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

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ABSTRACT

The intestinal microbiota may play important roles in regenerating intestine of the sea cucumber *Apostichopus japonicus*, the underlying mechanism remains unclear. In the present study, a germ-free sea cucumber model was developed, and the intestinal microbial differentiation of faster and slower regenerating *A. japonicus* individuals during intestine regeneration was analyzed. The results revealed that depletion of the intestinal microbiota resulted in elevated abundance of the potential key players Flavobacteriaceae and Rhodobacteraceae during intestine regeneration and thus promoted the intestine regeneration 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 organ regeneration.

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 [1]. The intestinal microbiota can promote hematopoietic recovery after bone marrow transplantation [2], affect the bone marrow niche [3], and promote the generation of hematopoietic stem cells [4]. The gut microbiota influences skeletal muscle growth and function in mice [5] and significantly influences the larval growth rate of *Melitaea cinxia* [6]. In addition, intestinal microbiota can promote cell proliferation in the developing vertebrate intestine [7]. The gut microbiota can increase Paneth cell proliferation in the small intestine [8]. Moreover, the gut microbiota was correlated with the shrimp body weight and disease severity [9,10]. It is thus well established that functional interactions between the gut microbiota and the host are important for host 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 [11]. *A. japonicus* ingests organic matter, protozoa, microbes, algae and aquatic animal detritus, and thus plays an important role in benthic biogeochemical cycles [11,12]. Some microbes can evade digestion and populate in the intestine of sea cucumbers [13]. In particular, sea cucumbers 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 [14–16]. The lost organs can concurrently regenerate within a few weeks [17,18]. Thus, it is an excellent model to study host-microbiota interactions in the developing intestine. Actually, a lack of studies on factors affecting the intestine

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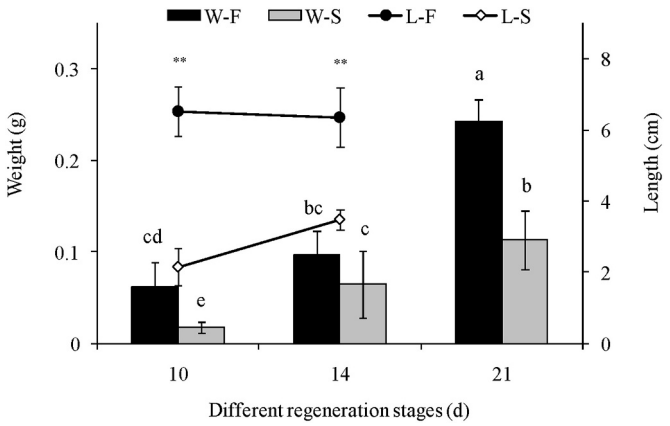

Fig. 1. The intestine length and weight of the faster and slower regenerating *Apostichopus japonicus* individuals during the different regeneration stages. The differences in the intestine length averages of the two groups, "faster" and "slower", were statistically significant. ** indicates a significant difference in the intestine length averages of the two groups at the $P < 0.01$ 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.

regeneration rate of *A. japonicus* is an obstacle to further success in this field. Extreme gap of intestine regenerating rate among sea cucumbers is 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 Fig. 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 [19,20], the physiological characterization of culturable bacteria in the intestine of the sea cucumber [21,22], and the effects of intestinal microbiota on *A. japonicus* growth and health [11,23,24]. They found that the intestinal microbiota might play an important role in sea cucumber growth. Moreover, previous studies revealed that intestinal microbiota of sea cucumbers had significantly different community structures and functions during intestine regeneration [13,25,26], and that Rhodobacteraceae and Flavobacteriaceae are potential keystone taxa in the intestinal microbial community of *A. japonicus* during intestine regeneration [26]. However, 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 the role of the microbiome in the process of tissue and organ regeneration.

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 gene 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.

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. 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 [27,28]. 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 had no feeding during intestine regeneration in our experiments.

2.2. Experimental design and sample collection

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 [18,28]. The 10th d was therefore an appropriate time point for sampling the new intestine. At least 40 individuals of similar size 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.

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. A total of 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 [29]. 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. The other 80 sea cucumbers were cultured normally in seawater in the laboratory as the ex-germ-free (XGF) group. During the period of rapid regrowth, at least 10 individuals of similar size were randomly selected per regeneration stage (10 and 14 d) 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. Then the intestines were washed in sterile water, and immediately transferred into sterile tubes and measured the weight. The washing water and intestine were together preserved at −80 °C for 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 (GTGCCAGCMGCCGCG GTAA) 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; [30]) 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 [31], and reads derived from host contamination were filtered using bowtie2.2.4 [32]. Clean reads were assembled and analyzed by SOAPdenovo software [33]. 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 [34,35]. A gene catalog was constructed using the gene models predicted from each sample by CD-HIT-EST (version 4.6.6) [36] 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) [37] 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) [38], eggNOG database (version 4.5) [39], and CAZy database (version 20,150,704) [40] 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 bacteria 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 [41]. 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 the 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 individual was significantly longer than that of the slower regenerating individual during intestine regeneration (10–14 d). The intestine of the faster regenerating individual was also heavier than that of the slower regenerating individual (Fig. 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 3147 OTUs were obtained across all samples. The samples from the faster regenerating group contained 2373 OTUs (on average), while the slower regenerating group samples contained 2365 OTUs (on average) (Supplementary 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) (Supplementary Fig. S1).

Analysis of the intestinal microbiota revealed that the faster and slower regenerating individuals had distinct taxonomic compositions (Fig. 2a, Supplementary Fig. 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 (Fig. 2a, Supplementary Fig. 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 relative abundance of Flavobacteriales was significantly more abundant in the faster regenerating individual than in the slower regenerating individual (Fig. 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

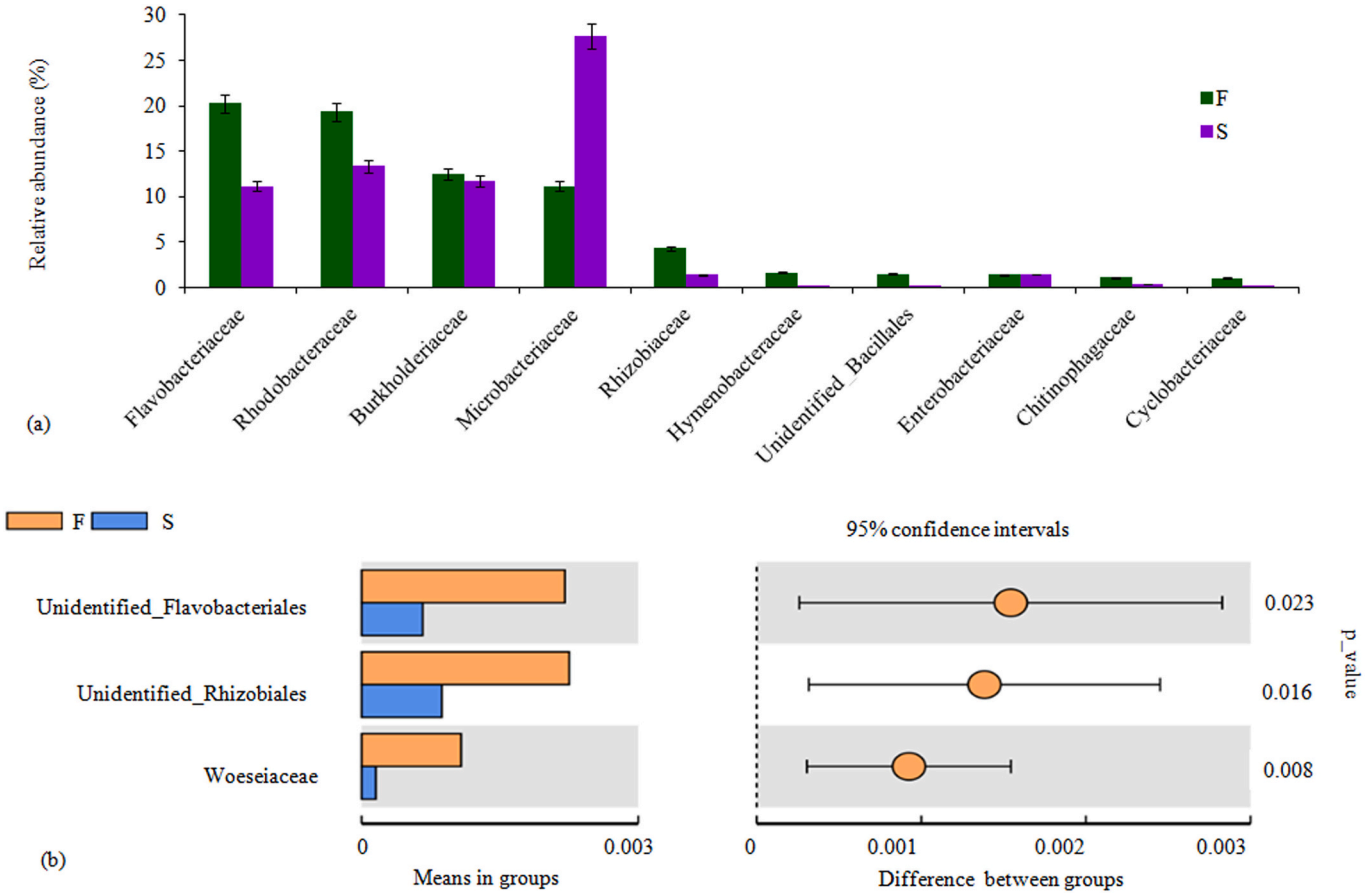

Fig. 2. The intestinal microbiota of the faster and slower regenerating *Apostichopus japonicus* individuals are different. (a) Comparison of the intestinal bacterial communities in the faster and slower regenerating *Apostichopus japonicus* individuals during different regeneration stages. (b) Differences 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.

representatives of the 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 individuals, 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 different functional levels were calculated, and a total of 300 functional genes were annotated to the metabolic pathways. The pathways of human diseases and metabolism contained the largest number of annotated genes, followed by pathways relevant to the organism system (Supplementary Fig. S2). The KEGG functional profiles in level 1 showed similarities in intestinal microbial functions in the faster and slower regenerating individuals, since the KEGG database does not represent invertebrates particularly well. However, there were still differences in some KEGG functional categories between the faster and slower regenerating individuals (Supplementary Fig. S3). The analysis demonstrated that 20 functional features in the subsystem category (level 2) were more abundant in one of the samples (Fig. 3). In detail, the genes for the 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' (Supplementary Fig. S4). There were no significant differences between the gene abundance of eggNOG functions when comparing faster and slower regenerating *A. japonicus* individuals.

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

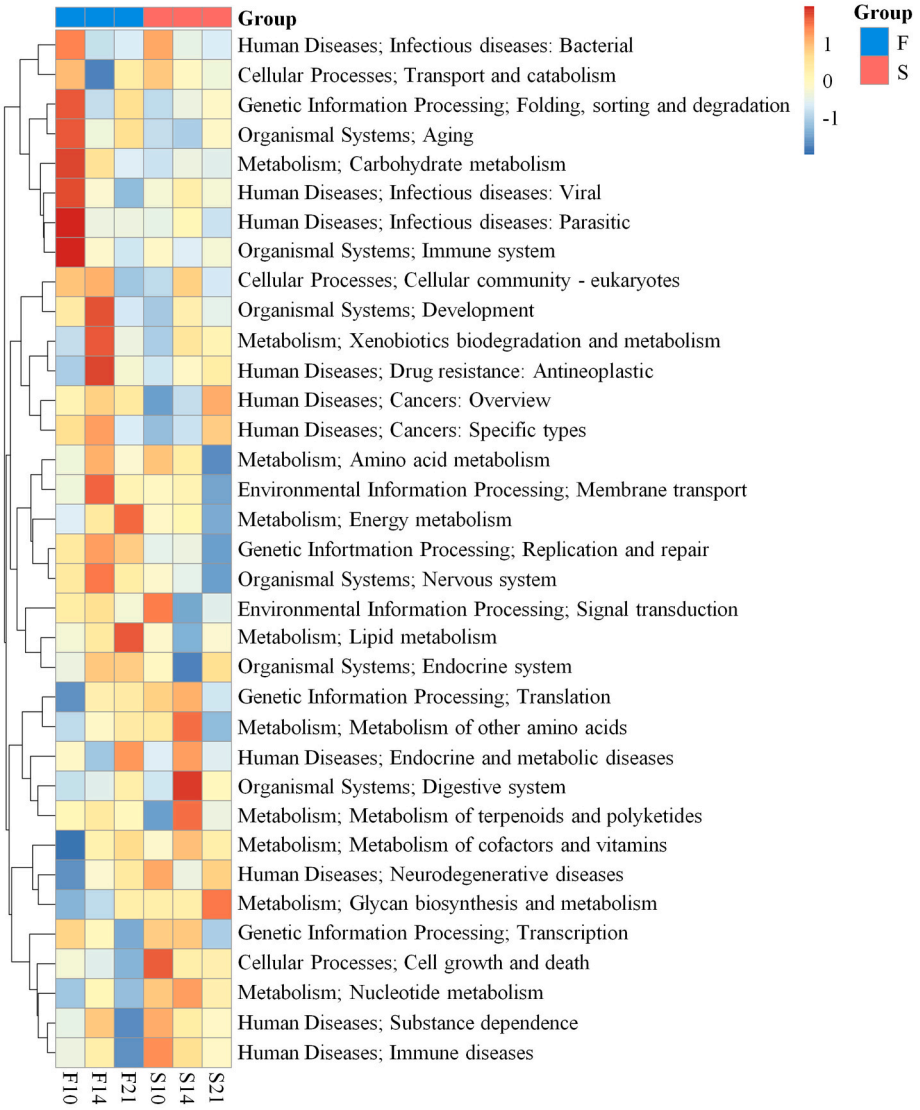

Fig. 3. Heatmap of the KEGG analysis of the intestinal microbiota in samples from the faster and slower regenerating *Apostichopus japonicus* individuals during the different regeneration stages.

observed a substantial variation in the copy numbers of the bacterial 16S rRNA genes between the faster and slower regenerating individuals. The abundances of the bacterial 16S rRNA gene were 8.9×10^7, 1.13×10^9 and 1.96×10^9 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 1.05×10^8, 1.5×10^9 and 2.99×10^9 copies per sea cucumber in the slower regenerating individuals in the 3 stages, respectively (Fig. 4). Additionally, the numbers of 16S rRNA gene copies/g (wet weight of intestine) were significantly different between the faster and slower regenerating individuals during intestine regeneration (Supplementary Fig. S5). The bacterial 16S rRNA genes were more abundant 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 gene 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 2.47×10^7 and 2.44×10^7 copies per sea cucumber in CV animals in the 10 d and 14 d stages, respectively. The bacterial 16S rRNA gene abundances were 8.23×10^6 and 8.18×10^6 copies per sea cucumber in GF animals in the 2 stages, respectively (Fig. 5). The number of bacterial 16S rRNA gene 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 (Fig. 6). The intestinal bacterial 16S rRNA gene abundance correlated with 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 1877 OTUs, the samples from the XGF group contained 1620 OTUs, and the GF samples contained 1228 OTUs (Supplementary Table S2). We analyzed

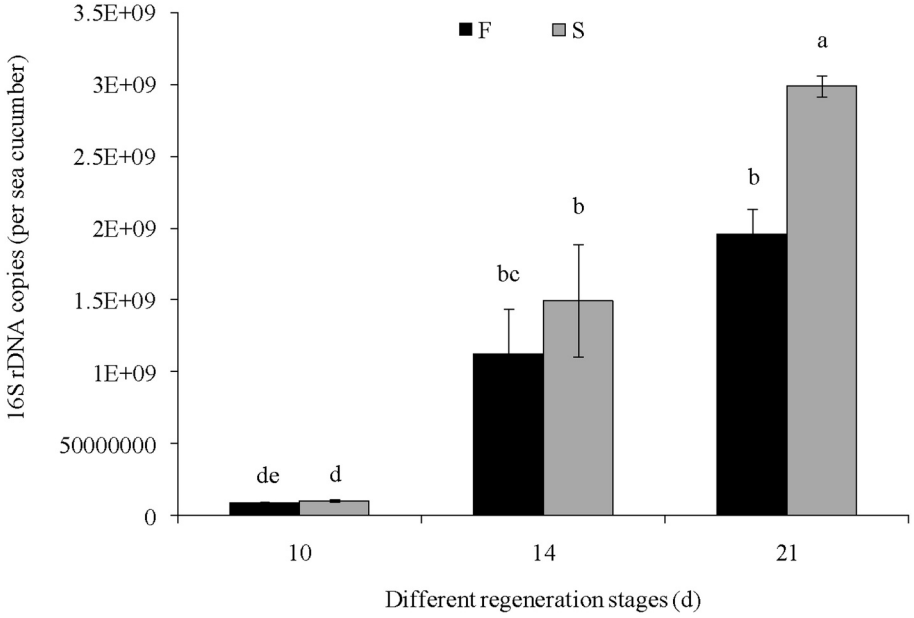

Fig. 4. Quantification of 16S rRNA gene copies/sea cucumber in the faster and slower regenerating *Apostichopus japonicus* individuals during the different regeneration stages. qPCR was performed with bacterial-specific primers on DNA extracted from the F or S groups. Different letters indicate significant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

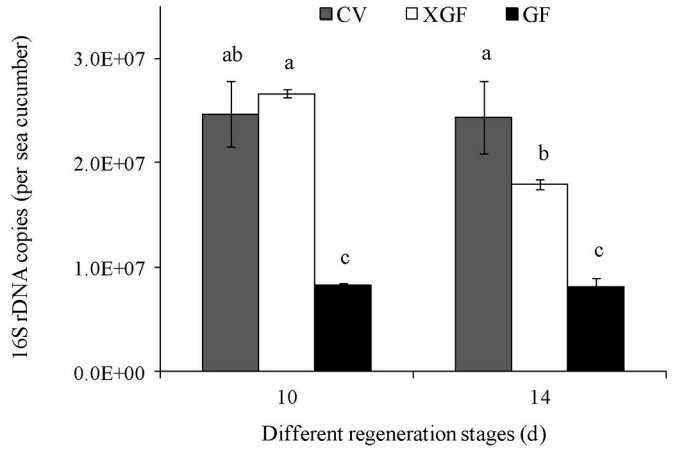

Fig. 5. Quantification of 16S rRNA gene copies/sea cucumber during the different regeneration stages. qPCR was performed with bacterial-specific primers on DNA extracted from the CV, XGF, or GF groups. Different letters indicate significant differences ($P < 0.05$).

genus 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 numbers of OTUs and genera 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 the *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, Supplementary 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, Supplementary Table S3–4).

The average abundances of Flavobacteriaceae (4.42%) and Rhodobacteraceae (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 to play an important role in sea cucumber health, growth and function [11,42]. 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 Rhodobacteraceae and Flavobacteriaceae may function as keystone taxa in the intestinal microbial community of *A. japonicus* during intestine regeneration [26]. 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 regenerating intestine in *A. japonicus*.

We demonstrated that decreased microbial quantity were observed in the intestine of faster regenerating *A. japonicus* individuals, who contained large fractions of Rhodobacteraceae 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 Rhodobacteraceae and then to promote the intestine regrowth rate of *A. japonicus*, which was observed

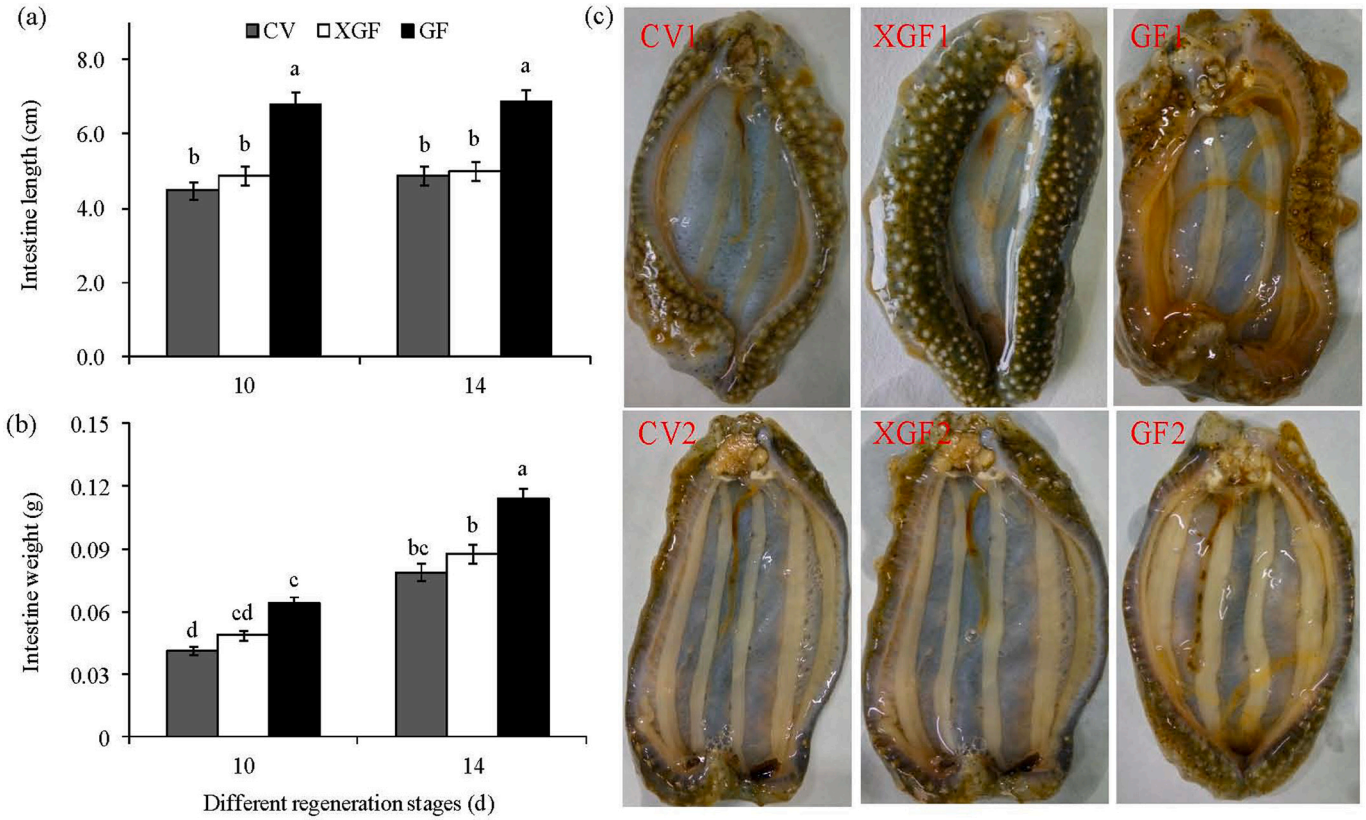

Fig. 6. The intestine length (a) and weight (b) of samples from the CV, XGF, or GF groups during the different regeneration stages. (c) Images of regenerating intestines from the CV, XGF, or GF groups at 10 or 14 d. Different letters indicate significant differences ($P < 0.05$).
Note: CV1-XGF1-GF1: 10 d; CV2-XGF2-GF2: 14 d.

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

Order	Genus	CV	GF
Flavobacteriales	***Flavobacterium***	**0.002**	**41.78**
Burkholderiales	*Ralstonia*	7.29	7.92
Pseudomonadales	*Psychrobacter*	0.002	6.46
Rhodobacterales	**Rhodobacteraceae_unclassified**	**1.12**	**3.05**
Flavobacteriales	*Polaribacter*	1.24	2.85
Flavobacteriales	**Flavobacteriaceae_uncultured**	**0.46**	**1.50**
Alteromonadales	*Colwellia*	5.15	1.21
Alteromonadales	*Pseudoalteromonas*	7.11	1.20
Flavobacteriales	*Crocinitomix*	**0.42**	**1.08**
Rhizobiales	*Pelagibacterium*	2.74	0.97
Sphingobacteriales	*Sphingobacteriales_norank*	5.18	0.71
Oceanospirillales	*Halomonas*	41.03	0.43
Vibrionales	*Vibrio*	3.46	0.31
Methylophilales	*Methylotenera*	1.21	0.27

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 the GF zebrafish model [7,43], and secretory cells were less abundant in the gut epithelium of GF zebrafish and rats [29,44]. In contrast to previous findings, our data suggest that depletion of the intestinal microbiota changed the microbial composition, and thus promoted the intestine regeneration rate of *A. japonicus*. The intestine regeneration rate was significantly faster in GF sea cucumbers than in CV sea cucumbers. Notably, the relative abundances of Flavobacteriaceae and Rhodobacteraceae were significantly increased in GF samples compared to 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 [26].

Flavobacteriaceae present low pathogenicity [45], and can produce carotenoids that have antioxidative activities [46]. More importantly, the marine Flavobacteriaceae usually produce enzymes that degrade agars, fucoidan, fucose, laminarin, xylan, and carrageenans from micro- or macroalgae [47]. Within Flavobacteriaceae, the predominant genus was *Flavobacterium*, which presents high levels of resistance to a wide range of antibiotics [48]. 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 [49]. 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* [19,23,25,26,50]. Rhodobacterales retaining polyhydroxybutyrate (PHB) metabolism genes, as a PHB producer, promoted the growth of sea cucumber *A. japonicus* [11]. In addition to our research, Rhodobacteraceae were identified as keystone taxa in the microbial community associated with *Nannochloropsis salina* in aquatic ecosystems [51]. 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 [11]. PHB accumulates in commonly nutrient-limited bacterial cells [52], and the bacterial PHB, especially that produced by Rhodobacterales, might also serve as an energy source for the sea cucumber [11].

Together, our results indicate a direct link between intestinal bacterial 16S rRNA gene 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 a 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 first assessed possible links between intestinal microbial quantity and intestinal microbiome features and the intestine regeneration rate of *A. japonicus*. 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 the special life event of the host and provide new insights into the host-microbiota interaction related to organ regeneration.

Date accessibility

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

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.

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Declaration of Competing Interest

The authors declare that no conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2020.09.017.

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