Comparative analysis of the bacterial community in the cuttlefish Sepia pharaonis with different statuses

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Abstract

The cuttlefish *Sepia pharaonis* is rich in nutritional value, and thus has been cultured in large-scale artificial culture. During its culture process in 2021 and 2022, a contagious disease with a mortality as high as 90% occurred when the dorsal mantle length of the cuttlefish was approximately 2.5 cm, which presented as obvious symptoms of small white spots on their dorsal mantle surface. To address the cause of the disease outbreak from a microbiome perspective, the bacterial communities in the healthy, infected, and dead cuttlefish *S. pharaonis* were assessed using bacterial culturing and 16S rRNA sequencing technology. Cultivable bacteria showed that there was an antagonistic phenomenon in the healthy *S. pharaonis*, and the highest diversity of bacteria was found in the dead individuals. The bacterial community was distinctly different among the three groups of samples, characterized by decreased alpha diversity and alteration of the dominant bacteria and bacterial community structures as the disease progressed. In addition, bacterial genera can be used as biomarkers for this disease, including *Photobacterium*, *Bacillus*, and *Halarcobacter*. In conclusion, the balance of the microbiome is necessary for *S. pharaonis* to remain healthy, and biomarker bacteria can aid in the early detection of occurrences of disease.

1. Introduction

Cuttlefish are found in the Indian Ocean and western Pacific Oceans, distributed from the Red Sea and southern Arabian Sea to the Andaman Sea, and from the South China Sea to Taiwan, Japan, eastern Indonesia, and northern Australia(Roper et al. 2010). The cuttlefish *S. pharaonis* is a cephalopod species that broadly inhabits shallow coastal waters up to a depth of 130 m (Domingues et al. 2005a). *S. pharaonis* is a culture species with a high spawning rate, fast growth rates, short lifespan, tolerance of high population density, disease resistance and adaptable feeding habits; moreover, it is considered a high-quality food because it contains high protein and low fat(Xiamin Jiang et al. 2019). Therefore, this species has been proposed as an excellent candidate for large-scale artificial culture(Nesis 1987; Minton et al. 2001; Domingues et al. 2005b; Barord et al. 2009).

Several diseases have been described during the culture of cephalopods, caused by a wide variety of pathogens belonging to different phyla, including fungi, viruses, bacteria, and protozoan and metazoan parasites (Gestal et al. 2019). In previous reports, parasites and bacteria isolated from individuals of cultured *S. pharaonis* cuttlefish were the main pathogens (Sangster and Smolowitz 2003; Tao et al. 2016; Lv et al. 2019). Miamiensis avidus (Ciliophora: Scuticociliatida) was reported to be associated with skin ulcers from reared *S. pharaonis* (Tao et al. 2016), and a virulence-related Vibrio *alginolyticus* pathogen has been isolated in *S. pharaonis* (Lv et al. 2019). In addition, Sangster (Sangster and Smolowitz, 2003) reported cases of V. *alginolyticus* infection in cephalopods, including cultured *S. pharaonis*, *Sepia officinalis* and *Sepia apama*. During the recent *S. pharaonis* cuttlefish culture in our lab, previous breeding trials have laid a good foundation for the industrialized culturing of *S. pharaonis* (Xiamin Jiang et al. 2019). However, a serious disease outbreak occurred in May 2021 in the Xiangshan Laifa Aquatic Nursery (Ningbo, China), which caused huge losses to the nursery.

All cultured organisms live in close association with the surrounding microbes (Sekirov and Finlay 2006). Microbial balance plays critical roles in maintaining the healthy status of the host(Iebba et al. 2016). The eubiosis state is beneficial for host defenses against infections since these microbes are involved in protection from pathogens and immune system development(Iebba et al. 2016).

Currently, several approaches such as culturing and high-throughput sequencing are being used to identify and describe organism microbiota(Lagier et al. 2018). Culturing is the oldest technique used to isolate and identify bacterial colonies(Lagier et al. 2015). In addition to identifying bacteria, culturing can provide bacterial strains that can be used in vitro to confirm the roles of specific bacterial species in disease occurrence using animal models(Lagier et al. 2018). Furthermore, bacterial species that are identified using culturing may provide health benefits and therefore can be potential candidates for bacteriotherapy as probiotics (Lagier et al. 2018). However, the majority of environmental bacteria remain unculturable (Steen et al. 2019). Nevertheless, high-throughput sequencing methods enable unculturable bacteria to be identified and microbial signatures to be associated with a particular physiological state or disease. In addition, sequencing enables the function of microbial communities to be inferred through the analysis of genomes and the coding potential(Lagier et al. 2018). With advances in sequencing techniques, some scientific communities have proposed that culture methods are no longer needed(Lagier et al. 2012, 2015); however, several studies have shown that sequencing methods cannot discriminate between live bacteria and transient DNA and are also defective in detecting minority populations (Vilanova and Porcar 2016; Bilen et al. 2018; Lagier et al. 2018). Thus, a combination of both techniques to study the organism microbiota can offer more information than the use of either method alone.

In this study, bacterial community succession and the infection process in the cuttlefish *S. pharaonis* were investigated by integrating bacterial culture and 16S rRNA amplicon sequencing approaches. This is the first study to explore the bacterial community of the cuttlefish *S. pharaonis*. The results of this study provide a reference for the prevention and control of disease in *S. pharaonis*.

2. Materials And Methods

2.1. Sample collection

The *S. pharaonis* cuttlefish were collected from Xiangshan Laifa Aquatic Nursery (Ningbo, China). The cuttlefish were aseptically sampled by first cutting off the head, leaving only the dorsal mantle portion, followed immediately by washing with phosphate buffered saline (PBS). One part was used for bacterial culture, and another part was preserved in liquid nitrogen.

2.2. Bacterial culture

The dorsal mantle was collected from healthy, infected, and dead eviscerated cuttlefish. The tissues were homogenized in PBS, serially diluted 10-fold, and 50 µL was coated onto 2216E agar under aseptic conditions and incubated overnight at 27°C.

2.3 Sample collection for microbiome analysis

The *S. pharaonis* cuttlefish were obtained from Xiangshan Laifa Aquatic Nursery (Ningbo, China). Three healthy, infected, and dead individuals were separately collected, and their dorsal mantle was aseptically collected into a 2 mL sterile plastic tube under sterile conditions and immediately stored in liquid nitrogen.

2.4. 16S rRNA gene library preparation and sequencing

The genomic DNA of the samples was first extracted, followed by agarose gel electrophoresis to detect the purity and concentration of the extracted DNA. Then, an appropriate amount of DNA solution was transferred into a centrifuge tube and diluted to 1 ng/mL with sterilized ddH2O. Phusion® High-Fidelity PCR Master Mix with GC Buffer and high-efficiency high-fidelity enzymes were used for PCR according to the amplification region to ensure amplification efficiency and accuracy. The highly variable regions (V3 to V4) of the bacterial 16S rRNA gene were amplified with primers 338F (5'-GTACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Kozich et al., 2013). PCR was performed with initial denaturation at 95°C for 2 min and 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension step at 72°C for 10 min. The PCR products were detected by electrophoresis on a 2% agarose gel, and the target bands were recovered using the gel recovery kit provided by Qiagen. The library was constructed using the NEBNext® Ultra™ IIDNA Library Prep Kit, and the constructed library was quantified by Qubit and Q-PCR; after the library was qualified, it was sequenced using NovaSeq6000.

2.5 Read assembly and taxonomic classification

The samples were split from the downstream data based on the barcode sequences and PCR amplification primer sequences, and the reads were spliced using FLASH (V1.2.11, http://ccb.jhu.edu/software/FLASH/) software to obtain raw tags. The clean tags were compared with the database using Usearch software to detect and remove chimeras, resulting in the final effective data, i.e., effective tags. Noise reduction was performed using the DADA2 module or deblur in QIIME2 software (DADA2 was used by default), and sequences with abundance less than 5 were filtered to obtain the final amplicon sequence variants (ASVs) as well as the feature list. Subsequently, the ASVs were compared with the database using the classify-sklearn module in QIIME2 software to obtain the species information of each ASV.

2.6 Statistical analysis

QIIME2 software was used to calculate the observed OTUs and Shannon, Simpson, and Chao1 indices and plot the boxplot. Microbial communities at the phylum and genus levels were represented by stacked columnar charts by using the relative abundance of the raw number of reads. Nonmetric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) were performed to evaluate the overall differences in bacterial community structures. LDA effect size analysis (LEfSe) is an analytical tool to determine and interpret biomarkers (taxonomic units, pathways, or genes) of high-latitude data,

enabling comparisons between two or more subgroups, as well as comparative analysis between subgroups within subgroups to determine species that differ significantly between abundance between groups (i.e., biomarkers)(Segata et al. 2011). To further explore the differences in community structure among different groups, LEfSe was selected to test the significance of differences in species composition and community structure of grouped samples(Segata et al. 2011).

3. Results

3.1. Symptoms of the diseased S. pharaonis cuttlefish

During the artificial culture of *S. pharaonis* in April 2021 and 2022, small white spots appeared on the dorsal mantle surface of individuals in Xiangshan Laifa Aquatic Nursery (Ningbo, China) (Fig. 1). The diseased cuttlefish lost appetite and lacked stability in the water. The disease was highly contagious, and once one individual in the cement pond began to show this symptom, the whole population in the same pond would soon be infected, and the mortality rate was as high as 90%.

3.2. Differences in the cultivable bacteria

Different phenomena were observed among the cultivable bacteria that appeared on 2216E media. Interestingly, one type of bacterium (H1) with high motility covered the whole plates in the samples collected from the healthy *S. pharaonis* cuttlefish and was irregularly scattered with several antagonistic circles, and the bacterium (H2) within the antagonistic circles was judged as the antagonistic bacteria of H1 (Fig. 2A). Bacterium similar to H1 also appeared in the infected individuals, but the antagonistic bacterium H2 was not detected, and more kinds of bacteria with different appearances were observed, named I1-4 (Fig. 2B). Bacteria similar to H1 also appeared in the dead individuals, but other bacteria were also dominant (Fig. 2C). This result suggests that there were different bacteria in healthy, infected, and dead individuals, which were associated with the disease outbreak.

3.3. Alpha diversity analysis

Assessment of the microbial diversity in the healthy, infected, and dead *S. pharaonis* cuttlefish was conducted by using α-diversity indices. The Chao1 index represents the species richness, and Fig. 3A shows that the healthy group had the lowest microbial abundance, followed by the infected and dead groups. The Shannon index represents the species diversity. The species diversity of the healthy group was lower than those of both the dead and infected groups (Fig. 3B).

3.4 Beta diversity analysis

Applying NMDS analysis, the information about the species in different samples is reflected in the multidimensional space in the form of points, while the degree of variation between different samples is reflected by the distance between points and the inter- and intragroup differences of the samples. As shown in Fig. 4, the stress was 0.018 (< 0.02), indicating that NMDS could accurately reflect the degree of variation between samples. Based on the ASVs among different samples, the NMDS ordination indicates

that there were significant differences in the bacterial communities of the healthy group and the other two groups. This variation pattern is further confirmed by the ANOSIM results, indicating significant differences (ANOSIM P = 0.015 < 0.05) among bacterial communities from healthy, infected, and dead individuals.

3.5 Composition and diversity of bacterial communities in the dorsal mantle of healthy, infected, and dead S. pharaonis

To characterize the microbial communities in the healthy, infected, and dead *S. pharaonis* cuttlefish, nine dorsal mantle samples were collected for 16S rRNA gene sequencing (3 specimens in each group). The results show that Proteobacteria was the main dominant phylum (91.43 ± 4.64%) in the healthy individuals (Fig. 5A). The mean relative abundance of the phylum Proteobacteria in the dorsal mantle decreased significantly from 91.43 ± 4.64% to 39.37 ± 6.21% as the disease progressed, and Firmicutes became the dominant phylum in the diseased individuals (Fig. 5A). It is noteworthy that the mean relative abundance of Campilobacterota was significantly higher in the dead group than in the other two groups (Fig. 5A). Notably, the infected and dead groups contained the Cyanobacteria phylum, which was absent in the healthy group (Fig. 5A). At the genus level, the average relative abundance of *Photobacterium* was 44.32 ± 12.27%, which was significantly higher in the healthy group than in the other two groups (Fig. 5B). The presence of *Photobacterium* was barely observed in the infected and dead groups (Fig. 5B). The average relative abundance of *Bacillus* spp. was 23.82 ± 13.30%, which showed the most abundant genus in the infected group, and the average relative abundance of *Halarcobacter* spp. was 22.79 ± 14.10%, which was the most abundant species in the dead group (Fig. 5B).

3.6 LEFSe Analyses

The histogram of the distribution of LDA values shows the species with LDA scores greater than 4. These species were significantly different among groups. The length of the bar chart represents the effect size of the differential species. As shown in Fig. 5A and Fig. 6, at the phylum level, the significant difference in the healthy group was observed for the phylum Proteobacteria; the significant difference in the dead group was observed for the phylum Campylobacteria. At the genus level, the significant difference in the infected group was observed for the genus *Bacillus*; the significant differences in the dead group were observed for the genera *Halarcobacter* and *Psychrilyobacter* (Fig. 6). At the species level, the significant differences in the healthy group were observed for the species Photobacterium_swingsii and Photobacterium_jeanii; the significant difference in the dead group was observed for the species Sutterella_wadsworthensis (Fig. 6). These dominant biomarkers contributed to the differences among the dorsal mantles of the healthy, infected, and dead *S. pharaonis* cuttlefish.

4. Discussion

Dysbiosis in the microbiome has been associated with numerous diseases (Bäckhed et al. 2012; Petersen and Round 2014; Trompette et al. 2014; Lloyd-Price et al. 2016) and could be considered a perturbation that departs from an otherwise balanced ecology to prolong, exacerbate, or induce a detrimental health

effect(Petersen and Round 2014). Thus, finding features that broadly distinguish the microbiomes of healthy and unhealthy individuals will aid in the diagnosis of microbiology-related diseases and could potentially provide new means to prevent disease (Lloyd-Price et al. 2016).

Cultivable bacteria from the cuttlefish *S. pharaonis* of different statuses showed obvious differences in our study (Fig. 2). The antagonistic activity between bacteria plays an important role in host resistance to disease(Peterson et al. 2020). In our study, the bacterium H2, which showed obvious antagonistic activity to the bacterium H1, only appeared in the healthy individuals, which may indicate that antagonism plays important roles in maintaining the stability of bacterial communities(Waksman 1941). We speculate that the homeostasis of the bacterial community of infected cuttlefish *S. pharaonis* was affected. In future studies, we will further investigate the antagonistic mechanisms of H1 and H2 in *S. pharaonis* and the application of antagonistic bacteria as probiotics.

The microbial diversity was lower in healthy conditions than in infected conditions in the *S. pharaonis* cuttlefish in our study when using both culture methods and microbiome measurements, and similar findings have been confirmed in the diseased shrimp bacterial community(Yao et al. 2018). This result indicates that a reduction in diversity could lead to a decrease in the functional stability of a bacterial community (Yiu et al. 2017). Combined with the present results, the lower bacterial richness and diversity may be maximized to mitigate the risk of disease occurrence. In addition, disease-influenced host health status might alter bacterial compositions and reduce the diversity of the bacterial community(Yiu et al. 2017). Additionally, our comprehensive comparison revealed that the structures of the bacterial community differed substantially between health status categories in the current study (Fig. 4), which was similar to a study of shrimp samples whose bacterial community was clustered by health state (Yao et al. 2018).

In the present study, significant differences in the composition of microbial communities were observed across the cuttlefish (Fig. 5). Our results show that for *S. pharaonis*, the genus *Photobacterium* accounted for the largest proportion in the healthy group (Fig. 5B), which indicates that there is a possible link between *Photobacterium* and the health of the cuttlefish *S. pharaonis*. *Photobacterium* species are Gram-negative coccobacilli that are distributed in marine habitats worldwide(Moi et al. 2017). They are unique because of their capability of producing luminescence and are important symbionts (Urbanczyk et al. 2011; Moi et al. 2017). Previous studies have demonstrated the crucial role of *Photobacterium* in cephalopods(Bloodgood 1977; Barbieri et al. 2001; Collins et al. 2012; Gromek et al. 2016). Therefore, *Photobacterium* may be a beneficial bacterium for *S. pharaonis* cuttlefish. Additionally, Bacillus was the predominant genus in the infected *S. pharaonis* (Fig. 5B). The genus *Bacillus* is the largest, most diverse, and most prominent genus of aerobic endospore-forming bacteria(Fritze 2004). The genus *Bacillus* includes species with diverse natural histories, including free-living nonpathogenic heterotrophs such as B. subtilis and host-dependent pathogens such as Bacillus anthracis (the etiological agent of the disease anthrax) and Bacillus cereus(Chang et al. 2021). This result is also supported by previous studies showing that the genus *Bacillus* was also detected in infected Cephalopoda (Hanlon and Forsythe 1990). Moreover, B. cereus has been reported to cause disease in a variety of aquatic organisms, such as soft-

shelled turtle (Trionyx sinensis) (Zhang et al. 2022) and Litopenaeus vannamei(Velmurugan et al. 2015). Our results indicate that the presence of *Bacillus* spp. may be associated with the disease outbreak of the cuttlefish *S. pharaonis*.

A biomarker is a characteristic that can be used to evaluate and measure disease (Moreno-Arrones et al. 2020). LEfSe is used to identify biomarkers that determine the features most likely to explain differences between classes (Segata et al. 2011). *Photobacterium* is one of the oldest discovered genera in the family of Vibrionaceae under the class Gammaproteobacteria of the phylum Proteobacteria (Moi et al. 2017). According to Fig. 6, *Photobacterium* is likely a biomarker bacteria of the healthy cuttlefish *S. pharaonis*. The genus *Bacillus*, a member of the phylum Firmicutes (Logan and Vos 2015), are a bacterial biomarker bacteria of the infected *S. pharaonis* cuttlefish. *Halarcobacter*, a genus in the family Arcobacteraceae under the class Campylobacteria of the phylum Campilebactereta(On et al. 2020), are a bacterial biomarker bacteria of the dead *S. pharaonis* cuttlefish. Therefore, the possible use of these bacteria as predictive biomarkers of disease (i.e., speculating the healthy states of *S. pharaonis* cuttlefish) should be studied in the future.

In conclusion, we found that there were different bacterial community compositions among the healthy, infected, and dead *S. pharaonis* cuttlefish, in both the culturable bacteria and microbiome. Taken together, concomitant with disease outbreaks, the bacterial community of the cuttlefish *S. pharaonis* experienced a series of variations: different cultivable bacteria appeared on media, the α-diversity decreased, and the taxonomic structure of the *S. pharaonis* cuttlefish bacterial community composition became significantly different along with the changes in the bacterial biomarkers. These findings indicate that the health of the *S. pharaonis* cuttlefish is highly relevant to the homeostasis of its bacterial community. Preservation and restoration of the bacterial community equilibrium offer an effective strategy for *S. pharaonis* cuttlefish disease prevention.

Declarations

Ethical Approval and Consent to participate

The cuttlefish *Sepia pharaonis* were raised in Laifa Aquaculture company (Ningbo, Zhejiang). All experiments were carried out in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The research was conducted according to the Experimental Animal Ethics Committee of Ningbo University, China.

Consent for publication

With the submission of this manuscript, I would like to confirm that the above-mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute's Ningbo University representative is fully aware of this submission.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Competing interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Comparative analysis of the bacterial community in the cuttlefish Sepia pharaonis with different statuses”.

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Authors' contributions

Liting Xu: Conceptualization, methodology, software, formal analysis, validation, investigation, resources, writing - original draft, writing - review & editing

Yilong Ruan: Significantly contributed to the experiment

Maowang Jiang: Contributed to the conception of the study

Ruibing Peng: Contributed to the conception of the study

Xiamin Jiang: Conceptualization, methodology, visualization, supervision, project administration, funding acquisition

Weiwei Zhang: Conceptualization, methodology, visualization, supervision, paper revision

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Figures

Figure 1

The exterior appearance of the healthy cuttlefish *S. pharaonis* and infected cuttlefish *S. pharaonis.*

Figure 2

Cultivable bacteria from the cuttlefish *S. pharaonis*. (A) The bacteria from the tissues of the healthy individuals, H1, H2 and H3 are the three kinds of bacteria observed with naked eyes; (B) The bacteria from the tissues of the infected individuals, I1, I2, I3, I4 and I5 are the five kinds of bacteria observed with naked eyes; (C) The bacteria from the tissues of the dead individuals, D1 and D2 are the two kinds of bacteria observed with naked eyes.

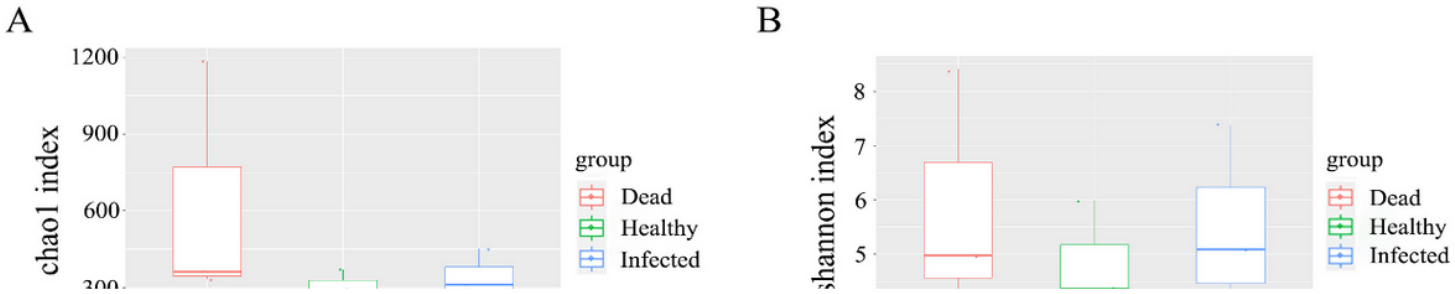

Figure 3

The boxplot of α- diversity indices. (A) Chao1 index of three groups of samples; (B) Shannon index of three groups of samples.

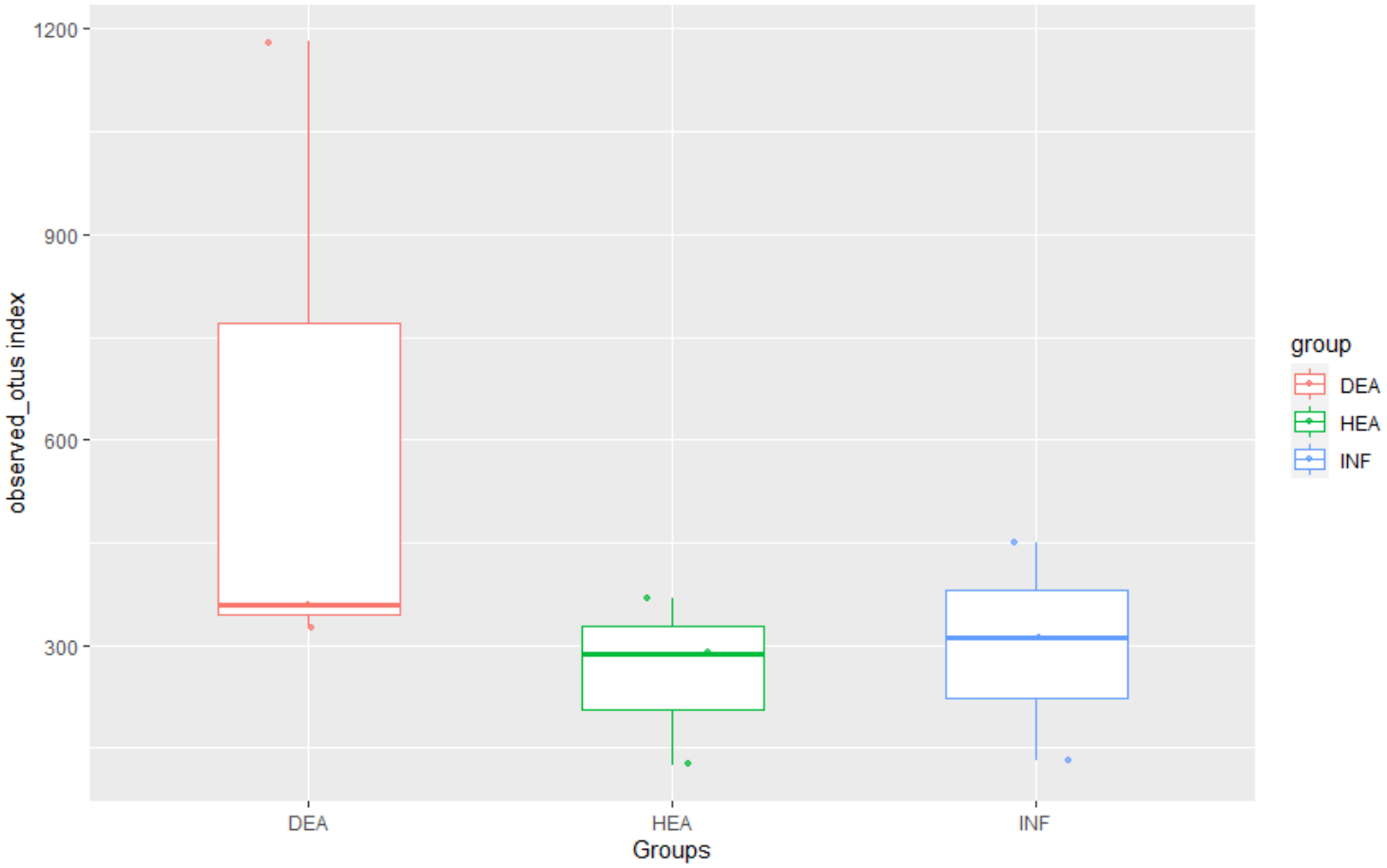

Figure 4

NMDS plot visualized the dissimilarities of bacteria from healthy, infected, and dead cuttlefish *S. pharaonis.*

Figure 5

Relative abundance histogram of the three samples at the phylum and genus levels. (A)Relative abundance of the top 10 bacteria at the phylum level. The other phyla are included as “Others” (B)Relative abundance of the top 10 bacteria at the genus level. The other genera are included as “Others”.

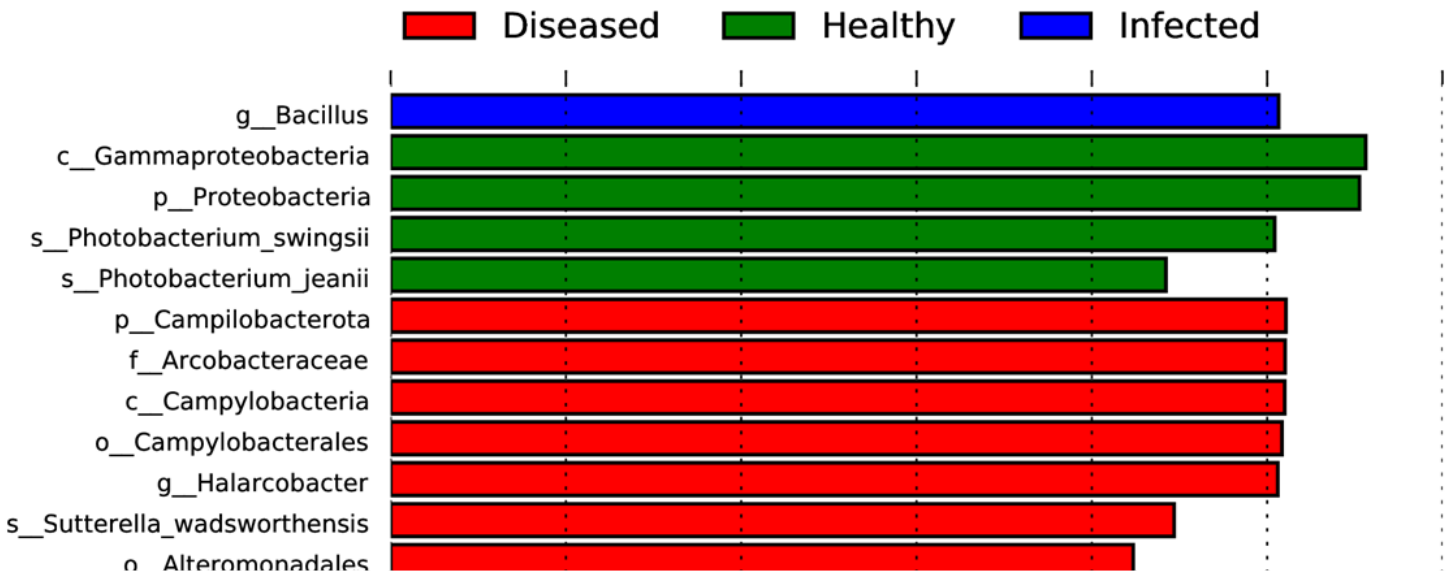

Figure 6

The different biomarker bacteria in the three groups.