with increasing number of samples and Chao 1 estimation of total OTU richness in microbiomes of *Haliotis fulgens* (HF) and *H. corrugata* (HC). The vertical line indicates the actual sample size above which the curve is extrapolated to reach Chao 1 estimator.

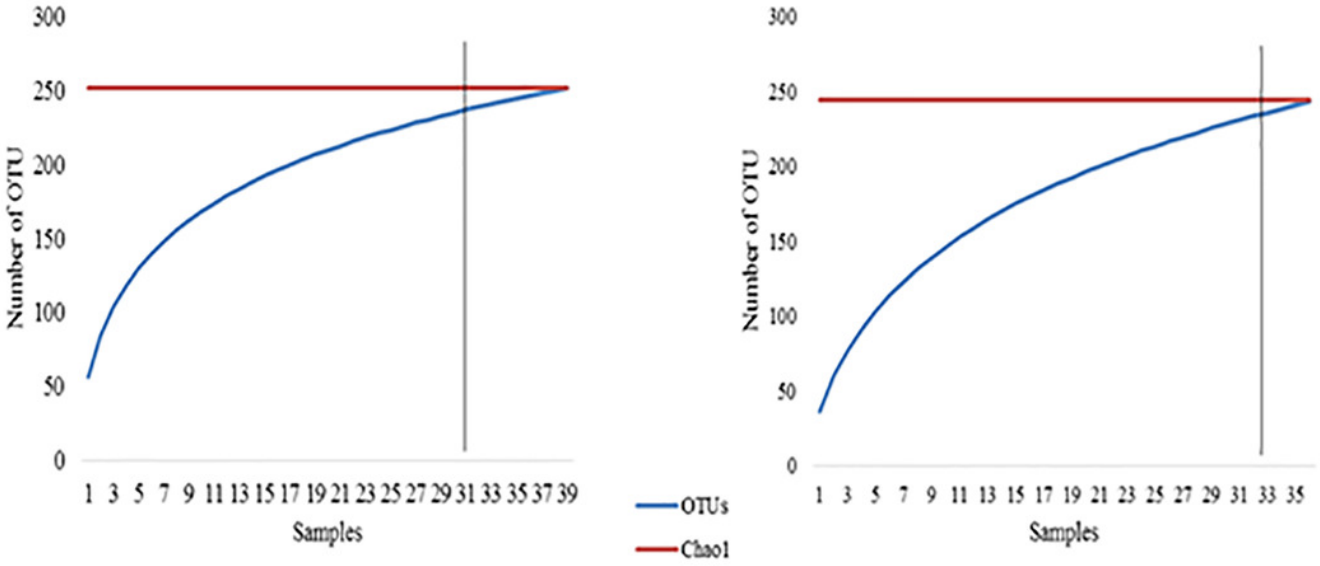

Figure 2

Abundance of detected bacterial classes

Major bacterial classes comprising the gut microbiota of *Haliotis fulgens* (HF) and *H. corrugata* (HC).

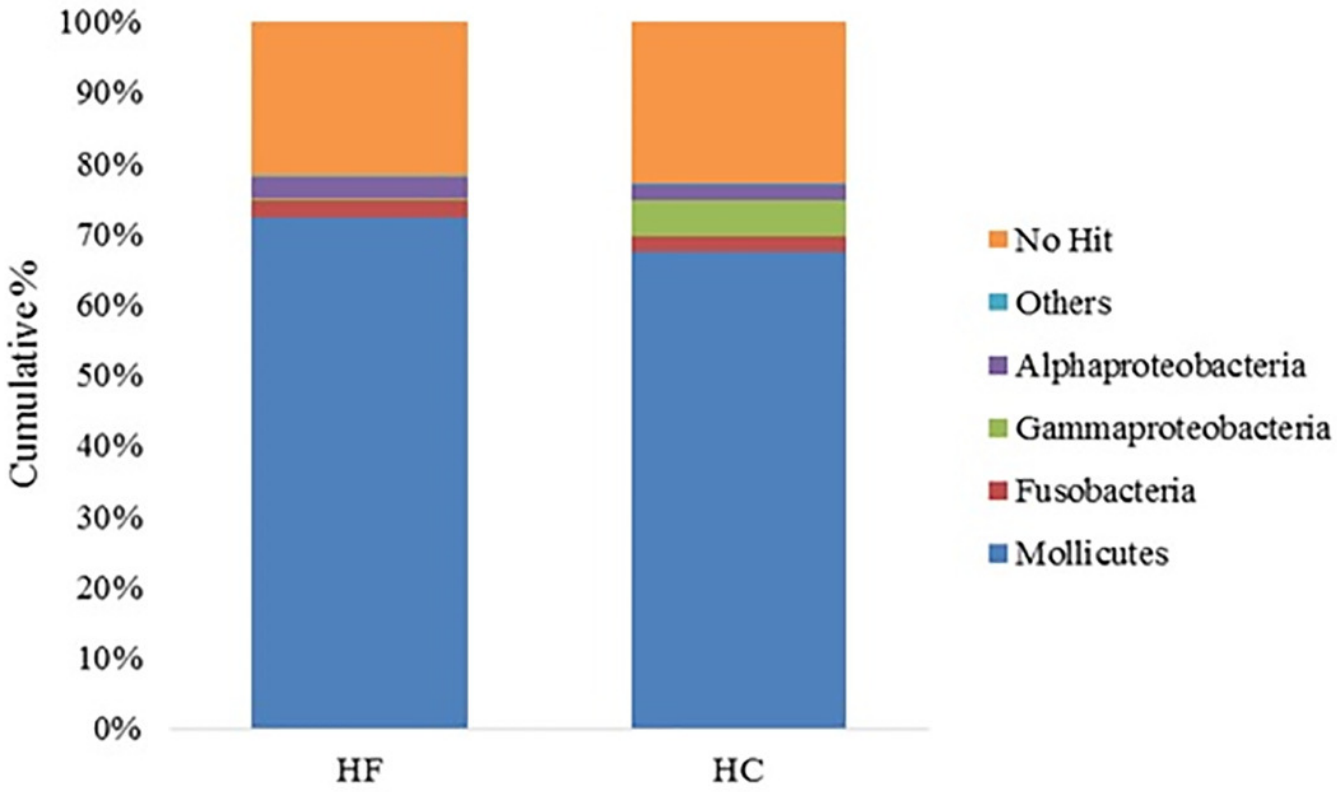

Figure 3

Microbiome taxonomic comparison

Non-metric multidimensional scaling (MDS) of Bray Curtis similarity index using log(x+1) transformed data based on read abundance of OTUs assembled at 97% similarity cut-off of the gut microbiota of *Haliotis fulgens* (HF) and *H. corrugata* (HC).

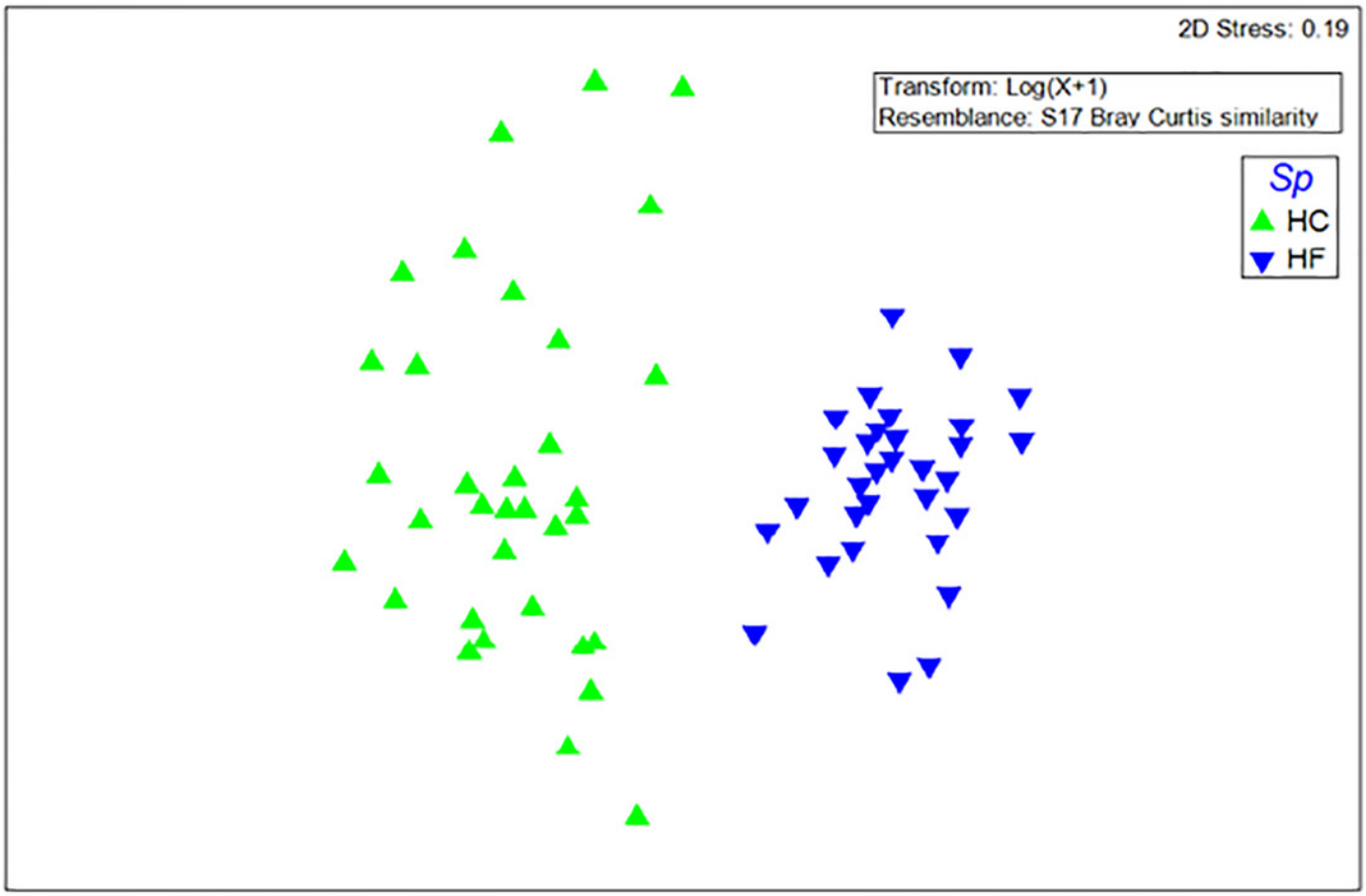

Figure 4

Bacterial interactions

Regression analysis of average Jaccard distance based on all pairwise relationships as a function of the number of OTUs (log scale) in microbiomes of *Haliotis fulgens* (HF) and *H. corrugata* (HC).

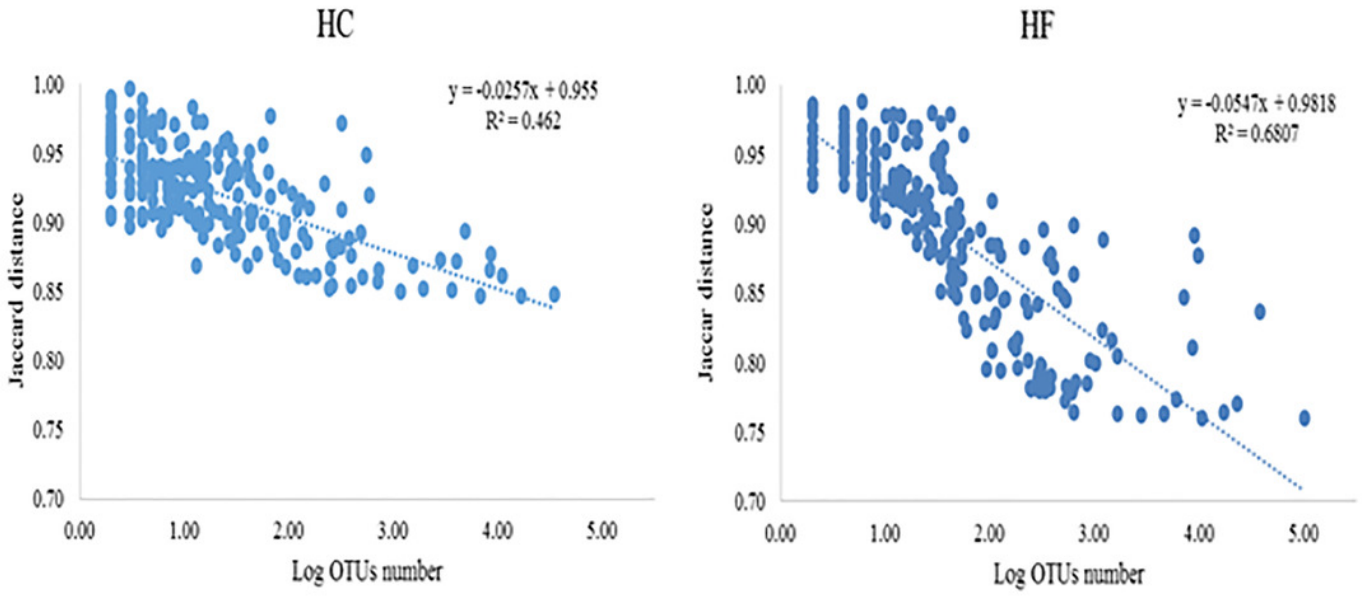

Figure 5

Bacterial functional ecology contributions

Bar plots of the relative bacterial gene count contributions of ten KOs with in the gut microbiomes of *Haliotis fulgens* (HF) and *H. corrugata* (HC) chosen at random among the 89 most abundant KOs (see text for details).

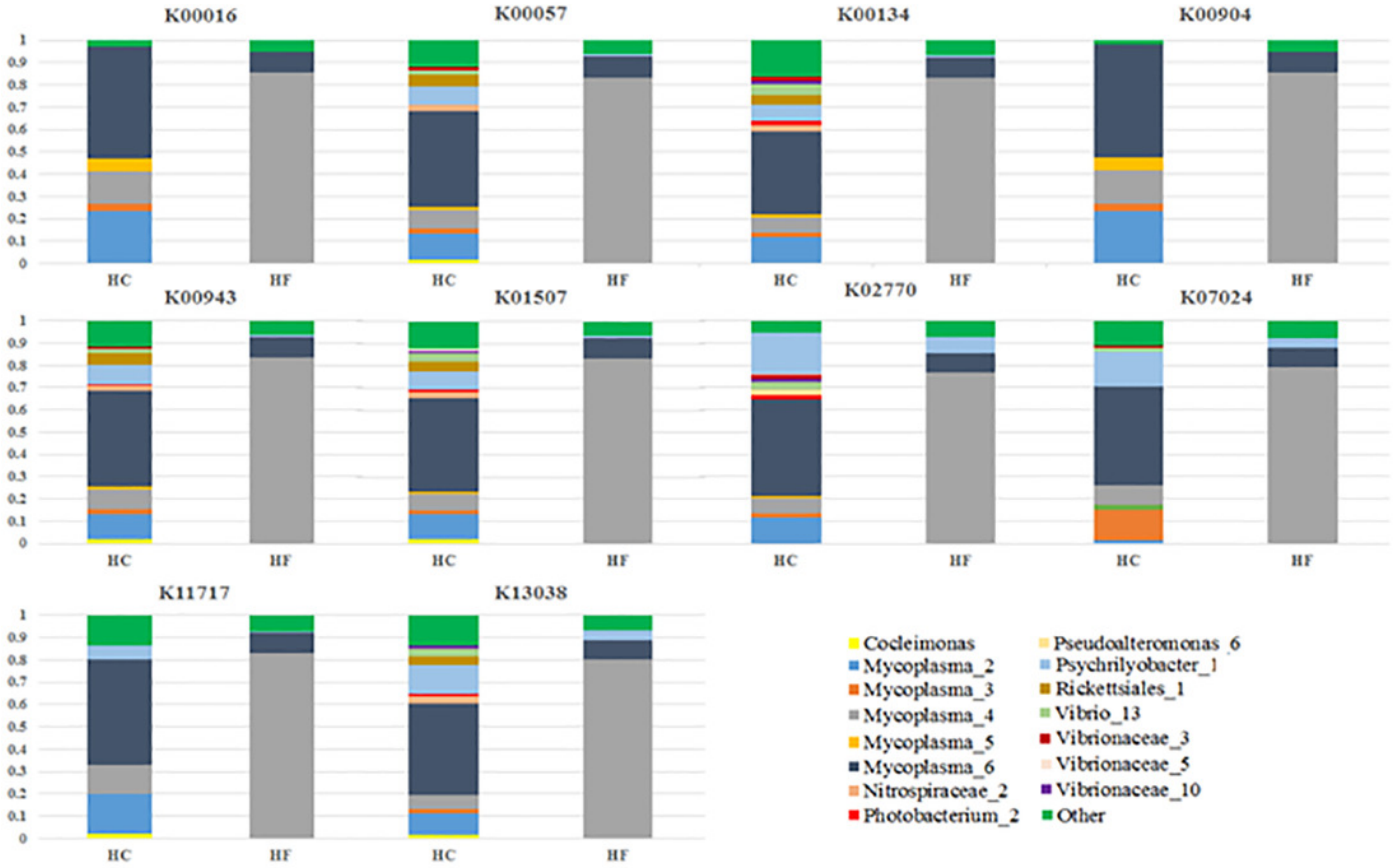

Figure 6

Comparison of predicted functional ecology

[A] Non-metric multidimensional scaling (MDS) of Bray Curtis similarity using log(x+1) transformed data of predicted ecological functions in the gut microbiomes of *Haliotis fulgens* (HF) and *H. corrugata* (HC). **[B]** MDS based on Bray Curtis similarity using log(x+1) transform data of KEGG orthologue counts in the gut microbiomes of *Haliotis fulgens* (HF) and *H. corrugata* (HC).