國立臺灣大學理學院海洋研究所

碩士論文

Institute of Oceanography
College of Science
National Taiwan University
Master Thesis

# 二氧化碳引起的海洋酸化對萊式擬烏賊生理及腸胃道微生物群的影響

# Effects of $CO_2$-induced ocean acidification on the physiological responses in association with digestive tract microbiota in bigfin reef squid

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# 國立臺灣大學碩士學位論文
# 口試委員會審定書
## MASTER'S THESIS ACCEPTANCE CERTIFICATE
## NATIONAL TAIWAN UNIVERSITY

二氧化碳引起的海洋酸化對菜式擬烏賊生理及腸胃道微生物群的影響

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本論文係游智堯 R09241208 在國立臺灣大學海洋研究所完成之碩士學位論文，於民國 111 年 6 月 20 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Department / Institute of Oceanography on 20 June 2022 have examined a Master's thesis entitled above presented by You, Jhih-Yao R09241208 candidate and hereby certify that it is worthy of acceptance.

口試委員 Oral examination committee:

（指導教授 Advisor）

系主任/所長 Director:



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# 摘要

自工業革命之後，人類排放過多的二氧化碳到大氣中，使部分二氧化碳溶入海水中，並造成海水的酸鹼值逐漸下降。過去的研究指出：海洋酸化會造成海洋動物的消化能力下降以及體內的微生物組成改變。腸胃道微生物群的是一群生活在動物的腸胃道中的細菌，這些細菌對宿主的神經、消化和免疫系統有著巨大的影響。然而，相關的研究在頭足類中所知甚少。本研究運用萊式擬烏賊(*Sepioteuthis lessoniana*)，探討其長期於高碳酸環境中，其消化生理與腸胃道微生物群是否會改變。本研究運用剛孵化的萊式擬烏賊，分別飼養於正常海水 (控制組，pH～8.1) 與高碳酸 (酸化組，pH～7.8) 的海水中三個月。本研究發現相較於控制組，酸化組的萊式擬烏賊血液樣本中，會累積較高濃度的碳酸氫根離子；而在消化生理方面，海洋酸化會顯著提升萊式擬烏賊的澱粉酶、胰蛋白酶以及酯酶的活性。此外，本研究運用一種較傳統短片段定序更為精準的合成長片段 (SLR) 定序 LoopSeq™ 技術，並進一步在 Illumina NovaSeq 平臺上解析細菌的 16S rRNA，以研究萊式擬烏賊的腸胃道微生物群。根據微生物群落結構以及螢光原位雜合 (FISH) 解析得知：黴漿菌 (*Mycoplasma*) 是盲腸中最主要的屬，而弧菌 (*Vibrio*) 是腸道中最主要的屬。此外，跟據主座標散點圖 (PCoA) 以及置換多元方差分析 (PERMANOVA) 顯示：控制組和酸化組樣本間的微生物群在盲腸與腸道均沒有成分差異。Alpha 多樣性分析顯示酸化組的盲腸微生物群的豐富度與多樣性，與在控制組相較產生明顯的差異，但在腸道中並無觀察到此差異。本研究首次深入解析萊式擬烏賊的腸道微生物群，並進一步探討海洋酸化對萊式擬烏賊消化生理功能和腸道微生物群的組成變化。研究結果顯示：萊式擬烏賊可能可以藉由有效調節其生理功能，以及維持腸胃道微生物群的穩定，以適應海洋酸化現象。

**關鍵字**：海洋酸化、萊式擬烏賊、消化系統、消化酵素、腸道微生物群



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# Abstract

After the industrial revolution, the partial pressure of $CO_2$ in the ocean increased from 280 atm to 400 atm, causing ocean acidification (OA). Ocean acidification has been shown to alter the digestion and composition of the microbial communities associated with marine animals. Gut microbes are a group of bacteria that colonize the digestive tracts of animals. Microbes in the gut may also play a crucial role in the nervous, digestive, and immune systems of the host; nevertheless, the related research on cephalopods is still limited. Under control (pH = 8.1) and OA (pH = 7.8) conditions, this study used the bigfin reef squid (*Sepioteuthis lessoniana*) to estimate host physiology and digestive tract microbial community. There was evidence that ocean acidification can result in bicarbonate accumulation in the blood of OA squids than in control ones. The enzymatic activities of amylase, trypsin, and lipase were higher in the OA group than in the control counterpart. Additionally, LoopSeq$^{TM}$, a synthetic long-read (SLR) sequencing technology, was applied to generate highly accurate long reads of bacterial 16S rRNA gene, and this was further performed on an Illumina NovaSeq platform. The microbial community structure and fluorescence *in situ* hybridization indicate that *Mycoplasma* is the most dominant genus in the caecum, while *Vibrio* is the most dominant genus in the intestine. The principal coordinate scatter plot (PCoA) and the permutational multivariate analysis of variance (PERMANOVA) indicate no



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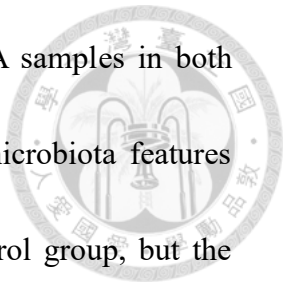

differences in microbial communities between the control and OA samples in both caecum and intestine. Alpha diversity showed that the caecum microbiota features under OA conditions differed significantly from those in the control group, but the difference could not be observed in the intestine. The present study is the first to examine the gut microbiota of bigfin reef squids and further estimate the possible impacts of ocean acidification on the gut microbiota, which could be important for bigfin reef squids to overcome the challenges induced by ocean acidification.

**Keywords:** ocean acidification, bigfin reef squid, digestive system, digestive enzyme, gut microbiota, physiological adjustments



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# Table of Contents





doi:10.6342/NTU202300020

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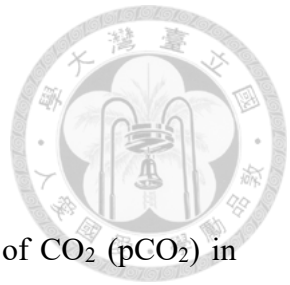
# Introduction



## Ocean acidification

Anthropogenic $CO_2$ emissions have increased the partial pressure of $CO_2$ ($pCO_2$) in seawater (Melzner et al., 2020). It is predicted that by the end of the century, the current level of $pCO_2$ in seawater will increase from 400 μatm to approximately 600-1000 μatm, resulting in a pH reduction (~ pH7.8) and bicarbonate accumulation (Melzner et al., 2020). Ocean acidification has resulted in a different environment for marine species, and the public is concerned about the loss of biodiversity and the chemical state of the marine ecosystem as a result of ocean acidification (Clark et al., 2020).

## The impacts of $CO_2$-induced ocean acidification on marine organisms

In light of the prominent increase in ocean acidification (OA), many marine organisms have been reported to be affected. Due to their calcified exoskeletons, reef-building corals, bivalves, and echinoderms are vulnerable to ocean acidification (Melzner et al., 2020). It has been found that active marine organisms (*e.g.*, teleosts, cephalopods) are more tolerant to environmental acidification due to their high metabolic rates (Hu et al., 2011). Nevertheless, several studies have suggested that ocean acidification has some physiological effects on these organisms. In a previous study, the splitnose rockfish, *Sebastes diploproa*, showed more anxiety behaviors in acidic water (Hamilton et al.,



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2014). The metabolic rate of bigfin reef squids, *Sepioteuthis lessoniana*, decreased significantly, and bicarbonate ions accumulated in their hemolymph (Hu et al., 2014). Furthermore, ocean acidification inhibited the immune responses of sea urchins (Figueiredo et al., 2016). In an acidified environment, the digestion ability of European bass, *Dicentrarchus labrax*, decreased significantly (Cominassi et al., 2020). Ocean acidification affects not only animal behavior and physiology, but also the bacterial community associated with animals. In oyster *Crassostrea virginica*, there was a noticeable change in the microbial community from the order *Vibrionales* and *Alteromonadales* to *Oceanospirillales*, *Cellvibrionales* and *Campylobacteriales* during ocean acidification (Unzueta-Martínez et al., 2021). Additionally, the composition and metabolic function of sponge-associated microbial communities were also altered by an acidified environment (Botté et al., 2019).

**The digestive system of cephalopods**

As a rule, cephalopods digest both extra- and intracellularly (Boucaud-Camou and Boucher-Rodoni, 1983). The process of extracellular digestion begins when the cephalopod bites its prey and secretes saliva containing digestive enzymes. The food passes through the esophagus and then enters the stomach, where it is temporarily stored. As the partially digested foods pass to the caecum, they are filtered, preventing



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gross particles from entering the intestine directly. By way of the hepatopancreatic ducts, sized foods enter the digestive glands, where intracellular digestion occurs (Anadón, 2019; Boucaud-Camou and Boucher-Rodoni, 1983; Linares et al., 2015).

Cephalopods are a group of important predators in marine ecosystems. Cephalopods have a very high metabolic rate as a result of their active locomotion, which leads to a high protein requirement (Potts, 1965; Boucher-Rodoni, 1987). In addition to protein, carbohydrates and lipids assimilated and processed through the digestive system are also important energy sources for animals (Ibarra-García et al., 2018). Therefore, digestive enzymes are essential for cephalopods to remain at the top of the food chain and maintain a high metabolic rate. Studies have been conducted on several digestive enzymes in octopus, including trypsin, chymotrypsin, cathepsins, amylase, and lipase (Aguila et al., 2007; Cárdenas-López and Haard, 2009; Cárdenas-López and Haard, 2005; Ibarra-García et al., 2018). However, research on bigfin reef squid is still limited, and even less research has been conducted on how ocean acidification affects the digestion of bigfin reef squid.

**Gut microbiota**

The digestive tract of animals contains a complex and dynamic ecosystem of microorganisms, which is composed of a wide variety of aerobic, facultative anaerobic



doi:10.6342/NTU202300020

and obligate anaerobic microbes (Nayak et al., 2010). The majority of these microorganisms interact with each other and their hosts (Nayak et al., 2010). The composition of gut microbiota is primarily host diet, lifestyle and habitat (Zhao et al., 2018). In addition to regulating gut motility, intestinal environment homeostasis, nutrient digestion and metabolism, the gut microbiota plays an important role in the development and function of the immune system (Cryan et al., 2012; Round et al., 2010; Olszak et al., 2012; Backhed et al., 2004; Bercik et al., 2012). Numerous studies have revealed the gut microbiome of marine animals. In marine carnivore fish gut microbiota, genera *Vibrio* and *Photobacterium* are the most frequently reported bacteria (Egerton et al., 2018). Additionally, other bacteria groups are found in the gut of marine carnivore fish, including *Pseudomonas*, *Clostridium*, *Aeromonas*, *Cetobacterium*, *Bacillus*, *Mycoplasma* and *Acinetobacter* (Egerton et al., 2018). The dominant groups in the gut microbiota of marine shrimp are *Photobacterium*, *Vibrio*, *Aeromonas*, *Xanthomonas*, *Agrobacterium* and *Bacillus* and the family *Enterobacteriaceae* (Wan et al., 2006; Odeyemi et al., 2021). In bivalve gut microbiota, *Mollicutes*, *Planctomyctes*, *phylotypes*, *Cristispira*, *Vibrio*, and *Stappia* were predominant (King et al., 2012; Mayasich and Smucker 1987; Odeyemi et al., 2021). There is little knowledge about the gut microbiota of cephalopods compared with other marine animals.



doi:10.6342/NTU202300020

**Cephalopods and microorganisms**

Diverse associations with microorganisms are known from cephalopods. A notable example is an association between the Hawaiian bobtail squid (*Euprymna scolopes*) and the marine bacterium *Vibrio fischeri*, which produces bioluminance (Jones et al., 2004). This association, which has been studied intensively since 1989, provides insights into how symbiotic bacteria influence all aspects of host biology, including evolution and ecology as well as the cellular, biochemical and molecular characteristics of the partners (Nyholm et al., 2021). Another association is the colonization of the accessory nidamental gland (ANG) of cephalopods by *Alpha-* and *Gammaproteobacteria* (Pichon et al., 2007). In marine environments, these bacteria produce secondary metabolites that are involved with quorum sensing and enter the ANG of cephalopods (Gromek et al., 2016). Furthermore, these symbionts play a critical role in the excretion of egg jelly coats, which protects the offspring from pathogenic microorganisms in marine environments (Cornet et al., 2015). Despite the close relationship between microorganisms and cephalopods, further research on the cephalopod gut microbiome is still needed to understand further the interaction between microorganisms and digestive mechanisms.

**Loop-seq**

Loop-seq is a high-throughput technique which is used to measure the mesoscale



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mechanical properties of DNA. The purpose of the loop-seq assay is measuring the cyclizabilities of ~ 100,000 short 50-bp reference DNA sequences simultaneously (Basu, 2021). The reference sequences are first designed in silico, which attached by two 25 base adaptor sequences on either end of every sequence to permit subsequent PCR amplification (Basu, 2021). This set of ~ 100,000 100-nucleotide (nt) sequences can be chemically synthesized commercially as an oligo pool of single-stranded (ss) DNA (Basu, 2021). The 100-nt ssDNA sequences in the pool are converted to 120-bp double stranded (ds) DNA with a biotin molecule attached to a modified thymine base via PCR amplification using primers that add the overhangs and introduce the biotin (Basu, 2021). The amplified dsDNA library is immobilized on the surface of streptavidin-coated beads. Subsequently, the immobilized library is chemically modified, resulting in each construct having a central 100-bp duplex region (of which the central 50 bp are variable, and the flanking 25-bp regions are identical among every molecule), flanked by two complementary 10-nt ssDNA overhangs (Basu, 2021). The bead-immobilized pool of dsDNA with flanking ssDNA overhangs is then exposed to 1 M NaCl for 1 min (Basu, 2021). This high salt concentration permits looping of individual constructs by allowing for stable annealing of the 10-nt ssDNA overhangs (Basu, 2021). Within 1 min, most copies of the very flexible sequences in the pool would have looped, while most copies of the most rigid sequences would have remained



doi:10.6342/NTU202300020

unlooped (Basu, 2021). After a minute, RecBCD is introduced, which digests the as-yet unlooped molecules (Basu, 2021). This results in the library being enriched for the more cyclizable sequences (Basu, 2021). A side-by-side control is performed involving all steps except RecBCD digestion (Basu, 2021). The two resulting libraries (RecBCD digested and control) are subject to deep-sequencing to count the relative population of every sequence in each of the libraries (Basu, 2021). Finally, cyclizability of each sequence is defined as the log of the ratio of the relative population of that sequence in the RecBCD digested library, to than in the control library (Basu, 2021). It is thus a measure of the probability that a DNA molecule bearing that sequence would have looped in under a minute, and thus survived the subsequent digestion step (Basu, 2021).

**Bigfin reef squid**

The bigfin reef squid, *Sepioteuthis lessoniana*, is a nektonic marine animal that can be found in the coastal waters of the Indian and West Pacific Oceans and represents one of the most important economical fishery products in Taiwan. Considering its wide distribution and pelagic lifestyle, *S. lessoniana* is an ideal model organism for examining how cephalopods cope with the challenges posed by global climate change, such as ocean warming and acidification. The bigfin reef squid has evolved advanced sensory and locomotory abilities as an apex predator, which may be convergent



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evolutionary traits for competition with teleosts in marine environments (Hu et al., 2014). With its well-developed nervous system, digestive system, and specialized locomotion, the bigfin reef squid is able to outcompete most marine invertebrates, or even teleosts (Hu et al., 2014). On the other hand, the bigfin reef squid has a relatively high metabolic rate and a locomotion type characterized by energy (Potts, 1965). Because of their high energy requirement, nutrients obtained from food resources are important, which are highly related to gut microbiome composition and digestive enzyme activity. The digestive enzymes of bigfin reef squid have been reported, including trypsin, amylase and lipase (Satjarak et al., 2022). Microbiome related research on bigfin reef squid focus on accessory nidamental gland associated microbiome, including *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* (Yang et al., 2021). But, the research on bigfin reef squid gut micobiome composition are limited. In addition, the effects of ocean acidification on bigfin reef squid enzyme activity and gut microbiome composition are still unknown. In order to cope with ocean acidification, bigfin reef squid should possess remarkable characteristics in gut microbiome composition and enzyme activity.

**Aim of this study**



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In this study, an analysis of the three-month-long effects of ocean acidification was carried out by comparing the high $CO_2$ treated bigfin reef squids with the control group. This study aims to determine whether the high $CO_2$ environment will alter the physiology of the bigfin reef squid, including its hemolymph status, digestive enzyme activity, and gut microbial composition.



doi:10.6342/NTU202300020

# Materials and methods

## Experimental animals

The new born *Sepioteuthis lessoniana* juveniles (Control group, CT group) were kept in a filtered and aerated circulatory seawater system (approximately total water volume 1000L, 10/14 light-dark photoperiod) at the Jiao-Shi Marine Research Station of the Institute of Cellular and Organismic Biology, Academia Sinica. CT group hatched at February 8 and kept in a filtered and aerated circulatory seawater system until March 1. OA group hatched at March 8 and kept in a filtered and aerated circulatory seawater system until April 10. After 1 month of breeding, these juveniles were transferred to 30,000 L pools (10/14 light-dark photoperiod) and kept for another 2 months (CT group: March 1 ~ May 13, OA group: April 10 ~ Jun 15). The survival rate of CT group was 23.75%. The survival rate of $CO_2$-induced acidified conditions (OA group) was 8.5% (Fig. 1). These squids were fed by Pacific white shrimp (*Litopenaeus vannamei*), *Neocaridina denticulate* and goldfish (*Carassius auratus*) 4 times a day. All these animals, which mantle sizes between 98 mm and 154 mm, were sacrificed by decapitation after anesthesia with 90% ethanol.

## $CO_2$-induced acidified seawater

In this study, the control group was set up with natural seawater (salinity = 33.7 ‰,



doi:10.6342/NTU202300020

temperature = 28.4°C, pH = 8.061 ± 0.04, $pCO_2$ = 412.23 ± 39.23 μatm; Fig. 2A). As for the acidification treatment, the acidified group was immediately transferred into the $CO_2$-induced acidified conditions (salinity 33.6‰, temperature 28.3°C, pH 7.78 ± 0.07, $pCO_2$ = 890.87 ± 126.83 μatm; Fig. 2B) after hatching. All the acidification treatment systems were prepared by aerating the water with $CO_2$ under the control of a pH-stat system.

**Squid hemolymph status**

The squid mantle and funnel were dissected on the ventral side. Hemolymph samples were extracted from the vena cava via Hamilton syringe, then transported to a new microtube. Determination of extracellular fluid pH was measured by a microelectrode (WTW Mic-D) and a WTW pHi 340 pH meter with 1000 μl volume per sample. Total dissolved inorganic carbon ($C_T$) was determined in triplicate (10 μl each) through a Corning 965 carbon dioxide analyzer (precision ± 0.1 mmol $L^{-1}$; Olympic Analytical Service, England) calibrated by generating a sodium bicarbonate standard curve with a fresh dilution series of 10, 5, 2.5, 1.25, 0.625 mM bicarbonate in distilled water. $pCO_2$ in *S. lessoniana* hemolymph was calculated from pH and $C_T$ measurements according to the Henderson-Hasselbalch equation:

$$pCO_2 = C_T \left(\alpha\left(10^{(pH-pK_1)}+1\right)\right)^{-1}$$



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$$e[HCO_3^-] = C_T - (\alpha pCO_2)$$

α is the solubility coefficient of $CO_2$ (0.039) in seawater, e is Euler's number and $pK_1$ is the dissociation constant of carbonic acid at a salinity of 33.67 ‰ and a temperature of 28 ℃. (Hu et al. 2014)

**Squid trypsin activity assay**

Trypsin Activity Colorimetric Assay Kit (Sigma-Aldrich, Inc. St. Louis, MO, USA) was used for squid trypsin activity assay following the manufacturer's protocol. Approximately 80 mg of each squid digestive gland, mixed with 200 µl Trypsin Assay Buffer, was used for trypsin activity assay. Samples were homogenized by a sterile grinding rod in a microtube. Samples were centrifuged for 10 minutes at 4℃ at top speed using a cold microcentrifuge to remove insoluble material. The supernatant was transferred to a new microtube. Samples were transferred into the 96 well plate, with 20, 30, 40 µl/well and adjusted the volume to 50 µl/well with Trypsin Assay Buffer. For positive control, 5 µl of positive control were added to the well and adjusted volume to 50 µl with Trypsin Assay Buffer. 0, 4, 8, 12, 16, 20 nmole/well p-nitroaniline (p-NA) standard (2 mM) were generated by adding 0, 2, 4, 6, 8, 10 µl p-NA standard into a series wells and adjusting the volume to 50 µl/well with Trypsin Assay Buffer. All the p-NA standard, samples and positive control were prepared in duplicate. 50 µl of



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Reaction Mix, prepared by the mixture of 48 µl Trypsin Assay Buffer and 2 µl Trypsin Substrate, were added to each well mixed with samples, p-NA standards and positive control. 96 well plate was put into SpectraMax i3 colorimetric microplate reader (Molecular Devices, LLC. San Jose, CA, USA) to measure absorbance at 405 nm in a kinetic mode, every 2 – 3 minutes, for 1 – 2 hours at 37℃ protected from light.

**Squid amylase activity assay**

Amylase Assay Kit (Colorimetric) (Abcam PLC, UK, Cambridge) was used for squid amylase activity assay following the manufacturer's protocol. Approximately 100 mg of each squid digestive gland, mixed with 500 µl Amylase Assay Buffer, was used for amylase activity assay. Samples were homogenized by a sterile grinding rod in a microtube. Samples were centrifuged for 10 minutes at 4˚C at top speed using a cold microcentrifuge to remove insoluble material. The supernatant was transferred to a new microtube. Samples were transferred into the 96 well plate, with 20, 30, 40 µl/well and adjusted the volume to 50 µl/well with Amylase Assay Buffer. For positive control, 5 µl of Amylase Positive Control was added to wells and adjusted the volume to 50 µl with Amylase Assay Buffer. 0, 4, 8, 12, 16, 20 nmole/well nitrophenol standard were generated by adding 0, 2, 4, 6, 8, 10 µl nitrophenol standard (2 mM) into a series wells and adjusting volume to 50 µl/well with Amylase Assay Buffer. All the nitrophenol



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standard, samples and positive control were prepared in duplicate. 100 µl of Reaction Mix, prepared by the mixture of 50 µl Amylase Assay Buffer and 50 µl Substrate Mix, were added to each well mixed with samples, nitrophenol standards and positive controls. 96 well plate was put into SpectraMax i3 colorimetric microplate reader (Molecular Devices, LLC. San Jose, CA, USA) to measure absorbance at 405 nm in a kinetic mode, every 2 – 3 minutes, for 30 – 60 minutes at 25°C protected from light.

**Squid lipase activity assay**

Lipase Activity Assay Kit (Colorimetric) (Abcam PLC, UK, Cambridge) was used for squid amylase activity assay following the manufacturer's protocol. Before the experiment, 2 ml of Calcium Chloride (3 mM) was mixed with 25 ml Lipase Assay Buffer. Approximately 40 mg of each squid digestive gland, mixed with 100 µl Lipase Assay Buffer, was used for lipase activity assay. Samples were homogenized by a sterile grinding rod in a microtube an. Samples were centrifuged for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove insoluble material. The supernatant was transferred to a new microtube. Samples were transferred into the 96 well plate, with 20, 30, and 40 µl/well and adjusted the volume to 50 µl/well with Lipase Assay Buffer. For positive control, 5 µl of Lipase Positive Control was added to wells and adjusted the volume to 50 µl with Amylase Assay Buffer. 0, 4, 8, 12, 16, 20 nmole/well



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glycerol standard were generated by adding 0, 2, 4, 6, 8, 10 µl glycerol standard (100 mM) into a series wells and adjusting volume to 50 µl/well with Lipase Assay Buffer. All the glycerol standard, samples and positive control were prepared in duplicate. 100 µl of Reaction Mix, prepared by the mixture of 93 µl Amylase Assay Buffer, 2 µl OxiRed Probe, 2 µl Enzyme Mix and 3 µl Lipase Substrate, were added to each well mixed with samples, nitrophenol standards and positive controls. 96 well plate was put into SpectraMax i3 colorimetric microplate reader (Molecular Devices, LLC. San Jose, CA, USA) to measure absorbance at 570 nm in a kinetic mode, every 2 – 3 minutes, for 60 – 90 minutes at 37°C protected from light.

**DNA extraction**

Esophagus, stomach, caecum and intestine were collected from each mature big fin reef squid. All samples were stored at -80°C. All DNA samples were extracted from approximately 80 mg of each squid gastrointestinal organ and food resource using the EasyPrep Stool Genomic DNA Kit (BIOTOOLS CO., LTD, New Taipei City, Taiwan) following the manufacturer's protocol. 2 L water samples were filtered by Filter Cup 500 ml RTYPE (GeneDireX, Taoyuan, Taiwan) and Aspirator A-1000S (EYELA, Tokyo, Japan). The filter was removed from the filter cup and cut to several small pieces for DNA extraction by DNeasy Power Water Kit (QIAGEN, Düsseldorf, Germany).



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The DNA concentration was measured by spectrophotometry under the absorbance of 260 nm and 280 nm of each sample (ND-2000c, NanoDrop Technol, Wilmington, DE, USA) to quantify the total amount of and quality of DNA.

**16S ribosomal RNA gene amplification and sequencing**

Total of 48 DNA extracted samples were utilized for bacterial 16S rRNA gene amplification and sequencing. LoopSeq 16S Long Read Kit (Loop Genomics, San Jose, CA, USA) was used for the 16S rRNA gene from bacterial DNA in all samples, following the manufacturer's protocol. The first section was Sample Indexing. 2.5 µl samples were added to each well of Sample Indexing Plate (96 well) and perform PCR program in a thermal cycler. Indexed samples were diluted by adding 150 µl of EB buffer, which were referred as 80FD. 528 µl of the Sample Normalization Mix and 316.8 µl Nuclease free water were mixed in a new 1.5 mL microcentrifuge tube. 8 µl of mixture was added into each well of a new 96 well PCR plate and mixed with 2 µl of each 80FD, followed by PCR program. The PCR products were preformed a 0.6x SPRIselect Cleanup and eluted in 50 µl of EB buffer. Cleanup products were transferred to a new PCR tube. The second section was Barcode Enrichment. 8 µl of Enhancer Reagent A and 2 µl of each Cleanup product were mixed in each well of a new 96 well plate. The mixtures were performed a PCR progrom, which is followed by 0.6x



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SPRIselect Cleanup. The third section was Barcode Calibration. The amount of DNA sample was measured by using a Fluorometric Instrument for DS DNA quantification. The Calibration Analysis was performed on the Loop Genomics website to calculate calculate the number of molecules per microliter in undiluted SPRI cleaned samples. This number is the molecule count per microliter for the samples. Carry forward the volume of sample appropriate for HiSeq/NextSeq/NovaSeq or MiSeq into the single sample Barcode Amplification step depending on the intended sequencer. The forth section was Barcode Amplification. 18 µl of Amplification Mix B and 2 µl of Cleanup dilution, based on calibration worksheet instructions, were added to each well of 96 well PCR plate and perform a PCR program. 10 µl of each sample were pooled in a new 1.5 mL microcentrifuge tube and performed 0.6x SPRIselect Cleanup. Sample sequencing was performed using paired-end 150-base reads on Illumina NovaSeq sequencing platform with sequencing depth at 100~150M PE reads (Illumina, San Diego, CA, USA), which generated 739718 sequences.

**16S RNA gene and microbial community statistical analysis**

Sequenced data were adaptor-trimmed online at the Loop Genomics website (loopgenomics.com). Trimmed data were performed using the Taxonomic Analysis function in the Microbial Genomic Module of CLC Genomics Work Bench. The



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taxonomic analysis included data quality control and taxonomic profiling, which aligned sequences to the database (NCBI Microbial Genomes database, 2019-08-30 released). 291143 sequences matched to database and generated 767 species. The sequence number and species number in each sample were showed in Table 3 ~ 6. The data of microbial composition, alpha- and beta-diversity were produced by CLC Genomics Work Bench. Venn diagrams were created in R using the ggVennDiagram package.

**Sample fixation and sectioning**

Esophagus, stomach, caecum and intestine tissue samples were immersed for 24 hours in Bouin's Fixative solutions and rinsed in 75% ethanol. Samples were dehydrated into 100% ethanol by an ethanol series (80%, 90%, 100%) and rinsed in histoclear. Samples were infiltrated by the Paraplast-histoclear series and embedded in paraplast (Paraplast Plus, Sigma, P3683). Sections were cut by a Leica RM2265 microtome (20 μm) and collected on poly-L-lysine coated slides. Each slide was dewaxed and rehydrated by histoclear and ethanol washing series (100%, 75%, 50%, 25%, PBS).

**Fluorescence *in situ* hybridization**

After rehydration, slides were incubated in 20 mM Tris-HCl (pH 7.4) for 10 minutes at



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room temperature. Protease K (10 μg/ml) and lysozyme (10 mg/ml) were mixed together in the same volume, then applied to slides for 1 hour at 37°C for sample permeabilization. After discharging the protease K and lysozyme, slides were incubated in 20 mM Tris-HCl (pH 7.4) for 10 minutes at room temperature. Slides were washed by Hybridization buffer (900 mM NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% SDS, 30% [vol/vol] formamide, 10% blocking solution). Hybridization buffer (900 mM NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% SDS, 30% [vol/vol] formamide, 10% blocking solution, with each probe at final concentration of 20 ng/ml) was applied to slides and incubated at 59°C for 3 hours, after hybridization buffer washing. Wash buffer (112 mM NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% SDS, 30% [vol/vol] formamide, 5 mM EDTA) was applied to slides and incubated at 48°C for 10 minutes. Slides were incubated in 1x PBS for 10 minutes at room temperature. Slides were incubated in DAPI (4', 6-diamidino-2-phenylindole [2.5 μg/ml]) and for 15 minutes at room temperature in the dark. Eub338 probe-applied slides were incubated in wheat germ agglutinin (20 μg/ml ) conjugated with Alexa Fluor 488. Slides were dipped in 1x PBS and mounted with a no. 1.5 cover glass (22 x 30 mm, Corning), and cured in the dark at 4 °C before imaging. The probes used in this study are listed in Table 7, including E338 (Target: Bacteria, 5'- GCTGCCTCCCGTAGGAGT -3', Amann et al., 1990), Myc 1-1 (Target: genus *Mycoplasma*, 5'- GCGGTA ATACATAGGTYGCAAGCG – 3', Cheaib et al. 2020 )



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and GV841 (Target: genus *Vibrio*, 5' – AGGCCACAACCTCCAAGTAG – 3').

**Hematoxylin and eosin staining**

The rehydrated samples were stained with hematoxylin for 1 ~ 4 minutes and eosin for 5 ~ 7 minutes, followed by dehydration through a progressive ethanol series (70%, 80%, 90%, 100%) for 5 minutes. Samples were mounted in Entellan new (Merck Millipore, Burlington, MA, USA) and covered with a no. 1.5 cover glass (22 x 30 mm, Corning).

**Statistical analysis**

Values were presented as the mean ± standard deviation (SD) and analyzed by using Mann Whitney U test. The significant difference was accepted at p value < 0.05. Linear regression was calculated to determine the correlations of digestive-somatic index (DSI) between CT and OA group. A permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity was used to determine the dissimilarity between each sample bacterial community.



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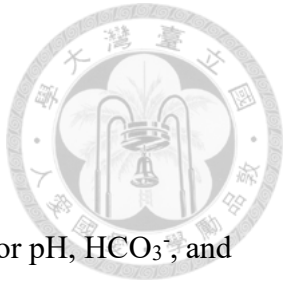
# Results



## Bigfin reef squid hemolymph status

The hemolymph sample was collected from the vena cava of squid for pH, $HCO_3^-$, and $pCO_2$ measurements (Fig. 4). In the control (CT) and ocean acidification (OA) groups, the hemolymph pH were 7.1083 ± 0.0464 and 7.074 ± 0.1026, respectively (Fig. 4A). The Mann Whitney test did not reveal a significant difference in hemolymph pH between CT and OA squids (p value = 0.1081). The hemolymph $HCO_3^-$ of CT group was 1.1662 ± 0.1096 mM, whereas that of the OA group was 1.6604 ± 0.3068 mM which was about 1.4-fold higher than that of the CT (Fig. 4B). Mann Whitney test results showed significant differences between control and OA squids in hemolymph $HCO_3^-$ (p value = 0.0101). These results suggest that squid can effectively compensate for the hemolymph acidosis induced by ocean acidification.

## Bigfin reef squid body status

The body status of CT squid was presented in Table. 1, including total body weight (W), mantle length, digestive gland weight (DG), and digestive-somatic index (DSI), which represents the proportion of DG weight in total body weight. The average total body weight was 115.1 g. The average mantle length was 12.67 cm. The average DG weight was 3.37 g. The average DSI was 2.94% (Table 1). The body status of OA squid was



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presented in Table. 2. The average body weight was 179.4 g. The average mantle length was 13.98 cm. The average DG weight was 4.1867 g. The average DSI was 2.36% (Table 2). The correlation analysis showed that DG weight was positively correlated with body weight (CT correlation coefficient = 0.9762, OA correlation coefficient = 0.9503), which indicates that squid DG weight was positively correlated with total body weight (Fig. 5A). However, the Mann Whitney test showed that the DSI of OA group was significantly lower than that of the CT group (p value < 0.0001), which inferred that OA could alter the alter the morphology of the digestive glands of squid (Fig. 5B).

**Bigfin reef squid digestive enzyme activity assay**

The average amylase activity of CT group was 30.063 ± 12.569 mU / mL, while the average amylase activity of OA group was 67.200 ± 6.104 mU / mL (Fig. 6A). The Mann Whitney test showed that the amylase activity of OA group was significantly higher than that of the CT group (p value = 0.0002), which indicates that ocean acidification increased the amylase activity in the bigfin reef squid. The average trypsin activity of CT group was 15.324 ± 3.083 mU / mL, and the average trypsin activity of OA group was 20.514 ± 2.616 mU / mL (Fig. 6B). The Mann Whitney test showed that the trypsin activity of the OA group was significantly higher than that of the CT group (p value = 0.0152), which indicates that ocean acidification increased the trypsin



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activity of bigfin reef squid. The average lipase activity of CT group was 12.507 ± 2.795 mU / mL and the average lipase activity of OA group was 17.335 ± 2.755 mU / mL (Fig. 6C). The Mann Whitney test showed that the lipase activity of OA group was significantly higher than CT group (p value = 0.021), which indicates that ocean acidification increased the lipase activity in bigfin reef squid.

**Bigfin reef squid gut microbiota**

The average composition of CT caecum (n = 10) showed that the caecum microbiota was composed of four main bacterial groups: *Mollicute* (73%), *Alphaproteobacteria* (12%), *Gammaproteobacteria* (6.8%) and *Betaproteobacteria* (3.6%) (Fig. 7A). The top ten dominant bacteria species were *Mycoplasma hominis* (15.90%), *Mycoplasma mobile* (15.30%), *Mycoplasma anatis* (12.95%), *Mycoplasma spp.* (10.69%), *Mycoplasma moatsii* (10.02%), Zymomonas mobilis (9.74%), *Mycoplasma alkalescens* (6.52%), *Aquabacterium sp. NJ1* (1.52%), *Acientobacterr sp. P8-3-8* (1.33%) and *Acientobacter spp.* (1.11%). The average microbial composition of OA caecum (n = 9) revealed that the average community consisted of three bacterial groups: *Mollicute* (73%), *Alphaproteobacteria* (10%) and *Gammaproteobacteria* (8%) (Fig. 7B). The top ten dominant bacteria species were *Mycoplasma hominis* (18.91%), *Mycoplasma anatis* (16.70%), *Mycoplasma mobile* (15.70%), *Mycoplasma moatsii* (9.80%), *Zymomonas*



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*mobilis* (9.30%), *Mycoplasma spp.* (8.11%), *Mycoplasma alkalescens* (5.16%), *Mycoplasma auris* (2.70%), *Vibrio parahaemolyticus* (1.78%) and *Mycoplasma arthritidis* (1.61%). The abundance variance of the top ten dominant bacterial species in squid caecum was low, indicating that individual differences from the host were not significant (Figure 7). Genus *Mycoplasma* was the most dominant bacteria group in the caecum (CT = 71.38%, OA = 78.69%). Genus *Zymomonas* was the second dominant bacteria group in caecum (CT = 10.14%, OA = 9.82%), the representative species was *Zymomonas mobilis*. However, the less abundant bacteria group in CT group, genus *Acientobacter* (5.94% → 0.23%) and *Aquabacterium* (2.72% → 0.34%), were diminished in OA group and replaced by genus *Vibrio* (0.52% → 6.61%). The Venn diagram (Fig. 8A) showed that there were 90 bacteria species appeared in CT and OA bigfin reef squid caecum. 31.1% of them only appeared in CT caecem. 20% of them only appeared in OA caecum. 48.9% of them appeared in both CT and OA caecum. The CT group showed higher alpha diversity than OA group (Simpson' s index p value = 0.0055, Shannon entropy p value = 0.0123) (Fig. 8B). A principal coordinate analysis (PCoA), based on Bray-Curtis distance, showed that the microbial community of CT and OA caecum was clustered together (Fig. 9). A permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity showed that there was no dissimilarity between the microbiota of CT and OA caeca (Pseudo-f statistic =



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2.45669, p value = 0.9979) (Table 8). This indicates that OA did not alter the main microbial composition of the caecum of bigfin reef squid. However, OA decreased microbial diversity by reducing the number of bacteria species with relatively low abundance.

The CT intestine was composed of three main bacterial groups: *Gammaproteobacteria* (53%), *Alphaproteobacteria* (24%) and *Mollicute* (14%) (Fig. 10A). The top ten dominant bacteria species were *Vibrio spp.* (19.27%), *Vibrio tubiashii* (10.86%), *Ruegeria conchae* (9.67%), *Vibrio alginolyticus* (5.03%), *Mycoplasma hominis* (4.82%), *Mycoplasma mobile* (4.54%), *Mycoplasma anatis* (3.93%), *Vibrio campbellii* (2.71%), *Ruegeria lacuscaerulensis* (2.56%) and *Acientobacter sp. P8-3-8* (2.01%) (Fig. 10A). The OA intestine was composed of three main bacterial groups: *Gammaproteobacteria* (57%), *Alphaproteobacteria* (23%) and *Mollicute* (17%) (Fig. 10B). The top ten dominant bacteria species were *Vibrio spp.* (23.37), *Vibrio tubiashii* (14.85%), *Mycoplasma hominis* (7.12%), *Mycoplasma mobile* (6.03%), *Mycoplasma antis* (5.81%), *Ruegeria conchae* (4.77%), *Labrenzia sp. DG1229* (4.46%), *Vibrio sp. ER1A* (2.69%), *Pseudovibrio sp. FO-BEG1* (2.46%) and *Vibrio anguillarum* (2.10%) (Fig. 10B). The abundance variance of the top ten dominant bacterial species in squid intestines was high, indicating that individual differences from the host were significant (Figure 10). In CT and OA intestine, genus *Vibrio* was the most dominant bacteria group



doi:10.6342/NTU202300020

(CT = 44.85%, OA = 53.75%), which was affiliated with class *Gammaproteobacteria*. Genus *Ruegeria* was the second most abundant bacteria group in the intestine (CT = 16.97%, OA = 9.11%), affiliated with class *Alphaproteobacteria*. The decreased abundance of *Ruegeria* was replaced by *Labrenzia* and *Pseudovibrio*, which were affiliated with *Alphaproteobacteria*. Genus *Mycoplasma* was the third abundant bacteria group in the intestine (CT = 14.2%, OA = 17.42%). The Venn diagram showed that there were 178 bacteria species appeared in CT and OA bigfin reef squid intestine (Fig. 11A). 40.4% of the only appeared in CT group intestine. 20.8% of them only appeared in OA group intestine. 38.8% of them were shared in both CT and OA intestine (Fig. 11A). OA could not change the diversity of CT and OA intestine microbiota (Simpson's index p value = 0.8891, Shannon entropy p value = 0.7639) (Fig. 11B). A principal coordinate analysis (PCoA) showed that CT and OA group intestine microbial communities were clustered together (Fig. 12). Based on a permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity, no significant differences were found between CT and OA caecum microbiota samples (Pseudo-f statistic = 0.51724, p value = 0.79512) (Table 8). The results demonstrate that OA did not affect the microbial composition and diversity of the intestine of bigfin reef squids.

**Microbiota in bigfin reef squid food resources and water samples**



doi:10.6342/NTU202300020

The microbial composition of food resource samples was shown in Figure 13. In goldfish samples (Column A and B), the top three dominant bacteria species were *Exigubacterium* sp. MH3 (16%), *Parabacteroides* sp. ASF519 (14.5%) and *Vibrio mimicus* (10.2%). In Pacific white shrimp samples (Column C, D and E), the top three dominant bacteria species were *Photobacterium damselae* (18.3%), *Elizabethkingia anopheles* (12.4%) and *Vibrio tubiashii* (11.1%). In *N. denticulata* sample (Column F), *Elizabethkingia anopheles* (5.6%), *Haliscommenobacter hydrossis* (5.4%) and *Rubrivivax gelatinosus* (5.3%) were the top three dominant bacteria species. In Table 9, the alpha diversity index of food resource samples was presented.

The microbiota of water samples was shown in Figure 14. The main composition of the water sample was *Flavobacterium sp. SCGC AAA160-P02* (17.8%), *Rhodobacteraceae bacterium HIMB11* (7.97%), *Flavobacteria bacterium MS024-3C* (7.56%) and *Alteromonas macleodii* (7.24%). The Venn diagram showed that there were 278 bacteria species appeared in CT and OA seawater samples (Fig. 15A). 18.3% of them only appeared in CT seawater. 33.8% of them only appeared in OA seawater. 47.8% of them were shared with CT and OA seawater. The alpha diversity analysis did not reveal any differences between the microbial communities of CT and OA water samples (Shannon entropy p value = 0.3333, Simpson's index p value = 0.3333) (Fig. 15B).



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In the Bray-Curtis dissimilarity-based PCoA, food resource samples were significantly separated from the squid gut microbial community (PERMANOVA: Pseudo-f statistic = 9.08875, p value = 0.0001) (Fig 16 and Table 10). In the Bray-Curtis dissimilarity-based PCoA, CT and OA water samples were clustered together (PERMANOVA: Pseudo-f statistic = 16.09788, p value = 0.3333) (Fig 16 and Table 10). The water samples were, however, separated from the squid gut microbiota (PERMANOVA: Pseudo-f statistic = 12.25097, p value = 0.0001) (Fig 16 and Table 10). Venn diagram analysis revealed that there were 536 bacteria species in food resource samples, 87 (48+39, 16.23%) of them were shared with squid gut microbiota (Fig. 17).

**Hematoxylin and eosin stain (HE stain) and Fluorescence *in situ* hybridization (FISH)**

The nuclei of bigfin reef squid were visualized using HE stain and DAPI. The spatial distribution of bacteria in the digestive tract of the bigfin reef squid was determined by FISH. The caecum of the bigfin reef squid was characterized by a large number of leaf-like structures and epithelium cells (Fig. 18). Figure 19 showed that Eub338 signal located in the epithelium cells of caecum. As a result of FISH, numerous *Mycoplasma* signals were located inside the epithelium cells (Fig. 20A). However, *Vibrio* signals,



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located in the leaf-like structure axis, were less than *Mycoplasma* signals (Fig. 20B). Villi were found in abundance in the intestine of the bigfin reef squid (Fig. 21A). The villi epithelium consists of numerous mucus cells and epithelium cells. The inner layer of the villi is composed of muscle cells, connective tissue, and vessels (Fig. 21B). Figure 22 showed that Eub338 signals located in the mucus muscle cells, connective tissue, and vessels. Moreover, the majority of *Mycoplasma* signals are located in the inner layer of the villi (Fig. 23A). *Vibrio* signals were detected in both the fold inner layer and the villi epithelium (Fig. 23B). Partially colocalization of *Mycoplasma* and *Vibrio* signals was observed (Fig. 23F).



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# Discussion

**Bicarbonate accumulation in bigfin reef squid *S. lessoniana***

In the present study, a long-term (3 months) treatment of ocean acidification (OA) was utilized to investigate the effect on squids by comparing the changes in squid hemolymph pH, $HCO_3^-$ and partial pressure of $CO_2$ between control (CT) and OA groups. Previous studies hypothesized that excessive environmental $CO_2$ diffuses into the squid and teleost gill epithelial cells where $CO_2$ and $H_2O$ are catalyzed by carbonic anhydrase (CA) to form $H^+$ and $HCO_3^-$ (Lin et al., 2008; Lee et al., 2011; Hu et al., 2014). As a result, the epithelial cells discharged excessive $H^+$ through $Na^+/H^+$ exchanger 3 (NHE3) in order to protect animal cells (Shih et al., 2012; Hu et al., 2014). In addition, apical Rhesus proteins (RhP) release $NH_3$ into the luminal space of the gills (Hu et al., 2014). $NH_3$ is then reacted with $H^+$ to form $NH_4^+$, which is then released by NHE3 (Hu et al., 2014). Upon exchange of $Na^+$ with $H^+$ by NHE3, imported $Na^+$ is co-transported with $HCO_3^-$ into hemolymph by $Na^+$/ $HCO_3^-$ cotransporter (NBC) (Lee et al., 2011; Shih et al., 2012; Hu et al., 2014). As a result, $HCO_3^-$ accumulates in the squid hemolymph for pH stabilization (Hu et al., 2014). The hemolymph pH stabilization was accompanied by an increase in $HCO_3^-$ level, which indicates that the bigfin reef squid can effectively compensate for internal acidosis induced by an acidified environment up to pH 7.8. In fish, crustaceans, and cephalopods, this mechanism is conserved and



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efficient in countering respiratory acidosis (Hu et al., 2014; Pörtner et al., 1991; Henry et al., 2012).

**OA effect on bigfin reef squid morphology and digestive system**

The total weight, mantle length, and digestive-somatic index (DSI) of the bigfin reef squid were measured to determine whether ocean acidification alters the morphology and digestive system of the species. Several studies have shown that acidified environments result in smaller body sizes in marine animals (Kelly et al., 2013; Pimental et al., 2015). Nevertheless, in this study, the CT group was lighter and had a shorter mantle than the OA group. This opposite result may be due to the breeding density (CT = 9.5 individuals/box net, OA = 6.5 individuals/box net). In addition, OA influences the digestion of marine animals (Khan et al., 2020; Xu et al., 2020). Therefore, this study calculated the digestive-somatic index (DSI) of bigfin reef squid, including CT and OA groups, since the activity of digestive enzymes is related to DSI (Hani et al., 2018). The acidified group showed a lower DSI, which indicates that OA may affect the development and function of the digestive system in bigfin reef squid because the morphology of the digestive gland plays an important role in detoxification, digestive enzymes secretion, and nutrient storage, (Ramón Anadón 2019; Rodrigo et al., 2017). Cephalopods have been reported to contain digestive enzymes, such as trypsin,



doi:10.6342/NTU202300020

amylase, and lipase (Ibarra-García et al., 2018). A comprehensive analysis of the enzyme activity of trypsin, amylase, and lipase in the digestive gland was conducted in order to address this issue. Interestingly, all three enzymes had significantly elevated activities in the OA group. Except for trypsin and lipase, it is uncommon to find high levels of amylase activity in bigfin reef squid as a carnivore animal, whereas similar findings have been reported for octopus and slipper lobster (Ibarra-García et al., 2018; Johnston et al., 1998). In cephalopods, amylase has been reported to break down the α-1-4 linkages in polysaccharide storage molecules present in prey muscle tissue (Ibarra-García et al., 2018). The higher digestive enzyme activity in bigfin reef squids suggests that they can efficiently absorb nutrients from food to maintain high metabolic rates. In this study, acidic conditions led to the development of a smaller but more active digestive gland in the squid. In previous research, the acid treatment decreased the activity of digestive enzymes in Korean mussels, *Mytilus coruscus*, including amylase, lipase, trypsin, trehalase, and lysozyme (Khan et al., 2020). Trypsin activity also decreased in European sea bass *Dicentrarchus labrax* during OA (Cominassi et al., 2020). However, the bigfin reef squid showed an opposite response in this study. Similarly, amylase and trypsin activities were also found to be higher under pH = 7 conditions than pH = 8 conditions in the California two-spot octopus *Octopus bimaculoides* (Ibarra-García et al., 2018). Cephalopods are likely to adapt to acidified



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environments by activating digestive enzymes. This study is the first to provide information regarding the effect of OA on digestive enzyme activity in cephalopods. Despite this, the nutrient content of the digestive gland, such as sugar, protein, and lipid, has not been measured. There is a need for more experiments to be conducted in the future.

**OA effect on gut microbiota structure in bigfin reef squid**

Previous studies have revealed the gut microbiome of several cephalopods. The genus *Vibrio* was the dominant bacterial group in the European common cuttlefish *Sepia officinalis*, which forms a highly simplified microbiota (Lutz et al., 2019). *Mycoplasma* and *Photobacterium* were the two most common genera in the gut microbiota of wild cephalopods, including cuttlefish, beka squid, inshore squid, Japanese flying squid, common octopus, and whiparm octopus (Kang et al., 2021). For marine carnivore fish, *Photobacterium*, *Vibrio, Mycoplasma* and *Acinetobacter* are common colonizers in the gut (Egerton et al., 2018). Likewise, these genera were all identified in the bigfin reef squid. The high degree of overlap between bacteria groups in gut microbiota may be evidence that cephalopods and marine carnivore fish evolved convergently to compete with each other (Hu et al., 2014). Moreover, cephalopods are also known to have diverse associations with microorganisms. A famous symbiosis is the one between the



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bioluminescent producer *Vibrio fischeri* and the Hawaiian bobtail squid (Jones et al., 2004; Nyholm et al., 2021). Another symbiosis is the colonization of the accessory nidamental gland (ANG) by *Alpha-* and *Gammaproteobacteria*, which enables the host to secrete egg jelly coat to protect the offspring (Pichon et al., 2007; Lutz et al., 2019). The identified bacteria described above are likely to form a symbiotic relationship with the bigfin reef squid in the digestive system, thus performing probiotic functions and facilitating digestion.

In this study, the gut microbiota of bigfin reef squids, including the caecum and intestine, was further examined. Both 16S ribosomal gene sequencing and FISH images indicated that *Mycoplasma* and *Vibrio* are two predominant bacterial genera in the digestive tract of bigfin reef squid. Although the acidic treatment did not alter the main microbial composition in the bigfin reef squid's caecum, it decreased the microbial diversity due to a reduced number of bacterial species and relatively low abundance. On the other hand, OA did not affect the microbial composition and diversity of the intestine. The FISH image revealed numerous *Mycoplasma* signals within the epithelial cells. Furthermore, *Mycoplasma* signals were mostly found in the inner layer of the villi in the intestine. *Vibrio* signals were detected in both the fold inner layer and the villi epithelium with partial colocalization with *Mycoplasma* signals.

*Mycoplasma* is an obligate parasitic bacterium found in the gut of many marine



doi:10.6342/NTU202300020

animals, such as Norway lobster, jellyfish, and Atlantic salmon (Kang et al., 2021; Meziti et al., 2010; Star et al., 2013; Viver et al., 2017). The intestinal ecosystem typically contains pathogenic bacteria related to *Mycoplasma* (Waites et al., 2004). There is, however, little information available on their roles within invertebrates. Based on the FISH image (Fig. 20 and 23), the *Mycoplasma* signals were primarily located inside the host cell. The *Mycoplasma* signals in the caecum are distributed in the epithelium, suggesting that *Mycoplasma* would also be opportunistic bacteria in the bigfin reef squid. This intracellular *Mycoplasma* can form endosymbiotic relationships with protists (Margarita et al., 2022). There is a possibility that these intracellular *Mycoplasma* are endosymbionts of the bigfin reef squid. The investigation is necessary to determine the characteristics of *Mycoplasma* in the digestive tract of bigfin reef squid.

Besides, it has been reported that *Vibrio* produces chitinase, protease, amylase and lipase, which suggests the possibility that the *Vibrio* colonization of the digestive tract could aid in digestion (Egerton et al., 2018; Lutz et al., 2019). The presence of *Vibrio* in the intestine of bigfin reef squid may serve to absorb nutrients from the caecum's food residue. Otherwise, *Vibrio*-related species are known as pathogens in corals, fish, diverse marine organisms and humans (Lutz et al., 2019; Pruzzo et al., 2005; Takemura et al., 2014), and sometimes result in skin lesions or mortality in cephalopods (Hanlon et al., 1990; Forsythe et al., 1990; Ford et al., 1986; Lutz et al., 2019). Whether the



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genus *Vibrio* is a part of the physiology of bigfin reef squids or whether they represent pathogenic and opportunistic microorganisms that colonize the digestive tract of the squids remains to be determined.

According to a previous study, ocean acidification has altered the structure of mussel gut microbiomes, resulting in a pathogen-dominated community (Khan et al., 2021). Furthermore, an acidified environment altered the composition and metabolic function of sponge-associated microbial communities (Botté et al., 2019). Nevertheless, the same phenomenon was not observed in the gut microbial community of bigfin reef squids. The results of the beta diversity analysis showed that ocean acidification did not affect the microbial community in the caecum and intestine of bigfin reef squids (Fig. 9, 12 and Table 8). In a similar fashion, ocean acidification did not change the composition of marine fish *Sparus aurata* (Fonseca et al., 2019). In other hand, the less abundant bacteria could be affected by ocean acidification. In caecum, genus *Acinetobacter* and *Aquabacterium* were replaced by *Vibrio*. In intestine, the abundance of genus *Ruegeria* decreased, at the same time, the abundance of genus *Labrenzia* and *Pseudovibrio* increased. The main composition of the gut microbiota did not change, but the minor composition of the gut microbiota changed. The metabolic profile of the bacterial community might change in the digestive tract of bigfin reef squids during ocean acidification (Botté et al., 2019). In order to reveal the physiological and



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pathogenic characteristics of bacterial colonizers in the digestive tract of bigfin reef squid further investigation is needed.



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# Conclusion

In the present study, we first reveal the impacts of ocean acidification on the gastrointestinal system, including the digestive enzymes and the microbial community in the gut of the bigfin reef squid *Sepioteuthis lessoniana*. During ocean acidification, the activity of digestive enzymes in bigfin reef squid, including amylase, trypsin, and lipase, increases, which is different from the activity of other marine animals. Additionally, *Mycoplasma* and *Vibrio* are the most dominant bacteria groups in the digestive tract. According to the results of this study, ocean acidification did not change the gut microbiota structure of *S. lessoniana*. It is still possible that the increased digestive activity is related to *Vibrio*, which is known to produce several digestive enzymes. Accordingly, symbiotic bacteria may be involved in digestion, supplying sufficient nutrients to both the host and the microbiota to overcome the challenges posed by ocean acidification. In order to understand how the ocean and its inhabitants will cope with an acidified environment in the future, more comparative studies based on these systematic results from bigfin reef squid are still necessary.



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# Tables and Figures



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# Table 1

**Table 1.** The body status of control (CT) group bigfin reef squids.

| Number | Weight (W, g) | Mantle length (cm) | Digestive gland weight (DG, g) | Digestive-somatic index (DSI, %) |
|---|---|---|---|---|
| 1 | 87 | 11 | 2.68 | 3.08 |
| 2 | 116 | 12.5 | 3.53 | 3.04 |
| 3 | 86 | 11.6 | 2.52 | 2.93 |
| 4 | 90 | 11.9 | 2.78 | 3.09 |
| 5 | 122 | 12.8 | 3.42 | 2.80 |
| 6 | 110 | 12.5 | 3.11 | 2.83 |
| 7 | 133 | 13.1 | 3.81 | 2.86 |
| 8 | 133 | 14.5 | 4.06 | 3.05 |
| 9 | 138 | 13.6 | 4.05 | 2.94 |
| 10 | 136 | 13.2 | 3.79 | 2.78 |
| Average | 115.1 | 12.67 | 3.37 | 2.94 |



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# Table 2

**Table 2.** The body status of ocean acidification (OA) group bigfin reef squids.

| Number | Weight (W, g) | Mantle length (cm) | Digestive gland weight (DG, g) | Digestive-somatic index (DSI, %) |
|---|---|---|---|---|
| 1 | 193 | 13.1 | 4.31 | 2.23 |
| 2 | 137 | 13.2 | 3.12 | 2.28 |
| 3 | 124 | 12.2 | 3.39 | 2.73 |
| 4 | 134 | 13.5 | 3.52 | 2.63 |
| 5 | 216 | 14.6 | 5.25 | 2.43 |
| 6 | 221 | 14.7 | 4.78 | 2.16 |
| 7 | 168 | 13.9 | 3.78 | 2.25 |
| 8 | 224 | 15.4 | 5.21 | 2.33 |
| 9 | 198 | 15.2 | 4.32 | 2.18 |
| Average | 179.4 | 14.0 | 4.19 | 2.36 |



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# Table 3

**Table 3.** The sequence number and species number of CT group squid gut microbiome.

| Sample | Number of sequences | Number of sequences (Match to database) | species |
|---|---|---|---|
| CT-1-Ce | 13855 | 2460 | 21 |
| CT-2-Ce | 10485 | 1841 | 26 |
| CT-3-Ce | 7245 | 1293 | 22 |
| CT-4-Ce | 28579 | 5213 | 29 |
| CT-5-Ce | 14582 | 2541 | 24 |
| CT-6-Ce | 7705 | 1275 | 20 |
| CT-7-Ce | 33175 | 5891 | 29 |
| CT-8-Ce | 20953 | 1787 | 20 |
| CT-9-Ce | 8262 | 1555 | 19 |
| CT-10-Ce | 4607 | 815 | 15 |
| CT-1-In | 25262 | 5065 | 35 |
| CT-2-In | 8160 | 1340 | 19 |
| CT-3-In | 12393 | 5225 | 24 |
| CT-4-In | 9036 | 6506 | 28 |
| CT-5-In | 33516 | 6728 | 34 |
| CT-6-In | 16968 | 14348 | 44 |
| CT-7-In | 53973 | 23563 | 33 |
| CT-8-In | 9778 | 8518 | 31 |
| CT-9-In | 8262 | 1555 | 19 |
| CT-10-In | 4680 | 1769 | 19 |



doi:10.6342/NTU202300020

# Table 4

**Table 4.** The sequence number and species number of OA group squid gut microbiome.

| Sample | Number of sequences | Number of sequences (Match to database) | species |
|---|---|---|---|
| OA-1-Ce | 8210 | 1426 | 19 |
| OA-2-Ce | 11459 | 1850 | 18 |
| OA-3-Ce | 22246 | 3377 | 23 |
| OA-4-Ce | 22532 | 3746 | 23 |
| OA-5-Ce | 14703 | 2463 | 25 |
| OA-6-Ce | 29235 | 6579 | 29 |
| OA-7-Ce | 25645 | 4366 | 25 |
| OA-8-Ce | 16366 | 2597 | 24 |
| OA-9-Ce | 17785 | 3076 | 23 |
| OA-1-In | 9118 | 3257 | 21 |
| OA-2-In | 7953 | 1818 | 27 |
| OA-3-In | 21189 | 4541 | 34 |
| OA-4-In | 24539 | 6733 | 26 |
| OA-5-In | 17680 | 14183 | 34 |
| OA-6-In | 16180 | 13259 | 31 |
| OA-7-In | 11812 | 8983 | 30 |
| OA-8-In | 13858 | 2419 | 27 |
| OA-9-In | 13680 | 8295 | 33 |



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# Table 5

Table 5. the sequence number and species number of food resource.

| Sample | Number of sequences | Number of sequences (Match to database) | species |
|---|---|---|---|
| WS-1 | 18951 | 18558 | 52 |
| WS-2 | 13299 | 11985 | 71 |
| F-1 | 16738 | 15726 | 88 |
| F-2 | 17810 | 16924 | 93 |



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# Table 6

**Table 6.** The sequence number and species number of CT and OA water samples.

| Sample | Number of sequences | Number of sequences (Match to database) | species |
|---|---|---|---|
| Water-CT-1 | 8131 | 6438 | 78 |
| Water-CT-2 | 7840 | 6117 | 85 |
| Water-OA-1 | 15944 | 11792 | 103 |
| Water-OA-2 | 15339 | 11350 | 116 |



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# Table 7

**Table 3.** Fluorescence *in situ* hybridization (FISH) probes used in this study.

| Probe | Fluorophore | Target organism | Sequence (5' – 3') | Reference |
|---|---|---|---|---|
| Eub338 | Cy5 | Bacteria | GCTGCCTCCCGTAGGAGT | Amann et al., 1990 |
| Myc 1-1 | Cy5 | *Mycoplasma* | GCGGTAATACATAGGTYGCAAGCG | Cheaib et al. 2020 |
| GV841 | Alexa488 | *Vibrio* | AGGCCACAACCTCCAAGTAG | Giuliano et al. 1999 |



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# Table 8

**Table 8.** Permutational multivariate analysis of variance (PERMANOVA) result of CT and OA squid gut microbiota.

| Group 1 | Group 2 | Pseudo-f statistic | p-value | p-value (Bonferroni) |
|---|---|---|---|---|
| CT-Ce | OA-Ce | 2.45669 | 0.09979 | 0.09979 |
| CT-In | OA-In | 0.51724 | 0.79512 | 0.79512 |



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# Table 9

**Table 9.** Alpha diversity index of bigfin reef squid food resource, including 2 goldfish, 3 Pacific white shrimp and one *Neocaridina denticulate*.

| | **Simpson's index** | **Shannon entropy** |
|---|---|---|
| Gold fish-1 | 0.91 | 4.3 |
| Gold fish-2 | 0.91 | 4.31 |
| Pacific white shrimp-1 | 0.8 | 3.84 |
| Pacific white shrimp-2 | 0.9 | 4.09 |
| Pacific white shrimp-3 | 0.92 | 4.83 |
| *Neocaridina denticulate* | 0.97 | 5.9 |



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# Table 10

**Table 10.** Permutational multivariate analysis of variance (PERMANOVA) result of all squid gut microbiota, water and food resources.

| Group 1 | Group 2 | Pseudo-f statistic | p-value | p-value (Bonferroni) |
|---|---|---|---|---|
| Water-CT | Water-OA | 16.09788 | 0.33333 | 1 |
| Gut microbiota | food | 9.08875 | 0.0001 | 0.0003 |
| Gut microbiota | Water | 12.25097 | 0.0001 | 0.0003 |
| food | Water | 4.69534 | 0.00476 | 0.01429 |



doi:10.6342/NTU202300020

# Figure 1

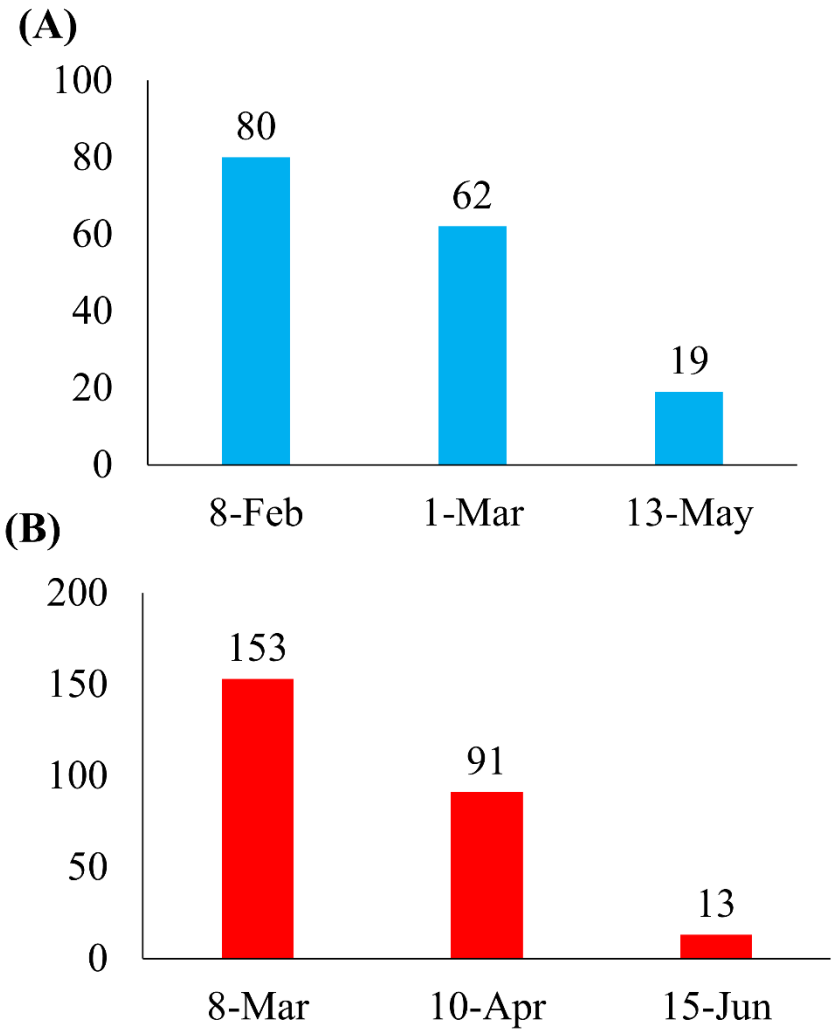


**Figure 1.** The individual number of bigfin reef squids during the experiment. **(A)** CT group hatched at February 8, was transferred from 1000L circulatory seawater system to 30,000 L pool on March 1 and sampled on May 13. **(B)** OA group hatched on March 8, was transferred from the 1000 L circulatory seawater system to the 30,000 L pool on April 10 and sampled on June 15.



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# Figure 2

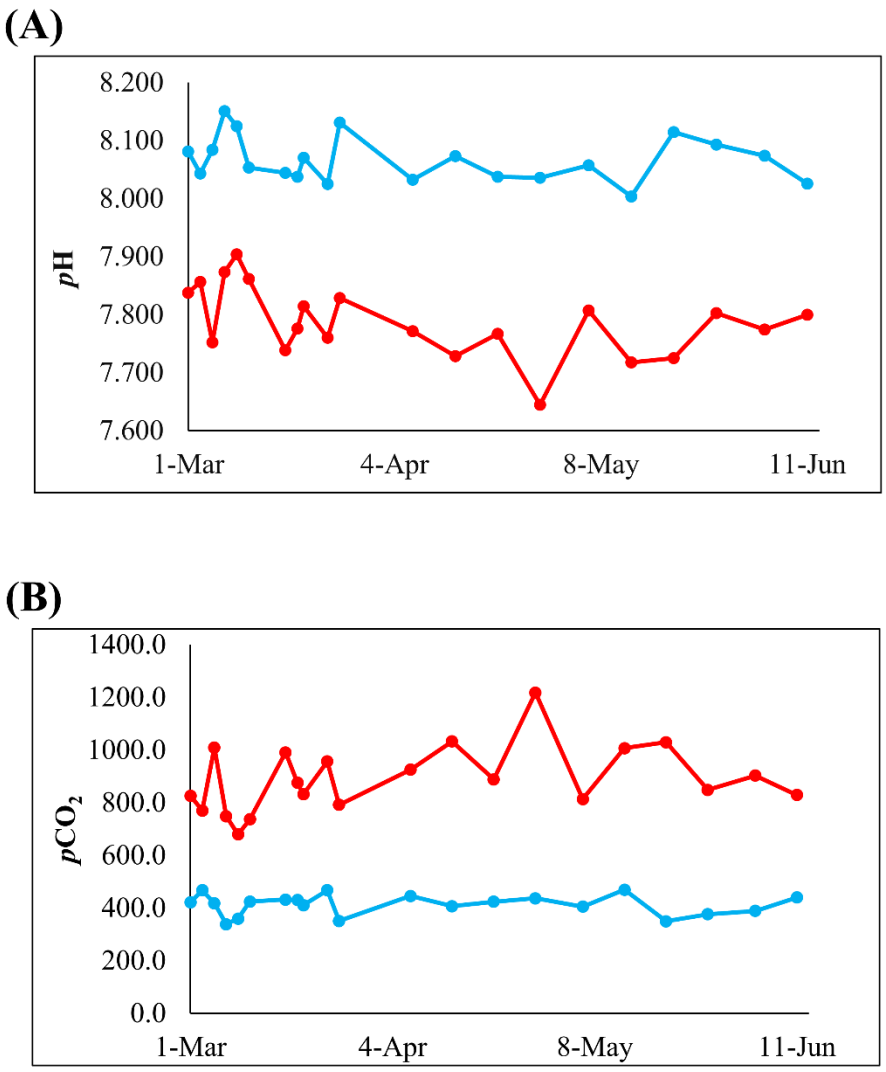


**Figure 2.** The water chemistry of the bigfin reef squid keeping space, including 1000L circulatory seawater system and 30,000L pool, during three-month ocean acidification (OA) experiments. **(A)** *p*H **(B)** $pCO_2$. Red dots are the OA group. Blue dots are the CT group.



doi:10.6342/NTU202300020

# Figure 3

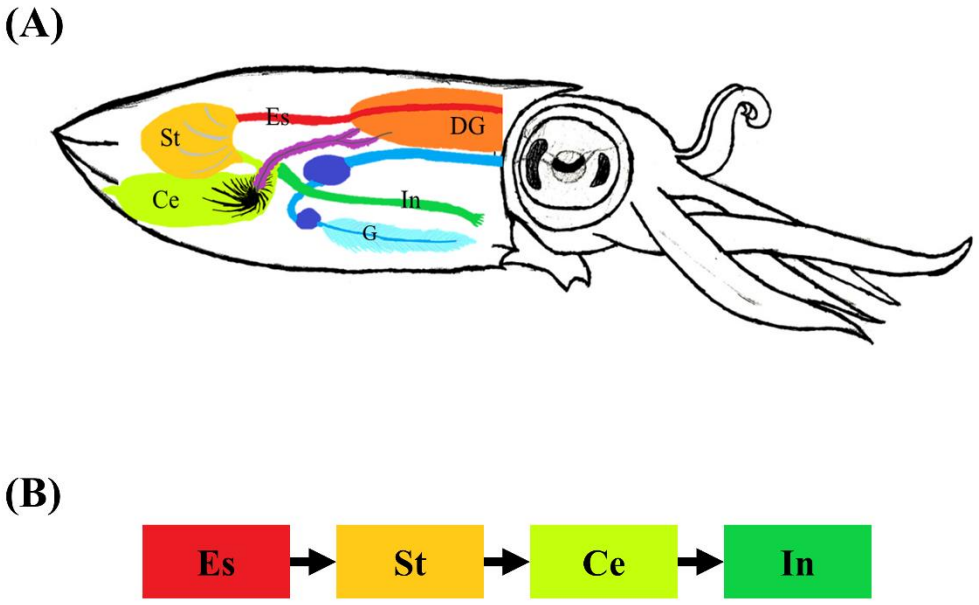


**Figure 3. (A)** The anatomy of bigfin reef squid. **(B)** The flow chart of food enters the bigfin reef squid digestive tract. Ce: Caecum, DG: Digestive Gland, Es: Esophagus, G: Gill, In: Intestine, St: Stomach.



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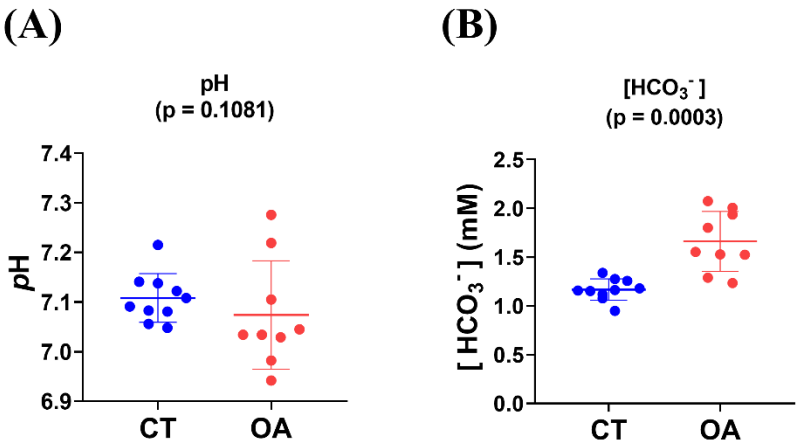
# Figure 4




**Henderson-Hasselbalch equation**

$$pCO_2 = C_T(\alpha\,(10^{(pH - pK1)} + 1))^{-1}$$

$$e[HCO_3^-] = C_T - (\alpha\, pCO_2)$$

**Figure 4.** The hemolymph status of bigfin reef squid. **(A)** pH and **(B)** pCO2. For the Henderson-Hasselbalch equation, CT is total dissolved inorganic carbon. Solubility coefficient of CO2 in seawater α = 0.039 μmol $L^{-1}$ Pa. The dissociation constant of carbonic acid at a salinity of 30 ‰ pK1 = 5.94. e is Euler's number = 2.718. Statistical analysis is presented by the Mann Whitney test. Dot represents as individual. Bar represents as mean ± SD.



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# Figure 5

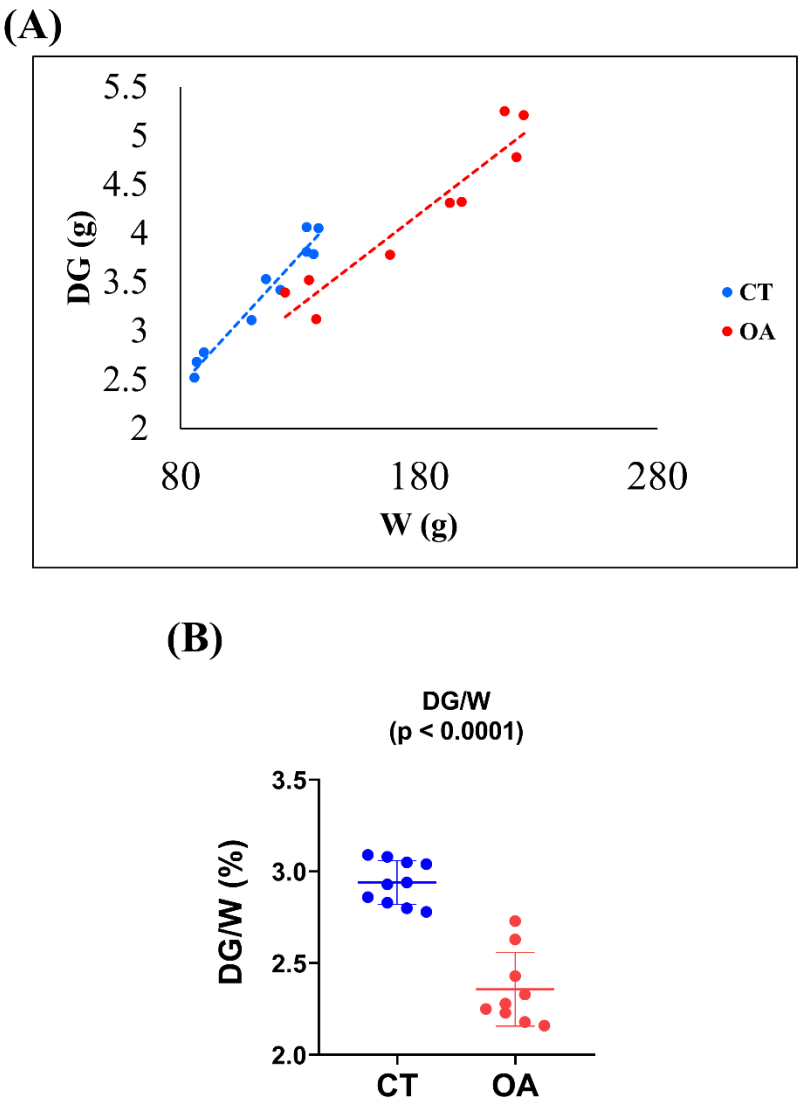


**Figure 5.** The proportion of Digestive Gland weight in total squid body weight, DG/W. **(A)** The correlation between DG weight and total weight (W). CT group correlation coefficient = 0.9762. OA group correlation coefficient = 0.9503. **(B)** Comparison of DG/W between CT and OA group. Statistical analysis is presented by the Mann Whitney test. Dots represent samples. Bar represents mean ± SD. The dash lines represent the trendline.



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# Figure 6

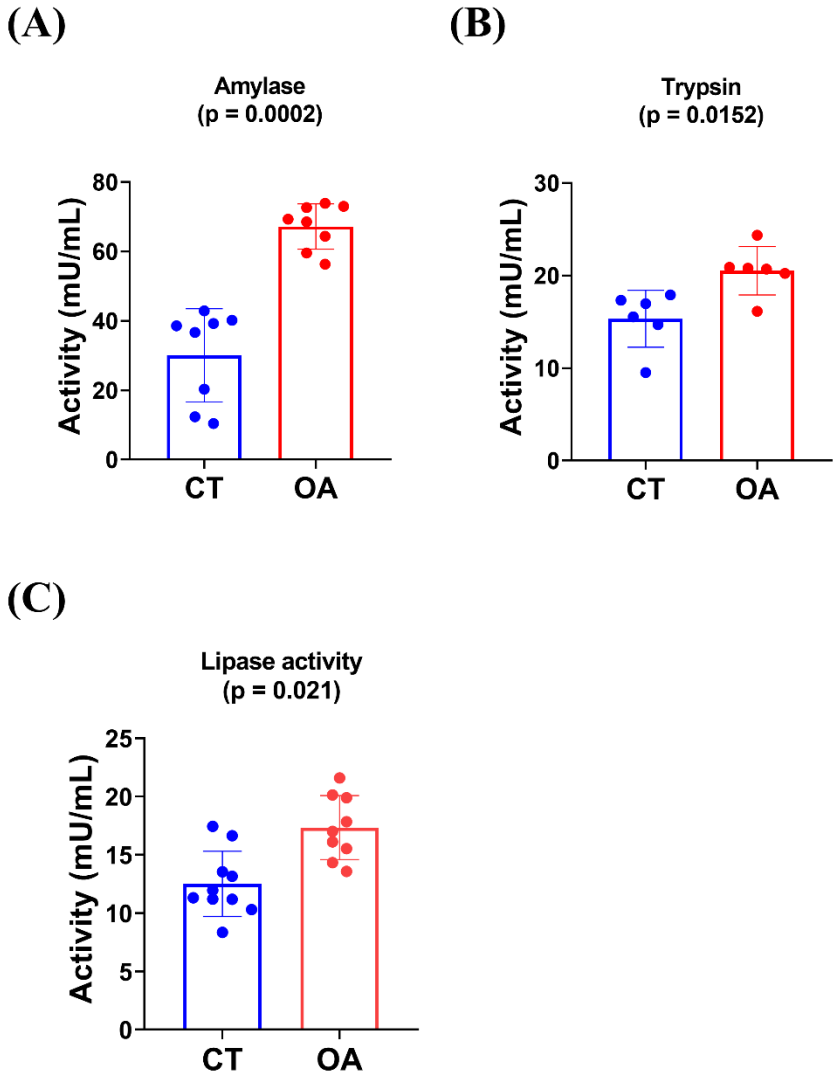


**Figure 6.** The enzyme activity of CT and OA group. **(A)** Amylase **(B)** Trypsin and **(C)** Lipase. Statistical analysis is presented by the Mann Whitney test. Dots represent samples. Bar represents mean ± SD.



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# Figure 7

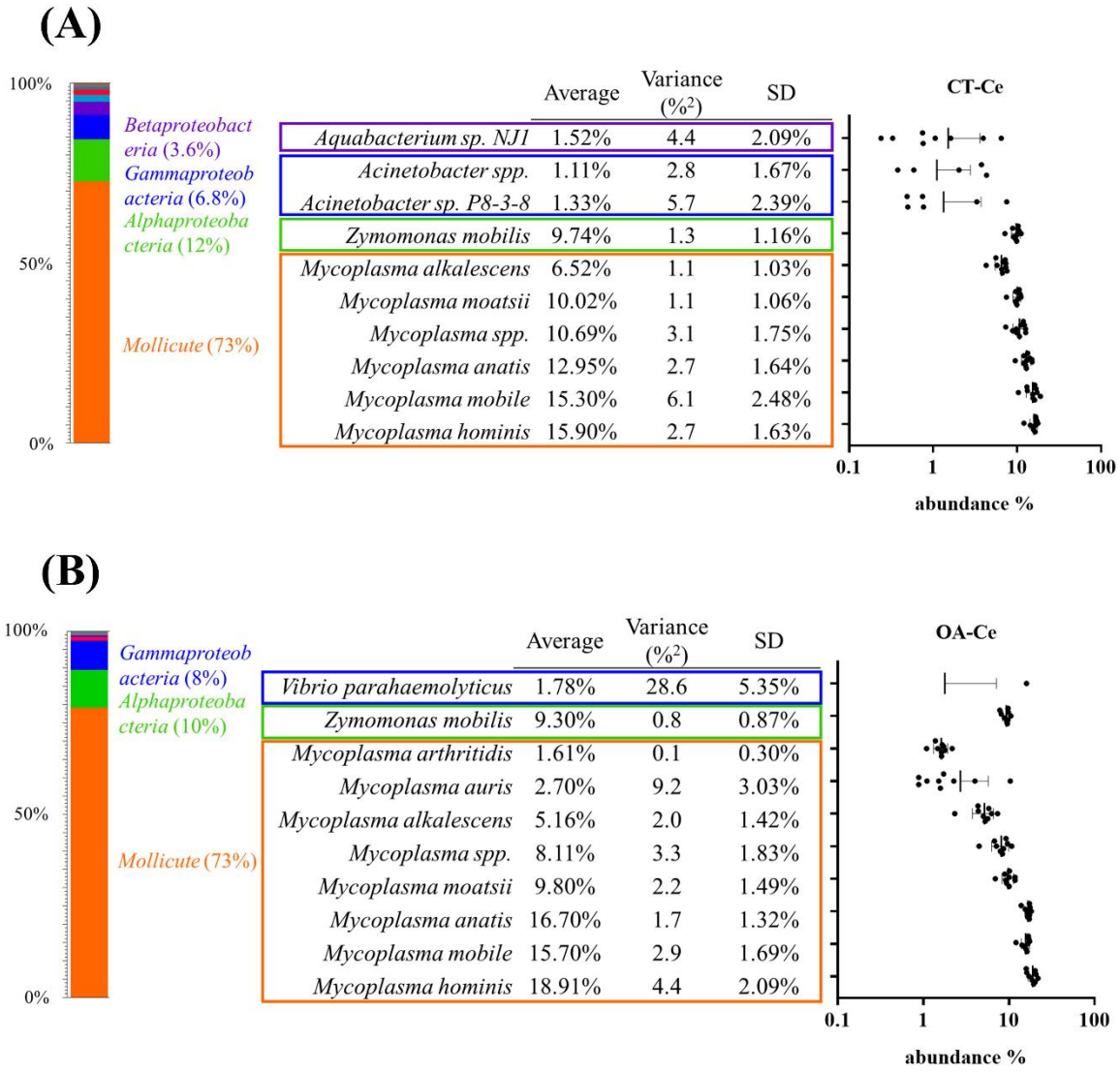


**Figure 7.** The average microbial composition in CT and OA group squid caecum (Ce). **(A)** control and **(B)** ocean acidification. Taxa is presented at the class level in the bar plot. Taxa is presented at the species level in the Table. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. A Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. Variance units are $\%^2$.



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# Figure 8

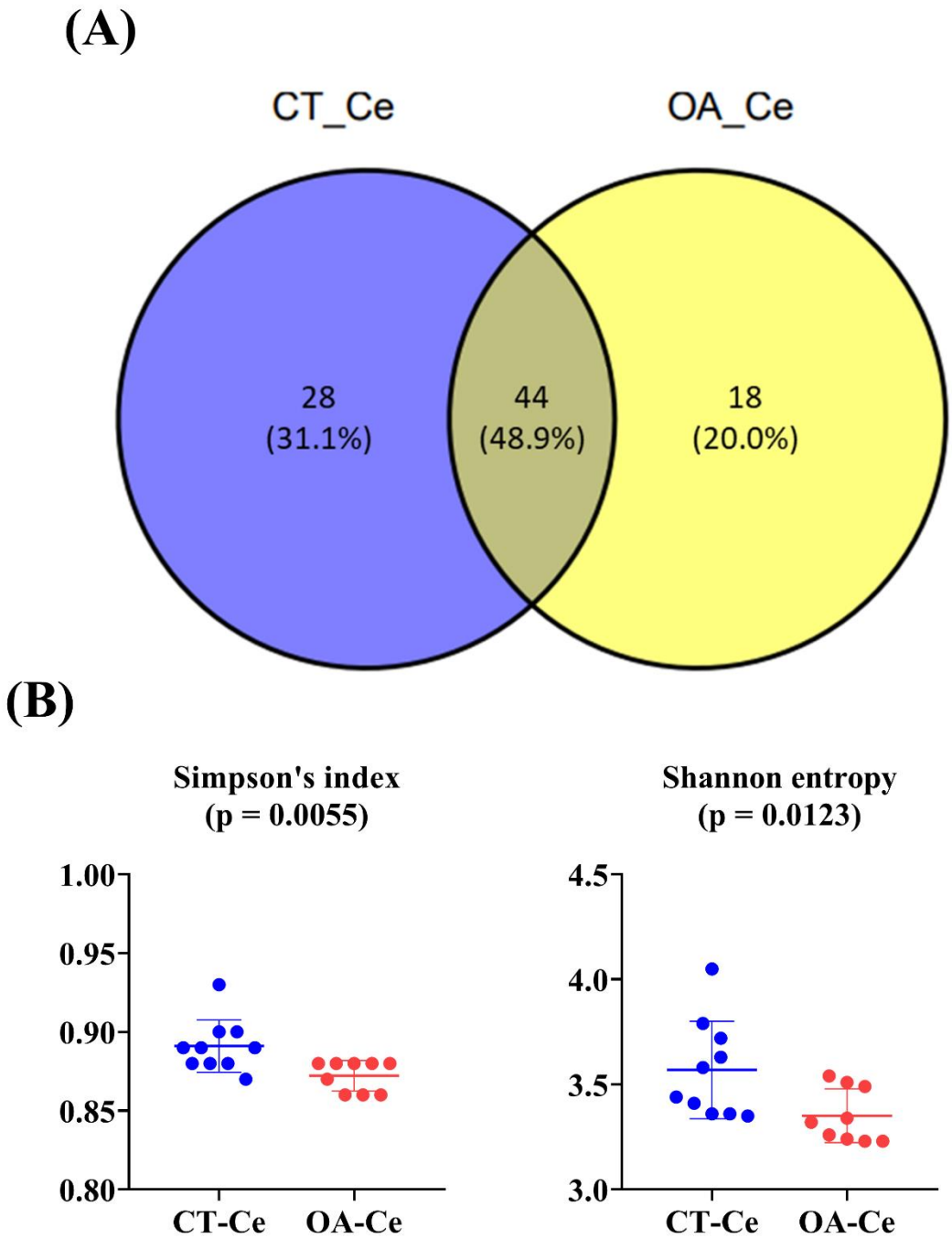


**Figure 8.** Comparison of the bacterial communities in CT and OA bigfin reef squid caecum. (A) Venn diagram and (B) alpha diversity. Numbers in the circle represent bacterial species numbers. Statistical analysis is presented by the Mann Whitney test.



doi:10.6342/NTU202300020

# Figure 9

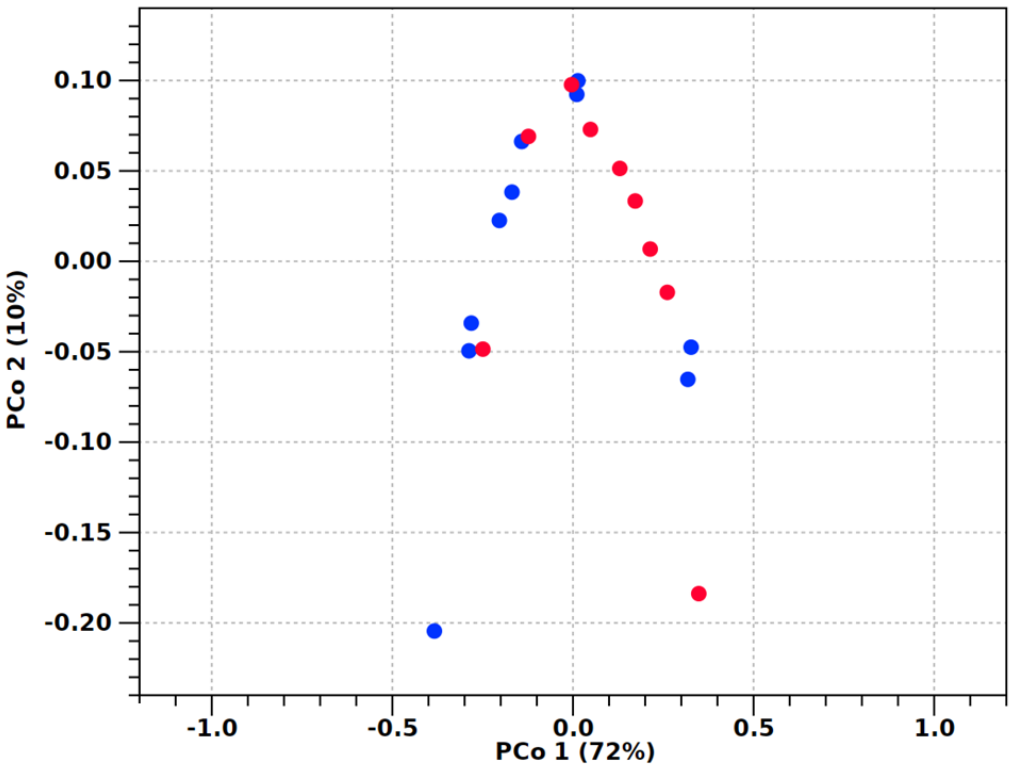


**Figure 9.** Principle-coordinate analysis (PCoA) of Bray-Curtis beta diversity comparing CT and OA caecum microbiota. Blue dots represent CT group. Red dots represent OA group.



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# Figure 10

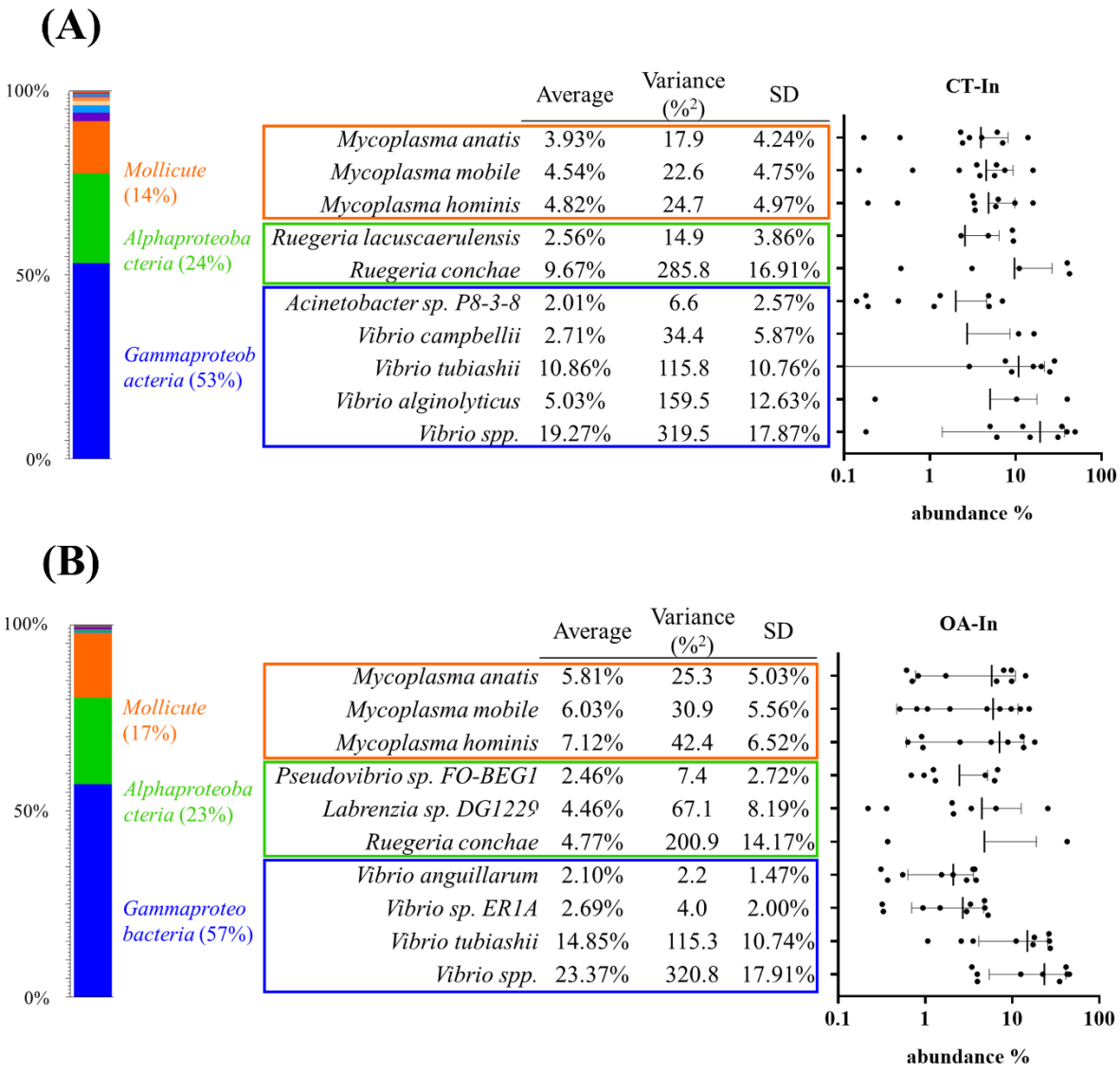


**Figure 10.** The average microbial composition in CT and OA group squid intestine (In). **(A)** control and **(B)** ocean acidification. Taxa is presented at the class level in the bar plot. Taxa is presented at the species level in the Table. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. A Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. Variance units are $\%^2$.



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# Figure 11

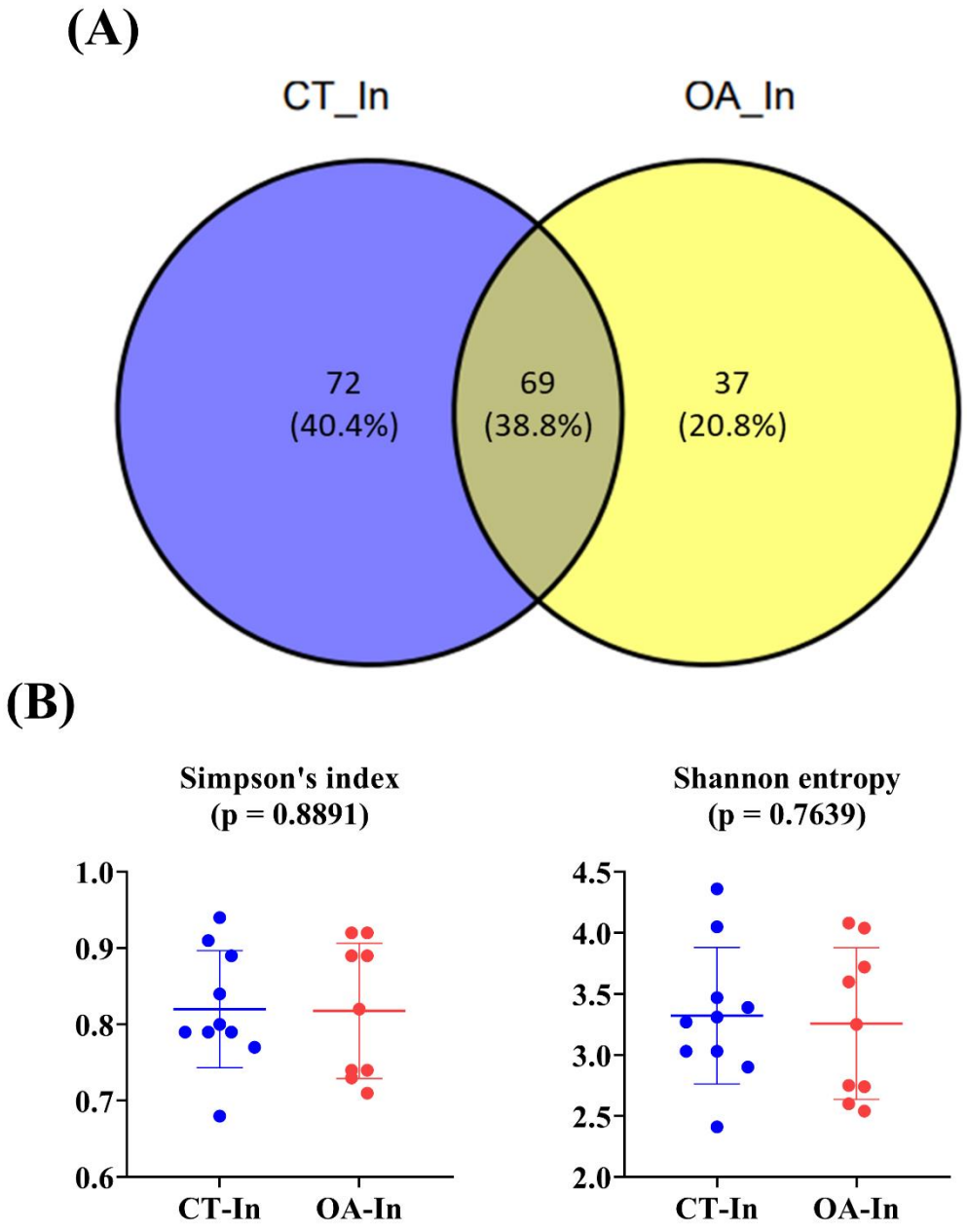


**Figure 11.** Comparison of the bacterial communities in CT and OA bigfin reef squid Intestine. (A) Venn diagram and (B) alpha diversity. Numbers in the circle represent bacterial species numbers. Statistical analysis is presented by the Mann Whitney test.



doi:10.6342/NTU202300020

# Figure 12

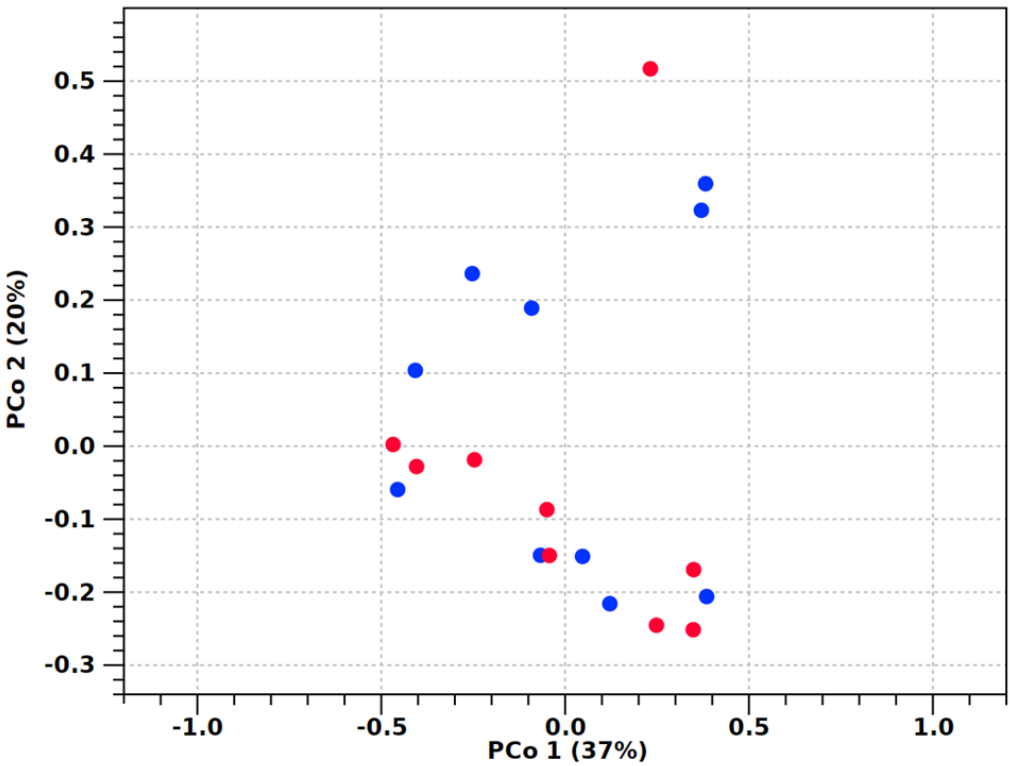


**Figure 17.** Principle-coordinate analysis (PCoA) of Bray-Curtis beta diversity comparing CT and OA interstine microbiota. Blue dots represent CT group. Red dots represent OA group.



doi:10.6342/NTU202300020

# Figure 13

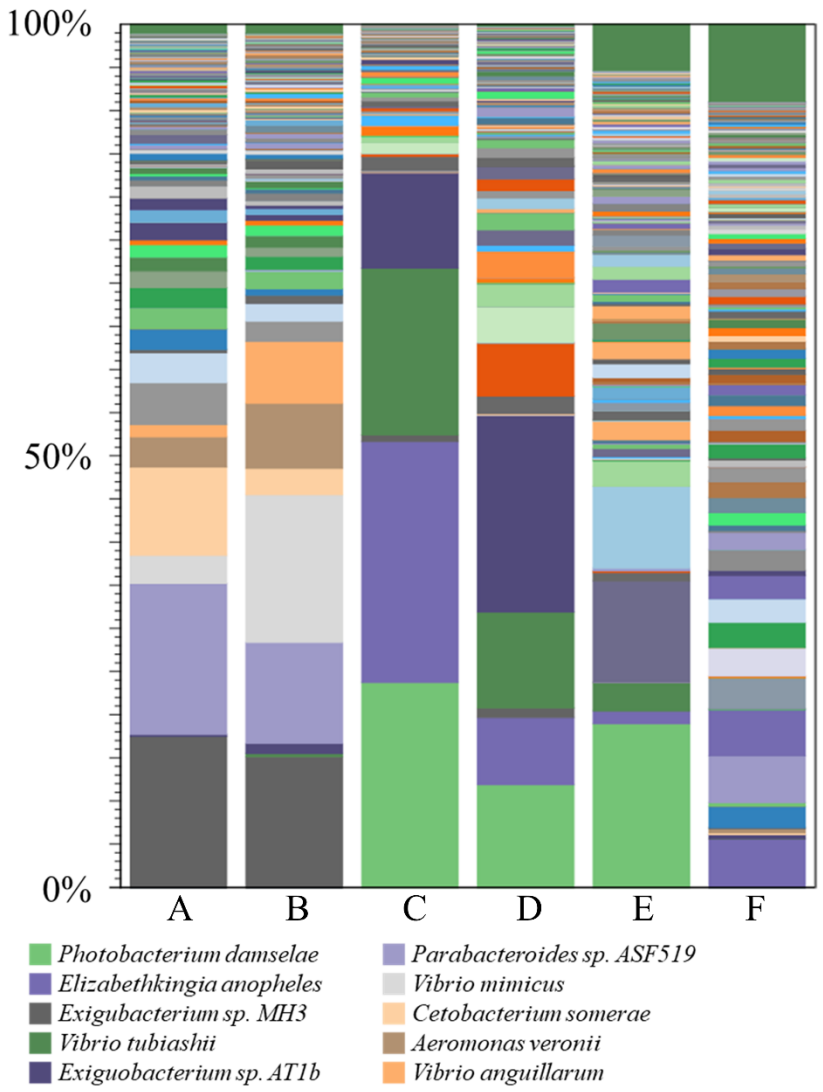


**Figure 13.** Bacterial composition in bigfin reef squid foods. Column A and B are goldfish (*Carassius auratus*). Column C, D and E are Pacific white shrimp (*Litopenaeus vannamei*). Column F is *Neocaridina denticulate*. Taxa is presented at the species level in this figure. Each column represent one sample.



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# Figure 14

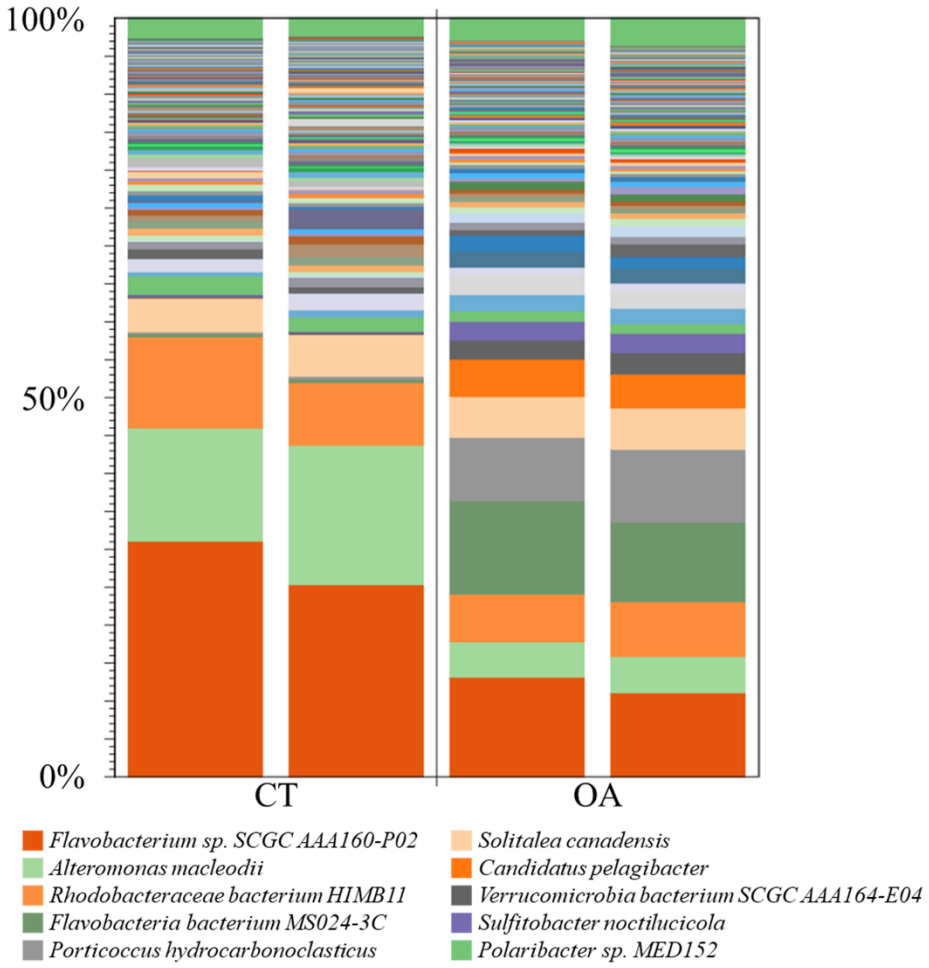


**Figure 14.** Microbiota in the water samples were collected from 30,000L pools and 1000L tanks. Taxa present at species level in this figure. Each column represent one sample.



doi:10.6342/NTU202300020

# Figure 15

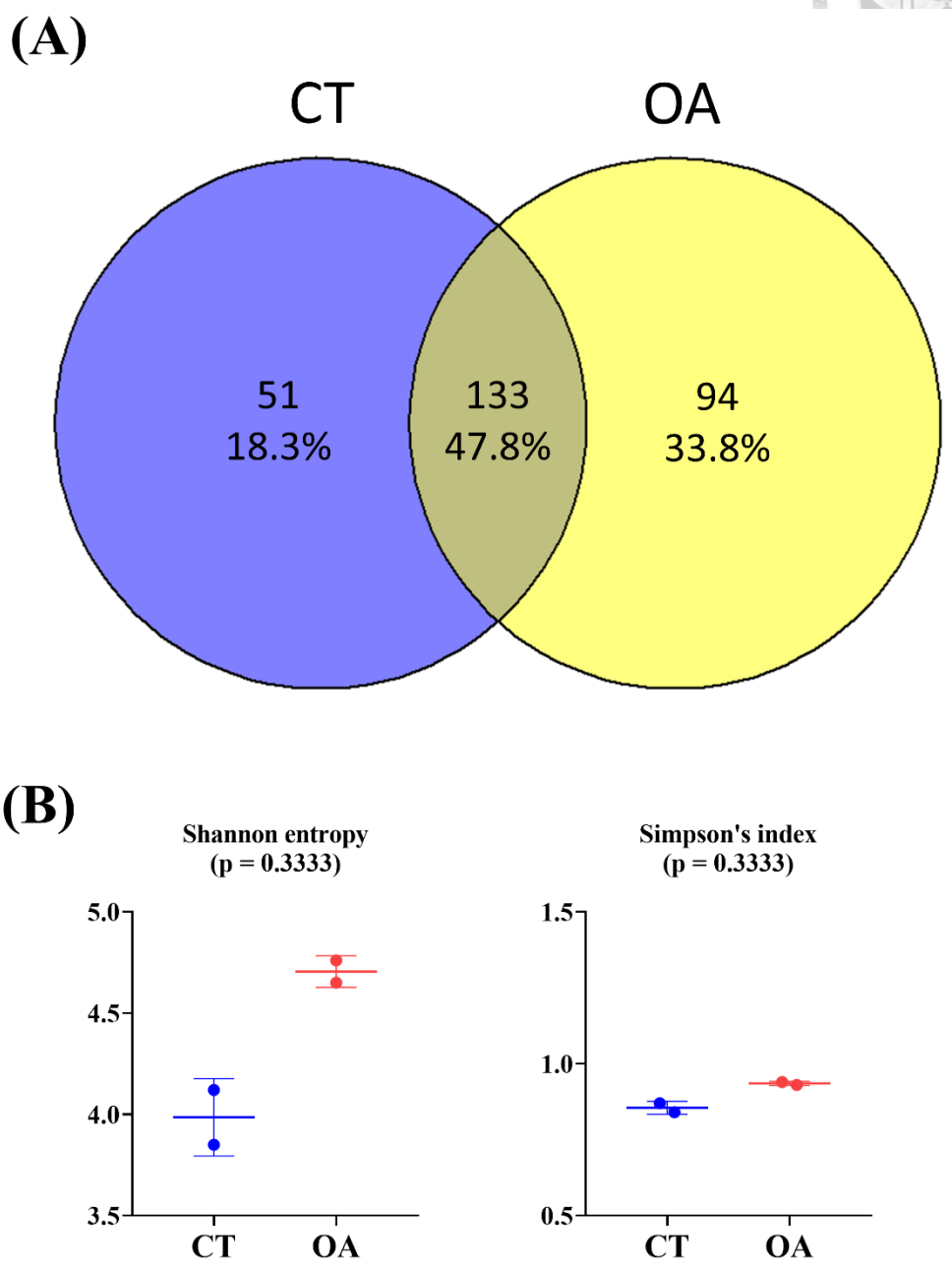


**Figure 15.** Comparison of the bacterial communities in CT and OA water samples. **(A)** Venn diagram and **(B)** alpha diversity. Numbers in the circle represent bacterial species numbers. Statistical analysis is presented by the Mann Whitney test.



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# Figure 16

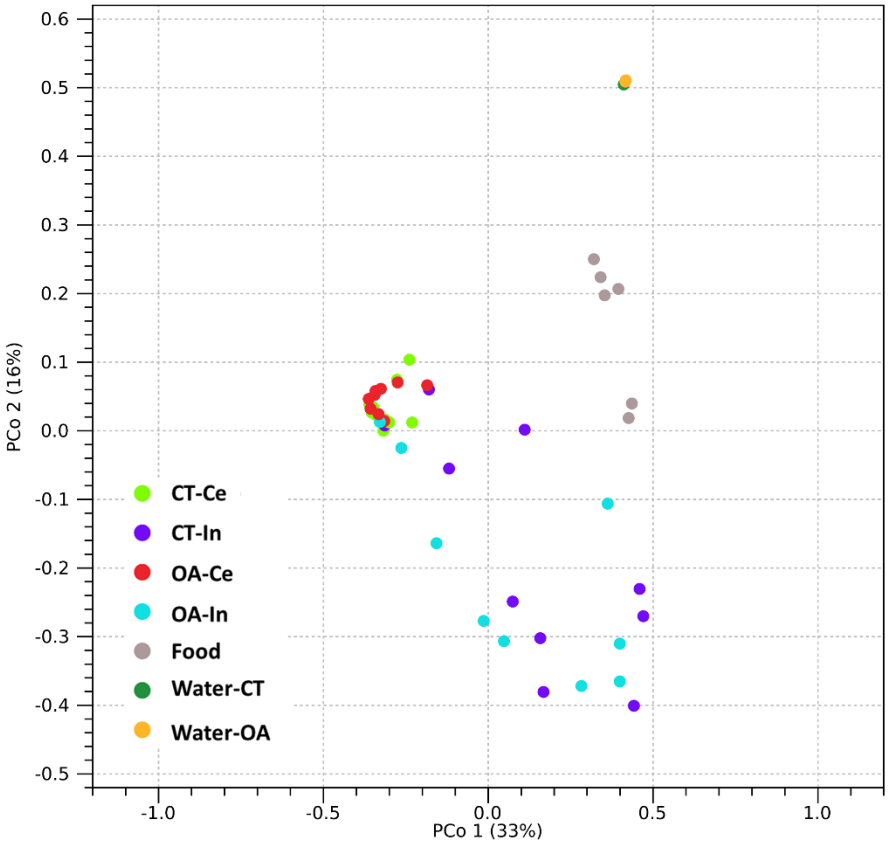


**Figure 16.** Principle-coordinate analysis (PCoA) of Bray-Curtis beta diversity comparing bigfin reef squid gut microbiota, food resource and water. Dots represent samples. PERMANOVA is presented in Table 10.



doi:10.6342/NTU202300020

# Figure 17

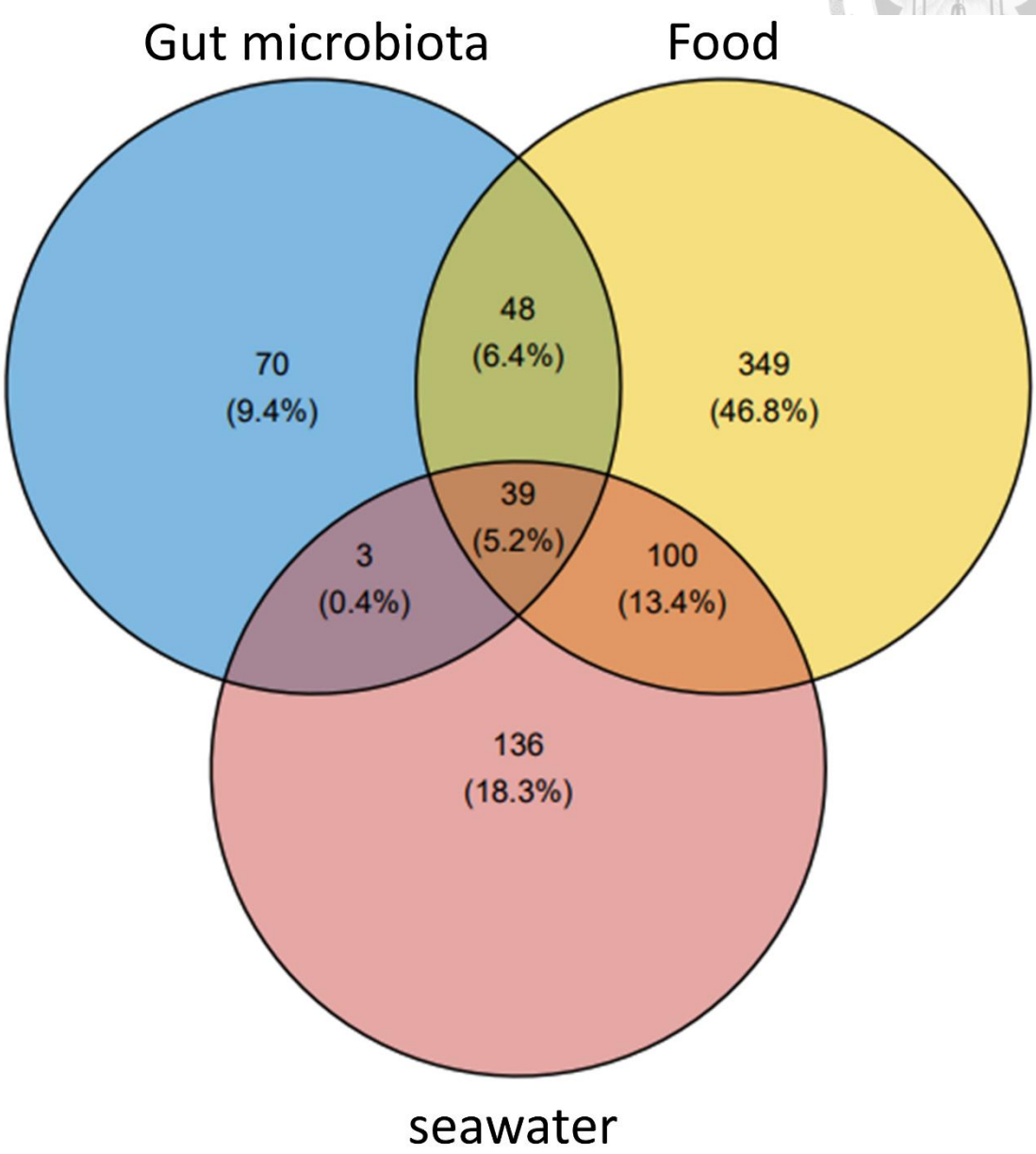


**Figure 17.** Comparison of the bacterial communities in bigfin reef squid gut microbiota, food resource and environment. Numbers in the circle represent bacterial species numbers.



doi:10.6342/NTU202300020

# Figure 18

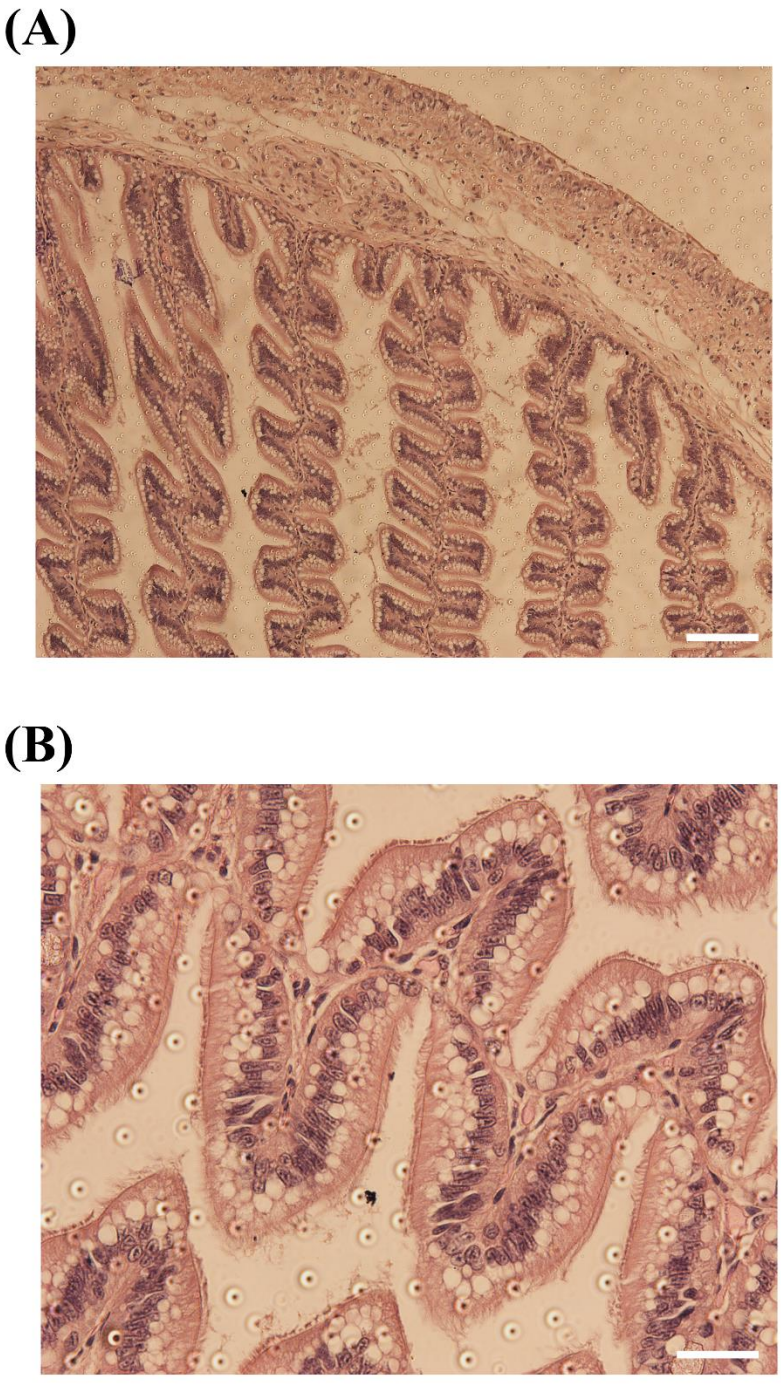


**Figure 18.** Hematoxylin and eosin stain in bigfin reef squid caecum. Scale bars = 250 μm in (A). Scale bar = 40 μm in (B).



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# Figure 19

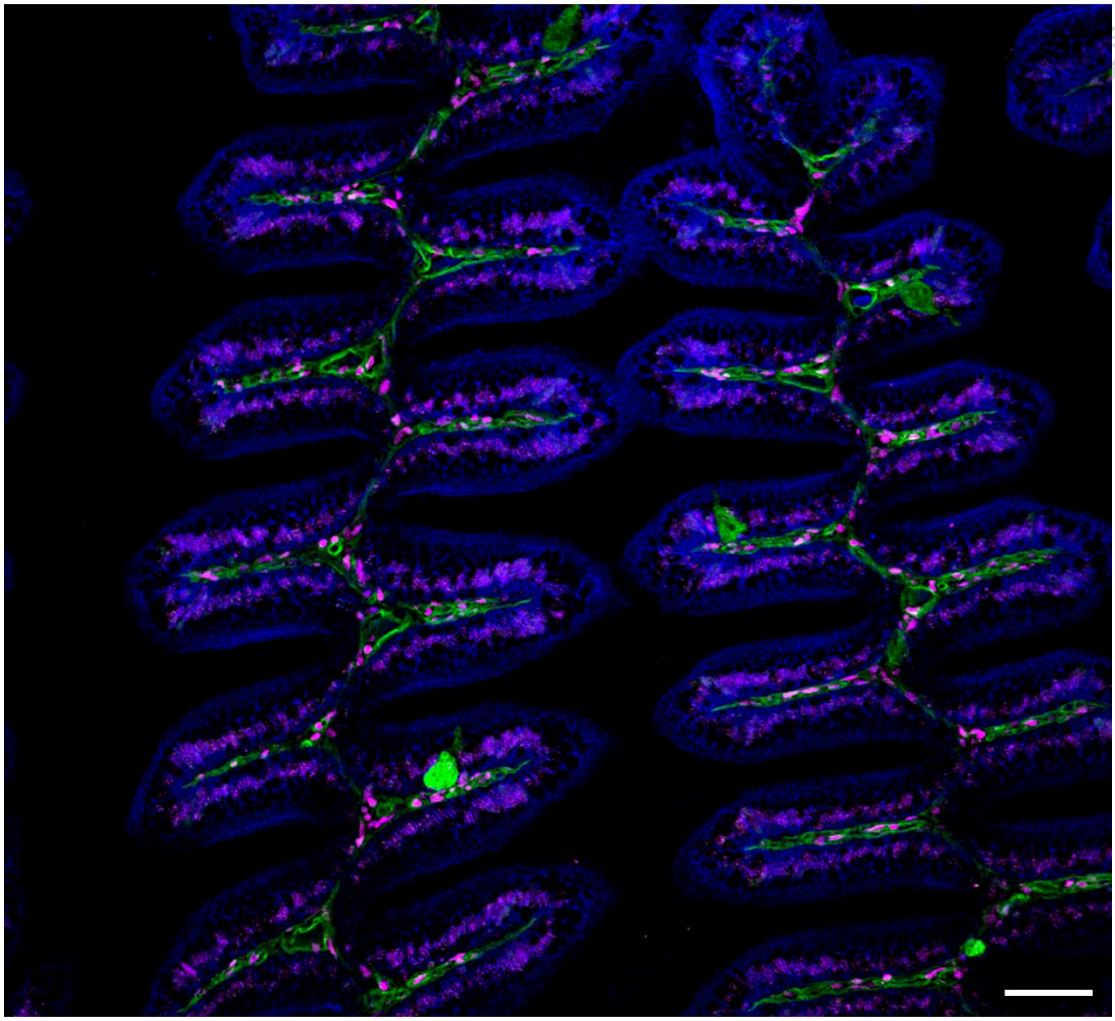

**Figure 19.** Identification of bacteria spatial distribution in bigfin reef squid caecum by utilization of Fluorescence *in situ* hybridization with near-universal bacteria probe (Eub338) and fluorophore-labeled wheat germ agglutinin (green) to visualize mucus. Scale bar = 50 μm.



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# Figure 20

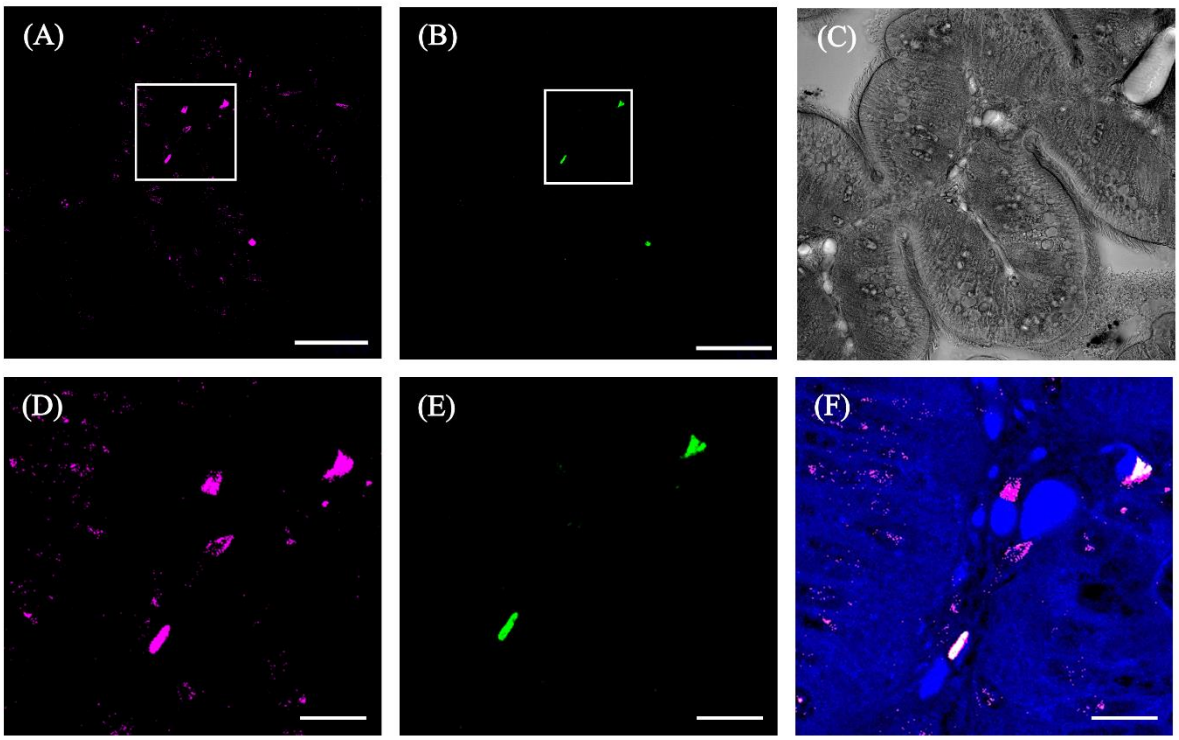


**Figure 20.** Identification of bacteria spatial distribution in bigfin reef squid caecum by utilization of Fluorescence *in situ* hybridization. **(A)** *Mycoplasma* genus-specific probe (Myc 1-1). **(B)** *Vibrio* genus-specific probe (GV841). **(C)** TD. (D) *Mycoplasma* genus-specific probe (Myc 1-1). **(E)** *Vibrio* genus-specific probe (GV841). **(F)** Merge. Scale bar in A, B and C = 40 μm. Scale bar in D, E and F = 20 μm.



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# Figure 21

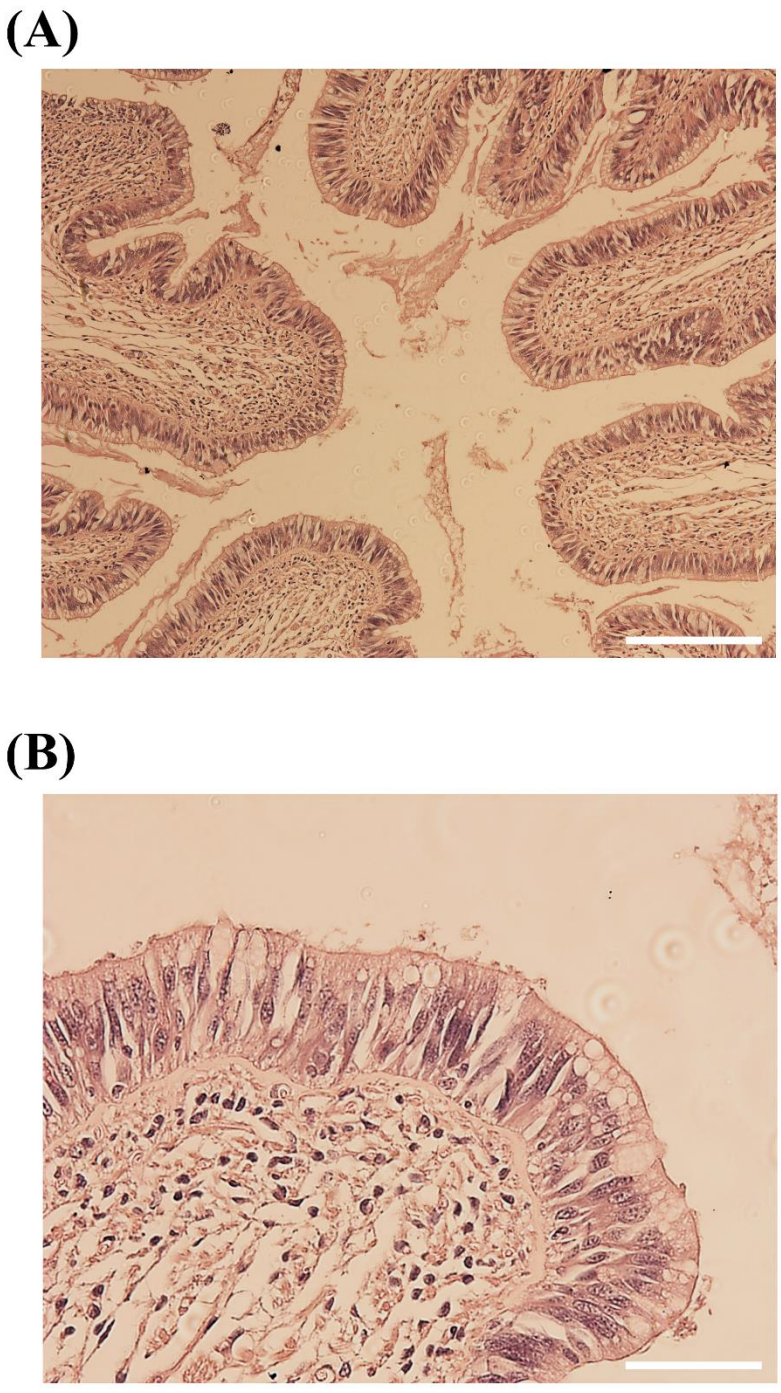


**Figure 21.** Hematoxylin and eosin stain in bigfin reef squid intestine. Scale bars = 250 μm in (A). Scale bar = 50 μm in (B).



doi:10.6342/NTU202300020

# Figure 22

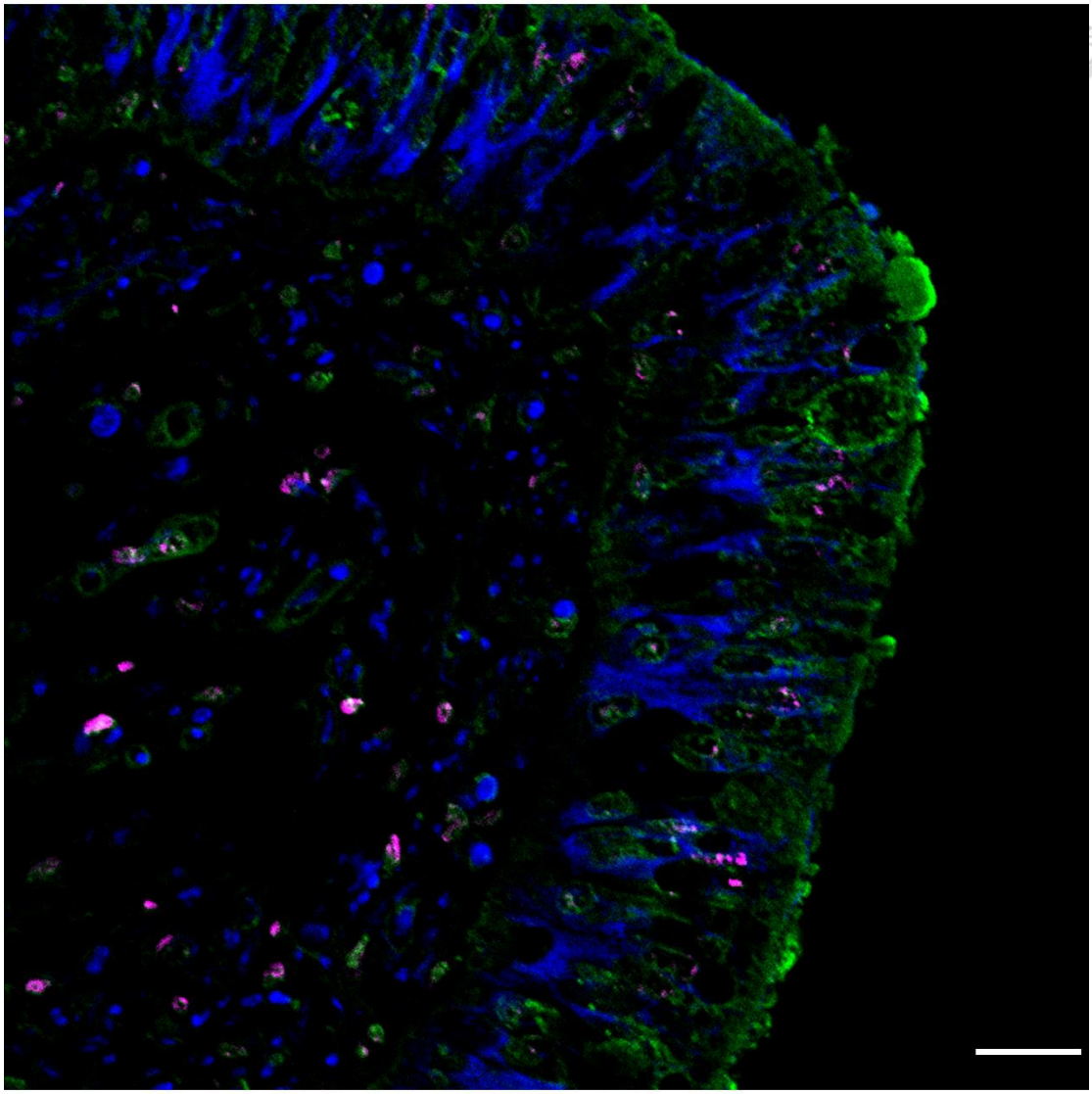

**Figure 22.** Identification of bacteria spatial distribution in bigfin reef squid intestine by utilization of Fluorescence *in situ* hybridization with near-universal bacteria probe (Eub338) and fluorophore-labeled wheat germ agglutinin (green) to visualize mucus. Scale bar = 30 μm.



doi:10.6342/NTU202300020

# Figure 23

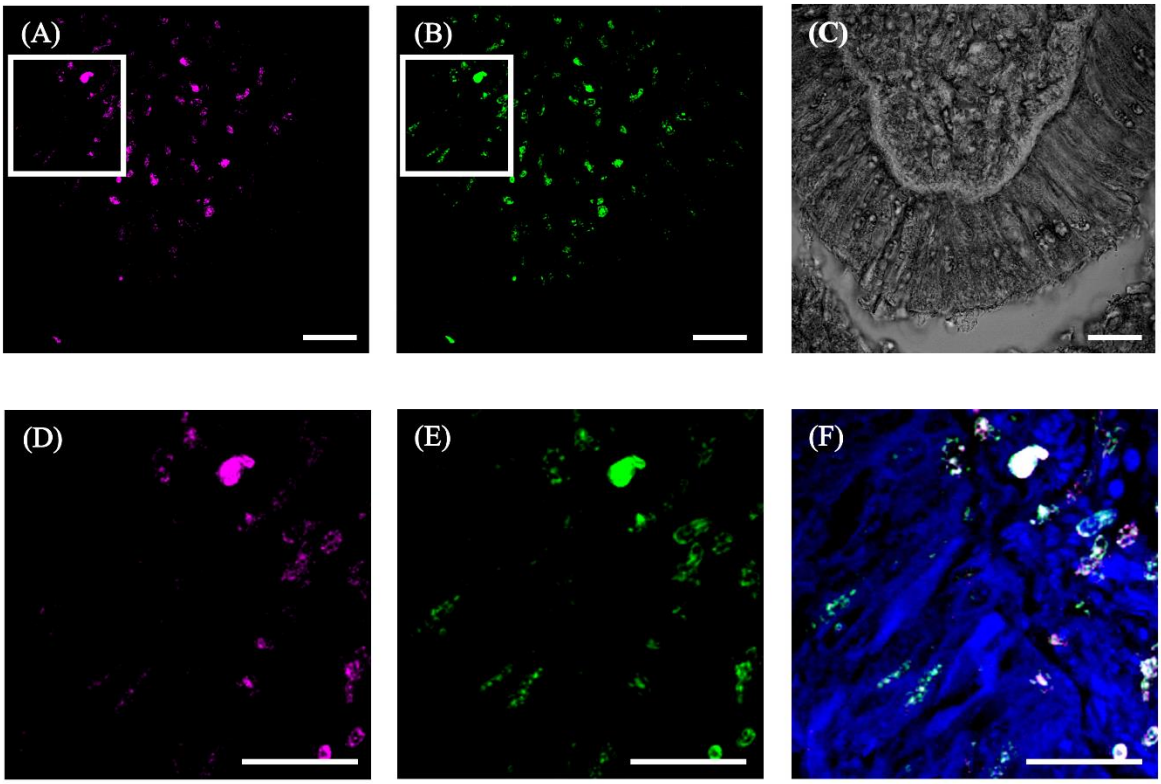


**Figure 23.** Identification of bacteria spatial distribution in bigfin reef squid intestine by utilization of Fluorescence *in situ* hybridization. **(A)** *Mycoplasma* genus-specific probe (Myc 1-1). **(B)** *Vibrio* genus-specific probe (GV841). **(C)** TD. **(D)** *Mycoplasma* genus-specific probe (Myc 1-1). **(E)** *Vibrio* genus-specific probe (GV841). **(F)** Merge. Scale bar in A, B and C = 40 μm. Scale bar in D, E and F = 20 μm.



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