Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Behavior and gut health of the sea cucumber Apostichopus japonicus with Frontonia infection

Yingzhuo Sun
Dalian Ocean University
Yunjie Yang
Dalian Ocean University
Xiajing Wang
Dalian Ocean University
Yang Yang
Dalian Ocean University
Sihan Wang
Dalian Ocean University
Chong Zhao
chongzhao@dlou.edu.cn
Dalian Ocean University

Research Article

Keywords: Sea cucumber, Frontonia, behavior, intestinal protease activity, intestinal microbiological

Posted Date: November 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10793942/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Behavior and gut health of the sea cucumber *Apostichopus japonicus* with *Frontonia* infection

Yingzhuo Sun, Yunjie Yang, Xiajing Wang, Yang Yang, Sihan Wang, Chong Zhao[1]

Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture and Rural Affairs, Dalian Ocean University, Dalian 116023, China.A

Abstract

Adverse aquaculture environments frequently induce diseases in the sea cucumber *Apostichopus japonicus*, and *Frontonia* infection is a prevalent condition that severely impairs land-based sea cucumber culture efficiency. The present study clarified the effects of *Frontonia* infection on the behavioral traits and intestinal health of sea cucumbers. Behavioral observations showed that infected sea cucumbers significantly reduced feeding capacity, sharply increased wall-climbing tendency, and disturbed adhesion and movement-related behaviors, while their righting and foraging behaviors remained unaffected, with abnormal foraging movement trajectories as a typical characteristic. Physiologically, *Frontonia* infection altered the intestinal morphological structure of sea cucumbers, but caused no significant difference in intestinal pepsin-like protease activity between infected and healthy individuals. 16S rRNA gene sequencing revealed that the infection only induced few changes in individual intestinal bacterial taxa, without affecting the overall richness, diversity and core structure of the intestinal microbiota, which is quite different from the intestinal microbiota imbalance caused by sea cucumber enteritis. These results indicate that *Frontonia* infection is probably not a typical intestinal infectious disease, but a stress response of sea cucumbers to parasitic infestation. This study provides new insights for the accurate diagnosis of *Frontonia* infection and scientific guidance for targeted aquaculture prevention and control. We suggest aqua-farmers adopt

relieving stress management measures and take effective preventive measures against the infected surface of sea cucumbers with *Frontonia* infection.

Keywords: Sea cucumber, *Frontonia*, behavior, intestinal protease activity, intestinal microbiological

---

* Corresponding author: Chong Zhao: chongzhao@dlou.edu.cn

1. Introduction

Sea cucumbers (*Apostichopus japonicus*) are recognized for their high edible value and important medicinal properties (Li et al., 2016). Consequently, the sea cucumber industry has been regarded as a highly valued and rapidly developing new economic growth point in the aquaculture sector of northern China. Substantial contributions to the local economy are made by the production efficiency of sea cucumber farming. However, with the development of the sea cucumber aquaculture industry, the efficiency and economic benefits of sea cucumber aquaculture have been severely affected by various diseases (Han et al., 2016). Infection by *Frontonia* is considered as a common disease in sea cucumber aquaculture. This disease has been widely detected in many sea cucumber farming regions across China (Wang et al., 2018).

Frontonia is a typical parasitic ciliate that poses important threats to sea cucumber aquaculture. From a taxonomic perspective, it is classified under the phylum Ciliophora and class Scuticociliatea, among which *Frontonia labialis* is identified as the dominant disease species infecting sea cucumbers (Deng et al., 2005). The respiratory tree tissues and body surface folds of sea cucumbers are primarily colonized by this creature, which can invade internal tissues and cause mechanical damage in hosts. *Frontonia* disease is recognized as a common disease in major sea cucumber producing areas such as Liaoning and Shandong provinces in China. Both juvenile and adult sea cucumbers are susceptible, and no obvious size specificity is observed (Deng et al., 2024). Currently, numerous deficiencies still exist in domestic research on sea cucumber *Frontonia* disease. To date, the specific relationship between *Frontonia* parasitism and abnormal sea cucumber behavior has not been clarified. There is no definitive conclusion has been reached on whether this parasite interferes with the host's intestinal digestive capacity or disrupts the balance of the

intestinal flora. The insufficiency of basic mechanistic research makes it difficult for aqua-farmers to identify behavioral characteristics induced by *Frontonia* infection and to establish corresponding disease identification systems. To some extent, *Frontonia* infection has hindered the development of ecological and healthy sea cucumber farming models.

This study aims to investigate: 1) whether the feeding, foraging, adhesion, righting and wall-climbing behavior of sea cucumbers are affected by *Frontonia* infection, and 2) whether pepsin activity and intestinal flora structure change in sea cucumbers with *Frontonia* infection.

2. Material and method

2.1 Sea cucumbers

The sea cucumbers used in the experiment were collected from a sea cucumber hatching room in Wafangdian, Dalian (121º44′ E, 39º51′ N). The water source and aquaculture facilities at the collection site were free of contamination by *Frontonia*. The sea cucumbers were temporarily raised in water tanks and fed with self-prepared feed (a mixture of seaweed powder and sea mud in a ratio of 1: 4, with a daily feeding amount of 10% of their body weight) until the start of the experiment. During the temporary raising period, the sea cucumbers were kept in an environment free from light. The water used for raising was changed by half every three days to maintain its freshness and cleanliness, and was aerated throughout the process. The portable water quality monitor (HACH, HO40d, USA) measured the water temperature at 16 °C ± 0.5 °C and the salinity at 31.3 ± 0.15.

2.2. Behavioral experiments

This experiment comparatively studied the foraging, feeding, righting and adhesion behaviors of infected and normal sea cucumbers to obtain accurate information on the

impact of *Frontonia* on the behaviors of sea cucumbers. All behavioral experiments were conducted between sea cucumbers parasitized by *Frontonia* (group A) and normal sea cucumbers (group B). The method of infecting the sea cucumbers in group B with parasites is as follows. We first obtained the *Frontonia* population from the infected live sea cucumbers, and expanded the culture in the culture solution containing live sea cucumber tissue. During the temporary rearing period in group B, we continuously contaminated the rearing water with the water containing *Frontonia* to parasitize the healthy sea cucumbers. Before the experiment began, we sampled and tested the sea cucumbers, dissected them and removed the water lung tissue, selected the inner wall to make slides, and checked the sections under a microscope (Nikon SMZ1500, Nikon, Japan). Judging criteria were as follows: under a microscope at 40x magnification, if the number of *Frontonia* in one of view was more than 30, it was considered a severe infection (Deng et al., 2024). After each experiment, we dissected the sea cucumbers to ensured that all the data in group B came from severely infected sea cucumbers. The methods for measuring foraging, feeding, righting and adhesion behaviors are described as follows.

2.2.1 Foraging behavior

Sea cucumbers were selected from groups A and B. We placed the sea cucumbers in the middle of a tank (length × width × height: 70 cm × 30 cm × 50 cm), and the food was placed at the other end of the tank. We used a digital camera with infrared function (Legria, HF20, Canon) to record the foraging time and crawling track of the sea cucumbers within 30 minutes. Foraging time refers to the time it takes for an individual sea cucumber to successfully reach the area of the food (Zhao et al., 2023). If the sea cucumber failed to find the food within 30 minutes, the foraging time is calculated as 1800 seconds (Sun et al., 2023). The experiment was conducted at 8

p.m., with only a weak light source maintained for shooting. The temperature was 16 °C ± 0.5 °C and the salinity was 31.1 ± 0.15. Based on the recorded videos, we analyzed the foraging process of the sea cucumbers. We used the ImageJ software (version 1.51n) to analyze the movement of the sea cucumbers. In each video, an image was captured every 5 seconds. We used the plugin in the ImageJ software (version 1.51n) to extract the head and tail coordinates of the sea cucumbers in each image to track their head and tail positions and movements. The experiment was repeated 15 times using different sea cucumbers. (N = 15). The formula for the movement speed (v) is rooted from Sun et al. (2021):

$$v_{\alpha}=\sqrt{[x_{\alpha}(t)\text{-}x_{\alpha}(t\text{-}1)]^2+[y_{\alpha}(t)\text{-}y_{\alpha}(t\text{-}1)]^2}\times\frac{\beta}{5}$$

($x_{\alpha}(t)$, $y_{\alpha}(t)$) = the coordinates of sea cucumber α at image t; β = the scale of the picture.

To quantify the level of movement randomness, the experiment uses the mean result length (R) as an assessment metric. R falls within the range [0, 1], where a value of 0 indicates absolute randomness in movement, and a value of 1 indicates absolute directionality (Mardia et al., 2009; Dam-Bates et al., 2016). The formula is as follows:

$$R=\sqrt{[\frac{1}{n}\sum_{i=1}^{n}\cos(\theta_i)]^2+[\frac{1}{n}\sum_{i=1}^{n}\sin(\theta_i)]^2}$$

Theta i represents the turning angle at time i.

To measure the level of movement linearity, the experiment uses the linearity index (L) for assessment. L in [0,1], where 0 indicates a completely non-linear trajectory (irregularly tortuous) and 1 indicates a completely linear trajectory (straight-line movement). The formula as follows.

149 $$L=\frac{D}{S}=\frac{|\sum_{i=1}^{n} d_i|}{\sum_{i=1}^{n} |d_i|}$$

150 d_i is the displacement vector of the i-th step, D is the straight-line distance from the
151 start point to the end point, and S is the total path length of the movement.

152 *2.2.2 Feeding behavior*

153 Feeding behavior refers to the repetitive process of sea cucumbers spread their
154 tentacles in food, grasping food particles, retracting the tentacles into their mouths, and
155 swallowing the food particles (Cameron and Fankboner 1984). This cycle is called
156 tentacle activity frequency. The strength of the tentacle activity frequency can reflect
157 the feeding ability of sea cucumbers (Tian et al., 2023). We evenly sprinkled a layer of
158 food particles at the bottom of a plastic box and then placed sea cucumbers from group
159 A and group B in the middle of the box (length × width × height: 24 cm × 12 cm × 40
160 cm), respectively. We used a digital camera with infrared function (Legria, HF20,
161 Canon) to record the tentacle activity cycles of the sea cucumbers within 30 minutes
162 and counted the number of cycles based on the video. The experiment was repeated 15
163 times using different sea cucumbers (N = 15).

164 *2.2.3. Wall-climbing behavior*

165 Wall-climbing behavior refers to the tendency of sea cucumbers to prefer the walls of
166 a pond over the bottom when seeking a place to attach (Hu et al., 2021). During the
167 experiment, all sea cucumbers were placed at the bottom of an opaque square plastic
168 container (length × width × height: 30 cm × 5 cm × 20 cm), filled with fresh seawater
169 and aerated for each group. One hour later, we recorded the number of sea cucumbers
170 that climbed up to the side walls of the container. The experiment was repeated 15
171 times with different sea cucumbers (N = 15).

172 *2.2.4. Adhesion behavior*

173 The adhesion of sea cucumber tube feet is related to their locomotion and feeding

behaviors (Dam-Bates et al., 2016). A lower adhesion force is a typical phenomenon when sea cucumbers are parasitized (Rong, 2005). We selected sea cucumbers from group A and group B and placed them in opaque plastic boxes (length × width × height: 30 cm × 20 cm × 6 cm), keeping them horizontal and allowing them to rest for 10 minutes to ensure a firm adhesion. Then, the plastic boxes were quickly and steadily inverted, and the adhesion time of the sea cucumbers was recorded. The adhesion behavior of the sea cucumbers was examined by calculating the adhesion index based on the adhesion time. The adhesion time refers to the duration from when the plastic box is slowly inverted until the sea cucumber loses its adhesion and falls to the bottom of the box. The adhesion index was calculated as follows:

$$Ai=\frac{At}{W}$$

Ai = adhesion index; At = adhesion time (s); W = wet weight of sea cucumbers (g).

Water quality was measured using a portable water quality monitor (HACH, HO40d, USA). group A had a salinity of 31.26 ± 0.15, and group B had a salinity of 31.24 ± 0.15. The experiment was repeated 15 times with different sea cucumbers (N = 15).

2.2.5. Righting behavior

The righting behavior refers to the time it takes for a sea cucumber to return to an upright position with its dorsal side up when its ventral side is fully in contact with the bottom of the tank (Hu et al., 2022). We selected sea cucumbers from group A and group B and placed them upside down in opaque plastic boxes (length × width × height: 30 cm × 20 cm × 6 cm). When a sea cucumber completed a full righting behavior, the time was recorded as the righting time. If a sea cucumber failed to right itself within 5 minutes, the time was recorded as 300 seconds (Hu et al., 2022). The experiment was repeated 15 times with different sea cucumbers (N = 15).

2.3 Experiment 2: Intestinal health

This study investigated the intestinal health of sea cucumbers by examining the effects of posterior mouth worm parasitism on their pepsin and intestinal flora. All experiments were conducted on healthy sea cucumbers (group A) and those parasitized by *Frontonia* (group B). The methods for determining pepsin and intestinal flora are described below.

2.3.1 Pepsin

Six sea cucumbers were randomly selected from group A and group B. We dissected the sea cucumbers and collected fresh intestinal tissues using sterile instruments and collected in 1.5 mL centrifuge tubes. The samples were stored at -20 °C for further analysis. The pepsin enzyme-linked immunosorbent assay kit (Mlbio, ml560431) was used (Sun et al., 2015). The kit instructions were provided by Bioengineering (Shanghai) Co., Ltd. The specific determination method for pepsin-like protease activity was used, and each group was measured in U/mg prot according to the pepsin kit instructions. Each sample was analyzed using a 450 nm wavelength microplate reader (Nanjing Jianguo Bioengineering Research Institute, Nanjing, China) (Sun et al., 2015). The pepsin activity of the two groups of sea cucumbers was compared (N = 3).

2.3.2 Intestinal flora

Sea cucumbers were randomly selected from groups A and B. Sea cucumbers were fasted for 48 hours before the experiment. Fresh intestinal tissues were dissected using sterile instruments and stored in 1.5 mL centrifuge tubes at -20 °C.

The diversity and abundance of intestinal flora in normal and parasitized sea cucumbers were detected. The intestinal flora of sea cucumbers was detected by Seville Bioengineering Co., Ltd. The extraction was performed using an extraction kit, and the bacterial DNA was enriched using a bacterial DNA extraction kit (Bioengineering (Shanghai) Co., Ltd.). High-throughput sequencing was performed using the Illumina

MiSeq platform to determine the species composition of each sample (Yang et al., 2015). Finally, the data were sequenced and subjected to bioinformatics analysis. Random forest analysis was used to check which bacteria caused the differences between the groups, and post hoc analysis was conducted to compare the abundance of different groups in species classification. Principal component analysis (PCA) revealed the relationships between different samples (N = 3).

2.4 Statistical analysis

Independent sample T-tests were used to analyze the foraging speed, tentacle activity, and wall climbing rate of sea cucumbers in experiment 1. Since the data did not meet the normality and/or homogeneity of variance, Mann-Whitney U test was used to analyze the adhesion behavior of sea cucumbers. The Alpha diversity of Chao, ACE, Simpson, and Shannon was analyzed using the Mann-Whitney U test, as the data did not meet the normality and/or homogeneity of variance. In the beta diversity analysis, PCA was used to evaluate the similarity of microbial community structures between group A and group B, and analysis of variance was used to evaluate the differences in microbial community structures.

All data were statistically analyzed using SPSS 26.0 software. When the probability level $P < 0.05$, it was considered significant.

3. Results

3.1 Experiment 1: Behaviors

3.1.1 Righting behavior

There was no significant difference of the righting time between group A (40.5 s ± 11.49 s) and group B (37.81 s ± 12.97 s) (Mann-Whitney $U = 95.00$, $P = 0.540$, Fig. 1A).

3.1.2 Wall climbing Behavior

249 The wall-climbing rate in group A (60.89% ± 14.00%) was significantly higher than
250 that in group B (24% ± 17%) (t = 6.445, $P < 0.001$, Fig.1B).
251 *3.1.3 Adhesion behavior*
252 The adhesion index of group A was significantly lower than that of group B (group
253 A: 16.59 ± 15.17, group B: 31.86 ± 14.81, $t = -2.788$, $P = 0.009$, Fig. 1C).
254 *3.1.4 Foraging behavior*
255 There were no significant difference between group A and group B in foraging time
256 (group A: 1390.00 s ± 620.71 s, group B: 1253.75 s ± 632.59 s, Mann-Whitney $U =$
257 53.00, $P = 0.600$, Fig. 2A) and foraging speed (group A: 1.61 mm/s ± 0.56 mm/s,
258 group B: 1.20 mm/s ± 0.77 mm/s, Mann-Whitney $U = 49.00$, $P = 0.147$, Fig. 2C).
259 However, the movement distance during foraging behavior was significantly longer in
260 group A compared to group B (group A: 2102.08 mm ± 950.63 mm, group B: 1164.29
261 mm ± 626.70 mm, $t = 2.853$, $P = 0.009$, Fig. 2B). There was also no significant
262 difference between group A and group B in staying time (group A: 133.75 s ± 142.67
263 s, group B: 319.17 s ± 361.70 s, Mann-Whitney $U = 65.00$, $P = 0.685$, Fig. 2D).
264 No significant differences were found between the two groups in terms of head
265 movement speed (group A: 1.09 ± 0.48 mm/s, group B: 1.44 ± 0.77 mm/s,
266 Mann-Whitney $U = 58.00$, $P = 0.200$, Fig. 3A) or tail movement speed (group A: 0.86
267 ± 0.39 mm/s, group B: 1.34 ± 1.12 mm/s, Mann-Whitney $U = 66.00$, $P = 0.178$, Fig.
268 3B).
269 There was no significant difference of the linear rate between group A (0.17 ± 0.16)
270 and group B (0.30 ± 0.29) (Mann-Whitney $U = 52.00$, $P = 0.248$, Fig. 3A). However,
271 the randomness degree was significantly higher in group A compared to group B
272 (group A: 0.31 ± 0.03, group B: 0.45 ± 0.16, $t = -2.976$, $P = 0.017$, Fig. 3B).
273 *3.1.5 Feeding behavior*

The tentacle activity frequency in group A (10.53 times ± 5.69 times) was significantly lower than that in group B (20.93 times ± 5.16 times) (t = -5.242, P < 0.001, Fig. 1D).

3.2 Experiment 2: Gut health

3.2.1 Pepsin-like protease activity

No significant difference in pepsin-like protease activity was found between group A and group B (group A: 1.36 ± 0.37, group B: 2.03 ± 0.82, Mann-Whitney U = 8.00, P = 0.097, Fig. 4).

3.2.2 Intestinal microbiological 16S rRNA gene sequencing

In the Venn diagram illustrating the gut microbiota (Fig. 6), the orange circle represents the gut microbiota of healthy sea cucumbers, occupies a unique microbial count of 8,987. The overlapping region between the two circles, comprising a unique microbial count of 766, shows that the shared gut microbiota common to both infected and healthy sea cucumbers.

No significant difference was observed between group A and group B in terms of ACE richness estimator (P = 0.394), Chao1 richness estimator (P = 0.394), Simpson diversity index (P = 0.454), Shannon-Wiener diversity index (P = 0.522), phylogenetic diversity (P = 0.522), or coverage (P = 1.000). The coverage indices of both groups were close to 1, indicating a high sequencing depth and that the sequencing results adequately reflected the actual composition of the gut microbiota.

Principal Component Analysis (PCA) was used to assess the similarity in microbial community structures between group A and group B (Fig. 7A). The species composition exhibited considerable similarity between the two groups. For a more intuitive representation, analysis of variance (ANOVA) was used to evaluate differences in microbial community structure (Fig. 7B). No significant difference was

found in the gut microbiota composition between group A and group B.

At the phylum level, the gut microbial composition of sea cucumbers was largely consistent in groups A and B, with Firmicutes, Proteobacteria, Bacteroidota, and Synergistota identified as the dominant phyla (Fig. 8A). At the genus level, the gut microbiota composition of group A and group B was basically similar, with *Muribaculaceae* and *Lachnospiraceae* showing dominance. Specifically, *Lachnospiraceae*_NK4A136_group showed generally higher relative abundance in group B samples. The relative abundance of *Halorubrum* was elevated in group B. In contrast, *Natronomonas* consistently demonstrated higher levels in group A, while maintaining a stable but lower relative abundance in other samples of group B. However, *Cyanobacteriales* was distributed relatively evenly across both groups (Fig. 8B).

4. Discussion

4.1 Behavioral differences

Righting behavior is frequently used to characterize the adaptability of echinoderms to environmental conditions (Hu et al., 2022). The study found no significant difference in the righting behavior between infected and healthy sea cucumbers. Accordingly, we infer that *Frontonia* infection does not impair the basic adaptability of sea cucumbers. However, the wall climbing rate of sea cucumbers infected with *Frontonia* was significantly higher than that of healthy sea cucumbers. This behavior is consistent with a stress response, and is similar with the yellow intestinal disease (Zhao et al., 2026). But contrasts sharply with the symptoms of enteritis, in which wall climbing behavior is notably weakened and most individuals separate from the tank (Feng et al., 2017). Therefore, we suppose that *Frontonia* infection leads to a stress response rather than intestinal infection.

In this study, the activity times of tube feet was significantly lower in *Frontonia* infected sea cucumbers than in healthy individuals. Furthermore, no significant differences were observed in foraging speed, foraging time, head and tail movement speed, or staying time between infected and normal sea cucumbers. However, the foraging distance of infected sea cucumbers was significantly longer than that of healthy individuals. Similarly, their moving randomness exhibited higher. These results indicate that *Frontonia* infection does not infect the movement ability of sea cucumbers, but increases the difficulty of foraging and correspondingly reduces the feeding ability. The reduced feeding ability is a typical response to parasitic infection (Han et al., 2016). Similarly, this finding is consistent with the reduced frequency of tube feet of sea cucumbers under various stressors, including air exposure, mechanical disturbance, medicinal bath, and seawater flushing (Yang et al., 2022; Wang et al., 2023). This reduction may be related to the reallocation of energy under stress. Sea cucumbers under stresses preferentially allocate energy toward the synthesis of numerous stress hormones (Tian et al., 2023). Thus, we speculate that *Frontonia* infection may cause a stress response similar to that induced by stresses. This stress response may directly reduce the energy allocated to feeding-related behaviors and increase the energy devoted to alarm signals.

Tube foot adhesion determines a number of behavioral processes in sea cucumbers (Dam-Bates et al., 2016; Wang et al., 2023). In the present study, the adhesion index of *Frontonia* infected sea cucumbers was significantly lower than that of healthy individuals. This is consistent with the declined adhere ability observed in sea cucumbers suffering from infectious diseases like skin ulcer syndrome (Rong, 2005), suggesting that *Frontonia* infection may predispose sea cucumbers to secondary bacterial infections. This result also corroborates the phenomenon described by Deng

(2024), in which *Frontonia* infection leads to epidermal ulceration in sea cucumbers. Interestingly, despite weakened the adhere ability, the foraging speed of infected sea cucumbers remained unaffected. Instead, their foraging distance was significantly longer and moving randomness shows higher. One possible explanation is that intense mechanical disturbance of internal tissues by *Frontonia* makes the sea cucumbers overcomes the reduced adhere ability and triggers a stronger moving drive. This heightened that moving motivation likely makes the sea cucumbers more sensitive to the environment and impels them to escape residual food and feces, which are perceived as alarm signals (Zhao et al., 2023). We therefore propose that the effects of *Frontonia* infection on sea cucumber movement ability are triggered by stress responses.

In summary, this experiment clarified the behavioral characteristics of sea cucumbers infected with *Frontonia*. We found that righting and foraging behaviors were not significantly affected by *Frontonia* infection. However, feeding behavior was significantly decreased, adhesion and moving-related behaviors were significantly disturbed, and the wall climbing rate was significantly increased. These findings greatly differ from the common perception among aqua-farmers that *Frontonia* does not affect the growth and development of sea cucumbers or just causing gut infection, while further reveal the impacts of *Frontonia* on sea cucumber behavior. In addition, abnormal foraging movement track and a high tendency for wall climbing were typical characteristics of *Frontonia* infection. We recommend that aqua-farmers closely monitor sea cucumbers with abnormal moving track or increased wall adhesion, and promptly remove residual food and feces during the period when *Frontonia* is prone to proliferation, so as to prevent the formation of adverse environmental conditions.

374 *4.2 Effect of Frontonia infection on intestinal protease activity*

375 In sea cucumber intestine, protease is among the most active digestive enzymes,
376 and its activity serves as a key indicator of digestive and absorptive efficiency (Sun et
377 al., 2015). In the present study, despite significant alterations in intestinal morphology
378 and structure in sea cucumbers affected by *Frontonia* infection, no significant
379 difference in intestinal protease activity was observed. Yang et al., (2015)
380 demonstrated that modulation of the intestinal microbiota influences digestive
381 physiology in sea cucumbers, including the activity of key digestive enzymes. Sun et
382 al., (2015) further highlighted that intestinal protease activity, a core measure of
383 digestive capacity, is closely linked to the metabolic function of the intestinal
384 microbiota in these animals. Synthesizing these findings, we hypothesize a close
385 association between the intestinal microbiota and protease activity. In our study, the
386 structure of the intestinal microbiota in diseased sea cucumbers remained largely
387 unchanged, which likely explains the absence of significant differences in protease
388 activity.

389 *4.3 Effect of Frontonia infection on intestinal microbiota*

390 The biological barrier composed of intestinal microbiota plays an important role in
391 maintaining intestinal homeostasis, and is closely associated with host health and
392 disease infection (Koboziev et al., 2014). Microbial community richness is commonly
393 represented by the Chao1 and ACE indices, while diversity is often assessed using the
394 Shannon and Simpson indices (Yu et al., 2022). α-diversity and β-diversity are
395 commonly used to characterize the diversity and discrepancy of intestinal microbiota
396 (Yu et al., 2022). Surprisingly, no significant differences were detected in bacterial
397 community richness (ACE, Chao) and diversity (Shannon, Simpson) in the intestines
398 of sea cucumbers infected with *Frontonia*. The results demonstrated that *Frontonia*

infection did not impair the basic richness and evenness of the intestinal microbiota in sea cucumbers, which was different from the decline in microbial diversity commonly found in typical intestinal diseases such as enteritis (Takashima et al., 2008).

In addition, the analysis of microbial community structure (principal coordinate analysis, PERMANOVA/ANOSIM) showed no significant difference in the overall structure of the intestinal microbiota between the two groups. The intestinal microbiota composition at the phylum level was similar between healthy and infected sea cucumbers, which were both dominated by Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteriota based on 16S rRNA gene sequencing. This result was consistent with the intestinal microbiota of healthy sea cucumbers in normal water conditions reported by Liu et al. (2023), indicating that *Frontonia* infection exerted little effect on the taxonomic structure of the intestinal microbiome. Among them, Bacteroidetes was identified as the key and dominant active microbial phylum in the intestine of sea cucumbers (Liu et al., 2023). Overall, *Frontonia* infection had limited impacts on the intestinal microbiota structure of sea cucumbers.

Frontonia infection induced few changes in a few bacterial taxa, rather than causing overall dysbiosis at specific taxonomic levels. At the phylum level, the relative abundance of Crenarchaeota was significantly higher in the intestine of sea cucumbers infected with *Frontonia* than in the control group. At the genus level, the abundance of *Facklamia* was significantly increased, while the abundances of *Aadhiraibacter* and *Gemella* were decreased. However, these changes did not affect the stability of the core functional microbiota, including Firmicutes and Bacteroidetes. In addition, Wang et al. (2025) reported that enteritis caused significant changes in specific bacterial groups at the family and genus levels. For instance, this research find that the relative abundances of Vibrionaceae and Rickettsiaceae significantly

increased in the intestines of sea cucumbers with enteritis. At the genus level, the abundances of *Alivibrio* and *Vibrio* were also higher than those in healthy individuals. Nevertheless, these changes were not observed in sea cucumbers infected with *Frontonia*.

Venn diagram analysis showed that the healthy group and the *Frontonia* infected group had 4010 and 3122 unique microbial species, respectively, with 604 core microbial species shared between the two groups. Despite the differences in specific microbial species, the stability of the overall microbial community structure was not altered. As the primary functional microbiota in the intestine of sea cucumbers, the relative abundances of Firmicutes and Bacteroidetes remained stable in both groups. These two phyla play critical roles in carbon metabolism and the decomposition of nutrients including polysaccharides and proteins (Koboziev et al., 2023). We therefore speculate that the stability of the core microbiota may be one of the reasons why the digestive enzyme activity of sea cucumbers infected with *Frontonia* was not significantly affected.

Furthermore, this study revealed that *Frontonia* infection had little effect on the intestinal microbiota structure of sea cucumbers, but could change the intestinal morphological structure. In aquaculture, *Frontonia* infection is usually regarded as a type of enteritis in sea cucumbers. However, the results of the present study suggested that *Frontonia* infection does not conform to the typical characteristics of enteritis. The intestine of sea cucumbers with enteritis appears white, which is different from that of sea cucumbers infected with *Frontonia*. Enteritis can lead to intestinal microbiota imbalance (Takashima et al., 2008), whereas the intestinal microbiota structure of sea cucumbers infected with *Frontonia* remained basically stable. It has been reported that enteritis significantly reduces digestive enzyme activity (Feng et al.,

2017), but this phenomenon was not observed in *Frontonia* infection, in which the pepsin activity of diseased sea cucumbers showed no significant change. This explains why the treatment strategies for enteritis are ineffective against *Frontonia* infection. It should be noted that this study did not conduct a direct comparison with sea cucumbers suffering from enteritis. We recommend that aquaculture farmers treat *Frontonia* infection as a stress response rather than a typical enteritis disease. Furthermore, the potential relationship between *Frontonia* infection and poor environmental conditions still needs further investigation.

Acknowledgements

This work was funded by Liaoning Province "Xingliao Talents Plan" project (XLYC2002107) and National Natural Science Foundation of Liaoning Province (2024-MSLH-042). We thank Zihe Zhao, Xiang Li for their assistance in this study.

References

Cameron, J.L., Fankboner, P.V., 1984. Feeding mechanics of Apostichopus californicus. Can. J. Zool. 62, 1012–1020.

Dam-Bates, P., Curtis, D.L., Cowen, L.L.E., Cross, S.F., Pearce, C.M., 2016. Assessing movement of the California sea cucumber Parastichopus californicus in response to organically enriched areas typical of aquaculture sites. Aquac. Environ. Interact. 8, 67–76.

Deng, P., Yu, Y., Zhao, Z., et al., 2024. Behavior, intestinal health, and growth of small sea cucumbers Apostichopus japonicus in different color morphs. Mar. Environ. Res. 193, 106300..

Feng, H., Liao, M., Li, B., et al., 2017. Sea cucumber enteritis disease during temporary rearing and its control methods. Prog. Fish. Sci. 38(3), 188–197.

473 Han, Q., Keesing, J.K., Liu, D., 2016. A review of sea cucumber aquaculture,
474 ranching, and stock enhancement in China. Rev. Fish. Sci. Aquac. 24(4), 326–
475 341.
476 Hu, F., Ding, P., Yu, Y., et al., 2021. Effects of artificial reefs on selectivity and
477 behaviors of the sea cucumber Apostichopus japonicus: new insights into the
478 pond culture. Aquac. Rep. 21, 100842.
479 Hu, F., Zhao, Z., Tian, R., et al., 2022. A cost-effective approach to decreasing the
480 disease transmission of the sea urchin Strongylocentrotus intermedius: new
481 information for seed production and longline culture. Aquaculture 548, 737569.
482 Koboziev, I., Webb, C.R., Furr, K.L., Grisham, M.B., 2014. Role of the enteric
483 microbiota in intestinal homeostasis and inflammation. Free Radic. Biol. Med.
484 68, 122–133.
485 Liu, Y., Zhang, J., Zhou, Y., et al., 2023. Bacterial community characteristics and
486 correlation analysis in the gut of cage-cultured Apostichopus japonicus and
487 ambient seawater from North China. Prog. Fish. Sci. 44(6), 190–202.
488 Mardia, K.V., Jupp, P.E., 2009. Directional Statistics, 2nd ed. John Wiley & Sons,
489 Chichester, UK.
490 Rong, X., 2005. Epidemiological and histopathological studies on major diseases and
491 parasitic pathogens of cultured Apostichopus japonicus. Master's thesis, Ocean
492 University of China, Qingdao, China.
493 Sun, J., Zhang, L., Pan, Y., et al., 2015. Feeding behavior and digestive physiology in
494 sea cucumber Apostichopus japonicus. Physiol. Behav. 139, 336–343.

Sun, J., Zhao, Z., Zhao, C., et al., 2021. Interaction among sea urchins in response to food cues. Sci. Rep. 11(1), 9985.

Tian, R., Hu, F., Wu, G., et al., 2023. An effective approach to improving fitness-related behavior and digestive ability of small sea cucumbers Apostichopus japonicus at high temperature: new insights into seed production. Aquaculture 562, 738755.

Takaishi, H., Matsuki, T., Nakazawa, A., et al., 2008. Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. Int. J. Med. Microbiol. 298(5–6), 463–472.

Wang, H., Wu, G., Ma, T., et al., 2023. Crevice is beneficial to fitness-related behaviors of small sea cucumbers Apostichopus japonicus exposed to handling stress. Aquac. Rep. 31, 101642.

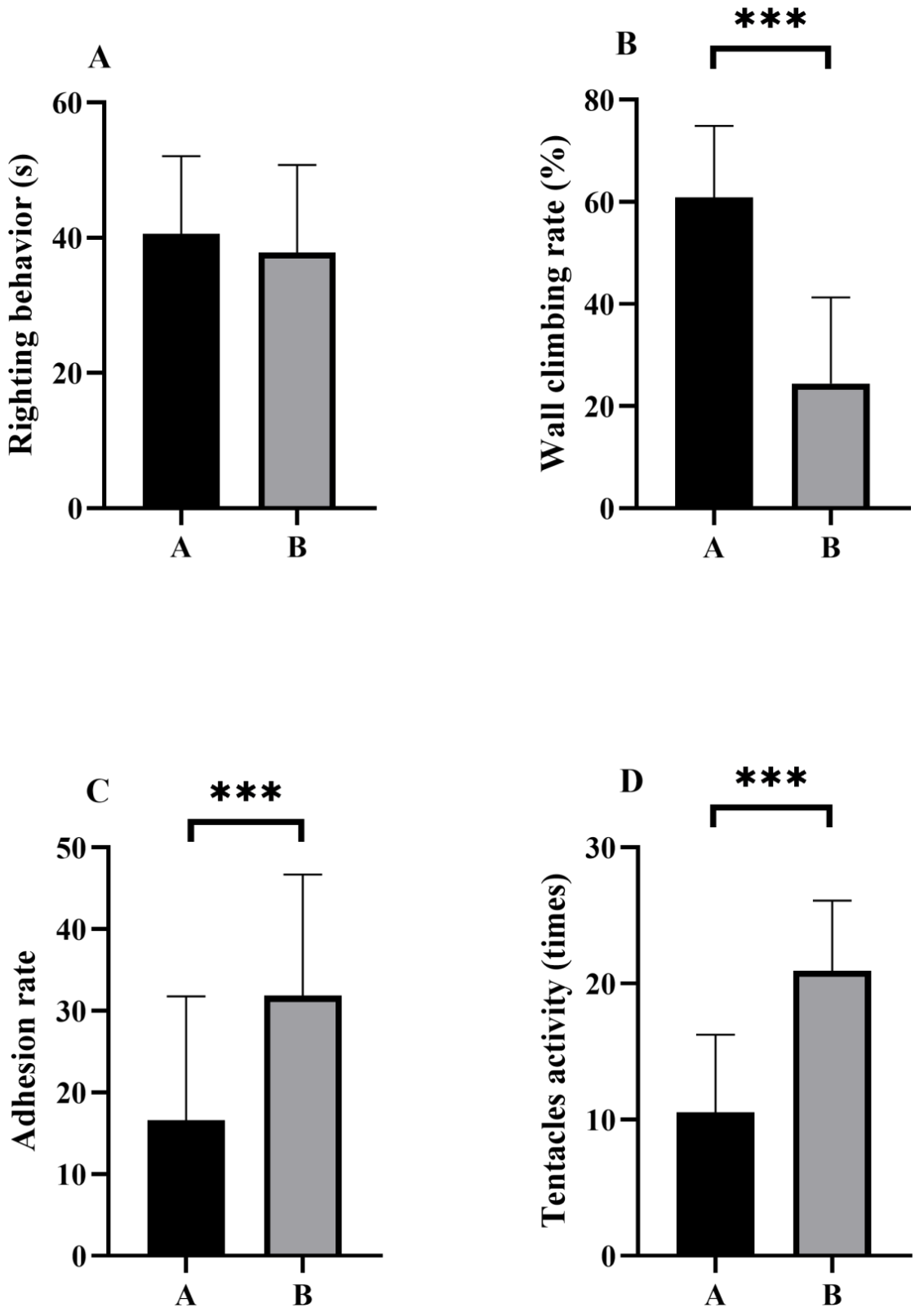
Wang, J., Wang, Y., Li, B., et al., 2025. Enteritis in cage-cultured Apostichopus japonicus from Dongwuyang Bay, Fujian: pathogenic characteristics and intestinal bacterial community structure. Prog. Fish. Sci. 46(4), 201–216.

Yang, G., Tian, X., Dong, S., Peng, M., Wang, D., 2015. Effects of dietary Bacillus cereus G19, B. cereus BC-01 and Paracoccus marcusii DB11 supplementation on growth, immune response and immune-related gene expression in coelomocytes and intestine of Apostichopus japonicus. Fish Shellfish Immunol. 45, 800–807.

Yang, M., Li, X., Hu, F., et al., 2022. Effects of handling stresses on fitness-related behaviors of small sea cucumbers Apostichopus japonicus: new insights into seed production. Aquaculture 546, 737321.

517 Yu, Y., Qiao, Y., Ding, P., et al., 2022. Effects of sea urchin feces on behaviors,
518 digestion ability, growth and disease resistance of the sea cucumber
519 Apostichopus japonicus. Front. Mar. Sci. 9, 967452.
520 Zhao, Z., Li, X., Sun, Y., Wang, S., Ding, J., Zhao, C., 2023. Effects of alarm cues on
521 behavior and physiology of the sea cucumber Apostichopus japonicus. Aquac.
522 Rep. 33, 101806.
523 Zhao, Z., Sun, Y., Li, X., Wang, S., Ding, J., Zhao, C., 2026. Behavior and gut health
524 of the sea cucumber Apostichopus japonicus with yellow intestinal disease. N. Z.
525 J. Mar. Freshw. Res. 60, e70001.

526 **Figure:**



527

528

529 **Fig. 1.** Righting behavior (A), wall climbing behavior (B), adhesion behavior (C), and

tentacles activity (D) of Apostichopus japonicus (mean ± SD). Note: The asterisks *** mean P < 0.001. Group A means Frontonia infected sea cucumbers, group B means normal sea cucumbers.

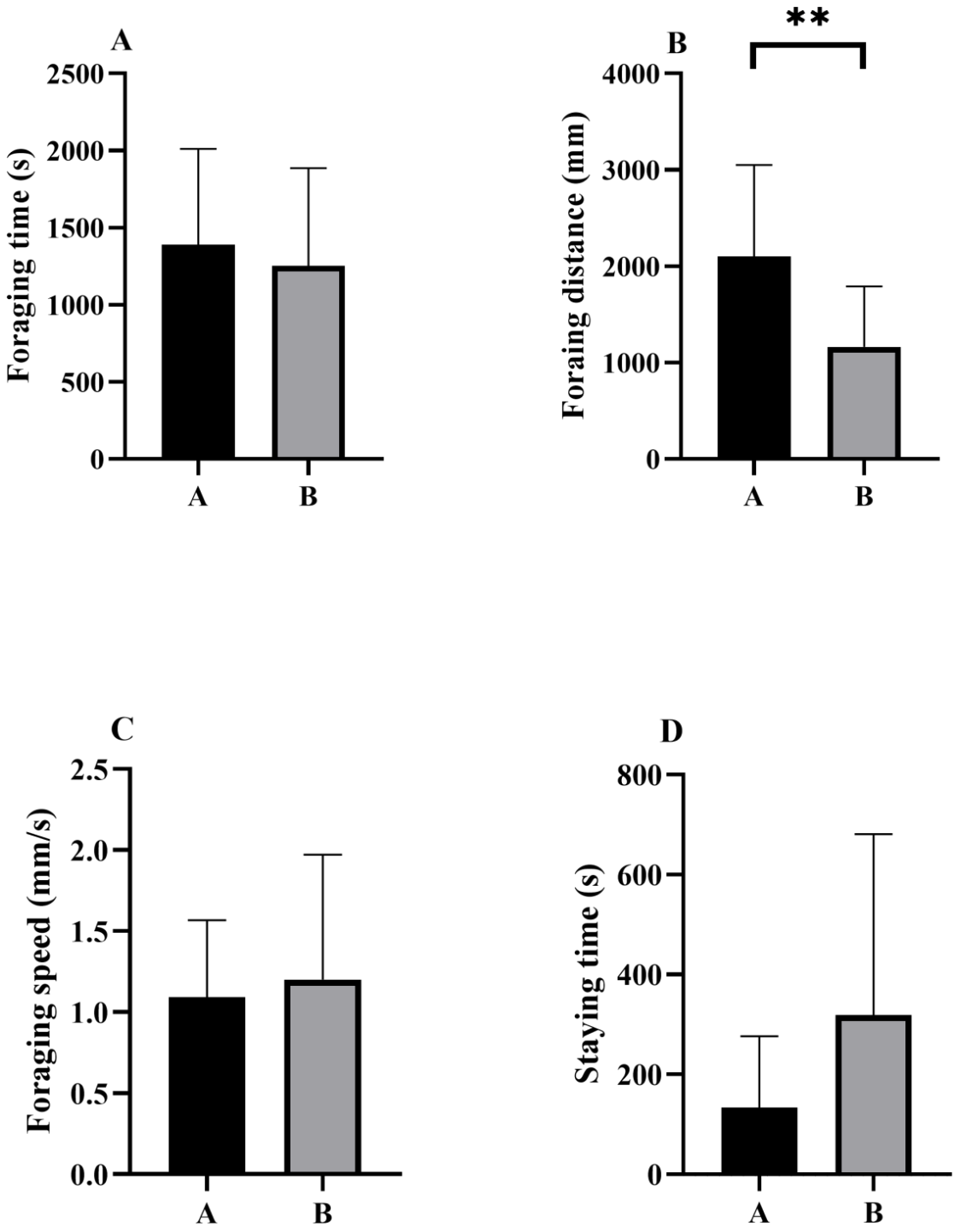


Fig. 2. Foraging time (A), foraging distance (B), foraging speed (C), staying time (D) of Apostichopus japonicus (mean ± SD). Note: The asterisks ** mean P < 0.01. Group

A means Frontonia infected sea cucumbers, group B means normal sea cucumbers.

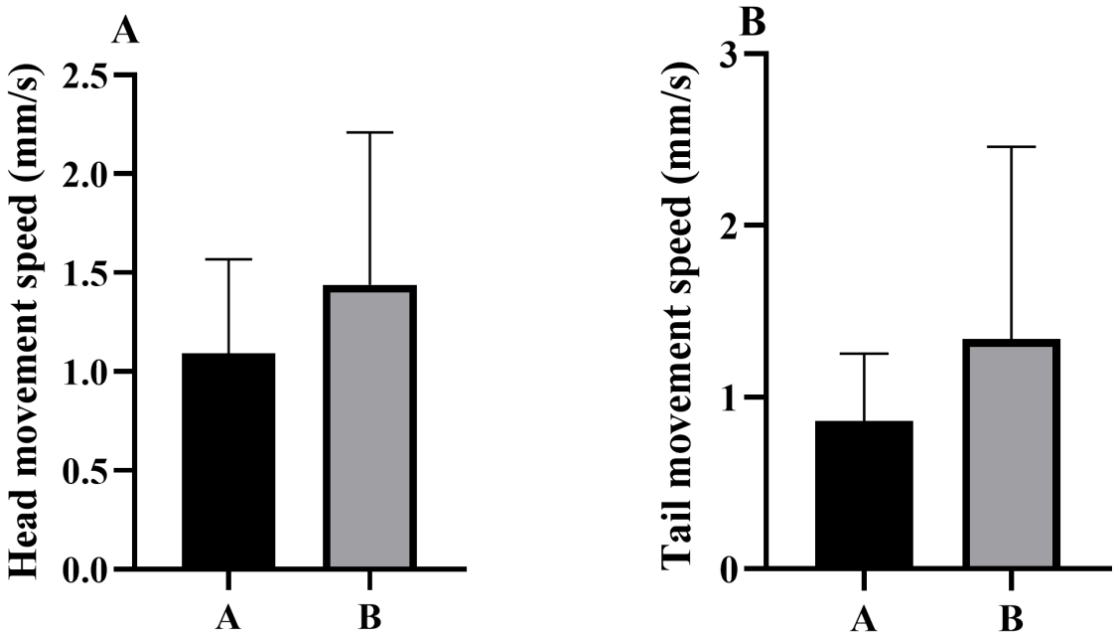


Fig. 3. Head movement speed (A) and tail movement speed (B) of Apostichopus japonicus (mean ± SD). Note: Group A means Frontonia infected sea cucumbers, group B means normal sea cucumbers.

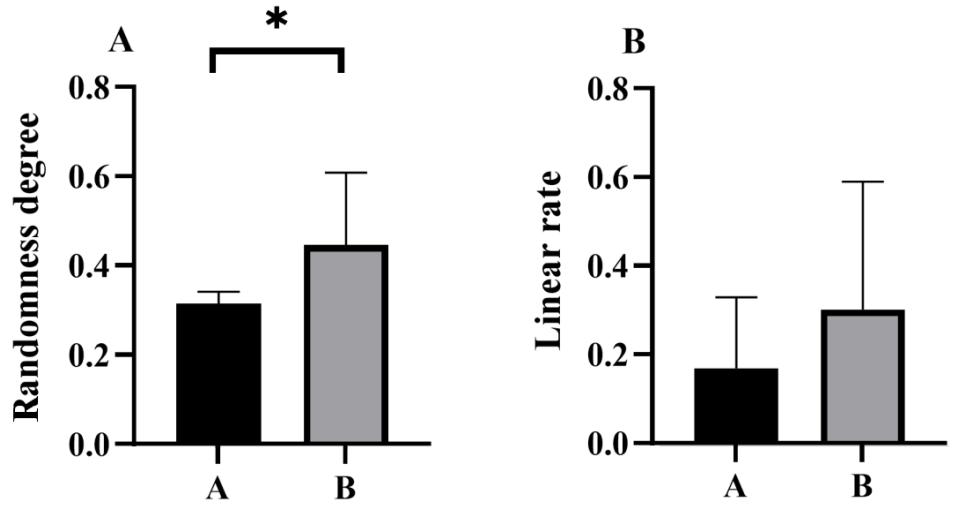
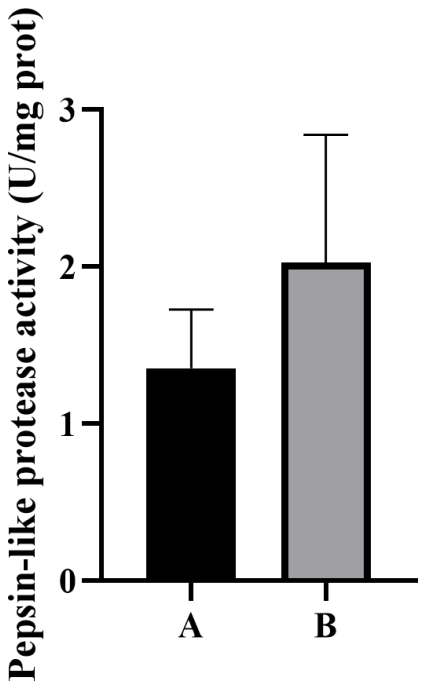
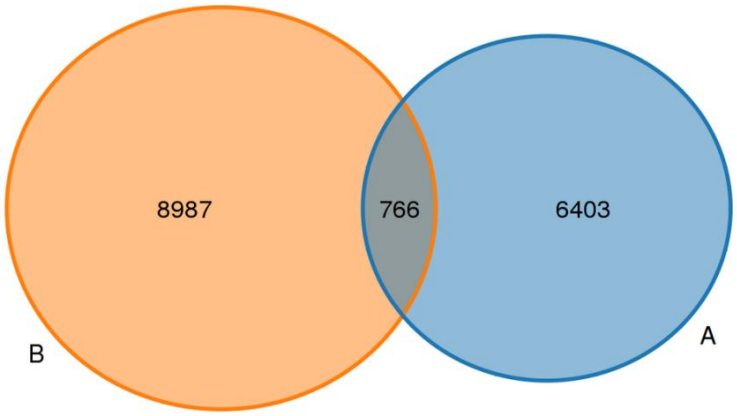


Fig. 4. Linear rate (A), and randomness degree (B) of Apostichopus japonicus (mean ± SD), group A means Frontonia infected sea cucumbers, group B means normal sea cucumbers. Note: The asterisks * mean $P < 0.05$. Group A means Frontonia infected sea cucumbers, group B means normal sea cucumbers.



546

547 **Fig. 5.** Pepsin-like protease activity of Apostichopus japonicus (mean ± SD). Note:
548 Group A means Frontonia infected sea cucumbers, group B means normal sea
549 cucumbers.



550

551 **Fig. 6.** Venn diagram. The colors of different ellipses represent different groups.
552 Group A means Frontonia infected sea cucumbers, group B means normal sea
553 cucumbers. The overlapping parts represent the number of features common to each
554 group, and the non-overlapping parts represent the number of features unique to each
555 group.

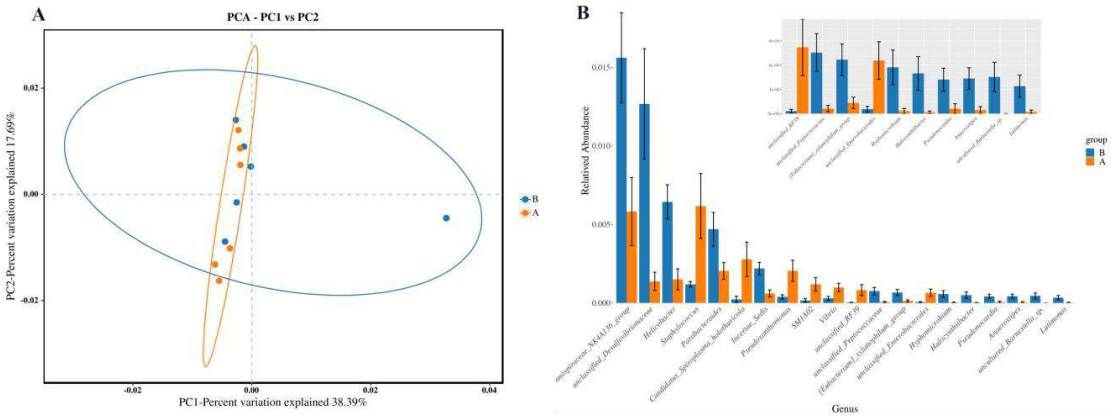


Fig. 7. PCA analysis (A), Anova analysis (phylum level) (B). In each figure, group A means Frontonia infected sea cucumbers, group B means normal sea cucumbers.

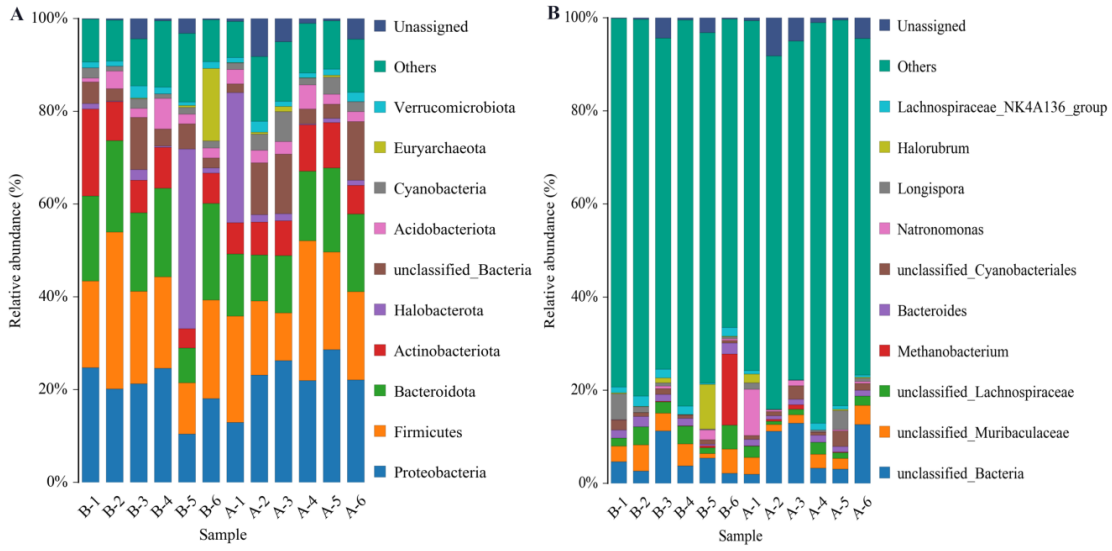


Fig. 8. Intestinal microflora of Apostichopus japonicus. The relative abundance histogram (phylum level) (A); The relative abundance histogram (genus level) (B). In each figure, group A means Frontonia infected sea cucumbers, group B means normal sea cucumbers.