

Surgeonfish feces increase microbial opportunism in reef-building corals

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ABSTRACT: Coral microbiomes are sensitive to multiple physical and biotic stressors that can increase host susceptibility to dysbiosis, leading to bleaching and mortality. While the factors that drive coral dysbiosis are complex and still not well understood, a number of mechanisms may facilitate transmission of opportunistic bacteria. For instance, several marine invertebrates and fishes are suspected to act as microbial vectors through corallivory, as well as via fecal transmission of microbes. Here, we used a factorial manipulative experiment to test the interactive effects of feces deposition by a common surgeonfish *Ctenochaetus striatus* and mechanical wounding (mimicking corallivory) on microbial communities of the coral *Porites lobata*. We found that exposure of unwounded and wounded corals to fish feces induced an increase in microbial richness and diversity of 1.5- and 2-fold, respectively, and resulted in greater abundance of potential opportunists (e.g. *Rhodobacteraceae*, *Verrucomicrobiaceae*, *Flavobacteriaceae*, *Vibrionaceae*, *Fusobacteriaceae*). Many of these opportunists were also found in *C. striatus* feces. While microbial communities in corals exposed to individual stressors tended to shift back to their original composition at the end of the 48 h experiment, the persistence of potential opportunists (e.g. *Vibrionaceae*, *Campylobacteraceae*, *Cohaesibacteraceae*) when feces and wounding were combined indicates that impacts to the coral microbiome may be exacerbated when stressors interact. Our results suggest that fish feces may represent a novel route of transmission of microbes and/or facilitate the enrichment of potentially opportunistic and pathogenic bacteria within reef-building corals. Further, interactions with other common stressors such as corallivory might worsen these impacts, increasing dysbiosis and ultimately affecting coral health.

KEY WORDS: Surgeonfish · *Ctenochaetus striatus* · Coral · Feces · Microbiome · Bacteria · *Vibrio* · 16S rRNA gene

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1. INTRODUCTION

Coral reefs are one of the most biologically productive ecosystems on Earth, despite inhabiting nutrient-poor surface waters across the world's ocean. Such high rates of productivity originate from complex symbiotic interactions between micro- and macroorganisms within the reef ecosystem (Muscatine & Weis 1992, Rosenberg et al. 2007b). For instance,

microbes such as dinoflagellate algae (Symbiodiniaceae) transfer sugar-rich molecules they produce via photosynthesis to their coral host (Muscatine 1990), providing the energy required to sustain calcification in corals and the production of the reef framework (Muscatine & Porter 1977). Additionally, reef-building corals co-diversified with a wide diversity of symbiotic bacteria in their tissues, mucus, and skeleton that contribute to essential host functions

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(Thompson et al. 2015, McDevitt-Irwin et al. 2017, Pollock et al. 2018). Although little is known about the functional roles of bacteria within the coral holobiont, they may provide several benefits, including the potential transfer of essential nutrients to the coral host, as well as production of antibiotic molecules and secondary metabolites (Zhang et al. 2015, Bourne et al. 2016). Thus, coral-associated bacteria are vital to functions ranging from coral nutrition to immunity that influence fundamental ecosystem processes within coral reefs (Rosenberg et al. 2007a,b).

Despite their importance, coral-associated bacterial assemblages are sensitive to multiple physical (e.g. temperature, nutrient pollution) and biotic (e.g. macroalgal competition, corallivory) stressors (McDevitt-Irwin et al. 2017). These stressors can drive dysbiosis in corals (i.e. the loss of beneficial microbes or increase of opportunists) that results in compromised health and mortality (Zaneveld et al. 2016, McDevitt-Irwin et al. 2017, Muller et al. 2018). Yet, the factors influencing the dynamics of coral dysbiosis are complex and not well understood. However, a number of mechanisms known to support transmission of opportunistic microbes and possible pathogens have been identified. For example, echinoids (Katz et al. 2014), gastropods (Bettarel et al. 2018, Nicolet et al. 2018), and fishes (Aeby & Santavy 2006, Chong-Seng et al. 2011, Nicolet et al. 2018) may be vectors of opportunists or pathogens to corals via oral transmission during corallivory. In particular, coral-feeding chaetodontid butterflyfishes have been associated with increased rates of black band disease in experimental plots (Aeby & Santavy 2006), and greater disease prevalence is observed on overfished reefs with elevated butterflyfish densities (Raymundo et al. 2009). These fishes often feed on infected coral lesions, then prey on healthy coral colonies (Chong-Seng et al. 2011). This feeding behavior likely makes corallivorous fishes capable of spreading putative pathogens among corals via their mouthparts.

Although most studies to date have focused on oral transmission of pathogens by corallivores to reef-building corals (Nicolet et al. 2018), fecal pellets deposited by fishes might represent an alternative and underappreciated route of transmission for microbial pathogens within reef ecosystems (Aeby & Santavy 2006, Garren et al. 2008, 2009). Due to abundant fishes, coral reefs experience a continuous 'rain of feces' (Smriga et al. 2010), which may provide key nutrients to corals that increase growth (Meyer et al. 1983, Meyer & Schultz 1985). However, the gut of many reef fishes supports a high abundance of diverse microbes (Smriga et al. 2010, Ghanbari et al.

2015, Nielsen et al. 2017, Jones et al. 2018) that may include parasites or pathogens that could adversely affect coral health (Clements et al. 2014, Hennersdorf et al. 2016). Two recent studies showed that effluent from fish farms alters coral microbiomes (Garren et al. 2008, 2009), with overwhelmingly negative effects on corals. However, no study to date has evaluated the effects of reef fish feces on coral microbiomes or their potential for transmitting opportunistic microbes to corals.

Here, we investigated how deposition of fish feces impacts coral microbial communities. We focused on the line bristletooth surgeonfish *Ctenochaetus striatus*, an abundant and functionally important surgeonfish known to deposit large amounts of fecal pellets on and around large reef-building corals such as *Porites* spp. (Krone et al. 2008). We conducted a mesocosm experiment to characterize how the deposition of *C. striatus* feces alters microbial community composition for *P. lobata* corals. *Porites* corals also experience moderate rates of predation from parrotfishes (Bonaldo & Bellwood 2011) and other corallivores that may initiate or exacerbate feces-induced changes to coral microbiomes. Thus, we employed a factorial experimental designed to evaluate the individual and interactive effects of corallivory and fish feces deposition on coral microbiomes. We tracked the temporal changes in microbial community structure and stability in both healthy and wounded corals to assess the potential role of *C. striatus* feces in the transmission of opportunistic microbes. We first hypothesized that surgeonfish feces could promote holobiont dysbiosis by acting as a vector and/or facilitating the enrichment of potential microbial opportunists and pathogens within reef-building corals. Specifically, feces exposure would likely cause (1) an increase in coral microbial community diversity and variability through time as well as (2) distinct shifts in community composition that favor opportunists over beneficial bacterial lineages. Additionally, we hypothesized that the interactive effects of fish feces exposure and wounding would exceed their individual effects on microbial community diversity, variability, and composition, resulting in increased dysbiosis in corals.

2. MATERIALS AND METHODS

2.1. Assessing feces deposition rates and fecal pellet residence time in the field

This study was conducted in Mo'orea, French Polynesia (17° 29' 26.0" S, 149° 49' 35.10" W) in August 2017.

Fish follows were performed to assess the rate of feces deposition by *Ctenochaetus striatus* in different back reef areas around the island. Fishes were followed for 20 min each ($n = 25$ individuals) and rates of egestion were recorded. Then, the residence time of *C. striatus* fecal pellets on corals was evaluated in the field via a manipulative experiment conducted in the back reef. Fecal pellets ($n = 5$) were collected following direct release by *C. striatus* and placed on coral heads using a spoon. The corresponding coral head was tagged using a buoy and GPS coordinates. Fecal pellet residence times were monitored via direct observation over 2 d at 1, 2, 6, 24, and 48 h by assessing the presence/absence of the fecal pellet. Finally, four 25 m transects (1 m width) were performed in different back reef areas to assess the number of fecal pellets observed per surface area of reef.

2.2. Field collections and manipulative mesocosm experiment

Thirty-two *Porites lobata* colonies of approximately the same size (~20 cm diameter) were collected at ~3 m depth in the back reef area along Mo'orea's north shore and transported in coolers back to the Gump South Pacific Research Station. There, they were placed in eight 25 l tubs ($n = 4$ per tub) and acclimated to comparable temperature ($26 \pm 1^\circ\text{C}$) and light intensity ($700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) regimes. Aquaria received a continuous supply of water from the reef at a flow rate of 20 l h^{-1} . Submersible pumps were placed in each tank to ensure proper water mixing.

To acquire fish feces, 15 individuals of the surgeonfish *C. striatus* (Fig. 1) of approximately the same size (~20 cm total length) were collected at ~3 m depth within the same back reef area using hand and barrier nets. Following collections, fishes were sacrificed and dissected to remove the gut. Fecal samples were retrieved from the lowest part of each individual intestine (3–7 cm) using a sterile scalpel blade and pooled in a sterile Whirl-Pak bag to obtain a homogeneous mixture prior to transferring feces onto coral fragments. The whole process was completed on the same day.

Each *P. lobata* coral colony was cut into 4 fragments using a band saw, producing a total of 128 coral fragments (~8 cm diameter). Corals were allowed to recover 24 h prior to the start of the experiment. Coral fragments were distributed evenly so that 1 fragment of each parent colony was placed into each of 4 treatments ($n = 2$ tanks per treatment, $n = 8$ tanks total with $n = 16$ fragments per tank). The 4 experimental treatments were (1) tanks that contained only untreated coral fragments (control), (2) tanks with coral fragments that were artificially wounded to mimic corallivory, (3) tanks where a fecal pellet was placed on each untreated coral fragment, and (4) tanks where a fecal pellet was placed on each artificially wounded coral fragment. Using a bone cutter, we created an artificial wound by mechanically injuring fragments prior to the start of the experiment. We created a lesion ~2 mm deep, 1 cm long and wide, which removed coral tissue and part of the skeleton, similar to a parrotfish feeding scar. For fecal exposure, each fragment received a 300 mg fecal pellet, visually similar in size to the fecal pellets we observed *C. striatus* creating in the field. Four coral fragments were then randomly selected within each tank at 3 time points post treatment: 3, 15, and 48 h.

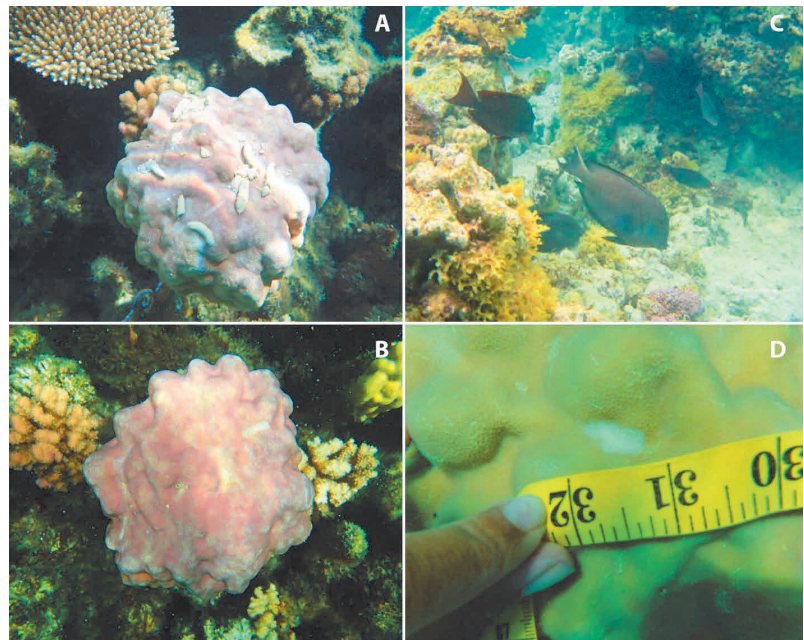


Fig. 1. Field observations of (A) fecal pellets deposited by *Ctenochaetus striatus* on a *Porites* coral in the back reef along the north shore of Mo'orea (image: Hailee Clover); (B) white spots of bleached tissue on the same coral as in (A) in areas where fecal pellets were previously observed (image: Hailee Clover); (C) individuals of *C. striatus* in the back reef along the north shore of Mo'orea (image: Cody Clements); and (D) areas of bleached coral tissue, probably indicative of hypoxia and tissue mortality, following removal of a fecal pellet from a coral surface (image: Katrina Munsterman)

Following each collection, coral fragments were rinsed with 0.2 µm filtered seawater to remove any residual fecal matter. From those fragments, we used a sterilized bone cutter to sample a portion of the coral tissue (tissue, mucus, and a small part of the skeleton) about 1 cm long and wide, where the treatment was applied (wound and/or fish feces). In addition to the coral fragments, 1 water sample of 1 l was collected from each tank at each sampling point and directly filtered onto a 0.2 µm filter (MilliporeSigma). Coral fragments and filters were then transferred into bead tubes (MoBio/Qiagen PowerSoil) and stored at –80°C until processing. The remaining fragments (n = 4 per tank) were discarded at the end of the experiment. In addition, in order to get more information regarding the microbial community in *C. striatus* feces, 1 individual *C. striatus* (about 20 cm) was collected the year following the experiment (August 2018) in the same back reef area and processed as described above. Fecal matter was sampled from the lowest part of the fish intestine, collected in a bead tube (MoBio/Qiagen Power Soil), and directly stored at –80°C until processing.

2.3. DNA extraction, 16S rRNA gene amplification, and metagenome generation

Sequencing of the 16S rRNA gene was used to compare microbiome diversity, composition, and stability metrics. DNA extraction was successfully performed on coral tissue/mucus and fecal matter samples using DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. Together with AccuStart II PCR ToughMix PCR reagent (Quanta BioSciences), a 2-step PCR was performed on the V4 hypervariable region of the 16S rRNA gene using the primer pair 515F (5'-GTG YCA GCM GCC GCG GTA A-3') (Parada et al. 2016) and 806R (5'-GGA CTA CNV GGG TWT CTA AT-3') (Apprill et al. 2015) that target bacterial and archaeal communities. Each 1st-step (12.5 µl total reaction volume) included 6.25 µl AccuStart II ToughMix (2×), 1.25 µl forward primer (10 µM), 1.25 µl reverse primer (10 µM), 0.5 µl sample DNA, and 3.25 µl PCR-grade water. PCR amplification followed a 3 min denaturation at 94°C; 35 cycles of 45 s at 94°C, 60 s at 50°C, and 90 s at 72°C; ending with 10 min at 72°C. Amplified products were run on a 1.5 % agarose gel, and target fragment bands were manually excised and purified with Wizard® SV Gel and PCR Clean-Up System (Promega). Purified product was then barcoded with dual indices in a second 25 µl 12-cycle PCR reaction with 12.5 µl AccuStart II

ToughMix (2×), 9.5 µl PCR-grade water, 1 µl (10 µM) each of forward and reverse barcodes, and 1 µl of purified DNA template. Barcoded amplicons were combined in equivolume ratios, and the pools purified using Agencourt® AMPure XP beads. Relative libraries were submitted to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University (OSU) for sequencing on the Illumina MiSeq Platform using MiSeq reagent kit v.3 (2 × 300 bp paired-end reads). The sequences obtained are available from the NCBI Sequence Read Archive (SRA; www.ncbi.nlm.nih.gov/sra) under submission number SUB6085290.

In addition, the *C. striatus* feces sample for metagenomic sequencing was prepared using Illumina's Nextera XT DNA Library Preparation Kit and sequenced via Illumina paired-end 2 × 300 bp MiSeq kit v.3, yielding 5568226 paired-end reads. After adapter trimming and filtering to an average Phred quality score >20 with a minimum length of 150 nucleotides with Trimmomatic v.0.39 (Bolger et al. 2014), 3480810 paired-end reads remained for downstream analyses.

2.4. High-throughput sequencing data processing and statistical analyses

A total of 153 samples were sequenced, quality-filtered, and run through the Deblur workflow; however, only 120 samples were of interest and included in the present study. A total of 7359944 raw sequences were first demultiplexed using the fastq-multipx tool from ea-utils (<http://code.google.com/p/eautils/>), then trimmed of primers and adapters using Cutadapt v.1.12 (Martin 2011), resulting in 7264722 reads over 116 samples. Four samples were removed since they failed to sequence. The following quality-control steps were conducted using VSEARCH v.2.8.1 (Rognes et al. 2016). Sequences were truncated at the first position having a quality score ≤10, and paired-end reads were merged, resulting in 3001907 reads over 116 samples. Next, sequences with a total expected error >1 per base or with >1 N (unidentified base) were discarded, resulting in 2916927 reads. The Deblur workflow was used to trim quality-controlled sequences to 250 bp, to identify exact sequences with single-nucleotide resolution, and to filter de novo chimeras (Amir et al. 2017). Briefly, the Deblur workflow is a novel method for obtaining sequences that describe community composition at the sub-operational taxonomic unit (sOTU) level using Illumina error pro-

files. A total number of 882 460 reads was generated over 116 samples from the Deblur workflow. The loss of ~70 % of reads in the workflow likely reflects the large proportion of host coral mitochondrial sequences (<250 bp) amplified by the primers. The QIIME2 pipeline (<https://qiime2.org>; Caporaso et al. 2010; version qiime2-2019.1) was then used to process the sOTU table resulting from the Deblur workflow. Taxonomy was assigned against the GreenGenes database (v.13.8) (McDonald et al. 2012) that is commonly used in microbial analyses (Knight et al. 2018), using the classify-sklearn algorithm in QIIME2. Unassigned sOTUs, singletons, and mitochondria or chloroplast amplicons were removed from the sOTU table. Moreover, samples failing to reach 1000 sequences were discarded. One sample was removed due to a low number of sequences. Filtering resulted in a total of 828505 sequences for 115 samples. The number of sequences per sample type varied from 1468 to 14067 for coral tissue and from 5103 to 14999 for water samples. The pre-filtered unprocessed sOTU table can be found in Table S1 (in Supplement 1 at www.int-res.com/articles/suppl/m631p081_suppl1.xlsx). All samples were then rarefied to a depth of 1468 sequences. Rarefaction was performed using the package phyloseq (v.1.26.1) using the command `rarefy_even_depth` in R (v.3.5.3). Overall, all steps removed only 5 samples from the 120 total samples. From the rarefied sOTU table, 3 alpha diversity metrics were calculated: the observed species richness, the Shannon-Wiener index, and the Pielou index. The effects of the experimental treatments, sampling time, and their interaction on alpha diversity metrics were assessed based on linear mixed effect (LME) models using the R package lme4 (v.1.1-21). Models were fitted using restricted maximum likelihood, with 'treatment' and 'time' and their factorial interactions included as fixed factors, while individual 'tank' and 'colony' were treated as random factors to account for the hierarchical structure of the data and the fact that there were multiple fragments of each treatment in each tank. When 'treatment' and/or 'time' or their interactions were significant, pairwise comparisons among group levels were performed based on differences of least square means (LSM) as implemented in the `diffsmeans` function from the R package lmerTest (v.3.0-1). Normality and homoscedasticity of the data residuals were tested using Shapiro-Wilk and Levene tests. If required, data were transformed using the Box-Cox transformation.

Redundancy analyses (RDA; Legendre & Legendre 2012) were used to investigate how compositional

variation in the bacterial community (beta diversity) varied as a function of (1) 'treatment', 'time', 'tank', and 'colony' for coral samples, and (2) 'treatment', 'time', 'tank', and 'sample type' between coral and water samples. Bacterial sOTUs were Hellinger-transformed to preserve the Hellinger distance among samples in the RDA (Legendre & Gallagher 2001). Significant explanatory variables were identified using a forward-selection procedure (Blanchet et al. 2008), and the significance of the canonical relationship between bacterial composition and selected explanatory variables was tested based on 999 permutations (Legendre & Legendre 2012, Borcard et al. 2018). Because the factors 'tank' and 'colony' were not selected following forward selection for the coral dataset ($p_{\text{colony}} = 0.25$ and $p_{\text{tank}} = 0.97$; Text S1 in Supplement 1), final results only included 'treatment', 'time', and their interaction. For the model based on coral and water samples, the factor 'tank' was not significant ($p_{\text{tank}} = 0.99$; Text S2 in Supplement 1), and results were presented according to 'sample type', 'treatment', and 'time' as well as their interactions. Pairwise differences between factor levels were tested using the function `pairwise.adonis` in R (Martinez Arbizu 2017) based on the Hellinger distance, and p-values were adjusted for multiple comparisons using the method of Benjamini & Hochberg (1995) with the false discovery rate (FDR).

In addition, to assess differential abundance of sOTUs across treatments within time periods and through time, we used the R package DESeq2 (v.1.22.2) (Love et al. 2014) on a pre-filtered unrarefied sOTU table. From the unrarefied sOTU table, taxa were agglomerated at the genus level using the `tax_glom` function within the phyloseq package (v.1.26.1). DESeq2 integrates a model that uses the negative binomial distribution and a Wald post hoc test for significance testing. Since this package does not account for random effects, a mixed-effect model was not performed. The p-values were adjusted for multiple testing using the Benjamini & Hochberg (1995) procedure.

Microbial community variability was compared between experimental treatments at each time point via analysis of multivariate homogeneity of group dispersions (Anderson 2006) using the R function `betadisper` in the package `vegan` (v.2.5-4). Community variability was computed based on Hellinger distance. When community variability significantly differed across treatments or time, pairwise comparisons among group levels were assessed based on Tukey's HSD between groups.

Finally, for the metagenome of *C. striatus* feces ($n = 1$ sample), quality-filtered paired-end sequence reads (3.5×10^6) were taxonomically classified using the tetramer-based classification algorithm Kraken (v.1.0, default parameters). Reads were classified against the full Kraken database (Genbank release 224), which included sequences from bacterial, archaeal, and viral taxa. The relative abundance of each taxon was calculated by dividing taxa read counts by the total number of classified reads ($\sim 1.3 \times 10^6$). Taxa relative abundance and counts were visualized using ggplot2 (v.3.2.0) and R (v.3.5.3).

3. RESULTS

3.1. Feces deposition rates and fecal pellet residence time

We observed that a single individual of *Ctenochaetus striatus* deposits fecal pellets at a rate of 3.9 ± 0.34 (SD) pellets over 20 min (~ 12 fecal pellets h^{-1} ind. $^{-1}$). On average, 1 out of every 30 fecal pellets lands on a live coral. However, this rate can vary widely according to the environment where the fish live. Specifically, *C. striatus* often create latrines located on coral heads, resulting in the deposition of fecal pellets every ~ 5 min on *Porites lobata*. Moreover, we monitored the residence time of $n = 5$ fecal pellets of *C. striatus* during 48 h when disposed randomly on coral heads in the field. Four pellets out of 5 were still present on *P. lobata* corals after 48 h at the end of our survey, showing that the time period of our manipulative experiment was ecologically relevant. Finally, from our field surveys of fecal pellet distribution, we observed 22.8 ± 3.3 fecal pellets disposed on live corals across 25 m^2 of reef (Fig. 1).

3.2. Bacterial assemblages differed between coral and water samples

Bacterial communities in tank water were mainly dominated by *Cyanobacteria* and *Proteobacteria* (Fig. 2A). Family-level assignment showed the dominance of, among others, *Synechococcaceae* (30.7%), *OCS155* (10.5%), *Rhodobacteraceae* (5.5%), *Flavobacteriaceae* (5.1%), and *A714017* (5.1%) (Fig. 2B; Table S2 in Supplement 1). Further, we confirmed that the composition of bacterial communities associated with *Porites lobata* was significantly different from the bacterial communities associated with tank water throughout the experimental period (Fig. S1

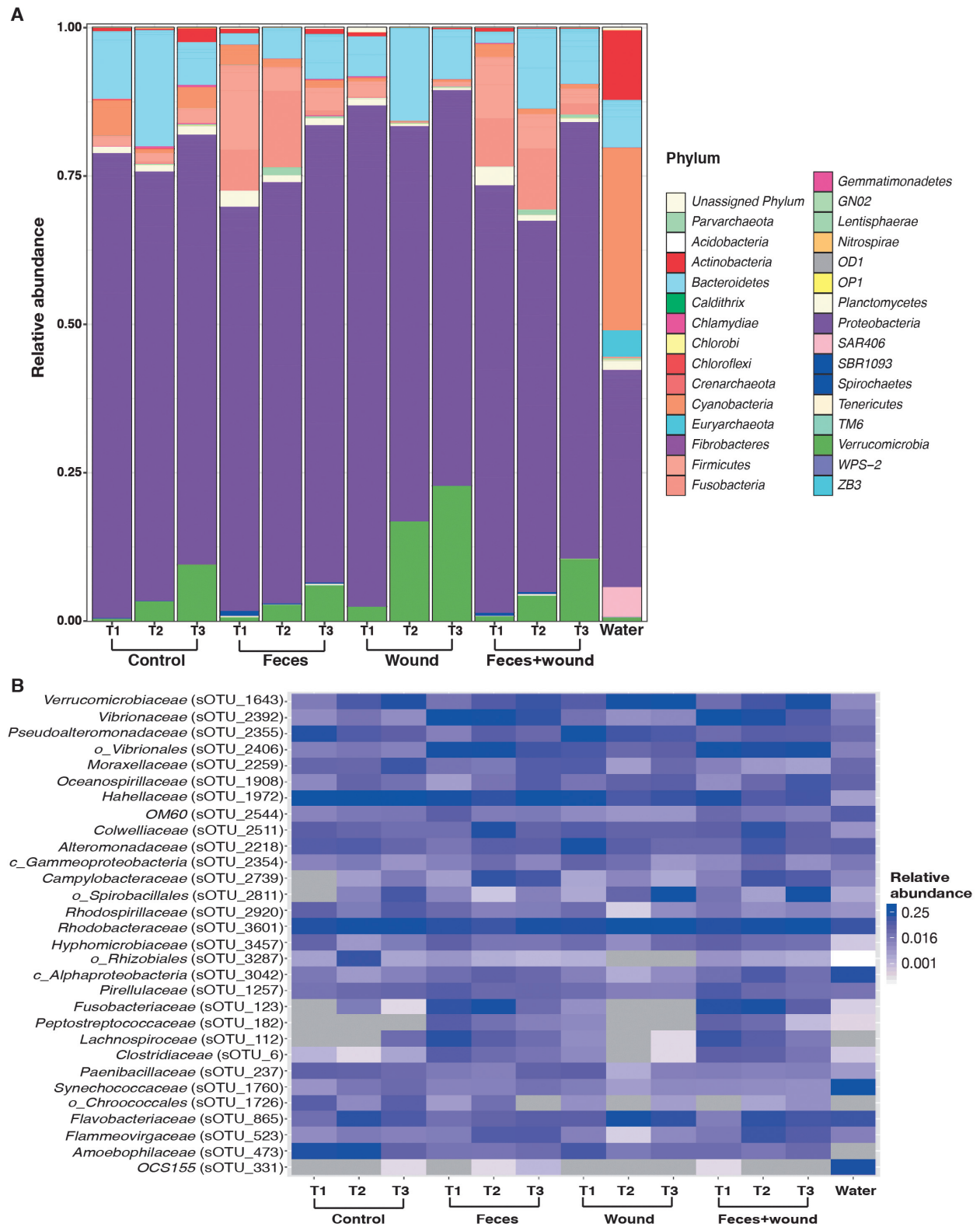
in Supplement 2 at www.int-res.com/articles/suppl/m631p081_supp2.pdf; Text S2, Tables S3 & S4 in Supplement 1; RDA: $F = 2.38$, $p_{\text{sampletype:time}} = 0.004$; pairwise: $p < 0.01$) and across the different treatments (Tables S3 & S4; RDA: $p_{\text{sampletype:treatment}} = 0.001$, pairwise: $p < 0.01$). In addition, we confirmed that microbial communities in tank waters were similar between treatments (Table S4; pairwise: $p > 0.07$).

3.3. Composition of coral microbiomes dominated by opportunists

Phylum-level classification showed the dominance of *Proteobacteria* in coral samples (Fig. 2A). Within this phylum, a single sOTU from the family *Hahellaceae* (sOTU_1972) was the most abundant taxon represented, with an average relative abundance of 17.8% (Fig. 2B; Table S5 in Supplement 1). Across all phyla, 160 families were detected but only a few were present at relatively high abundance, including *Rhodobacteraceae* (14.8%), *Vibrionaceae* (7%), *Verrucomicrobiaceae* (6.4%), *Pseudoalteromonadaceae* (4%), *Flavobacteriaceae* (3.9%), *Fusobacteriaceae* (3.6%), *Alteromonadaceae* (2.9%), *Amoebophilaceae* (2.7%), and *Colwelliaceae* (2.5%) (Fig. 2B, Table S5). These aforementioned families also included the most abundant sOTUs, such as *Hahellaceae* (sOTU_1972), *Rhodobacteraceae* (sOTU_3601), and *Vibrionaceae* (sOTU_2392) (Fig. 2B, Table S5).

3.4. Feces exposure increased bacterial richness and diversity

Microbial alpha diversity did not vary over time regardless of metric (observed richness, Shannon-Wiener, and Pielou's evenness indices; Table S6 in Supplement 1; $p > 0.3$ for all tests). However, there was a significant effect of the experimental treatments on both observed richness and Shannon-Wiener indices, with control corals characterized by lower sOTU richness (69.75 ± 9.45 [SD]) and diversity (2.5 ± 0.2) compared to corals exposed to fish feces (either unwounded or wounded corals) (Table S7 in Supplement 1; Fig. 3; observed richness: pairwise, $p < 0.001$; Shannon-Wiener: pairwise, $p < 0.001$). Additionally, wounded corals exposed to feces showed greater microbial richness (154.9 ± 8.8) and diversity (3.6 ± 0.5) compared to corals that were only wounded (97.6 ± 9.9 and 2.8 ± 0.1 , respectively) (Table S7, Fig. 3; observed richness: pairwise, $p < 0.001$; Shannon-Wiener: pairwise, $p < 0.001$).



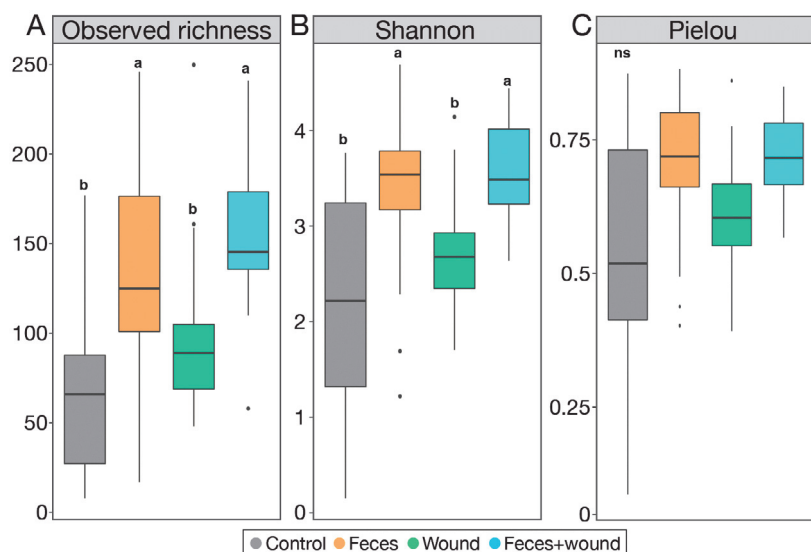


Fig. 3. Alpha diversity indices for coral-associated bacteria across the different treatments described as boxplots: (A) observed species richness, (B) Shannon-Wiener index, and (C) Pielou index. The horizontal line represents the median, box height is the interquartile range, whiskers extend 1.5× beyond the interquartile range, and dots are outliers. Different letters above plots indicate significant differences in each panel based on pairwise comparisons ($p < 0.05$). ns: not significant. Note the different y-axis scales

3.5. Feces exposure and wounding induced compositional shift in coral microbiome

We observed distinct differences in bacterial community assemblages that varied according to experimental treatment and time (Fig. 4; Table S8 in Supplement 1; RDA: $F = 6$, $p < 0.001$). Overall, corals exposed to the various treatments exhibited distinct bacterial assemblages from one another throughout the experiment (Table S9 in Supplement 1; pairwise, $p < 0.005$), except for wounded and un-wounded corals exposed to feces, which shared similar microbial assemblages at 3 and 15 h (Table S9; pairwise, $p > 0.3$). Control corals showed similar assemblages throughout the duration of the experiment (Table S9; pairwise, $p > 0.05$).

The RDA biplot shows that each experimental treatment and time period was associated with a distinct microbial community composition (Fig. 4). The first axis (Table S8; $R^2_{\text{adj}} = 16.3$; $F = 28.6$; RDA: $p = 0.001$) captured significant differences between the microbial composition of corals that were exposed to feces vs. those that were not. From the biplot, we observed that corals exposed to feces exhibited significantly higher abundance of sOTUs in the groups including *Vibrionaceae* (genus *Vibrio*: sOTUs_2386, 2392 and *Photobacterium*: sOTU_2432), *Fusobacteriaceae* (genus *Propionigenium*: sOTUs_123, 124),

Lachnospiraceae (genus *Epulopiscium*: sOTU_112), *Flammeovirgaceae* (genus *Persicobacter*: sOTU_523), and *Colwelliaceae* (genus *Thalassomonas*: sOTU_2511) in comparison with corals that were not exposed to fecal material (Species score; Table S8). The second axis (Table S8; $R^2_{\text{adj}} = 9.1$; $F = 15.9$; $p = 0.001$; Fig. 4) mainly captured significant differences between control and wounded corals. Higher prevalence of *Hahellaceae* (sOTU_1972) and *Amoebophilaceae* (sOTU_473) were associated with control corals, while *Verrucomicrobiaceae* (genus *Rubritalea*: sOTU_1643), *Rhodobacteraceae* (sOTUs_3592, 3601), *Flavobacteriaceae* (genus *Tenacibaculum*: sOTU_865), and bacteria from the order *Spirobacillales* (sOTU_2811) were more prevalent in wounded corals not exposed to feces according to the biplot (Species score; Table S8, Fig. 4).

3.6. Interaction of fish feces and wounding drove unique microbiomes

3.6.1. Evolution of the treatments through time

All results regarding differential abundance analyses included in this section applied to individual sOTUs with an adjusted $p < 0.05$. Regarding control corals, only 1 taxon (sOTU_1643) from the genus *Rubritalea* increased in abundance between 3 and 15 h (Table S10 in Supplement 1; $\log_2$ -fold change: 4.1). For wounded corals, a significant increase in the abundance of 8 sOTUs was observed between 3 and 15 h (Fig. 4A, Table S10; $\log_2$ -fold change: 3.5 to 6.2), including members from the families *Rhodobacteraceae* (sOTUs_3592, 3598, 3601, and 3632), *Verrucomicrobiaceae* (sOTU_1643), *Flavobacteriaceae* (sOTUs_840, 865), *Oceanospirillaceae* (sOTU_1995), and *Alteromonadaceae* (sOTU_2218). At 48 h, the abundance of 5 sOTUs decreased compared to 15 h, including taxa from the families *Flavobacteriaceae* (sOTU_865), *Oceanospirillaceae* (sOTU_1899), *Alteromonadaceae* (sOTU_2218), and *Rhodobacteraceae* (sOTU_3601) (Table S9; $\log_2$ -fold change: -2.5 to -5.1).

For corals exposed to feces, we observed an increase in the abundance of 18 sOTUs; among others, members from the groups *Rhodobacteraceae*

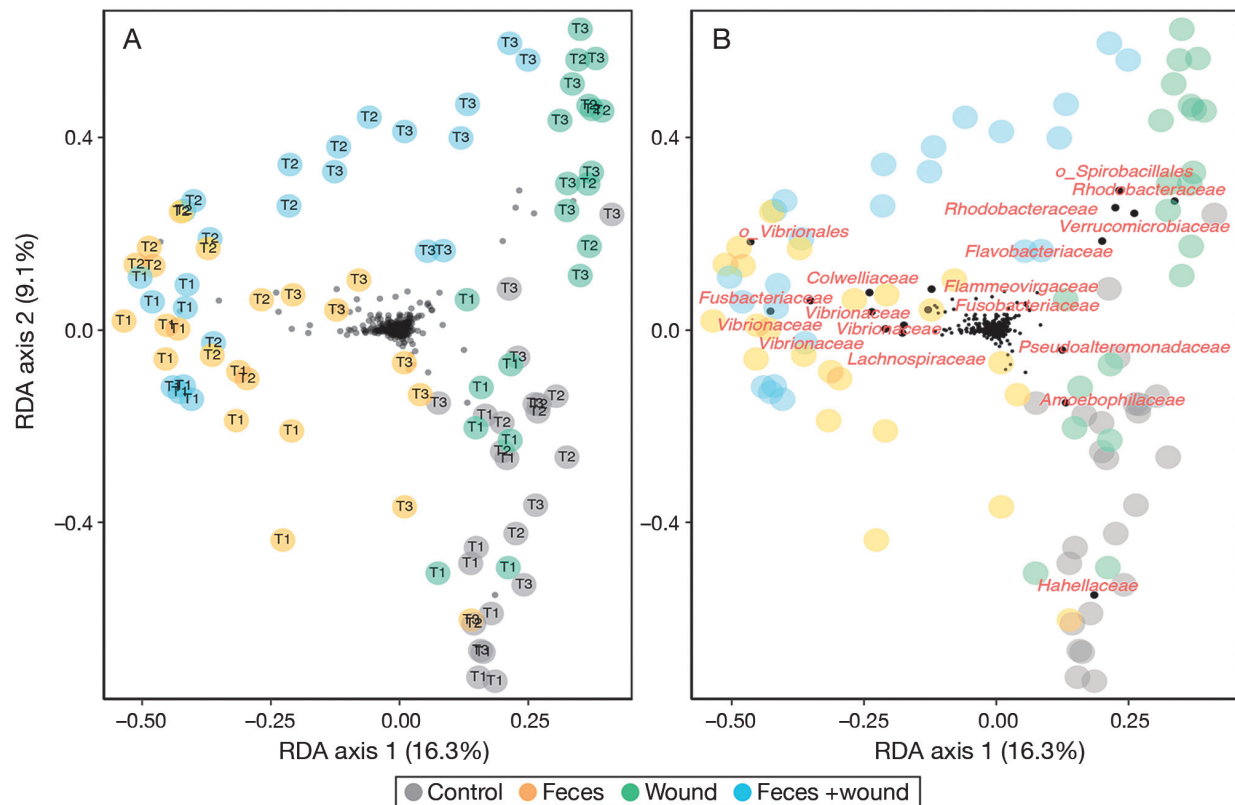


Fig. 4. Redundancy analysis (RDA) biplot illustrating the relationship between coral microbial composition and experimental treatments. (A) RDA biplot with samples colored by experimental treatments and divided by time periods (T1: 3 h; T2: 15 h; T3: 48 h). Individual sub-operational taxonomic units (sOTUs) pictured as small dots. (B) sOTU contribution to each RDA axis labelled as family or order (the latter starting with 'o_')

(sOTU_3601), *Campylobacteraceae* (sOTU_2739), *Colwelliaceae* (sOTU_2511), *Verrucomicrobiaceae* (sOTU_1649), *Flavobacteriaceae* (sOTU_849), and *Flammeovirgaceae* (sOTUs_514, 523) between 3 and 15 h (Table S10; $\log_2$ -fold change: 1.97 to 8.5). At 48 h, significant decreases in the abundance of 10 sOTUs were observed compared to 15 h (Table S10; $\log_2$ -fold change: -2.1 to -7.2), including members from the families *Fusobacteriaceae* (sOTU_123), *Flavobacteriaceae* (sOTU_811), *Vibrionaceae* (sOTU_2392, 2432), *Ferrimonadaceae* (sOTU_2451), and *Colwelliaceae* (sOTU_2511). Wounded corals exposed to feces showed increased abundance of >39 sOTUs between 3 and 15 h (Fig. 5A, Table S10; $\log_2$ -fold change: 1.7 to 8.9). At 48 h, they exhibited a significant decrease of 21 sOTUs compared to 15 h ($\log_2$ fold change: -2.5 to -6.8), and increased abundance of 8 taxa including members from the *Co-haesibacteraceae* (sOTU_3441), *Rhodobacteraceae* (sOTU_3592), and *Desulfovibrionaceae* (sOTU_2616) families (Table S10; $\log_2$ -fold change: 1.8 to 8.5).

3.6.2. Comparison of treatments within each time period

For clarity, experimental treatments were compared to control corals only. At 3 h, wounded and unwounded corals exposed to feces showed a net decrease in the abundance of taxa from the family *Hahellaceae* (sOTU_1972) (Table S11 in Supplement 1; $\log_2$ -fold change: -2.4). Corals exposed to feces (unwounded and wounded corals) showed an increase in abundance of 30 and 36 taxa respectively, including members from the families *Vibrionaceae* (sOTUs_2427, 2432), *Peptostreptococcaceae* (sOTUs_175, 182), *Fusobacteriaceae* (sOTUs_123, 130), *Clostridiaceae* (sOTU_6), *Rhodobacteraceae* (sOTU_3467), *Desulfobulbaceae* (sOTUs_1873, 1874), and *Lachnospiraceae* (sOTU_112) compared to control corals (Table S11). At 15 h, wounded corals exhibited an increased abundance of 4 taxa, including members from the families *Verrucomicrobiaceae* (sOTU_1643; genus *Rubritalea*) and *Rhodobacteraceae* (sOTUs_3601, 3632; genera *Shimia* and *Ruegeria*)

compared to control corals (Table S12 in Supplement 1; $\log_2$ -fold change: 3.4 to 4.2). Corals exposed to feces (unwounded and wounded corals) exhibited an increase in abundance of 36 and 44 taxa respectively compared to controls, including members from the *Flavobacteriaceae* (sOTUs_811, 849, 870) *Vibrionaceae* (sOTUs_2392, 2432) *Rhodobacteraceae* (sOTU_3647), *Campylobacteraceae* (sOTU_2739), and *Flammeovirgaceae* (sOTUs_508, 514, 523) (Table S12).

Marked differences were observed among wounded corals, with corals exposed to both wounding and fish feces showing a greater abundance of 40 sOTUs compared to only wounded corals, such as members from the families *Fusobacteriaceae* (sOTUs_123, 130), *Rhodobacteraceae* (sOTU_3647), *Vibrionaceae* (sOTUs_2385, 2392, 2432), *Clostridiaceae* (sOTUs_6, 36), *Flammeovirgaceae* (sOTUs_508, 514, 523), and *Colwelliaceae* (sOTU_2511) (Table S12; $\log_2$ -fold change: 1.9 to 11.5).

At the end of the experiment (48 h), corals only exposed to fish feces showed greater abundance of 4 taxa compared to controls, including members from the families *Vibrionaceae* (sOTUs_2392, 2432; genera *Vibrio* and *Photobacterium*), *Flammeovirgaceae* (sOTU_523, genus *Persicobacter*), as well as *Verrucomicrobiaceae* (sOTU_1649) (Fig. 5A,B; Table S13 in Supplement 1; $\log_2$ -fold change: 4.7 to 9.7), while only wounded corals presented higher abundance of 2 taxa from the families *Cryomorphaceae* (sOTU_888, genus *Fluviicola*) and *Cohaesibacteraceae* (sOTU_3441, genus *Cohaesibacter*) compared to controls (Fig. 5A,B, Table S13; $\log_2$ -fold change: 5.99 and 8.3).

Finally, corals exposed to both fish feces and wounding still differed in the abundance of 15 sOTUs compared to control conditions (Fig. 5A,B, Table S13; $\log_2$ -fold change: 3.97 to 10.2). Corals exposed to both stressors differed in the abundance of 9 sOTUs compared to corals only exposed to fish feces, including taxa from the families *Cohaesibacteraceae* (sOTU_3441), *Verrucomicrobiaceae* (sOTU_1643), *Lachnospiraceae* (sOTU_105), and *Rhodobacteraceae* (sOTU_3765) (Fig. 5B, Table S13; $\log_2$ -fold change: 4.75 to 8.1). Corals that were strictly wounded differed in the abundance of 18 sOTUs compared to corals exposed to both feces and wounding, including members from the families *Flammeovirgaceae*, *Verrucomicrobiaceae* (sOTU_1649, 1650), *Vibrionaceae* (sOTU_2432), *Desulfovibrionaceae* (sOTU_2616), *Lachnospiraceae* (sOTU_105), *Fusobacteriaceae* (sOTU_123), and *Flavobacteriaceae* (sOTU_523) (Table S13; $\log_2$ -fold change: 1.93 to 9.4).

3.7. Wounding decreased compositional variability in bacterial communities

Bacterial community variability did not significantly differ between experimental treatments at 3h (distance to centroid varied from 0.49 ± 0.09 [SD] to 0.62 ± 0.14) (Tables S14 & S15 in Supplement 1; $p > 0.05$). At 15 h, wounded corals exhibited significantly less microbial dispersion (0.38 ± 0.09) than control corals (0.57 ± 0.13) and corals exposed to both feces and wounding (0.56 ± 0.05) (Table S15; $p < 0.004$). At 48h, wounded corals (0.51 ± 0.07) again exhibited significantly less microbial dispersion than controls (0.72 ± 0.15), as well as unwounded corals exposed to feces (0.7 ± 0.1) (Table S15; $p < 0.01$).

3.8. *C. striatus* feces' bacterial communities characterized by opportunists

Surgeonfish feces metagenomic reads were classified into 323 unique bacterial, archaeal, and viral families. Read recruitment was heavily skewed, with 95 % of all reads classified as 10 taxa. Of these ten, 91 % were classified as the family *Burkholderiaceae* (Fig. S2A in Supplement 2). The remaining 9 % of the metagenomic reads included taxa of interest present in relatively low abundance, such as $5.9 \times 10^{-1}\%$ of *Vibrionaceae* (including *Vibrio* and *Photobacterium* genera), $7.6 \times 10^{-2}\%$ of *Flavobacteriaceae*, $4.6 \times 10^{-3}\%$ of *Campylobacteraceae* (including *Arcobacter* genus), $2.3 \times 10^{-3}\%$ of *Fusobacteriaceae*, $1.9 \times 10^{-3}\%$ of *Flammeovirgaceae* (including *Flammeovirga* genus), $1.8 \times 10^{-3}\%$ of *Oceanospirillaceae*, $5.4 \times 10^{-4}\%$ of *Verrucomicrobiaceae*, $4.6 \times 10^{-4}\%$ of *Ferrimonadaceae* (including *Ferrimonas* genus), $2.3 \times 10^{-4}\%$ of *Desulfobulbaceae*, $1.5 \times 10^{-4}\%$ of *Cryomorphaceae*, $7.7 \times 10^{-5}\%$ of *Cohaesibacteraceae* (including *Persicobacter* genus) (Fig. S2; Table S16 in Supplement 1).

4. DISCUSSION

Dysbiosis of coral-associated microbial communities can occur in response to numerous environmental and biotic factors (McDevitt-Irwin et al. 2017), including interactions with reef fishes, which are hypothesized to act as microbial vectors of pathogens through their mouth parts via predation but also via deposition of fecal material (Aeby & Santavy 2006, Chong-Seng et al. 2011). Our findings indicate that 48 h exposure of *Porites lobata* to either *Ctenochaetus striatus* feces or wounding induced blooms of

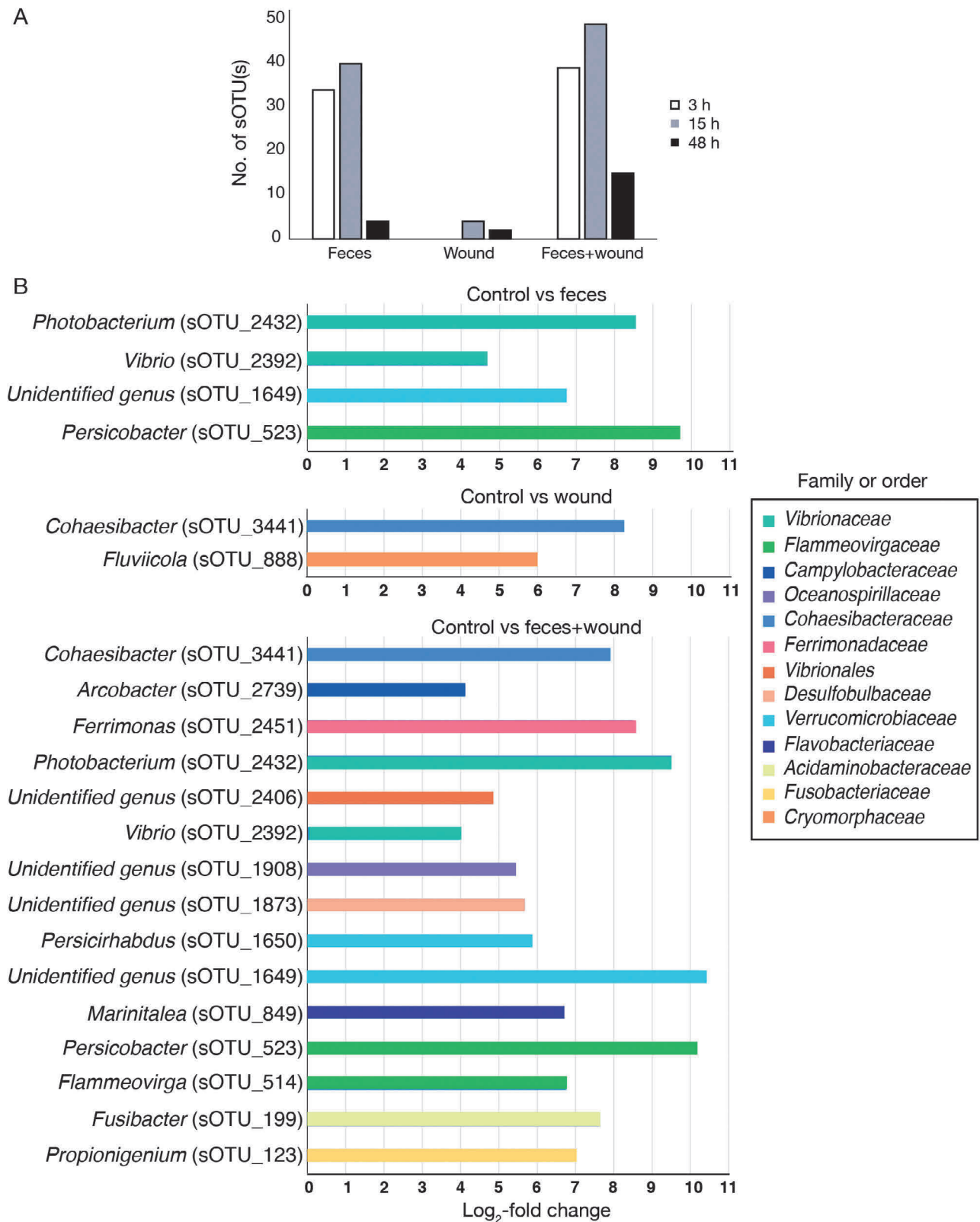


Fig. 5. Differential abundance analysis based on DESeq2 modeling. (A) The number of sub-operational taxonomic units (sOTUs) that differed significantly between control corals and experimental treatments at time points 3, 15, and 48 h. (B) The sOTUs labeled as genus and family or order that differed significantly between control corals and experimental treatments at time 48 h

opportunists and potential coral pathogens, with some taxa being directly associated with fish feces. We suggest that abundant reef fishes could potentially act as vectors of microbes to corals and/or favor shifts in microbial communities within corals through the deposition of fecal pellets. In addition, the abundance of potentially harmful taxa (e.g. *Vibrionaceae*, *Flavobacteriaceae*, *Clostridiaceae*, *Verrucomicrobiaceae*, *Cohaecibacteraceae*, *Campylobacteraceae*) when corals were exposed to both fish feces and wounding suggests that multiple stressors may interact to exacerbate alterations to the microbiome.

4.1. *C. striatus* feces facilitated enrichment of opportunists in reef-building corals

Reef fishes are known to deposit considerable compact amounts of fecal pellets in the surrounding reef environment (Krone et al. 2008) or directly on large coral heads (Fig. 1). While fish feces may represent an important source of nutrients to corals and other marine organisms (Robertson 1982, Meyer et al. 1983, Meyer & Schultz 1985), they may also support high abundances of pathogens that could adversely affect coral health (Sutton & Clements 1988, Smriga et al. 2010, Hennersdorf et al. 2016). We found that exposure of *P. lobata* corals to *C. striatus* fecal pellets led to increased richness and diversity of bacterial communities over 48 h compared to control conditions. Increased alpha diversity has been associated with a wide range of stressors, including water pollution (Röthig et al. 2016, Ziegler et al. 2016), temperature stress (Tout et al. 2015, Lee et al. 2016), ocean acidification (Meron et al. 2011), and algal competition (Barott et al. 2011, Vega Thurber et al. 2012); and may be related to dysfunction of the coral microbiome (McDevitt-Irwin et al. 2017, Welsh et al. 2017).

In our study, increased bacterial richness and diversity associated with feces deposition coincided with a broad shift in microbial community composition within the first 3 h in comparison with control corals. This included blooms of microbial taxa from families such as *Vibrionaceae* (genera *Vibrio* and *Photobacterium*), *Fusobacteriaceae* (genera *Propionigenium* and *Cetobacterium*), *Lachnospiraceae* (genus *Epulopiscium*), *Desulfobulbaceae*, as well as *Clostridiaceae* and *Campylobacteraceae* (genus *Arcobacter*). The absence of these taxa in the control corals and the presence of several taxa (families *Clostridiaceae*, *Desulfobulbaceae*, *Lachnospiraceae*; genera *Vibrio*, *Photobacterium* and *Arcobacter*) in the fish feces metagenome sample suggest that

Ctenochaetus striatus feces may potentially vector these bacteria to coral surfaces or facilitate their enrichment. The genera *Epulopiscium*, *Clostridium*, *Vibrio*, and *Photobacterium* are indeed known to populate the gut of some surgeonfish species (Montgomery & Pollak 1988, Sutton & Clements 1988, Flint et al. 2005, Smriga et al. 2010, Romero et al. 2014, Miyake et al. 2016). In addition, some strains of the genera *Vibrio* and *Photobacterium*, as well as bacteria from the families *Clostridiaceae* and *Campylobacteraceae* are often associated with infectious coral diseases, bleaching, and mortality (Kushmaro et al. 2001, Thompson et al. 2005, 2006, Sato et al. 2009, Sunagawa et al. 2009, Ushijima et al. 2012).

In contrast, putative beneficial microbial symbionts such as members of the *Hahellaceae* family (previously named *Endozoicimonadaceae*) were less prevalent in corals exposed to fish feces at the beginning of the experiment (3 h), although their populations recovered quickly—a common observation in other studies where corals were subjected to stress (Apprill et al. 2013, McDevitt-Irwin et al. 2017). Previous work investigating the effects of fish farm effluents on coral microbiomes reported similar shifts in community composition (Garren et al. 2009) that were largely driven by the presence of taxa associated with coral and other animal diseases, including *Desulfovibrio* and *Fusobacterium* (Nagaraja et al. 2005, Viehman et al. 2006), in sites characterized by high discharge of effluent. Ultimately, we demonstrate that common surgeonfishes such as *C. striatus* may act as a vector and/or promote shifts in coral-associated microbial communities through fecal pellet deposition.

Changes in the microbial community of corals exposed to feces were apparent within the first 3 h of the experiment and persisted throughout the 48 h experimental period. That said, microbial assemblages associated with unwounded corals exposed to feces tended to shift back to their original composition at the end of the experiment, suggesting the resilience of the holobiont through time. However, abundances of potential microbial opportunists such as members from the families *Verrucomicrobiaceae*, *Vibrionaceae* (genera *Photobacterium* and *Vibrio*), and *Flammeovirgaceae* (genus *Persicobacter*) remained significantly greater compared to control corals until the end of the experiment. The persistence of these microbial taxa, which were also found in the *C. striatus* feces metagenome, may be a cause or consequence of adverse health effects observed among corals in the tank experiment and the field, such as visual signs of bleaching or tissue mortality in the area of previous feces deposition (i.e. hypoxia;

Weber et al. 2012, Jorissen et al. 2016). Finally, it is worth noting that both the abundance of bacterial opportunists and related adverse health effects on corals could vary according to the studied reef areas, as surgeonfish feces residence time and abundance of *C. striatus* individuals are likely greater in back reef compared to fore reef areas (Brooks 2019). More work is required to understand the potential role of surgeonfish feces in contributing to bleaching, tissue necrosis, and/or dysbiosis in corals.

4.2. Mechanical wounding decreased bacterial community variability and favored opportunism

Although corallivores play essential roles in reef community dynamics (Enochs & Glynn 2017, Hoey & Bonaldo 2018), corallivory may represent an underappreciated stressor for corals, by providing entry points for microbes to proliferate and invade the resulting lesions. In our study, mechanical wounding significantly shifted microbial communities towards a more homogeneous state dominated by opportunistic microbes. This shift included an increase in a number of taxa from the families *Rhodobacteraceae* (genera *Shimia* and *Ruegeria*) and *Verrucomicrobiaceae* (genus *Rubritalea*) that were already present on corals but in low abundance. Members of the *Verrucomicrobiaceae* family are known to use specific sugars present on bleached corals and often defined as opportunistic and/or potentially pathogenic bacteria (Glasl et al. 2016, Lee et al. 2016). Thus, wounding may have released sugars or other nutrients from the coral that facilitated these opportunistic taxa. Moreover, although taxa from the *Rhodobacteraceae* family are commonly found in healthy corals (Sunagawa et al. 2009, Ceh et al. 2012, Bayer et al. 2013), they can also be associated with stressed and diseased holobionts (Sunagawa et al. 2009, Meron et al. 2011, Kellogg et al. 2013).

Differences between the microbial communities of wounded and control corals were less pronounced by the end of the 48 h experiment, with a marked decrease in the abundance of some potential opportunistic taxa (time point 48 h vs. 15 h: *Flavobacteriaceae*, *Oceanospirillaceae*, *Alteromonadaceae*, and *Rhodobacteraceae*). This dynamic likely indicates that the holobiont is relatively resilient to wounding (at least sterile, artificial sources of wounding) and that initial changes to the microbiome are relatively transient in the absence of additional stressors. Our results concur with previous work that did not observe any differences in bacterial community in

corals 10 d after being wounded (van de Water et al. 2015). Yet, some opportunistic protozoans and bacteria, such as those that characterize brown band disease and skeletal eroding band (i.e. ciliates) as well as black band disease (i.e. *Cyanobacteria*) preferentially target coral tissues following corallivory or mechanical injury, and in certain cases can compromise coral health and survival (Rützler & Santavy 1983, Page & Willis 2008, Katz et al. 2014). Thus, the effects of wounding on the coral microbiome may be highly context-specific depending on the coral, the type of wound, and the microbes present.

At the end of the experiment, wounded corals still exhibited a greater differential abundance of taxa including *Cohaesibacteraceae* (genus *Cohaesibacter*) and *Cryomorphaceae* (genus *Fluviicola*) compared to controls. The absence of the latter in control corals suggests that wounding may have induced the enrichment of new bacterial strains, with some associated with diseased and stressed corals (i.e. *Cohaesibacter*) (Kellogg et al. 2013). In addition, the microbial compositional variability (dispersion) significantly decreased at 15 h and 48 h in wounded corals compared to controls. It is worth noting that this latter effect might have slightly contributed to the explained differences in community composition. That said, our findings differ from previous studies which found that microbial communities associated with a variety of animals, including reef-building corals, showed increased dispersion and stochasticity during periods of stress (i.e. the Anna Karenina principle; Zaneveld et al. 2017). Here, reduced microbial dispersion among wounded corals may have possibly coincided with a shift in microbial community towards a state dominated by a few numbers of potential opportunistic taxa, as previously observed by Glasl et al. (2016) in other poritid species.

4.3. Interaction of fish feces and wounding drove unique bacterial assemblages

The combination of both feces and wounding resulted in unique dynamics of the bacterial community when compared to either feces or wounding alone. Exposure to fish feces, regardless of wounding, induced an increase in richness and diversity as well as a rapid shift in bacterial assemblages. This shift coincided with a decrease in the relative abundance of *Hahellaceae* and the proliferation of potential opportunists (e.g. *Vibrionaceae*, *Fusobacteriaceae*, *Rhodobacteraceae*, *Clostridiaceae*, *Lachnospiraceae*, *Campylobacteraceae*) (Ben Haim et al.

1999, Frias-Lopez et al. 2002, Luna et al. 2010, Sweet & Bythell 2012) not previously found in control corals. As time progressed, corals subjected to both stressors individually began exhibiting reductions in potential harmful taxa. However, the community composition of corals exposed to both wounding and feces simultaneously remained significantly distinct when compared to all other treatments. When compared to controls, these 2 stressors combined resulted in greater differential abundance of 15 sOTUs by the end of the experiment, including potential bacterial opportunists and coral pathogens (i.e. *Arcobacter*, *Cohaesibacter*, *Photobacterium*, *Vibrio*) not previously found in control corals but all present in the *Ctenochaetus striatus* feces metagenome. Further, the number of differentially abundant sOTUs in the combined treatment exceeded differences between controls and corals that were exposed to either stressor alone. Although wounding alone can alter microbial communities, it may also facilitate microbial proliferation from other external sources such as fish feces. Our data suggest that the interaction of these stressors provides unique conditions for microbial opportunists, facilitating taxa not present on corals exposed to either stressor in insolation.

5. CONCLUSION

These findings provide evidence that fish feces may be important vectors of bacteria to coral surfaces and create novel coral microbiomes. We found that *Porites lobata* exposed to either *Ctenochaetus striatus* fish feces or wounding (mimicking corallivory) induced a rapid shift in coral bacterial assemblages that coincided with blooms of opportunists and potential coral pathogens, with some also present in *C. striatus* feces. We suggest that fish feces could potentially act as a vector and/or promote shifts in coral-associated microbial communities through fecal pellet deposition. Although the impact on coral microbiomes decreased throughout the duration of the 48 h experiment, the persistent abundance and blooms of potential opportunistic microbial lineages when both feces and wounding were present indicates that these negative effects may be exacerbated when stressors interact. Our findings provide an underappreciated pathway that may link reef fish to microbial dysbiosis in corals, and possibly to the spread of pathogens and disease. Future work examining how fish feces interact with abiotic stressors such as increased temperature, hypoxia, or ocean acidification to impact coral microbiomes will be

important for understanding how global change will impact this important biotic interaction.

Authors' contributions. Conceived the experiment: R.V.T., L.E., D.E.B.; collected the samples: K.S.M., K.M.L., L.E.; performed tank experiment: L.E., K.S.M., K.M.L.; performed surveys and field experiments: K.S.M., L.E., K.M.L.; performed laboratory work: L.E., E.S.; performed data analysis: L.E., T.L., R.L.M., C.A.G.; wrote the manuscript: L.E., R.V.T., D.E.B.; revised the manuscript: L.E., R.L.M., C.A.G., E.S., D.E.B., R.V.T.

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