Coral co-culture alters bacterial community structure by altering rare bacterial species

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Abstract

Coral symbionts are important members of the coral holobiont, and the coral bacterial flora is essential in host health maintenance and coral conservation. Coral symbionts are affected by various environmental factors, such as seawater temperature, pH, and salinity. However, the effect of communication between different coral species and water flow against coral bacterial flora on its community structure is not well understood. In this study, we designed an artificial rearing environment to examine the impact of environmental and biological factors on Acropora tenuis, one of the major coral species in Okinawa, and Montipora digitata, from their co-culture. We intervened with the water flow to reveal that the movement of the rearing environment alters the bacterial flora of A. tenuis. During the bacterial community reconstruction, the alpha diversity of the coral microbiota increased, suggesting the establishment of rare bacteria from the ocean. No differences in the bacterial composition between the control and water flow groups were observed under the rearing conditions. However, the structure of the bacterial flora was significantly different in the co-culture group. Comparison among each bacterial community succession strongly suggest that the difference was due to the suppressed transmission of bacteria from the ocean in the co-culture group. These results contribute to the understanding of communication between corals and shed light on the importance of regional differences and bacterial composition of coral flora.

Introduction

Although coral reefs cover less than 0.2% of the ocean worldwide, they are home to more than 25% of marine life and are the foundation of marine ecosystems (Fisher et al. 2015). Corals maintain their activity by forming holobionts comprising symbiotic associations of bacteria, zooxanthellae, archaea, fungi, and viruses (Bourne et al. 2016). Symbiotic bacteria perform various functions, including defense against pathogenic bacteria, production of antibiotics, and supply of nutrients (Bourne et al. 2016; Rosado et al. 2019). Some symbiotic bacteria can improve coral health, and programs are underway to release these bacteria into corals for coral conservation (Rosado et al. 2019; Peixoto et al. 2020).

Therefore, clarifying the relationship between coral flora and habitat is essential for understanding coral ecology. Coral flora depends on pH (Thurber et al. 2009; Meron et al. 2011), sea temperature (Thurber et al. 2009; Webster et al. 2011; Tracy et al. 2015), salinity (Randle et al. 2020), and eutrophication (Jessen et al. 2013). Additionally, it has been shown that water flow is one of the most critical factors that affects the marine ecosystem. Furthermore, water flow inhibits bleaching in corals exposed to thermal stress (Lee et al. 2017).

Kevin Walsh et al. 2017 showed that corals influence the bacterial composition and function of water columns around coral reefs by forming a bacterial "aurabiome" of the surrounding seawater (Walsh et al. 2017). Laura Weber et al. 2019 also showed a bacterial exchange between corals and seawater by determining the bacterial composition of seawater proximal and distal to the coral (Weber et al. 2019). Additionally, coral symbiotic bacteria are transmitted from the surrounding seawater (Damjanovic et al.

2019), suggesting that water currents and hydrodynamic boundary layers around corals influence nutrient supply to corals (Shashar et al. 1996).

Acropora tenuis, one of the major coral species in Okinawa Prefecture, Japan, and is found in both inner and outer reefs (Omori, 2011). Our previous studiesas well as those by others have confirmed the abundance of *Acropora* and *Montipora* corals in Sesoko Island waters (Cabaitan et al. 2012; Maruyama et al. 2021). With little changes in water flow in the inner reef, *Montipora digitata* surround *A. tenuis*. In contrast, the outer reef is covered by *Acropora* corals and is exposed to continuous water flow. The effect of communication between different coral species and water currents on the bacterial community structure is not well understood. Therefore, in this study, we examined the impact of environmental and biological factors on *Acropora tenuis* and *Montipora digitata*, which were selected for our experiments based on the above findings. The findings from this study will provide useful insights into the conservation of coral ecosystems and design of coral plantings for reef restoration.

Materials And Methods

Coral branch sampling and Rearing

Coral samples were obtained on November 24, 2016; *A. tenuis* samples were obtained from a colony inhabiting the southern area of Sesoko Island, Okinawa Prefecture (Sesokominami, 26°53.05’ N, 127°85.77’ E), whereas *M. digitata* samples were obtained from six colonies inhabiting the northern area of Sesoko Island, Okinawa (Ishikawabaru, 26°67.51’ N, 127°87.00’ E). The coral samples were transported to the Sesoko Station, the Tropical Biosphere Research Center, at the University of the Ryukyus, and was allowed to stand for a day in a tank with external seawater inflow. Approval for collecting coral samples was obtained from Okinawa Prefecture by Okinawa Prefecture Fishing Regulation.

Experimental design

To evaluate the effect of environmental factors on the bacterial composition of *A. tenuis*, we divided them into three groups: a control group, water flow control group, and co-culture group (Fig. 1). The rearing environment simulated the coral reefs around the Sesoko Island in Okinawa Prefecture. *A. tenuis* occupy the Sesokominami field with the water flow averaged to 15 cm/s in the natural environment. Therefore, it was referred to as a water flow control group in the rearing environment. In contrast, the Ishikawabaru field is occupied by *Montipora digitata* surrounding *A. tenuis*, and water currents averaged less than 5 cm/s in the natural environment. Therefore, they were referred to as the co-culture group in the rearing environment (Supplemental figure 1). As a control group, we also established an environment with only *A. tenuis*, which was not exposed by water flow. In all three groups, nets were set up in the aquarium, and six coral branches (5–10 cm) of *A. tenuis* originated from one community, were placed on the nets. All groups were equipped with aerators to supply oxygen, and the inflowing seawater was the same as the seawater outside the experimental site. In the water flow group, a pump was used to generate a water flow of 15 cm/s in the tank based on the average water flow in the sea around Okinawa (Kitada et al. 2006). In the co-culture group, six branches (5–10 cm) of *M. digitata* originating from several colonies

were placed around the branches of *A. tenuis*. To determine the floral community structure at the beginning of the experiment, we collected *A. tenuis* from the control group, *M. digitata* branches from the co-culture group, and seawater from the three tanks. After four weeks of rearing, branches of *A. tenuis* and *M. digitata* were also collected from each group to obtain the bacterial flora after the experiment.

The intervention groups were divided into control, flow control, and co-culture groups. All the tanks were well aerated and supplied with seawater. The water flow was adjusted to 15 cm/s in the flow control using a pump. The intervention experiment lasted four weeks, and sampling for 16S rDNA sequencing was performed three times: when the experimental corals were obtained from the natural environment, at the beginning of the experiment, and at the end of the experiment.

DNA extraction and library preparation from coral branch

For 16S rDNA gene amplicon sequencing, coral branches were transferred into a 5 mL tube (Eppendorf Ltd., Hamburg, Germany) containing 4 mL RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and then frozen with liquid nitrogen and stored in a -80°C deep freezer until analysis. The frozen samples were thawed, and the coral branches were then picked with tweezers and washed with artificial seawater. Next, in a sterilized zipper bag, the coral tissue was blown off using a Waterpik (EW-DJ61-W, Panasonic Corp., Japan), and the suspensions were collected into a 50 mL tube (Nippon Genetics Co., Ltd., Tokyo, Japan). After centrifugation at 10,000 × g, 4 °C for 30 min, the supernatant was removed, and the pellet was resuspended in 500 µL of artificial seawater. The suspension was transferred into a 1.5 mL tube (SSIBio, Lodi, CA, USA) and centrifuged at 15,000 × g, 4 °C for 15 min. After the supernatant was removed, the pellet was stored at -80 °C until DNA extraction. DNeasy Plant Mini Kit (69104; QIAGEN, Hilden, Germany) was used for DNA extraction according to the manufacturer's instruction, with a minor modification. The thawed pellet was mixed with 400 µL of Buffer AP1 and transferred into 2 mL screw cap tubes with stabilized 0.1 mm zirconia beads (Yasui Kikai Corp., Osaka, Japan). The tubes were homogenized three times at 2,500 rpm for 60 s at 60 s intervals. After centrifugation, 4 µL of RNase A was added, and the sample was incubated at 65°C for 10 min before DNA extraction.

DNA extraction from seawater

One liter of seawater was collected from each tank and filtered through a 1.6 µm filter. The filtered seawater was filtered again using a 0.22 µm filter. The 0.22 µm filtrates after filtration were stored in RNA later (Thermo Fisher Scientific, USA). The filter was then cut with scissors, and DNA extraction was carried out through the same process as that for coral DNA extraction.

16S rDNA gene amplicon sequencing and bioinformatic analysis

The bacterial community was identified by targeting the variable region V1-V2 of the 16S rDNA gene using the primer set 27F and 338R. PCR amplification of the 16S rDNA gene amplicon (ca. 300 bp) was performed in a 25 µL mixture, and the amplicons were sequenced using Ion Torrent PGM with 318 Chip v2 (Thermo Fisher Scientific).

16S rDNA gene analysis was performed using QIIME2 2020.6 (Bolyen et al. 2019). For quality control, amplicon sequence variants (ASVs) were constructed using DADA2 (via q2-dada2) with default parameters (Callahan et al. 2016). All ASVs were aligned using mafft (q2-alignment) with default parameters (Katoh et al. 2002). fasttree2 (via q2-mask and q2-phylogeny) was used to construct a phylogenetic tree based on the masked ASV alignments with default parameters (Price et al. 2010). Taxonomic assignments were performed with 99% OTU full-length sequences of the silva database (version 138) using the q2-feature-classifier (Bokulich et al. 2018). Downstream analysis (α-diversity and β-diveristy analysis) was performed using R Bioconductor phyloseq (McMurdie and Holmes 2013) and MicrobiotaProcess package in R version 3.5.2 (Xu and Yu 2022). PERANOVA test was performed using a vegan package.

Results

Sequence data statistics and microbial diversity in corals

We conducted a 4-week rearing experiment using *A. tenuis* (collected from the natural environment) and compared the changes in symbiotic bacterial community structure before and after the experiment in the water flow control group and the co-culture group with the control group (no treatment). In all three groups (water flow control, co-culture, and control), no coral mortality or bleaching was observed, suggesting that fluctuations in the rearing environment did not cause fatal damage to the corals (data not shown).

The data consisted of 50 samples (seawater: 3 samples, coral: 44 samples (5 conditions, two coral species)), containing a total of 1,412,100 sequences (average length 294 bp). After exclusion of chimeric and host-derived sequences, 1,268,392 sequences were annotated to bacteria. A total of 7154 ASVs were constructed in total by 16S rDNA sequencing analysis, of which 5528 ASVs were detected in corals, and others were detected in seawater. Phylogenetic annotation showed that most (9.44–83.2%) of the bacterial composition in coral tissue belonged to the order Oceanspilaris (*Endozoicomonaceae*), followed by the orders Rhodobacteales, Flavobacterales, and Propiobacteriales (Fig. 2). In the following analysis, the data volume of all samples was adjusted to 10,000 reads by Rarefaction.

To measure the diversity of symbiotic bacteria in each coral, α-diversity (Observed ASVs, Shannon index, Simpson index) was calculated in *A. tenuis* and *M. digitata* after the experiment (Fig. 3). The number of ASVs detected in *A. tenuis* (observed ASVs) averaged 91.5 (SD: ±23.3) at the beginning of the experiment, compared to that observed in the control (316.5 (SD: ±109.1)), water flow control (396.8 (SD: ±181.2)), and co-culture (315.3 (SD: ±124.8)) groups. There was a significant increase in diversity in all experimental groups after the experiment (Fig. 3A). However, the Shannon index, which indicates the uniformity of α-diversity, showed a significant variation in the control group ($p = 0.041$) and the water flow group ($p = 0.0043$). In contrast, no significant variation was observed in the co-culture group using the Simpson's index ($p = 0.82$, Fig. 3A). Simpson index did not show significant variation in any sample, and

M. digitata in the co-culture group did not show significant variation the alpha diversity indices of all samples (Fig. 3B).

Variation in bacterial composition between the co-culture group and water flow control group

We compared the β-diversity among the experimental groups using weighted Unifrac and unweighted Unifrac distances to analyze the variation in bacterial flora structure among the groups (Fig. 4). In *A. tenuis*, a comparison between the three experimental groups at the beginning of the experiment and after four weeks of intervention showed a significant change in the bacterial composition (PERANOVA; p = 0.0027, F = 2.6474, supplemental fig. 2). Next, we analyzed β-diversity among the three groups after the experiment, and hierarchical clustering confirmed that the co-culture groups formed clusters at both weighted Unifrac and unweighted Unifrac distances (Fig. 4A, D). The bacterial flora of the control group and the two experimental groups were compared after the rearing experiment. The results showed that the intragroup distance of the co-culture group was significantly reduced compared with that of the control group (weighted Unifrac: p = 2.7e-07; unweighted Unifrac: p = 7.8e-05, Fig. 4C, F). Additionally, the intra-county distance of the co-culture group was significantly decreased by weighted Unifrac compared with that of the control group (weighted Unifrac: p = 0.0086; unweighted Unifrac: p = 0.081, Fig. 4C, F). These results suggest that the rearing environment in the co-culture group affects the structural variation of the bacterial flora of *A. tenuis*, resulting in the convergence of the flora structure. In contrast, the intra- and inter-county distances of both weighted Unifrac and unweighted Unifrac in the water flow control group did not change significantly compared to those of the control group (Fig. 4B, E). Therefore, the water flow adjustment did not have a substantial effect on the bacterial flora of *A. tenuis*.

Variation in rare bacteria between experimental groups

Next, we analyzed the presence and absence of ASVs in the bacterial flora of each experimental group to identify the bacteria involved in bacterial flora variation (Fig. 5, Table. 1). Of the 5528 ASVs in the three groups, at the beginning and end of the experiment, 30 ASVs (core ASVs) were commonly detected at the beginning of the investigation in all groups, with average relative abundance of 25.6% (0.118–36.3%) (Fig. 5A; upset groups: green). Most bacteria were members of the *Endozoicomonaceae* family (*Endozoicomonas* sp.) (Fig. 5B; upset groups: green). However, there were 192 ASVs commonly detected in the three experimental groups except at the beginning of the experiment, with an average relative abundance of 24.7% (0.047–78.1%) and the average relative abundance of 2.61% (0.00813.4%) for each ASV (Fig. 5A; red). Although the number of ASVs detected specifically in each group at the end of the experiment was high, they accounted for a low percentage of the total composition (Table. 1). Next, when we focused on the presence or absence of ASVs shared among the groups, we found 237 ASVs that were not present in the co-culture group exclusively (Fig. 5B; blue; total ASVs: 5.74 ± 4.63%, each ASV: 0.192 ± 0.381%). These ASVs had more than twice as much the ASVs absent in the control group (total ASV: 2.15 ± 1.85%, each ASV: 0.145 ± 0.620%) and the water flow control group (Fig. 5B; yellow; total ASV: 1.48 ± 1.06%, each ASV: 0.123 ± 0.178%). The results suggest that co-localization with M. digitata inhibits the establishment of many bacteria on A. tenuis compared to water flow and control conditions.

Bacterial species not detected at the beginning of the experiment but were commonly detected in the three groups at the end mainly belonged to the *Rhodobactraceae* family (Fig. 5B; red). The *Paraspirulinaceae* (genus unknown) and *Phycisphaeraceae* (*Phycisphaera* genus) families were specifically detected in the water flow control group. Bacteria that were not detected in the co-culture group included *Endozoicomonaceae* (*Endozoicomonas* genus), *Rhodobacteraceae* (*Limibaculum*, *Roseibacterium*, and *Silicimonas* genus), and *Piscirickettsiaceae* (*Piscirickettsia* genus) (Fig. 5B blue). In particular, Piscirickettsia was the only bacterium that disappeared under co-culture conditions while it was detected under other conditions.

Table. 1 Number of ASVs detected among each group

Groups	The number of ASVs	The total percentage of ASVs (%)	The average percentage of ASVs (%)
Control (beginning)	315	13.2±7.77	0.440±0.589
Control (Finished)	842	11.1±9.86	0.117±0.398
Water-flow	1,014	10.5±7.05	0.112±0.708
Co-culture	870	11.1±9.86	0.117±0.398
Control (Finished), Water-flow	237	5.74±4.63	0.192±0.381
Control (Finished), Co-culture	151	1.48±1.06	0.123±0.178
Co-culture, Water-flow	101	2.15±1.85	0.145±0.620

Discussion

The movement of corals to captive aquaria reconstructs bacterial composition

The bacterial flora in *A. tenuis* fluctuated wildly in all experimental conditions, throughout the 4-week period. Additionally, the alpha diversity (observed ASV) of the *A. tenuis* flora increased in all intervention groups. An increase in bacterial diversity has been reported in the bacterial flora of diseased corals (Morrow et al. 2012; Welsh et al. 2017). These results suggest that the bacterial composition restructuring in the captive environment of this experiment may have caused the corals to be exposed to stress. Additionally, this intervention increased the number of bacteril members in the *A. tenuis* bacterial flora, including members of the *Rhodobacteraceae* family, which increases during stress in bleaching, and the *Flavobacteriaceae* family includes pathogenic taxa. The abundance of these bacteria has also increased during previous *A. hemprichii* transplantation experiments (Ziegler et al. 2019) and has been reported to increase the bacteria in corals injured (Clements et al. 2020).

Co-localization with different coral species converges bacterial composition in corals

Surprisingly, the bacterial composition of *A. tenuis* co-culture with *M. digitata* converged to a unique structure compared to the composition in the control group (Fig. 4C). This suggests that co-culture with *M. digitata* had a substantial effect on the bacterial composition of *A. tenuis*. Additionally, alpha diversity results showed that the co-culture group had a lower mean Shannon index than others. Unweighted Unifrac analysis showed that the bacterial composition of the co-culture groups was similar.

These results suggest that the co-culture group formed a unique bacterial composition structure because certain bacterial species detected in the control and water-flow groups were not detected in the co-culture group due to the influence of *Montipora* corals. Among the bacteria that were not detected in the bacterial presence/absence comparison results, were those belonging to the genus *Piscirickettsia,* that has been reported as a pathogenic bacterium of a salmon but is present in the coral and seawater (Mauel and Fryer 2001; Sweet et al. 2017). It has been suggested that the bacterium amplifies its abundance in diseased corals (Sweet et al. 2017). These results indicate that co-cultured *Montipora* corals may reduce the establishment of pathogenic bacteria in the ocean.

A possible reason for the low alpha diversity and unique structure of coral flora, in the co-culture groups, is the influence of the ecosphere formed by *Montipora* corals. Communication between organisms via seawater in coral reefs has been reported between corals and other organisms such as seaweeds. The organisms that make up coral reefs release their microorganisms into the surrounding environment, creating an environment with a unique bacterial structure called the ecosphere. It has been reported that genes associated with resistance to antibiotics and toxins are enriched in seawater in the vicinity of *M. digitata* and *M. braziliensis*, a member of the same genus (Walsh et al. 2017). In our experiments, *M. digitata* may have inhibited the establishment of pathogenic bacteria in *A. tenuis* through its function in the ecosphere.

Impact of coral co-localization on coral conservation

Coral reefs are composed of various corals and are classified as inner and outer reefs, which differ in the environment and bacterial composition of the corals (Roder et al. 2015). *Acropora tenuis* is one of the important coral species in Okinawa, Japan, and is found in both inner and outer reefs (Omori 2011). Inner reefs have slight water flow variation and are surrounded by *Montipora digitata*. There is little change in water flow in the inner reef and corals surrounding *A. tenuis* (Supplemental figure 1).

In contrast, the outer reef is occupied by *Acropora* corals and is exposed to persistent water flow. Water flow and co-occurrence with different coral species have not been focused on factors affecting bacterial colonization in the reconstruction of coral bacterial composition. Several studies have suggested that corals under stress rebuild their bacterial flora. This study provides insights into coral conservation and stress tolerance by clarifying the influence of habitat on the rebuilding of the coral bacterial flora.

Conclusion

Previous studies on inter-coral communication have been limited to reconstructing the bacterial composition of coral neonates and the ecosphere. In this study, we used rearing experiments to replicate previously unnoticed factors of inner and outer reefs in the coral reef waters of Okinawa, Japan, to evaluate the effects of these factors on the bacterial composition of *A. tenuis*. This study suggests the importance of inter-coral communication in coral habitats and maintaining coral diversity within reefs for coral conservation.

Declarations

Data availability

The raw read data of 16S rDNA are available under BioProject number PRJNA821479.

Author Contributions

Y.Na., M.I., H.F. and H.T. designed the experiments. K.I., Y.Ni., and H.T. wrote the manuscript. M.I. and Y.Na. collected the data. K.I. performed all the bioinformatics analyses. All authors have read and approved the final manuscript.

Competing interests

There are no conflicts of interest to declare.

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Figures

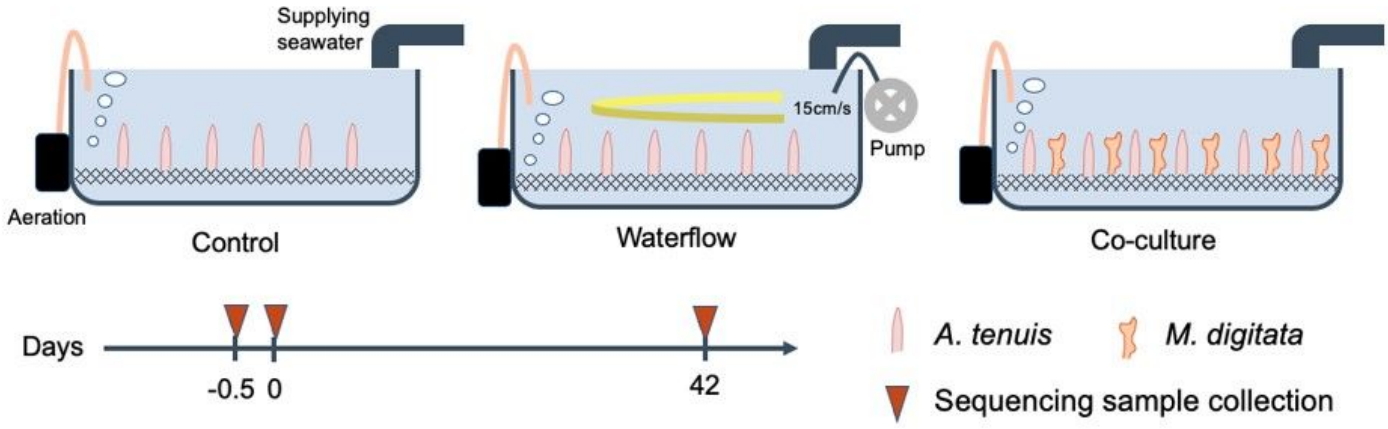

Figure 1

Overview of the experimental design.

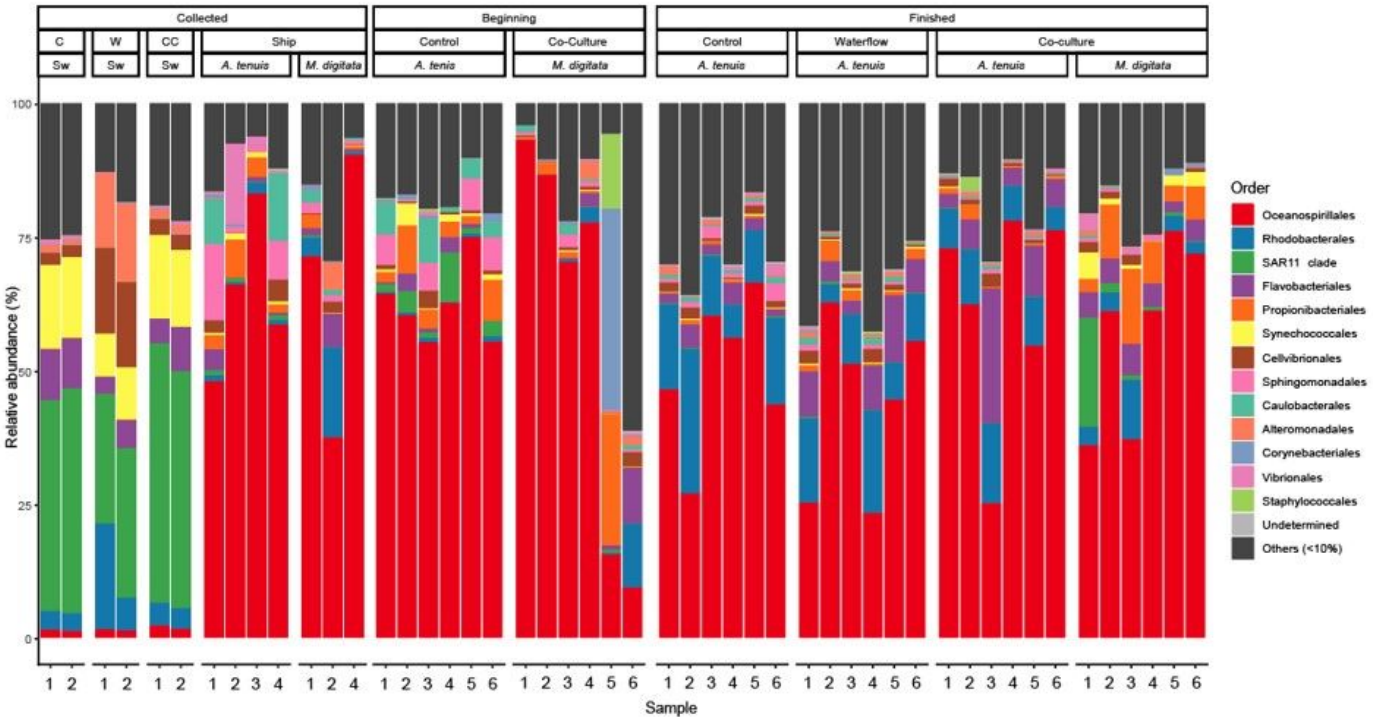

Figure 2

Coral bacterial flora during the intervention experiment and sampling

Results of bacterial composition at the order level by 16S rDNA of *A. tenuis* and *M. digitata* used in the experiment. The number on the X-axis indicates the branch number; all *A. tenuis* are from the same group, but M. digitata are from different groups; C; control group, F; water flow control group, CC; co-culture group.

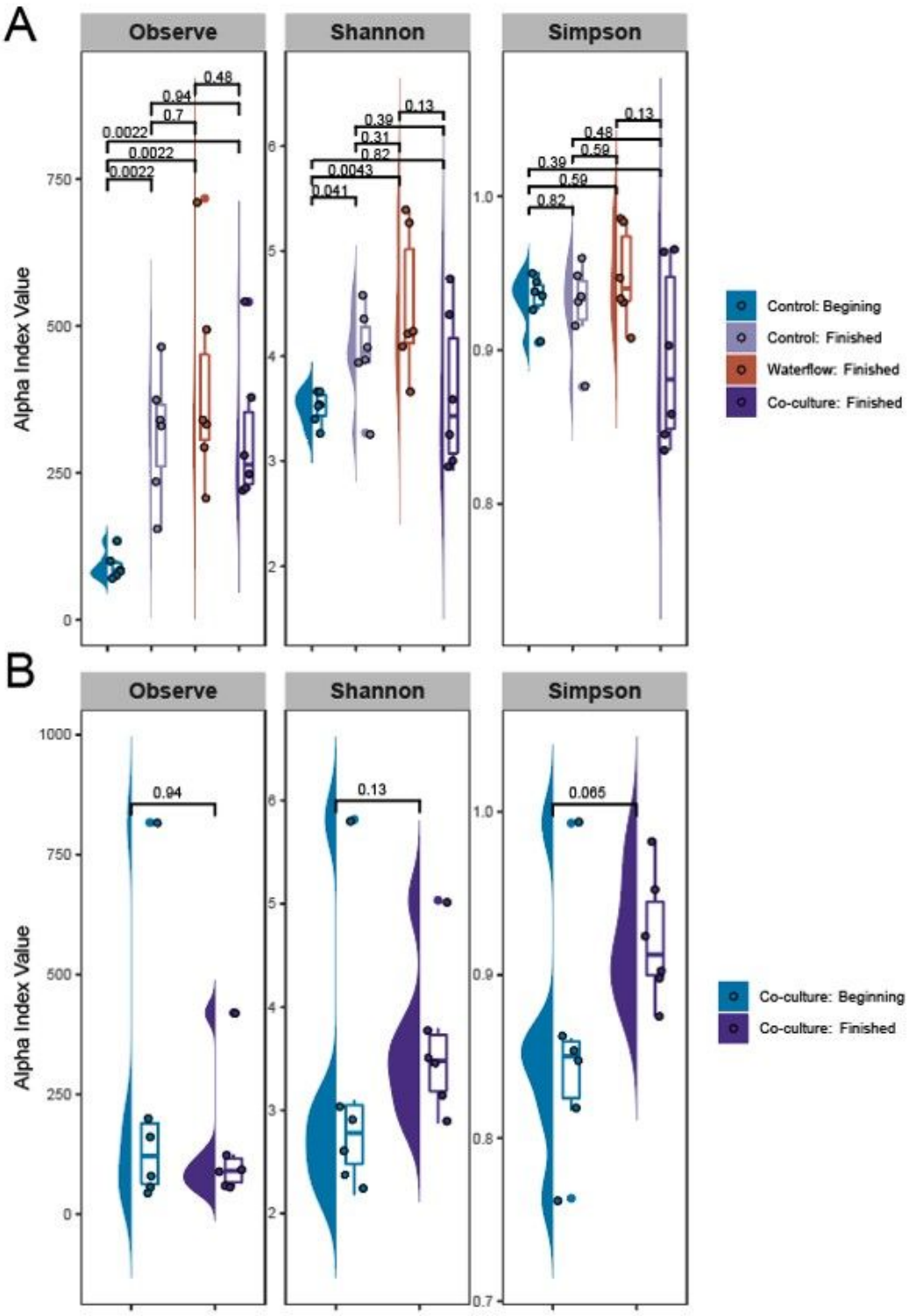

Figure 3

Comparison of alpha diversity between the intervention and experimental groups

Comparison of alpha diversity (Observed ASV, Shannon index, Simpson index) of control, flow control, and co-culture groups. Analyses were performed using the Wilcoxon rank sum test. A: Comparison of *A. tenuis* diversity in control (beginning and end of experiment), flow control, and co-culture groups. B: Comparison of *M.digitata* diversity in co-culture (beginning and end of experiment).

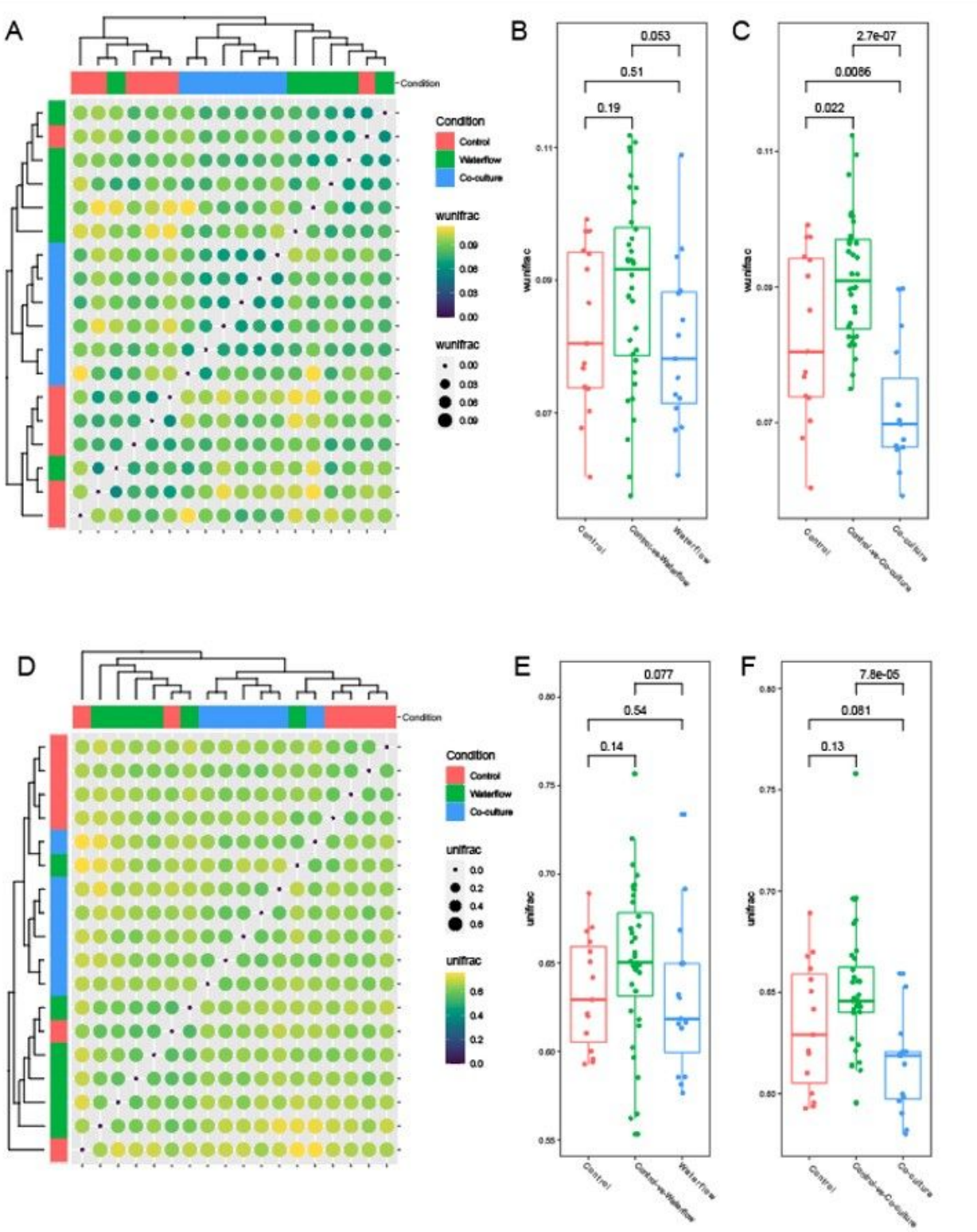

Figure 4

Clustering analysis of bacterial composition using weighted and unweighted UniFrac distances after intervention

A: Heat map using weighted UniFrac distance. Hierarchical clustering

B: Comparison of within-county and between-county distances between control and water flow control groups using weighted UniFrac distance. Analyses were performed using the Wilcoxon rank sum test.

C: Comparison of within-county and between-county distances between control and co-culture groups using weighted UniFrac distance. Analyses were performed using the Wilcoxon rank-sum test.

D: Heat map using unweighted UniFrac distance. Hierarchical clustering

E: Comparison of within-county and between-county distances between control and water flow control groups using unweighted UniFrac distance. Analyses were performed using the Wilcoxon rank sum test.

F: Comparison of within-county and between-county distances between control and co-culture groups using unweighted UniFrac distance. Analyses were performed using the Wilcoxon rank sum test.

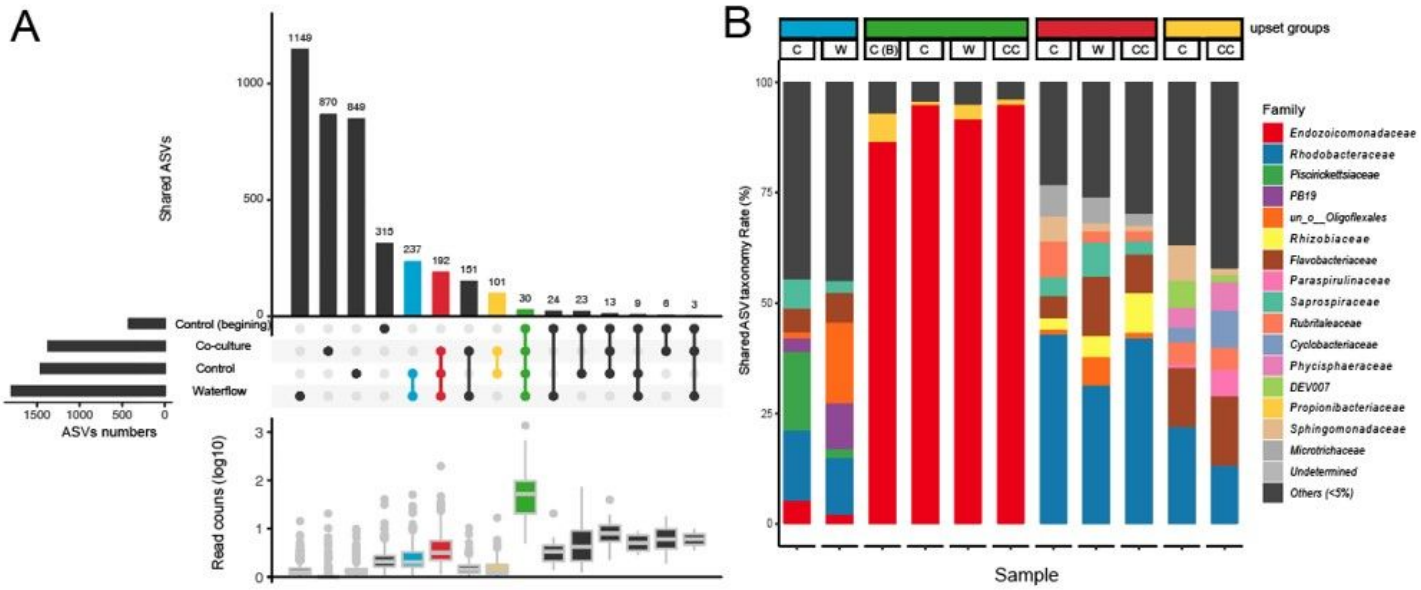

Figure 5

Detection of group-specific ASVs and confirmation of lineage

A: Comparison of ASVs shared between groups. The circles indicate their presence in the group and bars at the top indicate the number of shared ASV types. The bottom figure shows the number of reads (on a log scale) for each sample of ASV in each group. All samples were diluted to 10,000 reads. Each color indicates the upset groups of interest.

B: Identification of the phylogeny at the family level in each common ASV. This figure shows the phylogenetic distribution of the ASVs, not the bacterial composition. The upset groups at the top correspond to the colors in A. C; control group, C (B); control group at the beginning of the experiment, H; water flow control group, R; co-culture group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.docx