Title
The Coral Holobiont under Temperature Change and the Role of Local Acclimatization

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The Coral Holobiont under Temperature Change and the Role of Local Acclimatization

By

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DISSERTATION

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DAVIS

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Dedication

To Liz Dinsdale, my mentor and friend, for believing in me and providing unconditional support.

To every member of the Dinsdale lab and collaborators, because this dissertation is the result of the work of many hands and brains.

À minha família, acima de tudo meus pais, porque com eles esse título é maior que um diploma, é o resultado de uma caminhada que começou nas areias de Saquarema em direção a um mar sem fim.

Abstract

Corals host a highly diverse microbiome that provides key services, such as protection against pathogens and nutrient cycling, forming the coral holobiont. The coral surface mucus layer (SML) microbiome is very sensitive to external changes and constitutes the direct interface between the coral host and the environment. Environmental factors and microbe-microbe interactions act simultaneously on the microbial community structure, making the microbiome dynamics challenging to predict. The coral microbiome is essential to the health of coral reefs. Coral bleaching and disease outbreaks have caused an unprecedented loss in coral cover worldwide correlated to a warming ocean. Fortunately, acclimatization to local temperature increases coral thermal tolerance. My PhD investigates the role of the microbial community as a source of acquired heat-tolerance and an acclimatization mechanism for the coral holobiont. In chapter 1, I describe whether the bacterial taxonomic and functional profiles in the coral SML are shaped by the local reef zone and explore their role in coral health and ecosystem functioning. In chapter II, I develop a dynamic model to determine the microbial community structure associated with the SML of corals using temperature as an extrinsic factor and microbial network as an intrinsic factor. In chapter 3, I experimentally test whether physiological heat tolerance is higher among corals that are locally acclimatized to temperature fluctuations and whether heat stress has a deterministic or a stochastic effect on the coral SML microbiomes. The coral SML microbiome from *Pseudodiploria strigosa* was collected from two naturally distinct reef environments in Bermuda: inner reefs exposed to a fluctuating thermal regime and the more stable outer reefs. A laboratory experiment was conducted to compare the coral holobiont physiology and microbiome under heat stress. Shotgun metagenomics was used to describe the taxonomic and functional profiles and the microbial network of the coral SML microbiome. The coral SML microbiome from the thermally fluctuating inner reefs provides more gene functions that are involved in nutrient cycling, stress response, and disease protection. In contrast, the coral SML microbiome from outer reefs showed high proportions of microbial gene functions that play a potential role in coral disease. The SML microbiome was best predicted by model scenarios with the temperature profile that was closest to the local thermal environment, regardless of microbial network profile, concluding that the coral microbiome is primarily structured by seasonal fluctuations in temperature at reef-scale, while microbe-microbe interaction is a secondary driver. Corals from a more fluctuating environment maintained high photosynthesis to respiration ratios, showing tolerance to heat stress. The metagenomes of corals exposed to heat stress showed high similarity, indicating a deterministic and stable response of the coral microbiome to disturbance. In conclusion, my dissertation shows that reef-scale acclimatization to a more fluctuating temperature profile results in a more beneficial coral microbiome and physiologically resistant coral holobiont. Therefore, my study supports conservation efforts that focus on promoting and maintaining coral microbiome health to protect coral reefs.

The Coral Holobiont under Temperature Change and the Role of Local Acclimatization

Introduction

Reef-building corals create one of the most productive and biodiverse ecosystems on Earth. Coral reefs are home to one quarter of all known marine species (Bourne et al., 2016, Moberg and Folke, 1999, Silveira et al., 2017, Torda et al., 2017) and bring more than $3.4 billion into the U.S. economy each year (NOAA, 2013). Microbial ecology is at the core of the engine that makes coral reefs thrive. Reef-associated microbes provide key ecosystem services such as nutrient cycling (Kelly et al. 2019), facilitation of coral recruitment (Webster et al. 2011), and health of host macro-organisms (Ritchie 2006; Walsh et al. 2017; Vanwonterghem and Webster 2020). Corals associate with endosymbiotic dinoflagellates of the family *Symbiodiniaceae* and a diverse microbiome (e.g. bacteria, archaea, viruses) which function as a unit and form a holobiont (Rohwer et al. 2002). The coral surface mucous layer (SML) sustains a diverse and abundant community of these microbial partners (Koren and Rosenberg 2006; Sharon and Rosenberg 2008; Garren and Azam 2012; Ainsworth et al. 2015). The coral microbiome benefits from the high nitrogen content and organic matter in the SML (Wild et al. 2005; Rädecker et al. 2015) and provides protection against coral pathogens via production of antimicrobials (Ritchie 2006; Krediet et al. 2013b). However, coral-associated microbial communities are sensitive to environmental changes, especially to increased temperature, which disrupt the beneficial services provided to the holobiont (Thurber et al. 2009; Vega Thurber et al. 2014; Raina et al. 2016; Zaneveld et al. 2016).

Coral reefs are at great risk of collapse as coral bleaching (i.e., loss of algal symbionts) and disease outbreaks have become more frequent in the last two decades, particularly correlated to rising seawater temperature, leading to major losses in coral cover worldwide (Maynard et al.

2015; Heron et al. 2016; Precht et al. 2016; Muller et al. 2018). These losses are pronounced on shallow water reefs of the Caribbean, where an overall decline in coral cover of up to 59 % has occurred since 1984 (Jackson et al. 2014). Corals live at their upper thermal limits, and therefore thermal thresholds may not be able to adjust to projected rises in seawater temperature in times of rapid environmental change (Berkelmans and Willis 1999; Fitt et al. 2001; Palumbi et al. 2014; Lough et al. 2018).

Within the coral microbiome, there is a diversity of microbial taxa with traits that potentially improve coral fitness and resilience (Peixoto et al. 2017a). The foundation of coral homeostasis relies on the presence of *Symbiodinium* within the gastrodermis cells and provides 75 – 100 % of the daily metabolic requirements of the holobiont (Grottoli et al. 2006; Tremblay et al. 2012). *Symbiodinium* fix inorganic carbon via photosynthesis and translocate approximately 60 – 80 % of fixed carbon to the coral host, while the host exchanges nutrients, proteins, and lipids (Davy et al. 2012; Tremblay et al. 2012). This exchange of photosynthates and nutrients between the coral animal and the symbiotic algae is the basis of coral growth and calcification (Allemand et al. 2011). As a result, coral-algal physiological processes such as photosynthesis and respiration are used as indicators of coral health (Wooldridge 2014). The coral–algae physiology depends on nutrient cycling (e.g. nitrogen and sulfur cycling) mediated by bacteria, archaea and fungi (Wegley et al. 2007; Siboni et al. 2008; Raina et al. 2013; Rädecker et al. 2015). The coral SML microbiome protects the colony against opportunistic pathogens via competition and production of antibiotic compounds (Ritchie 2006; Shnit-Orland and Kushmaro 2009). Corals invest up to 50 % of fixed carbon on mucus production (Crossland et al. 1980; Crossland 1987) for physical protection and to trap organic matter that can be consumed via heterotrophy (Brown

and Bythell 2005). The coral SML is in intimate contact with the external environment and acts as a conduit between the water column and host physiology (Gates and Ainsworth 2011). Therefore, changes in coral host physiological parameters might be tightly correlated to the dynamics of the mucus microbiome.

The symbiotic relationships in the coral holobiont are environmentally sensitive. Coral bleaching is a typical stress response of corals, leading to an insufficient energy intake to maintain the holobiont homeostasis that may result in tissue mortality (Brown 1997; Lesser 1997; Warner et al. 1999). Bleached corals also experience changes in their mucus microbial composition, characterized by an increase in virulence genes and potentially pathogenic organisms (Littman et al. 2011). High temperatures, eutrophication, and other stress conditions shift the taxonomic and functional composition of the coral microbiome from a healthy to a disease-associated microbial community (Thurber et al. 2009; Bourne et al. 2016). This shift in the community structure due to environmental stressors is called microbial dysbiosis, which is considered the main trigger of disease in marine systems, rather than infection caused by a single pathogen (Egan and Gardiner 2016). One of the most virulent and widespread of all coral diseases is the black-band disease (BBD), a polymicrobial infection associated with a consortium dominated by cyanobacteria, sulfur- reducing and sulfur-oxidizing bacteria (Richardson 2004; Sato et al. 2016). The disease manifests as a dark microbial mat between living tissue and exposed skeleton resulting from tissue necrosis with progression rates of up to 2 cm per day, rapidly leading to partial or total colony mortality (Kuta and Richardson 2002). The cyanobacteria *Roseofilum reptotaenium* dominates BBD mats representing in average 20% of the community and it is also ubiquitously found in the microbiome of healthy corals in relative abundances not higher than 3% (Meyer et

al. 2016). The increase in *R. reptotaenium* abundance is potentially creating an advantageous environment to opportunistic pathogens by providing photosynthates and other metabolites to the disease consortium (Meyer et al. 2016). This is an example of how a dysbiotic microbial community can give rise to a disease infection, highlighting the importance of connecting coral physiological stress responses to shifts in the mucus microbiome.

Investigating the drivers of dysbiosis is a major priority in the microbiome research, currently viewed as a 'central environmental factor' that is associated with complex interactions of host-microbial phenotypes and genetics (Clemente et al. 2012). Coral microbiome dysbiosis caused by changes in the environment is a key mechanism to the unprecedent decrease in coral health worldwide (Ainsworth et al. 2010; Glasl et al. 2016; Haas et al. 2016; Zaneveld et al. 2016; Sweet and Bulling 2017). The exudates released by the benthic community can affect the microbial composition in the water column and have an impact on coral microbiome dysbiosis by increasing the abundance of heterotroph and virulent microbes (Haas et al. 2013; Walsh et al. 2017). Water quality also influences the reef-associated microbial community. Under eutrophic conditions caused by human impact, microbial communities in the reef water increased 10-fold in abundance (comparisons made on 60 reefs sites in 3 oceans) and there was a higher proportion of heterotrophic microbes with metabolic pathways associated with virulence activity (Dinsdale et al. 2008b; Haas et al. 2016). Coral reef trophic structure is shifting towards higher microbial biomass and energy, a phenomenon called microbialization, which aggravates the ecosystem phase shift from coral to macroalgae-dominated reefs (Kelly et al. 2014; Haas et al. 2016; Silveira et al. 2017). Loss in coral cover reduces the competitive ability of corals against fast-growing organisms such as turf algae, stablishing a positive feedback loop cascading to an overall decrease

in coral reef resilience (Smith et al. 2006; Barott and Rohwer 2012; Haas et al. 2013). To understand the underlying mechanisms of large-scale changes in ecosystem functioning caused by microbial dysbiosis, it is crucial to identify the stress responses at the coral holobiont level.

The effects of environmental disturbances on microbial communities can be challenging to predict. Establishing a robust methodological pipeline to sample and analyze the microbiome is the first issue that needs to be addressed. High-throughput sequencing has facilitated major advances in the field of microbial ecology by providing access to unculturable microbial taxa and genes (Streit and Schmitz 2004). The taxonomic composition and phylogenetic diversity of environmental samples can be characterized by using markers such as the 16S rRNA gene (16S) of bacteria and archaea (Case et al. 2007). Marker genes are frequently used in microbial ecology studies, but since this technology focuses on one or a few universal genes, the metabolic potential of functional genes cannot be directly identified (Teske et al. 1996; Langille et al. 2013). An alternative methodology to address this caveat is metagenomic sequencing. Environmental metagenomics using shotgun DNA sequencing is one of the most efficient techniques to analyze taxonomic and functional composition of the microbial genomes (Handelsman 2004; Riesenfeld et al. 2004). Metagenomics relies on deep sequencing to access rare organisms and genes across millions of reads to produce detailed functional profiles (Knight et al. 2012). Including functional data to the analysis of microbial communities under environmental stress allows the identification of changes in ecosystem functioning, an effect that taxonomic parameters alone may not be able to elucidate (Ainsworth et al. 2010; Shade et al. 2012; Kelly et al. 2014; Widder et al. 2016).

The acclimatization mechanisms of the coral holobiont to changing environmental conditions are not completely understood; however, the coral microbiome is recognized as a major player. The microbial-mediated transgenerational acclimatization (MMTA) theory hypothesizes that the coral holobiont benefits from inheritable microbial taxa and/or genes acquired and/or selected in the coral microbiome when exposed to environmental changes (Webster and Reusch 2017). Within the coral microbiome, there is a diversity of microbial taxa with traits that potentially improve coral fitness and resilience (Peixoto et al. 2017a). For example, the associated microbial community is a potential source of acquired heat tolerance (Ziegler et al. 2017). Corals develop resilience to stress factors by associating with certain microorganisms and maintaining their "health-state" microbial taxonomic composition under stress or rapidly recovering to the "health-state" microbes after disturbances (Garren et al. 2009). Microbial functional profiles also respond to environmental gradients and can be used to identify changes in host health and ecosystem functioning (Dinsdale et al. 2008b; Thurber et al. 2009; Kelly et al. 2014). Determining which microbial taxa and functional genes are available in the surrounding environment and how they are being selected in the coral microbiome is key to provide a foundation to theories such as MMTA applied to the coral holobiont.

A big challenge is the lack of datasets describing coral-microbe dynamics interfering with successful predictions of how environmental change will affect the coral holobiont (Bourne et al. 2016). Since resilience is relative to the normal operation range of a community, or to the pre-disturbance conditions, it can be hard to be defined in the absence of baseline data (Shade et al. 2012). Therefore, establishing coral microbiome baselines under natural fluctuations such as spatial variability is key. Mathematical models that use microbial growth rates as a function of

environmental temperature (Ratkowsky et al. 1982; Davey 1989; Rosso et al. 1993, 1995; Vaidya and Wahl 2015) and include microbial interactions derived from network analysis (Marino et al. 2014; Dam et al. 2016) can be a powerful tool to investigate the dynamics of microbial communities. However, this approach remains to be further adapted and applied to coral reef systems. The ecological interactions between the members of the microbiome are challenging to elucidate, but metagenomic sequencing (Dinsdale et al. 2008a, 2013; Haggerty and Dinsdale 2017) combined with network analysis has been able to reveal these relationships (Fath et al. 2007; Steele et al. 2011; Deng et al. 2012; Chow et al. 2014; Wang et al. 2015). Microbial networks constructed by correlation-based methods identify microbial interactions and the key taxa to the structure of the community by using measures of network centrality, such as eigenvector and betweenness centrality (Layeghifard et al. 2017; Röttjers and Faust 2018).

Another outstanding topic to be investigated is the relationship between stress and stability in the microbiome (Zanevald et al. 2017). According to the Anna Karenina principle for animal microbiomes, stress or disease will increase instability and result in low similarity among microbiomes exposed to the same disturbance (Zaneveld et al. 2017). Whether stress has a stable and deterministic effect on the microbiome, leading to a specific dysbiotic community or stochastic consequences that result in a variety of unhealthy community structures has not been determined. In some systems, microbiome stability is maintained in physiologically stressed corals (Röthig et al. 2016; Hadaidi et al. 2017). In contrast, loss in diversity and instability in the microbiome was found in corals showing reduced physiological performance under stress (Grottoli et al. 2018).

This dissertation aims to **investigate the role of local acclimatization to temperature fluctuations in shaping the coral microbiome and heat stress response**. This main objective is structured according to three chapters addressing specific aims:

- **Aim 1/Chapter 1) To compare the taxonomic and functional profiles of microbial communities in the coral mucus and seawater across reef zones**

Lima, Laís FO, Amanda T. Alker, Bhavya Papudeshi, Megan M. Morris, Robert A. Edwards, Samantha J. de Putron, and Elizabeth A. Dinsdale. **"Coral and Seawater Metagenomes Reveal Key Microbial Functions to Coral Health and Ecosystem Functioning Shaped at Reef Scale."** *Microbial Ecology* (2022): 1-16. (https://doi.org/10.1007/s00248-022-02094-6)

- **Aim 2/Chapter 2) To predict the taxonomic structure of the coral microbiome using local temperature profiles and microbial networks**

Lima, Laís FO, Maya Weissman, Micheal Reed, Bhavya Papudeshi, Amanda T. Alker, Megan M. Morris, Robert A. Edwards, Samantha J. de Putron, Naveen K. Vaidya, and Elizabeth A. Dinsdale. **"Modeling of the coral microbiome: the influence of temperature and microbial network."** *MBio* 11, no. 2 (2020): e02691-19. (https://doi.org/10.1128/mBio.02691-19)

- **Aim 3/Chapter 3) To test the effect of heat stress in the coral-algal physiology and microbial taxa and function of corals collected from different reef zones**

Lima, Laís FO, Amanda T. Alker, Megan M. Morris, Robert A. Edwards, Samantha J. de Putron, and Elizabeth A. Dinsdale. "**Heat stress drives a stable and potentially beneficial response in coral microbiomes"** *In review*

Chapter 1

Coral and seawater metagenomes reveal key microbial functions to coral health and ecosystem functioning shaped at reef scale

Lima, Laís FO, Amanda T. Alker, Bhavya Papudeshi, Megan M. Morris, Robert A. Edwards, Samantha J. de Putron, and Elizabeth A. Dinsdale.

Microbial Ecology (2022): 1-16. (https://doi.org/10.1007/s00248-022-02094-6)

Abstract

The coral holobiont is comprised of a highly diverse microbial community that provides key services to corals such as protection against pathogens and nutrient cycling. The coral surface mucus layer (SML) microbiome is very sensitive to external changes, as it constitutes the direct interface between the coral host and the environment. Here we investigate whether the bacterial taxonomic and functional profiles in the coral SML are shaped by the local reef zone and explore their role in coral health and ecosystem functioning. The analysis was conducted using metagenomes and metagenome assembled genomes (MAGs) associated with the coral *Pseudodiploria strigosa* and the water column from two naturally distinct reef environments in Bermuda: inner patch reefs exposed to a fluctuating thermal regime and the more stable outer reefs. The microbial community structure in the coral SML varied according to the local environment, both at taxonomic and functional levels. The coral SML microbiome from inner reefs provides more gene functions that are involved in nutrient cycling (e.g., photosynthesis, phosphorus metabolism, sulfur assimilation) and those that are related to higher levels of

microbial activity, competition, and stress response. In contrast, the coral SML microbiome from outer reefs contained genes indicative of a carbohydrate-rich mucus composition found in corals exposed to less stressful temperatures and showed high proportions of microbial gene functions that play a potential role in coral disease, such as degradation of lignin-derived compounds and sulfur oxidation. The fluctuating environment in the inner patch reefs of Bermuda could be driving a more beneficial coral SML microbiome; potentially increasing holobiont resilience to environmental changes and disease.

Keywords

Host-microbiome, acclimatization, resilience, environmental change, coral reefs

Introduction

Reef-building corals are considered model organisms to study host-associated microbiomes under environmental changes (Apprill 2017; Zaneveld et al. 2017). Coral colonies function as a holobiont in which the coral animal associates with endosymbiotic dinoflagellates of the family *Symbiodiniaceae* and a diverse community of bacteria, archaea, fungi, and viruses (Rohwer et al. 2002). The coral holobiont depends on nutrient cycling (e.g., nitrogen and sulfur cycling) mediated by the associated microbiome (Wegley et al. 2007; Siboni et al. 2008; Raina et al. 2013; Rädecker et al. 2015). The coral surface mucous layer (SML) sustains a high abundance (10^6–10^8 cells per milliliter) and diversity of these microbial partners (Koren and Rosenberg 2006; Sharon and Rosenberg 2008; Garren and Azam 2012; Ainsworth et al. 2015). Corals invest up to 50 % of fixed carbon on mucus production (Crossland et al. 1980; Crossland 1987) for physical protection and to trap organic matter that can be consumed via heterotrophy (Wild et al. 2004; Brown and Bythell 2005). The coral mucus and associated microbial community influences

nutrient fluxes into the benthos, water column, and sediment (Wild et al. 2004, 2005; Naumann et al. 2009; Rix et al. 2016; McNally et al. 2017; Hoadley et al. 2021) thus shaping the ecosystem functions. The coral microbiome benefits from the high nitrogen content and organic matter in the SML (Wild et al. 2005; Rädecker et al. 2015) and provides protection against coral pathogens via production of antimicrobials (Ritchie 2006; Krediet et al. 2013b). However, coral-associated microbial communities are sensitive to environmental changes, particularly to increased temperature and nutrient concentration, which disrupt the beneficial services provided to the holobiont (Thurber et al. 2009; Vega Thurber et al. 2014; Raina et al. 2016; Zaneveld et al. 2016). Therefore, the coral SML microbiome constitutes a direct interface between the coral host and the environment and is strongly influenced by the microbial community in the water column (Apprill et al. 2016; Pollock et al. 2018).

The acclimatization mechanisms of the coral holobiont to changing environmental conditions are not completely understood; however, the coral microbiome is recognized as a major player. The microbial-mediated transgenerational acclimatization (MMTA) theory hypothesizes that the coral holobiont benefits from inheritable microbial taxa and/or genes acquired and/or selected in the coral microbiome when exposed to environmental changes (Webster and Reusch 2017). Within the coral microbiome, there is a diversity of microbial taxa with traits that potentially improve coral fitness and resilience (Peixoto et al. 2017a). For example, the associated microbial community is a potential source of acquired heat-tolerance (Ziegler et al. 2017). Corals develop resilience to stress factors by associating with certain microorganisms and maintaining their “health-state” microbial taxonomic composition under stress or rapidly recovering to the “health-state” microbes after disturbances (Garren et al. 2009).

Microbial functional profiles also respond to environmental gradients and can be used to identify changes in host health and ecosystem functioning (Shade et al. 2012; Kelly et al. 2014; Widder et al. 2016). Determining which microbial taxa and functional genes are available in the surrounding environment and how they are being selected in the coral microbiome is key to provide a foundation to theories such as MMTA applied to the coral holobiont.

Coral reef microbial ecology has benefited from the advancement of shotgun metagenomics to provide an in-depth description of the microbial taxa and functional genes that play a key role in the health of reef ecosystems (Dinsdale et al. 2008b; Zaneveld et al. 2016; van Oppen and Blackall 2019; Cissell and McCoy 2021; Dong et al. 2022). Shotgun metagenomics is not restricted to marker genes such as 16S rRNA in amplicon metagenomics, which results in a more complete profile of the microbial taxa and metabolic potential of functional genes (Streit and Schmitz 2004; Brumfield et al. 2020). However, the use of shotgun metagenomics in coral reef microbiology has traditionally focused on sequencing the microbial communities in reef water (Dinsdale et al. 2008b; Thurber et al. 2009; Kelly et al. 2014, 2019; Haas et al. 2016; Coutinho et al. 2017; Silveira et al. 2017; Walsh et al. 2017). Consequently, the microbial functional profile in the coral holobiont is still underexplored (Robbins et al. 2019). Here we investigate whether the microbial taxonomic and functional profiles in the coral SML are shaped by their local reef environment and explore their role in coral health and ecosystem functioning.

Methods

Aim of the study. We compared the metagenomes associated with the brain coral *Pseudodiploria strigosa* (Dana, 1846) and the water column sampled *in situ* from two naturally distinct reef environments in Bermuda. The reef system in Bermuda is the most northern in the Atlantic and

experiences large seasonal variations in environmental conditions (Smith et al. 2013b). In addition, fine-scale variations in temperature, light, and seawater chemistry occur between the outer rim reefs at the edge of the platform and inner lagoon patch reefs (Courtney et al. 2017) with the inner patch reefs historically being warmer and more thermally variable (de Putron and Smith 2011; Smith et al. 2013b; de Putron et al. 2017; Courtney et al. 2020; Wong et al. 2021). We showed in Lima et al. 2020 (Lima et al. 2020) that the coral SML microbiome from the inner patch reefs and the outer rim reefs in Bermuda can be modelled according to the local annual thermal profile. Here, we expand the analysis to a fine-scale taxonomic level (i.e., microbial genera and metagenome assembled genomes – MAGs) and to the functional level (i.e., SEED subsystems and pathways) in the microbial communities from the coral SML and surrounding water across these reef zones.

***In situ* collections.** We selected *P. strigosa* as the coral host species because it is widely distributed across the Bermuda platform. The reef zones sampled were approximately 8 km apart (Lima et al. 2020) and *P. strigosa* is a broadcast spawner; therefore, there is a high likelihood that gene flow between the coral hosts colonizing inner and outer reefs is maintained and that the host genetics is not structured into different populations. Indeed, studies on other species have indicated high genetic exchange among reef sites in Bermuda (Serrano et al. 2014, 2016). The sampling period occurred between May 18th and May 22nd, 2017, late spring in the northern hemisphere, when environmental conditions between the two reef zones, especially temperature, are similar. The environmental gradient assessed here are based on the knowledge that these two reef zones are exposed to different regimes on a seasonal basis, with the most striking fluctuations occurring in the winter and summer months (de Putron and Smith 2011;

Smith et al. 2013b; Courtney et al. 2017). Therefore, we selected this period to capture a potential long-term acclimatization of the coral holobiont to their reef zones, and not their immediate response to acute temperature fluctuations. Each reef zone was replicated across three reef sites (Lima et al. 2020). The SML of *P. strigosa* was collected from six colonies (diameter, 10 to 15 cm) from the inner and outer reef zones (*n* = 12 colonies total) using a modified two-way 50-ml syringe filled with 0.02-µm-filtered seawater (Lima et al. 2020) that dislodges the microbes and recollects the microbial-mucus slurry in the backside of the syringe. We collected 200 ml of coral mucus-microbe slurry (four syringes applied to different parts of the colony's surface) per colony to increase DNA concentration per sample. The reef water (volume = 10 L per replicate) was collected about 1 m above the coral colonies from the inner and outer reef zones (*n* = 12 replicates total). Coral SML and water samples were pushed through a 0.22- µm Sterivex filter (EMD Millipore) for DNA extraction. The collections were performed via SCUBA diving at a depth of 4 to 6 m. A Manta2 Series MultiprobeTM was used to measure pH (0 -14 units), water temperature (˚C), chlorophyll concentrations (µg/L), and dissolved oxygen (% saturation and mg/L) across a 6 m depth profile at each sampling site. Our benthic survey methods were based on the Atlantic and Gulf Rapid Reef Assessment (AGRRA) Program protocols (Lang et al. 2015). The benthic cover was measured via 10-m line transects (n = 3 per site) using the point intercept method every 10 cm (100 points total). Corals were identified at species level and the other organisms categorized in the following groups: macroalgae, turf algae, crustose coralline algae, gorgonian, milleporid, sponge and other.

Metagenomic analysis. Microbial DNA from the coral mucus and seawater collected on the 0.22-µm Sterivex was extracted using a modified Macherey-Nagel protocol using NucleoSpin

column for purification. DNA was stored at - 20°C until quantification with Qubit (Thermo Fisher Scientific) (Dinsdale et al. 2008b). The Swift kit 2S plus (Swift Biosciences) was used for library preparation since it provides good results from small amounts of input DNA, characteristic of microbial samples collected from the surface of the host (Doane et al. 2017; Cavalcanti et al. 2018). All samples were sequenced by the Dinsdale lab on Illumina MiSeq at San Diego State University. The sequenced DNA was analyzed for quality control using PrinSeq (Schmieder and Edwards 2011) before annotation. The metagenomes were annotated through MG-RAST (Meyer et al. 2019), using the RefSeq database for taxonomic annotations and the SEED database for functional annotations. The number of sequence hits for each microbial taxon or function is represented as the relative abundance by calculating the proportion of sequence hits for that parameter over the total number of sequences annotated for that metagenome. Metagenomes were compared using proportional abundance, which is preferred to rarefaction (McMurdie and Holmes 2014; Quince et al. 2017; Luz Calle 2019). We used metagenomics to describe the abundance of genes in the microbiome as a proxy for gene expression: although it does not measure which functional genes are being expressed at the point the sample was taken, it measures which functional genes are important for the microbes in that environment (Dinsdale et al. 2008a; Coelho et al. 2022). There is a high level of correlation between the metagenomes and metatranscriptomes (Franzosa et al. 2014), where the abundance of a gene in metagenomes is a predictor of its expression level in the metatranscriptome and areas where the two analysis vary are associated with short term changes in expression rather than bacteria functions that are under strong selective pressure and are well adapted to their environment (Gilbert and Dupont 2010; Gilbert et al. 2012; Mason et al. 2012).

Metagenome assembled genomes (MAGs). MAGs were constructed to identify the level of shared taxa between the coral SML from the two locations. All the coral SML metagenomes post quality control using Prinseq (Schmieder and Edwards 2011) were cross assembled using megahit (Li et al. 2015) and spades (Prjibelski et al. 2020). To remove the redundancy in the assembled contigs, bbtools program (Bushnell et al. 2017) dedupe.sh script was to remove 15% of contigs that were exact duplicates. The resulting contigs were run through Metabat2 (Kang et al. 2019) and CONCOCT (Alneberg et al. 2014) binning tools to generate 38 MAGs and 167 MAGs respectively. DasTool (Sieber et al. 2018) was run on these bins to generate 82 non-redundant set of MAGs. CheckM (Parks et al. 2015) was run on these 82 MAGs to assess the completeness and contamination within each MAG. The MAGs were annotated through PATRIC version 3.6.9 using RAST tool kit (RASTtk) (Brettin et al. 2015). MAGs were described following the minimum standards for MAGs (Robbins et al. 2019; Nayfach et al. 2020).

Statistical analysis. Statistical analyses were conducted using PRIMER v7 plus PERMANOVA, Statistical Analyses of Metagenomic Profiles (STAMP) software (Parks et al. 2014), and R (R Project for Statistical Computing). Significant differences in the relative abundances of microbial genera and functions in the coral microbial communities sampled from inner and outer reefs were identified by permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis distances of normalized relative abundance obtained using a fourth-root transformation. The fourth-root transformation balances the effects of a community structured on a few abundant species and a community structured on all species, and thereby influenced by the occurrence of the rarest taxa (Clarke and Warwick 1994; Clarke et al. 2014). A principal

coordinate analysis was created to visualize the separation of the coral microbiome between inner and outer reefs. We also used PRIMER to calculate Pielou's evenness index (J') and Shannon's diversity index (H') of microbial genera. The multiple comparisons of either taxa or functions across the four groups of metagenomes (i.e., outer coral, outer water, inner coral, and inner water) were conducted in STAMP using ANOVA/Tukey-Kramer and Benjamini-Hochberg FDR corrections. We used R to test parametric assumptions of normality (Shapiro-Wilk's test) and homoscedasticity (Bartlett's test), and pairwise comparisons between relative abundances of gene pathways (Student's T-test).

Results

Taxonomic profile

The metagenomes associated with the coral SML of *P. strigosa* and the water column sampled from inner and outer reefs in Bermuda (n = 24) were sequenced at high coverage, ranging from 421,976 to 1,368,678 sequence counts. Bacteria accounted for approximately 99 % of the annotation (Table S1); therefore, we are only analyzing bacterial taxa and gene functions in this study. The metagenomes were assigned to four different groups (total n = 24 with 6 metagenomes in each group) according to their host medium and location: inner reef corals, inner reef water, outer reef corals, and outer reef water. Microbial richness did not vary significantly between groups or samples, ranging from 581 to 587 bacterial genera identified, including 23 taxa unclassified at genus level, across all metagenomes. Evenness (J') of bacterial genera was slightly lower in inner reefs (coral: 0.72 ± 0.03, water: 0.72 ± 0.02) when compared to outer reefs (coral: 0.75 ± 0.01, water: 0.75 ± 0.01), which translated in a higher diversity index

(H') in outer reef samples (coral: 4.78 ± 0.06, water: 4.80 ± 0.08) than in inner reef samples (coral: 4.56 ± 0.17, water: 4.59 ± 0.11).

In contrast to diversity metrics, the microbial community structure (i.e., relative abundance of taxa) was significantly different between the four groups (PERMANOVA, Pseudo-F = 10.8, $p < 0.001$). The metagenomes clustered according to the reef zone and were more similar to one another among the coral-associated samples than the water samples (Fig. 1A). Among the most abundant taxa (i.e., average relative abundance > 1% in a least one of the four groups), eight bacterial genera were significantly overrepresented according to their associated environment (Fig. 1B). The SML microbiome of corals from the inner reef zone had a greater relative abundance of the alphaproteobacterium Candidatus *Pelagibacter,* and of an unclassified genus, also belonging to the order *Rickettsiales*, compared to all other groups (ANOVA, Eta-squared = 0.93, $p < 0.001$). The relative abundance of cyanobacterium *Synechococcus* (ANOVA, Eta-squared = 0.62, $p < 0.001$) was greater in the water microbiome from inner reefs compared to the microbiome from both water and coral in outer reefs (Tukey-Kramer, $p < 0.01$). This overrepresentation was also reflected in the coral SML microbiome from inner reefs compared to the coral SML microbiome from outer reefs ($p < 0.05$). The SML microbiome of corals from outer reefs showed a greater abundance of alphaproteobacteria Candidatus *Puniceispirillum* (ANOVA, Eta-squared = 0.92, Tukey-Kramer, $p < 0.001$), *Ruegeria* (ANOVA, Eta-squared = 0.73, $p < 0.001$), and *Rhodospirillum* (ANOVA, Eta-squared = 0.92, Tukey-Kramer, $p < 0.001$) compared to all groups. The coral SML microbiomes from both reef zones were enriched with gammaproteobacteria of the genus *Pseudomonas* (ANOVA, Eta-squared = 0.61, $p<0.001$) when compared to the surrounding water microbiome from their respective local environment (Tukey-

Kramer, $p < 0.05$). In contrast, *Flavobacterium* had a greater representation in the microbial communities from the water of both reef environments than in the microbiome associated with corals from inner and outer reefs (ANOVA, Eta-squared = 0.61, Tukey-Kramer, $p < 0.01$).

MAGs indicated a clear separation between the coral SML microbiome from inner and outer reefs (Fig. 2). A total of 82 bins were constructed, and we selected eight MAGs with high levels of completeness (53 < 98%) and that represented a wide range of taxonomic variation according to the preliminary annotation from CheckM for further analysis. A hierarchical clustering tree separated the bins into two major clusters, each with four MAGs, including bacterial and archaeal taxa. The first cluster was formed by MAGs annotated as *Puniceicoccaceae* (Bin 16), *Synechoccocus* (Bin 2), *Flavobacteriaceae* (Bin 1), and Candidatus *Pelagibacter ubique* (Bin 22). The metagenomes that contributed to most to the bins in this cluster were samples from the SML of inner reef corals. The second cluster was comprised of MAGs annotated as *Alphaproteobacteria* (Bin 116), *Euryarchaeota* (Bin 159), and *Pseudomonas stutzeri* (Bin 8 and Bin 142). The metagenomes that contributed to each of the MAGs in this cluster were majorly samples from the SML of outer reef corals.

Functional profile

The microbial communities associated with the coral SML and water column from inner and outer reefs revealed specific functional traits. Bacterial genes classified at the broadest functional categories (SEED subsystem level 1) significantly varied across the four groups (PERMANOVA, Pseudo-F = 8.49, $p < 0.001$). From a total of 26 broad functional categories, 12 were significantly overrepresented according to their associated environment (Fig. 3). The microbiome of corals from outer reefs had a greater proportional abundance of functional genes

belonging to carbohydrate metabolism and to sulfur metabolism than all other groups (ANOVA, Eta-squared = 0.74 and 0.61, $p < 0.001$; Tukey-Kramer, $p < 0.05$). In contrast, protein metabolism functional genes were significantly lower in relative abundance in the outer coral microbiome when compared to all other groups (ANOVA, Eta-squared = 0.61, $p < 0.001$; Tukey-Kramer, $p < 0.01$). Functional genes involved in metabolism of aromatic compounds were overrepresented in the water and coral microbiome of outer reefs when compared to the microbiome in the water and coral microbiome of inner reefs (ANOVA, Eta-squared = 0.84, $p < 0.001$; Tukey-Kramer, $p < 0.001$).

The inner coral SML microbiome was overrepresented with genes involved in phosphorus metabolism and in secondary metabolism (ANOVA, Eta-squared = 0.61 and 0.84, $p < 0.001$, Tukey-Kramer, $p < 0.01$). Functional genes within cell division and cell cycle as well as cell wall and capsule were in higher abundance in the water microbiome from inner reefs compared to the microbiome from water and corals from the outer reefs and in the microbiome from inner corals compared to the outer coral SML microbiome (ANOVA, Eta-squared = 0.79 and 0.72, $p < 0.001$; Tukey-Kramer, $p < 0.01$). Photosynthesis functional genes were overrepresented in the water microbiome of inner reefs when compared to all other groups (ANOVA, Eta-squared = 0.70, $p < 0.001$; Tukey-Kramer, $p < 0.01$).

Bacterial respiration genes were overrepresented in the microbiome of corals from both reefs when compared to the microbiome in the water column from inner and outer reefs (ANOVA, Eta-squared = 0.76, $p < 0.001$; Tukey-Kramer, $p < 0.01$). Stress response genes showed higher relative abundance in the SML microbiome of inner corals than in the water microbiome of inner reefs, and similarly more of stress response genes in the microbiome of outer corals

when compared to the water microbiome from both reef zones (ANOVA, Eta-squared = 0.67, $p < 0.001$; Tukey-Kramer, $p < 0.01$). DNA metabolism genes were overrepresented in the microbiome from the water column in both reef zones when compared to the coral SML microbiome from inner and outer reefs (ANOVA, Eta-squared = 0.71, $p < 0.001$; Tukey-Kramer, $p < 0.01$).

The nine broad functional gene categories (SEED subsystem level 1) that varied significantly according to the reef zone were analyzed at a higher level of resolution (SEED subsystem levels 2 and 3) to illustrate which specific functions could be under selection at reef-zone level in the coral SML microbiome only (Fig. 4). Genes involved in central carbohydrate metabolism, one-carbon metabolism, and CO_2 fixation accounted for approximately 60 % of the total carbohydrate genes both in the inner and outer coral SML metagenomes (Fig. 4A). Protein biosynthesis genes (relative abundance = 70 %) dominated the protein metabolism, followed by protein degradation genes (relative abundance = 14 %) (Fig. 4B). Gram negative cell wall components (relative abundance = 32 %) and capsular and extracellular polysaccharides (relative abundance = 26 – 27 %), were dominant among cell wall and capsule genes (Fig. 4C). Phosphate metabolism and transporters genes together were approximately 75 % of the total phosphorus metabolism, whereas genes involved in phosphorus uptake by *Cyanobacteria* at 12 % relative abundance (Fig. 4D). Within cell division and cell cycle, two cell division clusters/chromosome partitioning genes were higher in inner coral SML metagenomes (relative abundance = 23%) compared to outer coral SML metagenomes (relative abundance = 19 %) (Fig. 4E). In the metabolism of aromatic compounds, n-Phenylalkanoic acid degradation and anaerobic benzoate genes were more represented in inner coral metagenomes (22 % in inner and 15 % in outer, and 11 % in inner and 9 % outer, respectively), while benzoate catabolism was higher in outer coral

metagenomes (6 %, compared to 4 % in inner), and cathecol branch was approximately 8 % in both groups (Fig. 4F). Proteorhodopsin genes accounted for 30 % of the photosynthesis and light-harvesting complexes in outer coral metagenomes, compared to 20 % in inner coral metagenomes, while photosystem II genes were lower in outer coral metagenomes (relative abundance = 22 %) compared to the inner coral metagenomes (relative abundance = 25 %) (Fig. 4G). In secondary metabolism, genes encoding auxin biosynthesis were higher in outer coral metagenomes than in the ones from inner reefs (relative abundances of 52 % and 38 %, respectively), contrasting with alkaloid biosynthesis from L-lysine genes that were more represented in inner coral metagenomes (28 % versus 10 %). Sulfur metabolism genes showed striking differences in proportions at subsystems level 3 (Fig 4I), where sulfur oxidation genes were almost three-fold more abundant in outer coral metagenomes than in inner coral metagenomes. Because of the differences in sulfur metabolism, in the next section, we will be focusing on the specificities of sulfur pathways and their associated taxa.

Sulfur metabolic pathways in the coral SML microbiome

Sulfur oxidation, inorganic sulfur assimilation, and organic sulfur assimilation (including dimethylsulfoniopropionate - DMSP breakdown) were the three major sulfur subsystems in all metagenomes, accounting for approximately 90 % of total sulfur metabolism genes, but the proportions of sequences related to each subsystem varied between the two reef zones. In the microbiome of outer corals, the relative abundance of sequences from each of these subsystems were evenly distributed (sulfur oxidation 33.2 ± 3.7 %; inorganic sulfur assimilation 28.3 ± 2.5 %; and organic sulfur assimilation 29.8 ± 1.2 %). A similar pattern was detected in the water column of outer reefs (sulfur oxidation 28.5 ± 5.9%; inorganic sulfur assimilation 34.1 ± 4.1%; and organic

sulfur assimilation 26.8 ± 1.7 %). In contrast, in the metagenomes of inner corals, sulfur oxidation was underrepresented (12.5 ± 3.4 %), when compared to inorganic sulfur assimilation (40.8 ± 6.5 %) and organic sulfur assimilation (38.3 ± 1.0 %). The metagenomes from the water column of inner reefs were also low in sulfur oxidation genes (15.7 ± 2.1%), and high in inorganic sulfur assimilation (38.5 ± 3.0 %) and organic sulfur assimilation (34.0 ± 3.1 %). Within the organic sulfur assimilation cluster, DMSP breakdown was highest in the SML microbiome of corals from inner reefs (48 ± 8.4 %), followed by outer corals (33.2 ± 3.4 %), inner water (31.8 ± 7.8 %), and outer water (26.5 ± 7.6 %). Release of dimethyl sulfide (DMS) from DMSP accounted for less than 0.001 % of the sulfur metabolism genes in coral metagenomes from both reef zones.

The proportion of sequences within the sulfur metabolism cluster encoding the enzyme DMSP demethylase *dmdA* (EC. 2.1.210) was greater in the SML microbiome of corals from inner reefs (T-test, $t = 5.38$, $p = 0.001$; Fig. 5A), while those encoding the sulfur oxidation protein *soxB* were higher in corals from outer reefs (T-test, $t = -11.56$, $p < 0.001$; Fig. 5B).

The bacterial genera that contributed to DMSP breakdown belonged to the same five taxa between inner and outer coral metagenomes, but these were represented in different proportions (Fig. 5C). *Roseobacter* (ANOVA, Eta-squared = 0.634, $p < 0.001$), *Ruegeria* (ANOVA, Eta-squared = 0.625, $p < 0.001$), and *Dinoroseobacter* (ANOVA, Eta-squared = 0.545, $p < 0.001$) were the main contributors to the DMSP breakdown genes in outer metagenomes, while Candidatus *Pelagibacter* (ANOVA, Eta-squared = 0.849, $p < 0.001$), and *Roseovarius* (ANOVA, Eta-squared = 0.353, $p = 0.042$) showed greater proportions in the metagenomes of inner corals. Sulfur oxidation genes were encoded by 75 genera of bacteria and the twelve most abundant taxa showed different relative abundances between inner and outer coral metagenomes (Fig.

4C). *Rhodopseudomonas* (ANOVA, Eta-squared = 0.869, $p < 0.001$) accounted for about one quarter of all the bacterial genera encoding sulfur oxidation genes in outer coral SML, while in inner corals the highest abundances were distributed more evenly across *Rhodopseudomonas*, *Ruegeria*, and *Roseobacter*. *Azorhizobium* (ANOVA, Eta-squared = 0.73, $p < 0.02$) was overrepresented in the sulfur oxidation genes in outer coral SML, and *Chlorobium* (ANOVA, Eta-squared = 0.63, $p < 0.031$) in the microbiome of inner corals.

Discussion

The metagenomes associated with the SML of *P. strigosa* and the water column from inner and outer reefs in Bermuda had similar taxonomic diversity metrics (e.g., richness, Shannon's diversity index), corroborating that the coral SML microbiome is shaped by microbial communities in their surrounding environment (Apprill et al. 2016; Pollock et al. 2018). However, the microbial community structure (i.e., relative abundances of sequences) in Bermuda's reef system is simultaneously selected by the coral host versus water and the local environment (i.e., inner reefs versus outer reefs), both at taxonomic and functional levels. The coral SML microbiome of *P. strigosa* was dominated by taxa commonly present in seawater that are found in other coral species (Marchioro et al. 2020; Osman et al. 2020) and are selectively trapped and consumed by the coral host (McNally et al. 2017; Hoadley et al. 2021). In this study, *P. strigosa* from each reef zone had different microbial genera filling similar niches. For example, alphaproteobacterial metabolic generalists were the most abundant genera in both reef zones, represented by SAR11 Candidatus *Pelagibacter* in inner corals and SAR116 Candidatus *Puniceispirillum* in outer corals. Among phototrophs, cyanobacterium *Synechococcus* was a signature genus in inner corals and *Rhodospirillum* in outer corals. At the microbial metabolism

level, the microbiome is providing key functions for coral holobiont health and ecosystem functioning; specific to each reef zone (Fig. 6).

The coral SML microbiome from a fluctuating environment provides more services related to nutrient cycling, stress tolerance, and disease protection

The coral and water microbiomes from inner reefs reflect a highly productive and fluctuating system when compared to outer reefs. The overrepresentation of photosynthetic bacteria in the water column and the coral SML of inner reefs mirrored the elevated abundance of functional genes related to photosynthesis and phosphorus metabolism. *Synechococcus* is a main primary producer in the picoplankton, reaching the highest concentrations off Bermuda during the spring bloom (DuRand et al. 2001); the same season as this study. *Synechococcus* was highly abundant in the metagenomes and MAGs from inner reef corals and, therefore, could be the main contributor to photosynthesis and phosphorus metabolism genes. We used metagenomics to describe the abundance of genes in the microbiome which identifies functional genes that are important for the microbes in that environment (Dinsdale et al. 2008a; Coelho et al. 2022) although it does not measure which functional genes are being expressed at the point the sample was taken. In the coral SML metagenomes, we identified phosphorus metabolism was mostly comprised of genes involved in phosphate metabolism and phosphorus uptake by *Cyanobacteria* (e.g., *Synechococcus*). The coral SML is rich in phosphate when compared to the water column (Wild et al. 2005); contributing to primary productivity in benthic and pelagic reef ecosystems (Huettel et al. 2006). The coral SML efficiently traps *Synechococcus* from the pelagic picoplankton, which contributes to the flux of particulate organic matter (POM) from the water column to benthos (Naumann et al. 2009). Corals selectively remove *Synechococcus* and other

pelagic microbes via feeding, and promote the growth of diverse picoplankton, shaping the microbial community in the surrounding reef water (McNally et al. 2017; Hoadley et al. 2021). Heat-stressed corals preferentially fed on *Synechococcus* to access the high nitrogen content in their cells and to compensate for the loss of nitrogen from algal endosymbiont *Symbiodiniaceae* during recovery from bleaching (Meunier et al. 2019). The inner lagoon patch reefs in Bermuda are exposed to greater environmental fluctuations, particularly changes in temperature (de Putron and Smith 2011; Smith et al. 2013b; Courtney et al. 2017; Lima et al. 2020). Therefore, the high abundance of *Synechococcus* in the water column and in the SML of *P. strigosa* could be contributing to the energy transfer from pelagic to benthic trophic levels, and to the coral thermal tolerance in the inner lagoon reefs of Bermuda.

Microbial activity, growth, and competition are higher in the inner reefs than in the outer reefs in Bermuda, as suggested by the functional profiles from the coral SML and water column. Functional genes related to cell division and cell cycle, such as those encoding two cell division and chromosome partitioning, are in greater abundance in inner coral metagenomes. In addition, there is a high relative abundance of cell wall and capsule functional genes, including those encoding capsular and extracellular polysaccharides in the microbial communities of inner reefs. Microbial extracellular polymeric substances (EPS) play a crucial role in marine environments; increasing dissolved organic carbon (DOC) levels, binding and removing heavy metals from the water column, and influencing oxygen levels (Bhaskar and Bhosle 2005). Microbial growth rates in the coral SML are higher under elevated DOC levels (Kline et al. 2006); therefore, the abundance of genes related to EPS suggests an increased microbial activity in the SML of corals from inner reefs. DOC levels are also associated with larger quantities of exudates released by

benthic macroalgae in coral reefs (Haas et al. 2011). Even though both reef zones showed similar coral cover; turf and macroalgae were more abundant in inner reefs (Figure S1), indicating that the DOC levels induced by macroalgae exudates could be higher in this reef zone in Bermuda. The microbial communities associated with inner corals are enriched with genes belonging to secondary metabolism, including a high relative abundance of genes encoding alkaloid biosynthesis from L-lysine. *Cyanobacteria* are key producers of marine alkaloids (Zotchev 2013), which could be contributing to the high levels of these functional genes in coral metagenomes from inner reefs. Alkaloids function as antimicrobials (Lozano et al. 2019; Othman et al. 2019); therefore, the overrepresentation of alkaloid biosynthesis genes indicates greater microbe-microbe competition in the coral SML microbiome from inner reefs. Microbial competition and production of antimicrobial compounds offer protection against opportunistic pathogens to the coral host (Shnit-Orland and Kushmaro 2009; Kvennefors et al. 2012; Krediet et al. 2013a; Othman et al. 2019) and thus promoting a more beneficial SML microbiome on *P. strigosa* colonies inhabiting inner reefs compared to outer reefs.

Dimethylsulfoniopropionate (DMSP) breakdown genes (e.g., *dmdA*) belong to the organic sulfur assimilation subsystem and were more abundant in the SML microbiome of inner corals across all metagenomes. DMSP is a valuable component in marine environments, with high turnover rates, and is an important link between primary production and bacterial activity (Yoch 2002). *Pelagibacter ubique*, for example, exclusively assimilates sulfur from organic sources such as DMSP (Tripp et al. 2008), and was a key taxon associated with DMSP breakdown in inner reefs. The coral metagenomes had greater proportions of *Pelagibacter* than the water metagenomes suggesting the coral SML is providing a DMSP-rich environment for bacterial growth. DMSP is

considered an antioxidant (Sunda et al. 2002; Deschaseaux et al. 2014), and increased levels of this compound have been associated with stress response in the coral holobiont (Garren et al. 2009; Yost et al. 2010; Aguilar et al. 2017). DMSP that reaches the coral SML is produced by the coral-algal symbiont (Broadbent et al. 2002) and the coral animal, especially under thermal stress (Raina et al. 2010, 2013). Bacteria subsequently use this compound as a sulfur and carbon source, relying on the *dmdA* gene to encode DMSP methyltransferase to incorporate sulfur to amino acids (e.g., methionine) (Visscher and Taylor 1994; Tripp et al. 2008). Sulfur as a product of DMSP breakdown can also be used by bacteria to form sulfur-based antimicrobial compounds such as tropodithietic acid (TDA), which protects the coral host by inhibiting the growth of pathogens (Raina et al. 2016). Therefore, DMSP breakdown is considered one of the main beneficial services provided by the coral microbiome to the holobiont, because it is linked both to disease protection and nutrient cycling (Peixoto et al. 2017a). The sulfur metabolism of the microbiome of inner corals, which prioritizes sulfur assimilation and DMSP breakdown, is another indicator that the coral holobiont from inner reefs is responding to a more fluctuating thermal environment and potentially is associating with a microbiome that is more beneficial for this environment.

The coral SML microbiome from a stable environment indicates less exposure to stress, but is potentially under nutrient limitation and more prone to coral disease

The microbial functional profile in outer reefs was characterized by a carbohydrate-dominated metabolism, and a reduction in protein metabolism genes and is indicative of the variation of the SML composition between corals from the two reef zones. Corals secrete a polysaccharide protein lipid complex that is colonized by an abundant microbial community (Brown and Bythell 2005). The proportions of carbohydrates, proteins, and lipids in the coral

mucus vary according to factors such as coral species (Meikle et al. 1988; Wild et al. 2010; Hadaidi et al. 2019), stress (Lee et al. 2016b) and reef environments (Wild et al. 2010). The coral SML microbiome is strongly shaped by the mucus composition (Lee et al. 2016a); therefore, the high relative abundance of microbial genes involved in carbohydrate metabolism and the loss of protein metabolism genes is consistent with corals from outer reefs producing mucus with a higher carbohydrate to protein ratio. Heat-stressed corals had an increase in protein content, and higher microbial activity, compared to healthy corals under mild temperature conditions (Wright et al. 2019). Corals from the outer reefs in Bermuda are less exposed to thermal fluctuations (de Putron and Smith 2011; Smith et al. 2013a; Courtney et al. 2017) and the microbial community structure from their mucus can be modelled according to their local thermal environment (Lima et al. 2020). The reduction in protein metabolism genes and overrepresentation of carbohydrate metabolism genes suggest that the mucus composition of corals from outer reefs is characteristic of corals under low exposure to thermal stress.

Metabolism of aromatic compounds was a signature function both in the coral and water microbiomes from outer reefs. The gene encoding the enzyme muconate cycloisomerase (EC 5.5.1.1) is part of the catechol branch of beta-ketoadipate pathway and was found at lower relative abundance in the SML microbiome of inner corals (1 %), than in outer corals (8%). The beta-ketoadipate pathway is commonly present in soil microbes, involved in the degradation of lignin-derived aromatics such as cathecol to citric acid cycle intermediates (Harwood and Parales 1996), although lignin degradation genes are found in many marine bacterial strains of *Pseudoalteromonas*, *Marinomonas*, *Thalassospira,* among others (Lu et al. 2020). The sources of lignin that is being degraded by the microbiome of outer reefs is unresolved, as this compound

is characteristic of vascular land plants, but lignin has been described to be within the cells of one marine macroalga species, *Calliarthron cheilosporioides* (Martone et al. 2009). Interestingly, an increased relative abundance of genes responsible for lignin degradation in the coral mucus microbiome was associated to yellow-band disease and attributed to lysing of the coral tissue (Kimes et al. 2010). Therefore, the role of lignin degradation in the coral microbiome could be related to coral health and needs to be further investigated.

Outer reef corals showed a higher abundance of total sulfur metabolism genes in their SML microbiome when compared to the microbiome of inner corals. An increase in the relative abundance of sulfur metabolism genes in the coral microbiome has been associated with low pH, thermal stress (Thurber et al. 2009), and bleaching (Littman et al. 2011). However, the colonies were visually healthy, and the environmental conditions were mild during sampling collection (Table S2). The microbiomes of outer and inner corals adopted different sulfur metabolism strategies according to their local environment. Sulfur oxidation was overrepresented in the outer water and coral metagenomes, in comparison to metagenomes from inner reefs, which invested more in inorganic and organic sulfur assimilation. Sulfur oxidation in the coral microbiome is much less understood than sulfur assimilation and is usually studied in the context of black-band disease (BBD). BBD is one of the most virulent and widespread of all coral diseases and develops as a polymicrobial consortium dominated by cyanobacteria, sulfur- reducing and sulfur-oxidizing bacteria (SRB and SOB, respectively) that change in relative abundance across stages of infection (Richardson 2004; Sato et al. 2016). The disease manifests as a dark microbial mat between living tissue and exposed skeleton resulting from tissue necrosis with fast progression rates (Kuta and Richardson 2002). BBD prevalence in *P. strigosa* colonies from outer

reefs was the highest across Bermuda reef zones and among other coral host species, despite the pristine water quality and marine protected area status (Jones et al. 2012). Sulfur oxidation genes from *Rhodobacteraceae* were proportionally higher in outer coral metagenomes and were identified in BBD lesions (Bourne et al. 2013). However, SOB do not seem to be directly linked to BBD pathogenicity, but likely function as secondary colonizers (Bourne et al. 2013). The high sulfide concentrations created by SRB and loss of oxidizers within the BBD mat are linked to coral tissue degeneration (Meyer et al. 2016; Sato et al. 2017). Sulfur oxidation in outer reef corals could be part of a healthy coral microbiome metabolism; related to amino acid degradation as a sulfur source to bacteria. The *soxB* gene pathway is part of the Sox enzyme complex that allows a phylogenetically diverse group of SOB to convert thiosulfate to sulfate (Meyer et al. 2007) and was significantly more abundant in the outer coral SML microbiome. Thiosulfate can be a fermentation product of taurine (Denger et al. 1997). Taurine dioxygenases were present in MAGs associated with the coral microbiome, suggesting the microbes are using this amino acid as a nutrient source (Robbins et al. 2019), especially in more oligotrophic waters such as in the outer reefs of Bermuda. The role of sulfur metabolism in coral health and disease susceptibility needs to be further studied, and the Bermuda reefs provide a natural laboratory system for coral microbiome research.

The coral SML microbiome has distinct features from the water column microbiome independent of local reef zone

The coral SML microbiome of *P. strigosa* from inner and outer reefs shared some taxonomic and functional features, despite the strong effect caused by the local reef zone. *Pseudomonas* was the only genus that was overrepresented in the coral SML from both reef

zones in comparison to their local water microbiome. *Pseudomonas stutzeri* was identified by our MAGs particularly in outer reef samples. Marine strains of *P. stutzeri* have been isolated from the water column and sediment, and their major ecological roles are related to denitrification and sulfur oxidation (Lalucat et al. 2006). *P. stutzeri* could be playing an important nutrient cycling role in the coral SML and this relationship requires further investigation. At functional level, the coral SML microbiome showed greater proportions of respiration and stress response genes, independent of their local reef zone. The coral microbiome was dominated by heterotrophs that take advantage of the rich carbon sources in the mucus, therefore, increasing microbial respiration, i.e., oxygen consumption, when compared to the free-living, photosynthetic, and oxygen-producing microbial community in the surrounding water (Dinsdale et al. 2008b; Thurber et al. 2009). A greater relative abundance of pathways associated with stress response may indicate passive or active selection within the holobiont, which could be a source of resilience according to the hologenome theory of evolution, if these microbial genes can be vertically transmitted (Rosenberg and Zilber-Rosenberg 2018). This potential selection of microbial stress response genes relates to the MMTA theory that is yet to be corroborated and assumes that the coral holobiont benefits from inheritable microbial taxa and/or genes acquired and/or selected in the coral microbiome when exposed to environmental changes (Webster and Reusch 2017). Future research should investigate whether the coral holobiont is selecting microbial genes differently in response to environmental stress and whether they are passed on through generations.

Conclusion

Coral health has sharply decreased in the last two decades as coral bleaching and disease outbreaks have become more frequent worldwide, particularly correlated to rising seawater temperature (Maynard et al. 2015; Heron et al. 2016; Precht et al. 2016; Muller et al. 2018). Conservation efforts to improve coral health by promoting or maintaining a beneficial microbiome (e.g., development of probiotics) depend on a detailed understanding of the dynamics of microbial taxa and functional profiles (Damjanovic et al. 2017; Epstein et al. 2019; van Oppen and Blackall 2019).

Our results showed specific coral-microbial gene functions and taxa that are being selected, either passively or actively, according to the local environment, in response to primary productivity, stress, and nutrient cycles, particularly the sulfur cycle. The fluctuating environment in the inner patch reefs of Bermuda could be driving a more beneficial coral SML microbiome for the prevailing environment via local long-term acclimatization; potentially increasing holobiont resistance to thermal stress and disease. This reef zone could be a source of a coral holobiont that is more resilient to environmental changes in comparison to outer reefs. Coral restoration programs, especially when using transplantation of coral colonies across different areas of the reef, should design strategies that consider the trade-offs involving coral microbiome acclimatization at reef scale.

Figures

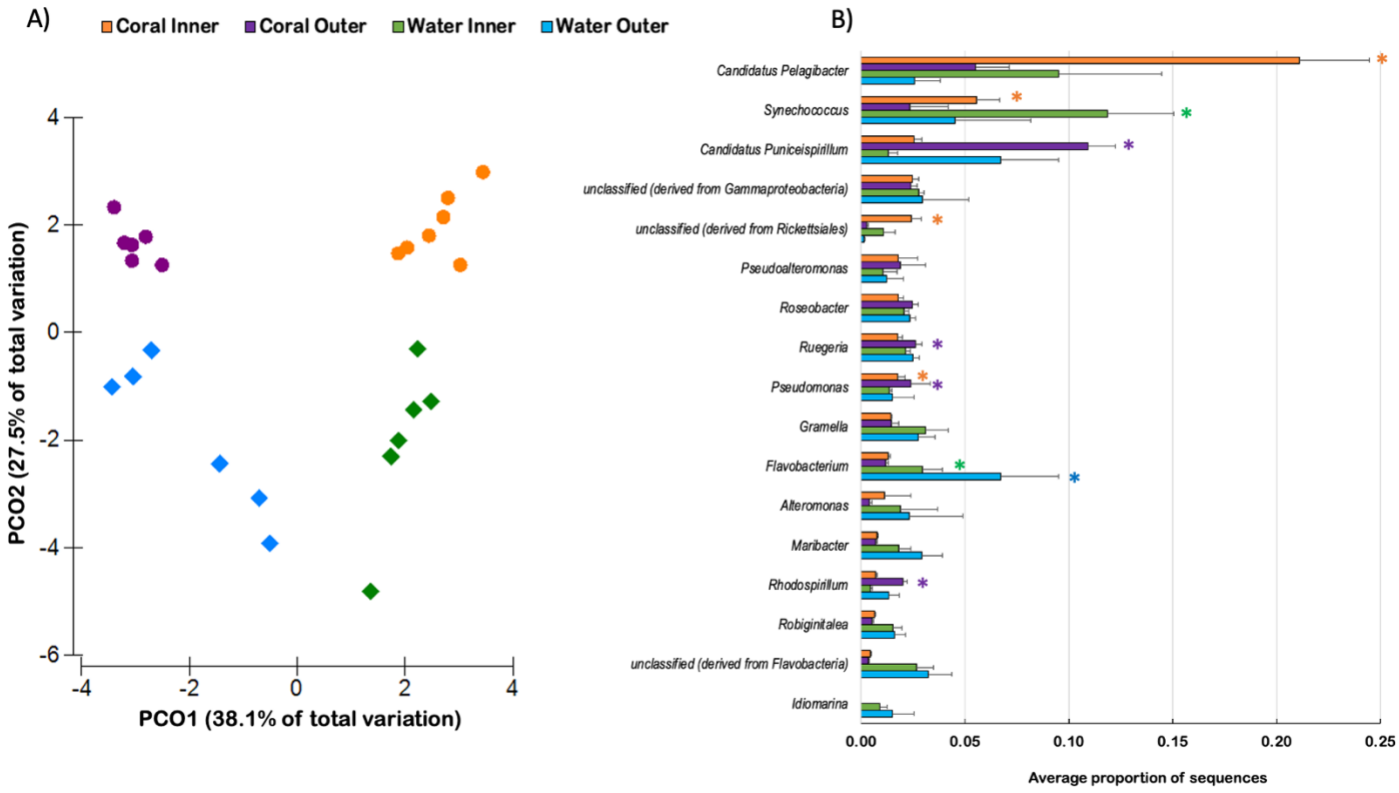

Figure 1. Clear differences in taxonomic make-up of the microbial community were shown using a Principal Coordinate Analysis (A) based on a Bray-Curtis similarity matrix of the relative abundance of bacterial genera associated with the SML microbiome of corals (circles) and the water column (diamonds) from inner and outer reefs. Bacterial genera (mean ± SD; average abundances > 1 %) showed significantly different proportions (B) according to multiple comparison Tukey-Kramer tests (asterisks indicate $p < 0.05$).

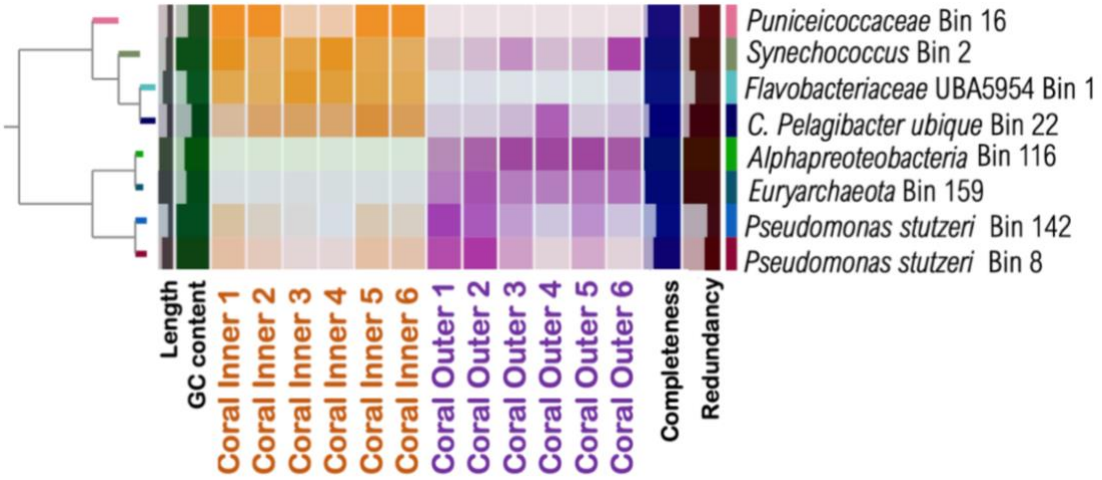

Figure 2. Metagenome Assembled Genomes (MAGs) of eight bins generated from the twelve coral SML metagenomic samples. The heatmap shows the contribution of each metagenome to the formation of each individual bin; organized by hierarchical clustering tree using Euclidean distance and Ward linkage.

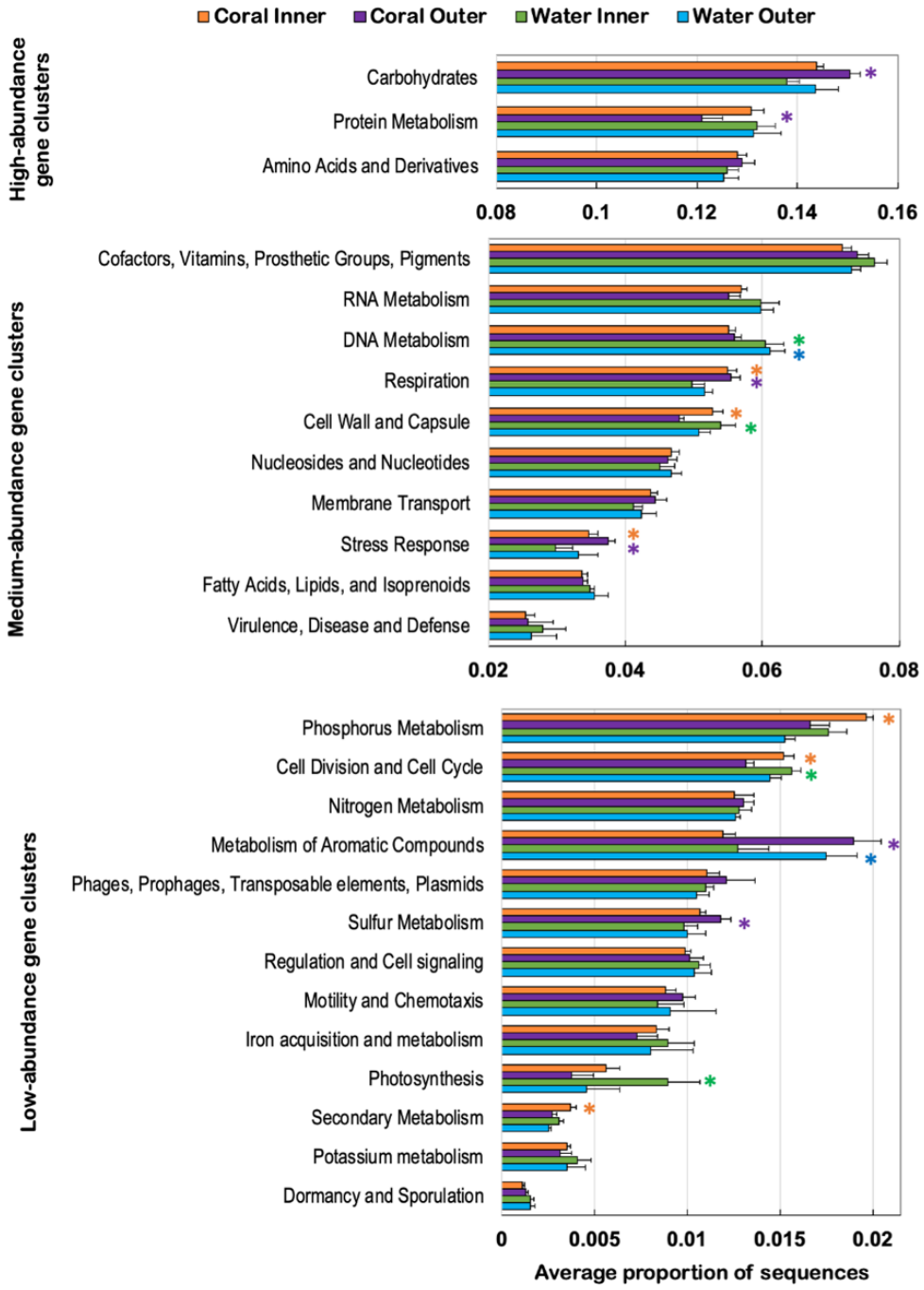

Figure 3. Bacterial broad functional gene categories (SEED subsystem 1) (mean ± SD; average abundances > 1%) associated with the SML microbiome of corals, and the water column from inner and outer reefs showed significantly different proportions according to multiple comparison Tukey-Kramer tests (asterisks indicate $p < 0.05$).

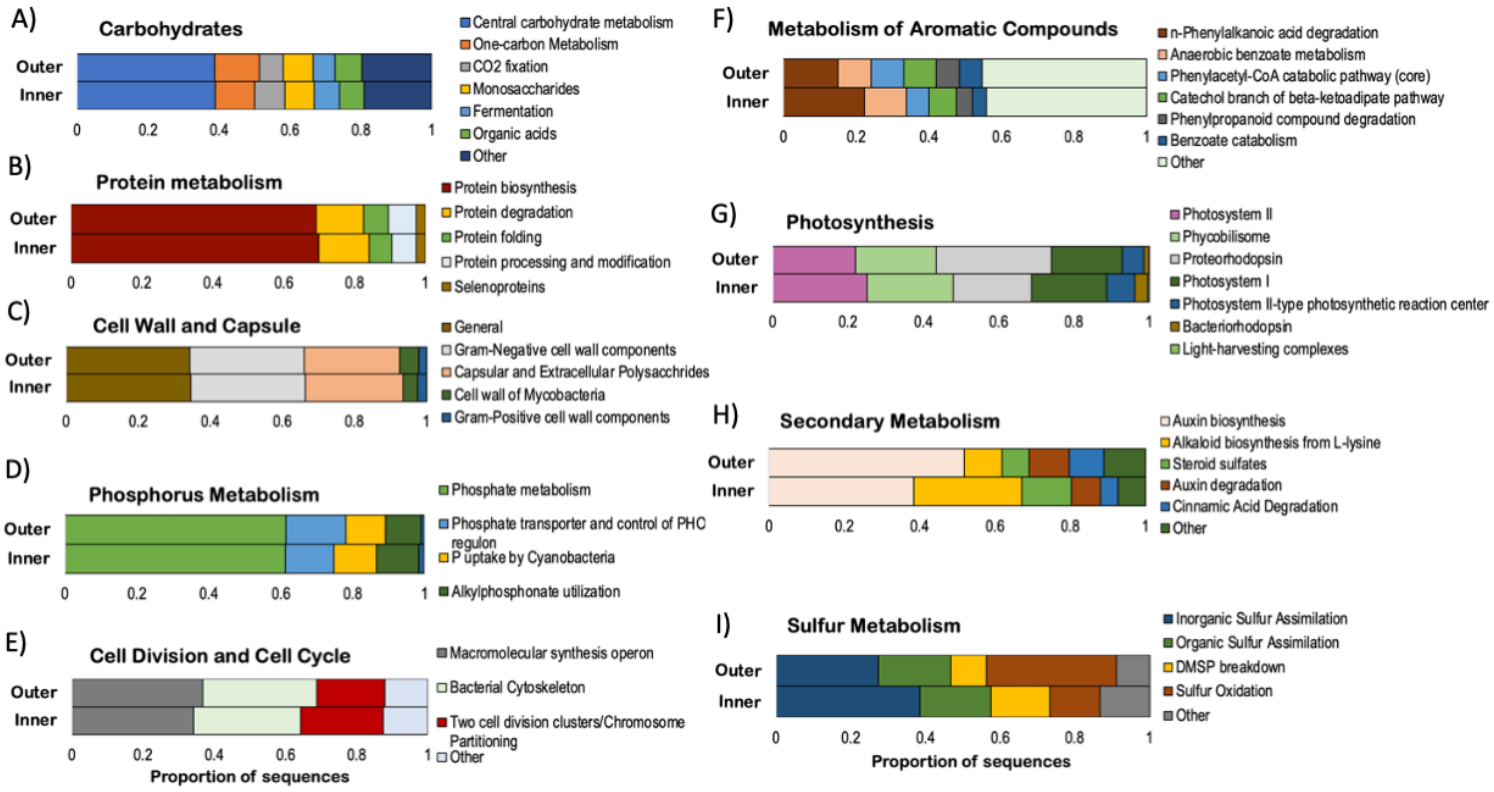

Figure 4. Relative abundance of bacterial functional gene subsystems (SEED subsystem 2: A - C, and subsystem 3: D - I) within their respective broad functional gene category (SEED subsystem 1 the bold heading) associated with the SML microbiome of corals from inner and outer reefs.

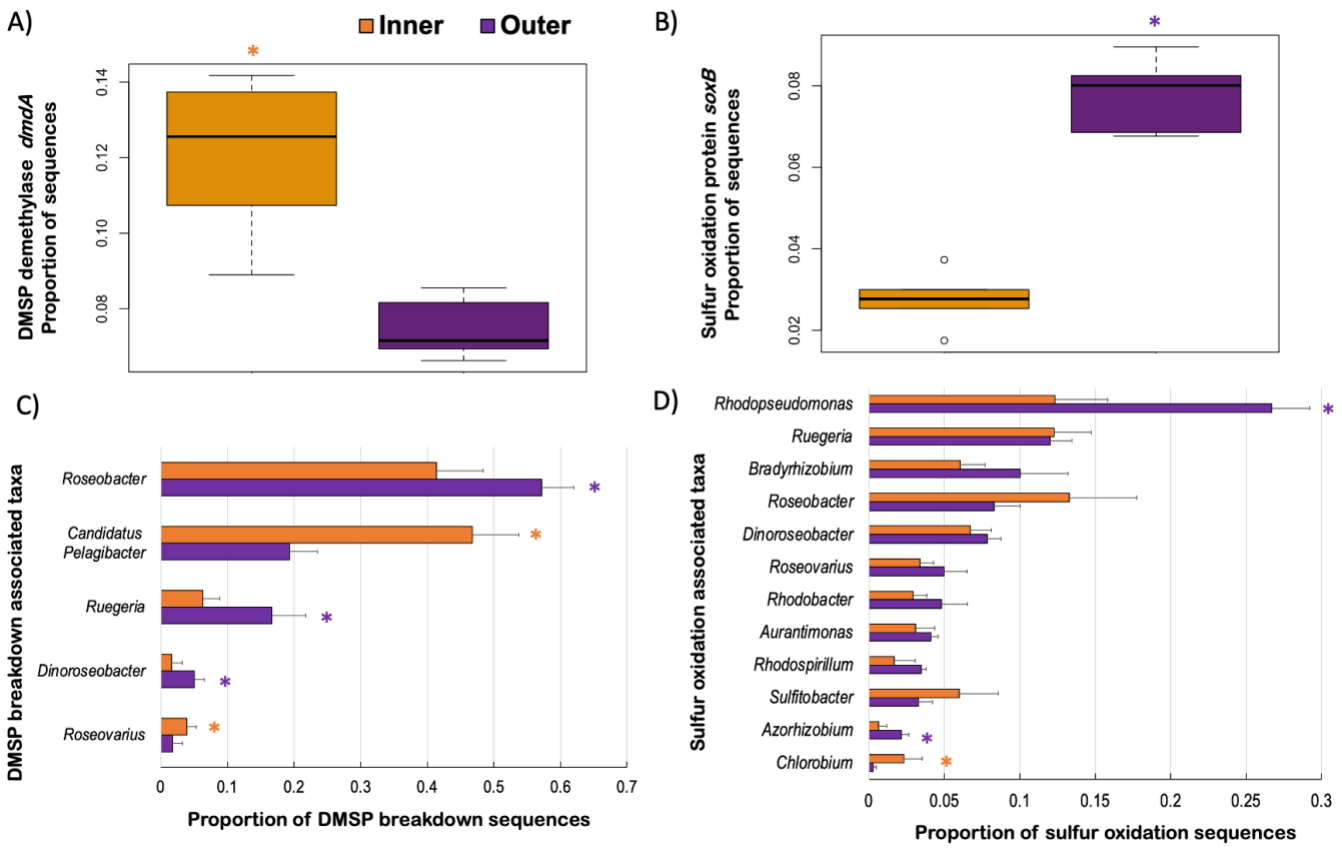

Figure 5. Sulfur metabolism gene pathways and respective taxa associated with the SML microbiome of *P. strigosa* from inner and outer reefs in Bermuda. Proportion of bacterial DMSP demethylase *dmdA* genes (A) and sulfur oxidation *soxB* genes (B) relative to the total sulfur metabolism genes, and of bacterial genera associated to DMSP breakdown (C) and to sulfur oxidation (D).

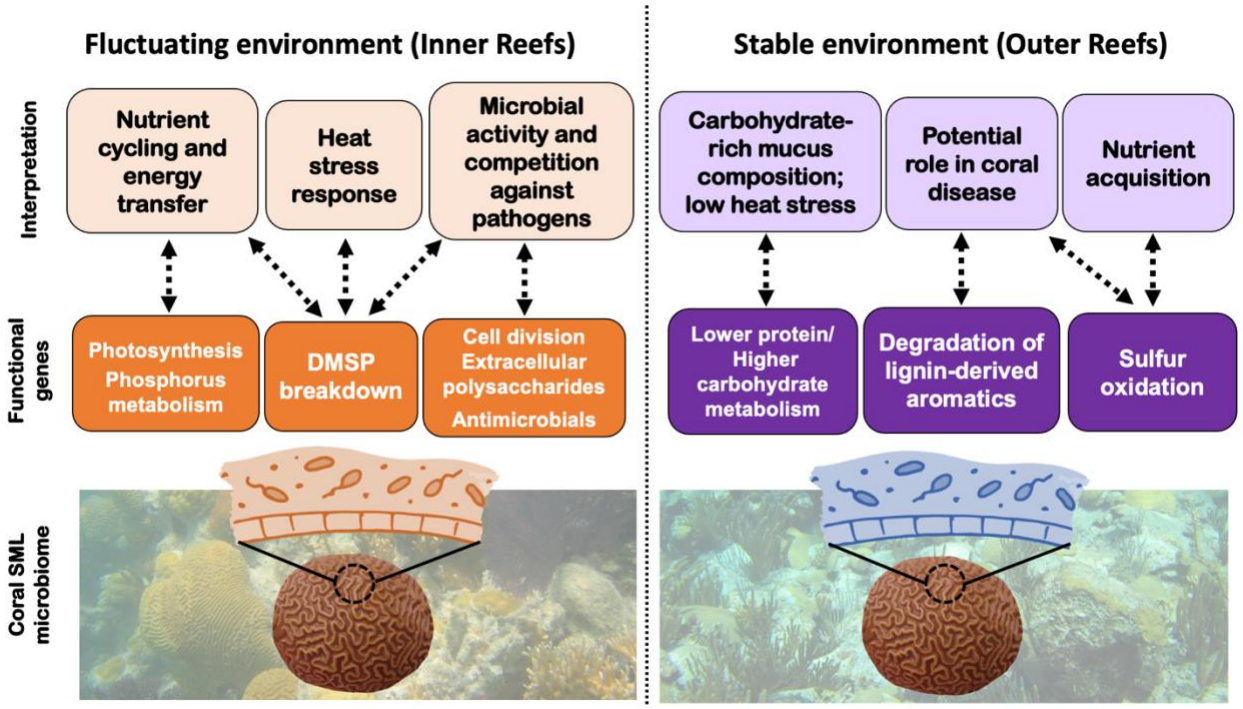

Figure 6. The functional metabolism of bacteria associated with the coral SML microbiome of *P. strigosa* varied across reef zones in Bermuda. In inner reefs, corals are exposed to a more fluctuating environment and their SML microbiome functional profile indicates that it provides more services related to nutrient cycling (e.g., carbon, phosphorus, sulfur), stress tolerance, and disease protection. In outer reefs, corals are exposed to a more stable environment and their SML microbiome is characterized by functional genes related to a mucus composition with a high carbohydrate to protein ratio (indicating low exposure to thermal stress), and involved in nutrient acquisition (i.e., taurine fermentation followed by thiosulfate oxidation) and coral disease (e.g., yellow-band and black-band diseases).

Supplemental Material

Table S1. Metagenomic sequences coverage and annotation hits through MG-RAST (as of April 8th, 2021).

Metagenome name	Sample ID	Total number of sequences	Bacterial sequence hits	Archaeal sequence hits
Inner_Reef_1_AA	mgs602127	932,522	409,892	2,090
Inner_Reef_1_LL	mgs602130	1,264,982	539,254	3,004
Inner_Reef_1_water_1	mgs602169	860,221	522,241	1,809
Inner_Reef_1_water_2	mgs602172	780,980	556,559	1,823
Inner_Reef_2_AA	mgs602133	1,115,369	473,425	2,116
Inner_Reef_2_water_1	mgs602175	897,812	594,281	2,055
Inner_Reef_2_water_2	mgs602178	968,692	746,806	2,501
Inner_Reef_3_AA2	mgs602145	626,624	262,136	1,522
Inner_Reef_3_LL1	mgs602142	898,828	354,127	1,543
Inner_Reef_3_LL2	mgs602148	870,627	343,187	2,212
Inner_Reef_3_water_1	mgs602181	642,681	478,392	1,768
Inner_Reef_3_water_2	mgs602184	752,643	584,613	1,604
Outer_Reef_1_AA	mgs602151	921,996	341,979	2,208
Outer_Reef_1_LL	mgs602154	864,577	609,245	3,968
Outer_Reef_1_water_1	mgs602187	985,985	610,602	2,575
Outer_Reef_1_water_2	mgs602190	1,221,790	705,749	2,735
Outer_Reef_2_AA	mgs602157	858,085	548,099	3,710
Outer_Reef_2_LL	mgs602160	1,368,678	906,029	5,470
Outer_Reef_2_water_1	mgs602193	1,025,086	759,119	4,161
Outer_Reef_2_water_2	mgs602196	526,746	455,237	3,295
Outer_Reef_3_AA	mgs602163	646,510	287,792	1,631
Outer_Reef_3_LL	mgs602166	684,623	486,791	3,102
Outer_Reef_3_water_1	mgs602199	421,976	250,062	1,020
Outer_Reef_3_water_2	mgs602202	657,501	424,819	1,960

Table S2. Environmental parameters (mean ± SD) in the water column (4 - 5m depth) of inner and outer reefs of Bermuda.

Reef Zone	Temperature (˚C)	pH	Chlorophyll-a concentration (µg/L)	Dissolved Oxygen (mg/L)	Dissolved Oxygen Saturation (%)
Inner Reefs	23.83 ± 0.21	8.27 ± 0.03	1.79 ± 0.23	7.22 ± 0.04	106.53 ± 0.53
Outer Reefs	23.15 ± 0.33	8.27 ± 0.03	1.32 ± 0.06	7.30 ± 0.34	106.53 ± 5.44

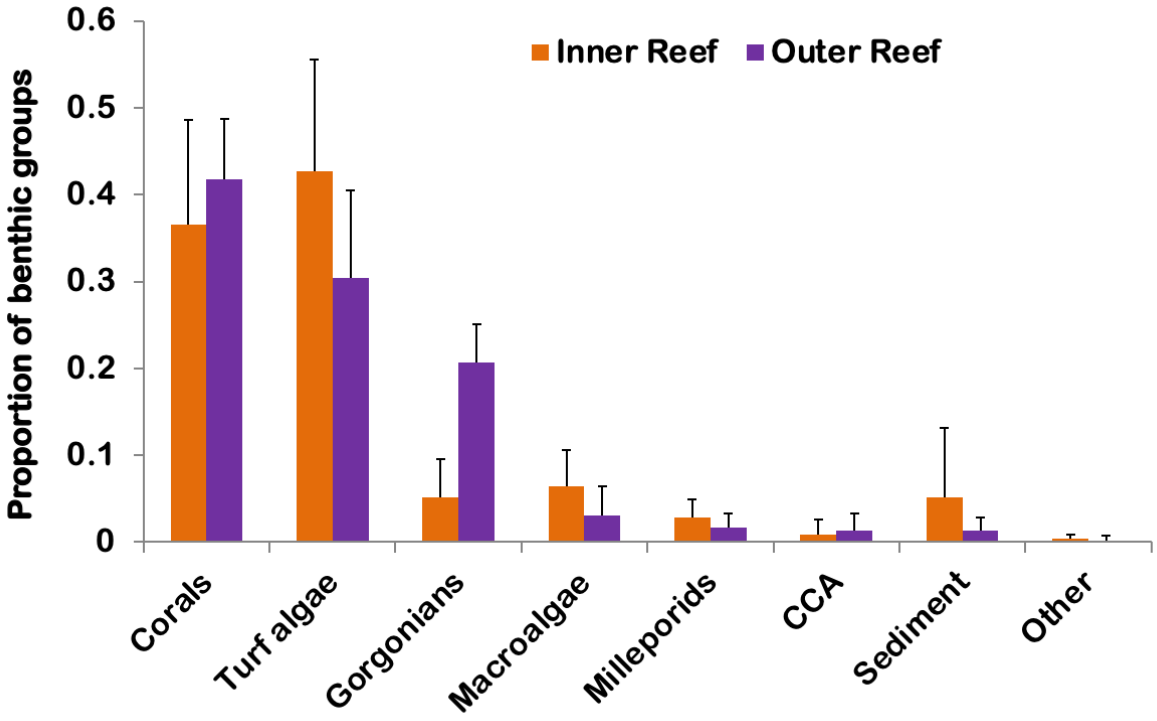

Figure S1. Benthic coverage (mean ± SD) of inner and outer reefs of Bermuda.

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Chapter 2

Modeling of the coral microbiome:

the influence of temperature and microbial network

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Abstract

Host-associated microbial communities are shaped by extrinsic and intrinsic factors to the holobiont organism. Environmental factors and microbe-microbe interactions act simultaneously on the microbial community structure, making the microbiome dynamics challenging to predict. The coral microbiome is essential to the health of coral reefs and sensitive to environmental changes. Here, we develop a dynamic model to determine the microbial community structure associated with the surface mucus layer (SML) of corals using temperature as an extrinsic factor and microbial network as an intrinsic factor. The model was validated by comparing the predicted relative abundances of microbial taxa to the relative abundances of microbial taxa from the sample data. The SML microbiome from *Pseudodiploria strigosa* was collected across reef zones in Bermuda, where inner and outer reefs are exposed to distinct thermal profiles. Shotgun metagenomics was used to describe the taxonomic composition and the microbial network of the coral SML microbiome. By simulating the annual temperature fluctuations at each reef zone,

the model output is statistically identical to the observed data. The model was further applied to six scenarios that combined different profiles of temperature and microbial network, to investigate the influence of each of these two factors on the model accuracy. The SML microbiome was best predicted by model scenarios with the temperature profile that was closest to the local thermal environment, regardless of microbial network profile. Our model shows that the SML microbiome of *P. strigosa* in Bermuda is primarily structured by seasonal fluctuations in temperature at reef-scale, while the microbial network is a secondary driver.

Importance

Coral microbiome dysbiosis (i.e. shifts in the microbial community structure or complete loss of microbial symbionts) caused by environmental changes is a key player in the decline of coral health worldwide. Multiple factors in the water column and the surrounding biological community influence the dynamics of the coral microbiome. However, by including only temperature as an external factor, our model proved to be successful in describing the microbial community associated with the surface mucus layer (SML) of the coral *P. strigosa*. The dynamic model developed and validated in this study is a potential tool to predict the coral microbiome under different temperature conditions.

Introduction

The community structure of a host-associated microbiome is shaped by factors that are both extrinsic (e.g. abiotic conditions, and community composition of micro- and macro-organisms in the surrounding environment) and intrinsic (e.g. microbial interactions, and host physiology) to the holobiont (Soen 2014; Bordenstein and Theis 2015; Foster et al. 2017; Walsh et al. 2017). Identifying the role that each factor plays in predicting the diversity and community structure in

the microbiome of host organisms is a major priority in microbial ecology, especially in the context of environmental changes (Spor et al. 2011; Kueneman et al. 2014; Adair and Douglas 2017; Apprill 2017).

Coral reefs are among the most productive, biodiverse and endangered ecosystems in the world (Cinner et al. 2016; Hughes et al. 2018) and the health of the reefs is directly mediated by the associated microbiota (Dinsdale et al. 2008b; Dinsdale and Rohwer 2011; Kelly et al. 2014; Haas et al. 2016). Corals host one of the most phylogenetically diverse microbiomes among animal hosts (Huggett and Apprill 2019), which is composed by endosymbiotic dinoflagellates (Symbiodiniaceae), bacteria, archaea, fungi and viruses (Rohwer et al. 2002). The coral microbiome provides essential services to the holobiont such as nutrient cycling (Wegley et al. 2007; Siboni et al. 2008; Raina et al. 2013; Rädecker et al. 2015) and protection against opportunistic pathogens via competition and production of antibiotic compounds (Ritchie 2006; Shnit-Orland and Kushmaro 2009; Peixoto et al. 2017b).

The symbiotic relationships in the coral holobiont are sensitive to changes in environmental conditions. Extrinsic factors, including eutrophication (Furby et al. 2014; Vega Thurber et al. 2014), salinity (Garren et al. 2009), pH (Thurber et al. 2009; Meron et al. 2011), neighboring macro-organisms (Walsh et al. 2017), herbivorous fish abundance (Zaneveld et al. 2016), copper concentration (Gissi et al. 2019), and temperature (Thurber et al. 2009; Maynard et al. 2015; Tracy et al. 2015; McDevitt-Irwin et al. 2017), alter the taxonomic and functional composition of the coral microbiome. Overall, the response of the coral microbiome to environmental disturbances is consistent across multiple stressors, characterized by an increase in the relative

abundance of *Vibrionales, Flavobacteriales, Rhodobacterales* and *Alteromonadales* (McDevitt-Irwin et al. 2017).

Corals are widely recognized as thermally sensitive organisms (Warner et al. 1996; Fitt et al. 2001; Lough 2012; Roth 2014) and elevated temperatures are correlated to coral bleaching (i.e. loss of the algal symbiont) and disease outbreaks in coral reefs worldwide (Dinsdale 2002; Willis et al. 2004; Harvell et al. 2007; Cróquer and Weil 2009; Ban et al. 2013; Maynard et al. 2015). High seawater temperature causes the greatest change in the functional metabolism of coral microbiome, when compared to eutrophication and low pH, as a result of an increase in the abundance of *Vibrio* spp. and other diseased-associated microbes (Thurber et al. 2009). Seawater temperature, therefore, is one of the most important drivers of the coral-microbial community composition (Bourne et al. 2016).

The surface mucus layer (SML) microbiome constitutes the direct interface between the coral host and the environment. Within the coral holobiont, the coral SML, tissue, and skeleton provide different microhabitats to the microbial community (Apprill et al. 2016; Pollock et al. 2018). Across the three coral microhabitats, the microbial composition of the SML is the most influenced by environmental factors (e.g. temperature, benthic coverage, geographic region) (Pollock et al. 2018) and by the microbial community in the water column (e.g. high similarity) (Apprill et al. 2016).

The influence of factors that are intrinsic to the coral holobiont on regulating the microbiome is less clear. Host genotype and Symbiodiniaceae phylotype are among intrinsic factors that do not correlate with the taxonomic composition in the coral microbiome, but instead the microbiome correlates with environmental factors such as habitat and seasonality (Pantos et al.

2015; Sharp et al. 2017; Ziegler et al. 2017). Microbe-microbe interactions (e.g. competition, predation, mutualism), however, are intrinsic factors that are potentially major drivers of the coral microbial community structure and holobiont homeostasis (Rypien et al. 2010; Krediet et al. 2013b; Rädecker et al. 2015; Sweet and Bulling 2017; Welsh et al. 2017). Coral-associated bacteria produce inhibitory compounds and have antagonist effects on each other, including *Pseudoalteromonas* spp. inhibiting the coral pathogen *Vibrio shiloi* (Rypien et al. 2010). High temperatures, however, can change the way microbes interact (Lin et al. 2016; Fujimoto et al. 2018). The number of coral-bacterial isolates inhibited by Alphaproteobacteria is drastically reduced when temperature increases from 25ºC to 31ºC (Rypien et al. 2010). Therefore, temperature and microbial interactions are interconnected and act simultaneously on shaping the community structure of the coral microbiome.

Mathematical models that use microbial growth rates as a function of environmental temperature (Ratkowsky et al. 1982; Davey 1989; Rosso et al. 1993, 1995; Vaidya and Wahl 2015) and include microbial interactions derived from network analysis (Marino et al. 2014; Dam et al. 2016) can be a powerful tool to investigate the dynamics of microbial communities. However, this approach remains to be further adapted and applied to coral reef systems. The ecological interactions between the members of the microbiome are challenging to elucidate, but metagenomic sequencing (Dinsdale et al. 2008a, 2013; Haggerty and Dinsdale 2017) combined with network analysis has been able to reveal these relationships (Fath et al. 2007; Steele et al. 2011; Deng et al. 2012; Chow et al. 2014; Wang et al. 2015). Microbial networks constructed by correlation-based methods identify microbial interactions and the key taxa to the structure of

the community by using measures of network centrality, such as eigenvector and betweenness centrality (Layeghifard et al. 2017; Röttjers and Faust 2018).

Here, we develop a new differential equation mathematical model to determine the community structure of the microbiome associated with coral SML using temperature as an extrinsic factor and microbial network as an intrinsic factor to the coral holobiont. To provide the input data for the model development and validation, we selected the coral reefs of Bermuda, where coral colonies are exposed to different thermal regimes at a reef-scale. The reef system in Bermuda is formed by distinct physiographic reef zones and there is a pronounced spatial gradient in temperature profiles across the inner and outer reef zones. The seawater temperature differences of the shallow inner lagoon reefs range between 13-15 ºC (winter averages of 16-17 ºC and summer averages of 30-31 ºC) whereas the outer reef temperature range is moderated with a 10 ºC temperature difference (seasonal averages of 19 and 29 ºC, respectively) (Smith et al. 2013a). The temperature profiles specific to each reef zone were simulated in the model. Metagenomic analysis was used to describe the taxonomic composition and generate the microbial network of the SML microbiome associated with the coral *Pseudodiploria strigosa* (Dana, 1846) from inner and outer reefs. The model was validated by comparing the predicted relative abundances of each microbial class to the measured relative abundances of each microbial class. Last, the model was applied to six scenarios that combine different profiles of temperature and microbial network, to investigate the drivers of the coral-microbial community dynamics. Our study shows that the SML microbiome of *P. strigosa* in Bermuda is primarily structured by reef-scale seasonal fluctuations in temperature, while the microbial network is a secondary driver.

Methods

In situ collections. The mucus from *P. strigosa* was collected from six colonies from the inner and outer reef zones (n = 12 colonies total) in May and June 2017. Each reef zone was replicated across three reef sites (Fig 1). The mucus microbiome of *P. strigosa* colonies (diameter 10-15 cm) was collected using a "supersucker", a two-way 50 ml syringe filled with 0.02 µm filtered seawater (Doane et al. 2017). The filtered seawater is flushed across the coral surface, dislodging the mucus and associated microbes, which are then sucked up via the recirculating tube, and the resulting sample pushed through a 0.22 µm sterivex (EMD Millipore) for DNA extraction. We collected 200 ml of coral mucus diluted in sterile seawater (four supersuckers) per colony to increase DNA concentration per sample. The reef water microbiome was also analyzed to control for contamination in the mucus samples (results not shown). The collections were performed via SCUBA diving at 4-6m depth.

Metagenomics analysis. Microbial DNA from the coral mucus was extracted using a modified Macherey-Nagel protocol from 0.22 µm sterivex using NucleoSpin® column for purification. DNA was stored at -20 °C until quantification with Qubit (Thermo Fisher Scientific) (Dinsdale et al. 2008b). The Swift kit 2S plus (Swift Biosciences) was used for library preparation as it provides good results from low amounts of input DNA, characteristic of microbial samples collected from the surface of the host (Doane et al. 2017; Cavalcanti et al. 2018; Minich et al. 2018). All samples were sequenced by the Dinsdale lab on Illumina MiSeq at San Diego State University (Edwards et al. 2013). The Illumina MiSeq is one of the best sequencing technologies for short genomes, such as those associated with bacteria and archaea and provides longer reads compared with Illumina

HiSeq (Hernandez et al. 2008; Kozarewa et al. 2009). We described the proportional abundance of Bacteria and Archaea in the coral mucus microbiome using shot-gun metagenomics (Dinsdale et al. 2008a; Haggerty and Dinsdale 2017). The sequenced DNA was analyzed for quality control using PrinSeq (Schmieder and Edwards 2011) before annotation. The forward and reverse reads were paired using PEAR (Zhang et al. 2013). Sequencing depth ranged from 582,582 to 1,256,934 reads per metagenome (Table S1). FOCUS (Silva et al. 2014), which is a K-mer based approach, was used to annotate taxa. FOCUS has been identified as one of the top profiling analysis tools by CAMI (Sczyrba et al. 2017). The number of sequence hits for each microbial taxon is represented as relative abundance by calculating the proportion of sequence hits for that class over the total number of sequences annotated for that metagenome.

Statistical analysis of the sample data. Statistical analyses were conducted using PRIMER v7 + PERMANOVA and R (The R Project for Statistical Computing). Significant differences in the relative abundance of classes in the coral microbial communities sampled from inner and outer reefs were identified by Permutational Multivariate Analysis of Variance (PERMANOVA) using Bray-Curtis distances of normalized relative abundance obtained using a fourth-root transformation. A Principal Coordinate Analysis was created to visualize the separation of the coral microbiome between inner and outer reefs and the most important taxa driving the cluster by plotting the vectors corresponding to Spearman's correlation indices. A SIMPER analysis was performed to identify the taxa responsible for the similarity of the microbiomes within reef zones and dissimilarity between reef zones. The microbial network was constructed for each metagenomic dataset the taxonomic pairwise Spearman correlation matrix calculated in R. The matrix was calculated for each reef zone, and the network correlation coefficients were used in

the dynamic model described in the section "*Mathematical Model*". The Python packages 'pandas' (McKinney 2011) and 'networkx' (Hagberg et al. 2008) were used to test for sub clustering of the networks and identified that each network remains a single connected component. The R package 'igraph' (Csardi and Nepusz 2006) was used to construct a network using the microbial taxa at class level as nodes and the Spearman correlation values as edge weights. The calculated diameter of the network was unweighted. The taxonomic co-occurrences that met or exceeded the pre-set correlation threshold were kept while all other values were transformed to 0. The 'psych' package (Revelle 2011) was used to calculate the p-value for all pairwise coefficients. All pairwise coefficients from which the p-value exceeded 0.001 were discarded from the analysis. To identify taxa that occupy important structures of the microbial network, the R package 'igraph' was used to calculate the eigenvector and betweenness centrality. Eigen centrality identifies highly connected nodes that are connected to other highly connected nodes (Bonacich 1972, 2007). Betweenness centrality calculates the shortest path through a network and keeps record of how many times a node in a network is traversed (Freeman 1977). If a node is traversed frequently, the node in the network is considered to sit at a position that is important in facilitating the connectivity of the network. If the taxon has a high betweenness centrality then it sits at a position in the network that is responsible for facilitating correlations between different taxa. Without the presence of that taxon, the network loses the architecture that binds it together in an ordered way.

Mathematical model. In an isolated environment, we assume that each microbial class, B_i, grows according to the classical logistic growth equations, however growth rate, $R_i(T(t))$, is represented as a function of environmental temperature $T(t)$, which changes over time. Since

each microorganism has a distinct range of ideal temperatures for its growth, we consider the growth rate, $R_i(T(t))$, to be normally distributed with mean at the midpoint of the range of the ideal temperature as follows.

$$R(T) = R_{max} \cdot \frac{1}{\sqrt{2\pi\sigma^2}} \cdot e^{-\frac{(T-\mu)^2}{2\sigma^2}}$$

Here, R_{max} is the maximum growth rate, μ is the mean ideal growth temperature, and σ is the range of ideal temperature. These values are specific to each microbial taxon are summarized in the Appendix S6. The values used in the calculation of maximum growth rates were obtained from the literature for cultured representatives of each microbial class.

Variation in temperature is captured using a sinusoidal function (Vaidya and Wahl 2015),

$$T(t) = M + A \cdot sin(\omega \cdot t + \theta),$$

where M and A represent the mean and the amplitude of the temperature profile. ω is related to the period of the periodic temperature profile, *i.e.* the period is $2\pi/\omega$, and θ represents the phase shift of the temperature used to make the temperature equation more accurately reflect the temperature conditions in the reefs of Bermuda according to the literature (de Putron and Smith 2011; Smith et al. 2013a; Courtney et al. 2017). The differences in reef zone environment are represented with different values of M and A, as described in the "*Network and temperature profiles"* section.

When the microorganisms reside in a community together, such as in the coral mucus, there are two major potential effects each microbial taxon faces due to presence of one another: (1) all microbes compete for the common resources denoted by the total carrying capacity k; and (2) interactions among microbes in a network alters the net growth rate of each other. To

introduce the first effect into the model, we assumed that all taxa compete identically for the common resources as their relative competition coefficients are not well established. The second effect is introduced by altering the net growth rate of each microbial taxa with all other components of the microbiome according to its network correlation coefficients. The model we use is as follows:

$$\frac{dB_i}{dt} = R_i\big(T(t)\big) \cdot B_i \cdot \left(1 - \frac{\sum_{j=1}^{n} B_j}{k}\right) \cdot \left(1 + \sum_{j=1}^{n} \eta_{ij} \cdot B_j\right), \;\; i = 1,2,\dots,N.$$

Here, N is the total number of taxa, k represents a carrying capacity and η_{ij} represents the network correlation coefficient between i^{th} class and j^{th} class of bacteria.

Network and temperature profiles. The model uses network correlation coefficients (η_{ij}) and temperature fluctuations over time (T(t)) to predict the structure of the microbial community associated with coral mucus. Therefore, different profiles of microbial network and seawater temperature were selected in an ecological context of the coral reef system. For both network and temperature, we explored the specificities of each reef zone by including a "Specific" profile. In addition, we also considered a "Generalized" profile that represents the coral reef system on a broader scale, instead of according to the local zonation patterns. "Specific" and "Generalized" profiles are used to evaluate whether the inclusion of values that represent the microbiome (network) and the environment (temperature) at a fine spatial scale are necessary to achieve accuracy of the model outputs.

The Specific Network profiles (SN) include the correlation coefficients (η_{ij}) that represent the sample data collected in the specific reef zone being modeled. For example, to model the microbiome associated with inner reefs, the correlation coefficient (η_{ij}) produced from all of the

six metagenomes collected in inner reefs is used in the SN profile. In contrast, the Generalized Network profile (GN) uses the correlation coefficient (η_{ij}) produced by all the metagenomes collected, from both reef zones (n = 12).

The Specific Temperature profiles (ST) are produced by using yearly mean (M) and amplitude (A) in degree Celsius that are representative of each reef zone to calculate the temperature fluctuations as a function of time (T(t)). In the ST profile for the inner reef, temperature mean, and amplitude are higher (M = 24, A = 7) than in the ST profile for the outer reef (M= 18, A = 5). Therefore, the ST profiles are developed to represent the local temperature regimes in terms of annual temperature fluctuations, in which the inner reef zone is a warmer and more fluctuating environment and the outer reef zone is a milder and more stable thermal environment. The Generalized Temperature profile (GT) used the average between the parameters in the ST profiles (M= 21, A = 6). In all the temperature profiles, we used $\omega = 2\pi/365$ to account for the annual variability of temperature according to seasons (Figure S3). Constant Temperature profiles (CT) are also considered to evaluate the effect of temperature fluctuations on the model outputs. The CT profiles use the mean temperatures specific to each reef zone, (inner: M = 24, A = 0; outer: M= 18, A = 0).

Model application to identify drivers of microbiome dynamics. The mathematical model we developed considers both intrinsic and extrinsic factors affecting the coral mucus microbiome. The intrinsic factor is the microbial interaction within the microbiome, characterized by the network analysis, and the extrinsic factor is environmental temperature. To determine the key drivers governing coral mucus microbiome composition across reef zones, we evaluate six different model scenarios (i.e. different combination of network and temperature profiles):

(i) Specific Network, Specific Temperature (SN-ST); Both temperature profile and network parameters used are the ones specific to each reef zone

(ii) Specific Network, Generalized Temperature (SN-GT); The network profile is specific from each reef zone, but the temperature profile is generalized

(iii) Specific Network, Constant Temperature (SN-CT); The network profile is specific to each reef zone, but temperature remains constant at the mean specified to each reef zone

(iv) Generalized Network, Specific Temperature (GN-ST); The network profile is generalized, but temperature profile is specific to each reef zone,

(v) Generalized Network, Generalized Temperature (GN-GT); The network profile and the temperature profile are generalized

(vi) Generalized Network, Constant Temperature (GN-CT); The network profile is generalized, and temperature remains constant at the mean specified to each reef zone

Statistical Analysis of the model output data. The microbial relative abundances generated from the model was compared against the sample data from inner and outer reefs using a linear regression (in R Project for Statistical Computing). A Wald Linear Hypothesis test was performed on the parameters generated by the linear regression analysis (i.e. slope and intercept). If the model is an accurate predictor of the coral microbiome, the slope will not be statistically different from one and the intercept will not be statistically different from zero. Each model scenario was tested by the fourth-root transformation of sample data and model relative abundances was applied to achieve normality (Shapiro-Wilk test), then a linear regression analysis was performed and tested. Model components associated with changes in accuracy (R^2) of the model outputs are considered key factors shaping the coral microbiome structure. For example, if all model

scenarios that include “ST” are more accurate than the others, regardless of the network profile used, then local temperature profile is a key factor, therefore, temperature is a primary driver and microbial interactions is a secondary driver shaping the coral microbiome structure. If there are no differences in accuracies across model scenarios, then it is assumed that all factors have the same impact on the microbial community. Therefore, by comparing the model outputs generated by different combinations of network and temperature profiles, the model was applied to investigate the drivers of the coral-microbial community dynamics.

Data availability. The metagenomic data from this study is publicly available in the SRA database as “BioProject ID: PRJNA595374” (https://www.ncbi.nlm.nih.gov/bioproject/595374) and in MG-RAST as public study “SDSU_BIOS_2017 (mgp81589)” (https://www.mg-rast.org/linkin.cgi?project=mgp81589). The scripts used for the statistical analysis in R, Python and PRIMER are publicly available as a GitHub repository “MichealBReed/Microbiome_model” (https://github.com/MichealBReed/Microbiome_model).

Results

Microbial community in the coral SML. The structure of the SML microbiome of *P. strigosa* was specific to each reef zone (Fig. 1) in terms of relative abundances and microbial network parameters. The SML microbiome of *P. strigosa* included 30 bacterial and archaeal classes (inner reefs = 23, outer reefs = 21), with high proportional abundances of Alphaproteobacteria, Bacilli and Gammaproteobacteria (Fig.2). Inner and outer reef microbial communities shared the same co-dominant classes, but the relative abundances of taxa were significantly different between reef types (PERMANOVA, Pseudo-F = 7.79; P(perm) = 0.004; Appendix S1). The sample metagenomes from inner and outer reefs formed separate clusters, indicating that *P. strigosa*

harbors a reef zone-specific SML microbiome (Fig 3). There was lower inter-colony variability in the SML microbial community within the inner reef corals compared to the outer reef corals (SIMPER, average similarity, inner = 92.9 %, outer = 84.7 %, Appendix S2a, b). Therefore, the coral SML microbiome structure is more homogenous across host individuals of the same species in a more fluctuating environment than in a more stable environment in Bermuda. The inner and outer coral-mucus microbiome had an average dissimilarity of 18.16 % (Appendix S2c). The main classes contributing to the dissimilarity between reef zones were Chlamydiia, Deinococci, and Flavobacteriia, which were overrepresented in the microbiome of corals from inner reefs.

The SML microbiome from each reef zone showed a specific network (Fig. 4). The microbial network from inner reefs had 23 nodes (i.e. microbial classes), 46 edges and diameter of 7 while the microbial network from outer reefs had 20 nodes, 94 edges and diameter of 3. The network in the outer coral SML microbiome was more tightly connected compared with the inner coral SML microbiome taxa (eigen centrality > 0.75, n = 8 classes in outer reefs and n = 3 classes in inner reefs; Fig. 4). High values of eigen centrality characterize a highly structured community network, in which the relative abundances of microbial taxa are tightly correlated (Borgatti 2005). On the other hand, the microbial network from inner reefs showed higher betweenness centrality and lower eigen centrality (maximum betweenness = 45 in outer reefs and 90 in inner reefs; Fig. 4). A microbial class with a high level of betweenness centrality sits at a position that is important in facilitating the connectivity of the network (Freeman 1977). Thermotogae, even though it was a rare class (average relative abundance <1%) showed high eigen and betweenness in both reefs. Methanobacteria is a key node to the network of coral microbiome in outer reefs, with both high eigen centrality and betweenness centrality (Fig. 4). In the microbiome of corals

from inner reefs, Gammaproteobacteria, Mollicutes, Bacilli, and Flavobacteriia showed the highest eigen centrality and Deinococci, Methanomicrobia, and Alphaproteobacteria showed the highest betweenness centrality in the community network (Fig. 4).

Modeling the coral microbiome. We developed a dynamic model based on differential equations to describe the relative abundances in the microbial community associated with the coral mucus in response to temperature and microbial network. The model uses classical logistic growth equations to calculate growth rates for each microbial class as a function of seawater temperature over time (Vaidya and Wahl 2015) and accounts for the effects of microbial interactions on growth rates using network correlation coefficients (Marino et al. 2014). The model was validated by comparing model prediction with sample data, using the classes identified in the metagenomes that showed an average relative abundance greater than 1 % in at least one of the two reef zones (n = 17 classes shared between reef zones, out of the total of 23 in inner reefs and 21 in outer reefs). For both the inner and outer reefs, we used the corresponding specific networks and corresponding temperature profiles into the model of the SML microbiome of *P. strigosa* and solved the model to predict the microbiome composition (Appendix S3). Linear regression of the sample data and the model prediction of abundance of each microbial class had a slope of 0.96 and an intercept of 0.45, which were not statistically significant different from one and zero, respectively (Wald Linear Hypothesis test, Sum of Squares = 8.52, F = 0.81, P = 0.55). Therefore, the mathematical model developed in this study was accurate in predicting the observed SML microbiome of *P. strigosa* from the sample data, and the approach implemented here is appropriate for modeling the coral microbiome.

Investigating the role of temperature and microbial network as model components for the coral microbiome. We used our model to determine the key drivers governing the community structure in the coral SML microbiome. Six different combinations of temperature profiles (T = fluctuating temperature; CT = constant temperature) and network structures (N) that were both either specific to the reef zone (S) or generalized to the coral reef system (G) were evaluated (Table 1). An example scenario is SN-ST, that combines specific network and specific fluctuating temperature. The same model scenarios were applied to the microbiome associated with each reef zone (inner and outer reefs) separately generating twelve corresponding model outputs total (Fig. 5). Ten out of the total of twelve model outputs analyzed had a significant linear regression between sample data and model predictions (Fig 5. a-d, g-l). Model outputs that significantly described the relative abundances of sample data produced R^2 values ranging from 0.51 to 0.70 (Appendix S4). Model scenarios that used constant temperature profiles were not significantly correlated to the coral microbiome from inner reefs (Fig. 5. e-f, Inner SN-CT, GN-CT, R^2 = 0.1). Therefore, the coral SML microbiome from inner reefs could not be successfully predicted by our model under constant temperatures, regardless of the network used. In contrast, when the temperature was kept constant in outer reefs, the model output accurately described the measured microbiome.

Among the ten model scenarios that fit the linear regression analysis, some combinations of microbial network and seawater temperature profiles generated more accurate outputs (higher R^2) than others. In inner reefs, greatest accuracy is achieved by using a specific network and an average fluctuating temperature profile (Fig. 5c, Inner SN-GT, R^2 = 0.64). Accuracy of the model output is lower when a generalized network and a warmer and more fluctuating

temperature profile is applied (Fig. 5b, Inner GN-ST, $R^2 = 0.51$). The microbiome of corals from the outer reefs of Bermuda was best predicted by the model when compared to inner reefs as all model scenarios produced significant linear regressions. The outputs produced by the model scenarios that used milder fluctuating temperature profiles (Fig. 5g-h, Outer SN-ST, GN-ST) and constant temperature profiles (Fig. 5k-l, Outer SN-CT, GN-CT) showed similar accuracies ($R^2 = 0.69$), regardless of the network profile. Model scenarios that used an average fluctuating temperature profile (Fig. 5i-j, Outer SN-GT, GN-GT) generated outputs with lower accuracy ($R^2 = 0.59$) in outer reefs.

The accuracy of the model relative abundances in the coral SML microbiome varied across taxa between the two reef zones (Fig. 6). Alphaproteobacteria, the most proportionally abundant taxon in the coral SML microbiomes of both reef zones, was more accurately predicted by model scenarios that used reef-specific temperature profiles (Fig. 6, SN-ST, GN-ST). On the other hand, model scenarios that used constant temperature profiles (Fig. 6, Inner SN-CT, GN-CT) were the least accurate when modelling the abundance of Alphaproteobacteria, causing underestimation in inner reefs and overestimation in outer reefs. Other co-dominant taxa, such as Bacilli and Mollicutes, were also underrepresented in the "CT" model scenarios in inner reefs. Actinobacteria was overestimated by approximately eight-times fold under constant temperatures in inner reefs (Fig. 6, Inner SN-CT, GN-CT). The model scenarios applied to the coral SML microbiome of outer reefs produced outputs that were within the range of the standard deviation from the mean relative abundances of the observed data for most of the microbial classes (Fig. 6).

Discussion

The mathematical model we developed predicted the microbial relative abundances in the SML microbiome of the coral *P. strigosa* and is a robust tool to investigate the effects of different profiles of temperature and microbial network on the model microbiome. We considered temperature as the major driver affecting the predictability of the coral SML microbiome of both reef zones, when compared to microbial network (Fig. 7). Differences in accuracies were driven by the distinct temperature profiles used across the model scenarios, while different microbial network profiles caused no apparent effect. The SML microbiome was best predicted by model scenarios that had a temperature profile that is closest to the local thermal environment. The coral SML microbiome in inner reefs in Bermuda is more exposed to natural temperature fluctuations (de Putron and Smith 2011; Smith et al. 2013a; Courtney et al. 2017). Therefore, a fluctuating profile is crucial to accurately describe the microbiome from that reef zone using temperature as an extrinsic factor. The coral SML microbiome of inner reefs is best predicted by the model scenarios that include high (SN-ST; GN-ST) or average temperature fluctuations (SN-GT; GN-GT), as there is a significant loss in model fit when temperature is kept constant (SN-CT; GN-CT), regardless of the associated network. In contrast, the SML microbiome of corals in the outer reefs were modeled using any temperature or network profile, yet less accurate when a warmer and more fluctuating temperature profile was applied.

The model shows that the coral SML microbiome from a fluctuating environment is more sensitive to the temperature profile used to achieve accurate predictions of the microbial relative abundances. This indicates that temperature is exerting a stronger and more directional pressure on the microbiome of inner reef corals, as they experience more pronounced temperature fluctuations than corals from the outer reefs (annual temperature range of 13-15 ºC in inner patch

reefs and of 10 ºC in outer reefs) (Smith et al. 2013a). The sample data shows that the coral SML microbiome structure is more homogenous across host individuals of *P. strigosa* in inner reefs than in outer reefs. Stability in the microbiome among colonies exposed to environmental stress is characteristic of a directional, rather than stochastic, response to pressure (Zaneveld et al. 2017). Microbiome stability under environmental fluctuations was an unexpected finding. Previously, the microbiome of *P. strigosa* showed high variability under stress conditions *in situ*, compared to *Diploria labyrinthiformis*, a closely related species (Pratte and Richardson 2018). However, the microbiome of *P. strigosa* shows low inter-colony variation within the same site over time in the Caribbean (Chu and Vollmer 2016), indicating that the microbiome associated with different colonies of *P. strigosa* acclimates similarly to temporal variability, which could explain the stability among inner reef corals. The local temperature profile may also be influencing the differences in the relative abundances between the microbiomes from inner and outer reefs. Chlamydiia and Flavobacteriia are driving the difference in the microbiomes between the two reef zones. The abundance of Flavobacteriia increased in the microbiome of *P. strigosa* in the summer when compared to winter (Pratte and Richardson 2018) and both taxa have been associated with elevated temperatures in coral reef environments (Thurber et al. 2009; Webster et al. 2011; Lee et al. 2015; McDevitt-Irwin et al. 2017; Glasl et al. 2019). The overrepresentation of members of these classes in the microbiome of corals from inner reefs of Bermuda may be related to the warmer local temperature profile but can also be simultaneously influenced by other factors (e.g. fluctuations in dissolved organic carbon) (Smith et al. 2013b; Pizzetti et al. 2016). The coral holobiont responds to fluctuations in abiotic factors such as light availability, pCO_2, total alkalinity, pH and dissolved inorganic carbon (DIC) (Courtney et al. 2017) and dissolved organic carbon (DOC) (Dinsdale et al. 2008b; Kelly et al. 2014; Silveira et al. 2017) . These could also be

driving the community structure in the SML microbiome of corals and be potential extrinsic factors to be added to our model. However, in Bermuda, many of these variables are correlated to seasonal temperature fluctuations (Courtney et al. 2017), which could partially explain why seawater temperature as the only abiotic factor is sufficient to accurately model the SML microbiome of corals.

The specificities of the microbial networks associated with each reef zone are also in accordance with trends shown in the model and the microbial community composition. Co-occurrence networks are able to detect small-scale environmental differences and show network specificity to each environment (Berry and Widder 2014). The coral SML microbiome from outer reefs was more variable between individual corals, but the microbial network was highly structured, because many microbial classes had high eigen centrality while betweenness centrality across taxa was low. High eigen centrality and low betweenness centrality are characteristic of keystone taxa in microbial networks (Berry and Widder 2014). The milder environment in outer reefs could be releasing the coral SML microbiome from constant microbial community turnover caused by external disturbances, allowing the microbial community to establish several different co-occurrence patterns and generating more hubs of keystone taxa in the network, e.g. in the microbial network of outer reefs 8 classes had eigen centrality > 0.75, compared to 3 classes in the network of inner reefs. A host-associated microbial network that is less disturbed is characterized by nodes that are more interdependent and is more vulnerable to targeted disturbances, as the removal of hub species caused a greater disruption of the network diameter (Baldassano and Bassett 2016). The microbial network associated with the SML microbiome of inner reef corals showed the opposite structure, composed of high values of betweenness centrality and low eigen centrality. Host-associated microbial networks characterized by high betweenness centrality and

low occurrence of large hubs of interconnected microbial taxa are considered resilient because the removal of nodes would not greatly impact the connectivity of the others (Estrada-Peña et al. 2018). Therefore, the coral SML microbiome in the inner reef zone in Bermuda is organized in a network structure that potentially confers resilience to the microbial community exposed to environmental disturbances, while the outer reefs provide a more stable environment that is conducive to a tightly connected microbial network.

Coral reefs provide a variety of habitats characterized by different environmental conditions, which affect the biological community from the scale of macro- to microorganisms (Haas et al. 2013; Kelly et al. 2014; Tout et al. 2014; Walsh et al. 2017). Our results are in agreement with studies that show that the reef zone in which the coral colony resides is a major factor shaping the coral microbiome composition (Pantos et al. 2015; Sharp et al. 2017), particularly across areas exposed to different thermal regimes (Ziegler et al. 2017). The spatial gradient in temperature profiles across the reef zones in Bermuda is coupled with documented variations in coral growth, calcification (Courtney et al. 2017) and reproductive processes (de Putron and Smith 2011; de Putron et al. 2017; Goodbody-Gringley et al. 2018). We showed that the coral SML microbiome responds to the local thermal environment in the coral reefs of Bermuda.

Conclusion

Coral microbiome dysbiosis (i.e. shifts in the community structure or complete loss of microbial symbionts) caused by changes in the environment is a key mechanism in the decrease of coral health worldwide (Haas et al. 2016; Zaneveld et al. 2016; Glasl et al. 2017; Sweet and Bulling 2017). The lack of datasets describing long-term coral-microbe dynamics is interfering with

successful predictions of how environmental change will affect the coral holobiont (Bourne et al. 2016). Metagenomics followed by modelling and prediction are highlighted as main analytical tools to disentangle coral disease causation and to identify successful application of mitigation strategies (Mera and Bourne 2018). The dynamic model we developed and validated using sampled metagenomes has the potential to be applied in microbial ecology research and coral reef management. A caveat to this study is that only one time point was used to validate the dynamic model. A time-series of the coral microbiome *in situ* will further improve the model with regards to the temporal fluctuations in the microbiome structure. However, the model produced accurate outcomes across multiple scenarios of temperature and network profiles, suggesting that the model is very robust. We modeled the coral SML microbiome at class level to obtain a number of taxa that is large enough to be representative of both two reef zones and achieve model accuracy, as all the differential equations for each taxon are solved simultaneously. After the rare taxa (relative abundance < 1%) were removed, we had more than 70% of the total richness identified in the metagenomes represented in our model. We note that our model can be implemented using other taxonomic levels (e.g. order, family, genera), if the parameters that are necessary to calculate growth rates $R(T)$ are known for each taxon. We recommend the use a cut-off value to remove the rare taxa and maintain accuracy of the predicted relative abundances.

The model is relatively simple to use and to interpret and can be used to simulate the changes in the SML microbiome in response to seasonal temperature fluctuations (Fig. 7). Corals species exhibit a wide variation in thermal resilience (Warner et al. 1999; Kemp et al. 2011; Roth et al. 2012) and mucus production and composition (Brown and Bythell 2005), which indirectly

shapes the associated SML microbiome. The coral *Porites astreoides*, for example, goes through cycles of mucus aging and shedding that affects the microbiome dynamics (Glasl et al. 2016). Therefore, the model developed here could be used to identify whether the microbiome of other species is affected by temperature like the *P. strigosa* SML microbiome. We recommend that at least one annual collection of the mucus microbiome for metagenomic analysis across different reef environments for model calibration. This approach will allow for a level of resolution specific to different areas across the reef that might require distinct management decisions. For example, the model can be used to evaluate whether different environments across the reef are more susceptible to disease or dysbiosis due to a predicted change in the relative abundances of microbial taxa under specific temperature conditions. Our model can also be used to describe the microbiome associated with the coral tissue and skeleton following the same workflow that we developed for the SML microbiome (Fig. 7). However, as each compartment provides a specific microhabitat to the microbial community (Apprill et al. 2016; Pollock et al. 2018), the coral tissue and skeleton microbiomes may respond differently to temperature fluctuations when compared to the SML microbiome (Pollock et al. 2018). We encourage the application of this model to other compartments of the coral microbiome (i.e. tissue and skeleton), as well as different coral species and coral reef systems, to compare whether temperature remains the primary ecological driver of the host-associated microbiome compared with microbial network.

Tables

Table 1. Model scenarios generated by different combinations of network parameters and temperature profiles.

	Network Parameters		Temperature Profiles		
Model Scenario	Specific	Generalized	Specific, fluctuating	Generalized, fluctuating	Constant
SN-ST	■		■		
SN-GT	■			■	
SN-CT	■				■
GN-ST		■	■		
GN-GT		■		■	
GN-CT		■			■

Figures

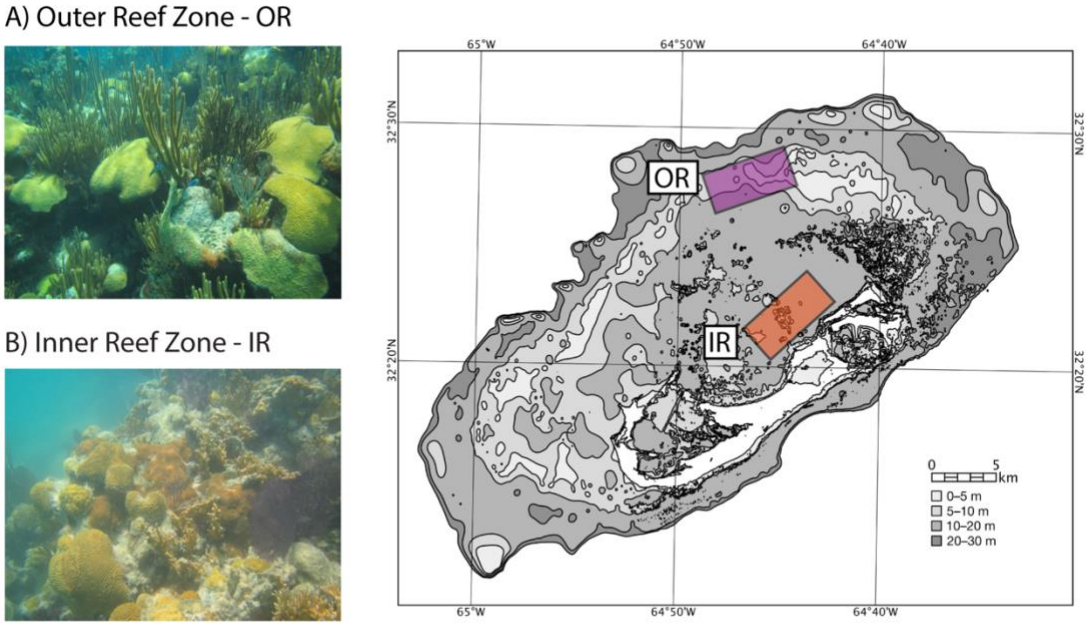

Figure 1. The coral reef in the Bermuda archipelago is composed of different reef zones across the platform. The outer rim reef (OR) is a relatively more stable thermal environment in comparison to the inner lagoon patch reefs (IR). Each reef zone was replicated (n = 6 corals per zone) in the colored areas.

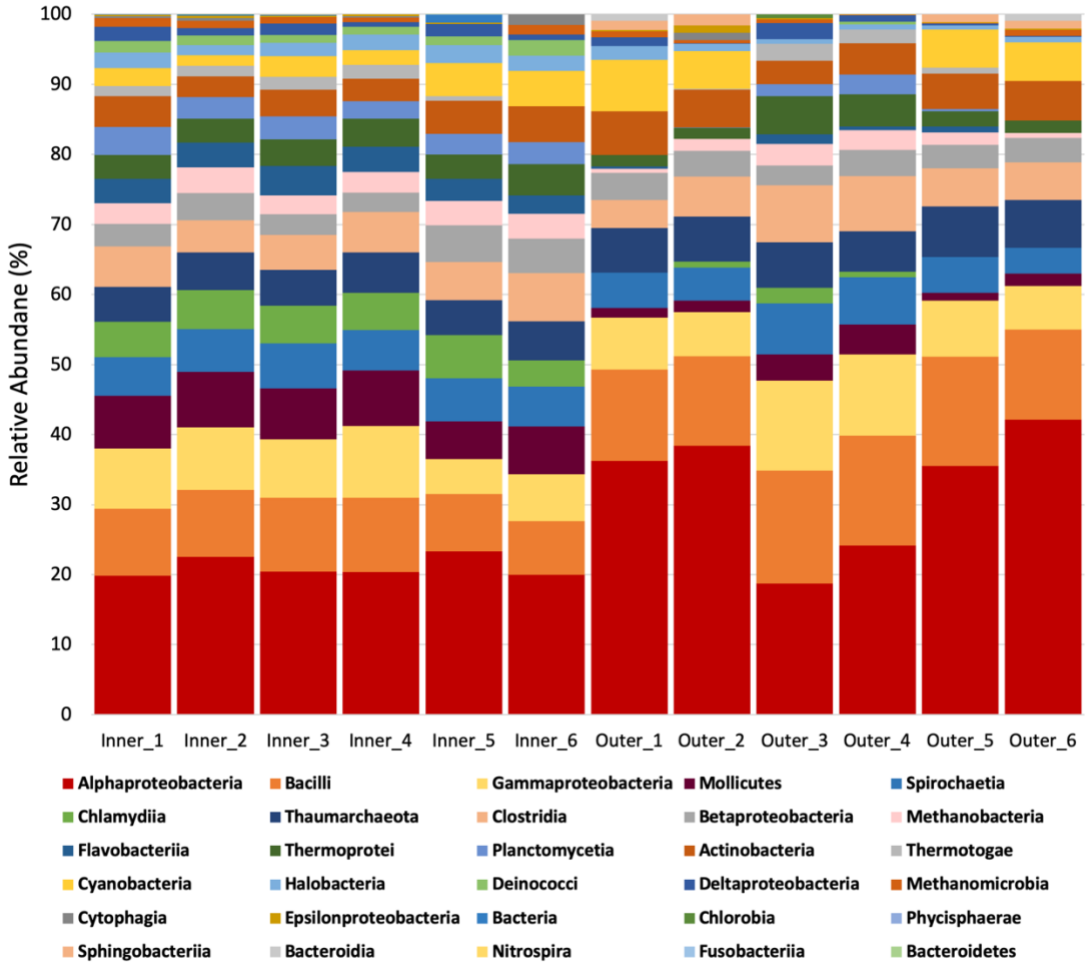

Figure 2. Relative abundances of microbial classes associated with coral mucus from inner and outer reefs.

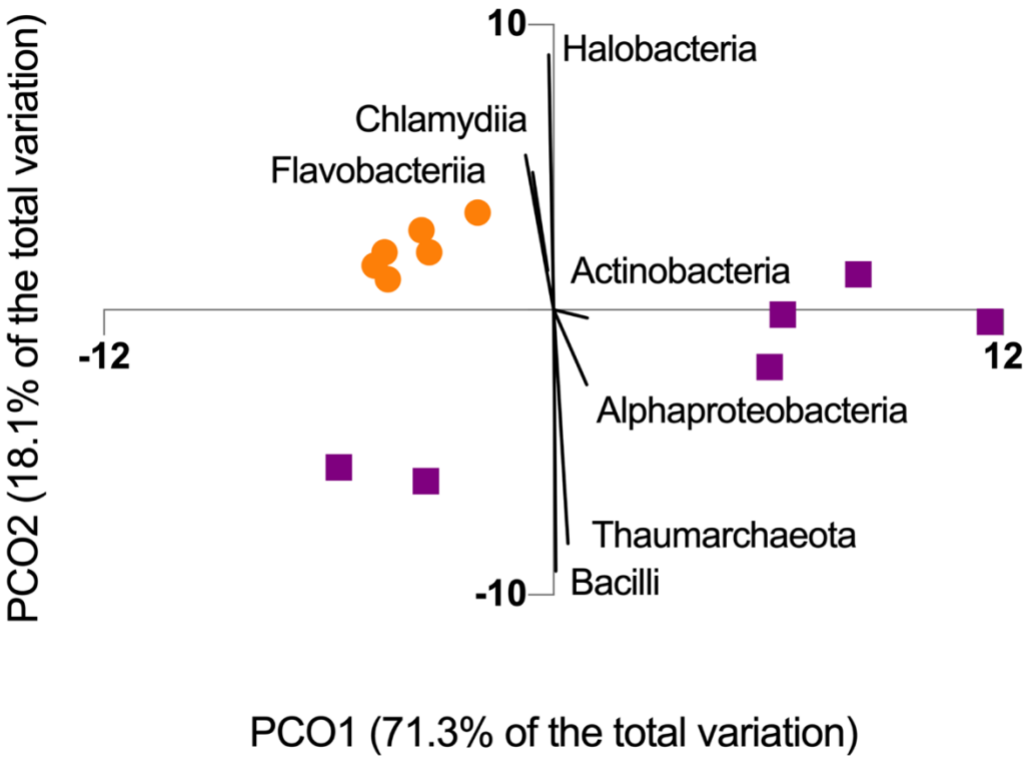

Figure 3. The mucus microbiome of *P. strigosa* corals from the inner reefs (circles) showed greater clustering than corals from the outer reef (squares), visualized using a principal coordinate analysis of relative abundance of microbial classes. Vectors correspond to Spearman's correlation indices higher than 0.9.

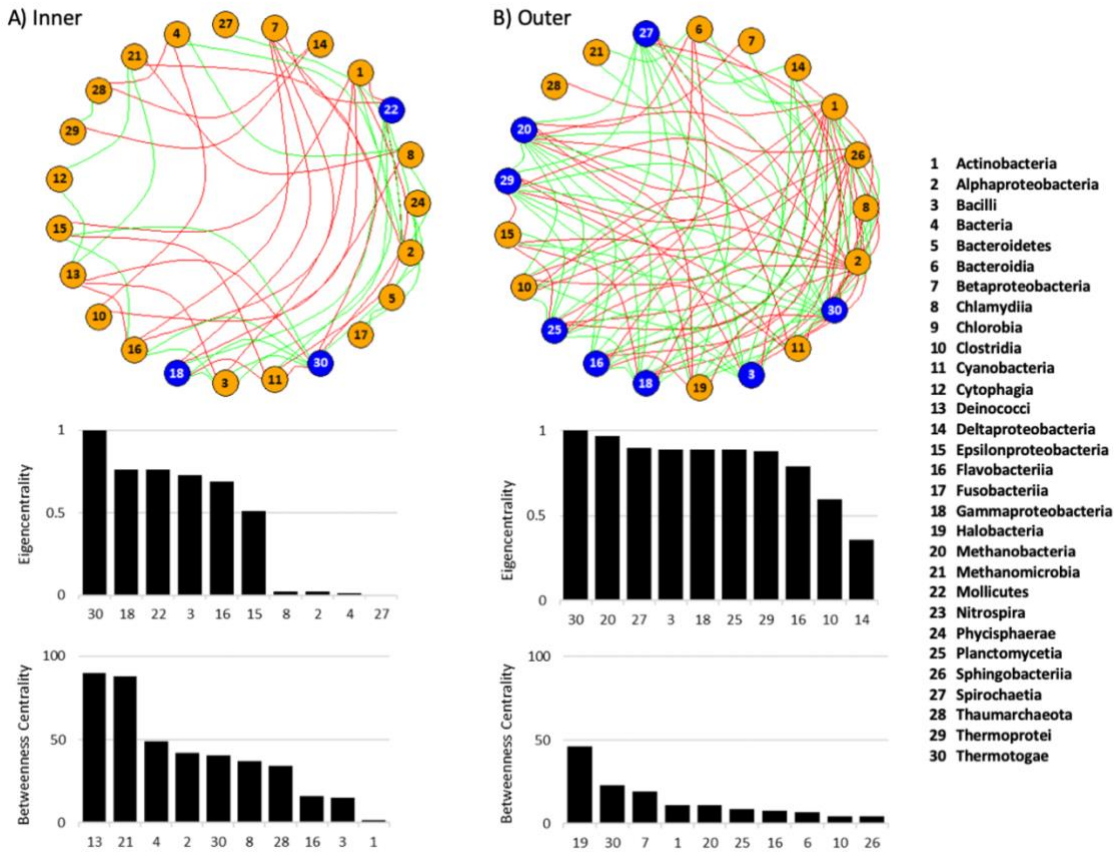

Figure 4. Network analysis of the coral mucus microbiome of *P. strigosa* from (a) inner and (b) outer reefs. Each node represents a microbial class interconnected by positive correlations (green) and negative correlations (red) (Spearman's rho > 0.7). Nodes that have eigencentrality higher than 0.75 is highlighted in blue. The top 10 values of eigencentrality and betweenness centrality across microbial classes are graphed below each network.

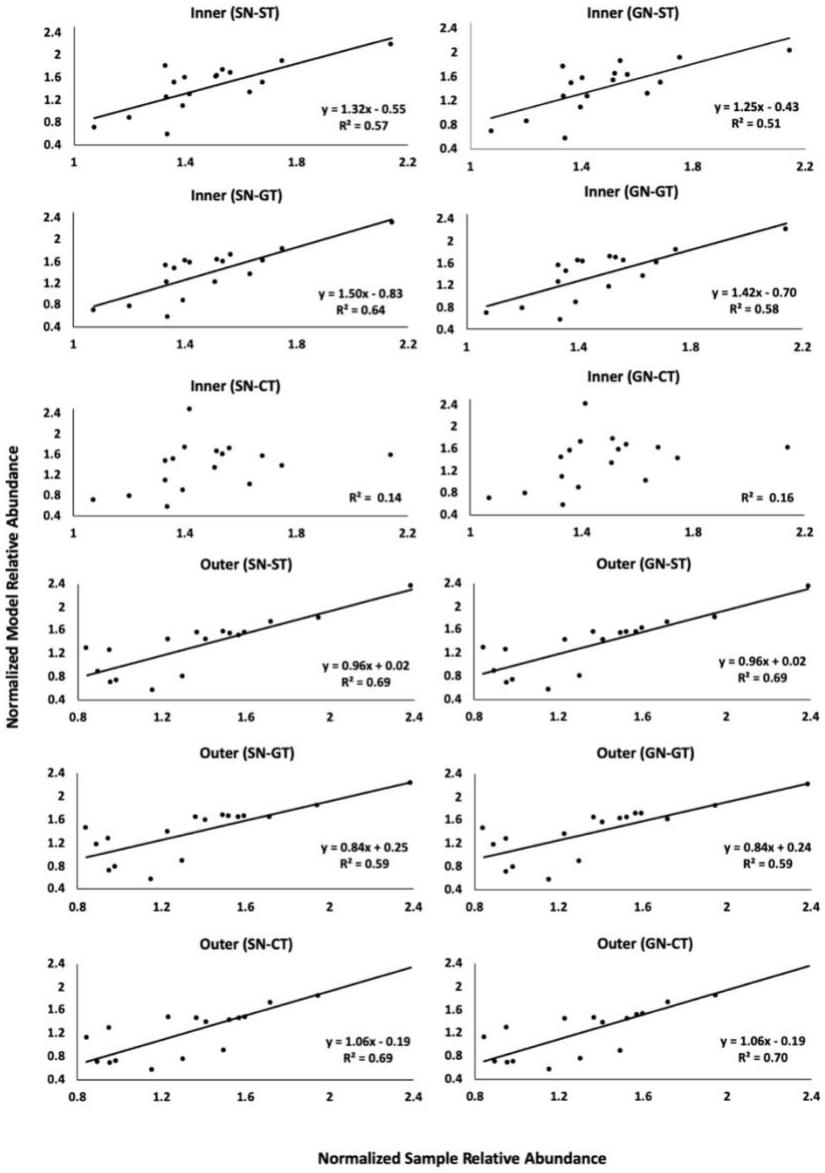

Figure 5. Linear regression analysis between sample and model data based on fourth-root transformed relative abundances. The sample data corresponds to the most abundant microbial classes (n = 17, average abundance >1%) in the metagenomes sequenced from surface mucus layer of the coral *P. strigosa* (n = 12 colonies; 6 per reef zone). The model abundances of these same classes were generated by the mathematical model for both inner (a-f) and outer reefs (g-l) using six different scenarios. The solid lines represent significant linear regressions (ANOVA, $p < 0.05$).

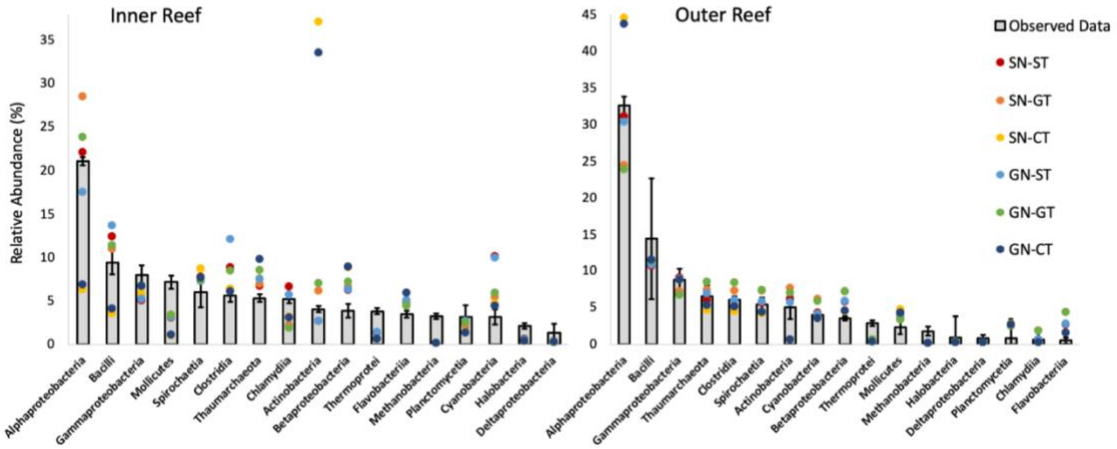

Figure 6. The model predictions of the relative abundances of seventeen microbial classes generated using the six scenarios (SN-ST, SN-GT, SN-CT, GN-ST, GN-GT, GN-CT) compared to the observed data (mean ± SD; n = 6 per reef zone) for the inner and outer reef zones respectively.

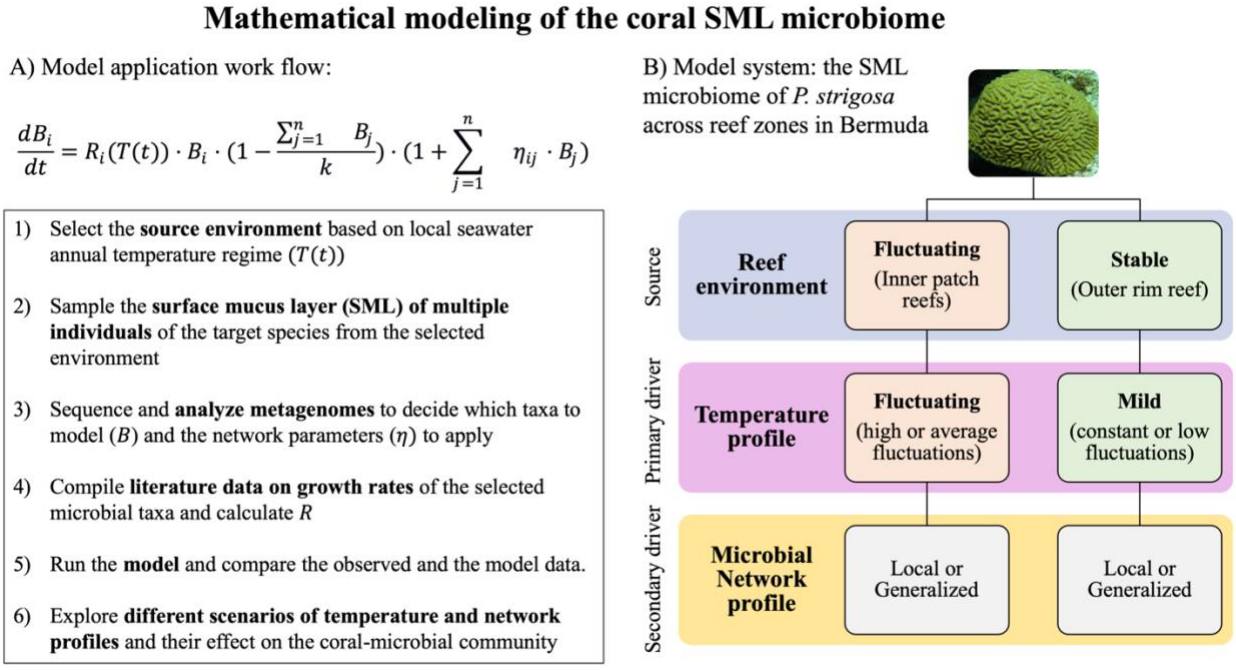

Figure 7. Modeling the coral surface mucus layer (SML) microbiome. On the left, a suggested workflow to apply the model developed in this study. On the right, a conceptual schematic of the drivers of the microbial community structure within the mucus of *P. strigosa* from each reef environment in Bermuda. The seawater temperature profile is the primary driver predicting the coral microbiome structure associated with different reef zones. Greater accuracy between the model and sample data was achieved when the model temperature profile depicts the natural temperature regimes. The network profile, used as a proxy for the microbial community interactions, is considered a secondary driver as it did not influence the accuracy of the model scenarios.

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Chapter 3

Heat stress drives a stable and potentially beneficial response in coral microbiomes

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Abstract

Coral reef health is tightly connected to the coral microbiome. Coral bleaching and disease outbreaks have caused an unprecedented loss in coral cover worldwide, and correlated to a warming ocean. Fortunately, acclimatization to local temperature can increase coral thermal tolerance. The acclimatization mechanisms of the coral holobiont are not completely understood, however, the associated microbial community is a potential source of acquired heat-tolerance. The relationship between stress and stability in the microbiome is key to the role that the coral microbiome plays in thermal tolerance. According to the Anna Karenina principle, stress or disease will increase instability and stochasticity among animal microbiomes. Here we investigate whether physiological heat tolerance is higher among corals that are locally acclimatized to temperature fluctuations and whether heat stress results in microbiomes that follow the Anna Karenina principle. We used shotgun metagenomics in an experimental setting to understand the dynamics of microbial taxa and genes in the surface mucus layer (SML) microbiome of the coral *Pseudodiploria strigosa* under heat treatment. The study was conducted in Bermuda, a marginal coral reef, where corals are naturally exposed to a wide annual range in

temperature fluctuations and reef-zone specific thermal regimes. We showed that thermal tolerance is shaped at reef-scale, since corals from a more fluctuating environment maintained high photosynthesis to respiration ratios under heat stress. The metagenomes of corals exposed to heat stress showed high similarity, indicating a deterministic and stable response of the coral microbiome to disturbance. The coral SML microbiome responded to heat stress with an increase in the relative abundance of *Ruegeria* and *Roseobacter*, and functional genes for nitrogen and sulfur acquisition. These consistent and specific microbial taxa and gene functions that significantly increased in corals exposed to heat appear beneficial to coral health and thermal resistance.

Key words: holobiont, acclimatization, thermal tolerance, stability, nutrient cycling

Introduction

Microbial symbioses are the engine of coral reef ecosystems. Corals associate with endosymbiotic dinoflagellates of the family *Symbiodiniaceae* and a diverse microbiome (e.g. bacteria, archaea, viruses) which function as a unit forming a holobiont (Rohwer et al. 2002). The coral holobiont depends on key services such as nutrient cycling (e.g., nitrogen and sulfur cycling) mediated by the associated microbiome (Wegley et al. 2007; Siboni et al. 2008; Raina et al. 2013; Rädecker et al. 2015; Lima et al. 2022). The coral surface mucous layer (SML) sustains a diverse and abundant community of these microbial partners (Koren and Rosenberg 2006; Sharon and Rosenberg 2008; Garren and Azam 2012; Ainsworth et al. 2015). The coral microbiome benefits from the high nitrogen content and organic matter in the SML (Wild et al. 2005; Rädecker et al. 2015) and provides protection against coral pathogens via production of antimicrobials (Ritchie 2006; Krediet et al. 2013). However, coral-associated microbial communities are sensitive to

environmental changes, especially to increased temperature, which disrupt the beneficial services provided to the holobiont (Thurber et al. 2009; Vega Thurber et al. 2014; Raina et al. 2016; Zaneveld et al. 2016).

Coral reefs are at risk of collapse as coral bleaching (i.e. loss of algal symbionts) and disease outbreaks have become more frequent in the last two decades, particularly correlated to rising seawater temperature, leading to major losses in coral cover worldwide (Maynard et al. 2015; Heron et al. 2016; Precht et al. 2016; Muller et al. 2018). These losses are pronounced on shallow water reefs of the Caribbean, where an decline in coral cover of up to 59 % has occurred since 1984 (Jackson et al. 2014). Corals live at their upper thermal limits, and therefore may not be able to adjust thermal thresholds to projected rises in seawater temperature (Berkelmans and Willis 1999; Fitt et al. 2001; Palumbi et al. 2014; Lough et al. 2018).

Acclimatization to local temperature regimes can increase coral thermal resilience irrespective of the coral host or the *Symbiodiniaceae* genotypes (Oliver and Palumbi 2011a; Howells et al. 2012). Thermal tolerance limits are affected by holobiont thermal history as shown by ‘resistance adaptations' in coral phenotypic response, i.e., where exposure to mildly stressful conditions increases resistance to lethal stress (Brown and Cossins 2011). For example, at inshore reef sites (e.g., lagoon patch reefs, tidal pools) coral populations are highly resilient to thermal stress as they are subject to more frequent and extreme temperature fluctuations (Castillo and Helmuth 2005; Oliver and Palumbi 2011b; Kenkel et al. 2015; von Reumont et al. 2016). The acclimatization mechanisms of the coral holobiont may involve the associated microbial community is a potential source of acquired heat-tolerance (Ziegler et al. 2017; Lima et al. 2020, 2022). There is also a potential evolutionary role of the microbiome as a source of genes (e.g.

stress response genes) that provide thermal resilience and disease protection via mechanisms such as horizontal gene transfer (Webster and Reusch 2017).

The role of the coral microbiome in thermal tolerance is understudied and the use of metagenomics associated with physiological data in experimental settings is a recommended approach (Bourne et al. 2016). The identification of dysbiosis in the coral-microbial communities facilitates diagnostic interpretations of environmental health applying microbial indicators to coral reef management (Glasl et al. 2017). Another outstanding topic is the relationship between stress and stability in the microbiome (Zanevald et al. 2017). According to the Anna Karenina principle for animal microbiomes, stress or disease will increase instability and result in low similarity among host-associated microbiomes exposed to the same disturbance (Zaneveld et al. 2017). Whether stress has a stable and deterministic effect on the microbiome leading to a specific dysbiotic community or stochastic consequences that result in a variety of unhealthy community structures has not been determined. In some systems, microbiome stability is maintained in physiologically stressed corals (Röthig et al. 2016; Hadaidi et al. 2017), whereas in others loss in diversity and instability in the microbiome occurred in corals under physiological stress (Grottoli et al. 2018).

Here we investigate whether physiological heat tolerance is higher among corals that are locally acclimatized to temperature fluctuations (inner reefs) compared to corals from a more stable environment (outer reefs).We describe whether heat stress results in unstable microbiomes through a stochastic process, as proposed by the Anna Karenina principle. We addressed these aims by exposing corals to a heat treatment and analyzing coral-algal physiological parameters and microbial taxa (genus level) and gene functions (stress response,

nitrogen metabolism, and sulfur metabolism) associated with the coral SML using shotgun metagenomics.

Methods

Field sampling

We selected *Pseudodiploria strigosa* (Dana, 1846) as the coral host species because it is widely distributed across the Bermuda platform. In Bermuda, a high-latitude subtropical reef system, corals are exposed to a wide annual range in temperature fluctuations and reef-zone specific thermal regimes ranging from fluctuating profiles in the inner lagoon patch reefs and the milder conditions in the outer reefs (de Putron and Smith 2011a; Courtney et al. 2017, 2020; Lima et al. 2020; Wong et al. 2021). The reef zones sampled were approximately 8 km apart (Lima et al. 2020) and *P. strigosa* is a broadcast spawner; therefore, there is a high likelihood that gene flow between the coral hosts colonizing inner and outer reefs is maintained and that the host genetics is not structured into different populations. Indeed, studies on other species have indicated high genetic exchange among reef sites in Bermuda (Serrano et al. 2014, 2016). The sampling period occurred between May 18th and May 22nd, 2017, late spring in the northern hemisphere, when environmental conditions between the two reef zones, especially temperature, are similar. These two reef zones are exposed to different regimes on a seasonal basis, with the most striking fluctuations occurring in the winter and summer months (de Putron and Smith 2011b; Smith et al. 2013; Courtney et al. 2017). Therefore, we selected this period to capture a potential long-term acclimatization of the coral holobiont to their reef zones, and not their immediate response to acute temperature fluctuations. The mucus from *P. strigosa* was collected from six colonies from the inner and outer reef zones (n = 12 colonies total) to describe

the natural spatial variability in the coral microbiome (Lima et al. 2020, 2022). After the mucus sampling, each coral colony was collected using a hammer and chisel to be used in the experiments to assess heat resistance. The colonies (n = 12 total, 6 from each reef zone) were carefully placed in a cooler with seawater and transported to the Bermuda Institute of Ocean Sciences. The collections were performed via SCUBA diving at 4-6m depth.

Colony preparation and acclimation

After collection, the coral colonies were placed in an outdoor tank with opaque roof supplied with unfiltered flowing seawater to acclimate for a week. Each colony was fragmented in four pieces using a tile saw, tagged and all non-coral tissue area was cleaned and covered in reef-safe epoxy. The colony fragments, here referred as "coral nubbins", were left acclimating in the indoor system for two weeks (ambient temperature 25°C ± 1°C). Using coral nubbins in laboratorial experiments is a way to expose the same colony and microbiome to different treatments, allowing for assessment of genetic variability among treatments, and reduces the number of collected coral colonies. The nubbins were assessed for recovery of the coring process (outward growth as opposed to tissue regression) and visual endosymbiont function as measured by color and fluorescence readings with a pulse amplitude modulated fluorometer (DIVING-PAM Walz Inc) and those that had tissue growth and a Fv/Fm > 0.6 were used in the experiments.

Temperature treatments

After the acclimation period, twelve coral nubbins (6 from each reef zone) were exposed to a high temperature treatment (29°C ± 1°C), while the remaining were maintained at ambient conditions (control, 25°C ± 1°C). Temperature was gradually increased from ambient to heat conditions over two days then maintained within the target range. The indoor temperature-

controlled tank system consisted in two fiberglass trays fed from a common header tank of filtered seawater. Each tray table was temperature controlled by aquarium chillers and heaters and light intensity maintained by aquarium lights (Storm and Storm X LED Controllers, CoraLux LLC) on a 12:12h photoperiod. Temperature was measured every 5 minutes throughout the experiment by pairs of HOBO data loggers (Onset Corp.) placed in each tray table and light intensity was measured by a LiCor PAR sensor.

Physiological measurements

Productivity and respiration rates were measured using dissolved oxygen probes in customized transparent glass chambers (approx. 4L) to detect oxygen evolution (production), and oxygen uptake (respiration rate). Each chamber had an oxygen sensor spot (PyroScience) glued to the inner chamber wall and a small in-line aquarium pump for water mixing during incubation. Oxygen concentration was monitored using a fiber optic sensor (PyroScience). Each individual nubbin was placed inside the chamber that was bathed in and filled with the filtered, temperature-controlled seawater from the experimental tray table. Dissolved oxygen concentration was measured in the chamber during a 1h-dark period to calculate dark respiration rates (R) and subsequently during a light exposure period of 1h to calculate net productivity (NP). The difference of dissolved oxygen concentration at incubation from the start and at the end was normalized over time and colony surface area as described by Schneider & Erez (2006). Surface area was determined by the aluminum foil technique (Marsh, 1970) and image analysis (Image J, US National Institutes of Health) of planar digital images. The dark respiration and photosynthesis rates were analyzed according to their linearity over time for quality control. Gross productivity (GP) was calculated (gross productivity = net productivity + dark respiration). Photosynthesis to

respiration ratios (GP:R) were calculated from the ratio of gross productivity (GP) to respiration (R) to estimate if production by the *Symbiodiniaceae* cells is exceeding maintenance requirements of both the symbiont and the coral host (Coles and Jokiel 1977). Maximum photochemical efficiency of photosystem II (effective quantum yield) was quantified daily via pulse-amplitude modulate (PAM) fluorometry (Warner et al. 1996; Jones et al. 1998). After overnight dark acclimation period, fluorescence (Fm and F0) was measured by saturation pulses at three random spots on each colony to calculate the colony's average Fv/Fm. Maximum photochemical efficiency of the PSII (Fv/Fm, Fv = Fm – F0) was calculated based on fluorescence measurements using a pulse amplitude modulated fluorometer (DIVING-PAM Walz Inc.).

Experiment design

The twelve acclimated coral nubbins (6 from each reef zone) were exposed to the high temperature treatment for one week while the remaining 12 nubbins were maintained at ambient conditions. Productivity and respiration rates and photochemical efficiency were measured the day before the heat treatment started (post-acclimation or pre-treatment) and on day 6, which is the final day of the experiment (post-treatment). The coral mucus was collected with a supersucker at the end of each incubation for respirometry using a "supersucker" (Lima et al. 2020, 2022). A supersucker is a two-way 50 ml syringe that is filled with 0.02 µm filtered seawater. The filtered seawater is squirted across the coral surface, dislodging the mucus and associated microbes, which are then sucked up via the recirculating tube, and resulting sample pushed through a 0.22 µm sterivex for DNA extraction. The SML was sampled using a supersucker to reduce stress and tissue damage to the coral during the experiment.

Metagenomic analysis

Microbial DNA from the coral mucus collected on the 0.22-µm Sterivex was extracted using a modified Macherey-Nagel protocol using NucleoSpin column for purification. DNA was stored at - 20°C until quantification with Qubit (Thermo Fisher Scientific) (Dinsdale et al. 2008b). The Swift kit 2S plus (Swift Biosciences) was used for library preparation since it provides good results from small amounts of input DNA, characteristic of microbial samples collected from the surface of the host (Doane et al. 2017; Cavalcanti et al. 2018). All samples were sequenced by the Dinsdale lab on Illumina MiSeq at San Diego State University. The sequenced DNA was analyzed for quality control using PrinSeq (Schmieder and Edwards 2011) before annotation. The metagenomes were annotated through MG-RAST (Meyer et al. 2019), using the RefSeq database for taxonomic annotations and the SEED database for functional annotations. The number of sequencess for each microbial taxon or function is represented as the relative abundance by calculating the proportion of sequence hits for that parameter over the total number of sequences annotated for that metagenome. Metagenomes were compared using proportional abundance, which is preferred to rarefaction (McMurdie and Holmes 2014; Quince et al. 2017; Luz Calle 2019). Bacteria accounted for approximately 99 % of the annotation; therefore, we are only analyzing bacterial taxa and gene functions in this study. We used metagenomics to describe the abundance of genes in the microbiome as a proxy for gene expression: although it does not measure which functional genes are being expressed at the point the sample was taken, it measures which functional genes are important for the microbes under those conditions (Dinsdale et al. 2008a; Coelho et al. 2022). There is a high level of correlation between the metagenomes and metatranscriptomes (Franzosa et al. 2014), where the abundance of a gene in metagenomes is a predictor of its expression level in the metatranscriptome and areas where

the two analysis vary are associated with short term changes in expression rather than bacteria functions that are under strong selective pressure and are well adapted to their environment (Gilbert and Dupont 2010; Gilbert et al. 2012; Mason et al. 2012). Metagenomes were sequenced from a subset of the coral nubbins used in the experiment (n = 8), representing four different coral colonies (n = 2 colonies per reef zone) that had their mucus sampled before and after the experimental treatments (n = 16 metagenomes total). The metagenomes from the experiment were compared with the "time zero" metagenomes collected from the same colonies *in situ* (Lima et al. 2022) prior to the experiment.

Statistical Analysis

Statistical analyses were conducted using PRIMER v7 plus PERMANOVA, Statistical Analyses of Metagenomic profiles (STAMP) software (Parks et al. 2014), and R (R Project for Statistical Computing). Significant differences in the relative abundances of microbial genera and functions in the coral microbial communities sampled from inner and outer reefs were identified by permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis distances of normalized relative abundance obtained using a square-root transformation. A cluster analysis was created to visualize the similarity of the coral microbiome between temperature treatments. We also used PRIMER to calculate Pielou's evenness index (J') and Shannon's diversity index (H') of microbial genera across metagenomes. The multiple comparisons of either taxa or functions across the three groups of metagenomes (i.e., pre-treatment (T1), ambient (T2), and heat (T2)) were conducted in STAMP using ANOVA/Tukey-Kramer, Welch's pairwise comparisons, and Benjamini-Hochberg FDR corrections. For the physiological state of the corals, we used R to test parametric assumptions of normality (Shapiro-Wilk's test) and homoscedasticity (Bartlett's test),

and differences in the Fv/Fm and GP:R rates across different temperature treatments (Factorial ANOVA, Tukey's post-hoc test).

Results

Coral holobiont physiology

Corals from both reef zones showed a lower photochemical efficiency (Fv/Fm) after exposure to the heat treatment (Fig. 1.A, Factorial ANOVA, $F_{1,40} = 8.612$, $p < 0.001$; Tukey's post hoc, $p < 0.001$). Coral nubbins from inner reefs showed more variability in Fv/Fm at the end of the ambient (ranging from 0.54 to 0.64) and heat ().52 to 0.62) treatments compared with corals from outer reefs (ambient: 0.60 to 0.63, heat: 0.57 to 0.59).

Gross Productivity to Respiration ratios (GP:R) significantly decreased on corals from the outer reef exposed to high temperatures (Fig. 1.B, Factorial ANOVA, $F_{1,40} = 5.369$, $p = 0.026$; Tukey's post hoc, $p = 0.035$), but not the inner corals. GP:R ratios were relatively stable (2 to 2.4) at the end of the ambient treatment in corals from both reef zones. After heat exposure, GP: R was highly variable among corals from inner reefs (1.2 to 2.6) and consistently lower in corals from outer reefs (1.3 to 2).

Coral SML microbiome

The coral SML microbiomes retained 74 % of the similarity (Bray-Curtis index) after being removed from their natural environments (Fig. 2). Heat-stressed coral SML metagenomes formed a cluster with the greatest similarity (Bray-Curtis index = 96 %) across all samples. Coral colonies showed different patterns of microbial succession throughout the experiment. For example, the microbiome of coral nubbins originating from the colony IR-2 started at 93 % similarity and after being exposed to different temperatures showed a 95 % similarity, indicating a strong colony

effect on maintaining a stable microbiome. In contrast, coral nubbins from colony OR-1 had 86 % similarity before the temperature treatments and after heat exposure shared 96 % similarity with a coral nubbin originated from a different colony and reef zone (IR-1.1), indicating a microbiome that responds strongly to external conditions.

Microbial richness (S, number of genera) ranged from 578 to 587 genera across all metagenomes and did not significantly change between microbiomes collected *in situ* and across the experimental treatments (Tab. 1). Diversity (H') was highest *in situ* due a decrease in evenness (J') under experimental conditions.

Pairwise PERMANOVAs showed that relative abundances of microbial genera ($t = 2.62$, $P(perm) < 0.03$), nitrogen metabolism genes ($t = 2.52$, $P(perm) = 0.03$), and sulfur metabolism genes ($t = 3.14$, $P(perm) = 0.03$) were significantly different between pre-treatment and heat-stressed microbiomes. The ambient temperature treatment did not affect the coral microbiomes, as metagenomes sampled from corals maintained at ambient conditions did not significantly change when compared to metagenomes from pre-treatment or heat treatments. Relative abundances of microbial stress response genes showed no significant change across treatments.

Heat exposure led to a significant increase in the relative abundances of *Ruegeria* ($t = -2.38$, corrected p-value < 0.02), *Roseobacter* ($t = -2.15$, corrected p-value < 0.001), *Oceanibulbus* ($t = -1.58$ corrected p-value < 0.03), *Chromohalobacter* ($t = -0.84$, corrected p-value < 0.02), and *Halomonas* ($t = -0.87$, corrected p-value < 0.02), according to Welch's pairwise comparisons among the top 20 most abundant taxa in the coral microbiome (Fig. 3). In contrast, there was a significant decrease in the relative abundances of *Shewanella* ($t = 0.82$ corrected p-value < 0.001),

Synechococcus (t = 0.88, corrected p-value < 0.04), and *Vibrio* (t = 0.284, p-value < 0.02) in the microbiome of corals exposed to heat treatment (Fig. 3).

The relative abundances of microbial gene pathways related to amidase with urea and nitrile hydratase (t = - 0.363, corrected p-value < 0.0001), alantoin utilization (t = - 1.079, corrected p-value = 0.042), and nitrogen fixation (t = - 0.130, corrected p-value = 0.049) increased in the coral microbiome under heat stress, while nitrosative stress (t = 1.628, corrected p-value = 0.033), and ammonia assimilation (t = 4.51 corrected p-value = 0.037) decreased (Fig. 4.B).

Sulfur metabolism microbial genes also changed in relative abundance after heat exposure of corals. There was an increase in glutathione utilization (t = - 1.70, corrected p-value < 0.0001), sulfur oxidation (t = - 8.08, corrected p-value < 0.0001), and taurine utilization (t = - 1.44, corrected p-value < 0.0001), and a decrease in inorganic sulfur assimilation (t = 8.04, corrected p-value < 0.0001) (Fig. 4.C).

Discussion

Coral holobiont physiology indicates heat resistance driven by local acclimatization

Pseudodiploria strigosa collected from the inner reef have an elevated thermal resistance when compared to corals from the outer reef, since they maintained a higher GP:R ratio even at reduced photochemical efficiency (Fig 1.). These results support the hypothesis that corals exposed to long-term temperature fluctuations show greater heat tolerance to acute heat stress (Castillo and Helmuth 2005; Oliver and Palumbi 2011b; Kenkel et al. 2015; von Reumont et al. 2016). Development of thermal tolerance in the coral holobiont is not completely understood. In some systems, heat resistance of the coral holobiont is explained by genetic parameters of the algal symbiont (Oliver and Palumbi 2011a; Hume et al. 2016) and/or the coral animal (Bay and

Palumbi 2014). In contrast, heat resistance sis correlated to a specific microbiome composition and uncorrelated to coral-algal features, suggesting that bacteria and other members of microbiome are also major players (Ziegler et al. 2017). The genomics of the coral host and *Symbiodiniaceae* were not addressed in this study, therefore, the specific role that each member of the holobiont played remains to be further investigated. Local temperature regimes are shaping microbiome of *P. strigosa* in Bermuda (Lima et al. 2020, 2022) and here we show that it is also driving the coral-algal physiology and heat resistance.

Stability of the coral SML microbiome under heat stress

Heat-stressed coral SML microbiomes formed a cluster with the highest similarity among all metagenomes analyzed (Fig. 2). The relative abundance of specific bacterial genera (Fig. 3) and gene functions (Fig. 4) changed consistently across coral replicates that were exposed to the heat treatment, while in the ambient treatment changes were more variable and not significantly different from pre-treatment coral microbiomes. Diversity (H') was maintained high both in ambient and heat conditions (Tab. 1). Therefore, the coral SML microbiome of *P. strigosa* showed a more deterministic and stable, rather than stochastic and unstable, response to heat stress. This result goes against the Anna Karenina principle for animal microbiomes, that predicts an unstable and stochastic microbiome composition on hosts that are exposed to acute stress or disease (Zaneveld et al. 2017). Microbiome dysbiosis would be a result of a lower ability of the host or its microbiome to regulate community composition under stress (Zaneveld et al. 2017).

The coral colonies *P. strigosa* from Bermuda used in this experiment showed an overall high resistance to heat stress as none of the coral nubbins bleached after exposed to high temperatures for one week. We hypothesize that the consistent changes in the microbiome of

corals under heat stress were beneficial to the holobiont and part of a healthy response to withstand high temperatures, not characteristic of a microbiome in dysbiosis.

Bacterial taxa

Ruegeria and *Roseobacter* significantly increased in relative abundance response heat stress (Fig. 3). High abundances of Rhodobacterales such as *Ruegeria* and *Roseobacter* in juvenile and adult corals suggest they play a key role in coral fitness (Ceh et al. 2013a; Zhou et al. 2017), nitrogen acquisition and remineralization (Ceh et al. 2013b; McNally et al. 2017), and sulfur cycling (Raina et al. 2013). *Ruegeria* are among the three most relative abundant genera that are associated with corals (Hugget and Appril 2019). *Ruegeria* strains have probiotic potential as they inhibit growth of pathogen *Vibrio coralliilyticus* (Miura et al. 2019) and help corals to withstand heat stress (Rosado et al. 2019, Kitamura et al. 2021, Santoro et al. 2021). In fact, *Vibrio* decreased in relative abundance in the coral microbiome under heat treatment (Fig. 3), indicating that *P. strigosa* was able to keep these potential pathogens in check. Lower abundances of *Synechococcus* after heat stress (Fig. 3) could indicate e that corals are using these cyanobacteria as an energy and nutrient source via heterotrophic feeding to compensate for lower productivity of the algal symbiont (Fig. 1). Corals preferentially feed on *Synechococcus*, especially to recover from heat stress and bleaching (McNally et al. 2017; Meunier et al. 2019; Hoadley et al. 2021).

Stress response

Bacterial stress response genes in the coral SML metagenomes were not affected by heat stress (Fig. 4A). Metagenomics describes the relative abundance of genes in the microbiome which identifies functional genes that are potentially being selected by the microbiome under those conditions (Dinsdale et al. 2008a; Coelho et al. 2022) but does not measure which

functional genes are being expressed at the point the sample was taken. Therefore, bacterial stress response gene expression could be increased under heat stress, although the metagenomes show that they are potentially not being selected by the coral SML microbiome over the experiment to respond to stress. Future studies coupling metagenomics to metatranscriptomics could elucidate the changes in stress response genes in the coral microbiome under heat stress. However, our metagenomes showed that bacterial genes related to nitrogen and sulfur metabolism strongly responded to heat stress and were potentially prioritized to be selected in the coral SML microbiome of *P. strigosa*.

Nitrogen metabolism

Ammonia assimilation genes decreased in relative abundance after heat stress exposure, indicating that less ammonia is being used in the biosynthesis of bacterial compounds by the microbiome (Fig. 4B). Coral-associated bacteria compete with *Symbiodiniceae* for host-generated ammonia, which is the preferred form of nitrogen of the symbiotic algae (Rädecker et al. 2015; Bourne et al. 2016). Lower ammonia assimilation by the coral SML microbiome under heat stress is potentially beneficial to the holobiont because more ammonia would be available to the algal symbiont to support photosynthesis and prevent coral bleaching.

Nitrosative stress genes allow bacteria to detoxify nitric oxide (NO) and reactive nitrogen species (RNS) involved in the denitrification process (Poole 2005). Nitrosative stress genes decreased in relative abundance under heat stress and denitrification and NO synthase genes did not change in proportion (Fig. 4B). Therefore, lower relative abundance of nitrosative stress genes could be a result of stability in the levels of NO and RNS in the coral SML microbiome.

Nitrogen incorporation, via bacterial genes related to allantoin utilization and nitrogen fixation, significantly increased in the coral SML post-heat stress (Fig. 4B). Allantoin is a urea-related compound produced by plants that can be a nitrogen source to bacteria and a form to transport fixed nitrogen to plants when nitrogen is limiting (Cobo-Díaz et al. 2015; Minami et al. 2016). Bacteria can provide about 11% of the nitrogen required by *Symbiodiniaceae* via nitrogen fixation in the coral holobiont (Bourne et al. 2016; Cardini et al. 2016). The significant increase in the proportions of allantoin utilization and nitrogen fixation genes could be a key beneficial service offered by the coral SML microbiome to supply nitrogen to the holobiont to withstand heat stress.

Sulfur metabolism

The relative abundances of bacterial genes related to glutathione utilization, sulfur oxidation and taurine oxidation, increased after heat stress in the coral SML microbiome (Fig. 4C). Glutathione is a key sulfur-based compound used by bacteria for protection against oxidative stress and by the coral holobiont as source of organic sulfur (Masip et al. 2006; Wegley et al. 2007; Bourne et al. 2016). Taurine oxidation could be coupled to sulfur oxidation to increase sulfur availability in the coral holobiont. The degradation of the amino acid taurine can produce thiosulfate, which is converted to sulfate via sulfur oxidation (Robbins et al. 2019, Lima et al. 2022). Sulfur oxidation genes increase in relative abundance under stress and bleaching (Wegley et al. 2007; Littman et al. 2011). Taurine oxidation by *Ruegeria* was coupled to productivity by planktonic dinoflagellates and plays a key role in the organic sulfur turnover in pelagic environments (Landa et al. 2019). Here, *Ruegeria* could be playing a similar role by increasing

sulfur availability via taurine oxidation to the dinoflagellates *Symbiodiniaceae* in the coral holobiont under stress.

Conclusion

The future of coral reefs depend on a healthy coral microbiome, as coral bleaching and disease outbreaks are some of the leading causes of an unprecedented loss in coral cover worldwide, particularly in response to rising seawater temperature (Miller et al. 2009; Maynard et al. 2015; Heron et al. 2016; Precht et al. 2016). We used shotgun metagenomics combined with physiology measurements, in an experimental setting to understand the dynamics of microbial taxa and genes in the coral microbiome under heat stress.

Thermal tolerance is shaped at reef-scale in Bermuda, where corals from a more fluctuating environment maintained high GP:R ratios under heat stress. The SML metagenomes of corals exposed to heat stress showed high similarity, indicating a deterministic and stable response of the coral microbiome to disturbance. The coral SML microbiome responded to heat stress with an increase in the relative abundance of Rhodobacterales and functional genes for nitrogen and sulfur acquisition into the coral holobiont. Our results showed coral-microbial gene functions and taxa that are potentially beneficial to corals under thermal stress and provide support to conservation efforts that focusing on promoting and maintaining coral microbiome health (Damjanovic et al. 2017; Epstein et al. 2019; van Oppen and Blackall 2019). Corals from marginal reefs may provide a refuge for the future of coral reefs and more experiments should investigate the microbiome of these corals and enhance the protection status of these reefs.

Tables

Table 1. Richness (S), Pielou's evenness index (J'), and Shannon's diversity index (H') of microbial genera from the coral SML metagenomes across different treatments.

Treatment	S (range)	J'	H'
In situ (T0)	582 - 586	0.74 ± 0.02	4.72 ± 0.14
Pre-treatment (T1)	578 - 585	0.55 ± 0.05	3.47 ± 0.32
Ambient (T2)	580 - 585	0.62 ± 0.04	3.93 ± 0.24
Heat (T2)	579 - 587	0.63 ± 0.004	4.04 ± 0.03

Figures

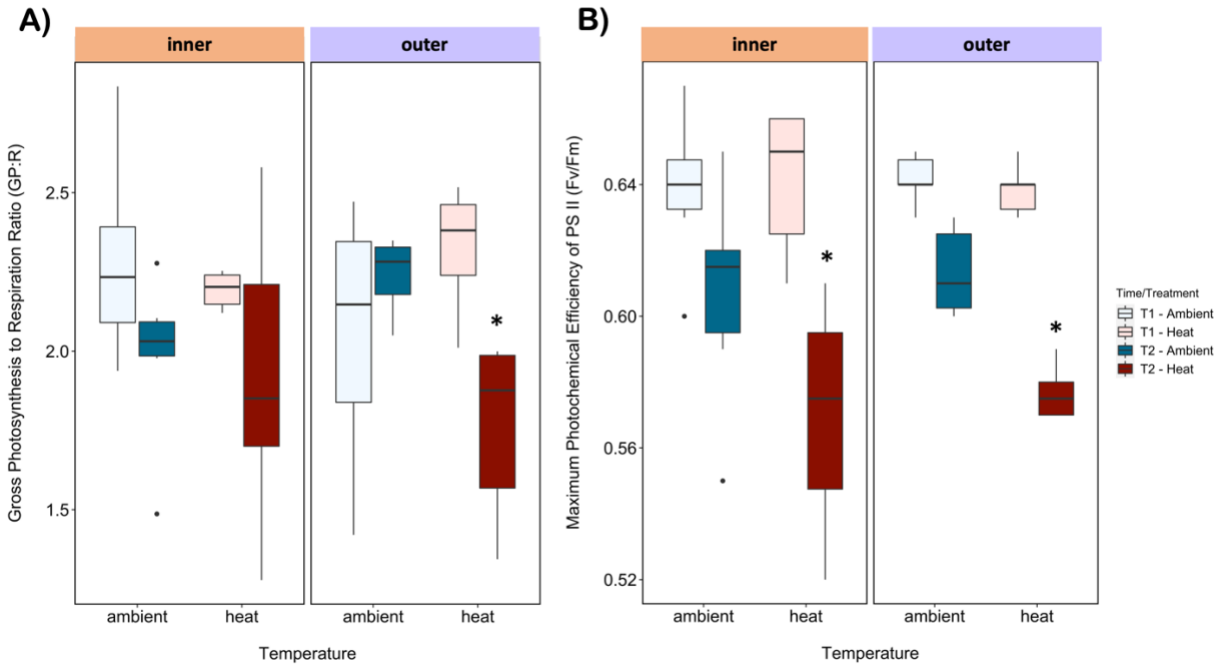

Figure 1. A) Gross productivity to dark respiration ratios (GP: R) and B) Maximum photochemical efficiency of photosystem II (Fv/Fm) of coral colonies (total n = 12, 6 per reef zone) collected from inner and outer reefs before (T1) and after (T2) exposure to heat and ambient temperatures for one week.

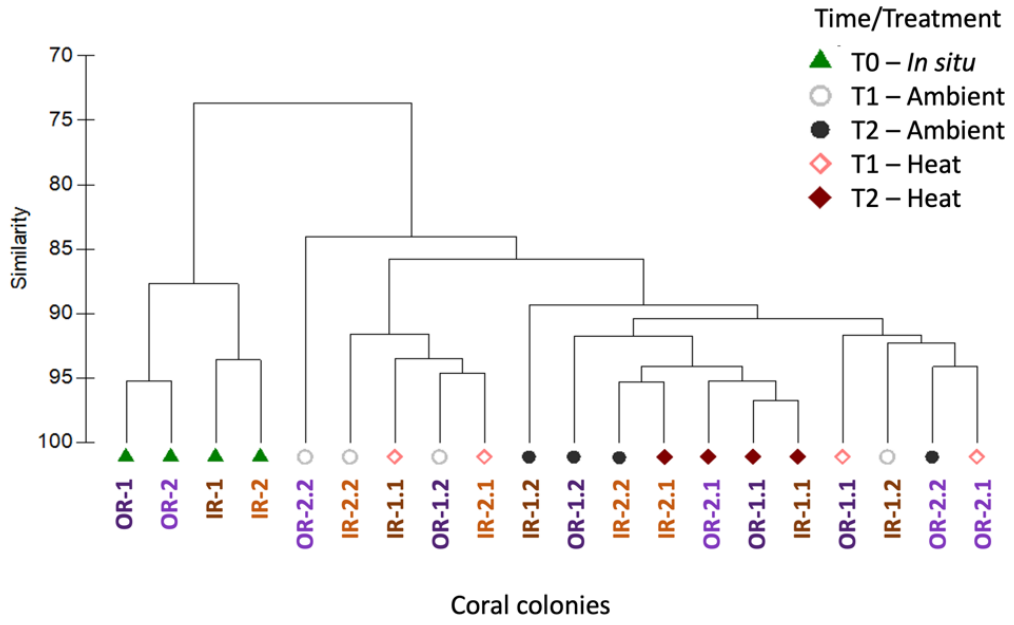

Figure 2. A hierarchical cluster analysis of the metagenomes associated with the coral SML microbiome of *P. strigosa* exposed to different temperature treatments. The microbiomes of four coral colonies (OR-1, OR-2, IR-1, IR-2) were sampled in their natural environment (T0) before the coral colonies were removed and replicated into two fragments (coral nubbins) each (OR-1.1, OR-1.2, OR-2.1, OR-2.2, IR-1.1, IR-1.2, IR-2.1, IR-2.2). The microbiomes of the coral nubbins were sampled before (T1) and after (T2) being exposed to ambient and heat treatments for one week. The analysis was based on a Bray-Curtis similarity matrix using group average of the relative abundances of the bacterial genera.

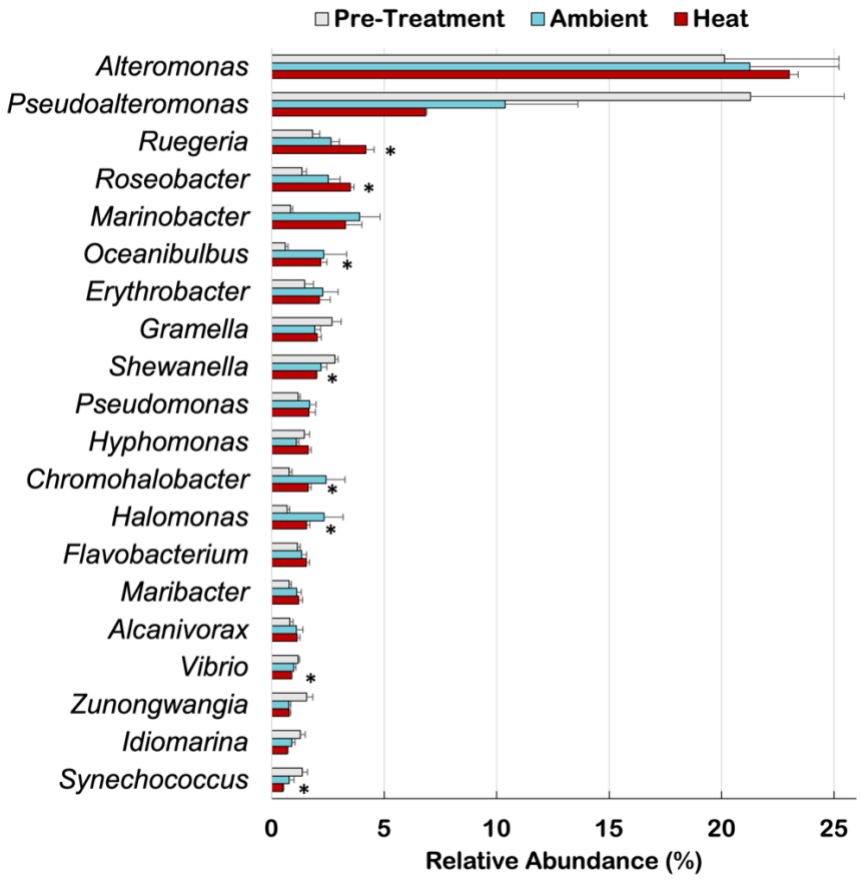

Figure 3. Top 20 most abundant bacterial genera (mean ± SE) across different temperature treatments. Asterisks indicate a significant difference between pre-treatment and heat-treatment metagenomes according to Welch's pairwise comparisons, and Benjamini-Hochberg FDR corrections.

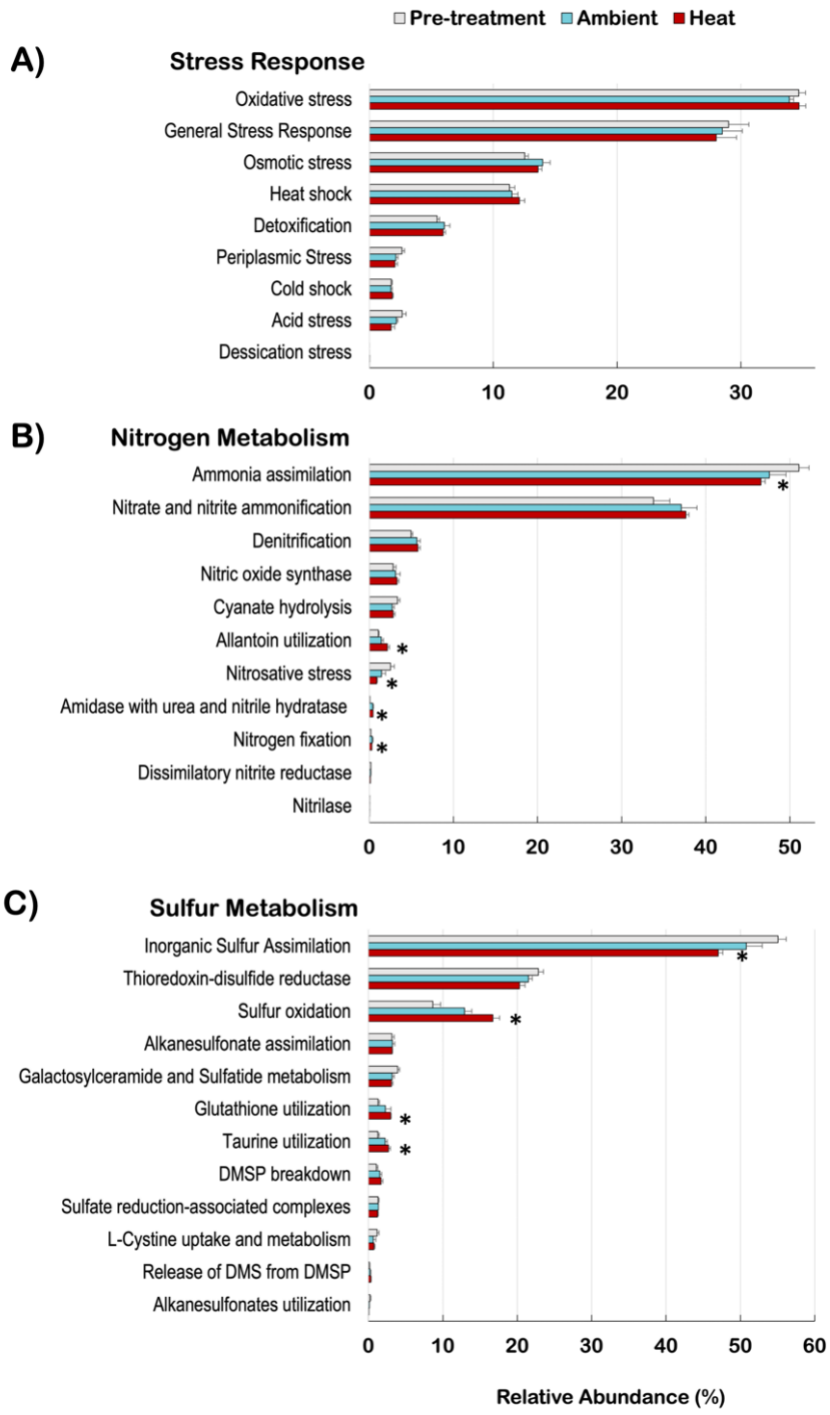

Figure 4. Relative abundances of bacterial genes related to stress response (A), nitrogen metabolism (B), and sulfur metabolism (C). Asterisks indicate a significant difference between pre-treatment and heat-treatment metagenomes according to Welch's pairwise comparisons, and Benjamini-Hochberg FDR corrections.

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