Community structure, function, and dynamics of sea cucumber gut microbiome

（ナマコ消化管マイクロバイオームの群集構造，機能およびダイナミクス）

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GENERAL INTRODUCTION

Holothuroidea (i.e. sea cucumber) belongs to the phylum Echinodermata, which is an early branched clade of an outgroup within Deuterostomia (Dunn *et al*., 2008). Currently more than 1,500 species have been described (Horton *et al*., 2018). Sea cucumbers typically have elongated tubular or flattened soft bodies ranging from 2 mm to 1 m in length covered by leathery skins. They are one of the most conspicuous marine benthos and are distributed from tropical to polar regions, or from coastal to deep-sea areas. Species belonging to the order Dendrochirotida are suspension-feeders using their dendric-shape tentacles, and those in the order Aspidochirotida are deposit-feeders using their shield- or dendric-shape tentacles (Hudson *et al*., 2005; Miller *et al*., 2017). By their feeding habits, deposit-feeding species significantly impact on biogeochemical processes and biomes in benthic environments (MacTavish *et al*., 2012; Purcell *et al*., 2016). In addition, sea cucumbers are important fishery resources in many Asian countries, especially in China, and over 70 species of sea cucumbers are commercially exploited (Anderson *et al*., 2011). Despite their ecological importance, wild stocks of some species have been dramatically reduced due to overfishing, including *Apostichopus japonicus* and *Thelenota ananas* (Conand *et al*., 2013a; Hamel and Mercier, 2013; Purcell *et al*., 2013). To sustain wild stocks, a stable and intensive seed production system needs to be developed based on biology of sea cucumbers. In the general introduction, current knowledge and problems in sea cucumber biology and in aquaculture are reviewed, and the direction of future studies is indicated.

Biology of Sea Cucumber

Characterization of Digestive System

Sea cucumbers have a long and looped digestive tract occupying most of the main body cavity. Morphology and structures of the digestive tracts are different dependent on each holothurian taxon, which might be reflected by a feeding type (Mashanov and García-Arrarás, 2011). Consensus nomenclature for each compartment of the digestive tube has been not accepted yet, however, based

on anatomical and histological observations, the digestive tract in many holothurian species can be separated into four parts; a pharynx, a short esophagus, a long looping intestine (composed of first and second descending intestines and ascending intestine), and a large thick-walled muscular cloaca. The digestive tube is suspended by the mesentery within the body cavity (Mashanov and García-Arrarás, 2011). The wall of the gut is composed of three layers; a folded innermost luminal (digestive) epithelium, an outermost complex muscular mesothelium (gut coelomic epithelium), and a connective tissue layer between the two. The luminal epithelium is composed of enterocytes which perform many roles including mucus production and secretion, nutrient absorption, synthesis of digestive enzymes, phagocytosis of food particles, and accumulation of nutrients (Mashanov *et al*., 2004).

Many species belonging to the orders Aspidochirota and Dendrochirota animals can autotomize their internal organs triggered by mechanical or physicochemical stimuli (Sun *et al*., 2013a). This visceral autotomy (i.e. evisceration) is controlled by the nervous system and involves separation of the anatomical components along predetermined breakage zones (Byrne, 2001; Wilkie, 2001). There are two types of evisceration in sea cucumbers. The first type is known as posterior evisceration and occurs mainly in the Aspidochirota sea cucumbers, which involves the detachment of the intestine from the esophagus and the cloaca, and also from the supporting mesenteria. The autotomized region of the digestive tract associated with visceral organs is then expelled though a rupture in the cloacal wall (García-Arrarás *et al*., 1998; Wilkie, 2001). The second type is known as anterior evisceration, which is unique in the order Dendrochirota, and it involves a more extensive loss of tissue than in posterior evisceration, because the whole anterior body end of the animal is discarded (Byrne, 2001; Mashanov *et al*., 2005; Mashanov and García-Arrarás, 2011).

Sea cucumbers can regenerate their intestines within two or three weeks after evisceration (Mashanov and García-Arrarás, 2011; Zhang *et al*., 2017). A wound closure at the end of body is the first response to injury. Although internal organs are removed from the body cavity through evisceration, most of the mesenteries remain intact, and the free edge of the mesentery plays a

pivotal role in gut regeneration (García-arrarás and Greenberg, 2001). The mesentery edge undergoes thickening and lengthening, and further the coelomic epithelial and muscle cells adjacent to the free edge dedifferentiate and migrate toward the mesenterial edge, where they form the intestinal primordium (García-arrarás and Greenberg, 2001). After the development of the intestinal primordium from the esophagus to the cloaca, the intestinal lumen starts to form. The luminal cells of Aspidochirota and Dendrochirota species have been shown to originate from the remnants of the digestive tract (cloaca and the esophagus), and these cells are likely to migrate and invade the primordium, thereby forming the digestive tract (García-Arrarás *et al*., 1998; García-arrarás and Greenberg, 2001; Mashanov and García-Arrarás, 2011).

Feeding Behavior and the Contribution to Benthic Biomes

Deposit-feeding sea cucumbers ingest huge amounts of sediment daily using tentacles surrounding the mouth, and the tentacles significantly influences the animal's feeding behavior (Uthicke, 1999; Slater *et al*., 2011; Sun *et al*., 2015). Sea cucumbers with stubby peltate tentacles (e.g. *Psychropotes longicauda*) collect sediment particles by sweeping, while sea cucumber with digitate tentacles (*Oneirophanta mutabilis*) collect sediment particles by raking (Roberts and Moore, 1997). Since sediments are a poor source of nutrients, sea cucumbers have evolved both physiological and behavioral strategies to adapt to such oligotrophic environments. In the former strategy, sea cucumbers developed a special connective tissue named "catch connective tissue", which enables them to change body shape with low energy consumption (Takemae *et al*., 2009). In the latter strategy, sea cucumbers show selective feeding being attracted to sediments contained higher organic matter (Uthicke, 1999; Uthicke and Karez, 1999). The selective feeding has been supported not only by behavior observations in the experimental aquarium but also by direct measurements of more organic matters or phytopigments contained in the gut contents compared to those in ambient sediments (Uthicke, 1999; Uthicke and Karez, 1999; Slater *et al*., 2011).

Sea cucumbers are considered to consume organic compounds in detritus and/or

microorganisms, and amylase, pepsin and lipase are detected as digestive enzymes (Yingst, 1976; Moriarty, 1982; Sun *et al*., 2015). Previous studies in *A. japonicus* reported that diatom is one of the valuable food sources based on fatty acid biomarkers (Gao *et al*., 2010), and that macroalgae were the principal food source based on carbon and nitrogen stable isotopes in a farm pond (Sun *et al*., 2013b). More recently, a 18S rRNA gene sequencing analysis revealed types of ingested eukaryotes by *A. japonicus*; over 800 operational taxonomic units (OTUs) of eukaryotes were detected in the guts, and these eukaryotic communities differed significantly between spring and winter (Zhang *et al*., 2016). As mentioned later, feeding experiments in aquaculture field showed that sea cucumbers have food preferences for specific algal species, and that macroalgae including *Saccharina japonica* and *Ulva lactuca* as well as microalgae, such *Cylindrotheca fusiformis*, increase sea cucumber growth compared to other algae (Xia *et al*., 2012a, b; Shi *et al*., 2013a; Anisuzzaman *et al*., 2017). However, no consensus has been reached in food preferences in eukaryotic taxa in *A. japonicus* in natural environments due to the difficulty in accurate eukaryotic identification using conventional microscopic observations.

Sea cucumbers rework huge amounts of sediments via ingestion and excretion (9-82 kg ind^{-1} y^{-1}) (Uthicke, 1999) and can extensively blend and reform sea floor substrata. Considering their high abundance in benthic habitats, their behaviors and biology have greatly affected physico-chemical processes in both soft-bottom and reef ecosystems (Uthicke, 2001a; Schneider *et al*., 2011; MacTavish *et al*., 2012; Purcell *et al*., 2016). There are five major ecological functions of sea cucumbers. The first one is their contributions to sediment condition; bioturbation and sediment cleaning are the two main activities of the animals in maintaining and improving the condition of sediments. Bioturbation, which is defined as biological reworking and mixing of sediments and soils, impacts on benthic primary producers, animals and microorganisms (Widdicombe and Austen, 1999; Meysman *et al*., 2006; Laverock *et al*., 2010; MacTavish *et al*., 2012). Sediment cleaning is performed by deposit-feeding sea cucumbers, which defecate less organic rich sand compared to those of the ingested sediments (Yingst, 1976; Moriarty, 1982; Mercier *et al*., 1999; Purcell *et al*.,

2016). The second function is recycling of nutrients; sea cucumber's feeding activity promotes efflux of inorganic nutrient (e.g. ammonium, phosphate) into seawater columns through organic matter mineralization in their guts, which has a positive impact on algal productivity (MacTavish *et al*., 2012; Purcell *et al*., 2016). This role is especially important in coral reef ecosystems, where inorganic nutrients are generally limited. The third function is to influence seawater chemistry (e.g. pH, alkalinity); based on studies in coral sea cucumbers, digestion of organic matter and release of ammonium can increase local seawater alkalinity and dissolved inorganic carbon, which may buffer pH decreases in coral ecosystems (Schneider *et al*., 2011). The fourth function is to form pathways of energy transfer in food chains; sea cucumbers are consumed by at least 19 species of sea stars, 17 species of crustaceans, several gastropods and around 30 species of fish, which transfer energy from organic matter detritus in sediments to higher trophic level animals (Francour, 1997; Purcell *et al*., 2016). The fifth function is to bolster biodiversity via symbiotic relationships; sea cucumbers provide both vertebrate and invertebrate species with a refuge and external food sources (Eeckhaut *et al*., 2004). Crabs, shrimps and ophiuroid sea stars are found on the body surface, and pearlfishes are found in the digestive tracts of sea cucumbers (Parmentier *et al*., 2010; Purcell *et al*., 2016).

Fisheries and Aquaculture of Sea Cucumbers

Sea cucumbers have been consumed as both food and medicine, and have been caught and traded for over one thousand years in the Indo-Pacific region (Conand and Byrne, 1993). They are consumed after being reconstituted from both dried and wet forms, with muscles cut in strips and boiled. Sea cucumber fisheries have social and economic importance to many coastal communities, especially in developing countries and regions including the Maldives, Solomon Islands and Sri Lanka (Anderson *et al*., 2011). Recently, various species of sea cucumbers within the order Aspidochirotida have been intensively harvested especially in the Asia (52 species) and Pacific (36 species) regions because of rich diversity of edible species in these regions. Total catches of sea cucumbers in the Asia Pacific region were estimated to be approximately 20,000 to 40,000 tons per year, and Hong Kong was

responsible for 58% of global sea cucumber imports by volume in 2006 (Anderson *et al*., 2011). Sea cucumbers are easily caught by hand or by snorkeling or SCUBA diving, and population replenishment is slow due to their slow growth and late age of maturity. Thus, wild stocks of the animals have been decreasing dramatically, and some of them, e.g. *A. japonicus* and *T. ananas*, are even listed in the IUCN Red List as "endangered species" (Conand *et al*., 2013b; Hamel and Mercier, 2013). There are three types of serial exploitation of sea cucumbers. First one is change of a fishing place from near shore to offshore. As stocks got depleted after harvesting by hand near shore, people move further offshore and harvest while snorkeling or SCUBA diving. This type is commonly observed in tropical countries (e.g. Maldives, Philippines and Sri Lanka). Second is change of sea cucumber species from high-value one to low-value one; catching low volumes of high-value species (e.g. *Holothuria scabra*, *H. whitmaei* and *H. fuscogilva*) changed to catching larger volumes of low-value species (e.g. *H. edulis*, *H. leucospilota*) because the high value species became depleted. This type is observed some counties, for instance, Malaysia and Madagascar. Third is reduction of body size of sea cucumbers caught. In the Gala´pagos, the mean fished size of *Isostichopus fuscus* decreased from 24.5 to 22.5 cm from 1999 to 2002 (Shepherd *et al*., 2004).

A. japonicus is one of the most important fishery resources in many Asian countries, especially in China (Lovatelli *et al*., 2004). To sustain wild stocks, many studies have attempted to develop and improve the sea cucumber aquaculture system. Most of the research has focused on the optimization of abiotic factors (seawater temperature and its fluctuations, salinity, material types of aquaria) on sea cucumber growth in aquaculture (Dong *et al*., 2006, 2008, 2010), as well as the assessment of preferable diet and its ration composition by observation of feeding preferences and by measurement of absorption using carbon stable isotopes and the effects of different diet and ration composition on their growth (Gao *et al*., 2011; Sun *et al*., 2012a; Xia *et al*., 2012a; Shi *et al*., 2013a, b). Many feeding experiments showed the macroalgae *S. japonica* and *U. lactuca* and the microalgae *C. fusiformis* are beneficial for growth in *A. japonicus* (Xia *et al*., 2012a, b; Shi *et al*., 2013a; Anisuzzaman *et al*., 2017). In addition, carbon stable isotopes showed *Gracilaria lemaneiformis* had

high absorption efficiency by *A. japonicus* and enhance their growth (Gao *et al*., 2011). However, skin ulceration and huge growth gaps of cultured sea cucumbers prevent establishment of the stable and intensive aquaculture system (Liu *et al*., 2010; Watanabe *et al*., 2014).

The rapid expansion in the sea cucumber aquaculture system has led to the occurrence of infectious diseases including skin ulceration (Deng *et al*., 2008, 2009). Antibiotics can be used as potential protective and therapeutic strategies to control pathogens. However, appearance of antibiotic-resistant bacteria, presence of antibiotic residues in seafood and destruction of microbial populations in the aquaculture environment are major risks (Zhang *et al*., 2010). Probiotics, dietary live microbial supplements, are expected to be an alternative therapeutic strategy. *B. subtilis* was known to be the first potential probiotic of sea cucumber *A. japonicus* based on growth promotion and immune stimulation (Zhang *et al*., 2010; Sun *et al*., 2012b; Zhao *et al*., 2012). In addition, Chi and colleagues isolated 224 exoenzyme-producing bacteria from the intestines of *A. japonicus*, and administration of three of these isolates, *Pseudoalteromonas elyakovii*, *Shewanella japonica* and *Vibrio tasmaniensis*, to sea cucumbers increased growth, immunity and pathogen resistance (Chi *et al*., 2014). More recently, *B. cereus* and *Paracoccus marcusii* were proposed as potential probiotics based on growth promotion and immune stimulation (Yan *et al*., 2014; Yang *et al*., 2015a). In addition to bacteria, yeast (e.g. *Rhodotorula benthica* and *Metschnikowia* sp.) has been proposed as a potential probiotic (Yang *et al*., 2014; Wang *et al*., 2015). However, these probiotics have been developed without evidences how probiotics affect the host physiology.

Recent Progress on Gut Microbiome Studies

Human and other animals are hosts to a huge number of microbes, represented by gut microbiota. Gene set of gut microbiota, defined as “Gut Microbiome”, encodes various types of enzymes which the host does not possess, contributing to host physiology and ecology. Currently, many studies posit that animal biology could not be evaluated properly without their associated microbiomes (McFall-Ngai *et al*., 2013), and host and associated microbes are even considered to be one

integrated lifeform, referred as “holobiont”. Furthermore, studies of gut microbiota have contributed to medical sectors in human and to industrial sectors in aquatic animals.

Transmission of Gut Microbes

Animals have evolved diverse modes of transmission of gut microbes. Acquisition of proper microbes is the first step for host animals to shape their own gut microbiota. Transmission of a specific symbiont from one generation to the next has been well documented. For example, *Ishikawaella capsulata*, colonized in the gut lumen of plant sap-feeding Heteroptera, *Megacopta punctatissim*, is vertically transmitted through consuming specialized symbiotic capsules on the outside of the egg case by juveniles following hatching (Hosokawa *et al*., 2006, 2010). Blood-feeding heteropterans, *Rhodnius prolixus*, acquires specifically associated actinomycete *Rhodococcus rhodnii* via coprophagy (Eichler and Schaub, 2002; Beard *et al*., 2002). Conversely, the bean bug, *Riptortus pedestris*, acquires a specific *Burkholderia* symbiont every generation (i.e. horizontally) possibly by selecting the symbiont from a range of ingested organisms (Kikuchi *et al*., 2005, 2007, 2011). Such horizontal transmission is also observed in *Aliivibrio fischeri* and bobtail squid symbiosis (Nyholm and McFall-Ngai, 2004).

Compared to the processes of single symbiont acquisition described above, those of gut microbiota are more complex. Shapira (2016) mentioned that the members of gut microbiota are in a multilayered structure composed of core microbiota, flexible microbiota, and environmental strains. Core microbiota is vertically transmitted, which have coevolved with the host and contribute to basic host functions. Indeed, the abundance of certain bacterial families in gut microbiota, for example, Christensenellaceae, has been shown to be strongly influenced by host genetics based on a large-scale twin study (Goodrich *et al*., 2014). Flexible microbiota is horizontally transmitted and exchanged with the environment, but this group is better adapted for host colonization than environmental strains and can facilitate host adaptation to new environmental conditions (Shapira, 2016). The prevalence of members of the Bacteroidaceae family was unlikely to be influenced by

genetic relatedness among individuals, but they are affected by diet and geography, suggesting that it is one of the flexible microbiota (Yatsunenko *et al.*, 2012). Environmental strains are environmentally acquired, which might be beneficial for host physiology and ecology but are not adapted to the host. Earthworms are unlikely to harbor resident bacteria, but instead they shape gut microbiota via selection from ingested soils (Egert *et al.*, 2004; Thakuria *et al.*, 2010; Aira *et al.*, 2015).

Factors that Affect Shaping of Gut Microbiota

There are intrinsic (host) and extrinsic (environmental) factors that shape animal gut microbiota. The former consists of genetics (Benson *et al.*, 2010; Goodrich *et al.*, 2014), age (Yatsunenko *et al.*, 2012; Stephens *et al.*, 2016), and developmental phase (Koren *et al.*, 2012). Fecal microbiome analysis using TwinsUK population, including 416 twin pairs, indicated that host genetics affect human gut microbiome composition (Goodrich *et al.*, 2014). In addition, co-speciation of predominant bacterial taxa (Bacteroidaceae and Bifidobacteriaceae) with the host hominids in strain-level might be another example of genetic effects on gut microbiota (Moeller *et al.*, 2016). A 16S rRNA gene sequencing analysis of human fecal microbiota collected from 326 individuals age 0-17 years, and 202 adults aged 18-70 years showed that the taxonomic composition of bacterial communities developed towards an adult-like configuration within the three-year period after birth (Yatsunenko *et al.*, 2012). Extrinsic factors include diet (David *et al.*, 2014) and temperature (Kohl and Yahn, 2016). Diet is one of the strongest drivers which rapidly and reproducibly changes gut microbiota (Muegge *et al.*, 2011; David *et al.*, 2014; Carmody *et al.*, 2015). Environmental temperature significantly impacts on gut microbiota of an ectotherm, tadpole (Kohl and Yahn, 2016). Surprisingly, even in a homeotherm, cold exposure markedly changed the composition of mouse gut microbiota, and “cold microbiota” increased the gut size and absorptive capacity (Chevalier *et al.*, 2015). The sum of host and environmental factors probably accounts for gut microbiota difference among geography and seasons (Yatsunenko *et al.*, 2012; Amato *et al.*, 2013; Linnenbrink *et al.*,

2013; Maurice *et al*., 2015).

Unveiled Novelties on Host-Microbe Interactions

In humans and mice, gut microbiota forms a complex ecological community that influences host physiology and is related to various diseases. Firmicutes and Bacteroidetes are dominant in the gut microbiota, and the ratio of Bacteroidetes to Firmicutes is related to obesity; colonization of germ-free mice with an "obese microbiota" results in a significant increase in total body fat compared to colonization with a "lean microbiota", suggesting that "obese microbiota" has increased capacity to extract more energy from diet (Ley *et al*., 2005; Turnbaugh *et al*., 2006). Segmented filamentous bacteria (SFB), which colonize in the guts of human infants within the first two years of life and also in mice, play a critical role in Th17 induction via adhesion to intestinal epithelial cells (Yin *et al*., 2013; Atarashi *et al*., 2015). Furthermore, autism spectrum disorder (ASD) model mice shows increased relative abundance of specific bacterial taxa like Lachnospiraceae (Clostridia), and it has been suggested that metabolites produced by abnormalized gut microbiota enter the blood from the gaps of cells with a leaky tight junction, which may cause behavior and physiological abnormalities (Hsiao *et al*., 2013). *Akkermansia muciniphila* (Verrucomicrobia), mucin-degrading bacteria in the human intestine, was isolated from feces in an anaerobic medium containing gastric mucin as the sole carbon and nitrogen source (Derrien *et al*., 2004), which is considered to be one of the key players in host-microbe interaction in humans. For examples, the decrease of *Akkermansia* proportion in mouse gut microbiota, triggered by low temperature, changes intestinal morphology and facilitates transformation of white adiposity cells to beige ones, which contributes to host adaptation to cold environments (Chevalier *et al*., 2015). *Akkermansia* bacteria also promoted the healing of mouse gut injuries by colonizing wound beds which are highly anaerobic conditions due to respiratory burst by immune cells (Alam *et al*., 2016). While contribution of gut microbes to wound healing has been reported, contribution to regeneration of host organs or body parts has not been investigated for animal taxa lacking model organisms.

Even in other vertebrates, there are many reports on findings on gut microbiomes. Zebrafish gut microbiota, especially Firmicutes groups, contributes to regulation of fatty acid absorption (Semova *et al*., 2012). Vultures have specialized gut microbiota with low bacterial diversity dominated by Clostridia and Fusobacteria, which are widely accepted pathogenic taxa to other vertebrates (Roggenbuck *et al*., 2014). Tannin-protein complex-degrading bacteria, *Lonepinella koalarum*, was isolated from Koala fecal microbiota, which can contribute to the degradation of Eucalyptus leaves (Osawa *et al*., 1995; Blyton *et al*., 2019).

In invertebrates, wood-feeding termites harbor bacteria retaining genes for cellulose and xylan hydrolysis (Warnecke *et al*., 2007). Gut microbes of the coffee berry borer (*Hypothenemus hampei*), the most devastating insect pest in coffee worldwide, mediate caffeine detoxification (Ceja-Navarro *et al*., 2015). In addition to such degradation and detoxification of diets, gut symbionts (e.g. *Ishikawaella capsulata*) of some heteropterans retain genes that enable nutrient provisioning to the hosts, which live on a restricted diet of plant sap (Hosokawa *et al*., 2006; Nikoh *et al*., 2011; Engel and Moran, 2013). Unlike these invertebrates, earthworm gut microbiota consists of ingested microbes from soils, but anaerobic habitats and mucus in their guts enrich denitrifying and fermentative bacteria, supporting digestion and assimilation of complex organic matter transiting through the guts, and thus is referred to as a "mutualistic digestive system" (Barois and Lavelle, 1986; Drake and Horn, 2007).

Bacteriotherapies

The idea of using fecal bacterial mixtures and probiotics to treat gastrointestinal (GI) diseases has existed for centuries (Baktash *et al*., 2018). "Bacteriotherapy" is an emerging technology to use microbes for therapy of diseased patients. Bacteriotherapies include probiotics (adding living, non-pathogenic bacteria thought to be generally healthful), prebiotics (dietary supplements that promote growth of beneficial bacteria), and fecal microbiome transplantation (FMT) (as capsules, through endoscopy, or via an enema), which is based on the central premise that restoration of the

gut microbiome to its original state after a perturbation (called gut dysbiosis) will relieve symptoms and potentially be curative (Severyn and Bhatt, 2018). Probiotics are one of the most commonly consumed dietary supplements. Recent estimates suggest that 3.9 million adults in the US consume prebiotic or probiotic supplements, and the probiotics market is projected to be over US $65.9 billion by 2024 (Zmora *et al.*, 2018). Bacteria used as probiotics belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus*. *Lactobacillus* and *Bifidobacterium* are frequently found in common food products such as yogurt and miso (Severyn and Bhatt, 2018). Healthy people consume probiotics aiming to strengthen the immune system (Fukushima *et al*., 1998), relieve gastrointestinal symptoms (Guyonnet *et al*., 2009), protect against infectious diseases (Panigrahi *et al*., 2017), mental and behavioral augmentation (McKean *et al*., 2016). Probiotics are also expected to be used in the treatment of infections or existing conditions such as acute, antibiotic-associated and *Clostridium difficile*–associated diarrhea, amelioration of inflammatory bowel disease and irritable bowel syndrome (IBS). In addition, other claims include eradication of *Helicobacter pylori*, reduction in incidence and severity of respiratory infections, alleviation of depression, prevention or treatment of atopic dermatitis and reduction of cardiovascular risk factors associated with the cardiometabolic syndrome (Crovesy *et al*., 2017; Rondanelli *et al*., 2017; Sniffen *et al*., 2018). However, there is a great need of additional evidence-based proof of such probiotics impacts on humans (Senok *et al*., 2005).

FMT is another treatment focusing on gut microbiota. In 1958 the first FMT for pseudomembranous colitis was formally reported (Baktash *et al*., 2018). FMT is expected to be a more effective treatment for gastrointestinal diseases including inflammatory bowel disease compared to probiotics (Suez *et al*., 2018; Zmora *et al*., 2018). Indeed, one's own FMT restores mucosal microbiome and gut transcriptome reconstitution, while probiotics delay gut microbiome and transcriptome reconstitution after antibiotics treatment (Suez *et al*., 2018). A major limitation in using FMT as a widely clinical application is that an appropriate stool sample must be collected in advance (Severyn and Bhatt, 2018). There are still many open questions, such as the optimal timing

and storage for baseline stool collection. Furthermore, safety must be confirmed; alterations in microbial composition can occur *ex vivo* (including horizontal gene transfer and contamination) and can thus compromise the "safety" of this product.

The application of probiotics has been widely studied also in aquaculture. Antibiotics have been used for long time to prevent infectious diseases in the aquaculture industry. However, recently many countries have banned antibiotics uses due to appearances of drug-resistant strains, the destruction of ecological balance and environmental pollution. Probiotics are proposed as an alternative. The microbiological additives are generally used as feed additives or are directly added to the water pond to improve the balance of digestive tract flora, the rate of feeding, the growth of aquatic animals, immunity, disease resistance and the quality of water by absorbing or degrading organisms or toxic substances (Liu *et al*., 2009; Wang *et al*., 2017). Numerous types of probiotic candidates have been proposed in aquaculture, including *Bacillus, Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella, Aeromonas, Vibrio, Enterobacter, Pseudomonas, Clostridium,* and *Saccharomyces* spp. (Wang *et al*., 2017). In particular, 1) Bacillus was used for fish including carp (*Labeo rohita*, *Catla catla*) (Newaj-Fyzul *et al*., 2007; Kumar *et al*., 2008; Bandyopadhyay and Das Mohapatra, 2009), rainbow trout (*Onchorhynchus mykiss*) (Bagheri *et al*., 2008) and *Tilapia* (*Oreochromis nilotieus*) (Aly *et al*., 2008), and for invertebrates including pacific white shrimp (*Litopenaeus vannamei*) (Gullian *et al*., 2004; Balcázar *et al*., 2007; Wang, 2007; Tseng *et al*., 2009) and black tiger shrimp (*Penaeus monodon*) (Rengpipat *et al*., 2000; Vaseeharan and Ramasamy, 2003); and 2) *Lactobacillus* was used for fish and shrimps including gilthead seabream (*Sparus aurata*) (Suzer *et al*., 2008), flounder (*Paralichthys olivaceus*) (Byun *et al*., 1997), giant freshwater prawn (*Macrobrachium rosenbergii*) (Venkat *et al*., 2004), gilthead seabream (Salinas *et al*., 2005), rainbow trout (*Oncorhynchus mykiss*) (Vendrell *et al*., 2008) and grouper (*Epinephelus coioides*) (Son *et al*., 2009).

Current Knowledge and Future Study Issues on Sea Cucumber Gut Microbiomes

The history of sea cucumber gut microbiome studies began in deep-sea adapted species which are prominent benthic members in bathyal depth, in terms of both abundance and biomass. In 1981, Deming and colleagues first cultured microbes from intestines of deep-sea holothurians (Deming *et al*., 1981). Deming and Colwell also analyzed total bacterial counts of sediments and sea cucumber guts, and reported that the gut contents had 1.5- to 3-fold higher bacterial abundance than in sediments around their habitats (Deming and Colwell, 1982). At the beginning of 21st century, Roberts *et al*. (2001) showed total bacterial counts and bacterial activities in the guts of abyssal holothurians, *Oneirophanta mutabilis*, *Psychropotes longicauda* and *Pseudostichopus villosus*, increased along with intestinal tracts. The culture-independent method, the molecular fingerprinting technique of Denaturing Gradient Gel Electrophoresis (DGGE) with band sequencing, was first used for abyssal sea cucumbers, and series of analyses suggested that *M. musculus* is unlikely to develop specialized gut microbiota in organic matter rich habitats, but they might develop such communities under organic matter-poor conditions (Amaro *et al*., 2009). More recently, prokaryotic abundance and bacterial diversity in sea cucumber guts were significantly higher than in ambient sediments of individuals, and suggested that archaea might be a key component within the gut of the holothurians (Amaro *et al*., 2012). They also suggested ca. 40% of bacterial operational taxonomic units were associated uniquely with the gut contents, based on terminal restriction fragment length polymorphism (T-RFLP) and an automated method of ribosomal intergenic spacer analysis (ARISA) as well as catalyzed reporter deposition (CARD) - fluorescence *in situ* hybridization (FISH) (Amaro *et al*., 2012).

Ward-Rainey *et al*. (1996) first analyzed “shallow-water” (i.e. coastal) sea cucumbers based on both culture-dependent and -independent methods and demonstrated that the number of culturable bacteria were greater in the hindgut than in the foregut, but in both cases less than those found in the surrounding sediments. Most of these gut isolates were identified as *Vibrio* and neighboring taxa (Ward-Rainey *et al*., 1996). Culture-independent 16S rRNA gene cloning further demonstrated that

many sequences were derived from members of the family Pasteurellaceae (Betaproteobacteria) (Ward-Rainey *et al*., 1996). Even in our laboratory, sea cucumber (*A. japonicus*)-associated microbiota was investigated using culture-dependent and -independent methods (Enomoto *et al*., 2012). Diverse and abundant heterotrophs, mostly Gammaproteobacteria members, were cultured (Enomoto *et al*., 2012). A 16S rRNA gene clone library method also showed difference in microbial communities between the different holothurian tissues (Enomoto *et al*., 2012). At the same time since 2012, the number of reports on coastal sea cucumber gut microbiota have increased. A total of 141 bacterial strains including *Bacillus* and *Vibrio* under aerobic conditions were isolated from guts of *Holothuria leucospilota*, and interestingly, the isolates degraded various polysaccharides including starch, alginate, xylan and agar, and were tolerant of anaerobic conditions (Zhang *et al*., 2012). Zhang *et al.* (2013) also reported that 1,133 strains showing various polysaccharide degradation activities, mainly affiliated to Firmicutes and Proteobacteria, were isolated under aerobic conditions from the guts of *A. japonicus*. Plotieau *et al*. (2013) reported that using several methods including culturing, DAPI, FISH and clone library, microbial communities from some gut compartments of *Holothuria scabra* and sediments identified a total 116 phylotypes mainly assigned to Gammaproteobacteria (60.5%) and Alphaproteobacteria (24.5%), and indicated that the number of bacteria is significantly greater in the foregut than in the ambient sediments and decreased in the mid- and hind-guts. Gao *et al*. (2014) performed the first meta16S rRNA gene sequencing to characterize the sea cucumber gut microbiota. They showed that bacterial diversity of gut microbiota of *A. japonicus* was higher than in the ambient sediments, and that community structures differed between guts and sediments, and between gut compartments, and Gammaproteobacteria and Deltaproteobacteria were abundant in the guts. More recently, dietary β-glucan supplementation increased Rhodobacteraceae abundance in the gut microbiota of sea cucumber *A. japonicus* and activated the host NF-κB signaling pathway, and it was then speculated that the activation of the signaling pathway was caused by the increased mass of the bacterial group (Yang *et al*., 2015b). In addition to these progresses, many reports have proposed candidates of probiotics for *A. japonicus*

based on feeding experiments described above sections. However, seasonal changes and processes of shaping in the sea cucumber gut microbiota, and host-microbe interaction and mechanisms by which probiotics affect the hosts have not been clear due to lack of methodologies to analyze individual sea cucumber gut microbiota without any sacrifice of valuable wild sea cucumbers as well as to track responses of individual gut microbiota to extrinsic (e.g. temperature, probiotics) or intrinsic (e.g. host physiology) stimuli or changes.

This thesis developed new methodologies to advance studies on sea cucumber gut microbiomes and attempted to fill the gaps in biology of sea cucumber holobiont described above. In CHAPTER 1, I developed the method to analyze individual sea cucumber gut microbiota without any sacrifice using cultured sea cucumbers. Furthermore, I compared fecal microbiota of larger and smaller sea cucumbers in body size to explore possible contribution of the gut microbiota to the growth gap in cultured *A. japonicus*. In CHAPTERs 2 and 3, I applied the non-destructive method to wild sea cucumbers and analyzed fecal and environmental metagenomes. In CHAPTER 2, I compared bacterial communities of sea cucumber's feces and adjacent sediments through one whole year to explore processes of shaping in the gut microbiota and potential contribution of the gut microbiota to the host ecological functions (e.g. sediment cleaning, nutrient cycling). In CHAPTER 3, I compared eukaryotic communities between the feces and adjacent sediments through one whole year to explore feeding behaviors of sea cucumbers against eukaryotes in natural environments. In CHAPTER 4, both caged mariculture and laboratory feeding systems for sea cucumbers were developed. Using these systems, I tracked sea cucumber gut microbiota derived from identical individuals during gut regeneration, used as the representative intrinsic change.

CHAPTER 1

Individual *Apostichopus japonicus* fecal microbiome reveals a link with polyhydroxybutyrate producers in host growth gaps

Abstract

Gut microbiome shapes various aspects of a host's physiology, but these functions in aquatic animal hosts have yet to be fully investigated. The sea cucumber *Apostichopus japonicus* (Selenka) is one such example. The large growth gap in their body size has delayed the development of intensive aquaculture, nevertheless the species is in urgent need of conservation. To understand possible contributions of the gut microbiome to its host's growth, individual fecal microbiome comparisons were performed. High-throughput 16S rRNA sequencing revealed significantly different microbiota in larger and smaller individuals; Rhodobacterales in particular was the most significantly abundant bacterial group in the larger specimens. Further shotgun metagenome of representative samples revealed a significant abundance of microbiome retaining polyhydroxybutyrate (PHB) metabolism genes in the largest individual. The PHB metabolism reads were potentially derived from Rhodobacterales. These results imply a possible link between microbial PHB producers and potential growth promotion in Deuterostomia marine invertebrates.

Introduction

Apostichopus japonicus (Selenka) is a temperate sea cucumber species occurring in the western Pacific Ocean, Yellow Sea, Sea of Japan, and Sea of Okhotsk (Purcell *et al*., 2012). It ingests organic matters, bacteria, protozoa, diatoms as well as algae and animal detritus and thus plays an important role in benthic nutrients recycling (Choo, 2008). *A. japonicus* is also an important fishery resource in many Asian countries, especially in China (Lovatelli *et al*., 2004). Wild stocks have drastically declined due to overfishing, and in 2013 this species was listed as "Endangered" on the IUCN Red List of Threatened Species (Hamel and Mercier, 2013). Wild stocks of sea cucumber species have

been sustained by seed production and aquaculture (Lovatelli *et al*., 2004), however a lack of information concerning ecology, physiology, and biochemistry of this species is an obstacle to further success in this field. Extremely large growth gap among the juveniles of *A. japonicus* (Fig. 1.1) is also a common problem in the seed production of sea cucumbers (Ramofafia *et al*., 1997; Battaglene *et al*., 1999; Watanabe *et al*., 2014). As a result, there is a right-skewed body size distribution among cultured animals even when raised in the same tank under identical conditions (e.g. temperature, light, animal density). This can be seen in Figures 1.1A and B in which the largest individual is ca. 50 times larger than the smallest one. Population densities, congenital hosts' traits and food competition have all been considered as possible causes, however, the cause of this huge growth gap is still unknown (Watanabe *et al*., 2014).

The increasing demand for *A. japonicus* livestock has led to further research seeking optimal conditions for seeding and farming. Most of the research has focused on 1) the effects of abiotic factors on sea cucumbers growth (Dong *et al*., 2006, 2008); 2) the effects of different diets and ration composition on growth (Xia *et al*., 2012a; Shi *et al*., 2013b); 3) the use of stable isotopes to assess carbon turnover, metabolism, growth and absorption of different food sources (Gao *et al*., 2011; Sun *et al*., 2012a); 4) the effects of potential probiotics on growth, immunity and resistance to pathogens (Zhao *et al*., 2012; Yang *et al*., 2014); and 5) the effect of stock density on growth rates (Dong *et al*., 2010). Holothurians are also used as model animals to study visceral regeneration of digestive systems (Ortiz-Pineda *et al*., 2009; Mashanov and García-Arrarás, 2011). Despite its importance, a few studies have assessed *A. japonicus* microbial diversity using either culture-dependent or independent methods (Zhang *et al*., 2013; Gao *et al*., 2014; Yang *et al*., 2015b).

The relationship between gut microbiota and hosts has been widely studied in many organisms. For instance, microbiome in ruminants and termites are largely responsible for the digestion of indigestible components such as cellulose and hemicellulose (Russell and Rychlik, 2001; Warnecke *et al*., 2007) and is also responsible for fatty acid absorption in zebra fish (Semova *et al*., 2012). Obesity has been associated with mouse gut microbiota (Ley *et al*., 2005); colonization of

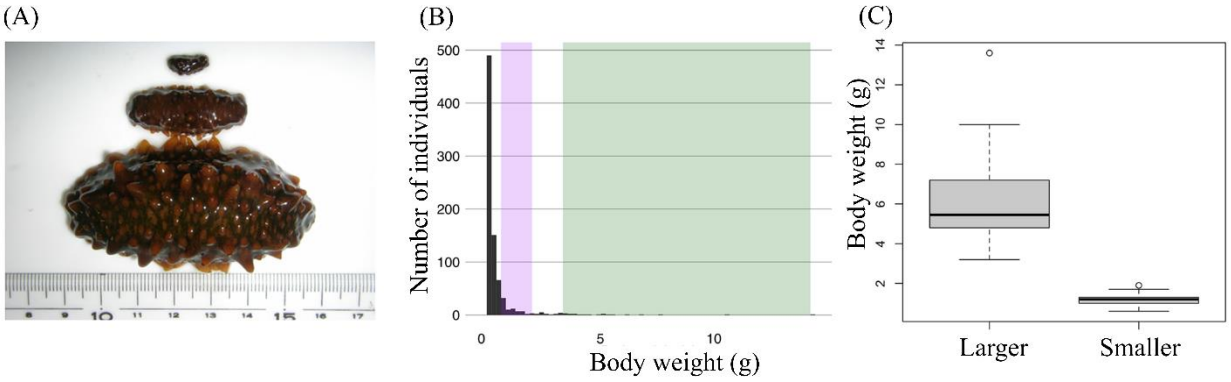


Fig. 1.1. Growth gaps observed in the cultured sea cucumbers. (A) Representatives of the larger and the smaller sea cucumbers cultured under identical conditions are displayed from lower to upper. (B) A histogram for body weight of sea cucumbers showed right-skewed size distribution. These specimens are 20 months old, and were raised on artificial diets for 17 months. For the final two months in the Kumaishi farm, the animals were fed with naturally occurred diatoms before being studied. Size distributions of the larger and smaller individuals used in this study were highlighted in green and purple, respectively. (C) Box plot for actual body weights form 10 larger and 10 smaller individuals used in this study. The body weight averages of two groups, “larger” and “smaller”, were statistically significant.

germ-free mice with an "obese microbiota" results in a significant increase in total body fat compared to colonization with a "lean microbiota" (Turnbaugh *et al*., 2006). Gut microbiota can improve immune system response (Ivanov *et al*., 2009) and can also modulate brain development and behavior (Collins and Bercik, 2009; Hsiao *et al*., 2013). These studies indicate a strong interaction between microbiota and host, however, little is known about that of *A. japonicus* sea cucumber, which is one of the phylogenetically close Deuterostomia species (Dunn *et al*., 2008).

We have observed a growth gap in juvenile sea cucumbers in Hokkaido hatcheries. Most previous studies have assessed the influence of abiotic and biotic factors on sea cucumber growth manipulating the concentrations, composition or intensity of the environmental factors (Dong *et al*., 2006, 2008, 2010; Gao *et al*., 2011; Xia *et al*., 2012a; Zhao *et al*., 2012; Shi *et al*., 2013b; Yang *et al*., 2014). To our knowledge, however, there have been no studies investigating the possible intrinsic causes of sea cucumber growth. Our hypothesis is that gut microbes play a major role on sea cucumber growth. To explore any possible contributions of the gut microbiome to its host's growth, individual fecal microbiome comparisons (both taxonomically and functionally) of both large and small individuals of sea cucumber *A. japonicus* were performed. To reach our goal, we developed a non-destructive (without animal's sacrifice) protocol for sea cucumber feces collection and DNA extraction to assess the gut microbial diversity in live specimens. We analyzed the microbial diversity taxonomically by pyrosequencing the V1-V2 region of 16S rRNA gene and functionally by massively parallel sequencing metagenomes. The non-destructive individual analyses we introduce here opens the way to both marine animal conservation and also to studies in the dynamics of host-microbes interaction.

Materials & Methods

Sea Cucumber Sampling

Cultured sea cucumber *A. japonicus* was used for this study. The animals were fertilized and grown in Hokkaido Fisheries Station, Muroran, Japan. The juveniles were fed with cultured diatoms and an

artificial diet (Norwegian *Ascophyllum nodsum* powder) for 18 months. These animals were then moved to a farm in Kumaishi, Japan, in June 2014, and then were acclimated with naturally occurring diatoms for two months in a 500 ton tank and reared in sand-filtered coastal seawater pumped up from 10 m depth. Seawater temperature in the tank ranged from 8.5ºC to 13ºC.

In order to verify the sea cucumber body weight distribution, 816 individuals from one tank were weighed (Fig. 1.1B). The mean body weight and the median were 0.41 g and 0.14 g, respectively. We categorized two groups of these individuals from the top 20% because the smaller individuals within this criterion were too small for feces sampling. We classified large individuals as weighing >2.0 g as "larger sea cucumbers" and small individuals as weighing <1.9 g as "smaller sea cucumbers".

Collection of Individual Sea Cucumber Feces and Seawater

For bacterial DNA preparation, we collected feces of individual sea cucumber to avoid sacrificing animals and seawater in a rearing tank under semi-aseptic conditions on site. All sampling procedures were performed inside an instant clean booth, illuminated by ultraviolet light for 15 minutes (GL-15, Panasonic, Osaka, Japan). For feces collection, we selected 10 individuals from each group (larger and smaller) (Fig. 1.1C). Sea cucumber individuals were cleaned using filter-sterilized seawater and moved individually into sterile beakers with 300 mL of filter-sterilized seawater until feces was released (~20 min). The filter-sterilized seawater was prepared as follows; natural seawater pumped 10 m depth was added in a pressure tank and filtered through a 0.22 μm Sterivex filter (Sterivex™-GV Sterile Vented Filter Unit 0.22 μm, EMD Millipore, Billerica, USA) by positive pressure using filtered (0.22 μm) high purity N_2 gas. Feces were collected into a 1.5 mL tube using an adopted 5 mL tip and preserved at -80°C until DNA extraction.

Five liters of the same seawater used in the sea cucumber rearing tank was filtered through a 0.22 μm Sterivex filter by positive pressure using filtered (0.22 μm) N_2 gas. The Sterivex filter was filled with SET buffer (sucrose 20%, EDTA 50 mM, Tris-HCl 50 mM) and preserved at -80ºC until

DNA extraction.

Microbial DNA Extraction

Microbial DNA extraction from sea cucumber feces was performed using the NucleoSpin Soil Kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacture's protocol. Microbial DNA extraction from seawater was performed using the NucleoSpin Tissue kit (MACHEREY-NAGEL), according to the modified manufacture's protocol. We used 20% SDS and proteinase K (20 mg mL^{-1}) for pre-lysis instead of buffer T1 and proteinase K. Also, we used 1 mL buffer B3 instead of 200 µL buffer B3.

16S rRNA Gene Pyrosequencing

The hypervariable V1-V2 region of the 16S rRNA gene was amplified by PCR with barcoded 27Fmod and 338R primers (Kim *et al.*, 2013). PCR was performed in 50 mL of 1 µL Ex *Taq* PCR buffer composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ in the presence of 250 mM dNTP, 1 U Ex *Taq* polymerase (Takara Bio, Ootsu, Shiga), forward and reverse primers (0.2 µM) and ~20 ng template DNA. Thermal cycling consisted of initial denaturation at 96ºC for 2 min, followed by 25 cycles of denaturation at 96ºC for 30 s, annealing at 55ºC for 45 s and extension at 72ºC for 1 min, and final extension at 72ºC on a 9700 PCR system (Life Technologies Japan, Tokyo, Japan). Negative controls were treated similarly, except that no template DNA was added to the PCR reactions. PCR products of ~370 bp were visualized using electrophoresis on 2% agarose gels, while negative controls failed to produce visible PCR products and were excluded from further analysis. PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amounts of each PCR amplicon were mixed and then sequenced using either 454 GS FLX Titanium or 454 GS JUNIOR (Roche, Basel, Switzerland). Based on sample specific barcodes, reads were assigned to each sample followed by the removal of reads lacking both forward and

reverse primer sequences. Data was further qualified by removal of reads with average quality values <25. Filter-passed reads were filed as FASTA for downstream analyses, after trimming off both primer sequences.

Taxonomic Assignment and Microbial Diversity Analyses

Eukaryotic (i.e. chloroplasts and mitochondria) sequences were removed from the dataset using Metaxa software (Bengtsson *et al.*, 2011). Sequence analysis was performed using Quantitative Insights Into Microbial Ecology (QIIME) 1.8 software package (Caporaso *et al.*, 2010a) as shown below; 1) Sequences were clustered into OTUs (similarity 97%) using uclust algorithm (Edgar, 2010). 2) Representative sequences of each OTU were assigned taxonomically using uclust consensus taxonomy assigner with default parameters (minimum percent similarity 90%) and Greengenes reference version 13.8. 3) Sequences were aligned to Greengenes Core reference using PyNAST algorithm (Caporaso *et al.*, 2010b) and phylogenic trees were generated using FastTree for tree based analyses. 4) Alpha diversity estimates, observed species, Faith's phylogenic diversity [PD] and Chao1 were calculated and compared between the larger and the smaller individuals using nonparametric test based on 999 iterations using a rarefaction of 1835 reads from each sample. 5) For beta diversity, even sampling of 1835 reads per sample was used, and we performed unweighted UniFrac analysis (Lozupone *et al.*, 2011) and visualized in PCoA plots (Vázquez-Baeza *et al.*, 2013). Beta diversity was compared in a pairwise fashion (larger vs smaller sea cucumbers, larger vs seawater, smaller vs seawater) using unweighted UniFrac distance matrixes, using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations to determine statistical significance. 6) Taxonomic relative abundances of fecal microbial communities between larger and smaller sea cucumbers were compared using Welch's t test with Storey's FDR (false discovery rate) for multiple test correction. Significance was assumed to be $p<0.05$ and $q<0.05$. Taxonomic affiliations of 16S rRNA gene reads below genus level could not be fully achieved due to the lack of marine microbial reference sequences even in this affiliation setting using QIIME, family table was

the lowest hierarchy analyzed in this study.

OTUs Relevant to Body Weight of Sea Cucumbers

To verify if specific OTUs abundance correlated to sea cucumber body weight, we performed multiple Pearson correlations, and *p* values were adjusted using the Holm method. We searched for highly ($r>0.7$) and significantly (corrected $p<0.05$) correlated OTUs against the body weight of the sea cucumbers. OTUs that occurred in only one sample were removed from this analysis. OTUs present in all larger individuals, in all smaller individuals, and all individuals tested were defined as "lager individuals core (LIC)", "smaller individuals core (SIC)", and "all individuals core (AIC)", respectively.

Metagenomic Sequencing

Each representative DNA samples of the largest and the smallest body weight sea cucumbers, from which sufficient volumes were available after 16S typing, were used for metagenome sequencing. Metagenomic sequencing was performed on HiSeq platform by Hokkaido System Science, Co. Ltd., Sapporo, Japan. The DNA quality and quantity were estimated using Nanodrop, Qubit Fluorometer and Agilent 2200 TapeStation System. By using TruSeq Nano DNA LT Sample Prep Kit, genomic DNA was fragment and insert DNA of 350 bp was selected and connected adapter sequences. Sixty nanograms of each DNA sample was amplified with nine cycling. Subsequently, 100 bp paired-end sequencing of two samples was performed on HiSeq platform. Using Chastity, sequences showing low fluorescence purity were removed.

Pre-processing and Data Annotation by MG-RAST

Sequencing data set in FASTQ format of samples (the largest and the smallest) was uploaded to MG-RAST server version 3.5. For quality control, firstly, dereplication (removing artificial replicate sequences) was performed using DRISEE (duplicate read inferred sequencing error estimation).

Secondly, the removal of low-quality sequences was performed by a modified DynamicTrim. After filtering, data gene calling was performed using FragGeneScan algorithm to predict protein or rRNA coding region. Clusters of proteins (similarity 90% or greater) were generated using uclust by BLAT analysis. The sequences assignments were conducted using the MG-RAST server and the following cut-off parameters: expected value less than $1x10^{-5}$, 60% minimum identity and 15 base pair or amino acid minimum alignment. Taxonomic annotation was performed using the National Center for Biotechnology Information (NCBI) GenBank database, and functional annotation was completed using the SEED database (Overbeek, 2005)

Metagenomic Analysis

Annotated metagenomes were analyzed using the MG-RAST analysis tools and STAMP software v2.0.9 (Parks *et al*., 2014). To test if the abundance of each functional feature of the largest and the smallest sea cucumbers were different, Fisher's exact test with Newcombe-Wilson confidence interval calculation method and Storey's FDR for multiple test correction method were used in STAMP software. *p*-values of <0.05 were considered statistically significant. To visualize metagenomic results, profile bar plots and extended error bar plots were generated at Subsystem Level 1 and 3. To plot data, results were filtered by 0.05 of *q*-value and 0.05 of effect size (Yadav *et al*., 2015).

To investigate the taxonomic affiliation of the significantly different functional features between the largest and the smallest sea cucumbers, we used the MG-RAST workbench tool implementing KEGG Orthology and GenBank databases to annotate with the functional features.

Data Deposition

All sequences generated in this study are deposited in BaMBa (Meirelles *et al*., 2015) under data package identification number pmeirelles.19.1, and DDBJ/GenBank/EMBL database under BioProject number PRJDB4366.

Results

Larger and Smaller Apostichopus japonicus *Have Different Fecal Microbial Communities*

We assessed fecal microbiota of the larger and smaller individual sea cucumbers using tag sequencing of the V1-V2 region of 16S rRNA gene. After trimming, 104,679 qualified reads from the sea cucumber samples (52,996 and 51,683 reads from 10 larger and smaller individual samples, respectively) and 5,241 qualified reads from the seawater sample were used to cluster into OTUs (similarity 97%) (Table 1.1). After removing Eukaryotic reads, we used 63,574 bacterial sequences for taxonomic assignment. Chloroplast sequences, which might be from ingested algae, were more frequently associated with smaller individuals [48.4±16% (mean±SD)] than larger individuals (37±15%), however, these were not significant (p>0.05). In total 1,976 OTUs with average Good's coverage of 93.5±1.8% were obtained. Eukaryotic diversity, consisting of >90% of stramenopiles, was not significantly different between the two groups.

The fecal microbiota of the larger and the smaller individuals were clustered separately from one another, and separated from the seawater microbiota from the rearing tank (Fig. 1.2A). We found two major taxa in the fecal microbiota. The most abundant phylum was Proteobacteria; 57.4±4.4% and 52.7±4.9% of reads in the larger and the smaller individuals, respectively. Proteobacteria accounted for 77.3% of reads in the seawater sample. Bacteroidetes was the second most abundant phylum in sea cucumber samples (34.0±4.8% and 37.8±6.3% in larger and smaller individuals, respectively). Only 11.4% of this phylum was observed in the seawater sample. Comparing classes among Proteobacteria in the fecal microbiota, the relative abundances of Alphaproteobacteria (Larger, 15.3±1.8%; Smaller, 11.5±2.8%) and Deltaproteobacteria (Larger, 2.3±0.7%; Smaller, 1.1±0.8%) were significantly different between the larger and smaller individuals (p<0.05, q<0.05). The relative abundances of minor phyla (Actinobacteria, Firmicutes, Fusobacteria and Spirochaetes) were significantly different between the two groups (p<0.05, q<0.05, see Table 1.2).

The most abundant order was Flavobacteriales (Larger, 29.0%±5.2%; Smaller, 33.5%±6.4%), followed by Alteromonadales (Larger, 23.9%±5.0%; Smaller, 25.1%±3.2%), but the

Table 1.1. Summaries of sequencing information. This table shows the information of 16S rRNA gene and shotgun metagenomic sequencing analyses; sample category, body weight of sea cucumbers, number of total sequenced reads, number of qualified reads, number of qualified reads without eukaryotic reads (16S only), qualified reads length average, Good's coverage (16S only) and number of annotated reads (metagenome only).

Category	Sample ID	Body weight (g)	No. of total reads	No. of reads passed quality check	No. of reads passed quality check (removed eukaryotic reads)	Reads length Average (bp)	16S rDNA Good's coverage (%)	Metagenimic annotated reads
Fecal 16S rRNA gene	Large.01	10	14658	5284	2970	294.74	92.8	-
	Large.02	5.1	13323	5181	3011	294.35	94	-
	Large.03	4	14174	5450	2846	292.82	94.6	-
	Large.04	5.8	14669	5483	4221	298.78	95.3	-
	Large.05	13.6	15153	6026	2800	291.79	92.3	-
	Large.06	3.2	13405	5438	3029	294.23	94.3	-
	Large.07	6.5	16141	5766	2998	293.72	92.9	-
	Large.08	7.2	14379	4838	3709	298.19	94.4	-
	Large.09	4.8	12883	4410	4116	302.33	94.5	-
	Large.10	4.8	13326	5120	3126	295.17	94.4	-
	Small.01	1.3	12520	5464	2005	289.6	92.3	-
	Small.02	1	12270	5053	3826	300.01	94.6	-
	Small.03	1.3	11429	5077	2178	291.54	93.1	-
	Small.04	1.7	11936	5656	1835	289.67	88.1	-
	Small.05	1.2	11661	4959	2403	291.96	94.6	-
	Small.06	1.2	12691	5001	3428	297.49	96	-
	Small.07	0.6	11550	5129	2955	295.04	94	-
	Small.08	0.9	11088	4501	2951	297.12	90.8	-
	Small.09	1.9	12574	5978	1900	289.43	93.1	-
	Small.10	1.1	11600	4865	2709	293.72	94.5	-
Seawater 16S rRNA gene	Water	-	13696	5241	4558	292.24	88.3	-
Fecal metagenomes	Large.05	13.6	22386506	18582516	-	101	-	6049986
	Small.03	1.3	22803396	18156817	-	102	-	4634631

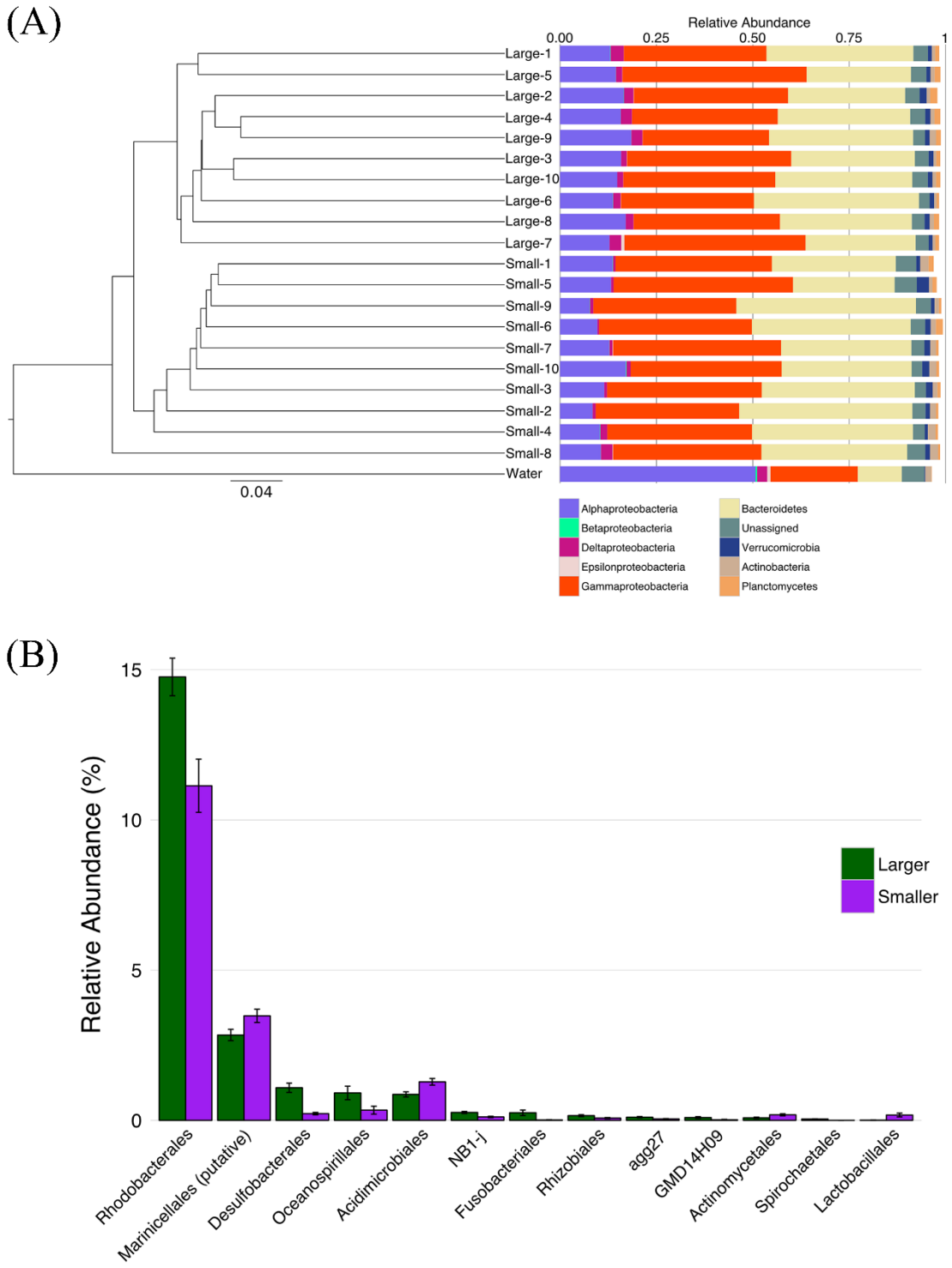


Fig. 1.2. The microbiota of larger and smaller sea cucumbers feces are different. (A) The larger and the smaller sea cucumbers clustered individually, and seawater sample differed from sea cucumber samples. (B) Ranking of the order level abundances in fecal microbiota of the larger (green) and the smaller (purple) individuals. Only significant taxa were shown in this bar plot; Rhodobacterales, Desulfobacterales and Oceanospirillales were significantly more abundant in the larger individuals, Marinicellales, Acidimicrobiales were more abundant in the smaller individuals.

Table 1.2 Taxa relative abundances between larger and smaller sea cucumber groups at phylum, class, order and family levels. Comparing taxa relative abundance between larger and smaller individuals by Welch's *t* test, only taxa with $p < 0.05$ and $q < 0.05$ (Storey's False Discovery Rate) were emphasized bold characters. NA means not applicable. We could not remove the sequences as Chloroplast by Metaxa software.

Phylum	Larger average	Smaller average	*p*-value	*q*-value
Proteobacteria	**0.574254**	**0.526678**	**0.033636**	**0.001801**
Bacteroidetes	0.340488	0.377979	0.150165	0.003787
Verrucomicrobia	0.012409	0.014875	0.305723	0.004909
Actinobacteria	**0.009633**	**0.015702**	**0.002457**	**0.000301**
Planctomycetes	0.0123	0.00874	0.052154	0.002105
WPS-2	0.006934	0.008117	0.30512	0.004909
Firmicutes	**0.001033**	**0.005035**	**0.037179**	**0.001801**
Fusobacteria	**0.002512**	**0.000143**	**0.031001**	**0.001801**
Chloroflexi	0.001029	0.000667	0.170349	0.003787
WS3	0.000924	0.000675	0.435711	0.005861
Acidobacteria	0.000342	0.000807	0.241274	0.004869
SBR1093	0.00053	0.000612	0.823583	0.009065
Lentisphaerae	0.00066	0.000391	0.474736	0.00605
Cyanobacteria	0.000318	0.000228	0.569334	0.006565
TM6	0.000262	0.000151	0.344626	0.004909
Spirochaetes	**0.000411**	**NA**	**0.002485**	**0.000301**
GN02	9.00E-05	0.000148	0.54818	0.006565
TM7	2.40E-05	0.000134	0.172025	0.003787
Fibrobacteres	0.000135	NA	0.101213	0.003064
Nitrospirae	0.000102	NA	0.081248	0.002811
Tenericutes	NA	4.60E-05	0.343436	0.004909
BRC1	3.30E-05	NA	0.343436	0.004909

Table 1.2. Continued

Class	Larger average	Smaller average	*p*-value	*q*-value
Gammaproteobacteria	0.396819	0.399983	0.863861	0.709386
Flavobacteriia	0.289925	0.334573	0.105446	0.241232
Alphaproteobacteria	**0.15251**	**0.114835**	**0.002643**	**0.034263**
Cytophagia	0.032055	0.031946	0.985793	0.723374
Deltaproteobacteria	**0.023212**	**0.010718**	**0.00128**	**0.034263**
Verrucomicrobiae	0.012148	0.014684	0.287557	0.349374
Acidimicrobiia	0.008656	0.012821	0.008466	0.082314
[Saprospirae]	0.011165	0.006852	0.052657	0.214801
Phycisphaerae	0.005483	0.006919	0.338937	0.349374
Bacteroidia	0.00626	0.003417	0.057595	0.214801
Actinobacteria	0.000865	0.002567	0.019679	0.136074
Clostridia	0.000967	0.002042	0.213715	0.349374
Fusobacteriia	0.002512	0.000143	0.031001	0.172239
Bacilli	6.60E-05	0.002467	0.038086	0.185152
BME43	0.00097	0.000947	0.949545	0.723374
OM190	0.001174	0.000519	0.020993	0.136074
Anaerolineae	0.001029	0.000589	0.083667	0.232423
Epsilonproteobacteria	0.001272	0.000339	0.280662	0.349374
PRR-12	0.000924	0.000675	0.435711	0.413302
Betaproteobacteria	0.000384	0.000694	0.248608	0.349374
[Lentisphaeria]	0.00066	0.000391	0.474736	0.439598
EC214	0.00041	0.000403	0.979941	0.723374
Erysipelotrichi	NA	0.000526	0.121205	0.261879
Sva0725	8.10E-05	0.000358	0.098499	0.241232
Chloroplast	0.000294	0.000134	0.236995	0.349374
Spirochaetes	**0.000411**	**NA**	**0.002485**	**0.034263**
Opitutae	0.000195	0.000191	0.969634	0.723374
SJA-4	0.000262	0.000118	0.216894	0.349374
Thermoleophilia	3.20E-05	0.000313	0.060754	0.214801
Acidobacteria-6	6.30E-05	0.00028	0.221426	0.349374
VHS-B5-50	0.00012	0.000209	0.538714	0.48724
C6	6.80E-05	0.000237	0.179943	0.349374
RB25	0.000132	8.10E-05	0.59026	0.510134
Sphingobacteriia	3.40E-05	0.000148	0.219747	0.349374
[Rhodothermi]	8.00E-05	9.60E-05	0.836451	0.707189
BD1-5	9.00E-05	8.10E-05	0.89722	0.712125
TG3	0.000135	NA	0.101213	0.241232
TA18	2.40E-05	8.40E-05	0.35035	0.349374
TM7-1	2.40E-05	8.10E-05	0.374014	0.363648
Nitrospira	0.000102	NA	0.081248	0.232423
AT-s2-57	6.70E-05	3.40E-05	0.562881	0.497527
4C0d-2	NA	9.40E-05	0.083606	0.232423
OPB41	8.10E-05	NA	0.343436	0.349374
Ellin6529	NA	7.90E-05	0.193872	0.349374
Verruco-5	6.60E-05	NA	0.343436	0.349374
Planctomycetia	2.70E-05	3.40E-05	0.875529	0.709386
BPC102	NA	5.40E-05	0.343436	0.349374
TM7-3	NA	5.30E-05	0.343436	0.349374
Mollicutes	NA	4.60E-05	0.343436	0.349374
SBRH58	NA	3.40E-05	0.343436	0.349374
NPL-UPA2	3.30E-05	NA	0.343436	0.349374
Pla3	NA	2.60E-05	0.343436	0.349374
Oscillatoriophycideae	2.40E-05	NA	0.343436	0.349374

Table 1.2. Continued

Order	Larger average	Smaller average	*p*-value	*q*-value
Flavobacteriales	0.289925	0.334573	0.105446	0.033251
Alteromonadales	0.239184	0.250987	0.538428	0.062472
Rhodobacterales	**0.147582**	**0.111355**	**0.004065**	**0.008427**
Thiotrichales	0.05282	0.049581	0.60935	0.068284
HTCC2188	0.036971	0.030522	0.09492	0.033251
Cytophagales	0.032055	0.031946	0.985793	0.09849
[Marinicellales]	**0.028409**	**0.034792**	**0.040825**	**0.027937**
Vibrionales	0.022384	0.018276	0.661444	0.073134
Verrucomicrobiales	0.012148	0.014684	0.287557	0.047466
Acidimicrobiales	**0.008656**	**0.012821**	**0.008466**	**0.014041**
[Saprospirales]	0.011165	0.006852	0.052657	0.030684
Desulfobacterales	**0.010853**	**0.002262**	**0.00032**	**0.002654**
Oceanospirillales	**0.00913**	**0.003392**	**0.043796**	**0.027937**
Phycisphaerales	0.005483	0.006919	0.338937	0.047466
Bacteroidales	0.00626	0.003417	0.057595	0.030684
Spirobacillales	0.004004	0.002775	0.376571	0.051124
Legionellales	0.002566	0.003504	0.317941	0.047466
Myxococcales	0.002894	0.00256	0.743589	0.07866
NB1-j	**0.002628**	**0.001142**	**0.001965**	**0.006869**
Clostridiales	0.000942	0.002042	0.203179	0.045537
Actinomycetales	**0.000833**	**0.001852**	**0.025095**	**0.025708**
Fusobacteriales	**0.002512**	**0.000143**	**0.031001**	**0.025708**
Chromatiales	0.00103	0.001449	0.434712	0.053524
Rhizobiales	**0.001571**	**0.000752**	**0.040952**	**0.027937**
Lactobacillales	**6.60E-05**	**0.001772**	**0.024059**	**0.025708**
Campylobacterales	0.001272	0.000339	0.280662	0.047466
GN03	9.00E-04	0.000675	0.463427	0.055695
Caldilineales	0.000996	0.000563	0.10472	0.033251
Bdellovibrionales	0.000842	0.000685	0.735661	0.07866
agg27	**0.001047**	**0.000464**	**0.030657**	**0.025708**
Syntrophobacterales	0.000673	0.000783	0.802773	0.083213
Thiohalorhabdales	0.000734	0.000602	0.542419	0.062472
GMD14H09	**0.000938**	**0.000199**	**0.030655**	**0.025708**
Lentisphaerales	0.00066	0.000391	0.474736	0.056239
BD7-3	0.000524	0.000286	0.275597	0.047466
Bifidobacteriales	3.20E-05	0.000716	0.083899	0.033167
Burkholderiales	0.00027	0.000456	0.388403	0.051124
Rickettsiales	0.00031	0.000364	0.831643	0.084589
Kiloniellales	0.000501	0.000157	0.12866	0.036026
HOC36	0.000177	0.00044	0.262759	0.047466
Rhodospirillales	0.000374	0.000223	0.434111	0.053524

Order	Larger average	Smaller average	*p*-value	*q*-value
Bacillales	NA	0.000566	0.137552	0.036795
Erysipelotrichales	NA	0.000526	0.121205	0.035896
Sva0725	8.10E-05	0.000358	0.098499	0.033251
Spirochaetales	**0.000411**	**NA**	**0.002485**	**0.006869**
Gaiellales	3.20E-05	0.000313	0.060754	0.030684
Pseudomonadales	NA	0.000299	0.062904	0.030684
Caulobacterales	0.000102	0.000165	0.687108	0.074972
Desulfuromonadales	0.000148	0.000109	0.749368	0.07866
Puniceicoccales	6.90E-05	0.000157	0.385716	0.051124
Neisseriales	NA	0.000211	0.108264	0.033251
d113	6.80E-05	0.000141	0.410952	0.053247
BPC015	NA	0.000189	0.130331	0.036026
Sphingobacteriales	3.40E-05	0.000148	0.219747	0.047466
CL500-15	0.000127	5.40E-05	0.436382	0.053524
[Rhodothermales]	8.00E-05	9.60E-05	0.836451	0.084589
Desulfovibrionales	2.40E-05	0.000135	0.438907	0.053524
TG3-2	0.000135	NA	0.101213	0.033251
Gemellales	NA	0.000128	0.083993	0.033167
Opitutales	6.80E-05	3.40E-05	0.550434	0.062527
Nitrospirales	0.000102	NA	0.081248	0.033167
MVS-107	NA	9.60E-05	0.168258	0.040672
MLE1-12	NA	9.40E-05	0.083606	0.033167
CCU21	NA	9.10E-05	0.176569	0.040672
Enterobacteriales	NA	8.40E-05	0.176438	0.040672
PHOS-HD29	NA	8.40E-05	0.176438	0.040672
WCHB1-41	6.60E-05	NA	0.343436	0.047466
iii1-15	6.30E-05	NA	0.172472	0.040672
B110	NA	5.40E-05	0.343436	0.047466
PB19	NA	3.40E-05	0.343436	0.047466
Methylococcales	NA	3.40E-05	0.343436	0.047466
Pasteurellales	NA	3.40E-05	0.343436	0.047466
Pirellulales	NA	3.40E-05	0.343436	0.047466
Desulfarculales	NA	3.40E-05	0.343436	0.047466
S0208	3.40E-05	NA	0.343436	0.047466
UA01	3.30E-05	NA	0.343436	0.047466
Planctomycetales	2.70E-05	NA	0.343436	0.047466
CFB-26	NA	2.60E-05	0.343436	0.047466
A21b	NA	2.60E-05	0.343436	0.047466
OPB54	2.40E-05	NA	0.343436	0.047466
Sediment-1	2.40E-05	NA	0.343436	0.047466
Chroococcales	2.40E-05	NA	0.343436	0.047466
CV90	2.40E-05	NA	0.343436	0.047466

Table 1.2. Continued

Family	Larger average	Smaller average	*p*-value	*q*-value
Flavobacteriaceae	0.250229	0.280026	0.230014	0.182131
OM60	0.210828	0.225557	0.421353	0.213347
Rhodobacteraceae	**0.147582**	**0.111009**	**0.003903**	**0.035572**
Piscirickettsiaceae	0.040435	0.038691	0.714755	0.298823
HTCC2089	0.035504	0.03031	0.168932	0.182131
Flammeovirgaceae	0.031834	0.031638	0.974185	0.37819
[Marinicellaceae]	0.028409	0.034792	0.040825	0.133232
Cryomorphaceae	0.024347	0.028741	0.282993	0.182131
Vibrionaceae	0.017315	0.012131	0.493006	0.234026
Verrucomicrobiaceae	0.012148	0.014684	0.287557	0.182131
C111	0.00726	0.010185	0.01665	0.094844
Desulfobulbaceae	**0.010787**	**0.002157**	**0.000324**	**0.007382**
Alteromonadaceae	0.003809	0.00799	0.201449	0.182131
Pseudoalteromonadaceae	0.005035	0.006145	0.643308	0.281883
Colwelliaceae	0.007007	0.002464	0.11625	0.165104
Oleiphilaceae	0.005111	0.001284	0.030891	0.129302
VC21_Bac22	**0.004885**	**0.00094**	**0.001675**	**0.025444**
Saprospiraceae	0.002302	0.002379	0.951694	0.376905
Moritellaceae	0.000305	0.0041	0.343716	0.182131
Halomonadaceae	0.001983	0.000739	0.034049	0.129302
Fusobacteriaceae	0.00248	0.000143	0.03377	0.129302
Coxiellaceae	0.000483	0.002138	0.050184	0.142932
JTB38	**0.001946**	**0.000314**	**4.70E-05**	**0.002142**
Oceanospirillaceae	0.001637	0.000598	0.262829	0.182131
Micrococcaceae	0.00069	0.001441	0.057879	0.155151
OM27	0.001431	0.00053	0.106266	0.165104
Psychromonadaceae	0.00129	0.000313	0.254449	0.182131
Caldilineaceae	0.000996	0.000563	0.10472	0.165104
Shewanellaceae	0.001249	0.00027	0.117582	0.165104
Phycisphaeraceae	0.000602	0.000904	0.279577	0.182131
Syntrophobacteraceae	0.000673	0.000783	0.802773	0.329574
Streptococcaceae	6.60E-05	0.001359	0.014708	0.094844
Prevotellaceae	NA	0.001398	0.19031	0.182131
KSB4	0.000683	0.000675	0.979285	0.37819
Campylobacteraceae	0.001102	0.000237	0.301831	0.182131
Phyllobacteriaceae	0.000926	0.000377	0.161999	0.182131
JdFBGBact	0.000177	0.000963	0.325498	0.182131
Bacteriovoracaceae	0.000633	0.000427	0.634917	0.280907
Lentisphaeraceae	0.00066	0.000391	0.474736	0.228763
Ruminococcaceae	0.000697	0.000268	0.040931	0.133232
Chitinophagaceae	0.000606	0.000354	0.310907	0.182131
Bifidobacteriaceae	3.20E-05	0.000716	0.083899	0.159483
Peptostreptococcaceae	5.40E-05	0.000669	0.36325	0.188108
Porphyromonadaceae	NA	0.000682	0.235229	0.182131
Chromatiaceae	0.00039	0.000246	0.465198	0.227949
Hyphomicrobiaceae	0.000445	0.000179	0.236738	0.182131
NB1-i	0.000203	0.000409	0.120991	0.165104
koll13	0.000193	0.000397	0.325279	0.182131
Clostridiaceae	0.000126	0.000454	0.111429	0.165104
Oxalobacteraceae	0.000102	0.000456	0.062403	0.156098
SB-1	0.000548	NA	0.007539	0.057259
Erysipelotrichaceae	NA	0.000526	0.121205	0.165104
[Amoebophilaceae]	0.000197	0.000307	0.631993	0.280907
Bdellovibrionaceae	0.000209	0.000257	0.709353	0.298823
Nannocystaceae	3.40E-05	0.000433	0.350907	0.183805
Planococcaceae	NA	0.00042	0.25392	0.182131
Spirochaetaceae	**0.000411**	**NA**	**0.002485**	**0.028311**
Ferrimonadaceae	0.000267	0.000121	0.264981	0.182131

Family	Larger average	Smaller average	*p*-value	*q*-value
Rickettsiaceae	0.000195	0.000172	0.874925	0.352838
Hyphomonadaceae	NA	0.000347	0.282469	0.182131
Lachnospiraceae	3.20E-05	0.000302	0.123184	0.165104
Legionellaceae	0.000273	4.20E-05	0.04816	0.142932
wb1_P06	0.000236	5.40E-05	0.162629	0.182131
Gaiellaceae	NA	0.00028	0.065083	0.156098
Helicobacteraceae	0.000171	0.000102	0.565695	0.260394
Veillonellaceae	NA	0.000272	0.075565	0.159483
Caulobacteraceae	0.000102	0.000165	0.687108	0.296999
Pseudomonadaceae	NA	0.000265	0.098594	0.165104
Desulfuromonadaceae	0.000148	0.000109	0.749368	0.310446
Kiloniellaceae	0.000229	NA	0.030031	0.129302
Saccharospirillaceae	NA	0.000227	0.137312	0.178782
Puniceicoccaceae	6.90E-05	0.000157	0.385716	0.197497
Neisseriaceae	NA	0.000211	0.108264	0.165104
Marinilabiaceae	6.90E-05	0.000131	0.598183	0.269896
Rhodospirillaceae	0.000137	6.30E-05	0.476899	0.228763
Bacteroidaceae	9.10E-05	0.000108	0.872049	0.352838
Desulfobacteraceae	6.60E-05	0.000105	0.690841	0.296999
Desulfovibrionaceae	2.40E-05	0.000135	0.438907	0.217404
Rhodothermaceae	5.60E-05	9.60E-05	0.597689	0.269896
Staphylococcaceae	NA	0.000146	0.209508	0.182131
Propionibacteriaceae	5.60E-05	8.10E-05	0.710969	0.298823
Burkholderiaceae	0.000135	NA	0.343436	0.182131
Actinomycetaceae	NA	0.000134	0.221429	0.182131
Enterococcaceae	NA	0.000134	0.221429	0.182131
Gemellaceae	NA	0.000128	0.083993	0.159483
Ectothiorhodospiraceae	3.30E-05	8.80E-05	0.437552	0.217404
Lactobacillaceae	NA	0.000112	0.082443	0.159483
Thiotrichaceae	0.000107	NA	0.343436	0.182131
[Weeksellaceae]	NA	0.000107	0.167923	0.182131
Opitutaceae	6.80E-05	3.40E-05	0.550434	0.258593
Nitrospiraceae	0.000102	NA	0.081248	0.159483
HTCC2188	5.10E-05	4.60E-05	0.934792	0.373675
Bradyrhizobiaceae	3.20E-05	6.30E-05	0.565131	0.260394
Enterobacteriaceae	NA	8.40E-05	0.176438	0.182131
Carnobacteriaceae	NA	6.80E-05	0.343436	0.182131
NS11-12	3.40E-05	3.40E-05	0.996429	0.381578
Mycobacteriaceae	NA	6.00E-05	0.171764	0.182131
S24-7	NA	5.20E-05	0.343436	0.182131
Pseudonocardiaceae	2.40E-05	2.60E-05	0.959415	0.376905
[Paraprevotellaceae]	NA	5.00E-05	0.343436	0.182131
[Mogibacteriaceae]	NA	5.00E-05	0.343436	0.182131
Corynebacteriaceae	3.60E-05	NA	0.343436	0.182131
Francisellaceae	NA	3.40E-05	0.343436	0.182131
Pasteurellaceae	NA	3.40E-05	0.343436	0.182131
Moraxellaceae	NA	3.40E-05	0.343436	0.182131
Pirellulaceae	NA	3.40E-05	0.343436	0.182131
Desulfarculaceae	NA	3.40E-05	0.343436	0.182131
Comamonadaceae	3.30E-05	NA	0.343436	0.182131
MND4	3.30E-05	NA	0.343436	0.182131
Leptotrichiaceae	3.20E-05	NA	0.343436	0.182131
Microthrixaceae	2.70E-05	NA	0.343436	0.182131
Planctomycetaceae	2.70E-05	NA	0.343436	0.182131
Nocardioidaceae	NA	2.60E-05	0.343436	0.182131
EB1003	NA	2.60E-05	0.343436	0.182131
[Balneolaceae]	2.40E-05	NA	0.343436	0.182131
PRR-10	2.40E-05	NA	0.343436	0.182131
Cyclobacteriaceae	2.40E-05	NA	0.343436	0.182131
Xenococcaceae	2.40E-05	NA	0.343436	0.182131
Methylobacteriaceae	2.40E-05	NA	0.343436	0.182131

data was not significantly different between the two groups (p>0.05). Rhodobacterales among Alphaproteobacteria was the third most abundant (Larger, 14.8±2.0%, and Smaller, 11.1±2.8%), and the relative abundances between larger and smaller individuals were significantly different (Fig. 1.2B and Table 1.2 (p<0.05, q<0.05)). Desulfobacterales (Larger, 1.1%±0.5%; Smaller, 0.2%±0.1%) (Deltaproteobacteria) and Oceanospirillales (Larger, 0.9%±0.7%; Smaller, 0.3%±0.4%), were more abundant in larger individuals (p<0.05, q<0.05), on the other hand Marinicellales (Larger, 2.8%±0.6%; Smaller, 3.5%±0.7%) and Acidimicrobiales (Larger, 0.9%±0.3%; Smaller, 1.3%±0.4%) were more abundant in smaller individuals (p<0.05, q<0.05). Relative abundances of some other orders, for instance Fusobacterales, Rhizobiales and Actinomycetales, were also significantly different between the two groups (p<0.05, q<0.05), but these were quite low occupation (<1%) (Fig. 1.2B).

Species richness and diversity were not statistically significant between the two groups of microbiota (Fig. 1.3A, B and C), on the contrary, the unweighted UniFrac analysis demonstrated clear clustering of the larger and smaller individual's microbiota (Fig. 1.3D).

Core Fecal Microbiota of A. japonicus *Juveniles and OTUs Correlated to Hosts' Body Weight*

We hypothesized that larger and smaller sea cucumbers could possess unique microbiota. Comparisons of individual fecal microbiota of *A. japonicus* demonstrated that 30, 12 and 35 OTUs were defined as larger individual core (LIC), smaller individual core (SIC), and all individual core (AIC) microbiota, respectively (Fig. 1.4A). The OTUs from LIC and SIC microbiota were further affiliated to 10 and 6 major bacterial orders, respectively. Rhodobacterales, Alteromonadales, Cytophagales, HTCC2188 (Gammaproteobcteria) and Myxococcales were absent in the SIC, and Marinicellales was not observed in the LIC microbiota (Fig. 1.4B). Those of the AIC microbiota were classified into nine orders (e.g. Rhodobacterales, Flavobacteriales, and Alteromonadales). A total of 1,728 OTUs were also defined as a pan fecal microbiota of the cultured juvenile *A. japonicus*.

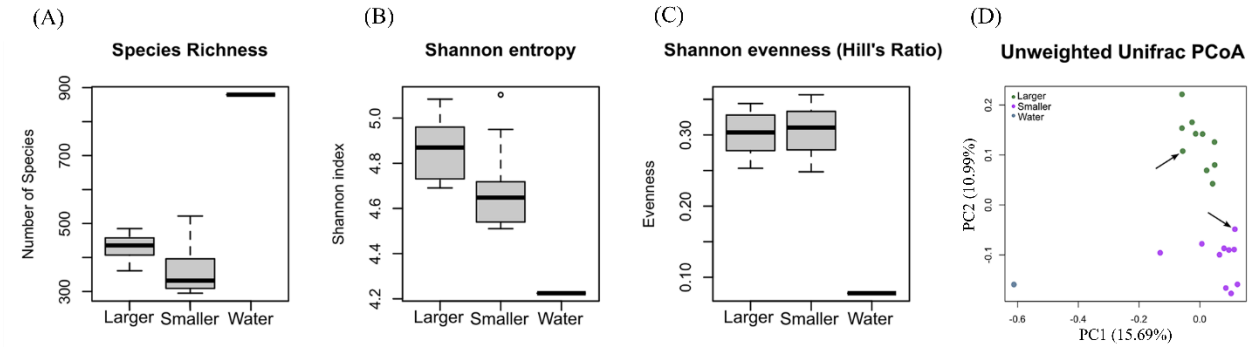


Fig. 1.3. Microbial diversity comparisons between larger and smaller *Apostichopus japonicus* individuals. (A) species richness, (B) Shannon index, (C) evenness, and (D) unweighted UniFrac-based 2D PCoA plot was based on all OTUs from the larger, the smaller sea cucumbers and seawater. Percent variation expected were PC1 15.69%, PC2 10.99%. Samples indicated with arrows were used for functional metagenomic analysis. The larger sea cucumbers, the smaller ones, and seawater sample are indicated in green, purple, and blue, respectively.

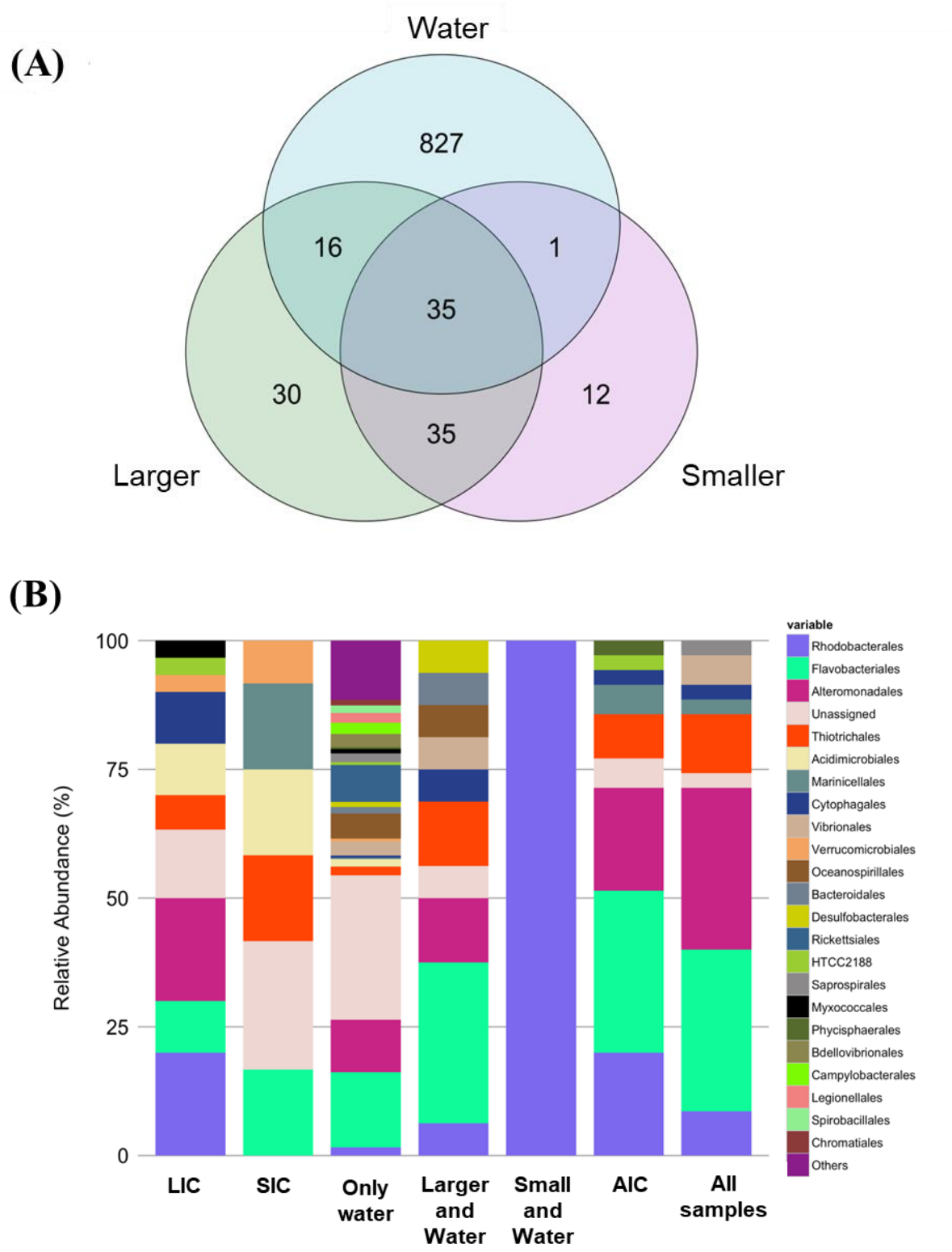


Fig. 1.4. Core fecal microbiota of cultured juvenile *Apostichopus japonicus*. (A) Venn diagram of core microbiota, and (B) the order level bacterial structure.

We further identified that 15 OTUs had strong positive correlation with sea cucumber body weight (r>0.7) with statistical significance (corrected p<0.05), respectively (Table 1.3). Two and six OTUs were affiliated to LIC and larger only, respectively. OTU584 (Legionellaceae) showed the highest positive correlation. OTU1150 (Alteromonadales OM60) was found both in all *A. japonicus* individuals used in this study and the seawater.

Comparative Metagenome Analysis of Fecal Microbiome

Metagenome sequencing using the HiSeq platform was performed on the largest and the smallest specimens (except specimens containing insufficient fecal DNA samples) as representatives of larger and smaller individuals used in the above analyses. From the largest and the smallest individuals, 22,386,506 reads (2.2 Gb) and 22,803,396 reads (2.3 Gb) were obtained, respectively. After quality filtering, 18,582,516 and 18,156,817 reads from the largest and the smallest individual, respectively, were used for MG-RAST annotation. The total number of annotated reads was 6,049,986 and 4,634,631 for the largest and the smallest individual, respectively. Bacterial reads occupied 92.6% and 87.6%, eukaryotic reads occupied 7.2% and 12.2%, and archaeal reads occupied 0.16% and 0.15% in the largest and the smallest library, respectively. Bacterial community based on the metagenomic reads was similar to those obtained by the 16S rRNA gene-based microbiota analysis (Fig. 1.5).

The metagenomic analysis demonstrated that 25 functional features on subsystem category (Level 1) were significantly more abundant in one of the samples. Microbial reads annotated to “carbohydrates”, “amino acids and derivatives”, “cofactors, vitamins, prosthetic groups, pigments”, “fatty acids, lipids, and isoprenoids”, “cell wall and capsule” and others were significantly more abundant in the largest individual, and “protein metabolism”, “RNA metabolism”, “respiration”, “virulence, disease and defense” and others were significantly more abundant in the smallest individual (Fig. 1.6A). In more detail, the reads affiliated to genes responsible for polyhydroxybutyrate (PHB) metabolism, n-phenylalkanoic acid degradation, fatty acid metabolism

Table. 1.3. OUTs showing strong correlations with *Apostichopus japonicus* body weight. Correlation coefficient was calculated by Pearson. Significance was justified by holm adjusted *p*-value. Numbers in parentheses mean the numbers of individuals sharing the OTUs, and L, S and W mean Larger individuals, smaller individuals and seawater sample, respectively.

OTU	Correlation coefficient	Adjusted *p*-value	Phylum	Class	Order	Family	Genus	Affiliation
585	0.816536	0.001271	*Proteobacteria*	*Gammaproteobacteria*	*Legionellales*	*Legionellaceae*		Larger only (5)
1884	0.800120	0.002555	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Alteromonadaceae*	*BD2-13*	Larger only (3)
2268	0.796084	0.002984	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Cryomorphaceae*		Larger only (8)
1150	0.791885	0.003494	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*OM60*		All samples
268	0.786718	0.004231	*Proteobacteria*	*Deltaproteobacteria*	*GMD14H09*			Larger only (2)
451	0.758249	0.011562	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*		N (L:3, W)
2068	0.758050	0.011531	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*			LIC
289	0.749621	0.015030	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*		N (L:5, S:1)
845	0.727699	0.029007	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Flammeovirgaceae*	*Roseivirga*	N (L:8, S:2)
1298	0.717973	0.037876	Unassigned					Larger only (4)
1956	0.715266	0.040431	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*		Larger only (3)
494	0.714639	0.040735	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Aliivibrio* (*fischeri*)	N (L:8, S:1, W)
1373	0.711881	0.043491	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*		N (L:9, S:1, W)
1353	0.711281	0.043767	Unassigned					LIC
1762	0.710193	0.044622	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Photobacterium* (*rosenbergii*)	N (L:6, S:2)

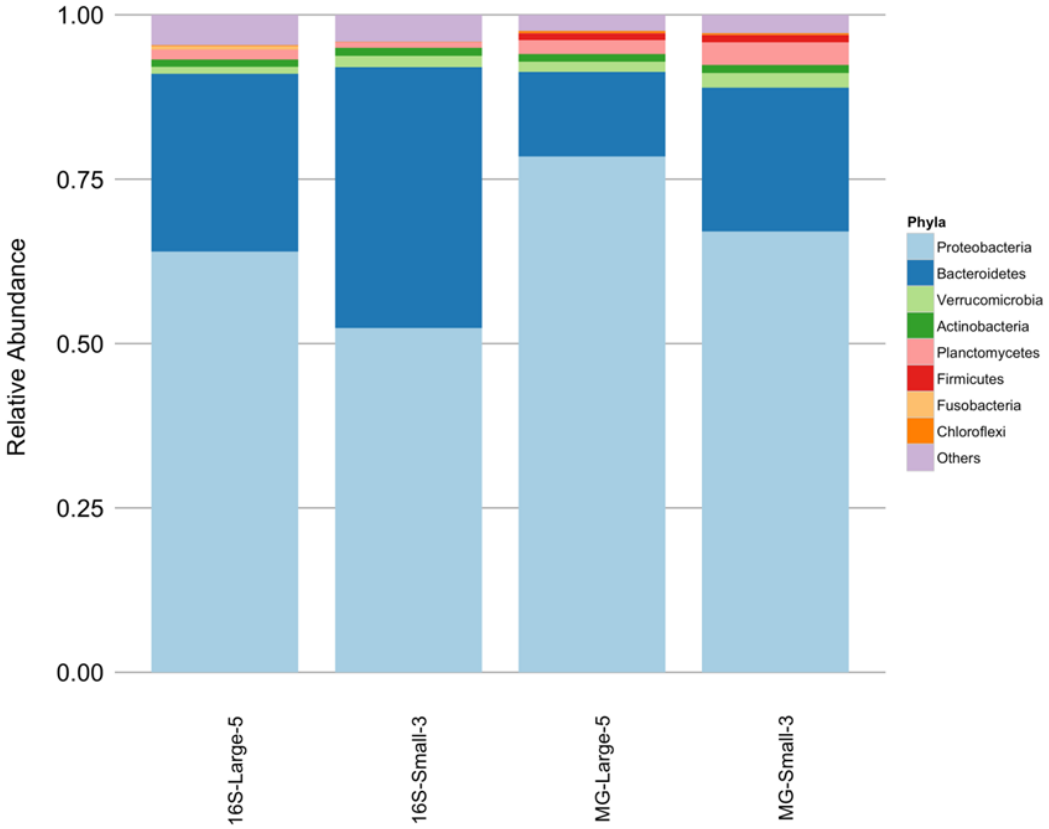


Fig. 1.5. Taxa comparison of representative fecal bacterial communities between 16S rRNA gene and shotgun metagenomic sequencing analyses. The largest and the smallest sea cucumbers were compared at phylum level. We used reference database Greengenes and GenBank for 16S rRNA gene-based analysis and metagenomic analysis, respectively.

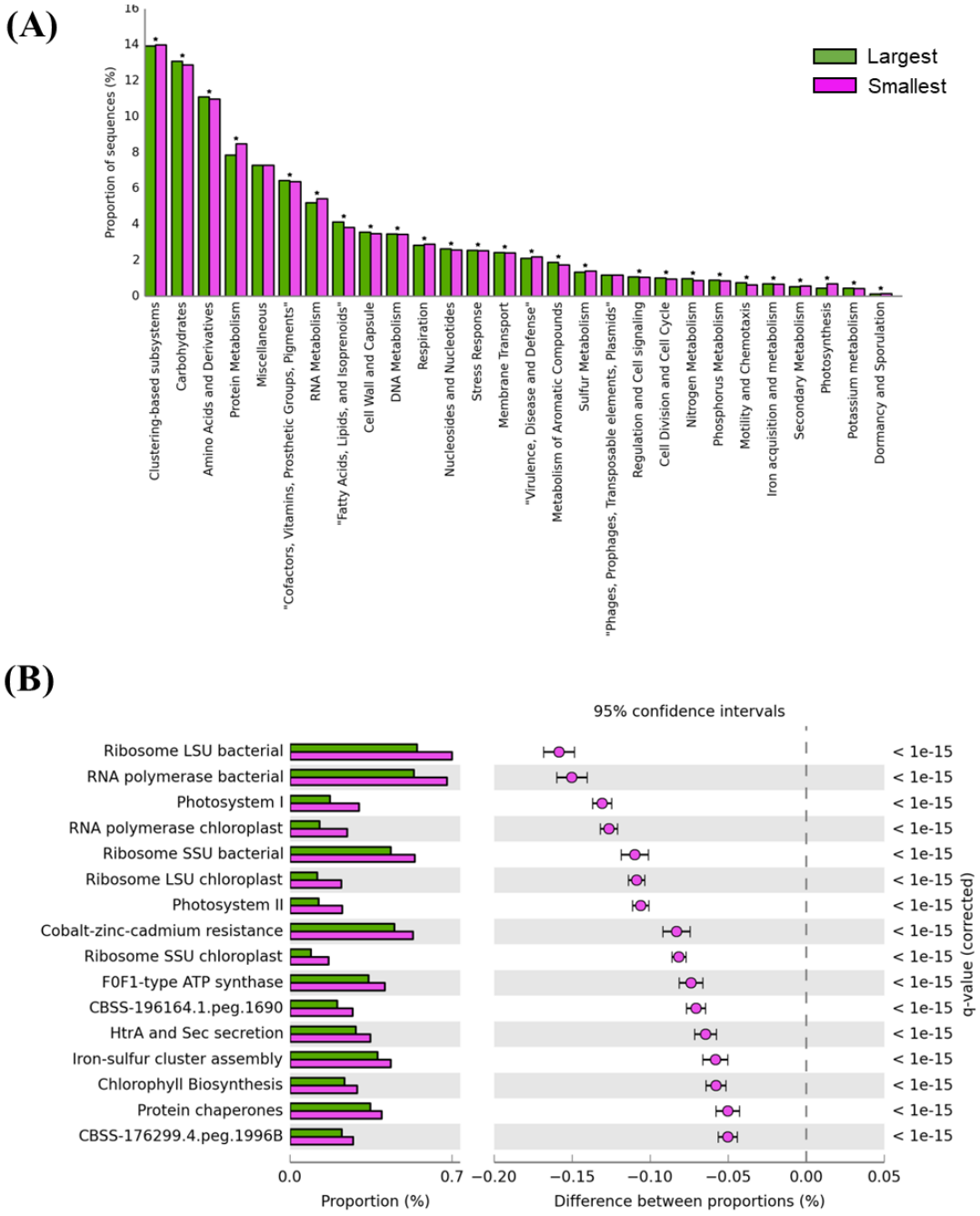


Fig. 1.6. Comparative metagenomic analyses at subsystem level 1 and 3. (A) Bar plot shows proportions of sequences for each functional feature at subsystem level 1. The largest indicated by green and the smallest indicated by purple color. Significantly different proportion of a feature between groups is indicated by an asterisk, respectively. (B) Only more abundant features in the smallest sea cucumbers are displayed. Green bars indicate the largest sea cucumber and purple indicate the smallest sea cucumber. Proportion (left side) means a possible abundance of microbes possessing each functional feature, and difference between proportions (= effect sizes) for each feature is indicated by a green dot. For this analysis, features were filtered by *q* value < 0.05 and effect size > 0.05. These functions were associated with ribosome, RNA polymerase and photosystems.

cluster, acetyl-CoA fermentation to butyrate, fatty acid degradation regulons, serine-glyoxylate cycle and butyrate metabolism in Level 3 subsystem category were significantly more abundant in the largest individual. Among those, the largest positive effect size (more abundant in the largest individual) was observed in reads affiliated to PHB metabolism (Fig. 1.7). Interestingly further KEGG mapping of the annotated reads into the PHB metabolism to validate which metabolic pathways are more prominent revealed *phaA* (acetyl-CoA C-acetyltransferase), *phaB* (acetoacetylCoA reductase) and *phaC* (PHB synthase), which are essential in PHB synthesis from acetyl-CoA to PHB, were more abundant in the largest individual's metagenomic library (Fig. 1.8A). In particular, the abundance of *phaA* gene (EC 2.3.1.9) reads impacted not only mapping into PHB metabolism but also that of other subsystem categories (Fig. 1.8B-G). The abundance of enoyl-CoA hydratase (EC 4.2.1.17), which is involved in butyrate metabolism, lysine and tryptophan synthesis and PHB degradation, also affected to positive effective size of most of the subsystem categories (Fig. 1.8A-E and G). Notably, 67% in average of total prokaryotic reads annotated to the *phaABC* genes were affiliated to Rhodobacterales, in accordance with the results obtained by 16S rRNA typing, where higher abundance of Rhodobacterales in the larger individuals' microbiota was found.

Reads affiliated to essential cellular functions in subsystem Level 3 categories (e.g. bacterial ribosome LSU or SSU, bacterial RNA polymerase, F0F1-type ATPase) were significantly more abundant in the metagenomic library from the smallest animal (Fig. 1.6B). Validation of the taxonomic affiliation showed, for example, bacterial RNA polymerase reads were consisted of diverse array of the sea cucumber gut microbiome.

Discussion

A. japonicus sea cucumber is a good candidate to study the evolution, dynamics and functions of gut microbiome in Deuterostomia invertebrates due to their unique ecophysiology (Ortiz-Pineda *et al.*, 2009; Mashanov and García-Arrarás, 2011), and it is in urgent need of both conservation and development of intensive aquaculture (Lovatelli *et al.*, 2004; Hamel and Mercier, 2013). To our

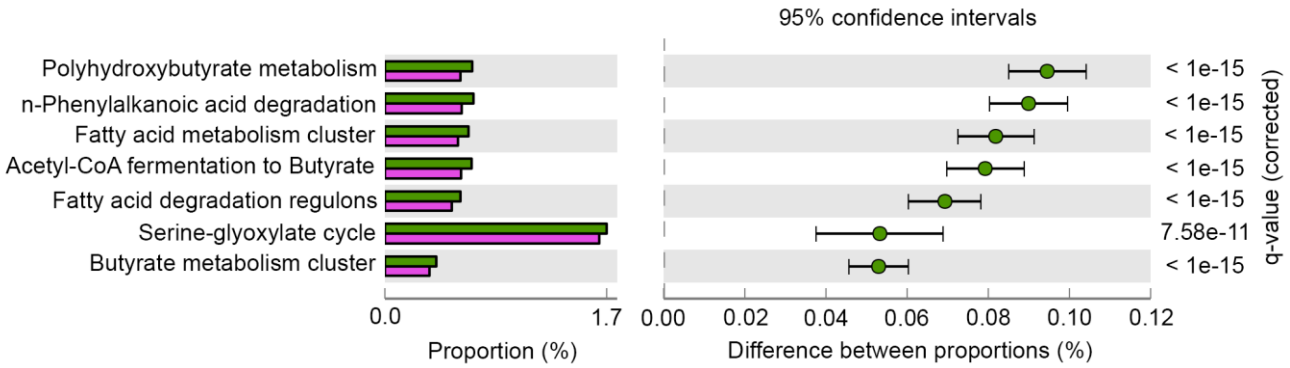


Fig. 1.7. An extended error bar plot showing more abundant features in the largest sea cucumbers. Green bars indicate the largest sea cucumber and purple indicate the smallest sea cucumber. Proportion (left side) means a possible abundance of microbes possessing each functional feature, and difference between proportions (= effect sizes) for each feature is indicated by a green dot. For this analysis, features were filtered by *q* value (0.05) and effect size (0.05). Relative abundances of metagenomic reads related to polyhydroxybutyrate (PHB) metabolism and butyrate metabolism were more abundant in the largest individual.

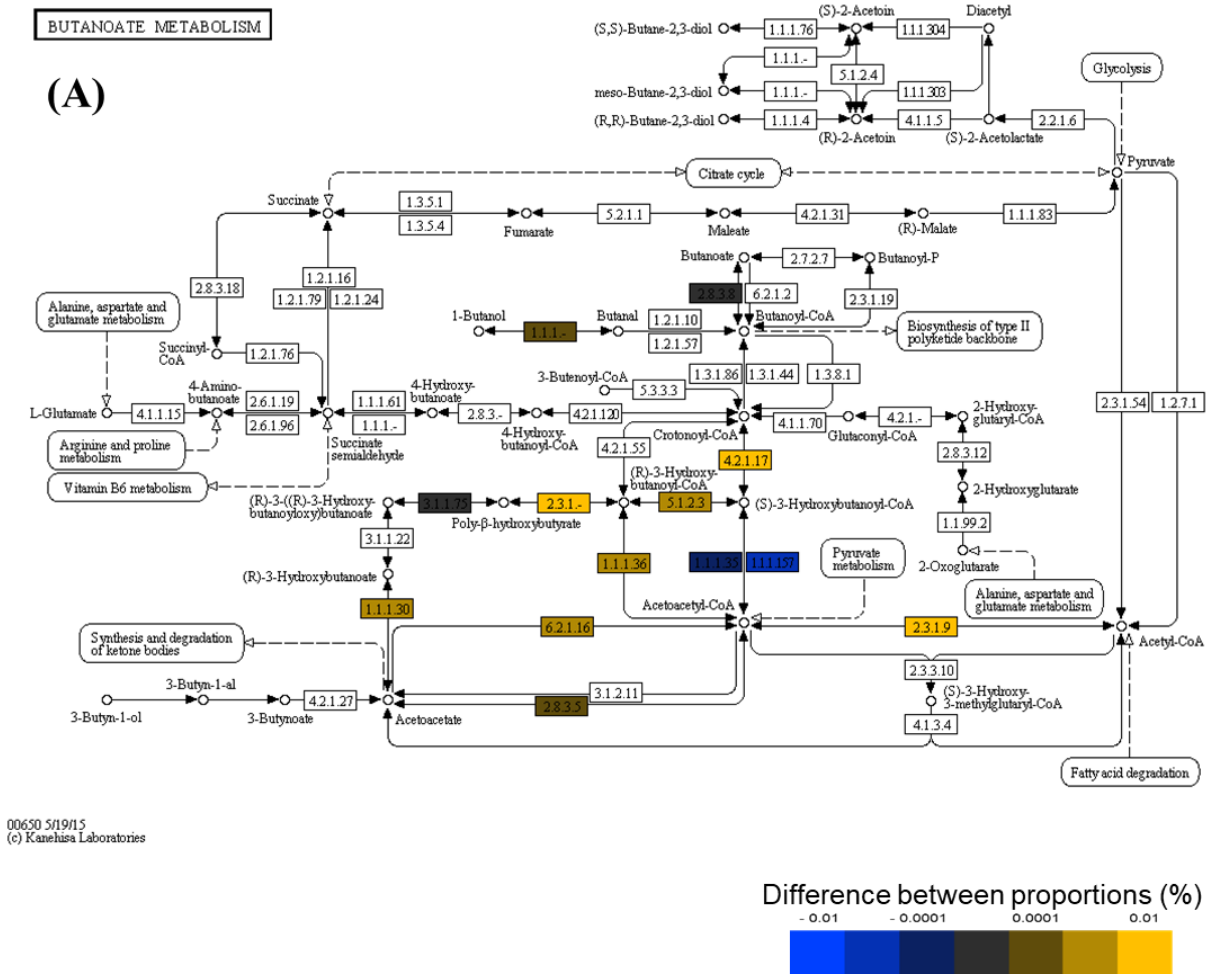


Fig. 1.8. KEGG mapping of metagenomic reads. Calculating the difference of read proportions between the largest and the smallest individuals for each gene, abundant genes in the largest individual was described by yellow color, conversely abundant genes in the smallest individual was described by blue color. Greater different genes between individuals were colored brighter. (A) PHB metabolism–annotated reads are mapped on KEGG pathway. The reads annotated as PHB metabolism in SEED-Subsystems were re-annotated by KEGG ORTHOLOG and mapped on butanoate metabolism pathway. The reads were mapped on genes required to synthesize PHB. Acetyl-CoA C-acetyltransferase (EC:2.3.1.9), 3-hydroxybutyryl-CoA epimerase (EC:5.1.2.3) and polyhydroxyalkanoate synthase (EC:2.3.1.-) were more abundant in the largest individual, conversely 3-hydroxybutyryl-CoA dehydrogenase (EC:1.1.1.157) was more abundant in the smallest individual.

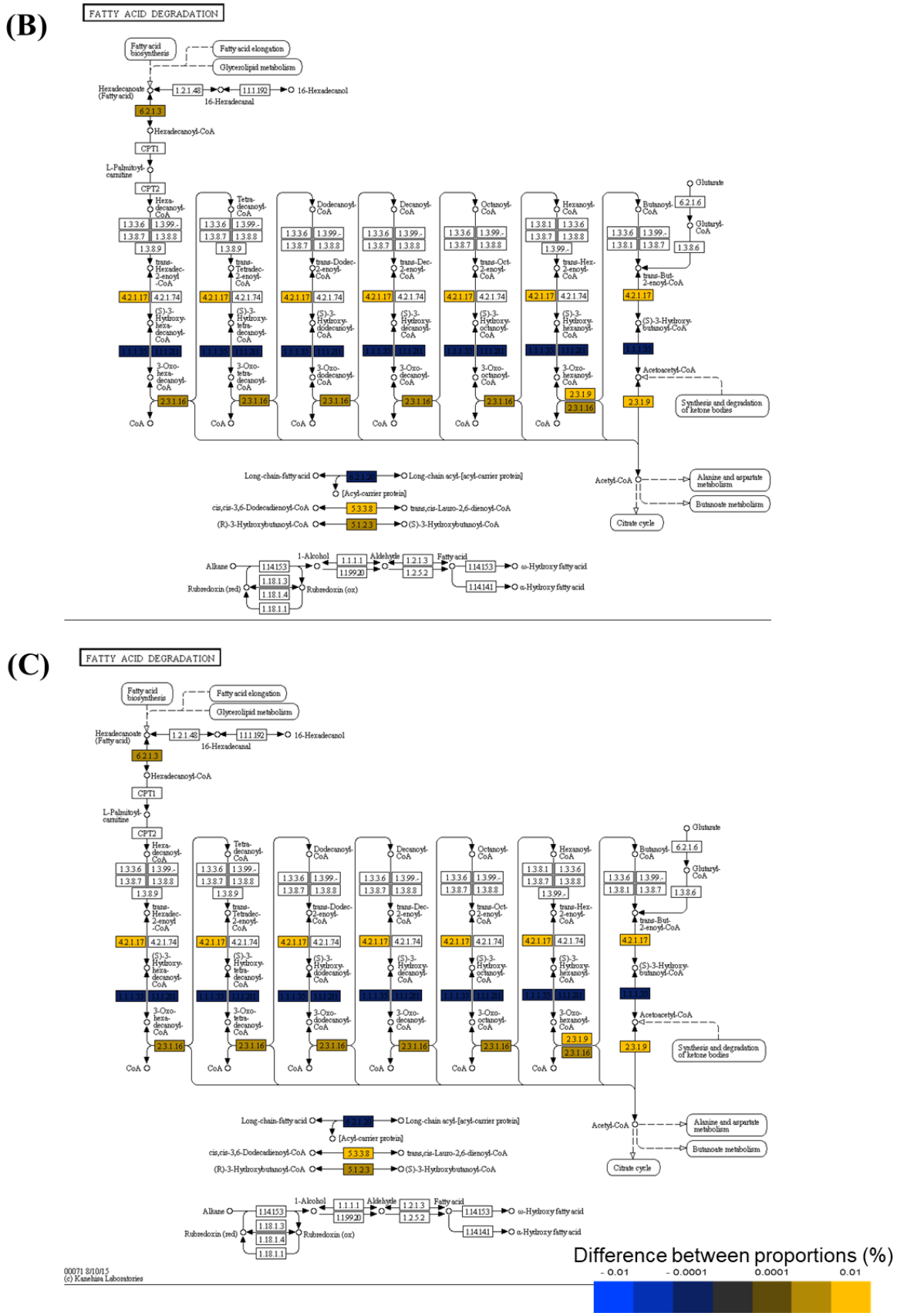


Fig. 1.8. Continued. (B) n-Phenylalkanoic acid degradation and (C) fatty acid metabolism cluster.

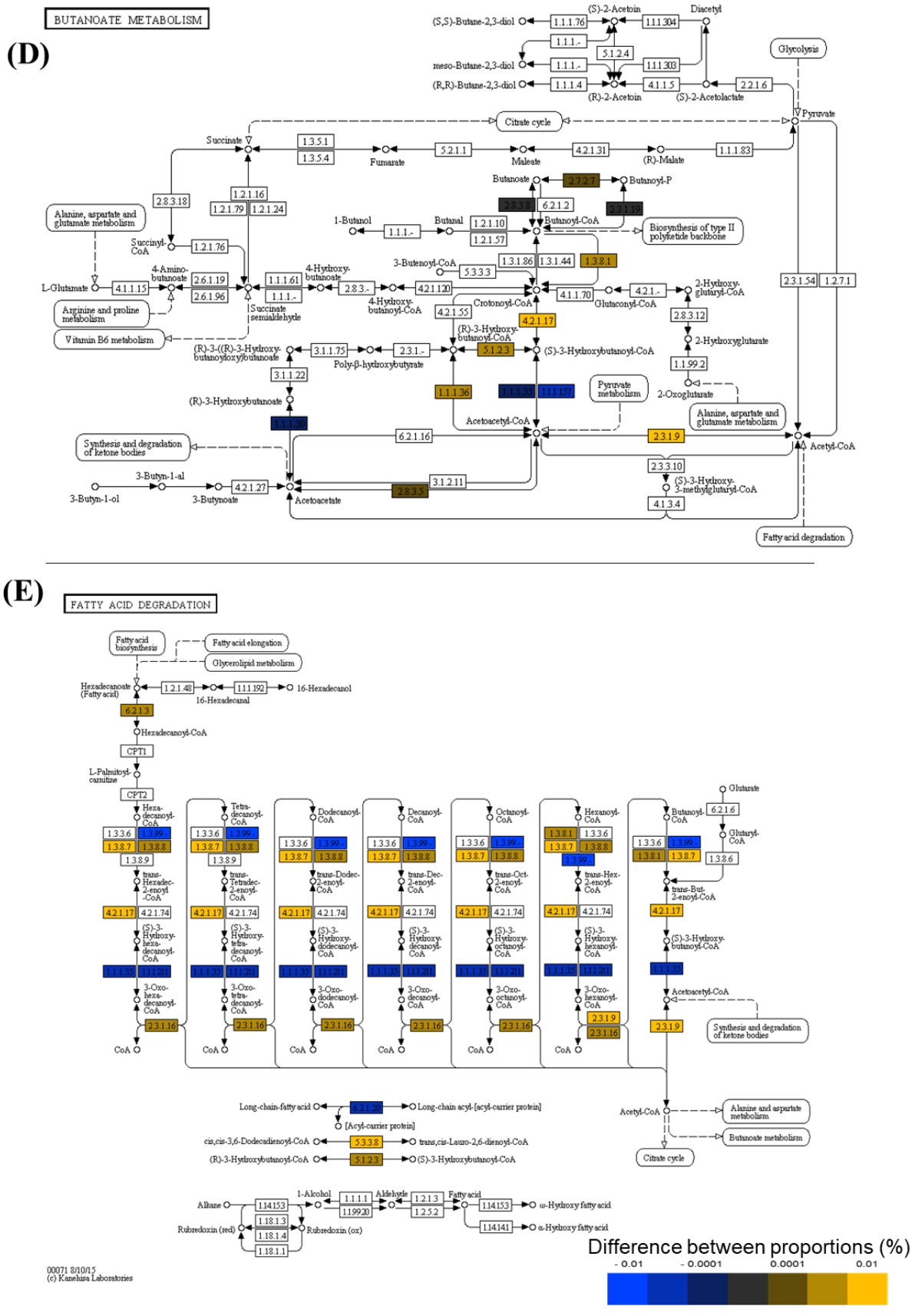


Fig. 1.8. Continued. (D) acetyl-CoA fermentation to butyrate and (E) fatty acid degradation regulons.

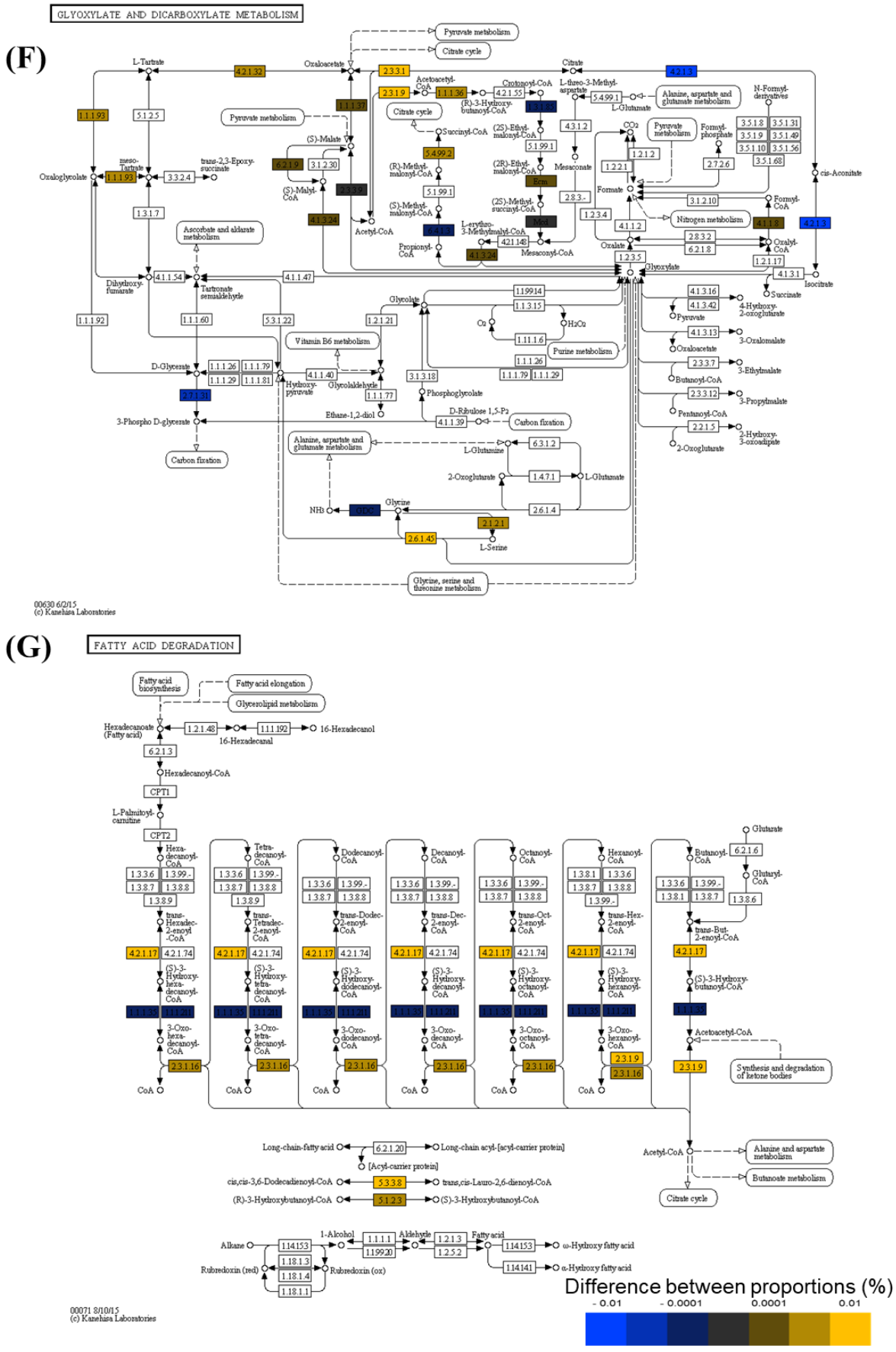


Fig. 1.8. Continued. (F) serine-glyoxylate cycle and (G) butyrate metabolism cluster.

knowledge, this is the first individual fecal microbiome analysis of *A. japonicus* to assess the possible interactions between the microbiome and the host animals' growth. Our analyses showed that the observed differences in fecal microbiota of larger and smaller individuals might be associated with PHB synthetic metabolism. Interestingly, both high-throughput sequencing approaches suggest that Rhodobacterales may play a key role in sea cucumber growth. Feeding trials assessing direct effect and causality of these gut microorganisms on their hosts' growth are necessary to support this idea, but our results provide a new insight into a link between PHB producing microbes and their potential contribution to sea cucumber growth. Only a few studies have investigated the gut microbiota of holothurians sea cucumbers, all of them using dissection techniques. These studies have assessed gut bacterial diversity and concluded selective feeding and proposed some potential probiotic bacteria (Plotieau *et al*., 2013; Gao *et al*., 2014). None of these previous studies have investigated the roles of bacterial communities on the host's health or growth. Using the non-destructive methodology presented here, live specimens can be used to monitor gut microbial dynamics and host-microbes interaction; for example, the dynamics of gut microbiome before and after evisceration, and after probiotic treatment could be monitored. Again, we would like to emphasize that sea cucumber is one of the best model animals to study in terms of organ regeneration among Deuterostome animals in both molecular and genetic basis. In fact, studies have revealed numbers of novel genes differentially expressed during the gut regeneration process (Ortiz-Pineda *et al*., 2009). It is interesting to understand how the gut microbiome, including PHB producers, is affected such examples of the regeneration process using the non-destructive fecal microbiome methodology. The strategy also aids to the "endangered" species conservation in the wild. Without unnecessary mortality, on site fecal collections of a Stichopodidae sea cucumber and further analyses are in progress.

We found a similar number of bacterial OTUs and diversity (Shannon index) in *A. japonicus* gut microbiota to other studies using similar DNA extraction and pyrosequencing strategies (Gao *et al*., 2014). The diversity indices of the *A. japonicus* fecal microbiota are similar to those observed in

Hydra microbiota but lower than those of fish and mammals (Hacquard *et al*., 2015). The fecal microbiota of juvenile *A. japonicus* is predominantly in Proteobacteria and Bacteroidetes, whereas Bacteroidetes were less dominant in maricultured adult specimens (Gao *et al*., 2014). It is well known that gut microbiota is influenced by food intake, by environmental factors and by developmental stages of hosts and their genetics (Benson *et al*., 2010; Hacquard *et al*., 2015). The observed differences in microbiota along with sea cucumber's body weight may be caused by one of (or synergy among) the above-mentioned factors. Similar to the previous report on host habitat selection of the gut microbiota of zebrafish and mouse (Rawls *et al*., 2006), 8 and 10 phyla detected in the sea cucumber microbiota were shared with those of mice/human and zebrafish, however the abundance and diversity below phylum level differed. The juvenile *A. japonicus* individuals also showed microbiotaignatures observed in both fish guts (Proteobacteria rich) and the human gut (Bacteroidetes rich) (Hacquard *et al*., 2015). In the previous report on gut microbiota transplantation experiments between zebrafish and mouse, a restoration tendency to the original composition of gut microbiota is observed; abundance of Proteobacteria increases, but that of Bacteroidetes drops to below the detectable limit in a mouse-microbiota transplanted zebrafish (Rawls *et al*., 2006). A hybrid type of sea cucumber microbiota observed here might be affected by not only their conserved status of these hosts in surviving in aquatic environments but also their host species specific selective pressure. Archaeal sequences were found to be rare in the metagenomic libraries. Similar results were found in a previous study (Enomoto *et al*., 2012), therefore it is not necessary to discuss their roles in the gut microbiota here.

Rhodobacterales, Desulfobacterales, and Oceanospirillales (in more detail Rhodobacteraceae and Desulfobulbaceae; see family table in Table 1.2) were significantly abundant in the larger individuals, and Marinicellaes and Acidimicrobiales were similarly abundant in the smaller. Rhodobacteraceae is one of the most diverse aquatic bacterial groups (>100 genera) consisting of aerobic photo- and chemoheterotrophs and a diverse array of polyhydroxyalkanoates (PHA), commonly PHB, producers isolated from marine environments (Cho and Giovannoni, 2004;

Zheng *et al*., 2011; Pujalte *et al*., 2014). Rhodobacteraceae sequences and isolates have frequently been observed in marine environments (Cho and Giovannoni, 2004) as well as in the gut microbiota of wild and maricultured *A. japonicus* (Enomoto *et al*., 2012; Gao *et al*., 2014). More recently, dietary β-glucan supplementation for *A. japonicus* triggered Rhodobacteraceae abundance in the gut microbiota and host NF-κB signaling pathway, and then it was speculated that the activation of the signaling pathway was caused by the increased mass of the bacterial group (Yang *et al*., 2015b). Rhodobacteraceae can be involved in sulfur and carbon biogeochemical cycling in water columns and on the photic sea floor due to their physiological traits. This bacterial group is being considered as transient fractions in the gut microbiota from the environments (Enomoto *et al*., 2012). Interestingly, the metagenomic comparisons described here reveal a further possible hypothesis that Rhodobacterales affects the host growth of *A. japonicus* as a PHB producer. PHB is known to be accumulate in commonly nutrient-limited bacterial cells, especially under increased carbon to nitrogen ratio conditions (Madison and Huisman, 1999; Jendrossek and Pfeiffer, 2014). There are three types of PHB, 1) high molecular weight storage PHB consisting of >1,000 3-hydroxybutyrate (3HB) residues, 2) low molecular weight PHB which has ca. 100 to 200 3HB units and 3) conjugated PHB (cPHB) consisting of low numbers of 3HB units (<ca. 30) which are covalently linked to proteins (Jendrossek and Pfeiffer, 2014). PHB-like granules have recently been observed in human cells (Elustondo *et al*., 2012). PHB degradation is also catalyzed by PHB/PHA depolymerase in the first step of biodegradation, and the degradation process can serve as energy sources for the host cells. PHB has been tested as feed additives promoting host growth in fish and crustacean aquaculture (De Schryver *et al*., 2010; Nhan *et al*., 2010; Najdegerami *et al*., 2012). The PHB also conferred protection to an *Artemia* host against a pathogenic vibrio by modulating the Hsp70-triggered innate immune response (Baruah *et al*., 2015). A PHB depolymerase gene (*phaZ*) was also retrieved from an EST library from the intestinal tissues of *A. japonicus* (GenBank JK730361.1). It is possible to consider that the bacterial PHB, especially that produced by Rhodobacterales, might also serve as an energy source for a host sea cucumber, causing the observed higher growth in the larger individuals.

PHB producing bacteria has also been found in termite guts, but its proper role has not been fully elucidated yet (Sudesh *et al*., 2008; Tay *et al*., 2010). This hypothesis further gives us a novel ecological insight into carbon cycling as a bridge between storage granule producing bacteria and extra-nutrition for benthic marine invertebrates. Considering our results, we suggest the use of PHB producers as probiotics in sea cucumber aquaculture. Future bioassays studies using isolated PHB producing bacteria or their PHB are necessary to confirm their probiotics potential in promoting sea cucumber growth.

Another group of bacteria more abundant in larger individuals was Desulfobulbaceae, which is a strictly anaerobic sulfate-reducing bacteria (SRB) (Kuever *et al*., 2005; Kuever, 2014). The presence of Desulfobulbaceae sequences suggests that anoxygenic environments are formed in the gut of *A. japonicus* juveniles. In a previous study, *Desulfosarcina*, a member of Desulfobacterales, was also predominant in the hindgut contents of the maricultured *A. japonicus* (Gao *et al*., 2014). The roles of SRB are presumed to be related to acetate production and nitrogen fixation (Gao *et al*., 2014). SRB colonizes ca. 50% of human guts, and are frequently detected in healthy adults fecal microbiota (Rey *et al*., 2013). SRB are known to be the most efficient hydrogenotrophs, they can utilize H_2 and some fermentation products (e.g. lactate, formate) produced in the guts as electron donors (Kuever, 2014). Thus, SRB contribute towards maintaining redox balance and maximizing microbial energy production. The higher abundance of SRB sequences observed in larger individuals' feces suggests higher redox states in their gut. Such conditions are more likely to be suitable for fermentation and PHB accumulation, which might affect the host's growth.

The lack of information on detection and/or isolation of Oceanospirillales, Marinicellales, and Acidimicrobiales in the growth of the host sea cucumber resulted the delays in discovering details in the structure-function relationships. *Halomonas meridiana* and *Marinomonas pontica* (Oceanospirillales), however, have been isolated from sea sediment of the host's habitat (Zhang *et al*., 2013), and the guts of *A. japonicus* (Enomoto *et al*., 2012). In particular, the *Halomonas* strain showed polysaccharide degrading abilities (starch and carboxymethyl cellulose sodium salt) (Zhang

et al., 2013), which expects the roles in supporting roles in digestion of ingested detritus by the host animals. Marinicellales reads were detected from hind gut of *A. japonicus* using 16S rRNA gene sequencing analysis (Gao *et al*., 2014), and Acidimicrobiales strains were isolated from the abdominal epidermis of a sea cucumber *Holothuria edulis* (Kurahashi *et al*., 2009). Both groups of bacteria actually might have associations with the sea cucumber, further ecophysiological and ecogenomics studies could infer the host effects on the unique host animals.

We found a strong positive correlation between OTUs affiliated to Legionellaceae and Alteromonadales OM60 to the body weight of *A. japonicus* juveniles. *Legionella* spp. are related to PHB production (James *et al*., 1999), and Alteromonadales OM60 (such as *Congregibacter* spp. (Spring *et al*., 2009)) is related to polyphosphate (polyP) production. Recently, in mammalian cells, cPHB has been found in a wide variety of tissues and in atherosclerotic plaques, and polyP has been linked to a variety of functions, including blood coagulation, cell proliferation, apoptosis and mitochondrial ion transport and energy metabolism (Dedkova and Blatter, 2014). Those bacterial polymers might trigger unique biological functions in host Deuterostomia invertebrates. Once the specific functions are unveiled, these bacteria could also be candidates for probiotics in sea cucumber aquaculture.

Conclusion

We firstly assessed possible links between gut microbiome and host growth in cultured *A. japonicus* juveniles. We suggest that Rhodobacterales bacteria retaining PHB metabolism genes might contribute to the production of larger individuals. Our data also revealed a healthy microbiome in larger individuals composed of SRB, commonly observed in human healthy adults, in balancing the redox state of gut environments. These results imply a link between microbial PHB producers and potential growth promotion in marine invertebrates. We need further studies using gut microbiota transplant from large to small sea cucumber gnotobiotic individuals to assess the direct effects of gut microbes on sea cucumbers' growth.

CHAPTER 2

Repeated selective enrichment process of sediment microbiota occurred in sea cucumber guts

Abstract

Deposit-feeding sea cucumbers repeat ingestion of sediments and excretion of feces daily and consequently increase bacterial abundance in sediments and promote organic matter mineralization. Such ecological roles are expected to be collaborative activities of sea cucumbers and the gut microbiota. Here, we performed a spatiotemporally-broad 16S rRNA gene analysis using 109 samples from sea cucumber feces and habitat sediments to explore potential contribution of their gut microbiota to the ecological roles. Most operational taxonomic units (OTUs) observed in the fecal samples were shared with the sediment samples, nevertheless fecal and sediment microbiota differed from each other in UniFrac analysis. Lower bacterial diversity and increased relative abundance of specific OTUs in the fecal microbiota strongly suggest selective enrichment of ingested sediment microbiota in their guts. Interestingly, representative fecal OTUs were more abundant in sea cucumber-populated sediments than in un-inhabited sediments, indicating bacteria selectively enriched in the guts were spread on ambient sediments via feces. Moreover, the predicted microbial community metabolic potential showed a higher abundance of genes related to carbohydrate and xenobiotics metabolisms in feces than in sediments. Our study suggests the repeated selective enrichment transforms ambient sediment microbial communities and maintains the host's ecological roles by promoting organic matter mineralization.

Introduction

Holothuroidea (i.e. sea cucumber), member of the phylum Echinodermata, is one of the most abundant animals in marine benthic biomes. Currently more than 1,500 species have been described (Horton *et al*., 2018). They are ubiquitous in marine environments, e.g. deep sea, coastal area, and

coral reefs (Purcell *et al*., 2012). Unlike other echinoderms (e.g. sea urchin, sea star, brittle star), a large number of sea cucumber species evolved as deposit-feeders consuming organic compounds derived from animal and plant detritus and microbial biomass (Yingst, 1976; Moriarty, 1982). Sea cucumbers rework huge amounts of sediments via ingestion and excretion (9-82 kg ind^{-1} y^{-1}) (Uthicke, 1999) and can extensively blend and reform sea floor substrata. Considering their abundance, sea cucumber's biological behavior has greatly affected physico-chemical processes of both soft-bottom and reef ecosystems (Uthicke, 2001b; Schneider *et al*., 2011; MacTavish *et al*., 2012; Purcell *et al*., 2016).

There are five major ecological functions of sea cucumbers: contributions to sediment condition, recycling of nutrients, influencing seawater chemistry (e.g. pH, alkalinity), forming pathways of energy transfer in food chains, and bolstering biodiversity via symbiotic relationships (Purcell *et al*., 2016). The first three are likely to be more important in maintaining environmental conditions in marine ecosystems. Bioturbation and sediment cleaning are the two main activities of the animals in maintaining and improving sediment condition. Bioturbation, which is defined as biological reworking and mixing of sediments and soils, impacts on benthic primary producers, animals and microorganisms (Widdicombe and Austen, 1999; Meysman *et al*., 2006; Laverock *et al*., 2010; MacTavish *et al*., 2012). Burying and non-burying sea cucumbers distribute sediments vertically and horizontally via their active ingestion and excretion of feces (Mercier *et al*., 1999; Purcell, 2010). Sediment cleaning is performed by deposit-feeding sea cucumbers, which defecate less organic rich sand compared to those of the ingested sediments (Yingst, 1976; Moriarty, 1982; Mercier *et al*., 1999; Purcell *et al*., 2016). Thus, sea cucumbers are used for integrated multitrophic aquaculture with other aquatic animals (e.g. bivalves, finfish) to reduce the accumulation of excess organic matter on the bottom of the farms (Slater and Carton, 2007, 2009; MacTavish *et al*., 2012; Yokoyama *et al*., 2015). This activity is analogous to the role of earthworms in soils (Drake and Horn, 2007). Moreover, sea cucumbers affect nutrient cycling by the conversion of organic nutrients into inorganic ones within their guts, and consequently influence the surrounding seawater chemistry

(Uthicke, 2001b; Schneider *et al*., 2011; MacTavish *et al*., 2012; Purcell *et al*., 2016).

Currently, many studies posit that animal biology and ecology could not be evaluated properly without their associated microbiomes (MacTavish *et al*., 2012). In this context, ecological roles, such as sediment cleaning and nutrient cycling promotion, are expected to be collaborative activities of sea cucumbers and their gut microbiota. Although previous studies showed community structures of sea cucumber guts differed from those of sediment microbiota (Plotieau *et al*., 2013; Gao *et al*., 2014), whether gut-unique microbes reside in the guts, and whether the gut microbes contribute to conversion of organic nutrients into inorganic ones, have not been evaluated. Furthermore, while it has been speculated that the deposition of sea cucumber's feces as a part of bioturbation could increase benthic bacterial abundance (MacTavish *et al*., 2012), effects of excreted gut microbiota via feces on benthic microbial community structures remain largely unexplored. To fill the gap between sea cucumber's ecological roles and the gut microbiota, in-depth comparison of gut and sediment microbiota is needed.

Sea cucumber wild stocks have been decreasing dramatically due to over-fishing, and some of them, e.g. *Apostichopus japonicus* and *Thelenota ananas*, are even listed in the IUCN Red List as "endangered species" (Conand *et al*., 2013b; Hamel and Mercier, 2013; Purcell *et al*., 2013). These species are also representative sea cucumbers distributed in the North (e.g. Menagawa) and South (e.g. Ishigaki) of Japan (Fig. 2.1). For their biological conservation, establishment of a seed production system for sea cucumbers is urgently needed. In addition to optimization of biotic and abiotic factors for sea cucumber farming (Dong *et al*., 2006, 2008; Xia *et al*., 2012a; Shi *et al*., 2013a), many studies have tried to develop probiotics to improve growth and immunity in the farmed individuals (Sun *et al*., 2012b; Zhao *et al*., 2012; Chi *et al*., 2014). Characterizing sea cucumber gut microbiota through comparison with sediment microbial communities could lead to the development of more effective probiotics for these endangered species. To characterize sea cucumber gut microbiota and to explore potential contribution of their gut microbiota to the host's ecological roles, we performed a spatiotemporally-broad assessment of sea cucumber fecal microbiota and sediment

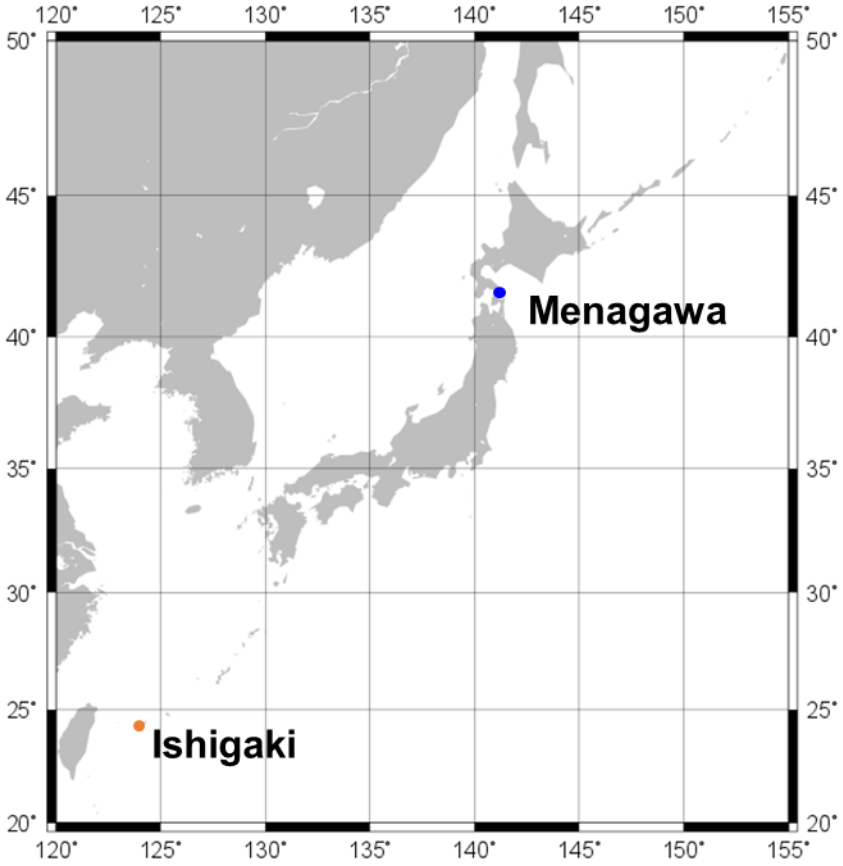


Fig. 2.1. Location of the Menagawa and the Ishigaki sites. The blue circle is the Menagawa site, Hokkaido, Japan (41°45'N, 141°5'E), where feces of *Apostichopus japonicus* and sediments were collected. The orange circle is the ishigaki site, Okinawa, Japan (24°21'N, 124°00'E), where feces of four species of sea cucumbers (*Holothuria edulis*, *H. atra*, *Stichopus chloronotus*, *Thelenota ananas*) and sediments were collected.

microbiota by 16S rRNA gene sequencing analyses without dissection of specimens (Yamazaki *et al*., 2016). Our results show sea cucumber gut microbiota is shaped by selective enrichment process of ingested microbes from sediments, and the repeated process might transform ambient sediment microbial community structures and promote organic matter mineralization.

Materials & Methods

Sampling at the Menagawa Site

We collected feces of *A. japonicus* and sediment surface samples by diving in Menagawa experimental aquarium (41°45N, 141°5E), Hokkaido, Japan (Fig. 2.1), in September, November and December 2016 and April, May, June and July 2017. To avoid bacterial proliferation on collected feces, we sampled it immediately after sea cucumbers' excretion. Thus, almost no time passed from the point the feces were deposited to the point when they were sampled. The environment in the aquarium resembles the natural habitat of sea cucumbers; Sea cucumbers fed on natural diet, and fresh natural seawater always entered the aquarium from a gate open to the sea (Fig. 2.2). The aquarium consists of four pools; sea cucumbers reside in the 1st to 3rd pools but not in the 4th one (Fig. 2.2). We collected feces in the 2nd and 3rd pools. Sediments were collected in the 2nd, 3rd and 4th pools. Seawater was collected in the 3rd pool in September 2016. Fecal and sediment samples were collected using a sterilized 10 mL syringe and subdivided to sterile 1.5 mL tubes and then immediately frozen on dry-ice and transported to our laboratory. 5 L of the seawater was collected and filtered through a 0.22 μm Sterivex filter. The Sterivex filter was filled with SET buffer (sucrose 20%, EDTA 50 mM, Tris-HCl 50 mM) and rapidly frozen on dry ice until transportation to our laboratory. All samples were stored at -80°C in our laboratory until DNA extraction.

Sampling at the Ishigaki Site

We collected feces of four species of tropical sea cucumbers (*Holothuria edulis*, *H. atra*, *Stichopus chloronotus*, *Thelenota ananas*) inhabiting the seafloor around Kayama island (24°21'N, 124°00'E),

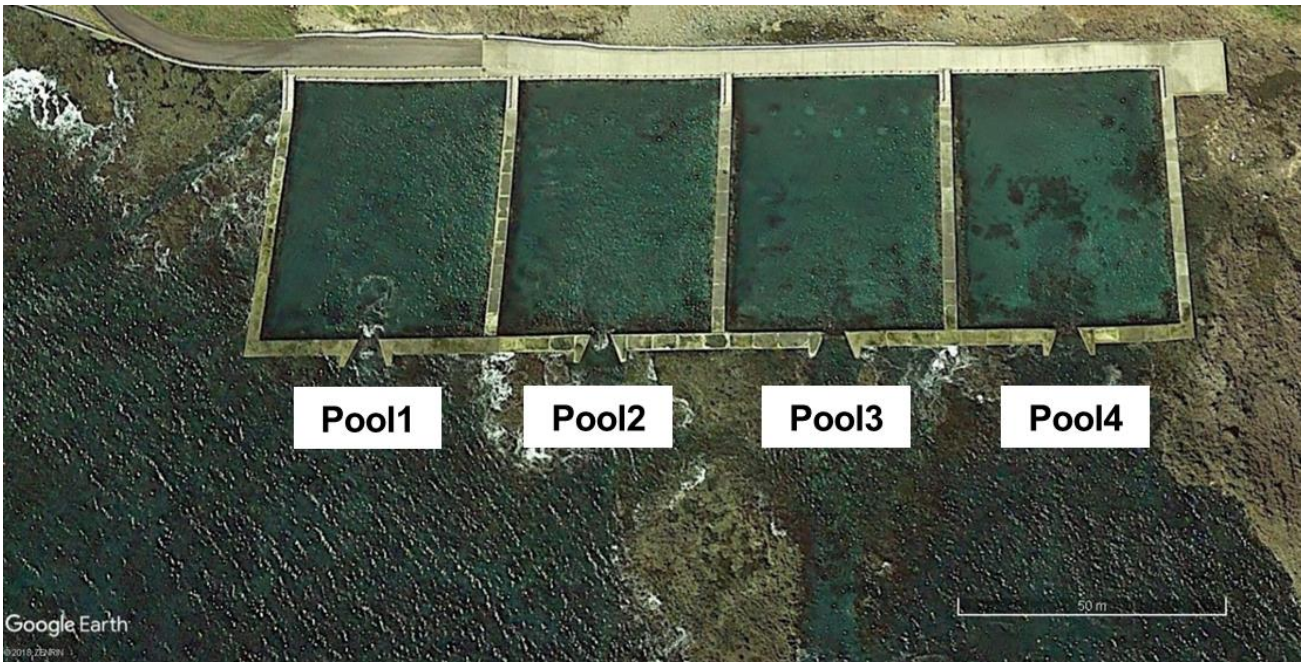


Fig. 2.2. A satellite image of Menagawa aquarium obtained using Google Earth. Pools second and third from left side were used for study fields.

Okinawa, Japan, in July 2015 (Fig. 2.1). Fecal samples of *H. edulis*, *H. atra* and *S. chloronotus* were captured and collected on board using sterile plastic bags contained 1 L of filter-sterilized seawater after rinsing the body surface with sterilized seawater. Feces of *T. ananas* were collected under water using a sterilized 50 mL tube due to the huge body size. To avoid bacterial proliferation on collected feces, we have sampled it immediately after sea cucumbers' excretion. Sediment samples were collected under water using a sterilized 50 mL tube. Samples were kept on ice on board, frozen in a local facility, and then transported to the laboratory. All samples were stored at -80°C until DNA was extracted.

Microbial DNA Extraction

After rapid thawing of the fecal and sediment samples, microbial DNA extraction from these samples was performed using the NucleoSpin Soil Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. After rapid thawing of seawater samples, the DNA was extracted using NucleoSpin Tissue Kit (MACHEREY-NAGEL, Düren, Germany), according to modified manufacturer's protocol (Yamazaki *et al*., 2016).

16S rRNA Gene Sequencing

16S rRNA gene sequencing was performed on extracted DNA samples (N=111; Table 2.1). For samples from the Menagawa site, the hypervariable V1-V2 region of the 16S rRNA gene was amplified by PCR with 27Fmod and 338R primers containing barcode and Illumina adaptor sequences. PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amount of each PCR amplicon was mixed and then sequenced using MiSeq Reagent Kit v3 (600-cycles) on the MiSeq Illumina platform. Based on sample specific barcodes, obtained reads were assigned to each sample and paired-end reads were merged using fastq-join program, and low-quality (average quality value <25) reads were removed.

Table 2.1. Sample information of 16S rRNA gene sequencing

Sample number	SampleID	Location	Sample type	Month/Year	No. of qualified reads	read length of qualified reads	No. of bacterial reads	No. of chloroplast reads	No. of bacterial OTUs	Bacterial Good's coverage (%)
1	Sep-62	Menagawa	Feces	Sep/2016	31188	346.6	30383	805	5604	88.2
2	Sep-63	Menagawa	Feces	Sep/2016	30949	343.55	29630	1319	5288	88.6
3	Sep-64	Menagawa	Feces	Sep/2016	31144	340.91	27718	3426	5417	87.1
4	Sep-65	Menagawa	Feces	Sep/2016	28824	344.68	27885	939	5147	88.7
5	Sep-66	Menagawa	Feces	Sep/2016	33051	343.05	30703	2348	5437	88.8
6	N2F	Menagawa	Feces	Nov/2016	22039	341.19	19957	2082	3819	87.0
7	DF17	Menagawa	Feces	Dec/2016	26287	341.82	24443	1844	3493	91.6
8	DF101	Menagawa	Feces	Dec/2016	25628	343.58	22989	2639	2745	93.4
9	DF102	Menagawa	Feces	Dec/2016	39531	343.06	36321	3210	4433	93.1
10	DF103	Menagawa	Feces	Dec/2016	30679	341.69	27734	2945	3423	93.0
11	ApF17	Menagawa	Feces	Apr/2017	20168	327.63	8723	11445	1756	88.0
12	ApF41	Menagawa	Feces	Apr/2017	23087	328.32	8885	14202	1259	91.6
13	ApF51	Menagawa	Feces	Apr/2017	24215	328.49	12485	11730	1834	91.5
14	MaF15	Menagawa	Feces	May/2017	38741	337.03	26261	12480	3895	92.0
15	MaF41	Menagawa	Feces	May/2017	27640	333.72	21086	6553	2529	92.9
16	MaF51	Menagawa	Feces	May/2017	21252	329.76	12984	8261	2096	90.1
17	JunF15	Menagawa	Feces	June/2017	28675	340.93	25723	2952	2300	95.3
18	JuneF17	Menagawa	Feces	June/2017	24659	341.36	22494	2165	2628	93.4
19	JuneF51	Menagawa	Feces	June/2017	23689	336.79	21032	2657	1823	95.1
20	JulyF15	Menagawa	Feces	July/2017	37905	340.16	35573	2332	4149	92.5
21	JulyF17	Menagawa	Feces	July/2017	33177	342.22	31895	1282	4085	91.8
22	JulyF41	Menagawa	Feces	July/2017	40394	342.54	37789	2605	6446	89.2
23	JulyF51	Menagawa	Feces	July/2017	32718	338.98	30217	2501	3567	92.4
24	JulyFK	Menagawa	Feces	July/2017	45350	339.49	41645	3704	4717	92.8
25	JulyFL	Menagawa	Feces	July/2017	43510	340.55	40134	3376	5337	91.3
26	JulyFM	Menagawa	Feces	July/2017	41812	337.79	38059	3753	5860	89.6
27	JulyFN	Menagawa	Feces	July/2017	34134	341.45	31934	2200	6321	86.8
28	Sep-69	Menagawa	Sediment	Sep/2016	33336	345.96	32284	1052	8452	83.6
29	Sep-70	Menagawa	Sediment	Sep/2016	28054	344.82	27376	678	6737	85.6
30	Sep-71	Menagawa	Sediment	Sep/2016	28605	344.68	26768	1837	6988	84.5
31	Sep-72	Menagawa	Sediment	Sep/2016	27064	343.49	25923	1141	6766	84.0
32	Sep-73	Menagawa	Sediment	Sep/2016	19508	340.36	17889	1619	5778	80.9
33	Sep-74	Menagawa	Sediment	Sep/2016	22679	339.61	20553	2126	6299	82.0
34	Sep-75	Menagawa	Sediment	Sep/2016	24863	338.97	22436	2427	6528	82.8
35	Sep-76	Menagawa	Sediment	Sep/2016	27272	339.51	25186	2086	6697	84.7
36	Sep-77	Menagawa	Sediment	Sep/2016	31424	337.26	29316	2108	6784	86.6
37	Sep-78	Menagawa	Sediment	Sep/2016	25718	341.03	23561	2157	6742	82.5
38	Sep-79	Menagawa	Sediment	Sep/2016	26364	340.88	24337	2027	6547	83.4
39	Sep-80	Menagawa	Sediment	Sep/2016	25342	341.18	23950	1392	6641	83.2
40	Sep-81	Menagawa	Sediment	Sep/2016	21633	341.6	20050	1583	6488	80.3
41	Sep-82	Menagawa	Sediment	Sep/2016	25857	340.84	22613	3244	6852	81.6
42	Sep-83	Menagawa	Sediment	Sep/2016	36133	335.35	21906	14227	5750	84.5
43	Sep-84	Menagawa	Sediment	Sep/2016	27697	337.16	18414	9283	5488	81.9
44	Sep-85	Menagawa	Sediment	Sep/2016	28775	333.9	15413	13362	4855	80.6
45	N2r1	Menagawa	Sediment	Nov/2016	22107	344.11	20353	1754	6633	78.9
46	N2r3	Menagawa	Sediment	Nov/2016	22314	341.78	19361	2953	6769	77.5
47	N2p1	Menagawa	Sediment	Nov/2016	23918	342.54	19876	4042	7174	76.6
48	N2p3	Menagawa	Sediment	Nov/2016	28670	341.53	25297	3373	7030	82.9
49	N4p1	Menagawa	Sediment	Nov/2016	26623	337.15	16846	9777	5438	79.6
50	N4p3	Menagawa	Sediment	Nov/2016	23679	343.94	19608	4071	6176	80.0
51	DS17	Menagawa	Sediment	Dec/2016	19395	342.36	17209	2186	5617	78.7
52	DS101	Menagawa	Sediment	Dec/2016	29388	342.98	26994	2394	3235	93.4
53	DS102	Menagawa	Sediment	Dec/2016	30000	346.33	28556	1444	7235	84.9
54	DS103	Menagawa	Sediment	Dec/2016	37484	342.56	32344	5140	6300	89.1
55	D2r1	Menagawa	Sediment	Dec/2016	30927	339.96	23241	7686	7629	79.3
56	D2r3	Menagawa	Sediment	Dec/2016	25921	344.02	22593	3328	6202	83.1
57	D2p1	Menagawa	Sediment	Dec/2016	23488	344.65	19936	3552	6783	79.0
58	D2p3	Menagawa	Sediment	Dec/2016	26303	344.65	22196	4107	6883	80.7
59	D4p1	Menagawa	Sediment	Dec/2016	22752	335.8	14820	7932	5138	77.9
60	D4p3	Menagawa	Sediment	Dec/2016	21687	340.84	16483	5204	6051	76.3
61	ApS17	Menagawa	Sediment	Apr/2017	22035	340.89	18306	3729	3909	87.3
62	ApS41	Menagawa	Sediment	Apr/2017	23134	342.95	20730	2404	6244	82.2
63	ApS51	Menagawa	Sediment	Apr/2017	27861	343.23	24652	3209	5014	88.0
64	MaS15	Menagawa	Sediment	May/2017	25464	343.41	21685	3779	5521	85.4
65	MaS41	Menagawa	Sediment	May/2017	22161	340.14	18584	3575	4565	85.5
66	MaS51	Menagawa	Sediment	May/2017	23861	337.34	16551	7310	3559	87.4
67	Ma2r1	Menagawa	Sediment	May/2017	26198	341.61	23088	3110	4971	87.9
68	Ma2r3	Menagawa	Sediment	May/2017	38947	342.67	34772	4175	6752	89.0
69	Ma2r5	Menagawa	Sediment	May/2017	37456	341.23	32170	5286	6687	88.7
70	Ma2p1	Menagawa	Sediment	May/2017	30389	343.75	28100	2289	6122	87.8
71	Ma2p5	Menagawa	Sediment	May/2017	46455	344.61	42429	4025	8009	89.8
72	Ma4p3	Menagawa	Sediment	May/2017	43538	343.18	37414	6124	7708	88.8
73	Ma4p5	Menagawa	Sediment	May/2017	43483	344.4	38217	5266	7556	89.1
74	JuneS15	Menagawa	Sediment	June/2017	48032	344.6	45366	2665	6992	91.7
75	JuneS17	Menagawa	Sediment	June/2017	21922	340.71	16826	5096	3333	88.8
76	JuneS41	Menagawa	Sediment	June/2017	31991	341.89	29345	2646	4601	90.9
77	JuneS51	Menagawa	Sediment	June/2017	38138	343.75	34328	3810	6405	89.5
78	June2r1	Menagawa	Sediment	June/2017	45422	344.57	41295	4127	6870	91.2
79	June2r5	Menagawa	Sediment	June/2017	21243	343.02	19762	1481	3946	88.8
80	June2p1	Menagawa	Sediment	June/2017	40064	344.75	37316	2748	7737	88.5
81	June2p5	Menagawa	Sediment	June/2017	39284	342.98	36838	2446	6409	90.6
82	June4p1	Menagawa	Sediment	June/2017	18585	347.55	17683	902	4655	84.5
83	June4p5	Menagawa	Sediment	June/2017	40016	345.32	37693	2323	7157	89.9
84	JulyS15	Menagawa	Sediment	July/2017	33185	338.23	29828	3357	6387	86.7
85	JulyS17	Menagawa	Sediment	July/2017	37329	343.88	35391	1938	6616	88.0
86	JulyS41	Menagawa	Sediment	July/2017	37811	343.59	35845	1966	7290	87.5
87	JulyS51	Menagawa	Sediment	July/2017	33643	342.81	31607	2036	7132	85.7
88	JulySK	Menagawa	Sediment	July/2017	34831	343.65	32493	2338	6064	88.0
89	JulySL	Menagawa	Sediment	July/2017	44671	343.49	42061	2610	8629	86.7
90	JulySM	Menagawa	Sediment	July/2017	45169	342.82	42397	2772	8243	87.7
91	JulySN	Menagawa	Sediment	July/2017	47681	344.4	45208	2473	10753	84.9
92	July2r1	Menagawa	Sediment	July/2017	45336	338.76	41452	3884	7779	88.2
93	July2r3	Menagawa	Sediment	July/2017	40832	341.84	38390	2442	7546	87.7
94	July2p1	Menagawa	Sediment	July/2017	43644	341.58	40283	3361	8052	88.1
95	July2p3	Menagawa	Sediment	July/2017	43126	340.81	40032	3094	8257	87.1
96	July2p5	Menagawa	Sediment	July/2017	54589	342.02	51230	3359	9736	88.6
97	July4p1	Menagawa	Sediment	July/2017	25686	341.76	23876	1810	6115	84.3
98	July4p3	Menagawa	Sediment	July/2017	23153	341.7	20834	2319	6314	80.7
99	July4p5	Menagawa	Sediment	July/2017	18230	342.34	17047	1183	5047	81.5
100	Sep-86	Menagawa	Seawater	Sep/2016	24558	333.68	22119	2439	1970	94.0
101	Sep-87	Menagawa	Seawater	Sep/2016	29798	332.7	26833	2965	2102	94.9
102	Ishigaki-30	Ishigaki	Feces	July/2015	10795	322.08	10508	287	2431	86.6
103	Ishigaki-32	Ishigaki	Feces	July/2015	11249	331.25	10871	378	3194	82.5
104	Ishigaki-34	Ishigaki	Feces	July/2015	9300	332.98	8954	346	3257	77.4
105	Ishigaki-36	Ishigaki	Feces	July/2015	10912	331.58	10702	210	3158	82.5
106	Ishigaki-38	Ishigaki	Feces	July/2015	10249	329.44	9803	446	2569	84.3
107	Ishigaki-31	Ishigaki	Sediment	July/2015	11762	336.47	10762	1000	5601	63.0
108	Ishigaki-33	Ishigaki	Sediment	July/2015	11158	333.83	10485	673	5028	66.8
109	Ishigaki-35	Ishigaki	Sediment	July/2015	11521	338	11167	354	5953	62.3
110	Ishigaki-37	Ishigaki	Sediment	July/2015	11914	336.3	11401	513	5957	63.2
111	Ishigaki-39	Ishigaki	Sediment	July/2015	13495	338.85	12735	760	6184	67.1

For samples from the Ishigaki site, the hypervariable V1-V2 region of the 16S rRNA gene was amplified by PCR with barcoded 27Fmod and 338R primers (Kim *et al*., 2013). PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amounts of each PCR amplicon were mixed and then sequenced using either 454 GS FLX Titanium or 454 GS JUNIOR (Roche, Basel, Switzerland). After demultiplexing of single-end reads according to their barcodes, low-quality (average quality value <25) and short (<250 bp) reads were removed.

Operational Taxonomic Unit (OTU) Clustering

Qualified reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9.1 (Caporaso *et al*., 2010a). Reads were clustered into OTUs (similarity 97%) using ucust_ref method (Edgar, 2010). Centroid sequences to cluster sequences were chosen from Greengenes database version 13.8, and failure sequences to cluster against the reference database were clustered newly each other. The latter OTUs (OTUs clustered based on only similarity among sequences) were obtained namely “denovo xxx”. Representative sequences of each OTU were assigned taxonomically using uclust consensus taxonomy assigner with default parameters (minimum percent similarity 90%) against Greengenes database version 13.8 and were aligned to Greengenes core reference using PyNAST algorithm (Caporaso *et al.*, 2010b).

After removing chloroplast and mitochondria sequences based on taxonomic annotation, we obtained 724,682, 1984,812 and 48,952 of bacterial reads from feces (27 samples), sediments (72 samples) and seawater (2 samples), from the Menagawa site, respectively (Table 2.1). We also obtained 50,838 and 56,550 of bacterial reads from feces (5 samples) and sediments (5 samples), from the Ishigaki site, respectively (Table 2.1).

Shared Bacterial OTUs between Fecal and Sediment Samples

To remove minor fraction of OTUs (i.e. OTUs observed in limited number of samples), we used

major fecal OTUs defined as those observed in all fecal samples by sampling points (Ishigaki July, Menagawa September, December, April, May, June and July) to calculate the number of shared OTUs between fecal and sediment samples. The fecal sample in November was excluded from this analysis due to limited available samples (N=1).

Diversity Analyses

Using 8000 of subsampled reads, Shannon index as alpha diversity was calculated and evaluated significance based on 999 Monte Carlo permutation tests with false discovery rate [false discovery rate (FDR)-corrected p<0.05]. Using the same number of subsampled reads, unweighted UniFrac distances as beta diversity were calculated and visualized in PCoA plots (Lozupone *et al*., 2011) based on phylogenic tree generated by FastTree. Significant difference of UniFrac distance was confirmed by permutational multivariate analysis of variance (PERMANOVA) (FDR-corrected p<0.05).

Relative Abundance Comparison

Linear Discriminant Analysis Effect Size (LEfSe) ver1.0 using Kruskal-Wallis Wilcoxon-rank sum tests and effect size threshold (LDA score>3.0) was used to identify which OTUs were more abundant in feces or sediment samples (Segata *et al*., 2011). The fecal sample in November was excluded from LEfSe analysis due to limited available samples (N=1). To visualize the difference of relative abundance of each OTUs between in feces and in sediments, we created a dendrogram-connected heatmap using iTOL v3 (Letunic and Bork, 2016). The dendrogram was constructed using MEGA7 (Kumar *et al*., 2016), after alignment using ClustalX (Larkin *et al*., 2007).

We selected six OTUs, denovo70836 (Flavobacteriales), denovo100805 (Rhodobacterales), denovo17525 (Flavobacteriales), 669813 (Flavobacteriales), denovo147395 (Rhodobacterales), and denovo98634 (Flavobacteriales), as representatives for this analysis, because these OTUs were more abundant in feces in the months of September and December in 2016, and May, June and July in

2017 based on the LEfSe analysis. Mean relative abundance of each representative OTU was calculated by feces, sediments where sea cucumbers were densely populated (populated sediments from pool2 and 3) and sediments with no record of habitation (control from pool4) by month. Statistical comparison of the relative abundance between populated sediments and controls were performed using Welch's *t* test corrected by FDR. Significance was assumed to be FDR-corrected $p<0.05$.

Metagenome Prediction

We applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) v1.1.1 (Langille *et al*., 2013) to predict functional composition of metagenome from 16S rRNA gene libraries. After removing non-reference based OTUs (i.e. "denovo xxx") from all libraries and normalizing read counts by samples based on known 16S copy number abundance, functional gene counts according to KEGG Orthology (KOs) were predicted using the normalized read counts by samples. Calculation of difference of predicted gene frequencies [i.e. effect size (%)] between feces and sediments by sampling points were performed by Welch's *t* test using STAMP software v2.1.3 (Parks *et al*., 2014). Significance was assumed to be FDR-corrected $p<0.05$ with an effect size threshold of 0.05. The effect size was visualized by heatmap generated by heatmap.2 function in R software version 3.5.1.

Data Deposition

All sequences were deposited in DDBJ/GenBank/EMBL database under the accession no. PRJDB7862.

Results & Discussion

The most abundant phylum observed in all samples was Proteobacteria; the relative abundance in feces (M-Feces), sediment (M-Sed), and seawater (M-SW) in Menagawa was 60.1±5.6%, 55.3

± 4.4%, 74.1% (mean ± SD), respectively (Fig. 2.3). Those from Ishigaki feces (I-Feces) and sediment (I-Sed) samples were 68.1±6.5% and 55.3±1.3%, respectively (Fig. 2.3). The second most abundant phylum was Bacteroidetes in the Menagawa samples (M-Feces, 25.8 ± 8.6%; M-Sed, 24.4 ± 5.0%; M-SW, 20.5%) and Ishigaki sediment (I-Sed, 9.8 ± 1.1%), and Cyanobacteria in the feces of the Ishigaki sea cucumbers (I-Feces, 8.6 ± 4.9%) (Fig. 2.3).

Sea Cucumber Fecal Microbiota Is Shaped by Selective Enrichment of Ingested Microbes from Sediments

We compared the microbiota of sea cucumber *A. japonicus* feces with sediments from Menagawa site through one whole year (from September 2016 to July 2017). Unweighted UniFrac analysis revealed that fecal microbiota differed from the sediment microbiota over the whole year tested [permutational multivariate analysis of variance (PERMANOVA)], and the fecal microbiota fluctuated in parallel with sediment microbiota along with seasonal changes based on UniFrac analysis (Fig. 2.4A). Such differences between gut and sediment microbiota have also been reported in *A. japonicus* maricultured in China (Gao *et al*., 2014). Interestingly, most major fecal OTUs, which were defined as OTUs observed in all fecal samples studied in each month, were detected in sediment microbiota (Table 2.2). Few bacterial OTUs were unique to fecal samples, compared to sediment samples. Additionally, these unique OTUs varied among sampling months and accounted for less than 0.1% of fecal bacterial communities in each month. Thus, we propose here that the sea cucumber fecal microbiota is occupied by transient fraction ingested with food (i.e. sediment).

Shannon indices of sediment microbiota were 1.21 ± 0.05 times higher than those of feces over the whole year (Monte Carlo permutation test) (Fig. 2.4B). We applied Linear Discriminant Analysis Effect Size (LEfSe), to compare which OTUs may be an indicator of fecal or sediment samples (Segata *et al*., 2011). LEfSe identified 110 OTUs (41 OTUs in September, 38 OTUs in December, 27 OTUs in April, 38 OTUs in May, 34 OTUs in June and 34 OTUs in July) whose relative abundance was higher in fecal microbiota than in sediment microbiota. Heatmap analysis

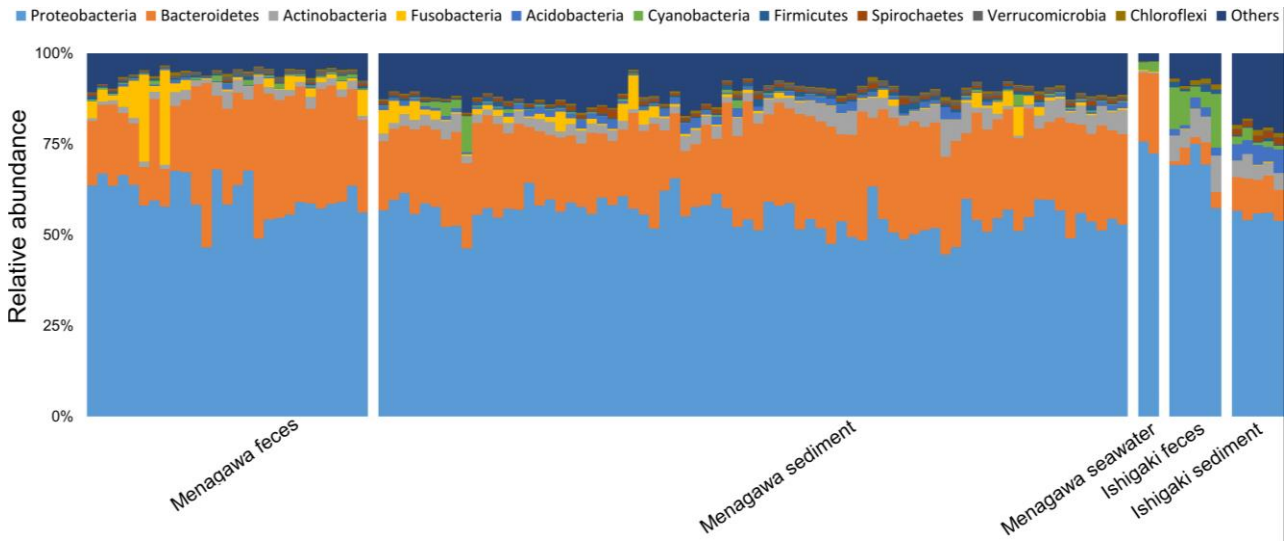


Fig. 2.3. Bacterial community structures (phylum level) of sea cucumber feces and their habitat's sediment and seawater. The Bar plot shows relative abundance of the top 10 phyla. Unassigned and under top 10 phyla were combined into others.

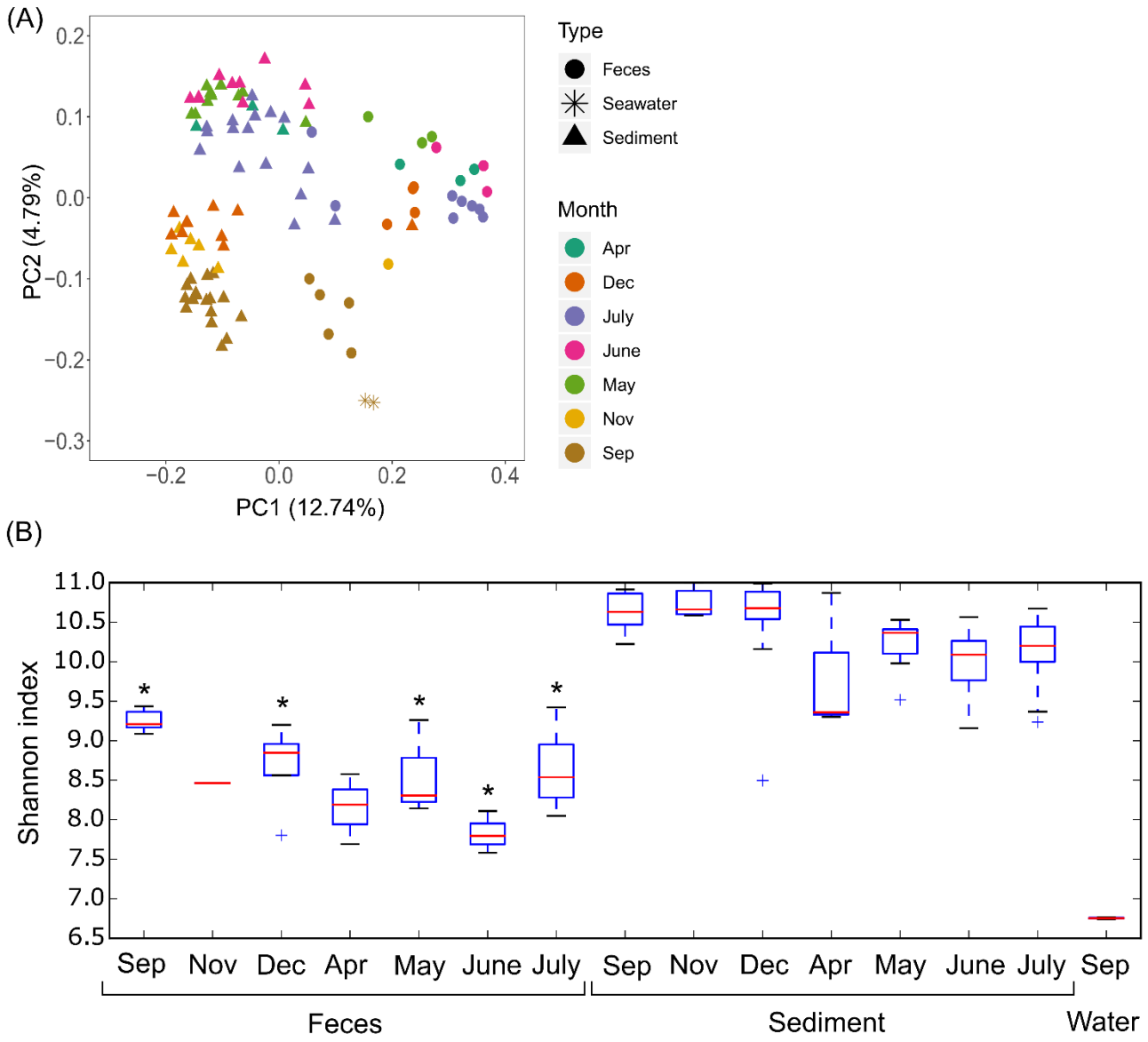


Fig. 2.4. Bacterial diversity analyses in the Menagawa site. (A) Unweighted UniFrac analysis was performed based on phylogenic tree using 8000 of subsampled reads. The PCoA 2D plot shows fecal, sediment and seawater microbiota in the Menagawa. Different shapes of samples indicate different sample types (i.e. feces, sediment, seawater), and samples were colored by months. Significant difference of UniFrac distance was confirmed by permutational multivariate analysis of variance (PERMANOVA) (FDR-corrected $p<0.05$). (B) Using the same number of subsampled reads, Shannon index of bacterial community diversity from sea cucumber fecal and sediment samples considering their sampling months was calculated. Asterisks show significant differences between mean Shannon indices within fecal and sediment samples in each month. Significance was evaluated based on 999 Monte Carlo permutation test with false discovery rate [false discovery rate (FDR)-corrected $p<0.05$].

Table 2.2. The number of major fecal OTUs shared by sediments. Major fecal OTUs are defined as OTUs observed in all fecal samples studied in each month.

		No. of major fecal OTUs	No. of major fecal OTUs shared by sediments	Proportion of major fecal OTUs shared by sediments (%)
Menagawa	September	998	992	99.4
	December	931	930	99.9
	April	441	429	97.3
	May	759	755	99.5
	June	728	719	98.8
	July	795	795	100.0
Ishigaki July		339	334	98.5

based on difference of relative abundance of the 110 OTUs showed that 1) in September, 14 OTUs belonging to Vibrionales were more abundant in fecal microbiota, 2) in November and December, 10, 8, and 6 OTUs belonging to Alteromonadales, Rhodobacterales, and Flavobacteriales, respectively, were more abundant in fecal microbiota, and 3) from April to July, Rhodobacterales (25 OTUs), Flavobacteriales (18 OTUs) and Alteromonadales (9 OTUs) were more abundant in feces than in sediments (Fig. 2.5A). These enriched bacterial groups, Vibrionales, Alteromonadales and Rhodobacterales, have already been proposed as probiotics candidates positively affecting physiology of *A. japonicus* in previous study (Chi *et al*., 2014; Yamazaki *et al*., 2016). Additionally, Flavobacteriales could also be a new target for probiotics in sea cucumbers. LEfSe also identified 46 OTUs (13 OTUs in September, 12 OTUs in December, 11 OTUs in April, 26 OTUs in May, 27 OTUs in June and 17 OTUs in July) which were more abundant in sediment microbiota than in fecal microbiota. The 46 OTUs were mainly affiliated to Flavobacteriales (16 OTUs) and Thiotrichales (9 OTUs), and notably Thiotrichales were more abundant in sediments than in feces over the whole year (Fig. 2.5B). These results indicate sediment bacteria were selectively enriched in the guts of *A. japonicus*.

To explore whether the above-mentioned process is common to other sea cucumber species living in natural environments, we analyzed bacterial communities of Ishigaki July samples including four species of sea cucumbers (N=5) and Menagawa July samples as a reference. Although Ishigaki fecal samples were taken from four different species of sea cucumbers, they were clustered together, and fecal and sediment microbiota differed from each other (PERMANOVA) (Fig. 2.6A). Most major fecal OTUs in Ishigaki samples were present in sediment samples (Table 2.2). Results of Shannon index comparison and LEfSe analysis were similar to those in *A. japonicus* (Figs. 2.6B and 2.7). The 38 OTUs including Rhodobacterales (15 OTUs), Desulfobacterales (7 OTUs), Chroococcales (4 OTUs) were more abundant in feces than in sediments at the Ishigaki site (Fig. 2.7A). On the other hand, the 9 OTUs including Thiotrichales (3 OTUs) and Flavobacteriales (2 OTUs) were more abundant in sediments than in feces at the site (Fig. 2.7B). Overall, our results

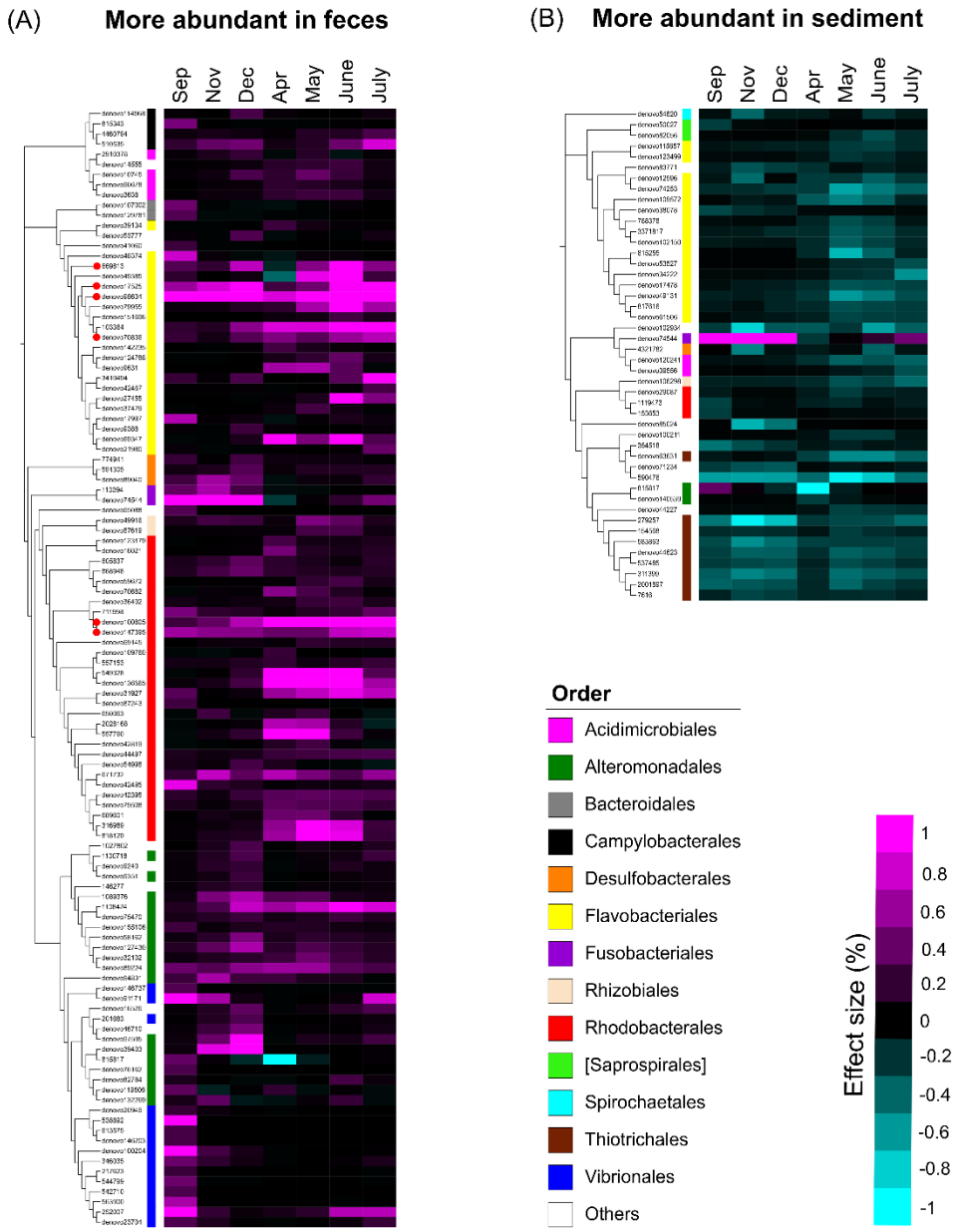


Fig. 2.5. Dendrogram-connected heatmaps of LEfSe-identified OTUs of the Menagawa site. Difference of mean relative abundance of each OTU between feces and sediments were calculated [= effect size (%)]. More vivid magenta and cyan corresponds to more abundant OTUs in feces and sediments, respectively. X axis of the heatmaps were aligned by time series of sampling. Y axis were aligned by the maximum-likelihood tree of representative reads of each OTU. Color bars show order level affiliation of each OTU. Unassigned and minor taxa (more abundant OTUs in feces or sediments than the other at one time only) were combined into others. The fecal sample in November was excluded from LEfSe analysis due to limited available samples (*N*=1). (A) The heatmap shows OTUs which were more abundant in fecal microbiota than in sediment microbiota. Red circles indicate six representative OTUs which were more abundant in feces than in sediments in the months of September and December in 2016, and May, June and July in 2017 based on LEfSe. (B) The heatmap shows OTUs which were more abundant in sediment microbiota than in fecal microbiota.

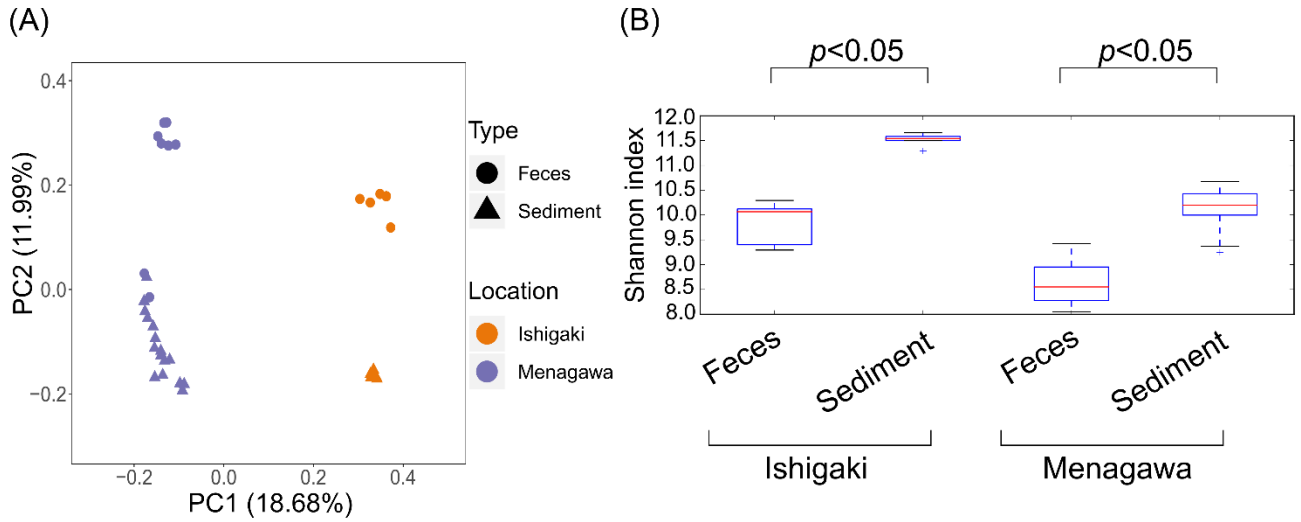


Fig. 2.6. Bacterial diversity analyses of Ishigaki July and Menagawa July samples. (A) Unweighted UniFrac analysis was performed based on phylogenic tree using 8000 of subsampled reads. The 2D PCoA plot shows fecal and sediment microbiota in the Ishigaki site in July and in the Menagawa site in July. Circles indicate fecal samples, and triangles are sediment samples. Ishigaki samples were colored by orange, and Menagawa samples were colored by dark purple. Significant difference of UniFrac distance was confirmed by PERMANOVA (FDR-corrected $p<0.05$). (B) Using the same number of subsampled reads, we compared bacterial community diversity (Shannon index) from sea cucumber fecal and sediment samples considering their geographical locations. Mean Shannon indices between fecal and sediment samples in each habitat were compared and evaluated based on 999 Monte Carlo permutation test with false discovery rate (FDR-corrected $p<0.05$).

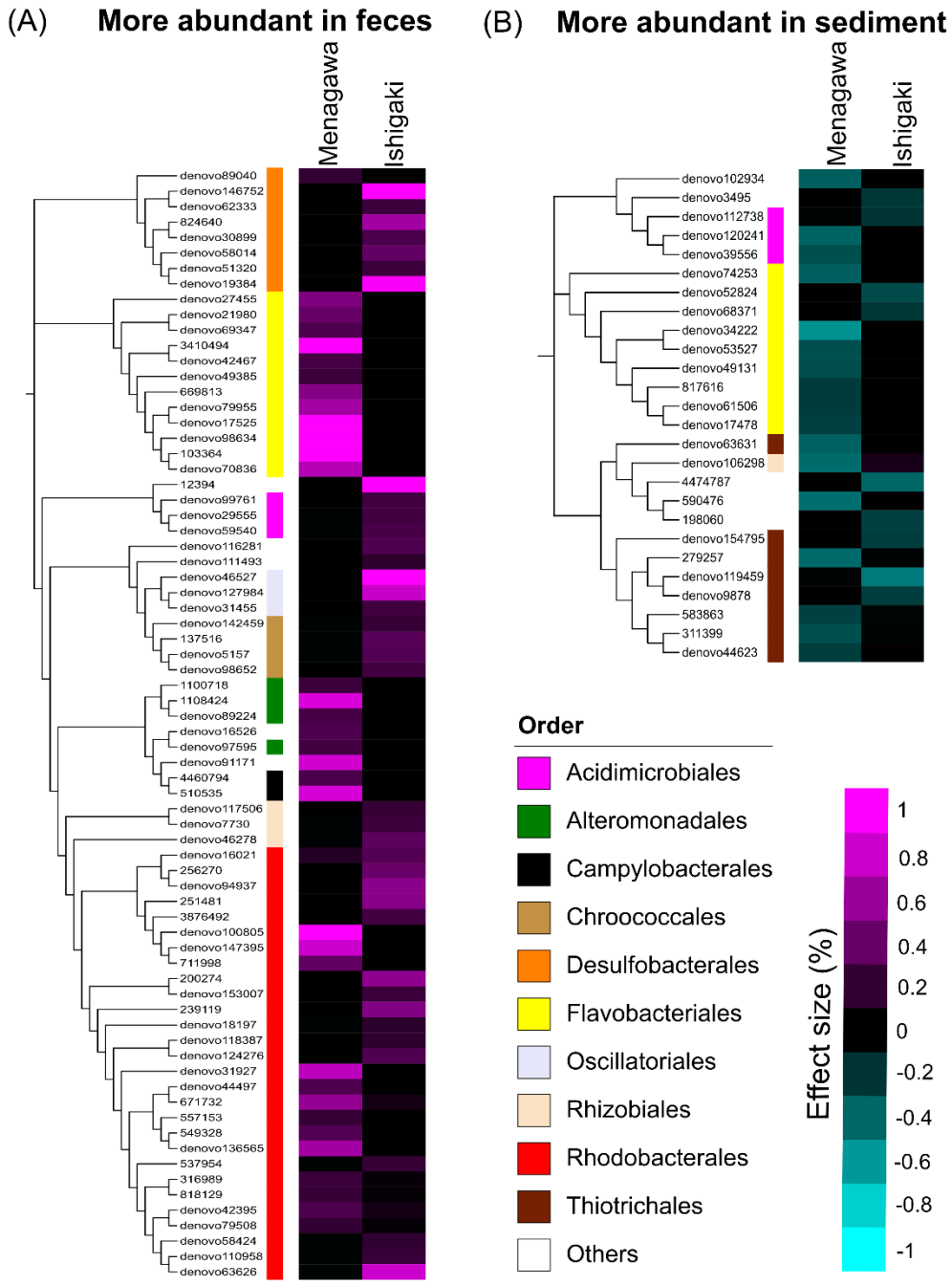


Fig. 2.7. Dendrogram-connected heatmaps of LEfSe-identified OTUs of Ishigaki July and Menagawa July samples. Difference of mean proportion of each OTU between feces and sediments was calculated [= effect size (%)] using samples collected in the Ishigaki site in July and the Menagawa site in July. More vivid magenta and cyan corresponds to more abundant OTUs in feces and in sediments, respectively. Y axis were aligned by Maximum-Likelihood tree of representative reads of each OTU. Color bars show order level affiliation of each OTU. Unassigned and minor taxa (more abundant OTUs in feces or sediments than the other at one time only) were combined into others. (A) The heatmap shows OTUs which were more abundant in fecal microbiota than in sediment microbiota. (B) The heatmap shows OTUs which were more abundant in sediment microbiota than in fecal microbiota.

suggest that selective enrichment of ingested microbes is the common process of shaping in fecal microbiota of coastal sea cucumbers.

The feeding habits and the ecological roles of sea cucumbers are similar to that of earthworms; they consume organic matter derived from bacteria, animals and plants with inorganic components (i.e. sediments and soils), and they promote organic matter mineralization (Drake and Horn, 2007; Purcell *et al*., 2016). Interestingly, our results suggest these animals share common process in shaping of fecal microbiota. In both animals, 1) community structures of fecal microbiota are different from that of ingested ones (e.g. sediment or soil), 2) high number of common microbes are detected in both fecal and ingested microbiota, and 3) bacterial diversity of fecal microbiota is lower than that of ingested microbiota (Thakuria *et al*., 2010; Gao *et al*., 2014; Aira *et al*., 2015). Although processes shaping gut microbiota of both animals share similar properties, taxonomic affiliation of gut microbiota differed from each other; In particular, Firmicutes are the dominant phylum in earthworms' gut microbiota (Wüst *et al*., 2011; Aira *et al*., 2015), but relative abundance of this phylum was rare in sea cucumbers' feces in all seasons tested. Our results suggest a convergence of process of shaping in fecal microbiota between marine and terrestrial invertebrates (Roberts *et al*., 2000; Drake and Horn, 2007).

The Repeated Selective Enrichment Process of Sediment Microbiota in Sea Cucumber Guts Transform Sediment Microbial Communities

A previous study reported deposition of sea cucumber's feces might increase benthic bacterial abundance (MacTavish *et al*., 2012), but it remains largely unexplored a possible linkage between sea cucumbers grazing and excretion activities with sediment microbial community structure shifts. To address the possible links, we selected six representative OTUs based on LEfSe: denovo70836 (Flavobacteriales), denovo100805 (Rhodobacterales), denovo17525 (Flavobacteriales), 669813 (Flavobacteriales), denovo147395 (Rhodobacterales), and denovo98634 (Flavobacteriales) (Fig. 2.5A). Relative abundance of these OTUs was compared between those in feces, in those sediments

where sea cucumbers were densely populated (host-populated sediments), and those in sediments with no record of habitation (control sediments). Relative abundance of these six representative OTUs was highest in feces, followed by the host-populated sediments (Fig. 2.8). Welch's *t* test showed significantly higher abundance of these six OTUs in host-populated sediments than in control ones (Fig. 2.8).

The above results suggest bacteria selectively enriched in sea cucumber guts were spread on ambient sediments of the animals together with feces and drove the transformation of the sediment microbial community structures. Sea cucumber guts may also serve as reservoirs of several types of microbes maintaining their abundance. Besides, after settlement, juvenile sea cucumbers might be able to construct gut microbiota easily by ingesting the sediment microbiota transformed by adult individuals in their habitat. Similarly, the transformed sediment microbiota could also help gut-regenerated individuals re-construct gut microbiota after the animals expel internal organs triggered by biotic and abiotic stress (Mashanov and García-Arrarás, 2011). Further studies are needed to examine whether heterogeneity in sediment microbial communities formed by sea cucumbers have positive impacts on recruitment of their juveniles and survival of gut-regenerated individuals.

Bacterial Metabolism in Sea Cucumber's Gut and Possible Contributions to the Host's Ecological Roles

To explore which bacterial functions increased in the feces through the selective enrichment process of sediment microbiota in the guts, we used PICRUSt software (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) which predicts functional metabolic potential of microbial communities from 16S rRNA gene libraries according to KEGG Orthology (KO) (Langille *et al.*, 2013). Although the PICRUSt prediction is limited to known microbial species described in the reference (e.g. Greengenes) and metagenome sequencing is more accurate to analyze functional profile in microbial community, PICRUSt is still useful to discuss potential metabolic

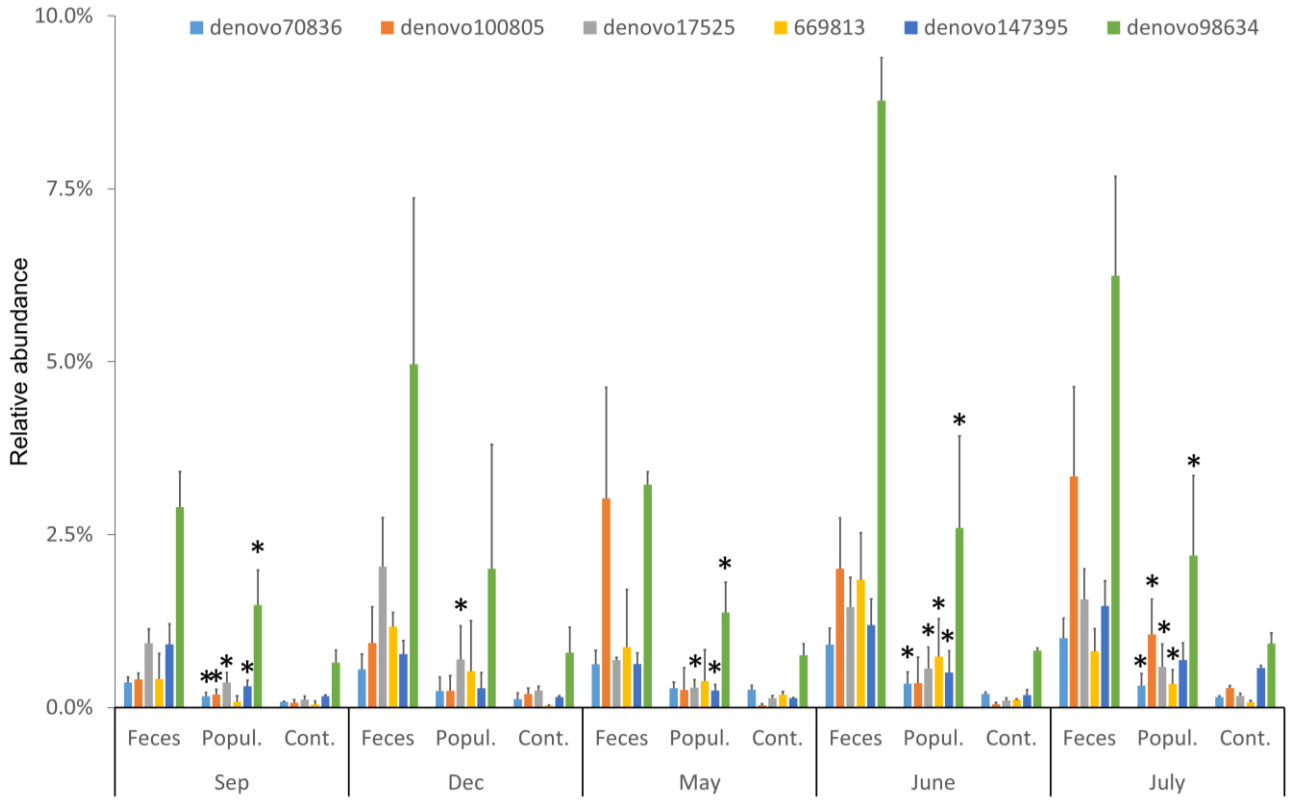


Fig. 2.8. Relative abundance of six representative OTUs by feces, host-populated sediments and controls. We selected the six representative OTUs based on LEfSe analysis in the Menagawa site. The bar plot shows mean relative abundance of the OTUs between those in feces, those in sediments where sea cucumbers were densely populated (host-populated sediments, i.e. Popul.) and those in sediments with no record of habitation (controls, i.e. Cont.). Error bars indicate standard errors. Bars marked by asterisks indicate the relative abundance of host-populated sediments which were significantly higher than those of control sediments in sampling months (Welch's *t* test, FDR-corrected $p < 0.05$).

features in microbial communities. The frequency of the bacterial functions was likely to be stable in all sample types (i.e. feces, sediment, seawater) collected in all seasons (Fig. 2.9).

We found that carbohydrates degradation metabolism potential (i.e. "Carbohydrate Metabolism" and "Xenobiotics Biodegradation and Metabolism" KEGG categories) was significantly more abundant in sea cucumbers feces than in sediment microbial communities (Fig. 2.10 and Table 2.3 for Welch's *t* test results). To confirm if the enriched microbial taxa in fecal microbiota were responsible for the increased carbohydrates degradation metabolisms, we removed all OTUs enriched in the feces identified by LEfSe from dataset, such as Vibrionales, Alteromonadales, Rhodobacterales, Flavobacteriales and Desulfobacterales. Interestingly, after removing the OTUs, "Carbohydrate Metabolism" had no significant difference, and the gene frequency of "Xenobiotics Biodegradation and Metabolism" was significantly more abundant in feces than in sediments in only one sampling point (Menagawa July) (Table 2.4). Therefore, these bacterial taxa enriched in sea cucumber guts might actively decompose carbohydrates and xenobiotics derived from animal and plant detritus thus contributing to organic matter mineralization of marine sediments.

The relative abundance of Thiotrichales, which consist of diverse sulfur oxidizing bacteria, was lower in sea cucumber guts than in sediments throughout the year (Figs. 2.5 and 2.7), suggesting anaerobic environments in their guts. In addition, Vibrionales known as facultative anaerobes and Desulfobacterales known as sulfate reducers were enriched in northern and southern sea cucumber guts, respectively (Figs. 2.5 and 2.7). Previous studies showed that Vibrionales is often dominant in sea cucumber guts (Enomoto *et al.*, 2012; Plotieau *et al*., 2013; Gao *et al*., 2017). These bacterial taxa cause fermentation within sea cucumber guts and may contribute to the decomposition of organic matter and further dissimilation processes in cooperation with other bacteria and host metabolism. Similar patterns were observed in organic matter mineralization by collaborative activity of earthworms and their gut microbiota (Horn *et al*., 2003; Drake and Horn, 2007; Wüst *et al*., 2011).

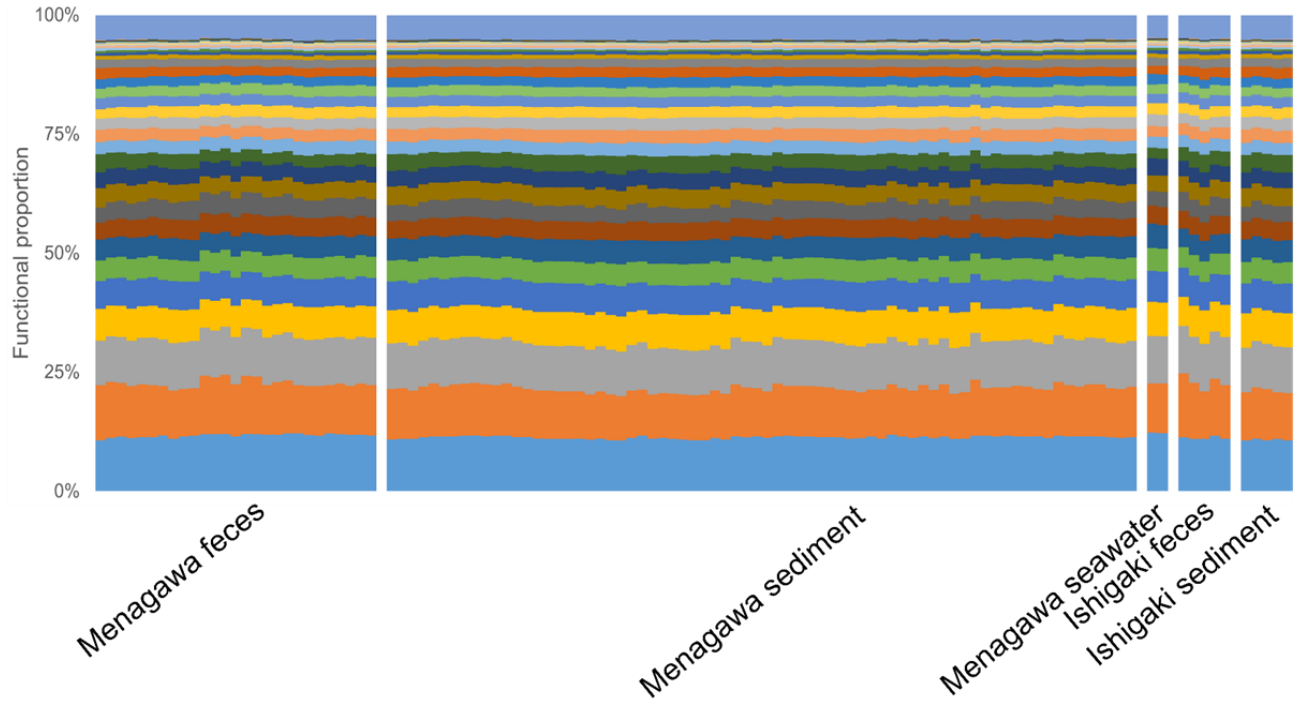


Fig. 2.9. Functional proportion of individual bacterial communities based on KEGG pathway.

- Amino Acid Metabolism
- Membrane Transport
- Carbohydrate Metabolism
- Replication and Repair
- Energy Metabolism
- Metabolism of Cofactors and Vitamins
- Translation
- Lipid Metabolism
- Xenobiotics Biodegradation and Metabolism
- Cellular Processes and Signaling
- Nucleotide Metabolism
- Cell Motility
- Metabolism
- Genetic Information Processing
- Folding, Sorting and Degradation
- Metabolism of Terpenoids and Polyketides
- Transcription
- Metabolism of Other Amino Acids
- Glycan Biosynthesis and Metabolism
- Signal Transduction
- Enzyme Families
- Biosynthesis of Other Secondary Metabolites
- Cell Growth and Death
- Neurodegenerative Diseases
- Infectious Diseases
- Endocrine System
- Transport and Catabolism
- Cancers
- Signaling Molecules and Interaction
- Environmental Adaptation
- Nervous System
- Metabolic Diseases
- Circulatory System
- Immune System
- Immune System Diseases
- Cardiovascular Diseases
- Digestive System
- Excretory System
- Cell Communication
- Sensory System
- Poorly Characterized

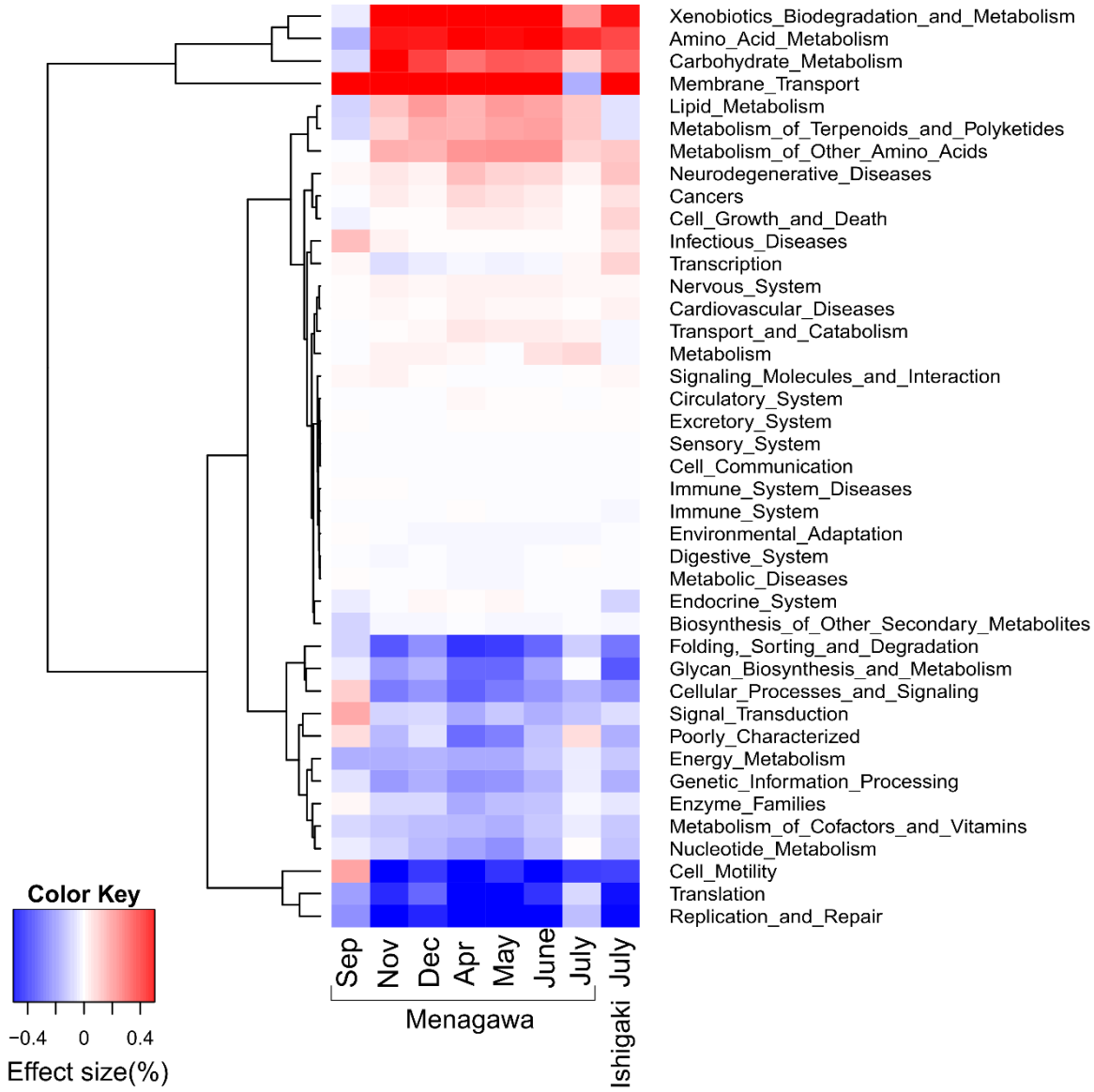


Fig. 2.10. Frequency of predicted KEGG functions. Row names indicate KEGG functions, and column names indicate sampling points. Difference of gene frequencies between feces and sediments was calculated [= effect size (%)]. Red and blue colors indicate functions which were more abundant in feces than in sediments and more abundant in sediments than in feces, respectively.

Table 2.3. Difference of frequencies of predicted KEGG functions between feces and sediments. "M-" and "I-" stand for the Menagawa and the Ishigaki sites, respectively. Proportion of predicted functions were compared between those in fecal and those in sediment samples, and twelve features (the left column) were significantly more abundant in feces than in sediments at least one sampling point. Yellow colors indicate statistical significance determined by Welch's *t*-test (FDR-corrected $p<0.05$). Numbers in cells show proportion of difference between those in feces and those in sediments by feature [effect size (%)]. Positive values indicate a feature is more abundant in feces than in sediments.

KEGG Functions	M-Sep	M-Nov	M-Dec	M-Apr	M-May	M-June	M-July	I-July
Amino Acid Metabolism	-0.14	0.45	0.44	0.51	0.48	0.62	0.40	0.36
Cancers	0.00	0.03	0.01	0.08	0.05	0.04	0.01	0.05
Carbohydrate Metabolism	-0.08	0.64	0.36	0.28	0.32	0.31	0.09	0.30
Cell Growth and Death	-0.03	0.01	0.00	0.03	0.04	0.03	0.00	0.08
Infectious Diseases	0.12	0.03	0.01	0.00	0.01	0.01	0.00	0.05
Lipid Metabolism	-0.09	0.11	0.20	0.15	0.20	0.18	0.10	-0.06
Membrane Transport	0.73	1.18	0.52	1.77	1.41	0.70	-0.15	1.45
Metabolism	-0.01	0.03	0.03	0.01	-0.01	0.05	0.08	-0.02
Metabolism of Other Amino Acids	-0.01	0.16	0.14	0.20	0.22	0.22	0.08	0.10
Metabolism of Terpenoids and Polyketides	-0.08	0.09	0.15	0.15	0.18	0.18	0.11	-0.05
Neurodegenerative Diseases	0.01	0.04	0.03	0.13	0.09	0.08	0.02	0.11
Xenobiotics Biodegradation and Metabolism	-0.03	0.64	0.58	0.75	0.81	0.65	0.19	0.47

Table 2.4. Difference of frequencies of predicted KEGG functions between feces and sediments using dataset excluding OTUs which were more abundant in feces than in sediments based on LEfSe analysis. "M-" and "I-" stand for the Menagawa and the Ishigaki sites, respectively. After excluding OTUs which were more abundant in feces than in sediments based on LEfSe analysis, proportion of predicted functions was compared between those in fecal and those in sediment samples. Five features were more abundant in feces than in sediments. Yellow colors indicate statistical significance determined by Welch's *t* test. Numbers in cells show proportion of difference between those in feces and those in sediments by feature [effect size (%)]. Positive values indicate a feature is more abundant in feces than in sediments.

KEGG Functions	M-Sep	M-Nov	M-Dec	M-Apr	M-May	M-June	M-July	I-July
Amino Acid Metabolism	0.10	0.26	0.30	0.46	0.21	0.15	0.15	0.31
Cancers	0.01	0.00	0.00	0.02	0.00	0.01	0.01	0.05
Carbohydrate Metabolism	0.12	0.84	0.36	0.03	0.12	0.10	0.05	0.13
Cell Growth and Death	0.01	-0.01	0.00	0.01	0.00	0.01	0.02	0.11
Infectious Diseases	0.03	0.01	0.01	0.00	0.00	0.03	0.02	0.06
Lipid Metabolism	0.03	0.05	0.18	0.21	0.18	0.01	0.07	0.02
Membrane Transport	0.66	1.05	0.64	0.44	0.56	0.61	0.43	1.19
Metabolism	0.03	0.06	0.02	0.13	0.03	0.03	0.02	-0.04
Metabolism of Other Amino Acids	0.06	0.07	0.10	0.15	0.10	0.10	0.07	0.15
Metabolism of Terpenoids and Polyketides	0.01	0.01	0.12	0.18	0.15	0.06	0.07	0.01
Neurodegenerative Diseases	0.01	-0.03	0.00	0.05	-0.01	0.01	0.01	0.11
Xenobiotics Biodegradation and Metabolism	0.22	0.44	0.49	0.55	0.46	0.27	0.27	0.39

One previous study found sea cucumbers facilitates organic matter mineralization in sediments through increasing bacterial abundance and decreasing microphytobenthos abundance in sediments, shifting the microbial balance from producers to decomposers (MacTavish *et al*., 2012). Our results showed sea cucumber feces contain more abundant Alteromonadales and Flavobacteriales, representative heterotrophic bacteria in marine environment, than in sediments (Figs. 2.5 and 2.7). The excreted feces could increase the abundance of such microbes in sediments and thus have a great impact on mineralization of sedimentary organic matters. This present study suggests a mechanism of increased bacterial abundance in sediments (MacTavish *et al*., 2012) and also a potential contribution of sea cucumber gut microbiota to sedimentary organic matter mineralization outside of their guts.

Conclusion

Our results indicate sea cucumber fecal bacterial communities are shaped by the selective enrichment of heterotrophic microbes acquired from ingested sediments. The repeated process transforms sediment microbial community structures around the host territory and can also promote organic matter mineralization inside and outside of the guts. Our study suggests, similar to earthworms, that sea cucumber gut microbiota can maintain the host's ecological roles including sediment cleaning and nutrient cycling promotion.

CHAPTER 3

An annual fecal metagenome of individual sea cucumber (*Apostichopus japonicus*) demonstrates the feeding behaviors against eukaryotes in natural environments

Abstract

Deposit-feeding sea cucumbers are important fishery resources in many Asian countries, and efficient aquaculture technology based on their ecology is urgently needed due to decreasing wild stocks. Wild animal's feeding behavior, however, is still poorly understood due to lack of methodology. To explore feeding behaviors of sea cucumber individuals against eukaryotes present in natural environments, we analyzed eukaryotic communities in feces of sea cucumber *Apostichopus japonicus* and sediments through one whole year based on 16S rRNA gene sequencing of the organelle's genomes. A total of 390 eukaryotic features were obtained, and 99.7% of the features were assigned to chloroplast. The eukaryotic communities in feces and sediments showed seasonal fluctuations through one year based on Bray-Curtis distance and community composition. Comparison of eukaryotic communities between feces and sediments showed that 12 families including Chaetocerotaceae and Laminariaceae were more abundant in feces than in sediments, suggesting that sea cucumbers may choose sediment containing these algal taxa more often compared to others. All features of Laminariaceae were assigned to *Saccharina japonica*, which is consistent with the fact that this alga is one of the most suitable diets in the aquaculture of *A. japonicus.* These metagenomic assessments could be an alternative candidate tool to monitor individual feeding behaviors and status for the bioresource conservation.

Introduction

Sea cucumbers are ubiquitous and abundant in marine benthic environments, from shallow to deep sea, worldwide, and ecologically and biogeochemically essential animal species in marine benthic biomes. Deposit-feeding sea cucumbers blend and reform the surface sediments by repeated

ingestion and excretion (Uthicke, 1999), and their feeding activity, i.e. bioturbation, greatly impacts on nutrient cycling and biomes including primary producers, animals and microorganisms in marine benthic environments (Widdicombe and Austen, 1999; Meysman *et al*., 2006; Laverock *et al*., 2010; MacTavish *et al*., 2012). Grazing by the animals decreases benthic microalgae, but the resultant nutrient release with the animals' feces through organic matter mineralization increases algal productivity (Uthicke, 2001a; MacTavish *et al*., 2012).

Sea cucumbers are also known as an important fishery resource in many Asian countries. These wild stocks have dramatically decreased due to over-fishing, so some coastal species living in Japan such as *A. japonicus* and *T. ananas* are listed in the IUCN Red List as "endangered species" in 2013 (Conand *et al*., 2013a; Hamel and Mercier, 2013; Purcell *et al*., 2013). To sustain wild stocks, many studies have attempted to improve sea cucumber's seed production: optimization of biotic and/or abiotic factors (e.g. animal density, seawater temperature and its fluctuations) on sea cucumbers growth (Dong and Dong, 2006; Dong *et al*., 2006, 2008, 2010) and assessment of suitable diets positively affecting their growth (Gao *et al*., 2011; Sun *et al*., 2012a; Xia *et al*., 2012a; Shi *et al*., 2013a).

Diatom and macroalgae are known as main food sources of sea cucmbers based on fatty acid biomarkers (Gao *et al*., 2010) or carbon and nitrogen stable isotopes (Sun *et al*., 2013b) analyses. Due to poor nutrition in dietary sediments, deposit-feeding sea cucumbers must consume huge amount of sediment daily (Uthicke, 1999; Slater *et al*., 2011). To efficiently obtain energy, it is hypothesized that these deposit-feeders show selective feeding behavior; they move around their territory to seek dietary sediments containing higher organic matter or microbial mat (Uthicke, 1999; Uthicke and Karez, 1999). In previous studies, relatively higher organic matter (OM) contents in the guts of several species than those of habitat sediments were reported (Uthicke, 1999; Slater *et al*., 2011). Some species of sea cucumbers actively selected sediments with the highest contents of microalgae and avoided sediment with the lowest pigment concentrations (Uthicke and Karez, 1999). In addition, laboratory feeding experiments of *A. japonicus* showed their selective feeding behavior

for sediment patches containing *S. japonica* from several artificial sediment patches containing different algal species (Xia *et al*., 2012b), suggesting a food preference for the brown algae. However, no consensus has been reached in food preferences in eukaryotic taxa in *A. japonicus* in natural environments due to the difficulty in accurate eukaryotic identification using conventional microscopic observations. Knowledge on seasonal changes of food source availability and those nutritional values for wild sea cucumbers are also unavailable until today due to lack of individual tracking methodologies.

Molecular biological technologies including a high-throughput sequencing are useful to assess possible dietary organisms in various wild animals such as centipedes (Günther *et al*., 2014), salps (Metfies *et al*., 2014), rays (Bade *et al*., 2014) as well as shrews and skinks (Brown *et al*., 2014) on the basis of internal transcribed spacers (ITS), cytochrome oxidase I (COI) and 18S rRNA gene analyses. In addition, chloroplast 16S rRNA gene sequence data was used to interpret potential food source information in monkey's gut microbiota (Trosvik *et al*., 2018). In sea cucumber species, Zhang and colleagues (2016) performed a 18S rRNA gene sequencing analysis of gut contents of *A. japonicus* populations and found over 800 operational taxonomic units of eukaryotes assigned to Dinoflagellata, Bacillariophyceae, Arthropoda, Mollusca, and Cercozoa in the guts. They also showed remarkable change in the eukaryotic communities between spring and winter (Zhang *et al*., 2016). However, the previous study could not compare eukaryotic communities between sea cucumber guts and sediments (Zhang *et al*., 2016).

Our aim is to explore the feeding behavior of wild *A. japonicus* against eukaryotes through one whole year. Based on the assumption that preferable eukaryotic organisms are more ingested by most of sea cucumber individuals, I compared eukaryotic communities of sea cucumber feces and sediments using 16S rRNA gene sequence datasets of mitochondrial and plastidial genomes obtained in our previous study (Yamazaki *et al*., 2019).

Materials & Methods

16S rRNA Gene Sequence Datasets and Benthic Biome Descriptions

The 16S rRNA gene sequencing datasets obtained in our previous study (Yamazaki *et al*., 2019) were used; feces of *A. japonicus* (27 samples), ambient sediments (72 samples) and seawater (2 samples). All sequences are available in DDBJ/GenBank/EMBL database under the accession number PRJDB7862.

In brief, I collected feces of *A. japonicus* and sediment surface samples by SCUBA diving in the Menagawa culture pond (41°45N, 141°5E), Hokkaido, Japan, in September, November and December 2016 and April, May, June and July 2017. Seawater samples were collected in this site only in September 2016. Size of the artificial tide pool pond studied is 55 x 46 x 4 m (in height, width and depth), where fish and benthic organisms (e.g. sea cucumbers, sea urchins, sea stars and abalones) and algae (*S. japonicus* and *Ulva* spp.) inhabit, and natural seawater is constantly exchange through flow paths equipped with these pools. Bottom features of these pools are sandy and rocky, thus sea cucumbers fed mainly sands and surface materials on rocks. Sea cucumbers grew well in autumn (September to early December) and in Spring (April to early May) in the pool. After DNA extraction from these samples, the hypervariable V1-V2 region of the 16S rRNA gene was amplified and sequenced using MiSeq Illumina platform (Yamazaki *et al*., 2019).

Quality Control and Taxa Assignment

The paired-end sequence data with quality scores (i.e. Fastq files) were analyzed using QIIME2 (Bolyen *et al*., 2019). Quality controls (e.g. trimming primers, denoising reads, removing chimeric reads) and merging paired-end reads were performed using DADA2 (Callahan *et al*., 2016). Reads with 100% similarity constituted a feature [i.e. amplicon sequence variance (ASV)]. Unlike the method used to cluster reads into operational taxonomic units (OTUs) with fixed threshold (usually 97%), this quality control method using DADA2 allows us to detect even single nucleotide differences. To detect eukaryotic (mitochondrial and chloroplast) reads, each feature was assigned to

taxonomy using the Naive Bayes classifier and Greengenes database version 13.8. Most eukaryotic reads were assigned to chloroplast (>99.99%), and thus only chloroplast sequences were used for the downstream analyses. The chloroplast reads were re-assigned to algal taxa using the Naive Bayes classifier and PhytoRef database, plastidial 16S rDNA reference sequences that originate from a large diversity of eukaryotes representing all known major photosynthetic lineages (Decelle *et al*., 2015).

Diversity Analysis

Using 1,000 subsampled reads, Bray-Curtis distance as beta diversity was calculated and visualized in principle coordinate analysis (PCoA) plots (Lozupone *et al*., 2011).

Relative Abundance Comparison

We assumed that preferable eukaryotic organisms as food sources of sea cucumbers is ingested more than other eukaryotes. To find which families were more abundant in feces than in sediment samples (Segata *et al*., 2011), Linear Discriminant Analysis Effect Size (LEfSe) ver. 1.0 using Kruskal-Wallis Wilcoxon-rank sum tests and effect size threshold (LDA score>3.0) was used (Segata *et al*., 2011). The fecal samples in November were excluded from this LEfSe analysis due to limited available samples (N=1). The differences in proportion between fecal and sediment samples were visualized using a heat map.

Results & Discussion

A total of 290,747 eukaryotic reads were obtained, and >99.99% of the eukaryotic reads were assigned to chloroplast based on Greengenes database. A total of 389 chloroplast features and 1 mitochondrial feature were retained. A total of 290,741 of chloroplast reads were re-assigned to each algal taxon based on the chloroplast database (Decelle *et al*., 2015) and then used for the downstream analyses (Table 3.1).

Table 3.1. Sample information

sample-id	Environment	Month	No. of input	No. of passed quality control	No. of mitochondrial reads	No. of chloroplast reads	No. of chloroplast ASVs
ApF17	Feces	April	23,501	15,851	0	9,613	22
ApF41	Feces	April	26,915	18,823	0	11,799	25
ApF51	Feces	April	28,261	19,561	0	9,820	27
ApS17	Sediment	April	25,552	15,945	0	2,800	29
ApS41	Sediment	April	27,213	14,328	0	2,404	20
ApS51	Sediment	April	32,590	19,812	0	2,516	26
D2p1-170126	Sediment	December	30,047	14,209	0	3,177	41
D2p3-170126	Sediment	December	33,234	16,278	0	3,281	41
D2r1-170126	Sediment	December	39,659	19,350	0	6,168	58
D2r3-170126	Sediment	December	32,733	16,651	0	2,848	34
D4p1-170126	Sediment	December	28,416	14,350	0	5,658	28
D4p3-170126	Sediment	December	27,608	12,807	0	3,862	30
DF101	Feces	December	30,207	19,626	0	1,869	27
DF102	Feces	December	46,624	30,189	0	2,385	43
DF103	Feces	December	36,146	23,995	0	2,158	34
DF17-170126	Feces	December	33,116	20,361	0	1,235	31
DS101	Sediment	December	34,315	22,366	0	1,729	31
DS102	Sediment	December	35,187	19,327	0	715	23
DS103	Sediment	December	43,773	26,584	0	3,778	59
DS17-170126	Sediment	December	24,475	12,047	0	1,275	36
July2p1	Sediment	July	54,738	32,249	0	2,323	36
July2p3	Sediment	July	54,450	31,923	0	2,010	30
July2p5	Sediment	July	68,905	40,411	0	2,286	37
July2r1	Sediment	July	56,736	32,933	0	2,668	45
July2r3	Sediment	July	51,316	30,060	0	1,744	30
July4p1	Sediment	July	32,702	16,622	0	1,197	23
July4p3	Sediment	July	29,102	15,034	0	2,207	25
July4p5	Sediment	July	23,008	11,686	0	744	20
JulyF15	Feces	July	47,288	31,362	0	1,922	24
JulyF17	Feces	July	41,111	26,809	0	807	14
JulyF41	Feces	July	50,814	30,560	0	1,925	33
JulyF51	Feces	July	40,304	26,958	0	2,030	22
JulyFK	Feces	July	55,905	37,704	0	3,098	28
JulyFL	Feces	July	54,196	35,492	0	2,763	25
JulyFM	Feces	July	51,752	32,438	0	2,816	29
JulyFN	Feces	July	42,645	25,726	0	1,507	21
JulyS15	Sediment	July	41,885	23,925	0	2,374	39
JulyS17	Sediment	July	46,786	28,012	0	1,360	20
JulyS41	Sediment	July	47,733	27,049	0	1,246	24
JulyS51	Sediment	July	42,250	24,093	0	1,464	21
JulySK	Sediment	July	43,874	27,056	0	1,999	18
JulySL	Sediment	July	55,883	33,483	0	2,009	26
JulySM	Sediment	July	55,463	33,972	0	2,085	26
JulySN	Sediment	July	59,686	32,119	0	1,851	27
Jun2p1	Sediment	June	50,161	27,451	0	2,636	22
Jun2p5	Sediment	June	48,648	28,396	0	2,322	22
Jun2r1	Sediment	June	56,649	33,460	0	3,746	28
Jun2r5	Sediment	June	26,844	15,615	0	1,524	14
Jun4p1	Sediment	June	23,682	11,595	0	584	13
Jun4p5	Sediment	June	49,480	27,290	0	1,591	29
JunF15	Feces	June	35,627	23,833	0	2,414	14
JunF17	Feces	June	30,872	19,632	0	1,419	19
JunF51	Feces	June	29,306	20,054	0	1,983	25
JunS15	Sediment	June	60,755	35,087	0	1,965	40
JunS17	Sediment	June	27,464	15,735	0	4,331	26
JunS41	Sediment	June	39,972	24,357	0	2,076	36
JunS51	Sediment	June	47,964	27,292	0	3,198	25
Ma2p1	Sediment	May	37,999	20,790	0	2,215	26
Ma2p5	Sediment	May	57,754	32,154	0	3,106	37
Ma2r1	Sediment	May	32,440	18,390	0	2,239	29
Ma2r3	Sediment	May	48,551	27,532	0	3,125	41
Ma2r5	Sediment	May	47,265	25,643	0	4,312	52
Ma4p3	Sediment	May	54,253	29,871	0	5,120	47
Ma4p5	Sediment	May	54,656	29,288	0	3,823	48
MaF15	Feces	May	48,389	30,634	0	10,578	54
MaF41	Feces	May	32,090	22,197	0	5,487	36
MaF51	Feces	May	24,671	16,726	6	6,980	36
MaS15	Sediment	May	31,814	16,629	0	3,299	19
MaS41	Sediment	May	25,860	15,188	0	2,681	36
MaS51	Sediment	May	27,990	17,942	0	6,398	34
N2F-170126	Feces	November	28,128	17,041	0	1,531	32
N2p1-170126	Sediment	November	30,580	13,963	0	3,309	45
N2p3-170126	Sediment	November	36,464	18,512	0	2,205	44
N2r1-170126	Sediment	November	27,968	13,553	0	846	26
N2r3-170126	Sediment	November	28,290	12,670	0	1,881	36
N4p1-170126	Sediment	November	33,663	17,699	0	7,752	39
N4p3-170126	Sediment	November	30,258	15,160	0	3,281	33
Sawabe-62-161114	Feces	September	42,551	23,195	0	431	9
Sawabe-63-161114	Feces	September	42,549	23,550	0	952	10
Sawabe-64-161114	Feces	September	41,231	23,677	0	3,424	10
Sawabe-65-161114	Feces	September	39,673	21,751	0	493	17
Sawabe-66-161114	Feces	September	45,610	25,117	0	1,518	35
Sawabe-69-161114	Sediment	September	45,334	22,707	0	519	15
Sawabe-70-161114	Sediment	September	39,374	18,667	0	202	12
Sawabe-71-161114	Sediment	September	39,980	17,547	0	1,153	24
Sawabe-72-161114	Sediment	September	37,600	18,397	0	706	16
Sawabe-73-161114	Sediment	September	26,673	11,603	0	1,120	28
Sawabe-74-161114	Sediment	September	30,605	13,760	0	1,353	37
Sawabe-75-161114	Sediment	September	34,221	15,838	0	2,092	33
Sawabe-76-161114	Sediment	September	36,746	16,398	0	1,211	33
Sawabe-77-161114	Sediment	September	42,770	21,038	0	1,120	28
Sawabe-78-161114	Sediment	September	35,408	16,194	0	1,322	26
Sawabe-79-161114	Sediment	September	35,805	17,235	0	1,335	27
Sawabe-80-161114	Sediment	September	34,650	15,923	0	807	21
Sawabe-81-161114	Sediment	September	29,878	13,031	0	861	22
Sawabe-82-161114	Sediment	September	35,639	15,909	0	2,810	30
Sawabe-83-161114	Sediment	September	48,574	25,247	0	11,406	51
Sawabe-84-161114	Sediment	September	37,816	18,160	0	7,814	34
Sawabe-85-161114	Sediment	September	38,641	19,648	0	10,691	39
Sawabe-86-161114	Seawater	September	32,581	14,628	0	1,163	32
Sawabe-87-161114	Seawater	September	39,868	17,794	0	756	24

Overview of Eukaryotic Communities of Sea Cucumber's Feces and Environmental Samples through One Whole Year

Clear seasonal fluctuation of the eukaryotic communities was observed through one whole year based on Bray-Curtis distance (Fig. 3.1). Significant differences in the eukaryotic community between sea cucumber feces and sediments were not detected at any time-points (Fig. 3.1). This contrasts strongly with the bacterial community, which shows significant dissimilarity between sediment and fecal microbiota (Yamazaki *et al*., 2019).

The eukaryotic communities appeared to be separated into three groups based on the PCoA plot: September to December, April to June, and July. From September to December, Cymbellaceae (class Bacillariophyceae) dominated [23.2 ± 13.3% (mean ± SD)], and notably Laminariaceae (class Phaeophyceae) reached maximally 93.3% in a fecal sample collected in September; from April to June, Chaetocerotaceae (class Bacillariophyceae) (29.5 ± 20.3%) and Cymbellaceae (14.2 ± 11.4%) were abundant, and Chaetocerotaceae reached a maximum of 78.9% in a fecal sample collected in April; In July, Cymbellaceae was abundant (16.6 ± 13.3%) (Fig. 3.2). The results support previous studies showing diatoms as major food sources for *A. japonicus* (Gao *et al*., 2010; Zhang *et al*., 2016). As propagation of alga in the gut of sea cucumber through the ingestion could be restricted under limited light conditions, the abundance of specific features in feces against those of ingested sediments could be a reasonable indicator of selective feeding by each individual instead of pigment analyses (Uthicke, 1999; Uthicke and Karez, 1999; Slater *et al*., 2011).

Identification of Over-represented Eukaryotic Families in Feces Compared to Sediments as an Indicator of Preferable Diet

I compared eukaryotic communities at family level between fecal and sediment samples using LEfSe software (Segata *et al*., 2011) to find which eukaryotic taxa were more abundant in feces compared to sediments as an indicator of selective feeding. LEfSe identified 12 families whose relative abundance was higher in feces than in sediments (Chlorellaceae and Laminariaceae in September;

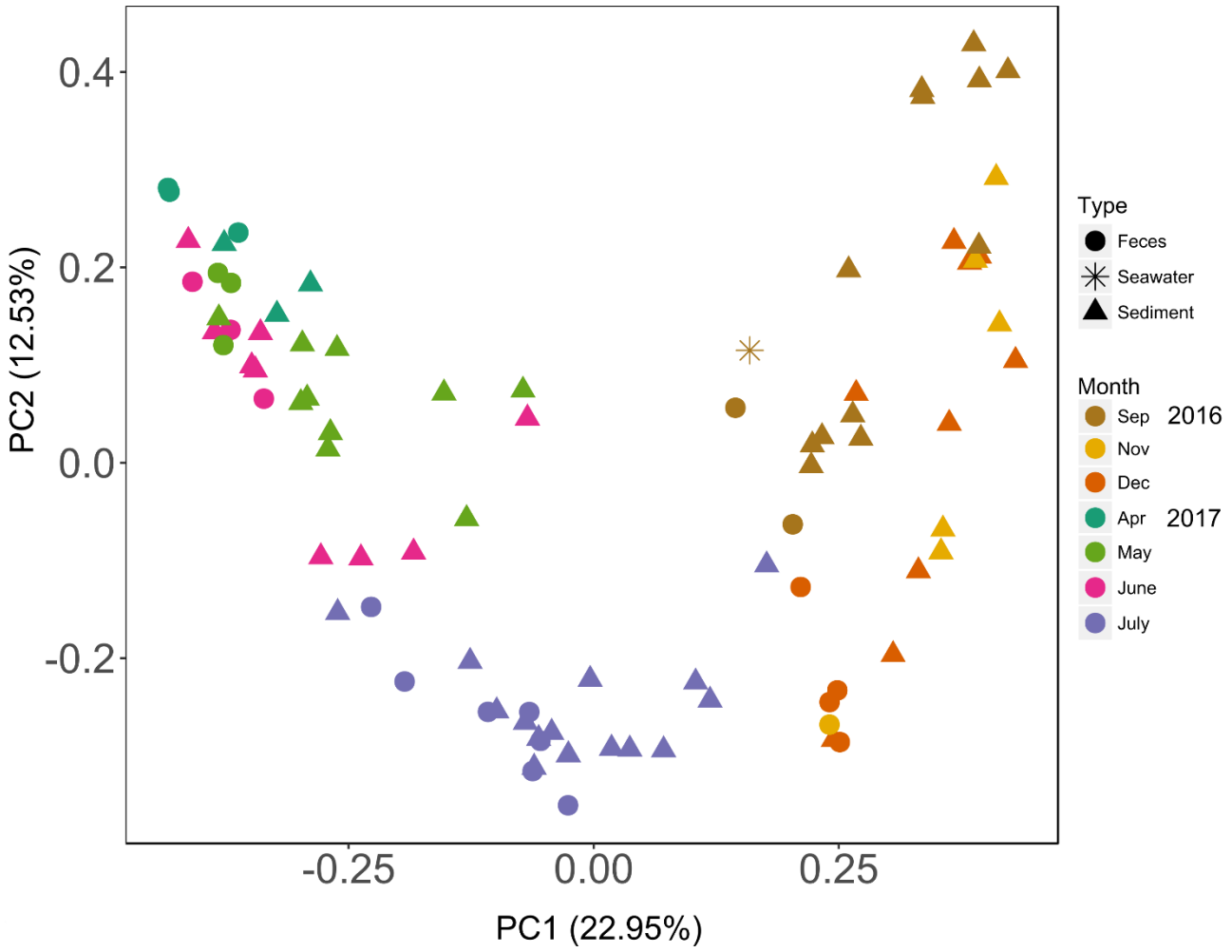


Fig. 3.1. Bray-Curtis-based 2D PCoA plot. The PCoA plot shows eukaryotic communities of sea cucumber's feces and environmental samples. Different shapes of samples indicate different sample types (i.e. feces, sediment, seawater). Samples were colored by sampling month.

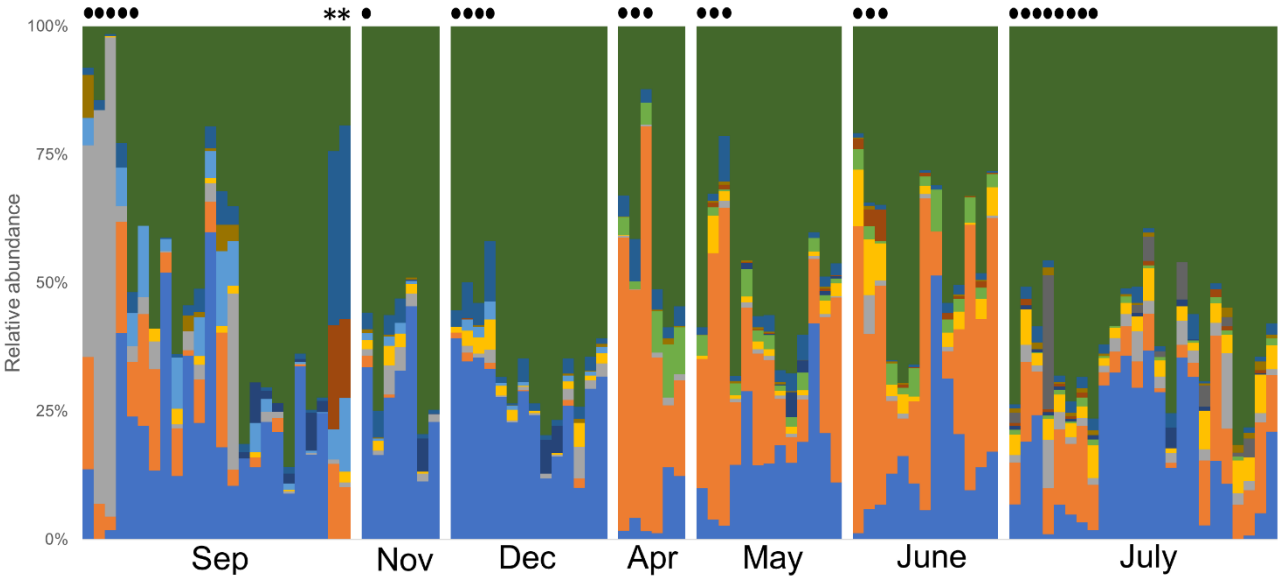


Fig. 3.2. Family Composition of eukaryotic communities. Top 10 families were shown in the bar plot. Minor taxa were clustered into others. Black filled circles are fecal samples, and black asterisks are seawater samples, and no marked bars are sediment ones.

Surirellaceae, Chlorellaceae, Hyalodiscaceae and Cymbellaceae in December; Chaetocerotaceae and Hyalodiscaceae in April; Chaetocerotaceae and Melosiraceae in May; Cymatosiraceae, Mamiellaceae, Chlorellaceae in June; Chaetocerotaceae, Chlorellaceae, Braarudosphaeraceae, Prasinophyceae and Chlorodendraceae in July) (Fig. 3.3). Chaetocerotaceae was more abundant in feces than in sediments in multiple months, in particular April to June (Fig. 3.3). Notably, relative abundance of Chaetocerotaceae, which is used as a common food source for planktonic larvae of *A. japonicus* in seed production, was over-represented in all sea cucumber individuals compared to its mean relative abundance in sediments; 57.1%, 44.5% and 78.9% in April feces and 21.6%±8.1% in April sediments on average; 25.5%, 51.9% and 62.0% in May feces and 16.4%±9.1% in May sediments on average; 59.7%, 34.1% and 42.7% in June feces and 25.9%±20.0% in June sediments on average (Fig. 3.2). In addition to Chaetocerotaceae, Laminariaceae was over-represented in feces compared to sediments in September (Fig. 3.2). Interestingly, Laminariaceae was more abundant in three sea cucumber individuals but not in two other individuals; 41.3%, 76.7%, 93.3%, 3.0% and 3.0% in September feces, and 3.4±8.1% in September sediments on average (Fig. 3.2). Previous 18S rRNA gene sequencing analysis also indicated huge interindividual variation in eukaryotic community compositions in sea cucumber gut contents (Zhang *et al*., 2016).

Cymbellaceae was over-represented in feces compared to sediment in winter (November and December), although the proportion of this family in feces or sediments was smaller compared to those of Chaetocerotaceae (April, May, June) and Laminariaceae (September) (Fig. 3.3). Conversely, Cymbellaceae was more abundant in sediments than in feces in other months (September, April to July), when Chaetocerotaceae or Laminariaceae were over-represented in feces (Fig. 3.3). In general, Cymbellaceae algae adhere benthic substrates, while Chaetocerotaceae algae are planktonic. Previous 18S rRNA sequencing analysis indicated that most diatom and pyrrophyte species detected in gut contents of *A. japonicus* were planktonic (Zhang *et al*., 2016). Thus, *A. japonicus* may prefer planktonic algae to benthic ones. In July, food preference was unlikely to be clear (Fig. 3.3). I observed sea cucumbers releasing sperm in the sampling site in this season, and feeding activity of

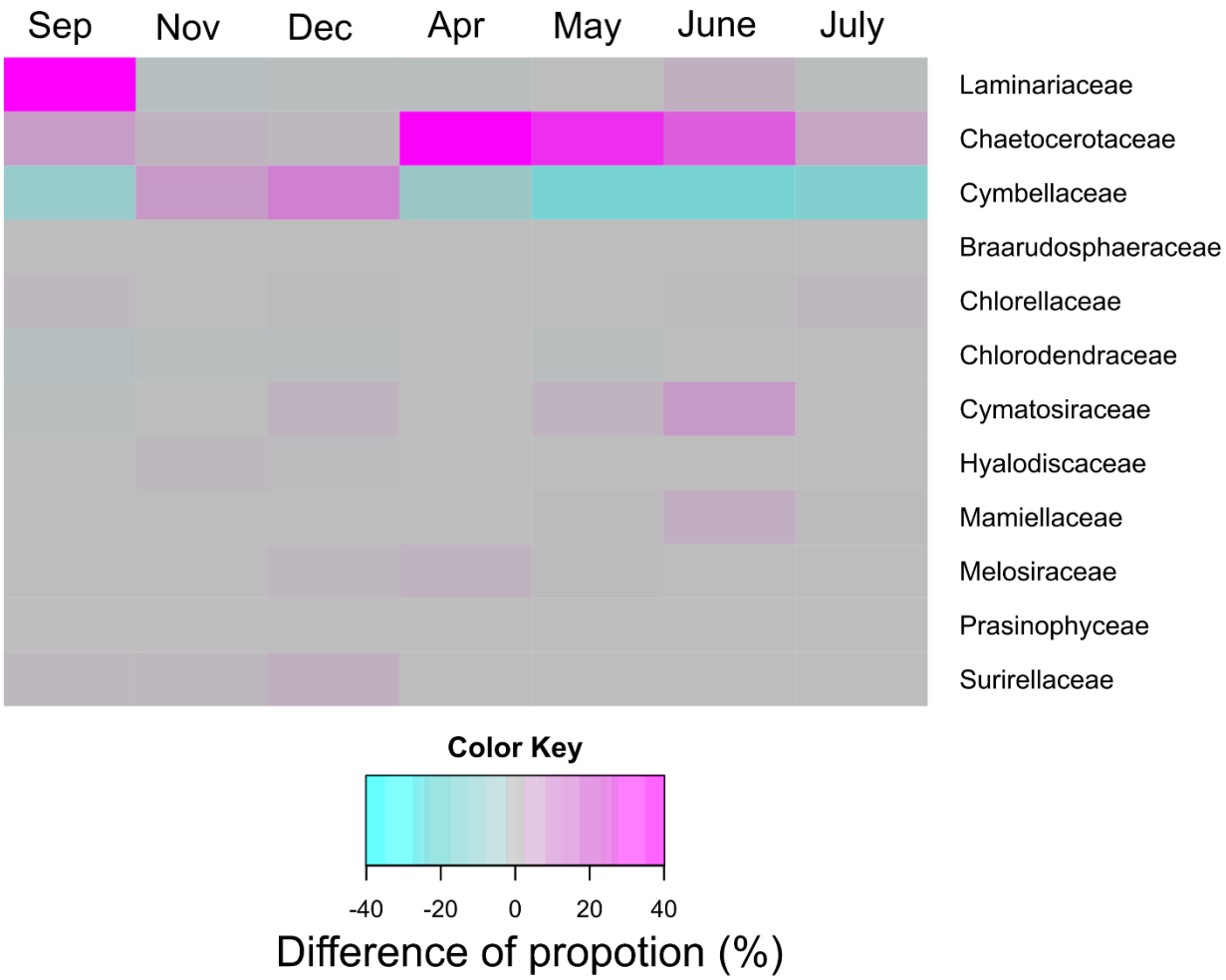


Fig. 3.3. Difference of proportion of 12 families based on LEfSe. Difference of relative abundance of 12 families identified by LEfSe between fecal and sediment samples by sampling month was shown in the heat map. Row names were families, and column names were months from September 2016 to July 2017. Magenta and cyan colors indicate families which were more abundant in feces than in sediments and more abundant in sediments than in feces, respectively.

A. japonicus could decrease in summer due to high temperatures (Gao *et al*., 2008). Seasonal physiological changes in individuals might directly affect their food preferences.

I further analyzed features of Chaetocerotaceae and Laminariaceae because they were abundant in fecal samples (Fig. 3.3). Chaetocerotaceae was consisted of 15 features, but below genus-assignment could not be achieved. On the other hand, Laminariaceae was consisted of three features, and all of the features were assigned to *S. japonica*. This brown alga is one of the most suitable feeds in aquaculture for *A. japonicus*, because growth and ingestion rate of powdered diets of this alga was much higher than in other algal species based on feeding experiments of *A. japonicus* (Xia *et al*., 2012a, b). These previous reports could justify that we used the abundance of specific features in feces against those of ingested sediments as an indicator of selective feeding. Interestingly, a previous study reported that *A. japonicus* chose sediment patches containing *S. japonica* from several artificial sediment patches containing different types of algae in a laboratory experiment (Xia *et al*., 2012b). Our results suggest that *S. japonica* might also be chosen by the sea cucumbers in natural habitats.

Conclusion & Future Prospects

This present study characterized eukaryotic community structures of sea cucumber's feces and sediment samples based on 16S rRNA genes retrieved from the organelle's genomes. Although few mitochondrial reads were detected, many chloroplast reads were obtained and assigned to algal taxa. The eukaryotic communities fluctuated throughout the whole year, and several eukaryotic families, in particular Chaetocerotaceae and Laminariaceae, were more abundant in the feces than in sediments, suggesting sea cucumbers may choose sediment containing high amounts of these algal taxa compared to others. More interestingly, the one-year analysis suggested that strength of feeding behavior of sea cucumbers against eukaryotic organisms might vary between individuals and between seasons. I assessed the feeding preferences based on abundance of specific features, but difference of digestibility of each algal species due to difference of structure and size of algal cell

walls could not be considered (Shi *et al*., 2013a). Future studies are thus needed to confirm feeding behavior of *A. japonicus* through multiple analyses including sequence analysis of eukaryotic communities, behavior tracking of wild sea cucumbers using loggers and bioassays using different types of algae in the laboratory.

Non-destructive methodology of individual sea cucumber gut microbiota I developed was used to analyze metagenomes of cultured and wild sea cucumbers up to here. I found several bacterial groups which may affect sea cucumber's growth and the host ecological functions in CHAPTERs 1 and 2, respectively. To examine effects of such microbes on sea cucumber's physiology and ecology, and to further understand host-microbe interaction in the guts, development of new methodologies is needed in the further study. In CHAPTER 4, I attempted to develop tracking systems of individual sea cucumber metagenomes both in the wild and in laboratory environments.

CHAPTER 4

Development of feeding systems to track dynamics of individual gut metagenome of sea cucumber *Apostichopus japonicus* during gut regeneration

Abstract

Animal species belonging to amphibians, urochordate, platyhelminths and echinoderms possess remarkable capacity to regenerate their body parts or organs. Regeneration of host organs and/or body parts involves reconstruction of these host associated microbiota, however, the dynamics and contribution of microbiota to the regeneration processes are largely unknown due to lack of experimental models. To establish an experimental model using sea cucumbers, I developed both caged mariculture and laboratory feeding systems of sea cucumbers to track dynamics of the gut metagenomes derived from identical individuals during gut regeneration, thereby performing longitudinal meta16S analyses. In the caged mariculture system, beta diversity analyses indicated that both bacterial and eukaryotic communities of sea cucumbers' guts were reconstructed within four months after evisceration. In the laboratory feeding system, a longitudinal Bray-Curtis analysis indicated that gut regeneration impacted on the gut bacterial communities. Consistently, relative abundance of several bacteria belonging to Alteromonadaceae, Rhodobacteraceae, Oceanospirillaceae and family unassigned Gammaproteobacteria was increased immediately after the gut regeneration and then decreased again, suggesting that these bacteria correspond to the gut regeneration process. The laboratory feeding system I developed might be useful to study the dynamics and contribution of microbiota to animal regeneration processes as a model.

Introduction

Regeneration is one of the fundamental fields in biology, and particularly, Spallanzani and Morgan had contributed to the progress (Nachtrab and Poss, 2012). Mammals (e.g. African spiny mouse), amphibians (e.g. newts), urochordate (e.g. sea squirt), echinoderms (e.g. sea stars, sea cucumbers)

and platyhelminths (e.g. Planaria) show remarkable regenerative capacities (Umesono *et al.*, 2013; Tanaka *et al*., 2016). Although exact definition of regeneration had been difficult due to a continuum from wound healing to organ regeneration regarding to mechanism, there is an emerging view to divide this continuum into three classes: wound healing, tissue repair, and regeneration (Galliot *et al*., 2017). The first one is the healing of wound with full or partial functional restoration. The second one causes functional restoration of the injured organ and/or body part with no patterned 3D reconstruction. The last one causes regrowth and 3D patterning of a complex structure, such as appendages or body parts, depending on blastema formation.

Regeneration of host organs and/or body parts involves reconstruction of host associated microbiota. Interestingly, a previous study in mice showed that colon wound beds are colonized by *Akkermansia*-enriched microbial communities due to the highly anaerobic condition, and indicated that *Akkermansia* bacteria promoted the healing of the injuries (Alam *et al*., 2016). Beyond such contribution of microbes to wound healing, there are two previous studies of gut microbiota during gut regeneration in sea cucumber *A. japonicus* (Wang *et al*., 2018; Zhang *et al*., 2019). One study indicated that bacterial community structures varied greatly in different stages in the gut regeneration (Wang *et al*., 2018). Another one performed both meta16S and metagenomic sequencing analyses and indicated that the abundance of Bacteroidetes and Rhodobacterales significantly increased in the gut microbiota during the gut regeneration process, and that the gut microbiota varied according to stages of the gut regeneration and may restore finally, and that gene frequencies of cell proliferation, digestion and immunity were increased in the microbiome of regenerated intestines (Zhang *et al*., 2019). These studies suggest that sea cucumbers could be used as a model to investigate responses and contribution of microbiota to organ and/or body part regeneration.

Since the sea cucumber gut microbiota is constructed by repeated selective enrichment of ingested microbiota from environments, the fecal microbiota obtained from different time axis appear to differ each other due to changed environmental microbiota (Yamazaki *et al*., 2019). Thus, microbial communities of regenerated guts need to be compared with that of normal guts constantly

at every stage of the gut regeneration. In addition, as different physiological condition of sea cucumbers including growth rate could affect the gut microbial structures, inter-individual differences in the gut microbiota need to be considered (Sha *et al.*, 2016; Yamazaki *et al.*, 2016). However, previous studies considered neither changes of environmental microbiota nor inter-individual variation of the gut microbiota during the gut regeneration. Here, I developed both caged mariculture and laboratory feeding systems of sea cucumbers to track dynamics of the gut metagenomes derived from identical individuals during the gut regeneration, thereby performing longitudinal meta16S analyses of the fecal microbial communities from regenerated individuals and control ones.

Materials & Methods

The Caged Mariculture System and Sample Collection at Menagawa Site

I collected feces of sea cucumber *A. japonicus* from seven individuals, three individuals as a regeneration group and four individuals as a control group, by diving in a culture pond at the Menagawa site (41°45N, 141°5E), Hokkaido, Japan in December 2016. Fecal samples were immediately frozen on dry-ice and preserved at -80℃ in our laboratory until DNA extraction. I then removed guts of sea cucumbers mechanically, and all animals were individually put in animal cages on the bottom of the sea, numbering to identify each specimen (Fig. 4.1). I re-started to collect their feces four months after the evisceration treatments; the one sample from the regeneration group and three samples from the control group were collected in April; each three (regeneration and control) samples in May; each three (regeneration and control) samples in June; three (regeneration) and four (control) samples in July (Fig. 4.2).

The Laboratory Feeding System and Sample Collection

Five specimens of *A. japonicus* as a regeneration group and three specimens as a control group were fed with artificial diets containing powdered algae and diatomaceous earth in an incubator (13℃)

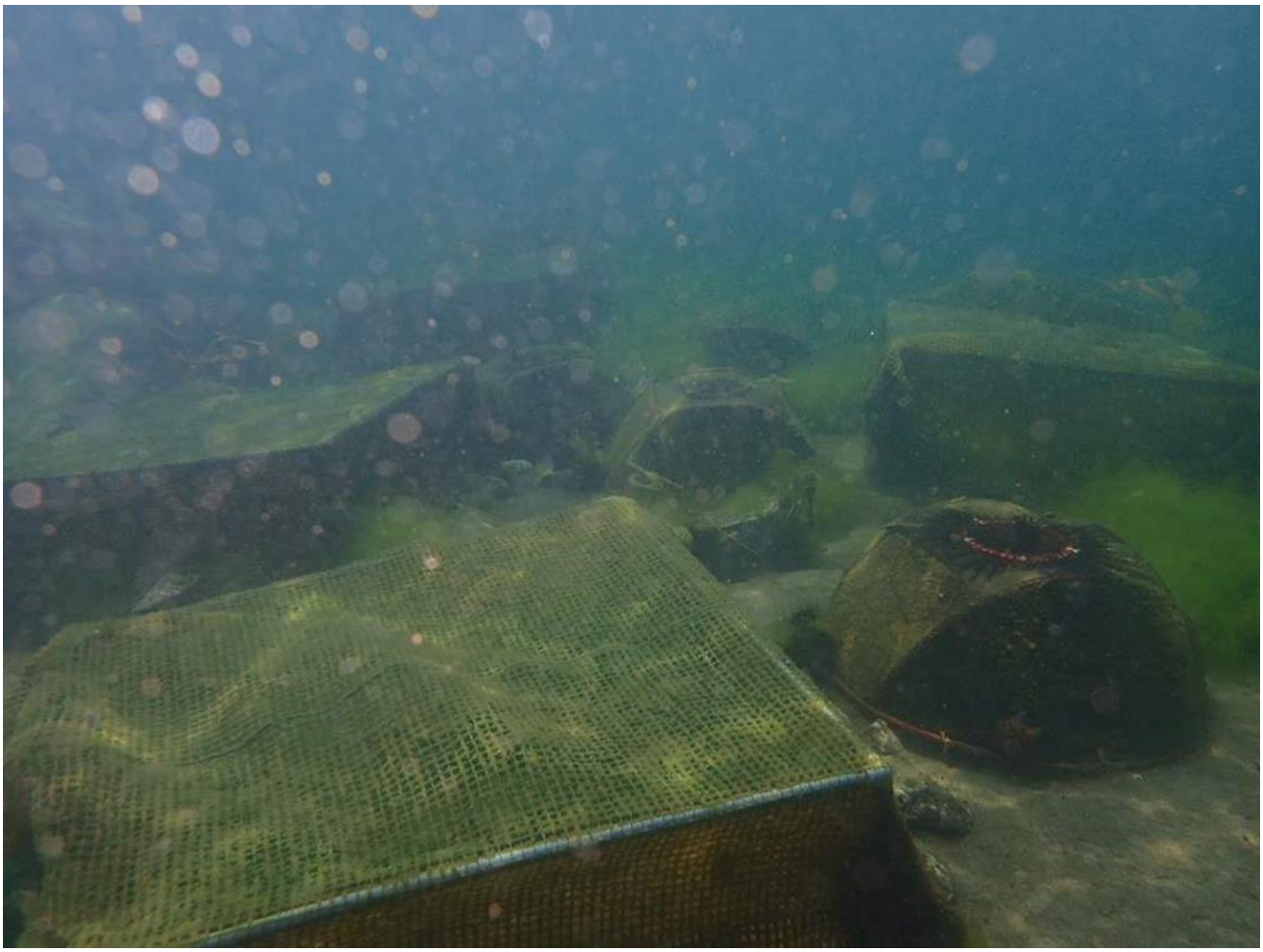


Fig. 4.1. The caged mariculture system of sea cucumbers in Menagawa.

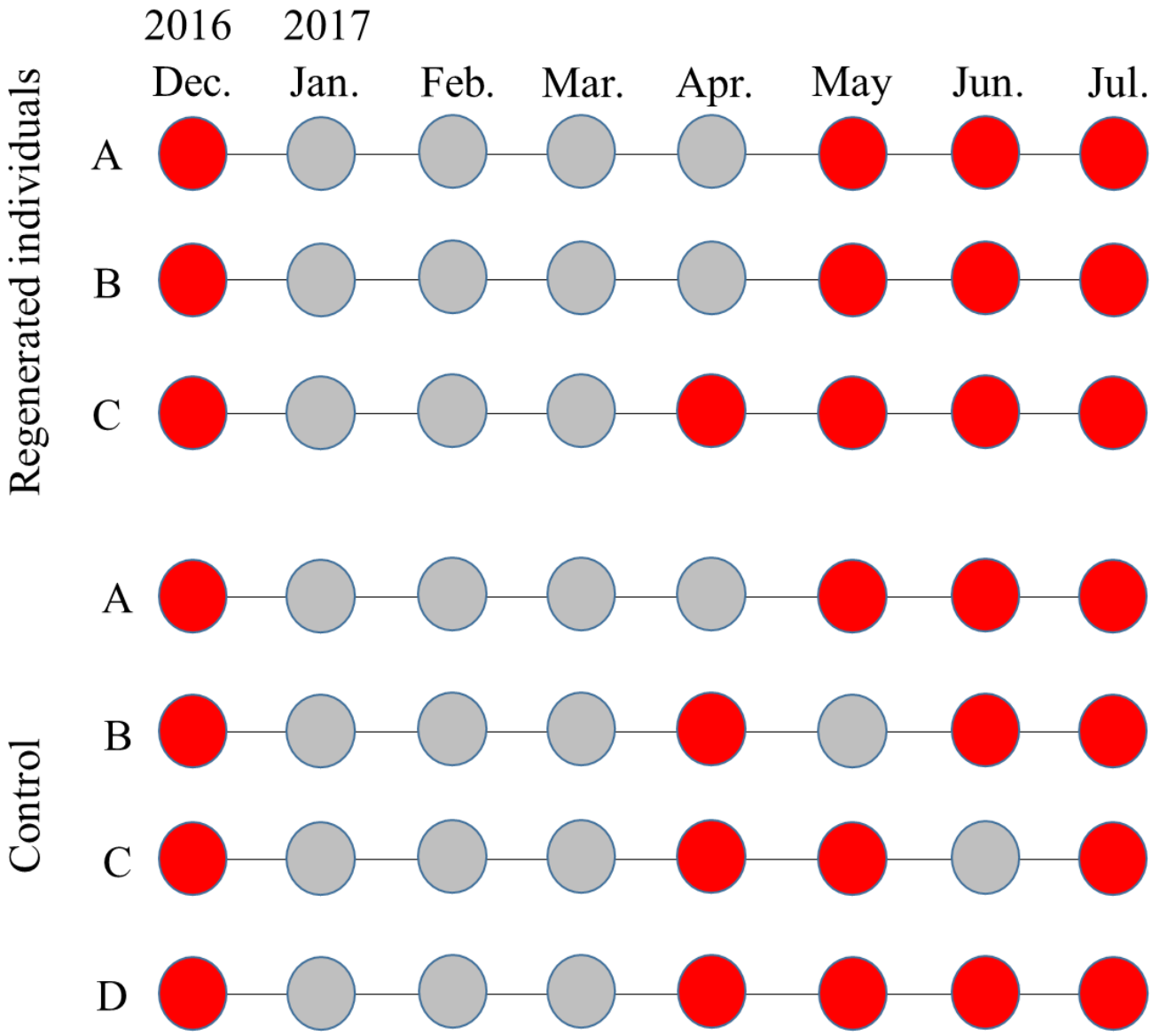


Fig. 4.2. Experimental design of the caged mariculture experiment. Fecal samples were collected in time points with marked red circles. I removed sea cucumbers' guts on December 2016. Their guts were already regenerated in April 2017.

(Unuma *et al*., 2013). Feces from these specimens were collected in 1.5 ml of sterilized tubes under a clean booth. These were immediately stored at -80°C until DNA extraction. After the sample collection, internal organs of the regeneration groups (*N*=5) were removed by injection of 0.35 M KCl into their body cavities. All specimens were reared in the incubator until gut is regenerated. Eviscerated guts were regenerated in 15 days (Regen-3, -4 and -5), 16 days (Regen-1) or 24 days (Regen-2) after the gut removal, and then I collected feces from eight specimens of sea cucumbers in two weeks (Fig. 4.3).

Microbial DNA Extraction

After rapid thawing of the fecal samples, microbial DNA extraction was performed using the NucleoSpin Soil Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol.

16S rRNA Gene Sequencing

16S rRNA gene sequencing was performed on extracted DNA samples. The hypervariable V1-V2 region of the 16S rRNA gene was amplified by PCR with 27Fmod and 338R primers containing barcode and Illumina adaptor sequences. PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amount of each PCR amplicon was mixed and then sequenced using MiSeq Reagent Kit v3 (600-cycles) on the MiSeq Illumina platform. Based on sample specific barcodes, obtained reads were assigned to each sample.

Quality Control and Taxonomic Assignment

The paired-end sequence data with quality scores (i.e. Fastq files) were analyzed using QIIME 2 (Bolyen *et al*., 2019). Quality controls (e.g. trimming primers and denoising sequences, removing chimeric sequences) and merging paired-end sequences were performed using DADA2 (Callahan *et al*., 2016).

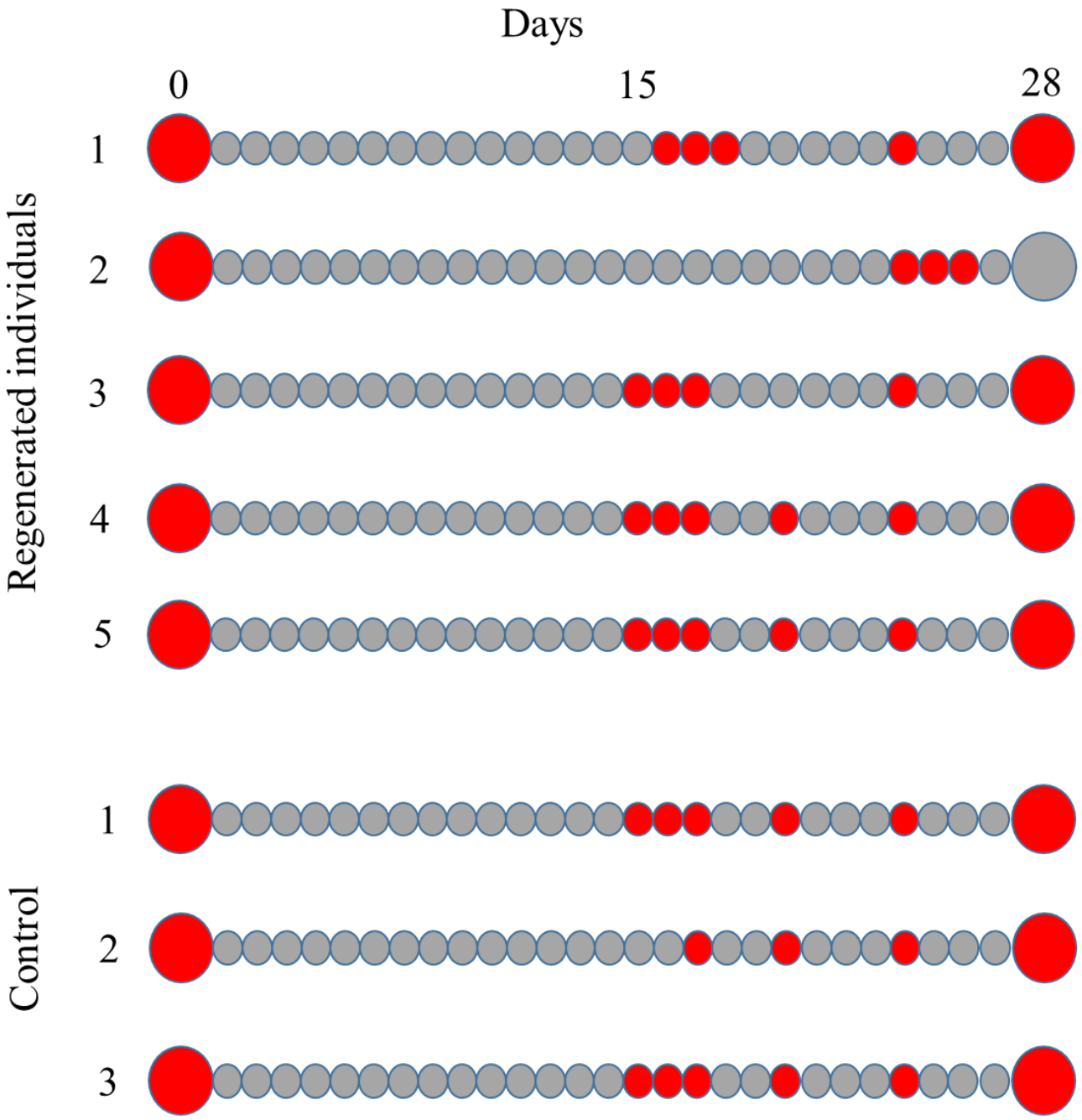


Fig. 4.3. Experimental design of the laboratory feeding experiment. Fecal samples were collected in time points with marked red circles. I removed sea cucumbers' guts at time-point 0. Guts of three specimens were regenerated at time-point 15, and those of other specimens were followed at later time points.

Reads with 100% similarity constituted an amplicon sequence variance (ASV). Unlike the method to cluster sequences into operational taxonomic units (OTUs) with fixed threshold (usually 97%), this quality control method using DADA2 allows us to detect even a single nucleotide difference. ASVs were assigned to taxonomy using the Naive Bayes classifier and Greengenes database.

In the caged mariculture experiment, a total of 1,049,891 paired-reads from 29 samples from seven individuals were obtained, and 519,815 bacterial reads (2,528 ASVs), 106,307 chloroplast reads (163 ASVs), and 5 mitochondria reads (1 ASV) were retained after quality control (Table 4.1). The chloroplast reads were further re-assigned to algal taxa using the Naive Bayes classifier and PhytoRef database (Decelle *et al*., 2015). The mitochondria reads were not used for the downstream analysis.

In the laboratory feeding experiment, a total of 2,470,596 paired-reads from 49 samples from eight individuals were obtained, and 1,767,900 bacterial reads, constituting 666 ASVs, were retained after quality control (Table 4.2). All eukaryotic reads were removed due to a small number of the reads (Table 4.2).

Diversity Analyses

Using subsampled reads (5,000 bacterial reads and 1,000 eukaryotic reads in the caged mariculture experiment, and 20,000 reads in the laboratory experiment), Bray-Curtis and unweighted UniFrac distances as beta diversity were calculated and visualized in PCoA plots (Lozupone *et al*., 2011) based on phylogenic tree generated by FastTree. Furthermore, in the laboratory experiment, I calculated Bray-Cutis distances of samples from time-point 0 to other time-points and compared them between regeneration and control groups by Mann-Whitney U test (FDR p-value < 0.05).

Relative Abundance Comparison

In the laboratory feeding experiment, features composed of over 500 reads were retained. I calculated difference of relative abundance of the features between time-point 0 and time-point 17 by

Table 4.1. Sample information in caged mariculture experiment

sample-id	specimen-id	Month	Regeneration	No. of raw reads	No. of qualified bacterial reads	No. of qualified chloroplast reads	No. of qualified mitochondria reads
ApF17	Cont-B	Apr	Control	23,501	5,859	9,461	0
ApF41	Cont-C	Apr	Control	26,915	6,463	11,577	0
ApF42	Regen-C	Apr	Regeneration	28,094	10,621	8,369	0
ApF51	Cont-C	Apr	Control	28,261	9,178	9,532	0
DF101	Cont-A	Dec	Control	30,207	16,717	1,847	0
DF102	Cont-C	Dec	Control	46,624	25,886	2,334	0
DF103	Cont-D	Dec	Control	36,146	20,189	2,184	0
DF16	Regen-A	Dec	Regeneration	25,152	15,012	499	0
DF17-170126	Cont-B	Dec	Control	33,116	16,720	1,117	0
DF18	Regen-B	Dec	Regeneration	24,115	14,450	819	0
DF42	Regen-C	Dec	Regeneration	26,471	15,628	342	0
JulyF15	Cont-A	July	Control	47,288	24,818	1,591	0
JulyF16	Regen-A	July	Regeneration	44,579	23,472	1,722	0
JulyF17	Cont-B	July	Control	41,111	21,876	723	0
JulyF18	Regen-B	July	Regeneration	41,880	21,375	1,369	0
JulyF41	Cont-C	July	Control	50,814	24,298	1,550	0
JulyF42	Regen-C	July	Regeneration	46,035	22,834	1,971	0
JulyF51	Cont-D	July	Control	40,304	21,342	1,816	0
JunF15	Cont-A	Jun	Control	35,627	19,486	2,311	0
JunF17	Cont-B	Jun	Control	30,872	16,565	1,301	0
JunF18	Regen-B	Jun	Regeneration	56,386	27,927	5,483	0
JunF42	Regen-C	Jun	Regeneration	60,535	33,770	2,534	0
JunF51	Cont-D	Jun	Control	29,306	16,520	1,817	0
MaF15	Cont-A	May	Control	48,389	17,776	9,745	0
MaF16	Regen-A	May	Regeneration	32,013	16,264	4,837	0
MaF18	Regen-B	May	Regeneration	27,195	13,164	3,604	0
MaF41	Cont-C	May	Control	32,090	15,751	5,413	0
MaF42	Regen-C	May	Regeneration	32,194	16,667	3,582	0
MaF51	Cont-D	May	Control	24,671	9,187	6,857	5

Table 4.2. Sample information in the laboratory feeding experiment

sample-id	Specimen-id	Group	Time-points	No. of raw reads	No. of qualified bacterial reads	No. of qualified eukaryotic reads
A10May19	Regen-1	Regeneraion	18	29,501	21,959	89
A16May19	Regen-1	Regeneraion	24	49,278	37,805	51
A20May19	Regen-1	Regeneraion	28	70,620	52,970	28
A22Apr19	Regen-1	Regeneraion	0	32,553	24,433	113
A8May19	Regen-1	Regeneraion	16	46,632	35,733	22
A9May19	Regen-1	Regeneraion	17	32,531	24,962	51
B16May19	Regen-2	Regeneraion	24	121,076	81,388	0
B17May19	Regen-2	Regeneraion	25	53,693	36,288	35
B18May19	Regen-2	Regeneraion	26	54,440	43,282	16
B22Apr19	Regen-2	Regeneraion	0	34,556	27,402	0
C16May19	Regen-3	Regeneraion	24	58,718	42,642	237
C20May19	Regen-3	Regeneraion	28	52,457	41,050	25
C22Apr19	Regen-3	Regeneraion	0	33,162	25,337	15
C7May19	Regen-3	Regeneraion	15	34,141	25,887	56
C8May19	Regen-3	Regeneraion	16	36,794	27,751	27
C9May19	Regen-3	Regeneraion	17	39,881	29,382	20
D12May19	Cont-1	Control	20	50,097	35,312	0
D16May19	Cont-1	Control	24	54,991	40,947	3
D20May19	Cont-1	Control	28	62,294	46,189	0
D22Apr19	Cont-1	Control	0	30,327	23,749	44
D7May19	Cont-1	Control	15	39,352	28,417	0
D8May19	Cont-1	Control	16	36,690	24,377	0
D9May19	Cont-1	Control	17	41,361	26,517	0
E12May19	Regen-4	Regeneraion	20	63,538	43,934	8
E16May19	Regen-4	Regeneraion	24	66,153	45,770	17
E20May19	Regen-4	Regeneraion	28	60,521	44,656	74
E22Apr19	Regen-4	Regeneraion	0	39,301	28,965	0
E7May19	Regen-4	Regeneraion	15	31,806	23,629	173
E8May19	Regen-4	Regeneraion	16	42,856	28,268	50
E9May19	Regen-4	Regeneraion	17	66,034	42,544	355
F12May19	Regen-5	Regeneraion	20	60,851	43,100	10
F16May19	Regen-5	Regeneraion	24	60,068	42,283	9
F20May19	Regen-5	Regeneraion	28	61,939	45,378	13
F22Apr19	Regen-5	Regeneraion	0	44,891	34,352	3
F7May19	Regen-5	Regeneraion	15	40,297	28,828	277
F8May19	Regen-5	Regeneraion	16	58,208	40,157	44
F9May19	Regen-5	Regeneraion	17	38,815	25,338	39
G12May19	Cont-2	Control	20	76,418	45,891	6
G16May19	Cont-2	Control	24	92,206	61,219	2
G20May19	Cont-2	Control	28	58,701	42,755	0
G22Apr19	Cont-2	Control	0	35,838	25,284	17
G9May19	Cont-2	Control	17	64,161	41,145	48
H12May19	Cont-3	Control	20	52,204	39,918	0
H16May19	Cont-3	Control	24	59,479	45,594	0
H20May19	Cont-3	Control	28	58,916	44,376	0
H22Apr19	Cont-3	Control	0	31,463	22,828	0
H7May19	Cont-3	Control	15	35,452	26,964	0
H8May19	Cont-3	Control	16	37,861	26,525	0
H9May19	Cont-3	Control	17	37,474	24,420	0

specimen, and visualized the difference using heat maps of the features aligned according to phylogenetic tree using FastTree. Specimen “Regen-2” was excluded in this analysis due to limited number of samples. Key features were defined as features of which the change in relative abundance from time-point 0 to time-point 17 were over 3.0% in at least two individuals from the regeneration group.

Results & Discussion

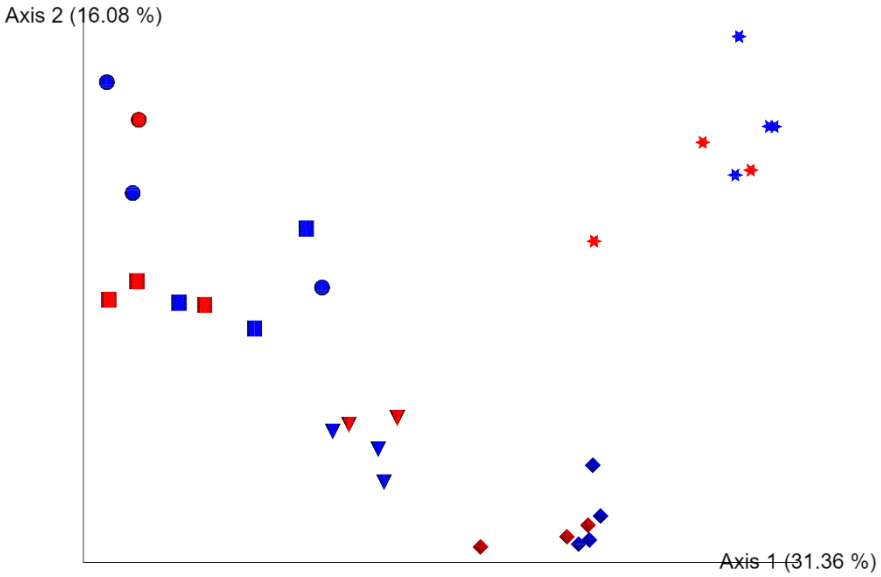
The Caged Mariculture Experiment

Bray-Curtis and unweighted UniFrac analyses showed that the fecal bacterial communities collected at same months were similar each other, while fecal bacterial communities of the regeneration group were not different from those of the control group (Fig. 4.4). Although I also compared fecal bacterial communities between the two groups by month, no significant differences were observed, which demonstrated that the normal bacterial communities were reconstructed within four months after the evisceration. Such short-time reconstruction of the gut bacterial communities might suggest immediate recovery of the holobiont functions to benefit the host from microbes in metabolizing ingested organic matter (Yamazaki *et al*., 2019). In addition to bacterial communities, gut eukaryotic communities were also not different between the regeneration and control groups based on both Bray-Curtis and unweighted UniFrac analyses (Fig. 4.5), indicating that feeding behaviors of sea cucumbers became active at that time. Unfortunately, a demerit of the cage mariculture system is limitation of sampling frequency. So, it is necessary to develop a laboratory feeding system to analyze early event occurred in the gut regeneration.

The Laboratory Feeding Experiment

I tracked dynamics of the fecal microbiota derived from identical specimens over one month and compared the results between regeneration (five individuals) and control groups (three individuals) (Fig. 4.3). Unweighted UniFrac analysis showed that bacterial communities were clearly different by

(A)



(B)

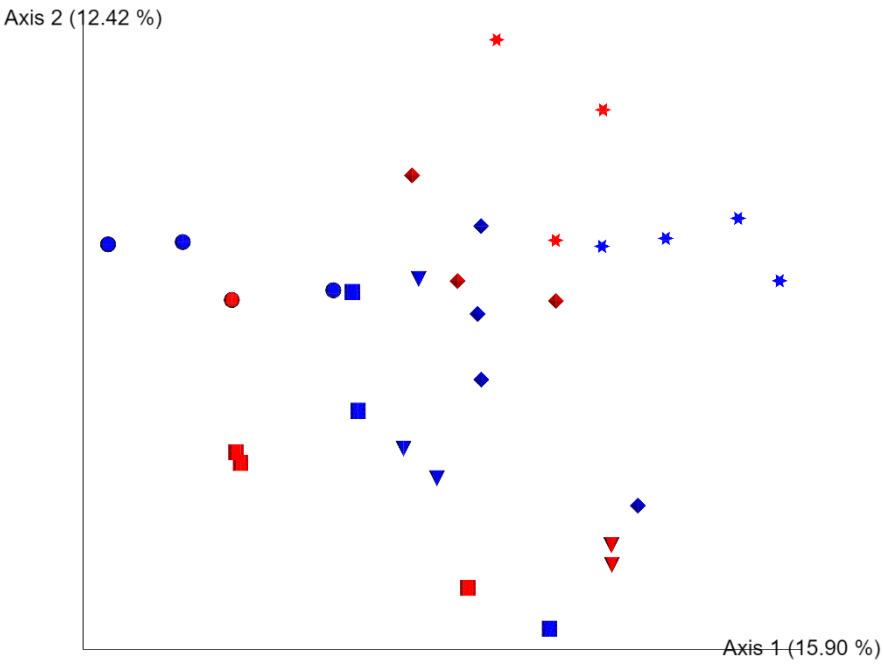


Fig. 4.4. Beta diversity analyses of the bacterial communities in the caged mariculture experiment. Red and blue colored symbols are regeneration and control groups, respectively. Star-shaped samples were collected in December, when we removed sea cucumber guts. Sphere-shaped samples were collected in April, and cylinders were in May, and cones were in June, and diamonds were in July. (A) Bray-Curtis PCoA plot. (B) Unweighted UniFrac PCoA plot.

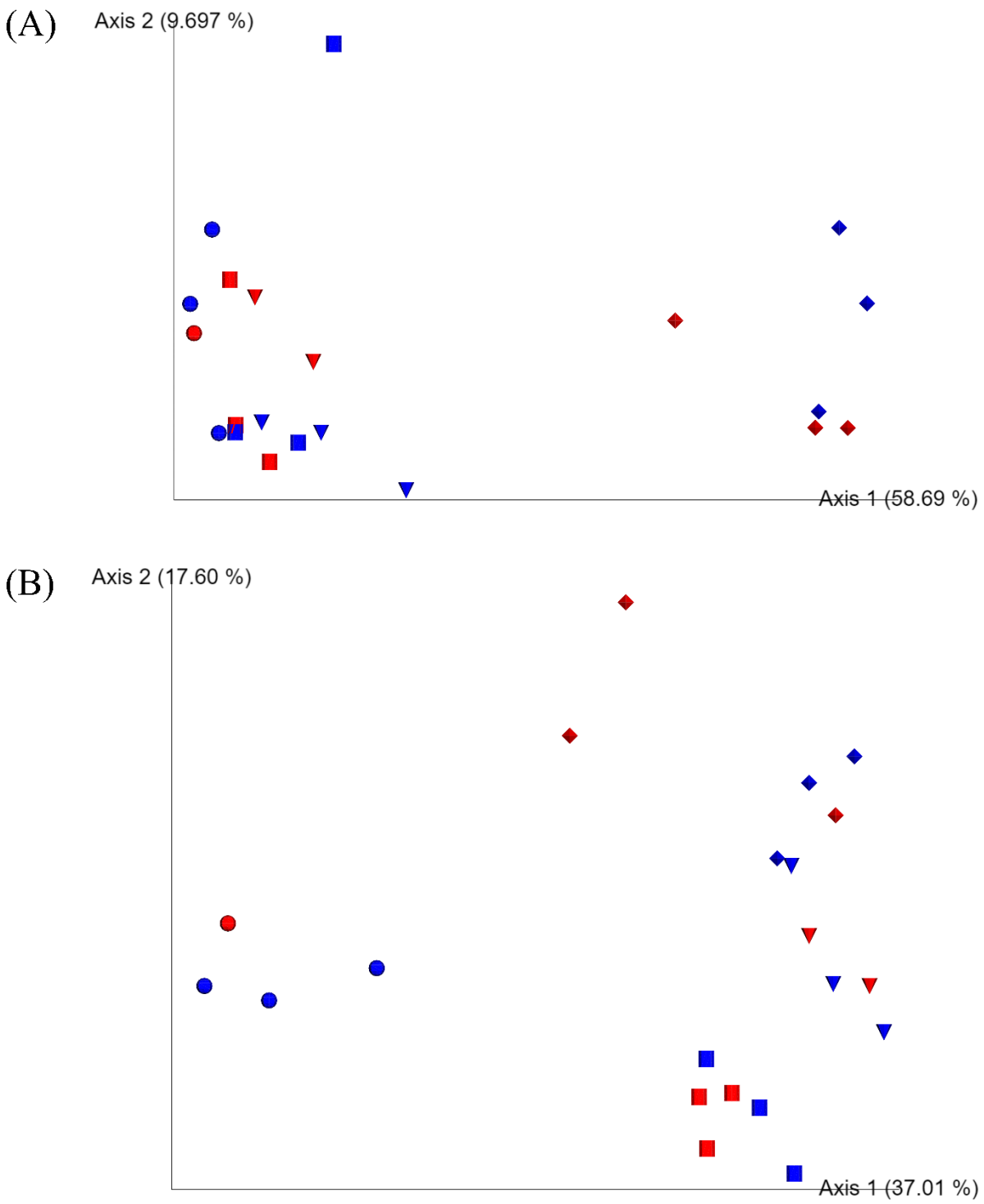


Fig. 4.5. Beta diversity analyses of eukaryotic communities in the caged mariculture experiment. Red and blue colored symbols are regeneration group and controls, respectively. Sphere-shaped samples were collected in April, and cylinders were in May, and cones were in June, and diamonds were in July. December samples were excluded due to limited number of eukaryotic reads. (A) Bray-Curtis PCoA plot. (B) Unweighted UniFrac PCoA plot.

specimen, and fecal microbiota collected before the evisceration were clearly different from those collected after the evisceration (Fig. 4.6). Although I expected that the individual fecal microbiota at same regeneration stage was similar each other as reported by Zhang *et al.* (2019), such trend was not observed in this study.

Comparison of the fecal bacterial composition by specimen at family level also showed inter-individual variation, although Rhodobacteraceae was the most abundant family in most specimens (Regen-1, 42.5±19.9%; Regen-2, 25.8±4.9%; Regen-3, 38.5±20.1%; Regen-4, 60.8±15.3%; Regen-5, 58.1±9.7%; Cont-1, 61.6±9.9%; Cont-2, 74.6±13.1%; Cont-3, 45.5±15.9%) (Fig. 4.7). Alteromonadaceae (Regen-1, 16.9±15.4%; Regen-2, 9.8±11.9%; Regen-3, 20.8±11.7%; Regen-4, 9.3±10.2%; Regen-5, 4.5±3.2%; Cont-1, 3.7±3.2%; Cont-2, 3.5±3.4%; Cont-3, 2.1±2.1%), Flavobacteriaceae (Regen-1, 2.0±2.2%; Regen-2, 18.2±28.2%; Regen-3, 12.6±13.8%; Regen-4, 0.2±0.3%; Regen-5, 3.0±3.8%; Cont-1, 0.6±0.5%; Cont-2, 0.2±0.2%; Cont-3, 4.8±9.1%), Oceanospirillaceae (Regen-1, 4.6±2.8%; Regen-2, 30.2±21.1%; Regen-3, 1.4±0.9%; Regen-4, 9.5±4.8%; Regen-5, 8.6±5.4%; Cont-1, 3.7±2.8%; Cont-2, 3.6±2.3%; Cont-3, 6.5±7.0%) were also abundant (Fig. 4.7). In addition, Colwelliaceae accounted for 43.1% of fecal microbiota of "Regen-1" at time-point 0, but this family decreased below 1% after the gut regeneration (Fig. 4.7). Similarly, Flavobacteriaceae and Rhodobacteraceae in specimens "Regen-2 and -3", respectively, were decreased after the gut regeneration. However, fecal microbiota of the control group were unlikely to be changed dramatically (Fig. 4.7).

In order to minimize the effects of inter-individual variation, I performed a longitudinal and paired sample comparison of the fecal microbiota. Calculation of Bray-Curtis distances from time-point 0 to other time-points as an indicator of a change of community composition showed that the mean distance in the regeneration group exceeded that in the control group at the all-time points (Fig. 4.8), suggesting that the gut regeneration impacted on the gut bacterial community structures. I also observed immediate increases after the gut regeneration (time-point 17) in eight key features, and notably, relative abundance of these features in the control group was not changed (Fig. 4.9).

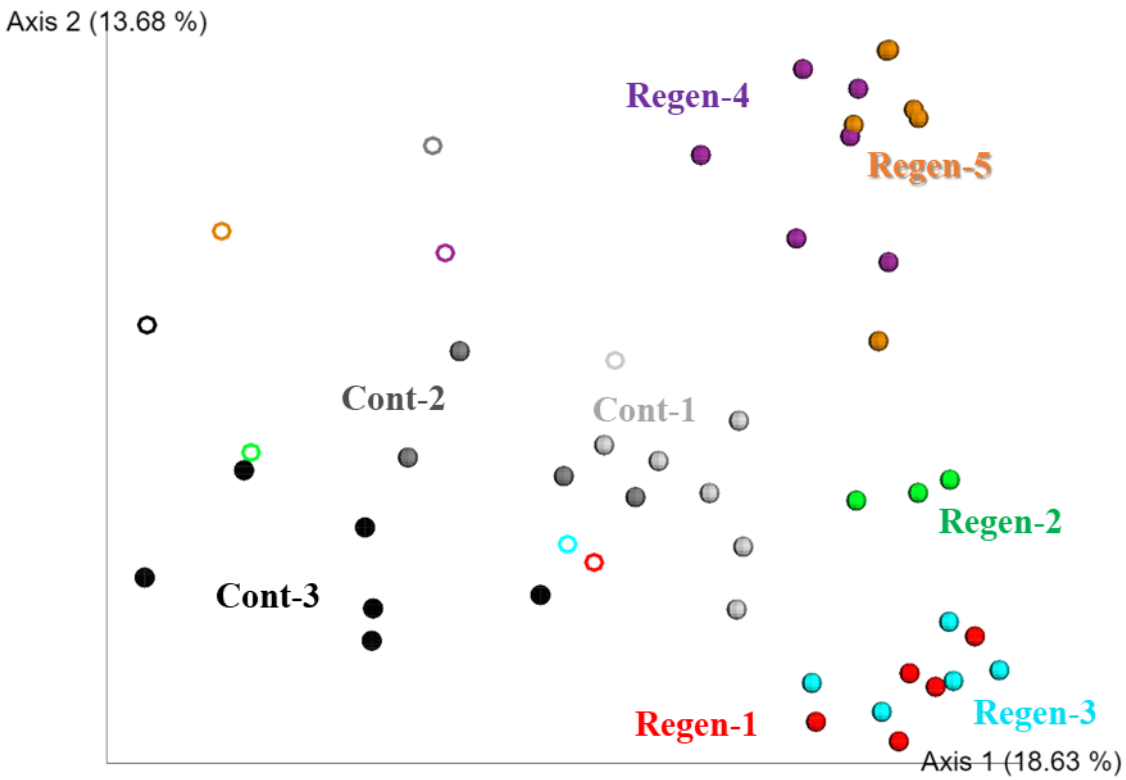


Fig. 4.6. Unweighted UniFrac distance-based 2D PCoA in the laboratory feeding experiment. Specimens in the regeneration group were indicated by "Regen-" and colored by different bright colors. Specimens in the control group were indicated by "Cont-" and in monochrome. Closed-circle samples are collected after regeneration, and open-circle samples are collected before evisceration.

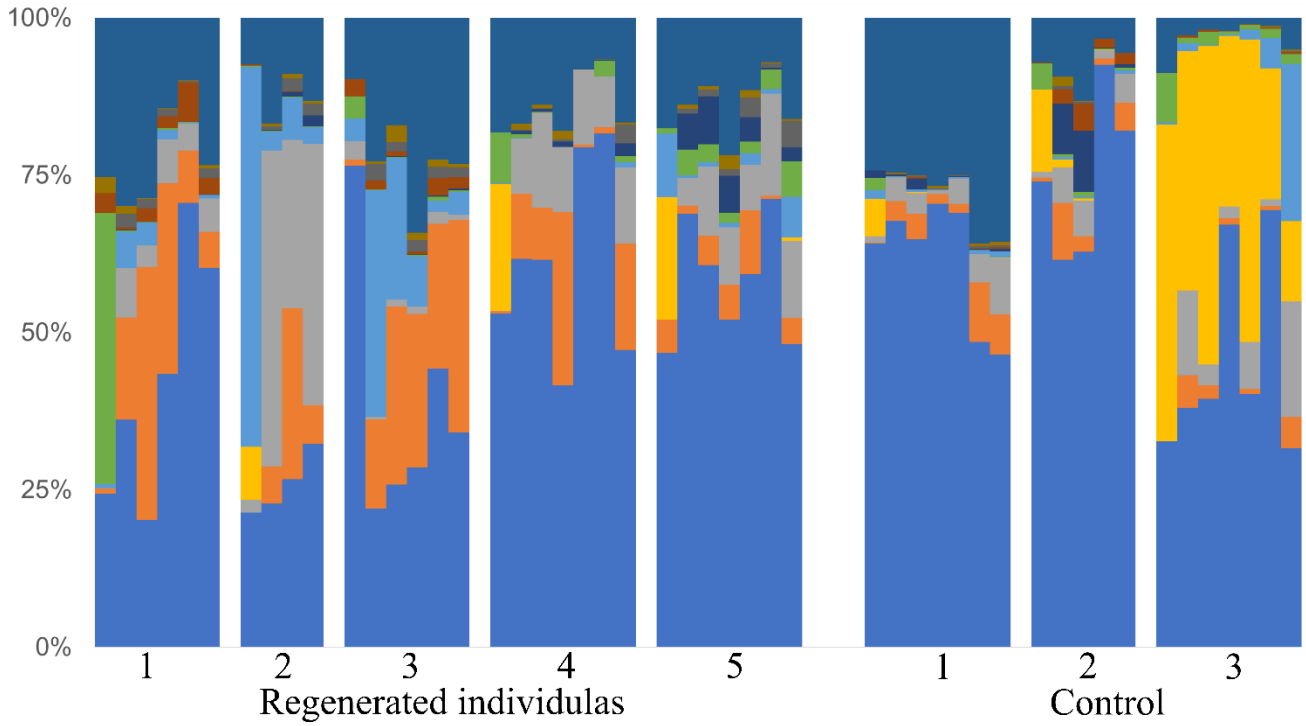


Fig. 4.7. Family Composition of bacterial communities. Top 10 families were shown in the bar plot. Minor taxa and unassigned reads were clustered into others.

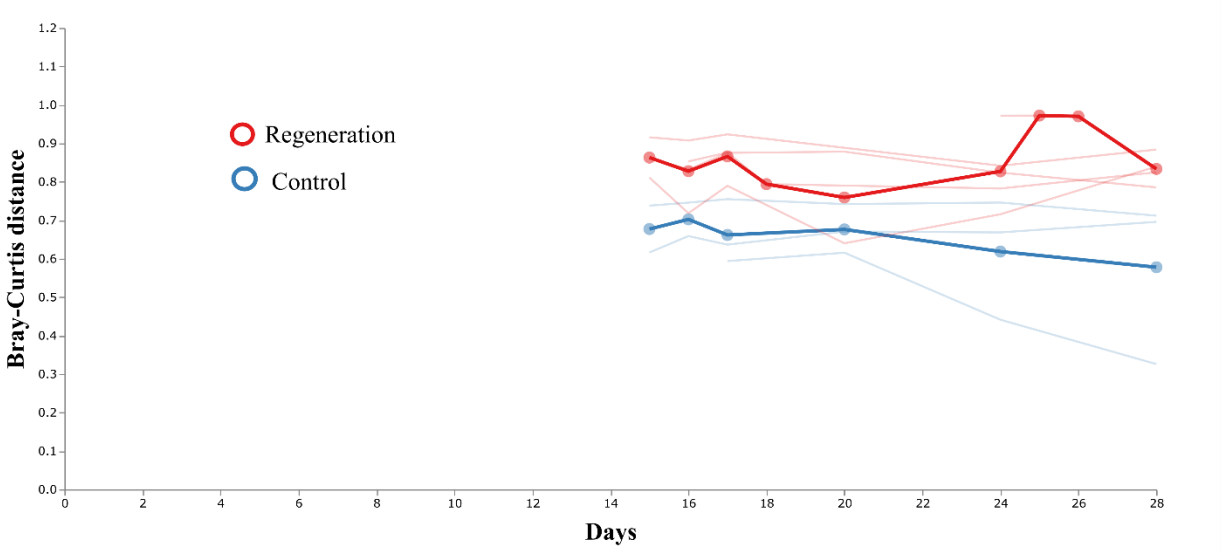


Fig. 4.8. Bray-Curtis distances of fecal microbiota from time-point zero to other time-points. Red and blue colored bold lines were mean Bray-Curtis distances from time point zero to other time points in regeneration group and control group, respectively. Pale colored lines showed each specimen.

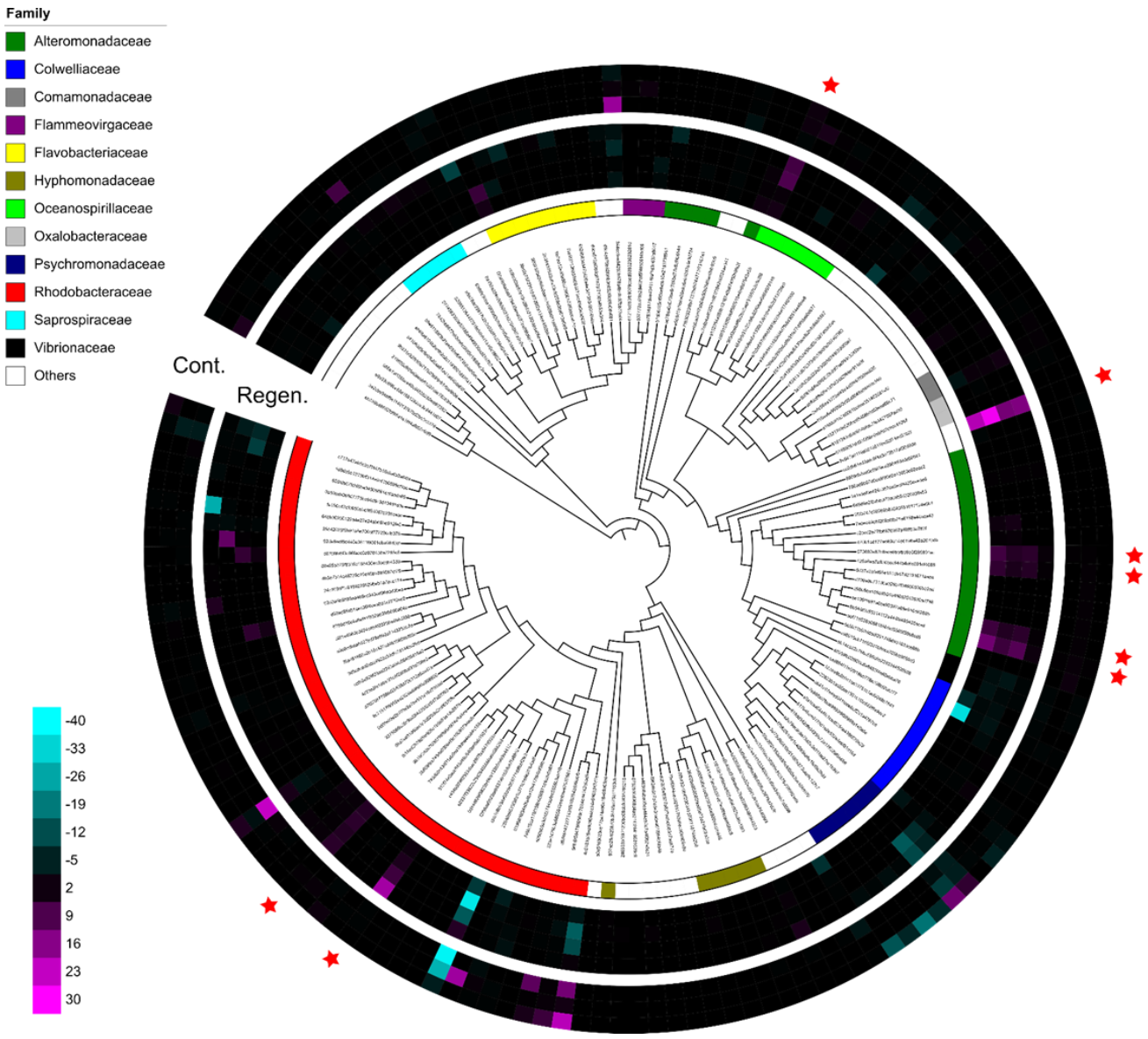


Fig. 4.9. Dendrogram-connected heatmaps features in the laboratory feeding experiment. Difference of relative abundance of features with > 500 reads between time-point zero and time-point 17 was calculated. The heat maps show four individuals from the regeneration group and three individuals from the control group, and specimen "Regen-2" was excluded due to limited number of samples. More vivid magenta corresponds to more abundant OTUs in time-point 17 than in time-point 0, and more vivid cyan corresponds to more abundant OTUs in time-point 0 than in time-point 17. Features were aligned by a maximum-likelihood tree of representative reads of each feature. The color bar shows family level affiliation of each feature. Unassigned and minor taxa were combined into others. Red stars indicate key features whose relative abundance was increased after evisceration in regeneration group.

A further longitudinal analysis showed that relative abundance of the key features was decreased at the late stage of the gut regeneration, indicating that these features correspond to the regeneration process (Fig. 4.10). One feature was assigned to Gammaproteobacteria (order unknown), two features were to Rhodobacteraceae, one was to Oceanospirillaceae and four features were to Alteromonadaceae (Fig. 4.10). Consistently, relative abundance of Rhodobacterales was significantly increased in gut microbiota of *A. japonicus* 14 and 21 days after evisceration in a previous study (Zhang *et al*., 2019). Rhodobacteraceae (order Rhodobacterales) was one of the key components of sea cucumber gut microbiota based on previous results that; Rhodobacterales bacteria were enriched in sea cucumber guts (Yamazaki *et al*., 2019); they were more abundant in larger size individuals than in smaller size ones (Yamazaki *et al*., 2016); potential probiotics, *Paracoccus marcusii*, have been reported based on effects on host growth and immune stimulation after treatment with diets (Yan *et al*., 2014; Yang *et al*., 2015a). The microbes might affect the gut regeneration process.

Conclusion

I developed both caged mariculture and laboratory feeding systems of sea cucumbers to track dynamics of the gut metagenome derived from identical individuals. In the caged mariculture experiment, I confirmed that bacterial and eukaryotic communities of sea cucumbers' guts are reconstructed within four months after evisceration. In the future, state-of-art biologging techniques will replace the caged mariculture tracking system. Moreover, the laboratory feeding experiment indicated that several bacteria belonging to Alteromonadaceae, Rhodobacteraceae, Oceanospirillaceae and family unassigned Gammaproteobacteria may correspond to the gut regeneration process. The results suggest that the laboratory experimental system might be a useful model to study dynamics and contribution of microbiota to animal regeneration processes. In the future, isolation and identification of uncultured microbes from the regenerating gut, and examination of effects of key microbes on the gut regeneration need to be performed. Furthermore, the individual tracking systems can be applied in studying host responses against environmental changes, and in mining probiotics candidates.

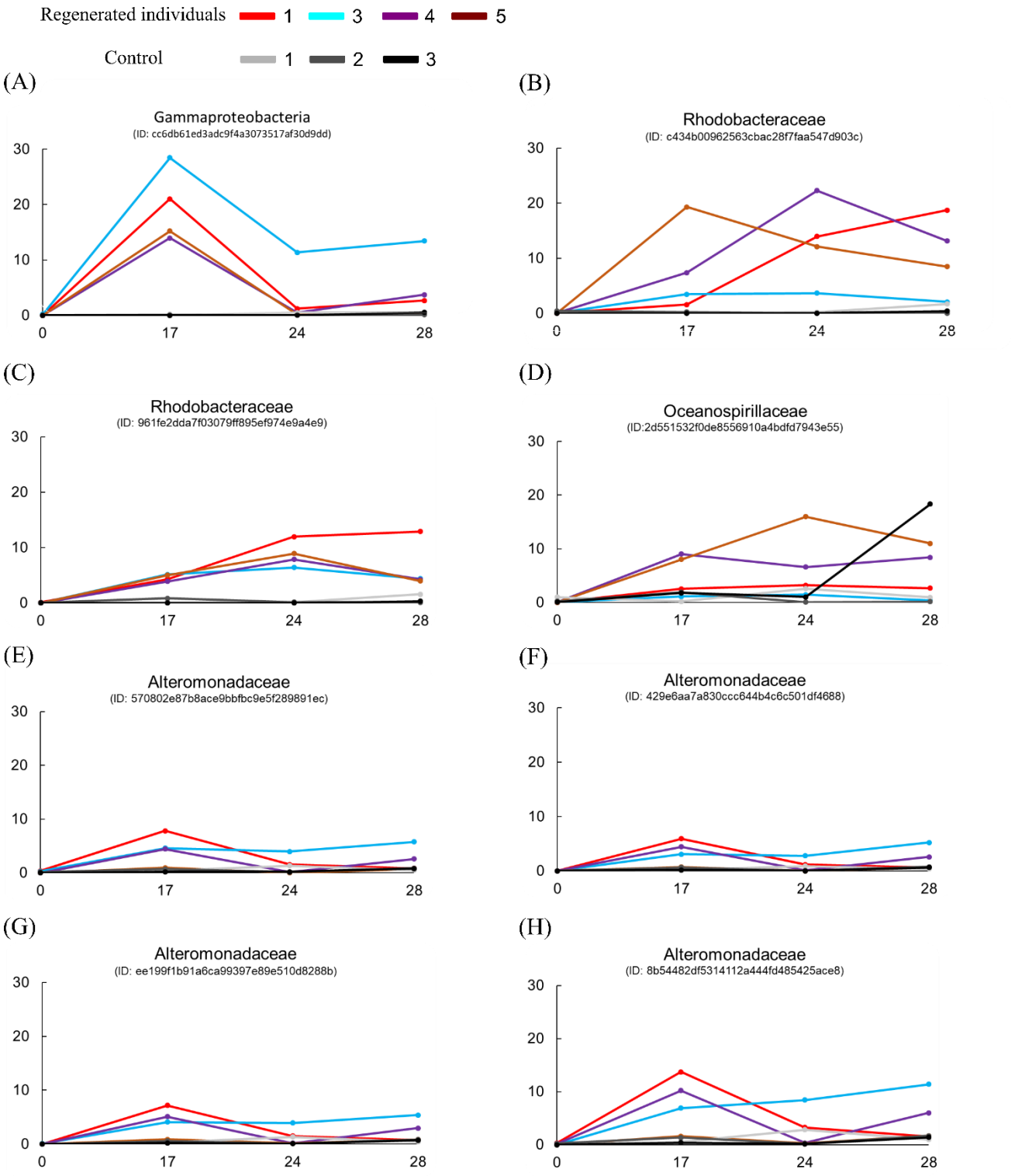


Fig. 4.10. Dynamics of relative abundance of key features based on the heatmap analysis. Eight key features were shown in (A)~(H), respectively. X axis is days, and Y axis is relative abundance (%) of each feature.

GENERAL DISCUSSION

Sea cucumbers are one of the most conspicuous marine benthos on sea floor and are distributed from tropical to polar regions, and from coastal to deep-sea areas. The animal is a well-known and excellent model of gut regeneration (Mashanov and García-Arrarás, 2011). Sea cucumbers rework huge amounts of sediments via ingestion and excretion (Uthicke, 1999), and their behaviors (i.e. bioturbation) have greatly affected biogeochemical processes and benthic biomes (Uthicke, 2001a; Schneider *et al*., 2011; MacTavish *et al*., 2012; Purcell *et al*., 2016). Despite of their ecological importance, wild stocks of some species, such as *A. japonicus* and *T. ananas*, have been dramatically reduced due to overfishing worldwide (Anderson *et al*., 2011; Conand *et al*., 2013a; Hamel and Mercier, 2013; Purcell *et al*., 2013). Although many studies have attempted to optimize feeding conditions on sea cucumber's growth to sustain wild stocks (Dong *et al*., 2006, 2008, 2010), huge growth variation of individuals and some diseases (e.g. skin ulceration) have prevented establishment of intensive and stable aquaculture. In addition, development of suitable artificial diets for sea cucumbers has not been advanced due to lack of knowledge on feeding behaviors of wild sea cucumbers (Gao *et al*., 2011; Sun *et al*., 2012a; Xia *et al*., 2012a; Shi *et al*., 2013a).

Recent studies demonstrate that gut microbiota play key roles in food digestion and absorption, nutrient supplement, energy homeostasis, immunity education and animal behaviors (Osawa *et al*., 1995; Ley *et al*., 2005; Turnbaugh *et al*., 2006; Hosokawa *et al*., 2006; Nikoh *et al*., 2011; Semova *et al*., 2012; Yin *et al*., 2013; Engel and Moran, 2013; Hsiao *et al*., 2013; Atarashi *et al*., 2015; Chevalier *et al*., 2015; Blyton *et al*., 2019). Moreover, based on accumulated knowledge on the human gut microbiota studies, bacteriotherapies including probiotics, prebiotics and more recently FMT have been developed to improve or maintain the human health and well-being (Suez *et al*., 2018; Zmora *et al*., 2018). Therefore, understating of sea cucumber gut microbiome could contribute to improvement of the aquaculture system.

Fundamental studies on the sea cucumber gut microbiota using a classical isolation strategy and clone library or 16S rRNA gene sequencing methodologies have been vigorously performed

(Roberts *et al*., 2001; Zhang *et al*., 2012, 2013; Plotieau *et al*., 2013; Gao *et al*., 2014; Yang *et al*., 2015b), and potential probiotics have been found to promote host growth and immunity (Sun *et al*., 2012b; Zhao *et al*., 2012; Chi *et al*., 2014; Yan *et al*., 2014; Yang *et al*., 2015a). However, seasonal changes and processes in shaping sea cucumber gut microbiota, and host-microbe interaction in the guts and mechanisms by which probiotics affect the host have not been clear due to lack of methodologies to analyze individual sea cucumber gut microbiota without any sacrifice of valuable wild sea cucumbers as well as to track responses of individual gut microbiota to extrinsic (e.g. temperature, probiotics) or intrinsic (e.g. host physiology) stimuli or changes.

In CHAPTER 1, I developed a non-destructive methodology to analyze sea cucumber gut microbiota using cultured animals. Using this method, I compared fecal microbiota of larger and smaller *A. japonicus* in body size and suggested that Rhodobacterales bacteria retaining PHB metabolism genes may contribute to promotion of the sea cucumber's growth. In CHAPTERs 2 and 3, I applied the non-destructive method to wild sea cucumbers. In CHAPTER 2, I compared bacterial communities of the feces and adjacent sediments through one whole year. The results showed that sea cucumber gut microbiota fluctuate by season according to changes of sediment microbiota and indicated that sea cucumber gut microbiota is constructed by repeated selective enrichment of ingested (sediment) microbiota and suggested that such enrichment process may contribute to the host ecological functions (e.g. sediment cleaning) through organic matter mineralization in their guts. In CHAPTER 3, I compared eukaryotic communities between feces and sediments through one whole year using datasets collected in the CHAPTER 2. The results suggested that *A. japonicus* may prefer Chaetocerotaceae and Laminariaceae algae rather than other algae. In CHAPTER 4, I developed caged mariculture and laboratory feeding systems of sea cucumbers to study responses of both the host and the microbiota to environmental changes or probiotics candidates. Focusing on the gut regeneration process in *A. japonicus*, I performed longitudinal analyses of gut microbiota derived from identical individuals during the regeneration process under the systems, suggesting that several bacteria belonging to Alteromonadaceae, Rhodobacteraceae, Oceanospirillaceae and family

unassigned Gammaproteobacteria correspond to the gut regeneration process.

Non-destructive Fecal Microbiome Analysis

Methodological innovation has been always required to open new doors in any academic fields. I developed a non-destructive methodology to analyze sea cucumber gut microbiota without any animal sacrifice. Notably, this method enabled us to analyze gut microbiota of endangered wild species i.e. *A. japonicus* and *T. ananas* without any sacrificing animals (Conand *et al*., 2013a; Hamel and Mercier, 2013). Furthermore, this method could be applied to the other aquatic animals to analyze and track their microbiota including sea urchins. However, on the other hand, this method could not analyze microbial community composition of gut mucosa or of different body parts. Indeed, recent human microbiota studies revealed that microbial communities of lumen and mucosa were different with feces, and that microbial communities varied through gastrointestinal tracts (Zmora *et al*., 2018).

Tracking Systems of Individual Sea Cucumber Gut Metagenome

I developed both caged mariculture and laboratory feeding systems to track sea cucumber gut metagenomes derived from identical individuals. The caged mariculture system is useful to study host-microbe interaction in the wild environment. As the system restrict animal behaviors in the cage, logger systems will replace caged mariculture for studies of wild sea cucumbers in the future. The laboratory feeding system enabled us to track dynamics of the gut microbiota during the gut regeneration and could be used to study host-microbe interaction in animal regeneration processes as a model. Furthermore, this system could be applied to test of probiotics effects on sea cucumber's growth and immunity and studies on host-microbe interaction during aestivation of *A. japonicus*. However, compared to individuals in the wild or in the farm, growth of individuals reared in this system is slow and not stable. Thus, optimization of feeding condition in this system on the growth is required.

Mode of Types Observed in Shaping Sea Cucumber Gut Microbiomes

Animals harbor resident microbial communities in the gut environment, which plays key roles in food digestion and absorption (Turnbaugh *et al*., 2006; Warnecke *et al*., 2007; Semova *et al*., 2012), immunity (Ivanov *et al*., 2009), development (Shin *et al*., 2011) and behavior (Hsiao *et al*., 2013). The first step of construction of the resident microbiota is the acquisition of microbes vertically (from parent) and/or horizontally (from environment) (Engel and Moran, 2013; Shapira, 2016). Next, matured gut microbiota develop through microbe-microbe and host (genetics)-microbe interaction (Benson *et al*., 2010; Goodrich *et al*., 2014). Exogenous microbes, e.g. food-borne and probiotic bacteria, can also be detected and distinguished from resident microbes (David *et al*., 2014; Zmora *et al*., 2018). On the other hand, this thesis indicates that sea cucumber fecal microbiota are shaped by repeated selective enrichment processes of ingested (sediment) microbes in their guts, suggesting that the fecal microbiota is mostly occupied by a transient population from the environment. Interestingly, the shaping process of sea cucumbers is likely to parallel with that of earthworms, which may suggest a variance of a convergence of construction in fecal microbiota between marine and terrestrial invertebrates, considering similar feeding behaviors between sea cucumbers and earthworms (Drake and Horn, 2007; Purcell *et al*., 2016).

Hammer and colleagues found that caterpillars lack a resident gut microbiome and proposed a “microbially independent lifestyle”, which may be widespread among animals (Hammer *et al*., 2017). Do sea cucumbers as well as earthworms also have microbially independent lifestyles like caterpillars? Both sea cucumbers and caterpillars harbor microbes ingested with food in their guts and are unlikely to harbor host-specific, resident symbionts. However, there are several different characteristics between sea cucumbers and caterpillars. Compared to the density of bacterial 16S rRNA gene copies in leaf caterpillars ingest, that in caterpillar feces decreased by one-hundredth, reflecting the high pH (>10) in the guts (Hammer *et al*., 2017). Total number of microbial cells in feces of sea cucumbers and earthworms was higher or equivalent compared to that in ingested sediments or soil (Drake and Horn, 2007; Plotieau *et al*., 2013). pH in sea cucumber’s guts has not

been reported, but pH in earthworm guts were 6.4-7.7 (Drake and Horn, 2007). In addition, proportion of plant sequences from caterpillar's feces accounted for >75% in average (Hammer *et al*., 2017). Although I also detected eukaryotic sequences in sea cucumber's feces, the eukaryotic fraction accounted for mean 15.8% (Yamazaki *et al*., 2019). Furthermore, Hammer and colleagues tested whether transient microbes might still contribute to feeding and development using antibiotics treatment, showing no significant effect on caterpillar weight gain, development, or survival. On the other hand, several bacteria isolated from the gut contents of a sea cucumber (*A. japonicus*) improved the host's growth and immunity, and these isolates produced extra-cellular amylase, lipase and protease (Chi *et al*., 2014). Therefore, I suggest that sea cucumbers are not microbially independent from the gut microbiota.

Key Bacterial Groups in Sea Cucumber Gut Microbiota

Rhodobacterales

Rhodobacteraceae, one of the major subdivisions of Alphaproteobacteria, include >100 genera and >300 species with highly diverse physiology (Pujalte *et al*., 2014). The first described members, *Roseobacter litoralis* and *Roseobacter denitrificans*, were pink-pigmented bacteriochlorophyll *a*-producing strains isolated from marine algae, although subsequent cultivation demonstrated that many strains within this clade are neither pink nor bacteriochlorophyll *a* producers (Buchan *et al*., 2005). Many Roseobacter-clade strains can metabolize dimethyl-sulfoniopropionate produced by algae, thus playing a key role in biogeochemical cycling (Buchan *et al*., 2005). Moreover, these bacteria are associated with algae (Ashen and Goff, 2000; Alavi *et al*., 2001; Wagner-Döbler *et al*., 2010; Sonnenschein *et al*., 2017). For instance, *Dinoroseobacter shibae* comprises symbionts of toxic dinoflagellates and provide the host with vitamins B1 and B12, which is essential for the host algae (Wagner-Döbler *et al*., 2010; Sañudo-Wilhelmy *et al*., 2014). The clade members are also associated with the reproductive accessory nidamental glands of the squid *Loligo pealei* and the cuttlefish *Sepia officinialis* and *Euprymna scolopes* (Grigioni *et al*., 2000; Barbieri *et al*., 2001;

Collins *et al*., 2015; Simon *et al*., 2017). More recent studies reported that *Octadecabacter* sp. was the dominant subcuticular symbiont in brittle star *Amphipholis squamata* (Morrow *et al*., 2018).

Consistent with previous studies (Enomoto *et al*., 2012; Yang *et al*., 2015b), Rhodobacterales was found to be an abundant member of sea cucumber gut microbiota in this study. Rhodobacterales bacteria might be ingested from algal detritus by sea cucumbers, because many *Roseobacter* species live as epibionts on marine algae (Buchan *et al*., 2005). Ingestion of a more algal diet in larger size sea cucumbers than in smaller ones may contribute to the difference in the relative abundance of Rhodobacterales between larger and smaller individuals in this thesis. In marine vertebrates, Rhodobacterales was not abundant in gut microbiota of well-studied fish including zebrafish and salmon (Llewellyn *et al*., 2016; Stephens *et al*., 2016), while Rhodobacterales bacteria were abundant in the gut of surgeonfish *Ctenochaetus striatus* (Miyake *et al*., 2015) and white leg shrimp *Litopenaeus vannamei* (Huang *et al*., 2016; Dai *et al*., 2018; Fan *et al*., 2019; Liu *et al*., 2019). Given that both *Ctenochaetus striatus* and *Litopenaeus vannamei* are referred to as detritus feeders (Varadharajan and Pushparajan, 2013), abundance of Rhodobacterales in animal gut microbiota might be related to feeding habits.

Arylsulfatase, which cleave sulfates from phenolic compounds, is a key characteristic of marine Rhodobacteraceae (Shvetsova *et al*., 2015; Simon *et al*., 2017). Marine Rhodobacteraceae colonize fucoidan-rich algae such as *Fucus* spp., and arylsulfatase could be used to breakdown fucoidan in detritus and algae. Rhodobacterales might promote the digestion of algae in sea cucumber's guts combined with the host enzyme, giving benefits to both the host and other microbes. Interestingly, a fucoidan is extracted from sea cucumber's body wall (Chang *et al*., 2010). Whether fucoidan is also extracted from the guts, and whether Rhodobacterales bacteria degrade the host polysaccharide need to be examined in further studies. Such host mucus degrading bacteria, for example *Akkermansia muciniphila*, have play key roles in energy homeostasis and wound healing in mouse guts (Chevalier *et al*., 2015; Alam *et al*., 2016).

Vascular plant-derived aromatic compounds, such as lignin, are often a significant

component of the carbon pool in coastal environments where roseobacters are abundant (Moran and Hodson, 1994). A gene encoding a key ring-cleaving enzyme of the β-ketoadipate pathway (*pcaH*) was identified in 16 of 19 roseobacter strains by a PCR assay (González *et al*., 1996; Buchan *et al*., 2000, 2001). In addition, the evidence of aromatic compound degradation has been uncovered in 7 of the 41 major roseobacter lineages (Buchan *et al*., 2001). These findings indicate that many roseobacters are able to utilize aromatic compounds as primary growth substrates (Buchan *et al*., 2005). As such aromatic compound-containing substrates are difficult to decompose for host animals, Rhodobacterales may play a key role in the digestion of aromatic compound-containing substrates in sea cucumber guts and contribute to the sediment cleaning function by sea cucumbers (Purcell *et al*., 2016).

Vibrionales

Vibrio, facultative anaerobic bacteria, is often recognized as a pathogen in aquatic animals. However, commensal or symbiotic interaction between vibrio and hosts could be established. *Aliivibrio fischeri* and bobtail squid symbiosis is the best-known example of such symbiosis; *A. fischeri* colonize the squid light-organs through the duct and produce luminescence (Nyholm and McFall-Ngai, 2004). *V. halioticoli*, the dominant bacterial species in the guts of abalone *Haliotis discus*, might contribute to digestion of alginate containing diets such brown algae in their guts (Sawabe *et al*., 1998; Tanaka *et al*., 2002). Abundance of Vibrionales in the gut microbial community of *A. japonicus* is controversial; some studies reported that vibrio was the dominant taxa (Enomoto *et al*., 2012; Gao *et al*., 2017), but others reported vibrio was absent or in low abundance (Zhang *et al*., 2013; Gao *et al*., 2014; Sha *et al*., 2016). Vibrionales bacteria were over-represented in sea cucumber guts in September but not in other months in this thesis (Yamazaki *et al*., 2019), suggesting that interesting results in the abundance of vibrios might arise from seasonal variation of the gut microbiota.

Vibrios might provide sea cucumbers with organic acids through fermentation in the guts. Short chain fatty acids (SCFAs), in particular, acetate, propionate and butyrate, are major products of

microbial fermentation. SCFAs are a major energy resource in ruminants, and previous estimations indicated SCFAs yields ~10% of human energy requirements (Bergman, 1990). In addition to serving as energy resources for animals, SCFAs function as ligands for G protein-coupled receptors (GPCRs) and as histone deacetylases (HDACs) inhibitors in humans, which play a key role in host metabolism, immunity and nervous systems (Koh *et al*., 2016). Furthermore, recent studies revealed lactate produced by gut microbiota, including *Lactobacillus* and *Bifidobacterium* spp., contributes to acceleration of intestinal epithelial cell regeneration (Lee *et al*., 2018). In earthworms which share similar processes of gut microbiota construction with sea cucumbers, the activity of fermentative bacteria is stimulated through providing anaerobic habitats and mucus, and support organic matter digestion (Horn *et al*., 2003; Drake and Horn, 2007; Wüst *et al*., 2011). Vibrios might play similar roles within sea cucumber guts.

Alteromonadales

Within Gammaproteobacteria, Alteromonadales was also abundant in sea cucumber guts, which includes various families such OM60, Psychromonadaceae, Colwelliaceae, Shewanellaceae, and Alteromonadaceae. *Shewanella* was the dominant genus in sea cucumber gut microbiota (>1%) in winter and spring in this thesis and was easily isolated from sea cucumber's feces and guts (Enomoto *et al*., 2012). Interestingly, a previous study proposed *Shewanella japonica* as potential probiotics in sea cucumber *A. japonicus* based on growth promotion and immune stimulation (Chi *et al*., 2014). Considering the heterotrophic metabolism in *Shewanella*, similar to *Vibrio*, they could contribute to detritus digestion through extracellular enzyme production in sea cucumber guts (Chi *et al*., 2014).

Sulfate-Reducing Bacteria

Sulfate-reducing bacteria (SRB) are facultative or strictly anaerobic microbes which use sulfate as a terminal electron acceptor in the decomposition of organic compounds (Muyzer and Stams, 2008). Previous estimations indicated that sulfate reduction can account for >50% of the organic carbon

mineralization in marine sediments (Jørgensen, 1982), which highlights the importance of SRB in both sulfur and carbon cycles. Desulfobacterales (Deltaproteobacteria), members of SRB, appear to be a key component in sea cucumber gut microbiota, as *Desulfosarcina* (Desulfobacterales) were abundant in the hindgut contents of *A. japonicus* (Gao *et al*., 2014), and Desulfobacterales bacteria were more abundant in sea cucumber's guts than in adjacent sediments (Yamazaki *et al*., 2019), and this order was more abundant in larger *A. japonicus* individuals than in smaller ones (Yamazaki *et al*., 2016). Conversely, Thiotrichales, consisting of diverse sulfur oxidizing bacteria, were more abundant in sediments than in the feces throughout one whole year. These findings indicate that sea cucumber guts form anaerobic environments, which is consistent with earthworm's gut referred to as a mobile anoxic microzone (Drake and Horn, 2007).

SRB are an indigenous member of human and mouse gut microbiota, the common genera of SRB in humans are *Desulfovibrio* (main genus), *Desulfobacter*, *Desulfobulbus*, and *Desulfotomaculum* (Rey *et al*., 2013; Barton *et al*., 2017). Few *Desulfovibrio*, *Desulfobacter* and *Desulfobulbus* bacteria were found in sea cucumber guts (Yamazaki *et al*., 2019). *Desulfococcus* were the most abundant in sea cucumber guts among Desulfobacteraceae (Desulfobacterales) (Yamazaki *et al*., 2019). Excessively high levels of H_2S produced by SRB contribute to slow transit, regressive autism, impaired maze performance in mice and colonic pathology including inflammatory bowel diseases (Finegold *et al*., 2012; Ritz *et al*., 2016, 2017; Barton *et al*., 2017), while recycling of sulfur contributed by SRB and continuous presence of H_2S at normal level triggers beneficial physiological responses in healthy tissues (Barton *et al*., 2017). Since H_2S per se is a highly reactive gas with various physiological functions in mammals, such ulcer healing, gastroprotection and suspended animation including hibernation (Blackstone *et al*., 2005; Wallace *et al*., 2007; Guo *et al*., 2014), future studies may disclose the contribution of SRB to such functions (Barton *et al*., 2017). In sea cucumbers, Desulfobacterales bacteria were more abundant in larger individuals than in smaller ones, and thus they are not recognized as pathogen or harmful microbes for sea cucumbers. Combined with digestive activity of Rhodobacterales bacteria probably using

arylsulfatase, SRB may contribute to the degradation of sulfur-containing detritus in sediments (Buchan *et al*., 2005). In addition, since aestivation occur in sea cucumbers at high water temperature (Gao *et al*., 2008), whether H_2S produced by SRB can induce the aestivation is an attractive topic for future studies (Blackstone *et al*., 2005).

Flavobacteriales

Flavobacteriales was one of the most dominant orders in sea cucumber gut microbiota in this thesis, which is consistent with previous studies of *A. japonicus* (Yang *et al*., 2015b, 2017). In other aquatic animals, Flavobacteriales was abundant in gut microbiota of oriental river prawn *Macrobrachium nipponense* and white leg shrimp *L. vannamei* (Chen *et al*., 2017; Fan *et al*., 2019). Interestingly, previous studies of crustacean *Daphnia magna* showed that "tolerant crustaceans" to toxic cyanobacteria were occupied by Flavobacteria (>70%), but "susceptible crustaceans" were done by Betaproteobacteria (>70%) (MacKe *et al*., 2017). The authors suggested that flavobacteria contribute to tolerance to toxic cyanobacteria through lysis of *Microcystis* cells and degradation of intracellular the components (Maruyama *et al*., 2003; Eiler and Bertilsson, 2004; MacKe *et al*., 2017). Moreover, such degradation of cyanobacteria in crustacean guts might provide additional nutrients to the host (MacKe *et al*., 2017). Since cyanobacteria is one of the important food sources of sea cucumbers (Purcell *et al*., 2016), Flavobacteria may also be beneficial for digestion of cyanobacteria in sea cucumber guts.

Towards the Application of these Scientific Observations

Bacillus subtilis is both the first and the most reported potential probiotic in sea cucumbers, which promotes growth and stimulates immunity (Zhang *et al*., 2010; Sun *et al*., 2012b; Zhao *et al*., 2012). This bacterial species was chosen based on beneficial effects on other aquatic animals as probiotics in previous studies, for example, in penaeid shrimp (Moriarty, 1998; Leonel Ochoa-Solano and Olmos-Soto, 2006), black tiger shrimp *Penaeus monodon* (Rengpipat *et al*., 1998, 2000), rainbow

trout *Oncorhynchus mykiss* (Kim and Austin, 2006) and Tilapia *Oreochromis niloticus* (Pirarat *et al*., 2006). Although *B. subtilis* has been isolated from sea cucumber's guts (Zhao *et al*., 2012), a previous meta16S analysis showed relative abundance of genus *Bacillus* was below 0.1% in fore- and hind-guts as well as sediments (Gao *et al*., 2014). Consistently, few members of the genus *Bacillus* or even Firmicutes were observed in *A. japonicus* guts over one whole year in this thesis. Thus, *Bacillus* may not be able to successfully colonize sea cucumber's guts. However, as many *Bacillus* spp. such as *B. amyloliquefaciens* DSM7, *B. laterosporus*, *B. licheniformis*, *B. macerans*, *B. cereus*, *B. circulans*, *B. firmus* G2, *B. subtilis* K8, *B. sphaericus* X3, *B. megaterium* Y6, *B. coagulans*, *B. brevis*, *B. sphaericus* ATCC 14577, *B. thuringiensis*, *B. mycoides* RLJ B-017 could produce PHA (Singh *et al*., 2009), *Bacillus* bacteria might provide additional energy source with sea cucumbers via PHA, similar to Rhodobacterales (Yamazaki *et al*., 2016).

Chi *et al*. (2014) selected three potential probiotics in sea cucumbers, i.e. *Pseudoalteromonas elyakovii*, *Shewanella japonica* and *Vibrio tasmaniensis* based on 1) extracellular-enzyme producing capacity (amylase, lipase and protease), 2) no pathogenicity both in hemolytic assays on sheep blood agar plates and in immersion of sea cucumbers in the bacterial suspension, and 3) beneficial effects on the host's growth, survival rate and immunity. These bacteria could contribute to detritus digestion through producing extracellular enzymes in the guts (Yamazaki *et al*., 2019). *Pseudoalteromonas* (Pseudoalteromonadales, Gammaproteobacteria) and also *Shewanella* (Alteromonadales, Gammaproteobacteria) are easily isolated from sea cucumbers' guts (Enomoto *et al*., 2012). The relative abundance of *Shewanella* was > 1% in sea cucumber guts in winter and spring in our previous studies (Yamazaki *et al*., 2019). *Vibrio* was abundant in fecal microbiota of wild sea cucumbers in summer and autumn (Yamazaki *et al*., 2019). Thus, these probiotics candidates may colonize sea cucumbers' guts more easily than *Bacillus*.

Paracoccus marcusii, belonging to Rhodobacterales, isolated from *A. japonicus* gut, has been proposed as a potential probiotic based on growth promotion and immune stimulation effects. In addition, within Rhodobacteraceae, *Phaeobacter* spp. (e.g. *P. gallaeciensis*) can be a new

candidate for sea cucumber probiotics, because these bacteria produce the antibacterial compound tropodithietic acid (TDA) and prevent vibriosis in cod larvae in the ponds (D'Alvise *et al*., 2012, 2013). Rhodobacterales is one of the most important components in sea cucumber gut microbiota. For example, this group is more abundant in the feces than in sediments and more abundant in larger sea cucumbers' guts than in smaller ones (Yamazaki *et al*., 2016, 2019), Rhodobacterales bacteria may affect the gut regeneration in *A. japonicus*. However, most Rhodobacterales sequences were not assigned to any known taxa below genus (Yamazaki *et al*., 2019). Thus, not-yet cultivated bacteria within Rhodobacteraceae may be attractive probiotics candidates, and they need to be isolated via new techniques such as culturomics (Lagier *et al*., 2018).

Not only bacteria itself but also its products could be used as a supplement to promote animal growth and/or strengthen immunity. The metagenome sequencing analysis in this thesis showed that the frequency of PHB metabolism genes was higher in the largest size sea cucumbers than in the smallest ones (Yamazaki *et al*., 2016). In other aquatic animals, PHB treatment resulted in beneficial effects including growth promotion, immune stimulation and pathogen resistance in giant freshwater prawn *Macrobrachium rosenbergii* (Nhan *et al*., 2010), *Artemia franciscana* (Defoirdt *et al*., 2007), blue mussel (*Mytilus edulis*) (Van Hung *et al*., 2019), European sea bass *Dicentrarchus labrax* (De Schryver *et al*., 2010), siberian sturgeon *Acipenser baerii* (Najdegerami *et al*., 2012), Pacific white shrimp *Litopenaeus vannamei* (Duan *et al*., 2017). PHB is known to be accumulated in commonly nutrient-limited bacterial cells and degrades to short chain fatty acid, β-hydroxybutyrate (Jendrossek and Pfeiffer, 2014). SCFAs produced by fermentative bacteria are important energy sources for humans and ruminants (Bergman, 1990; Koh *et al*., 2016). β-hydroxybutyrates, produced by PHB degradation in poly-β-hydroxybutyrate depolymerase enzymes produced by host animals or various microorganisms, could prevent growth of pathogenetic bacteria such *Vibrio campbellii* (Defoirdt *et al*., 2007, 2018). Moreover, the clear evidence indicating that PHB protects gnotobiotic *Artemia franciscana* from *V. campbellii* by increasing heat shock protein (Hsp) 70 were shown in the previous study (Baruah *et al*., 2015). PHB may be a candidate for food additives for sea cucumbers,

and mechanisms by which above mentioned probiotics affect sea cucumber's growth and immunity will be investigated using the laboratory feeding system I developed in the future.

Applications of probiotics for humans are underway but present challenges. First, a meta16S analysis could not distinguish resident and exogenous strains within the same species. Second, inter-individual variation of gut microbiota due to age, diet, sex and antibiotics usage is not considered. Third, variation of microbial communities through intestines is not considered. Fourth, mucosal samples are not obtained. Overcoming these challenges, recent studies showed that probiotic colonization in human mucosa is predictable by pre-treatment microbiome and host features, and that not probiotics but autologous FMT are useful to reconstitute gut microbiota after antibiotic treatment (Suez *et al*., 2018; Zmora *et al*., 2018). To establish probiotics in aquatic animals including sea cucumbers, mucosal microbiota analyses and FMT experiments need to be performed in the future.

Conclusion

I developed new technologies; non-destructive fecal microbiome analysis; and tracking systems of individual gut microbiome of sea cucumbers. Using these methodologies, I characterized community structure, function and dynamics of sea cucumber gut microbiomes. Further individual tracking experiments will be performed to understand host-microbe interaction and probiotics effects on sea cucumbers, which contributes to establishment of intensive and stable aquaculture systems.

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