Quantitative microbiome profiling links microbial community variation to the intestine regeneration rate of the sea cucumber Apostichopus japonicus

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Abstract

Intestine regeneration in the sea cucumber Apostichopus japonicus is a fascinating biological event and a typical example for studying host-intestinal microbiota interactions. The intestinal microbiota may play important roles in developing intestine promotion, but the underlying mechanism remains unclear. Notably, altered microbiota abundance may be a key marker of the observed ecosystem. To understand the role of the developing intestinal microbiota in intestine regeneration via quantitative data, we developed a germ-free sea cucumber model and analyzed the intestinal microbial differentiation of faster and slower regenerating A. japonicus individuals during intestine regeneration. The results revealed that depletion of the intestinal microbiota resulted in elevated abundance of the potential key players Flavobacteriaceae and Rhodobacterales during intestine regeneration and thus promoted the intestine regeneration rate of A. japonicus. These results first revealed a direct link between intestinal microbial quantity and microbiome features and the intestinal regrowth rate of A. japonicus. Metagenomic analysis revealed that the increased abundance of Flavobacteriaceae elevated the enrichment of genes associated with carbohydrate utilization, whereas the abundant Rhodobacteraceae -enriched genes were associated with polyhydroxybutyrate production. We identified microbiota abundance as a key driver of microbial community alterations, especially beneficial microbiota members, in the developing intestine of A. japonicus. This study provides new insights into the mechanism of host-microbiota interactions related to intestine development, and the understanding of molecular diversity to questions within intestinal ecology.

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Intestine regeneration in the sea cucumber *Apostichopus japonicus*is a fascinating biological event and a typical example for studying host-intestinal microbiota interactions. The intestinal microbiota may play important roles in developing intestine promotion, but the underlying mechanism remains unclear. Notably, altered microbiota abundance may be a key marker of the observed ecosystem. To understand the role of the developing intestinal microbiota in intestine regeneration via quantitative data, we developed a germ-free sea cucumber model and analyzed the intestinal microbial differentiation of faster and slower regenerating *A. japonicus* individuals during intestine regeneration. The results revealed that depletion of the intestinal microbiota resulted in elevated abundance of the potential key players Flavobacteriaceae and Rhodobacterales during intestine regeneration and thus promoted the intestine regeneration rate of*A. japonicus* . These results first revealed a direct link between intestinal microbial quantity and microbiome features and the intestinal regrowth rate of *A. japonicus* . Metagenomic analysis revealed that the increased abundance of Flavobacteriaceae elevated the enrichment of genes associated with carbohydrate utilization, whereas the

abundant Rhodobacteraceae -enriched genes were associated with polyhydroxybutyrate production. We identified microbiota abundance as a key driver of microbial community alterations, especially beneficial microbiota members, in the developing intestine of *A. japonicus* . This study provides new insights into the mechanism of host-microbiota interactions related to intestine development, and the understanding of molecular diversity to questions within intestinal ecology.

KEYWORDS: *Apostichopus japonicus* , intestine regeneration, intestinal microbiota, host-microbiota interactions, regeneration rate

1 | INTRODUCTION

The intestinal microbiota is increasingly recognized for its major roles in host health, growth, and mucosal and systemic immunity and for the important effect of resident intestinal bacteria promoting cell proliferation in the developing intestine. For instance, variation in the gut microbial structure can correlate with digestive enzyme activity and aid predigestion of the host nutrition (Holt, van der Giezen, Daniels, Stentiford, & Bass, 2019). Mite infection is significantly associated with altered microbial communities in *Urocyon littoralis catalinae* (DeCandia, Brenner, King, & vonHoldt). The intestinal microbiota can promote hematopoietic recovery after bone marrow transplantation (Staffas et al., 2018), affect the bone marrow niche (Y. Luo et al., 2015), and promote the generation of hematopoietic stem cells (Josefsdottir, Baldridge, Kadmon, & King, 2017). The gut microbiota influences skeletal muscle growth and function in mice (Lahiri et al., 2019) and significantly influences the larval growth rate of *Melitaea cinxia* (Ruokolainen, Ikonen, Makkonen, & Hanski, 2016). In addition, intestinal microbiota can promote cell proliferation in the developing vertebrate intestine (Cheesman, Neal, Mittge, Seredick, & Guillemin, 2011). Moreover, the gut microbiota can increase Paneth cell proliferation in the small intestine (Schoenborn et al., 2018). It is thus well established that functional interactions between the gut microbiota and the host are important for host physiology, growth, and sustained health.

Apostichopus japonicus is a temperate sea cucumber species, and has been exploited as an economically valuable fishery resource in many Asian countries, especially China. *A. japonicus* ingests organic matter, protozoa, microbes, algae and aquatic animal detritus, and thus plays an important role in benthic biogeochemical cycles (Choo, 2008; Yamazaki et al., 2016). In particular, sea cucumber possess a unique defense mechanism called evisceration. The organs, including the intestine, hemal system and respiratory trees can be eviscerated when they are subjected to natural or induced stimulation (Dolmatov & Ginanova, 2009; X. Li et al., 2017; L. Sun et al., 2017). The lost organs can concurrently regenerate within a few weeks (Shukalyuk & Dolmatov, 2001; L. Sun et al., 2011). Thus, it is an excellent model to study host-microbiota interactions in developing intestine. Actually, a lack of studies on factors affecting the intestine regeneration rate of *A. japonicus* is an obstacle to further success in this field. Extreme gaps of intestine regenerating rate among sea cucumbers are also a common problem in the study of animal organ regeneration. As a result, there is a right-skewed intestine length distribution among regrowth animals even when cultured in the same tank under identical conditions (e.g., temperature and animal density). This issue can be seen in Figure 1, in which the faster individuals are 1.83-3.03 times longer than the slower individuals. The cause of this regeneration gap is unknown.

In the past decade, research on the intestinal microbiota of the sea cucumber *A. japonicus* has rapidly accumulated and has been accompanied by increased interest in host-microbiota interactions as a means to modulate the animal's health and growth. Previous studies have focused on intestinal bacterial community structures (Gao et al., 2014; Gao, Li, Tan, Yan, & Sun, 2014), the physiological characterization of culturable bacteria in the intestine of the sea cucumber (L. Wang, Li, Hu, Lai, & Shao, 2015; X. Zhang et al., 2013), and the effects of intestinal microbiota on *A. japonicus* growth and health (Sha et al., 2016; Yamazaki et al., 2016; Z. Zhang et al., 2018). They found that the intestinal microbiota might play an important role in sea cucumber growth. However, few studies have addressed the question of the development of the intestinal microbiome composition and function of*A. japonicus* during intestine regeneration stages (Luo Wang et al., 2018; H. Zhang et al., 2019). Moreover, the effects of the intestinal microbiome and microbial quantity on intestine regeneration of *A. japonicus* are still unclear. Therefore, we are interested in the functional

interactions between the intestinal microbiota and intestinal regrowth rate of *A. japonicus* . Understanding the regulatory mechanisms by how the intestinal microbiota might influence the process is an important objective in improving outcomes during intestine regrowth, and may help in the development of a theoretical basis for further understanding intestinal ecology and the role of the microbiome in the process of intestine development.

We observed a regeneration gap during intestine regeneration in sea cucumbers *A. japonicus* . To our knowledge, there have been no studies investigating the possible effects of the intestinal microbiota on intestine regeneration of *A. japonicus* . Our hypothesis is that the intestinal microbiota plays an important role in intestinal regrowth of *A. japonicus* during intestine regeneration. To explore any possible contributions of the gut microbiome to its host's intestinal regrowth, individual taxon abundance and microbiome comparisons (both taxonomic and functional) of both the faster and slower regenerating sea cucumbers *A. japonicus* were performed. Deriving germ-free (GF) animals is a powerful experimental approach to investigating the function of intestinal microbiota. To further gain mechanistic insight into the interaction between the intestinal microbiota and the intestinal regrowth rate, we developed a GF sea cucumber model, quantified the intestinal bacterial 16S rRNA abundance by qPCR, and taxonomically analyzed the microbial diversity. The results will demonstrate the link between the microbiota quantity and microbial community variation and intestine development, which is of great theoretical and practical significance to the understanding of the dynamics of host-microbiota interactions, and to the cultural ecology and conservation of fishery resources.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Adult *A. japonicas* (100±10 g) were collected in April 2018 from the coast of Weihai, Shandong, China. In order to reduce the effect of genetic variation, sea cucumbers were taken from the same aquacultural area, and the animals breeding were at the same time. The animals were acclimated in seawater at 15±1°C for 2 weeks prior to treatment and were fed a formulated diet once per day. After acclimation, evisceration was induced by injecting approximately 2 mL 0.35 M KCl into the coelomic cavity (Lina Sun et al., 2017; Lina Sun, Yang, Chen, Ma, & Lin, 2013). Eviscerated animals were kept in well-aerated indoor seawater tanks. We began recording observations at the point when the sea cucumber had expelled the entire intestine. The sea cucumbers have no feeding during intestine regeneration in our experiments.

2.2 | Experimental design and sample collection

At least 40 individuals per regeneration stage [the beginning (10 d), middle (14 d) and end (21 d) of intestinal regeneration] were used for analyses. Ten individuals with the longest or shortest intestines were classified as the faster (F) and slower (S) regenerating individuals, respectively. The samples were labeled F10, S10, F14, S14, F21 and S21, respectively. After 7 d of intestine regeneration, lumen formation of the new intestine began; the intestine gradually developed to form a complete structure in which the digestive and absorptive functions were restored during 14-21 d of intestine regeneration (L. Sun et al., 2011; Lina Sun et al., 2013). The 10th d was therefore an appropriate time point for sampling the new intestine.

The GF and conventionally reared (CV) groups were subjected to two different treatments: 80 sea cucumbers were cultured normally in seawater in the laboratory as the CV group. 160 sea cucumbers were soaked for 3 h in sterile filtered seawater containing 100 U/ml penicillin and 100 μg/ml streptomycin, and were then cleaned twice in 0.003% sodium hypochloride (Bates et al., 2006). No developmental defects and similar rates of sterility were observed with the antibiotic mixtures used. Then, 80 sea cucumbers were transferred to sterile filtered seawater tanks at a density of 10 sea cucumbers per tank in a sterile room as the GF group; the tanks remained sterile for the duration of the experiment. And the other 80 sea cucumbers were cultured normally in seawater in the laboratory as the XGF group. At least 10 individuals of similar size were randomly selected per regeneration stage (10 and 14 d) were used for analyses.

In the experiments, individuals were rinsed with sterile seawater and moved into sterile plates. The coelomic fluid was withdrawn from the coelom of sea cucumbers using sterile syringes. The intestines were aseptically

dissected and measured the length, and immediately transferred into sterile tubes and measured the weight, and then preserved at -80°C until DNA extraction.

2.3 | DNA extraction and 16S rRNA gene sequencing

Total DNA was extracted from the gut contents of *A. japonicas* by using a FastDNA SPIN Kit for Feces (MP Biomedicals, Santa Ana, CA, USA) in accordance with the instructions provided by the manufacturer. The extracted DNA was dissolved in 50 μL of TE buffer, quantified using a NanoDrop spectrophotometer (NanoDrop, Thermo Scientific, USA) and stored at -20°C prior to analysis. PCR amplification of the bacterial 16S rRNA hypervariable V4-V5 regions was conducted using the universal primer set 515f (GTGCCAGCMGCCGCGGTAA) and 907r (CCGTCAATTCMTTTRAGTTT), with 5-bp barcodes fused to the forward primer to allow sample multiplexing. The purified PCR products with different barcodes were normalized in equimolar amounts, then prepared using an NEB Next® Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's protocol and sequenced on an Illumina HiSeq platform.

2.4 | Deep sequencing data processing

Raw deep sequencing data were processed using Quantitative Insights Into Microbial Ecology software (http://www.qiime.org, QIIME version 1.9.0; (Caporaso et al., 2010)) with the default parameters unless otherwise noted. After all chimeric and low-quality reads were removed, qualified sequences were clustered into operational taxonomic units (OTUs) at the 97% identity threshold level, and the most abundant sequence from each OTU was chosen as a representative sequence for that OTU. Taxonomic classification of each OTU was assigned using the Ribosomal Database Project classifier. The average relative abundance (%) of predominant genus-level taxonomic groups in each sample was estimated by comparing the number of sequences assigned to a specific taxon versus the total number of sequences obtained for that sample.

2.5 | Metagenomic sequencing and analysis

Each representative DNA sample from the sea cucumber intestine for which sufficient volumes were available after 16S typing was used for metagenomic sequencing. Metagenomic DNA paired-end libraries were prepared with an insert size of 350 bp and were quantified using a Qubit Fluorometer and an Agilent 2100 TapeStation system. Sequencing was performed on an Illumina HiSeq PE150 platform.

Raw reads were preprocessed using FasqMcf to exclude adapter sequences and low-quality sequences (Aronesty, 2011), and reads derived from host contamination were filtered using bowtie2.2.4 (Langmead & Salzberg, 2012). Clean reads were assembled and analyzed by SOAPdenovo software (R. Luo et al., 2012). Then, gene prediction was performed on contigs larger than 500 bp by MetaGeneMark software with the default parameter, and gene models with CDS lengths less than 100 bp were filtered out (Nielsen et al., 2014; Qin et al., 2010). A gene catalog was constructed using the gene models predicted from each sample by CD-HIT-EST (version 4.6.6) (Li & Godzik, 2006) with the parameters '-c 0.95 -n 10 -G 0 -a S 0.9', which adopts a greedy incremental clustering algorithm and criteria of identity $> 95\%$ and overlap $> 90\%$ of the shorter genes.

DIAMOND software (version 0.9.9) (Buchfink, Xie, & Huson, 2015) was used to align unigenes to the sequences of bacteria, fungi, archaea and viruses, which were all extracted from the NCBI nr database (version: 2018-01-02) with the parameter '-e 1e-5'. Functional assignments of protein sequences were made on the basis of DIAMOND alignment against the KEGG protein database (version 2018-01-01) (Minoru et al., 2006), eggNOG database (version 4.5) (Sean et al., 2014), and CAZy database (version 20150704) (Cantarel et al., 2009) by using the best hit with an e value $<$ 1e-5. For each sequence's BLAST result, the best BLAST hit was used for subsequent analysis.

Phylum, class, order, family, genus, species, KEGG orthology (KO), and orthologous group (OG) relative abundances were calculated by summing the abundance of the respective genes belonging to each category per sample based on the taxonomic assignments and KO and OG annotations. The relative gene abundance profile was also summarized into KEGG, eggNOG and CAZy functional profiles for functional analysis.

2. 6 | Quantitative real-time PCR (qPCR) assays

To assess the amounts of intestinal bacterial in different samples, we also quantified the bacteria through qPCR using the primers 341F and 518R, which are universal primers targeting a short fragment (ca. 171 bp) of the bacterial 16S rRNA genes. The qPCR assay was based on the fluorescence intensity of the SYBR Green dye and performed as previously described (F. Sun et al., 2015). Using the vector-targeted primers M13F/M13R, linear fragments were obtained from PCR amplification of circular plasmids (pTZ57R/T vector; Fermentas), which contained inserts of the bacterial 16S rRNA gene fragments. The standard DNA was also quantified using the PicoGreen dsDNA reagent kit (Invitrogen).

2.7 | Statistical analysis

The data were analyzed by a SPSS for Windows (Version 19.0) statistical package. Differences were determined by LSD test with P-values <0.05 being accepted as the statistical significance.

3 | RESULTS

3.1 | The faster and slower regenerating *Apostichopus japonicus* have different intestinal microbial communities

We assessed the intestine regeneration rate of the faster and slower regenerating *A. japonicus* individuals, including intestinal length and weight. The intestinal length of the faster regenerating individuals was significantly longer than that of the slower regenerating individuals during intestine regeneration (10-14 d). The intestine of the faster regenerating individuals was also heavier than that of the slower regenerating individuals (Figure 1). There were significant differences between the two groups. Thus, we assessed the intestinal microbiome of the faster and slower regenerating groups.

At 97% sequence identity, a total of 3,147 OTUs were obtained across all samples. The samples from the faster regenerating group contained 2,373 OTUs (on average), while the slower regenerating group samples contained 2,365 OTUs (on average) (Table S1). The Shannon indices were 5.69 and 4.77, and the Chao1 richness values were 897 and 688 OTUs in the faster and slower regenerating groups, respectively. The results indicated that the alpha diversity of the bacterial community was different between the faster and slower regenerating groups (F and S) (Figure S1).

Analysis of the intestinal microbiota revealed that the faster and slower regenerating individuals had distinct taxonomic compositions (Figure 2a, Figure S1). In the F samples, Flavobacteriales (average 21.3%) and Rhodobacterales (19.3%) were the most abundant orders, represented mainly by the Flavobacteriaceae (20.3%) and Rhodobacteraceae (19.3%) families, respectively. Compared to that in the F samples, a markedly varied profile was detected in the S samples (Figure 2a, Figure S1). The Micrococcales order prevailed (average 27.9%) and was represented mostly by the Microbacteriaceae (27.7%) family. Rhodobacterales (13.3%) was the next most abundant order, represented by the Rhodobacteraceae (13.3%) family. The reads affiliated with Flavobacteriales were significantly more abundant in the faster regenerating individuals than in the slower regenerating individuals (Figure 2b).

3.2 | Comparative metagenome analysis of the intestinal microbiome in the regenerating intestine of faster and slower regenerating *Apostichopus japonicus*

Metagenome sequencing using the Illumina HiSeq platform was performed on the fastest and the slowest regenerating specimens (except specimens containing insufficient intestinal DNA) as representatives of faster and slower regenerating individuals used in the above analyses. To detect the effects of the intestinal microbiota on host regeneration, 3 time points [the beginning (10 d), middle (14 d) and end (21 d) of intestinal regeneration] were chosen. From the fastest and the slowest regenerating individuals, across the 3 stages, 138,957,538 reads (20.8 Gb) and 129,641,842 reads (19.4 Gb) were obtained in total, respectively. After quality filtering and removing host sequences, 33,764 and 34,187 reads from the fastest and the slowest regenerating individual, respectively, were used for MG-RAST annotation.

The metagenomic data of the faster and slower regenerating individuals from different regeneration stages (10 d, 14 d and 21 d) were compared with data in the KEGG database. The relative abundances at differ-

ent functional levels were calculated, and a total of 300 functional genes were annotated to the metabolic pathways. The KEGG functional profiles also showed similarities in intestinal microbial functions in faster and slower regenerating individuals. The pathways of human diseases and metabolism contained the largest number of annotated genes, followed by pathways relevant to the organism system (Figure S2). However, there were still differences in some KEGG functional categories between the faster and slower regenerating individuals (Figure S3). The analysis demonstrated that 20 functional features in the subsystem category (level 2) were more abundant in one of the samples (Figure 3). In detail, the genes for immune system, carbohydrate metabolism, aging, and infectious diseases were more abundant in the faster regenerating individual than in the slower regenerating individual in the 10 d stage, and the genes for development, xenobiotic biodegradation and metabolism, drug resistance, and membrane transport were more abundant in the faster regenerating individual than in the slower regenerating individual in the 14 d stage. The genes annotated to energy metabolism and lipid metabolism were significantly more abundant in the faster regenerating individual than in the slower regenerating individual in the 21 d stage. Regarding the slower regenerating individual, the more abundant genes were annotated in cell growth and death, signal transduction, digestive system, and glycan biosynthesis and metabolism during intestine regeneration.

The genes were also annotated with eggNOG OGs to explore the difference in microbial functions between the faster and slower regenerating*A. japonicus* individuals during intestine regeneration. The functional features of the subsystem category at different levels were analyzed. The genes annotated to 'replication, recombination and repair', 'cytoskeleton' and 'amino acid transport and metabolism' were most abundant, followed by genes annotated to 'posttranslational modification, protein turnover, chaperones', 'cytoskeleton' and 'signal transduction mechanisms' (Figure S4). The gene abundance of eggNOG functions also changed between faster and slower regenerating *A. japonicus* individuals, but no significant differences were observed.

3.3 | Distinctive quantitative microbiota differences in the intestine of faster and slower regenerating *Apostichopus japonicus* individuals

The bacterial populations per animal were measured by qPCR amplification of the 16S rRNA gene with universal bacterial primers. We observed a substantial variation in cell counts between the faster and slower regenerating individuals. The abundances of the bacterial 16S rRNA gene were $8.9\text{x}10^7$, $1.13\text{x}10^9$ and $1.96\text{x}10^9$ copies per sea cucumber for the faster regenerating individuals in the 10 d, 14 d and 21 d stages, respectively. The bacterial 16S rRNA gene abundances were $1.05\text{x}10^8$, $1.5\text{x}10^9$ and $2.99\text{x}10^9$ copies per sea cucumber in the slower regenerating individuals in the 3 stages, respectively (Figure 4). Additionally, the numbers of 16S rRNA gene copies g^{-1}(wet weight of intestine) were significantly different between the faster and slower regenerating individuals during intestine regeneration (Figure S5). Higher bacterial populations were observed in the regenerating intestines of the slower regenerating *A. japonicus*individuals than in those of the faster regenerating individuals.

3.4 | Depletion of the intestinal microbiota improves the intestine regeneration rate of *Apostichopus japonicus*

The differences in bacterial 16S rRNA abundances in samples from different groups were determined by qPCR assays targeting the bacterial 16S rRNA gene. The abundances of bacterial 16S rRNA genes were $2.47\text{x}10^7$ and $2.44\text{x}10^7$ copies per sea cucumber in CV animals in the 10 d and 14 d stages, respectively. The bacterial 16S rRNA gene abundances were $8.23\text{x}10^6$ and $8.18\text{x}10^6$ copies per sea cucumber in GF animals in the 2 stages, respectively (Figure 5). The number of bacterial 16S rRNA copies per sea cucumber decreased significantly in GF samples during intestine regeneration and was reduced by 66% compared to that in CV samples. Notably, the intestinal growth of GF sea cucumbers was faster than that of CV animals during intestine regeneration. The average intestine lengths of CV animals in the 10 d and 14 d stages were 4.48 and 4.88 cm, and the average intestine weights were 0.041 and 0.079 g, respectively. The average intestine lengths of GF animals in the 2 stages were 6.8 and 6.86 cm, and the average intestine weights were 0.064 and 0.114 g, respectively (Figure 6). The intestinal bacterial 16S rRNA gene abundance correlated with significant influences on the intestine weight and intestine length of *A. japonicus* during intestine regeneration ($P < 0.05$).

3.5 | Intestinal microbiota depletion promotes the potential key players in the regenerating intestine of*Apostichopus japonicus*

At 97% sequence identity, the CV samples contained 1,877 OTUs, the samples from the XGF group contained 1,620 OTUs, and the GF samples contained 1,228 OTUs (Table S2). We analyzed both genus and species numbers in the samples from the 3 groups to further identify the differences in the bacterial communities of *A. japonicus*intestines during regeneration stages. The CV, XGF and GF groups contained 597, 578 and 446 genera, respectively. The number of OTUs and bacterial species from GF samples were significantly lower than those from CV samples. No developmental defects or regrowth rate differentiation were observed in the XGF samples compared to those in the CV samples; therefore, we compared and analyzed the CV and GF groups in this study.

Analysis of bacterial communities revealed distinct taxonomic compositions between the CV and GF samples at the order and genus levels. In CV samples, the Oceanospirillales order prevailed (average of 41.51% of all the reads) and was represented mostly by the Halomonadaceae (41.04%) family, mainly related to *Halomonas*(41.03%) genus. Alteromonadales (14.87%) was the next most abundant order, represented mainly by the Pseudoalteromonadaceae (7.74%) and Colwelliaceae (5.24%) families and the *Pseudoalteromonas*(7.11%) and *Colwellia* (5.15%) genera. The next most abundant order was Burkholderiales (8.08%), which was represented mainly by the Burkholderiaceae (7.41%) family and the *Ralstonia* (7.29%) genus (Table 1, Table S3-4).

In GF samples, the most abundant order was Flavobacteriales (52.68%), which was represented mostly by the Flavobacteriaceae (50.15%) family, with *Flavobacterium* , *Polaribacter* ,*Flavobacteriaceae_uncultured* and *Crocinitomix* comprising 41.78%, 2.85%, 1.5% and 1.08% of the total, respectively. Burkholderiales (average 9.25%) was the next most abundant order, represented mainly by the Burkholderiaceae (8.18%) family, mainly related to the *Ralstonia* (7.92%) genus. Rhodobacterales was also present (6.59%), mainly related to the Rhodobacteraceae (6.59%) family (Table 1, Table S3-4).

The average abundances of Flavobacteriaceae (4.42%) and Rhodobacterales (3.91%) (the potential key players) in CV samples were significantly lower than those in GF samples.

4 | DISCUSSION

The intestinal microbiota has been found that plays an important role in sea cucumber health, growth and function (C. Li et al., 2017; Yamazaki et al., 2016). The sea cucumber*A. japonicus* is one of the best model animals to study host-microbiota interactions during organ regeneration. We have previously reported that intestinal microbial composition and functional genes of *A. japonicus* are associated with intestine regeneration stages after evisceration and that Rhodobacterales and Flavobacteriaceae may function as keystone taxa in the intestinal microbial community of*A. japonicus* during intestine regeneration (H. Zhang et al., 2019). However, how the intestinal microbiota affects intestine regrowth is still unknown. Here, we present the first description of the effects of intestinal microbial quantity and microbiome features on developing intestine in *A. japonicus* .

We demonstrated that decreased cell counts were observed in the intestine of faster regenerating *A. japonicus* individuals, who contained large fractions of Rhodobacterales and Flavobacteriaceae. Given the importance of microbial quantity in regulating cell renewal in the intestine of the faster regenerating individuals, we asked whether microbial quantity could change the beneficial bacterial community and then promote the regrowth rate by depleting the intestinal microbiota in the GF group. We assessed the interactions between microbial quantity and potential key players with the intestinal regrowth rate by developing a GF sea cucumber model. We provide evidence that intestinal microbiota depletion is both necessary and sufficient to increase the abundances of Flavobacteriaceae and Rhodobacterales and then to promote the intestine regrowth rate of *A. japonicus* , which was observed in the GF samples and the slower and faster regenerating individuals, respectively.

Previous studies have reported that the gut microbiota influences cell proliferation in the gut epithelium.

Intestinal epithelial cell proliferation was reduced in GF zebrafish model (Cheesman et al., 2011; Rawls, Samuel, & Gordon, 2004), and secretory cells were less abundant in the gut epithelium of GF animals (Bates et al., 2006; Uribe, Alam, Johansson, Midtvedt, & Theodorsson, 1994). In contrast to previous findings, our data suggest that depletion of the intestinal microbiota promotes the intestine regeneration rate of *A. japonicus* . The intestine regeneration rate was significantly faster in the GF sea cucumbers than in the CV sea cucumbers. Notably, the relative abundance of Flavobacteriaceae was significantly increased in GF samples compared to that in CV samples, and Flavobacteriaceae was the most abundant family in GF samples. The possible mechanism for promoting the intestine regeneration rate could be that the reduced bacterial populations increased the abundance of potential functional microbes during intestine regeneration since Flavobacteriaceae and Rhodobacteraceae have been reported to be potential key players in the intestine of *A. japonicus* during intestine regeneration (H. Zhang et al., 2019).

Flavobacteriaceae present low pathogenicity (Jooste & Hugo, 1999), and can produce carotenoids that have antioxidative activities (Shindo et al., 2007). More importantly, the marine Flavobacteriaceae usually produce enzymes that degrade agars, fucoidan, fucose, laminarin, xylan, and carrageenans from micro- or macroalgae (Sakai, Kimura, & Kato, 2002). Within Flavobacteriaceae, the predominant genus was*Flavobacterium* , which presents high levels of resistance to a wide range of antibiotics (Thomson, 1988). Some *Flavobacterium* species play a role in mineralizing various types of organic matter (carbohydrates, amino acids, proteins, and polysaccharides) in aquatic ecosystems and are able to degrade various cellulose derivatives, such as carboxymethylcellulose (Bernardet & Bowman, 2006). The enzymatic abilities of Flavobacteriaceae may either directly or indirectly mineralize various types of organic matter from seawater and increase the production of carbohydrates, which is beneficial to the sea cucumber during the intestine regeneration process and thus promotes the intestinal regrowth rate.

Previous studies have shown that Rhodobacteraceae species are frequently found in the intestine of sea cucumber *A. japonicus*(F. Gao et al., 2014; Sha et al., 2016; Luo Wang et al., 2018; Yang, Xu, Tian, Dong, & Peng, 2015; H. Zhang et al., 2019). Rhodobacterales retaining polyhydroxybutyrate (PHB) metabolism genes, as a PHB producer, promoted the growth of sea cucumber *A. japonicus*(Yamazaki et al., 2016). In addition to our research, Rhodobacteraceae were identified as keystone taxa in the microbial community associated with *Nannochloropsis salina* in aquatic ecosystems (Geng, Tran-Gyamfi, Lane, Sale, & Yu, 2016). In the present study, the relative abundance of Rhodobacteraceae was obviously increased in GF samples. Thus, they might be important for the intestinal regrowth of *A. japonicus* .

In this study, quantitative analysis revealed that the abundance of the bacterial 16S rRNA gene was reduced in faster regenerating *A. japonicus* individuals during intestine regeneration. Meanwhile, the relative abundances of Flavobacteriaceae and Rhodobacteraceae in the faster regenerating individual were higher than those in the slower regenerating individual. Interestingly, metagenomic analyses suggested that genes annotated to carbohydrate metabolism were more abundant in the faster regenerating individual and that genes annotated to*pha* A (acetyl-CoA C-acetyltransferase) were also enriched, which is essential in PHB synthesis from acetyl-CoA to PHB (Yamazaki et al., 2016). PHB accumulates in commonly nutrient-limited bacterial cells (Jendrossek & Pfeiffer, 2014), and the bacterial PHB, especially that produced by Rhodobacterales, might also serve as an energy source for the sea cucumber (Yamazaki et al., 2016).

Together, our results indicate a direct link between intestinal bacterial 16S rRNA abundance to Flavobacteriaceae and Rhodobacteraceae and to the intestine regeneration rate of *A. japonicus* . Flavobacteriaceae is related to carbohydrate production, and Rhodobacteraceae is related to PHB production, which are beneficial to the regenerating intestine that has an disrupted function. We predict that depletion of the intestinal microbiota promotes the potential key players during intestine regeneration and thus promotes the intestinal regrowth rate of *A. japonicus* . In the present study, the most abundant species in GF sea cucumbers was*Flavobacterium_unclassified* . The lack of information on the detection and/or isolation of *Flavobacterium* in the regenerating intestine of *A. japonicus* resulted in delays in discovering details in the quantity-structure-function relationships. Hereafter, additional experiments should be conducted to further assess how the keystone taxa and functional genes respond to changes in the intestinal microbial quantity

and intestinal regeneration and to further study the ecophysiology and ecogenomics of the key players; then, we could infer their effects on host animals. Once the specific functions are unveiled, these bacteria could also be candidates for probiotics in sea cucumber aquaculture.

Broadly, this study contributes to the understanding of complex interactions between the components of intestinal ecology, which include intestinal microbial populations and developing intestine that has unsound function. By sequencing the intestinal microbiome of regrowth intestine, we identified microbiota abundance as a key driver of microbial community alterations, especially beneficial bacterial members, in the regenerating intestine of *A. japonicus* . The results indicated that intestinal microbial quantity and community structure exert regulatory mechanisms for organ development of the host and provide new insights into the host-microbiota interaction. Applying similar molecular techniques to wild and farmed systems around the world will not only further our understanding of the role of intestinal microbiomes in host health and development, but may also provide insights useful for improving intestinal ecology, management, and conservation of fishery resources.

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AUTHOR CONTRIBUTIONS

H.Z., L.Z., L.S. and H.Y. designed the study; Q.W., J.Z., and S.L. contributed new reagents or analytical tools; H.Z. conducted the laboratory work, analyzed data and took the lead in writing the manuscript; L.S. and H.Y. supervised the findings of this work. All authors provided critical feedback and helped to conduct the research, and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All 16S rRNA gene and metagenomic sequence raw data were submitted to the Sequence Read Archive (SRA) with project accession numbers PRJNA579220 and PRJNA512056.

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Table and Figure captions

TABLE 1 The relative abundance (%) of predominant genera in CV and GF samples

FIGURE 1 The intestine length and weight of the faster and slower regenerating *Apostichopus japonicus* individuals during the different regeneration stages. The differences in the intestine length averages of the two groups, "faster" and "slower", were statistically significant. ** indicates significant difference in the intestine length averages of the two groups at$P$ <0.01 level. Different letters indicate significant differences (P <0.05) in the intestine weight.

Note: W-F: intestine weight of the faster regenerating *A. japonicus* individuals; W-S: intestine weight of the slower regenerating*A. japonicus* individuals; L-F: intestine length of the faster regenerating *A. japonicus* individuals; L-S: intestine length of the slower regenerating *A. japonicus* individuals.

FIGURE 2 The intestinal microbiota of the faster and slower regenerating *Apostichopus japonicus* individuals are different. (a) Comparison of the intestinal bacterial communities in the faster and slower regenerating *Apostichopus japonicus* individuals during different regeneration stages. (b) Differences in bacterial communities at the family level between the faster and slower regenerating individuals (Wilcoxon rank-sum test, Storey's methods for multiple tests adjustment).

Note: F: faster regenerating group; S: slower regenerating group.

FIGURE 3 Heatmap of the KEGG analysis of the intestinal microbiota in samples from the faster and slower regenerating*Apostichopus japonicus* individuals during the different regeneration stages.

FIGURE 4 Quantification of 16S rRNA gene copies/sea cucumber in the faster and slower regenerating *Apostichopus japonicus* individuals during the different regeneration stages. qPCR was performed with bacterial-specific primers on DNA extracted from the F or S groups. Different letters indicate significant differences (P <0.05).

FIGURE 5 Quantification of 16S rRNA gene copies/sea cucumber during the different regeneration stages. qPCR was performed with bacterial-specific primers on DNA extracted from the CV, XGF, or GF groups. Different letters indicate significant differences (P <0.05).

FIGURE 6 The intestine length (a) and weight (b) of samples from the CV, XGF, or GF groups during the different regeneration stages. (c) Images of regenerating intestines from the CV, XGF, or GF groups at 10 or 14 d. Different letters indicate significant differences (P <0.05).

Note: CV1-XGF1-GF1: 10 d; CV2-XGF2-GF2: 14 d.

Supplementary Material

TABLE S1 Summary of the alpha diversity of bacterial communities in samples from F and S groups

TABLE S2 The numbers of OTUs, genus and species in CV, XGF and GF samples

TABLE S3 The relative abundance (%) of predominant orders in CV and GF samples

TABLE S4 The relative abundance (%) of predominant families in CV and GF samples

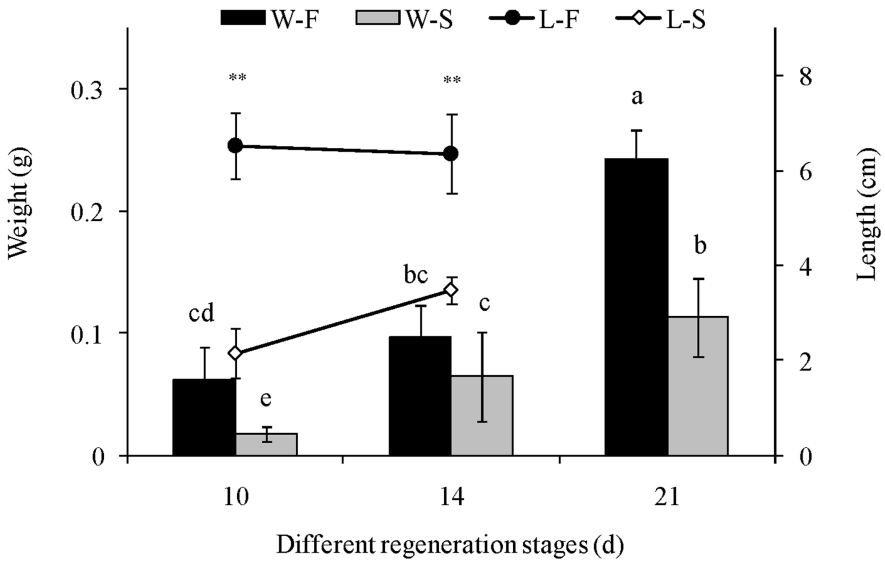
FIGURE S1 Relative abundance of bacterial communities at the order level in the intestine of the faster and slower regenerating*Apostichopus japonicus* individuals during regeneration stages.

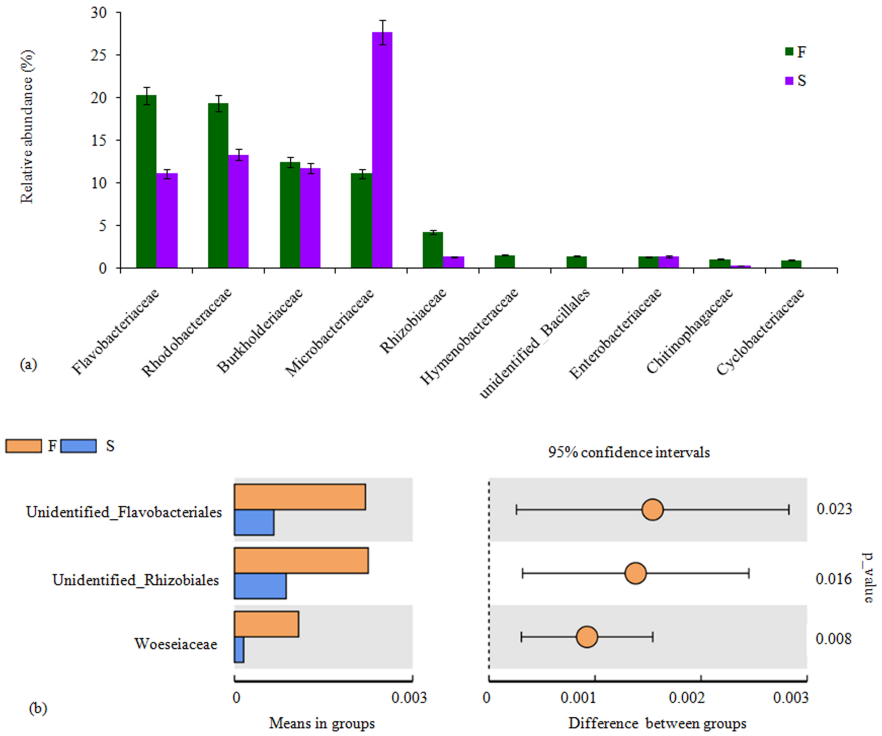
FIGURE S2 KEGG analysis of intestinal microbiota in the faster and slower regenerating *Apostichopus japonicus* individuals during regeneration stages.

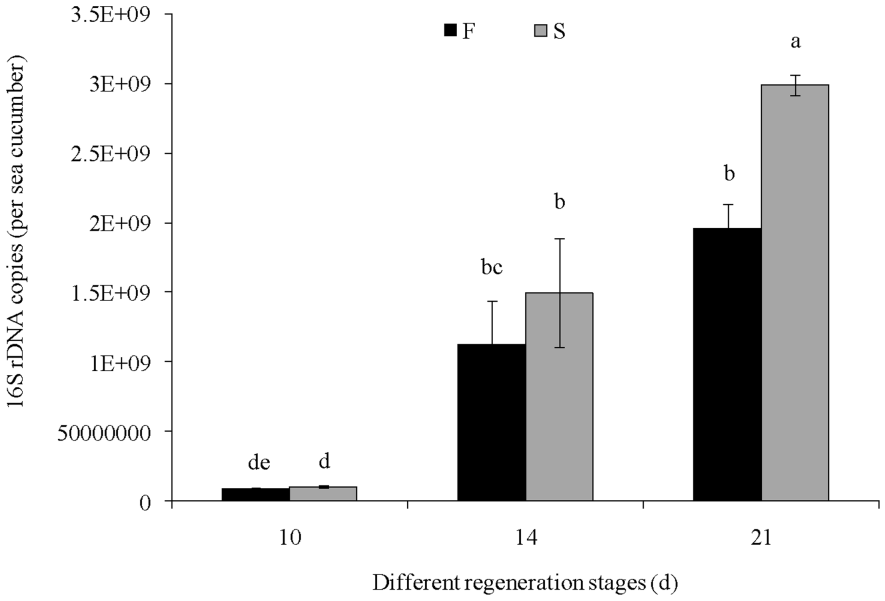
FIGURE S3 Comparison of the KEGG functional profiles of intestinal microbiota in the faster and slower regenerating*Apostichopus japonicus* individuals during regeneration stages.

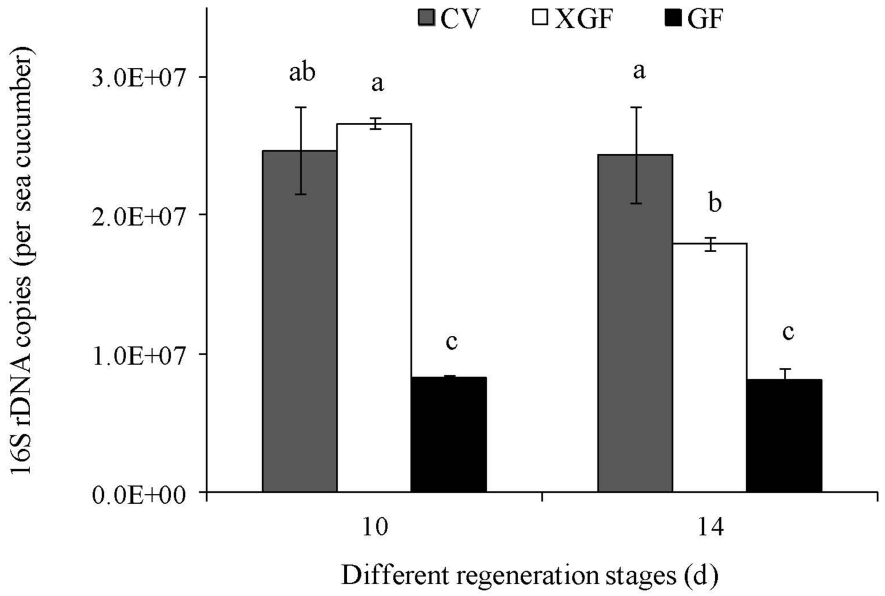
FIGURE S4 Comparison of eggNOG functional profiles of intestinal microbiota in the faster and slower regenerating*Apostichopus japonicus* individuals during regeneration stages.

FIGURE S5 Quantification of 16S rRNA gene copies/g of the intestine in the faster and slower regenerating *Apostichopus japonicus* individuals during the different regeneration stages. qPCR was performed with bacterial-specific primers on DNA extracted from F or S. Different letters indicate significant differences (P <0.05).









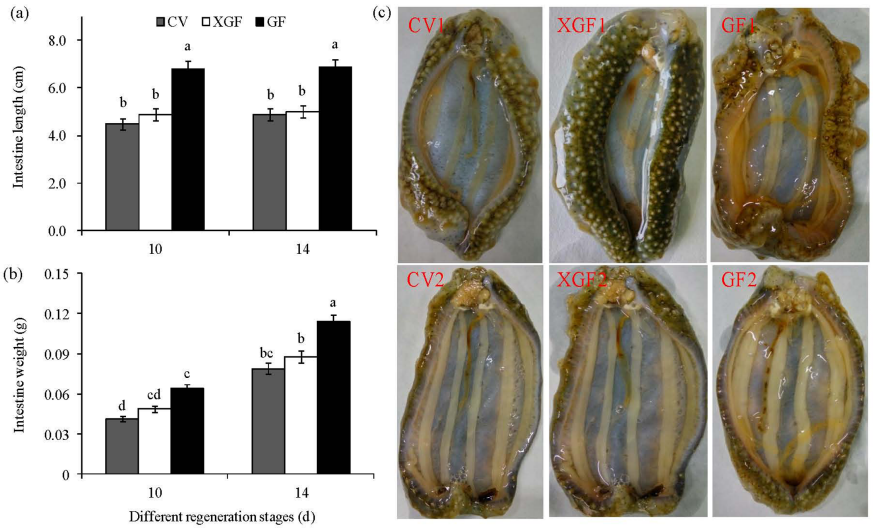


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Table 1.docx available at https://authorea.com/users/334554/articles/460522-quantitative-microbiome-profiling-links-microbial-community-variation-to-the-intestine-regeneration-rate-of-the-sea-cucumber-apostichopus-japonicus