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Comparative Physiological Study of Sea Cucumbers from Eastern Waters of United States

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Comparative Physiological Study of Sea Cucumbers from Eastern Waters of United States

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Abstract:

Sea cucumbers, belonging to the phylum Echinodermata, are known to possess valuable bioactive compounds that have medicinal properties. In several countries, such as Korea, China, and Japan, they are cultured in the aquaculture industries for food and medicinal purposes. To find the species with better physiological and immunological properties, we compared the physiological and immunological parameters of three species of sea cucumbers from the waters of the eastern United States namely, *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (*I. badionotus*), and *Pentacta pygmaea* (*P. Pygmaea*). When four different cells: phagocytic, red spherule, white spherule, and vibratile cells were counted, *C. frondosa* illustrated the highest concentrations of phagocytic cells, white spherule cells, and vibratile cells, compared to the two other species ($P<0.05$). Due to its high phagocytic cell concentration, the highest phagocytic capacity was seen in *C. frondosa* although it was not statistically significant ($P>0.05$). Apart from this, we also observed that *C. frondosa* had the highest total cell count ($P<0.05$). We also measured coelomic protein concentration and *C. frondosa* had the highest concentration of coelomic protein ($P<0.05$) among the three species. Lastly, when lysozyme activity was measured, the highest activity was also observed in *C. frondosa* ($P<0.05$). Looking at the results, we concluded that *C. frondosa* is the better species with immunological and physiological properties with the potential to be reared in the aquaculture system for use in the food and biomedicine industries.

Keywords: Sea cucumbers, *Cucumaria frondosa*, *Isostychopus badionotus*, and *Pentacta pygmaea*, Physiological and Immunological properties

Introduction

Sea cucumbers, also known as Holothurians, are invertebrates that belong to the phylum Echinodermata [1]. These animals, unlike others, have a unique elongated body structure that is supported by a hydrostatic skeleton, where the body muscle is shaped by a fluid-filled cavity, the coelom [2]. In Asian countries, species such as *Stichopus hermanni*, *Thelenota ananas*, *Thelenota anax*, *Holothuria fuccogilva*, and *Actinopyga mauritiana* are used as a functional food and traditional medication [3]. In recent studies, it was discovered that sea cucumbers possess bioactive compounds that have 16 valuable medicinal properties [4]. According to Xu et al. (2018), polysaccharides, triterpene glycosides, phenols, and lipids can be isolated from the invertebrate [5]. Several studies have shown that these compounds have anti-cancer, anti-inflammatory, anti-microbial, anti-angiogenic, anti-hypertension, anti-hyperglycemic, anti-oxidant, and immunomodulatory activities [6]. Due to this, sea cucumbers are considered to have the potential to be reared in the aquaculture system for use in the food and biomedicine industries.

Although some research has been conducted on several species of sea cucumbers around the world, not many studies have focused on the species of sea cucumbers in the waters of the United States. One of the species, the Orange-footed Sea cucumber, also known as *C. frondosa* can be found in the cold waters of the Atlantic Ocean in the United States. This species can be found in the benthic areas of the water, and they have an elongated leathery body [6,7]. According to a previous study, *C. frondosa* has the ability to grow up to 40-50cm in length and 10-15cm in width [6]. Due to its medicinal and nutritional benefits, *C. frondosa* is seen as a species to further culture in the aquaculture industry [8]. *I. badionotus* is a type of sea cucumber that is also called the chocolate chip sea cucumber and it can be found in the Gulf of Mexico [1,9]. According to Acosta et al. (2021), this species can be found primarily on rocky bottoms

between the cracks that are in 2.5m depth of water and they tend to have an average size and weight of 324mm and 628g respectively [10]. In recent studies, medicinal values such as anti-coagulant and anti-inflammatory properties have been found in this species, and in several countries, the animal is considered edible for nutritional purposes [11,12]. The last species in this study is *P. pygmaea* and it resides in the water of the Gulf of Mexico [13]. This species is brown in color and is considered to be small in size (4-6cm) with ossicles in its body wall that makes it stiffer than other sea cucumbers [14]. Although this species is not known to be edible, it has been found to have bioactive compounds with the potential to treat SARS-CoV-2 [15].

In many animals, it has been found that stress-induced changes can suppress the immune response in an animal [16]. For animals in an aquaculture system, one of the stressors that the animals often encounter is handling stress and this can reduce the quality of the species [17,18]. As a result, before culturing a species in the aquaculture system, it is important to discern the ability of the sea cucumber to mitigate stress without affecting the overall health and quality of the animal, or its nutritional and medicinal benefits. The immune responses of sea cucumbers depend on the coelomocytes that are in the coelomic fluid [19]. During stressful conditions and pathogenic exposures, these cells participate in maintaining homeostasis and eliminating pathogens [19].

As the sea cucumbers encounter stress or pathogens, the invertebrate undergoes cellular responses to maintain homeostasis and overcome the disease state [20]. In the coelomic fluid of sea cucumbers, there are four major types of coelomocytes, and they are, red spherule cells, white spherule cells, phagocytic cells, and vibratile cells [19]. They all have functions relating to antibacterial activity, inflammation, wound healing, encapsulation, graft rejection, and cytotoxic activity in the body of sea cucumbers [21]. The red and white spherule cells were found to secret

lipase, peroxidase, and serine proteinase resulting in the breakdown of materials after the phagocytosis [22,23]. Phagocytes contain lysosomal enzymes and have the ability to ingest and destroy unwanted organisms or particles that they encounter [24]. Vibratile cells are highly motile and known to assist in the circulation of the coelomic fluid [25,26]. The vibratile cells also can degranulate during the clotting process in the animal [25].

Although studies have been conducted on the medicinal and nutritional benefits of sea cucumbers, they have only been focused on species that reside in the East Asian and Middle Eastern countries [2,3,27-29]. Due to this, it is important to explore the medicinal properties of sea cucumber species in other parts of the world. One of the areas that were not studied extensively for sea cucumber species and their medicinal benefit is the United States. In this experiment, we study the immunological and physiological properties of coelomic fluid from three sea cucumber species collected from the waters of the Eastern United States: *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (*I. badionotus*), and *Pentacta pygmaea* (*P. Pygmaea*). The physiological parameters measured in this study will tell us about the overall health of the sea cucumbers.

Material and methods

Collection of Coelomic Fluid and Tissue

Three species of sea cucumbers, *Cucumaria frondosa*, *Isostychopus badionotus*, and *Pentacta pygmaea* were purchased from Gulf of Maine Inc (Pembroke, Maine) and Gulf Specimen Marine Lab (Panacea, Florida) respectively and reared in the aquaculture lab of Purdue University Fort Wayne. Coelomic fluid was collected through midsagittal dissection with a syringe after the animals were euthanized (N=6 for each species). The coelomic fluid was immediately kept on ice for analysis of coelomocytes, coelomic protein level, lysozyme activity, and phagocytic

capacity. The tissues (Body Wall, Viscera, and Tentacle) obtained from these invertebrates were kept at -80◦C after rinsing for future experiments.

Total and Differential Cell Count

After obtaining the coelomic fluid samples, 50µl of coelomic fluid was mixed with 50µl of the anticoagulant, Dipotassium Ethylenediamine Tetraacetate (Sequester-Sol, USA). Then, 25µl of the mixture was loaded into a hemocytometer. Four different types of cells, phagocytic cells - red spherule cells, white spherule cells, and vibratile - were counted to find the differential coelomocyte count (DCC) (Figure 2.1). Then the differential cell counts were summed up to find the total coelomocytes count (TCC). Top left and right 16 squares of the hemocytometer were counted and averaged. To obtain cells per milliliter, the following equation described by Yeh et al., (2004) was used [30]:

$$\frac{Cells}{mL} = \frac{cell\ count}{number\ of\ counted\ corners} \times dilution\ factor\ (2) \times 10^4$$

Total Coelomic Protein

Total coelomic fluid protein was measured using a Protein Refractometer (VEEGEE Scientific Inc. Kirkland, WA). Two to three drops of coelomic fluid without anticoagulant were added to the surface of the prism of the calibrated refractometer. Afterward, coelomic protein (g/100ml) was read holding the refractometer under the light.

Lysozyme Activity Assay

Before initiating the lysozyme assay, a suspension of *Micrococcus lysodeikticus* was made at a concentration of 0.2mg/ml using 0.05M (pH =6.2) sodium phosphate buffer. 25µl of collected coelomic fluid without anticoagulant was added into a cuvette with 1ml of the bacterial suspension. Then, the absorbance was measured at 1 minute and 5 minutes using a spectrophotometer at 540 nm. Lysozyme activity assay (LAA) was calculated according to the formula described by Mumu & Mustafa (2022)[31]:

$$LAA = \frac{(final\ absorbance - \ initial\ absorbance)}{total\ elapsed\ time\ (minute)}$$

Phagocytic Capacity

50µl of coelomic fluid was mixed with 50µl of the anticoagulant. Then, to a double cytoslide microscope slide, 50µl was pipetted into each circle [32]. The slide was incubated for 90-120 minutes at room temperature. After the first incubation, 50µl of formalin-killed bacteria (*Bacillus megaterium*) was added to each circle of the glass slide and was incubated again for 60 minutes at room temperature. When incubation was done, the slide was washed with phosphate buffer saline (PBS) for 1 minute. Then, it was fixed with methanol for 1 minute, stained with Wright-Giensa stain for 20 seconds, and rinsed with PBS. The slide was air-dried and counted under the microscope. Positive (≥5 engulfed bacteria) and negative phagocytic cells at a location were recorded to find the percent of phagocytic capacity [25].

Result

From the coelomic fluid obtained from each species, four different cell types were counted: phagocytic, red spherule, white spherule, and vibratile (Figure 2.2). We found that there was a

total of 127.83±21.11 x 10^4 cells/mL, 27.50±9.41 x 10^4 cells/mL, and 56.00±0 x 10^4 cells/mL of phagocytic cells for *C. frondosa*, *I. badionotus*, and *P. pygmaea* respectively. *C. frondosa,* when compared statistically to *I. badionotus* for phagocytic cells concentration, significant differences were seen between the species ($P<0.05$). On the other hand, when the phagocytic cell concentration of *C. frondosa* was compared to *P. pygmaea,* no statistical significance was found ($P>0.05$). For red spherule cells, there were 14.83±10.52 x 10^4 cells/mL for *C. frondosa*, 58.50±8.96 x 10^4 cells/mL for *I. badionotus*, and 13.00±0 x 10^4 cells/mL for *P. pygmaea*. No significant differences were found among the three species ($P>0.05$). The species, *C. frondosa*, *I. badionotus*, and *P. pygmaea* had 30.83±3.29 x 10^4 cells/mL, 25.50±8.96 x 10^4 cells/mL, and 15.00±0 x 10^4 cells/mL of white spherule respectively. No significant differences in white spherule cell concentrations were found among the groups ($P>0.05$). Lastly, there were 141.67±23.85 x 10^4 cells/mL, 27.50±11.08 x 10^4 cells/mL, and 63.00±0 x 10^4 cells/mL of vibratile cells for *C. frondosa*, *I. badionotus*, and *P. pygmaea* respectively. The concentration of vibratile cells between *C. frondosa* and *I. badionotus* were significantly different ($P<0.05$). When *C. frondosa* was compared to *P. pygmaea*, no statistical significance was found ($P>0.05$).

The total cell count was obtained for each type of species (Figure 2.3). The total cell count concentrations were 315.17±71.65 x 10^4 cells/mL for *C. frondosa*, 139.00±45.09 x 10^4 cells/mL for *I. badionotus,* and 147.00±0 x 10^4 cells/mL for *P. pygmaea*. When the statistical analysis was conducted, the total cell count concentration for *C. frondosa* was significantly different when compared to *I. badionotus* and *P. pygmaea* ($P<0.05$).

Coelomic fluid protein concentration was 1.85±0.09 g/100mL for *C. frondosa*, 2.80±0.07 g/100mL for *I. badionotus*, and 3.58±0.13 g/100mL for *P. pygmaea* (Figure 2.4). Coelomic protein concentrations were all significantly different from one species to another and *C.*

frondosa produced the lowest concentration of coelomic protein compared to *I. badionotus*, and *P. pygmaea* ($P<0.05$).

We also examined the lysozyme activity of the three species to measure the ability of lysozyme present in the coelomic fluid to break down bacterial cell walls over a period (Figure 2.5). We observed that the activities were $2.60 \times 10^{-3} \pm 5.60 \times 10^{-4}$ absorbance/minute for *C. frondosa*, $6.50 \times 10^{-4} \pm 7.4 \times 10^{-5}$ absorbance/minute for *I. badionotus*, and $1.25 \times 10^{-3} \pm 1.50 \times 10^{-4}$ absorbance/minute for *P. pygmaea*. After statistical analysis, lysozyme activity of *C. frondosa* was significantly different when compared to *I. badionotus* and *P. pygmaea* ($P<0.05$). Lastly, we looked at phagocytic capacity as a measure of immunological status (Figure 2.6). The percentages of phagocytic capacities were $85.86 \pm 4.30\%$, $76.25 \pm 3.33\%$, and $69.12 \pm 4.01\%$ respectively for *C. frondosa*, *I. badionotus*, and *P. pygmaea*. No statistical differences were found between the species ($P>0.05$).

Discussion

Based on the results obtained in this project, we were able to find the best species with physiological and immunological parameters. We can look at this by the differential cell count concentrations. Overall, we observed that *C. frondosa* had higher cell counts for all types that were counted, as well as the total cell count. In a study by Jobson et al. (2021), the authors also showed that higher coelomocyte counts were seen in *C. frondosa* when stress was induced [33]. Others have also shown that enhanced concentrations of phagocytes were seen over time as the sea cucumber was induced with a chemical stress [20]. In our result for differential cell count, we also saw that *C. frondosa* had a significantly high number of phagocytic cells, which could indicate a better immune response. Even though the data analysis showed no significant

differences, some phagocytic capacity was seen in *C. frondosa*. Although not all results for differential and total cell counts were significant, we can imply that *C. frondosa* has the highest number of counted immune cells in its coelomic fluid compared to *I. badionotus* and *P. pygmaea*.

During stressful and pathogenic encounters, proteins help maintain homeostasis and fight against pathogens are secreted into the coelomic fluid [34]. We measured the coelomic protein concentrations of the three species and found that *C. frondosa* had the highest concentration of coelomic protein among the three species ($P<0.05$). In a previous study, it has been shown that sea cucumbers produce an increased amount of proteins such as heat shock proteins and lysozymes to maintain homeostasis during stressful situations [35,36]. In addition to the coelomic protein, we also observed significantly different lysozyme activity when C. *frondosa* was compared to *I. badionotus* and *P. pygmaea* ($P<0.05$). We found that C. *frondosa* has the highest lysozyme activity and lysozyme activity was also seen in the coelomic fluid of *C. frondosa* in a previous study [37].

Conclusion

Based on these results, we can conclude that *C. frondosa* has the best immunological and physiological properties among the three species. It is important to find the best species for the aquaculture industry as species need to be able to handle stress and maintain healthy conditions in order to be cultured for nutritional and medicinal purposes.

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Declaration of interest

There was no conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Figure **Error! No text of specified style in document.**.1 Types of coelomocytes in echinoderms; red arrow= red spherule cells, white arrow= white spherule cells, blue arrow= vibratile cells, black arrow = phagocytic cells [26].

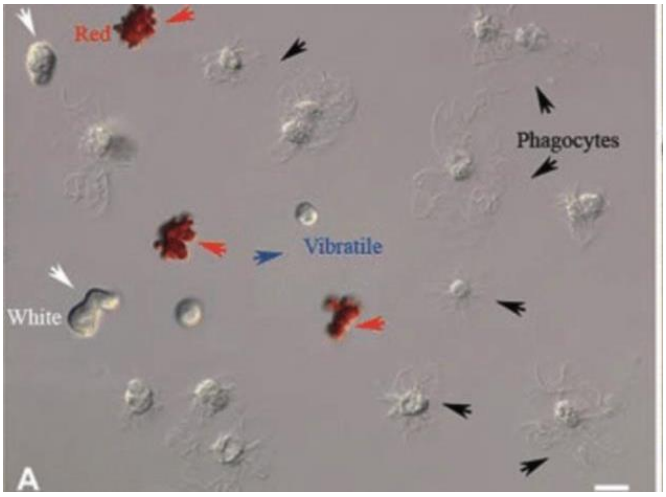

Figure **Error! No text of specified style in document.**.2 Differential cell count (cells/mL) of *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (*I. badionotus*), and *Pentacta*

pygmaea (*P. pygmaea*). The cell concentrations illustrated are averaged. Different alphabets represent significantly different concentrations among the species for each cell type (P<0.05).

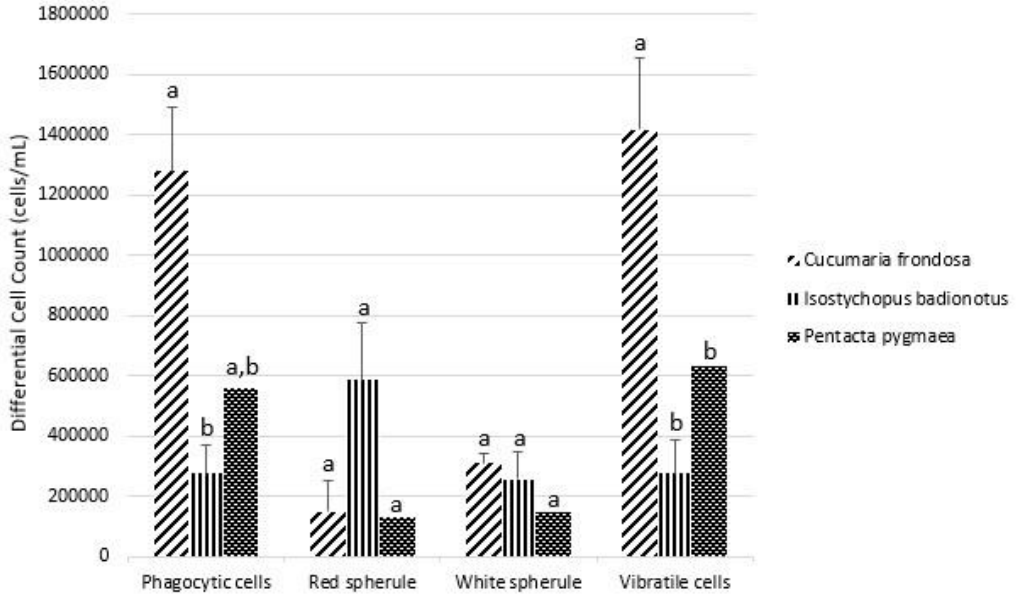

Figure **Error! No text of specified style in document.**.3 Total cell count (cells/mL) of *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (*I. badionotus*), and *Pentacta pygmaea* (*P. pygmaea*). The cell counts illustrated are averaged. Different alphabets represent significantly different concentrations of cells among the species (P<0.05).

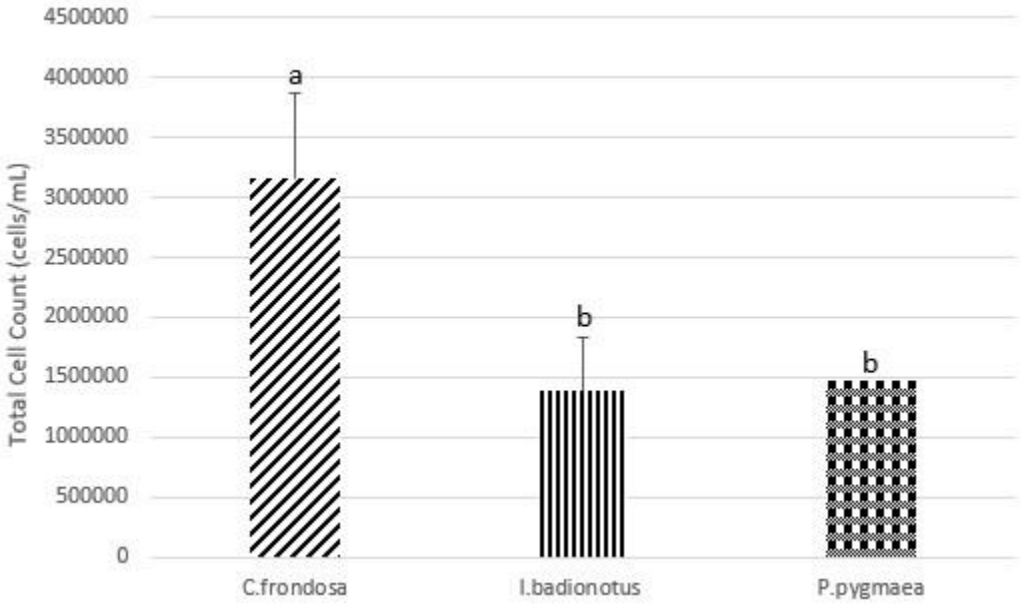

Figure **Error! No text of specified style in document.**.4 Coelomic protein (g/100mL) of *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (*I. badionotus*), and *Pentacta*

pygmaea (*P. pygmaea*). The concentrations are illustrated in mean +SEM. Different alphabets represent significantly different concentrations of coelomocyte protein among the species (P<0.05).

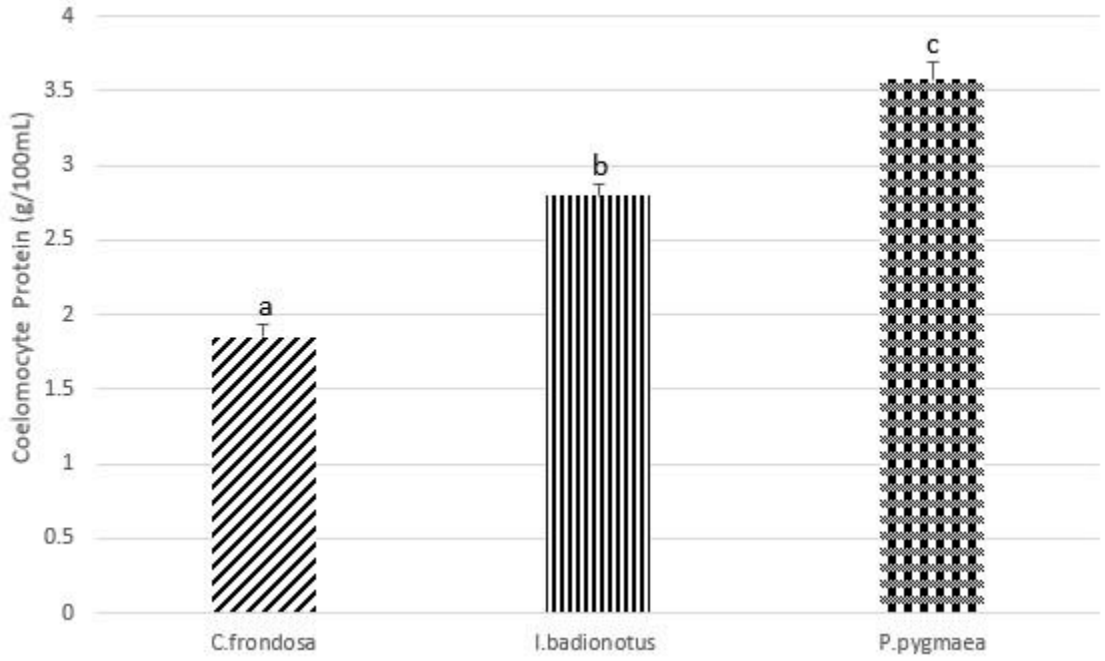

Figure **Error! No text of specified style in document.**.5 Lysozyme activity (absorbance/minute) of *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (I. badionotus), and *Pentacta pygmaea* (*P. pygmaea*). The activities are illustrated in mean +SEM. Different alphabets represent significantly different lysozyme activity among the species (P<0.05).

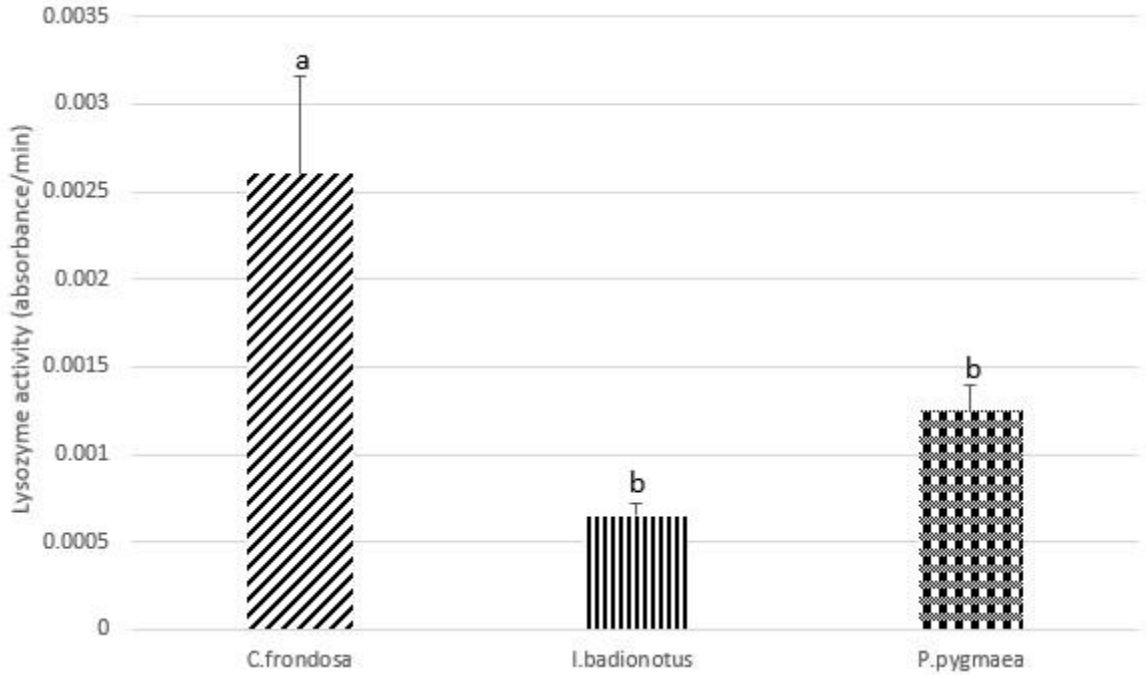

Figure **Error! No text of specified style in document.**.6 Phagocytic capacity (%) of *Cucumaria frondosa* (*C. frondosa*), *Isostychopus badionotus* (*I. badionotus*), and *Pentacta pygmaea* (*P. pygmaea*). The capacities are illustrated in mean +SEM. There were no significant differences among the three species ($P<0.05$).