SYMBIOSIS IN A CHANGING ENVIRONMENT

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SYMBIOSIS IN A CHANGING ENVIRONMENT

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EDITORIAL
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Editorial: Symbiosis in a Changing Environment

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Editorial on the Research Topic

Symbiosis in a Changing Environment

Symbiotic interactions are formed by the long-term intimate association of microorganisms and their host species. Such interactions are ubiquitous throughout the tree of life, and research suggests that they might have facilitated major evolutionary transitions and possibly the success of life on Earth (Moran, 2006, 2007; Raina et al., 2018; Kolodny et al., 2020). Symbionts owe their success to their ability to be sometimes "good" and sometimes "evil" (Jiggins and Hurst, 2011; Brannon and Mulvey, 2019; Newton and Rice, 2020). By manipulating their hosts' life history traits, fecundity, dispersal ability, and/or resistance to stresses caused by pathogens and environmental changes, these "Jekyll-and-Hyde" symbionts are promoting their own selfish fitness. The diversity of symbiont-induced phenotypes, the complexity of symbiotic interactions, their ecology and evolution through time and across environments has therefore unsurprisingly attracted the interests of a large scientific community. Under the current global context of fast environmental changes, one can predict that new and/or less predictable abiotic and biotic stressors will affect symbiotic interactions with potential cascading effects on the eco-evolutionary population dynamics of host species and the communities in which they are embedded (Pita et al., 2018; Trevathan-Tacket et al., 2019; Greenspan et al., 2020; Kolodny and Schulenburg, 2020).

Our Research Topic on *Symbiosis in a Changing Environment* aimed to bring together review articles, providing analyses of the state-of-the-art in the field, and experimental research articles, testing unique hypotheses on the interplay between host-symbiont interactions and their various environments. We called for submissions from a broad and diverse set of researchers, from international institutions worldwide ($N = 26$ institutions, 11 countries, 5 continents), to showcase their view and expertise on a range of symbiotic study systems, from the tropics to the Antarctic, from terrestrial arthropods and plants to marine invertebrates. The published articles are only a subset of the growing literature available in symbiosis research. However, the articles clearly present that a wide diversity of stressors, including changes in temperatures, in exposure to UV-radiations, or in metabolite provisioning, can severally affect symbiosis.

Based on the literature on terrestrial invertebrates and plants, Bénard et al., and Saikkonen et al., respectively, predict that by influencing the host's life-history traits and/or their host's response to natural stresses, Jekyll and Hyde symbionts can either exacerbate or ameliorate the effects of environmental stochasticity on their hosts. This is due to the essential role of microbial symbionts for ecological, evolutionary, and genetic processes in all higher organisms. Obligate symbioses can indeed turn into evolutionary traps or dead-ends, since the optimum ranges between hosts and their symbiont(s) may inevitably mismatch, whereas facultative symbioses can provide an adaptive solution to environmental changes and opportunities to novel niches. The mini-reviews agree that

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major progress has been made in (I) the description of microbial communities associated with organisms from different environments, although one can argue that unlike bacterial communities, fungal, and viral communities have been neglected, and (II) testing the role of various individual stressors on these interactions. However, much remains to be examined in understanding the organization and changes in these communities, and in their functionality for their hosts. Similarly, estimations of the adaptability of host-symbiont associations under multiple stressors, as it would be under natural conditions, remain scarce (Rondon et al.).

Carrier and Reitzel further synthesize the ecological importance of the host in the understanding of their associated symbiotic communities. They debate that, at least in echinoderms, the selection for bacterial communities occurs at the larval stage; however, the role of these selected bacteria for the larvae and/or for the adults of the same species remains unclear. Such gaps in the current knowledge is not restricted to studies on echinoderms. Numerous studies have shown the importance of the hosts' life-history strategies influencing the microbial communities, either because of changes in the host diet, hormones, or the environmental conditions faced at each life-stage (e.g., aquatic larvae vs. terrestrial adults in many insects or amphibians). Similarly, the health of individuals and species or populations can greatly affect the composition of their associated microbial community (Blanquer et al., 2016; Li et al., 2018), as pathogens replace or challenge symbiotic interactions in their hosts. Furthermore, the role of microbial communities is severely debated in some species. For example, the gut microbiota of *Lepidoptera* larvae is thought to be transient and non-functional, rather than resident and of any benefit to the host (Hammer et al., 2017; Duplouy et al., 2020).

One way to understand which symbiotic interactions are likely to thrive or disappear in changing environments, is through the investigation of the effects of individual and/or cumulated stressors on symbioses in the controlled conditions of laboratories. For example, Heyworth et al. investigate experimentally the responses of three facultative endosymbionts in the pea aphid (*Acyrthosiphon pisum*) to heat stress, and the influence on the host and an obligate symbiont (*Buchnera*). After exposing aphids to 38.5°C, they show that two of the facultative symbionts helped the recovery of both host and the obligate symbiont after heat stress, thus suggesting that under climate changes, the presence of these facultative symbionts may be of benefit to the host species. However, response to long-term heat exposure, rather than to short heat bursts further need to be investigated.

The Rondon et al. article provides insights on the combined effects of physical drivers associated with climate change (seawater warming and ice scour) in shaping the microbiome of the common sponge *Isodictya kerguelenensis* from the Western Antarctic Peninsula. Their findings based on a multi-stressors laboratory experiment demonstrates that disturbance produced by icebergs may have direct impacts on the microbiome of this sponge species. In this regard, the results highlight the importance of this relationship as the effect of both stressors are expected to increase under future climate change scenarios, hence having the potential of producing effects on the holobiont balance and also on the ecosystem.

Barrera et al. present the role of foliar endophytic fungi on the performance of their host plant, the Antarctic host plant *Colobanthus quitensis*, under high UV-B radiation. By comparing the expression levels of genes involved in UV-B photoreception, flavonoid accumulation, and physiological stress in fungus-infected vs. uninfected plants, their study provides evidence that Antarctic endophytic fungi minimize cell damage and boost physiological performance and tolerance to UV-B radiation in *C. quitensis*. Therefore, endophytic fungi could be effective partners in the context of increased UV-B radiation in the Antarctic. Future studies should highlight whether the current trend in climate change may already be selecting for such associations in different parts of the world.

Currin-Ross et al. use *Drosophila melanogaster* flies exposed to different levels of iron provisioning (i.e., deficiency, optimum, and excess), and evaluate the conflict that arose from this stress between the host flies and their facultative bacterial endosymbiont *Wolbachia*, as both rely on iron-provision from the environment. Through metabolomic methods, they show that flies exhibit variation in their metabolism when exposed to low or high levels of iron, and that these metabolic responses are differently activated upon infection with *Wolbachia*. The bacterium maintains oxidative metabolism in its *Drosophila* host. Whether this is a typical response for the symbiont in insects or in *Drosophila* hosts specifically remains to be investigated.

This set of experimental studies highlight the diversity of symbiotic systems, and the diverse changes in phenotypes between different treatment groups. They produce unique knowledge acquired within the controlled conditions of laboratories that inform about why host-symbiont interactions form, and how they maintain or decline through time. Such discoveries support and/or complement the interpretation of patterns of diversity and changes in symbiont communities associated to species across space and time in the field.

Based on a 2-year field monitoring study of two coral species in Kāneʻohe Bay, Hawaiʻi, Matsuda et al. characterize responses of colonies after a bleaching event produced by anomalous local seawater temperatures. Their results show that the two studies species exhibited either a bleaching-susceptible phenotype (bleached) or resistant phenotype (non-bleached). Colonies of *Porites compressa* show greater resilience following bleaching than *Montipora capitata*, despite having higher bleaching prevalence and severity. This is suggesting that bleaching susceptibility is not always a good predictor of mortality after a warming event and consequent bleaching events. The authors stress the importance of including monitoring not only at the population level but also at the individual level to better understand coral susceptibility to warming and to better predict responses to future scenarios.

Similarly, using a combination of *in situ* observations along with morphological and molecular approaches, van der Windt et al. study the relationship between bio-eroding sponges belonging to *Clionaidae* and *Symbionidiaceaea* from the Indo-Pacific. The findings of this study (I) suggest a high diversity of *Symbionidiaceaea* associated with bio-eroding species, and (II) highlight the potential importance of host identify and tolerance capacity to heat stress (up to 33°C) to maintain symbiotic interactions in these sponges. Furthermore, these results suggest that symbiotic interactions in bio-eroding sponges may greatly differ in their adaptive capacity from symbioses reported in other marine organisms such as corals (Baker et al., 2008). Another study from a contrasting environment by Papale et al. provides new information on microbial communities associated with different Antarctic sponge species. Their comparative study of sponges collected from 30 to 271 depth at Terra Nova Bay in the Ross Sea region provides new information on the diversity and functional profiles of microbial symbiont communities associated with such high-latitude marine organisms. They also show the presence of rich bacterial communities dominated by Proteobacteria and their functional prediction analyses expand the current knowledge of sponge-associated microbial communities, suggesting key roles played by the microbiome, including antibiotic biosynthesis, degradation of aromatic compounds, and methane metabolism. As sponges are considered an important reservoir of exceptional microbial diversity and a major contributor to marine microbial diversity (Thomas et al., 2016), these few studies allow for the comparison of research in symbiotic systems from different marine regions (tropics vs. Antarctic) that will complement our global knowledge of the role of symbionts in risks associated with global climate change scenarios in marine organisms.

The article collection from this Research Topic illustrates the already well-known ubiquitous character and the diversity of unique symbiotic systems, with a targeted focus on their responses to environmental change and stressors. These studies test just a few hypotheses, and introduce many more that remain and deserve to be investigated. Nonetheless, these studies clearly highlight that in order to fully grasp the role and responses of symbionts to environmental changes, more multidisciplinary and collaborative studies are needed, which will establish the essential mechanisms that forge the diverse associations between both marine and terrestrial hosts, their bacterial partners, and their environments.

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Symbiotic Life of Echinoderm Larvae

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Echinoderm larvae have served as a fundamental system for understanding development and life history evolution over much of the last century. In the last few decades, our understanding of echinoderm larvae has expanded to the microbiota that they associate with. These symbionts and the communities that they form in relation to echinoderm larval host are the focus of this review. Our synthesis of the literature suggests three primary themes. First, larval echinoderms associate with "subcuticle bacteria" that appear to colonize select tissue types. Second, the bacterial communities associated with larval echinoderms exhibit compositional shifts that are correlated with several fundamental properties of larval biology (e.g., development and morphological plasticity) and ecology (e.g., feeding environment). Third, echinoderm larvae exhibit specific responses to pathogenic bacteria that may aid in maintaining the symbiont community and avoid dysbiosis. To our knowledge, no studies have focused on whether climate-related stressors impact the composition of these symbiont communities or how changes in bacteria may modulate response by larvae to these environmental stressors. Lastly, we conclude by outlining techniques that need to be established in echinoderm larvae to transition from correlations between larvae and their associated microbiota to the function of these symbionts.

Keywords: microbiome, host-microbe, endosymbiont, marine invertebrate, development, life history, plasticity, evolutionary ecology

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ECHINODERMS AND THEIR LARVAE

The phylum Echinodermata is characterized by their pentaradial symmetry and global distribution in marine ecosystems. This group first appeared in the Cambrian and is composed of ∼7,000 extant species (Appeltans et al., 2012) that are grouped into five classes: Crinoidea (feather stars and sea lilies), Holothuroidea (sea cucumbers), Asteroidea (sea stars), Ophiuroidea (brittle stars), and Echinoidea (sea urchins). Echinoderm life cycles are primarily bi-phasic, where the adults reside on the sea floor and the embryonic stages are suspended in the water column (Thorson, 1950; Mileikovsky, 1971; Young and Chia, 1987). This phylum is, perhaps, most recognized for its remarkable diversity of larval forms that have fascinated biologists for more than a century (Levin and Bridges, 1995).

Echinoderm development, in general, follows either a lecithotrophic (non-feeding) or planktotrophic (feeding) trajectory (Thorson, 1950; Mileikovsky, 1971; Strathmann, 1985). Lecithotrophs develop from relatively large and energy-rich eggs (∼300 μm to 1 mm in diameter, or where $s \geq 1$; Vance, 1973; McEdward and Miner, 2006) with sufficient maternal investment to complete development and undergo metamorphosis. The developmental period for lecithotrophs typically lasts a few days and, due to a shorter pelagic larval duration, can result in marginal dispersal between populations (Thorson, 1950; Mileikovsky, 1971; Strathmann, 1985). Planktotrophs, on the other hand, develop from more energy-poor eggs (∼100 to ∼300 μm in diameter, or where $s < 1$; Vance, 1973; McEdward and Miner, 2006) with sufficient maternal input

to complete embryogenesis and develop into larvae with initial feeding structures. The remaining energetic supply required for larval development and metamorphosis is acquired from exogenous resources (e.g., phytoplankton, detritus, and other particles) that are concentrated by a cilia-lined feeding apparatus (Strathmann, 1987; Feehan et al., 2018). These particulates are often dilute, leading to a pelagic larval duration lasting weeks to months and, in some cases, years (Thorson, 1950; Mileikovsky, 1971; Strathmann, 1985; Olson and Olson, 1989).

Nearly four decades ago, Rivkin et al. (1986) recognized that the planktotrophic larvae of the asteroid *Porania antarctica* selectively interacts with the environmental microbiota through bacterivory. It has since been observed that additional planktotrophic echinoderms exhibit bacterivory and that this feeding mode is hypothesized to be important, but not essential, to the metabolic requirements of the larva (Pearse et al., 1991; Douillet, 1993; Ayukai, 1994; Moal et al., 1996; Gosselin and Qian, 1997). Based on maximum clearance rates and particle abundance, it is estimated that echinoderm larvae interact with ∼20 million bacteria each day by feeding alone (Carrier et al., 2018a). It is, however, unknown which bacterial (or other microbial) groups that echinoderm larvae may target and whether these microbes are selected strictly for bacterivory or as a symbiont that may be acquired by horizontal transmission.

Just prior to recognizing that echinoderm larvae were bacterivorous, Cameron and Holland (1983) observed that bacteria were living inside the tissues of healthy larvae. In this review we synthesize the properties of these bacteria and how they relate to the biology and ecology of the echinoderm larval host. In the first section, we provide an overview of our understanding of echinoderm larvae and their bacterial symbionts through the lens of microscopy and next-generation sequencing. In the second section, we summarize how larval-associated microbiota may be relevant in coping with anthropogenic stressors and outline the techniques needed to transition toward understanding the function of these symbionts.

BACTERIAL SYMBIONTS OF ECHINODERM LARVAE

Over the last few decades our understanding of echinoderm larvae and their microbes has gone through two primary phases. The first phase uses microscopy and focuses on "subcuticle bacteria" (or, due to their location within larval tissues, could be also be characterized as endosymbionts but this has not been explicitly tested). The second phase has developed in recent years and uses next-generation sequencing and other molecular tools to characterize larval-associated bacterial communities. This phase may be further divided into two focal points: the dynamics of these bacterial communities under different ecological conditions and the immune responses of the larval host when faced with pathogenic bacteria.

Subcuticle Bacteria

While determining how to preserve the thin cuticle overlying the epidermis for transmission and scanning electron microscopy, Holland and Nealson (1978) observed that adults for each of the five echinoderm classes had a high abundance of what they called 'subcuticle bacteria.' Holland and Nealson (1978) did not test whether embryonic or larval stages also contained subcuticle bacteria. They did, however, speculate on the nature of transmission for echinoderms, stating that because the eggs are "unattached to any follicle cells, and no bacteria have ever been observed on or in echinoderm eggs" that "it is probable that each new generation of such echinoderms acquires its subcuticular bacteria from the surrounding sea water." Moreover, Holland and Nealson (1978) suggested that, if acquired during the embryonic or larval stages, the echinoderm must select for these symbiotic bacteria from a diverse community of environmental microbiota.

Shortly thereafter and on multiple occasions since these original observations, studies have identified subcuticle bacteria in the developmental stages of echinoderms. These symbionts, thus far, have been found in three asteroids (Cameron and Holland, 1983; Bosch, 1992; Cerra et al., 1997), one ophiuroid (Walker and Lesser, 1989), and one echinoid (Heyland et al., 2018; Schuh et al., 2019) (**Figure 1**; **Table 1**). In these five species, subcuticle bacteria have been observed within the mouth and gut lumen, out-pockets of the extracellular matrix that surrounds the larval body, embedded in the inner layer of the secondary cuticle of the rudiment epidermis, and are engulfed and, in some cases, digested by epidermal cells (Cameron and Holland, 1983; Walker and Lesser, 1989; Bosch, 1992; Cerra et al., 1997; Heyland et al., 2018; Schuh et al., 2019).

The function of these subcuticle bacteria remains essentially unknown. Two cases, however, may suggest that these symbionts interact with and functionally benefit the larval host. First, the brittle star *Amphipholis squamata* has a "vestigial" pluteus (i.e., greatly reduced larval arms and lacking a ciliated mouth) that is brooded within their central plate. Using transmission and scanning electron microscopy, Walker and Lesser (1989) found that a rod-shaped *Octadecabacter* was abundant and actively dividing within the tissues of this "vestigial" pluteus (Morrow et al., 2018). This *Octadecabacter*, which strictly associates with these larvae and is not found in the environment, can uptake dissolved free amino acids that are then incorporated into bacterial proteins and increase the total amino acid uptake for *A. squamata* (Walker and Lesser, 1989; Lesser and Blakemore, 1990; Lesser and Walker, 1992). Second, clonal larvae of the sea star *Luidia* collected from the Gulf Stream had one to three rod-shaped morphotypes of subcuticle bacteria (Bosch, 1992). For *Luidia* as well as *Acanthaster*, some symbionts were located in the gut and auto-fluoresced (Bosch, 1992; Galac et al., 2016; Carrier et al., 2018b), suggesting the potential ability to be phototrophic. Collectively, these examples suggest, but do not show explicitly, that bacterial symbionts may have metabolic functions that could potentially benefit the larval host.

Bacterial Communities

The subcuticle bacteria discussed above are a portion of the collection of bacteria associated with echinoderm larvae. Using next-generation sequencing, larval-associated bacterial communities have been reported for six species of echinoderm

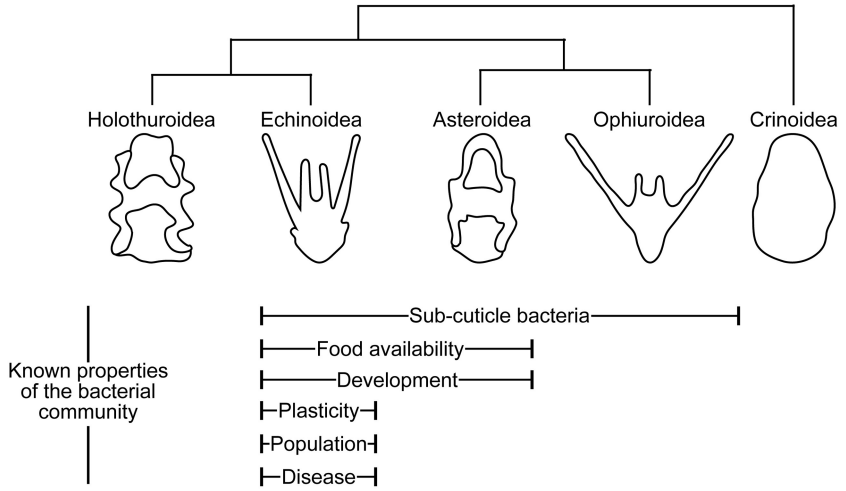

FIGURE 1 | Echinoderm larvae and their symbionts. Properties of echinoderm larval biology and ecology with known correlated shifts in the associated bacterial communities.

larvae: two asteroids (Galac et al., 2016; Carrier et al., 2018b) and four echinoids (Carrier and Reitzel, 2018, 2019a,b; Carrier et al., 2019) (**Figure 1**; **Table 1**). In general, these bacterial communities are composed of a couple hundred bacterial species (i.e., Operational Taxonomic Units or OTUs, as defined by ≥99% similarity of the phylogenetically-conserved 16S rRNA gene) (Galac et al., 2016; Carrier and Reitzel, 2018, 2019b; Carrier et al., 2018b, 2019). The predominant bacterial families encompassing these diverse communities are the α- and γ-Proteobacteria (Proteobacteria) and the Flavobacteriaceae (Bacteroidetes) (Galac et al., 2016; Carrier et al., 2018b, 2019; Carrier and Reitzel, 2019a,b).

Like many other studies of animal and plant microbes, the bacterial communities associated with echinoderm larvae are species-specific and taxonomically distinct from the environmental microbiota (Galac et al., 2016; Carrier and Reitzel, 2018; Carrier et al., 2018b), suggesting that these communities are, at least in part, selected by the host. This host-specificity, however, appears to be lost when larvae are cultured under traditional laboratory settings for rearing the developmental stages of marine invertebrates (i.e., fine-filtered or artificial saltwater) (Schuh et al., 2019). Specifically, *Strongylocentrotus purpuratus* larvae cultured under traditional laboratory settings associate with bacterial communities that are less diverse in total taxa and the phylogenetic breadth of those taxa, and retain ∼40% of the OTUs harbored by "wild-type" counterparts (Schuh et al., 2019). This implies that studying larval-associated bacterial communities is most accurately performed at near-natural conditions, such as by filtering ambient seawater to 5-μm to remove most debris and planktonic predators while retaining the environmental microbiota (Carrier and Reitzel, 2018; Hodin et al., 2019).

Larval-associated bacterial communities are variable in community membership and composition but exhibit non-random shifts that correlate with multiple components of larval biology and ecology. These communities, for example, are established on unfertilized eggs but not the sperm of sea urchins (Carrier and Reitzel, 2019b; Schuh et al., 2019). When cultured using coarsely (5-μm) filtered seawater, echinoderm larvae exhibit a development-based succession in symbiont composition and, using fluorescent *in situ* hybridization, these bacteria localize in the mouth and gut lumen (Carrier and Reitzel, 2019b; Schuh et al., 2019). Following fertilization, the diversity of these communities increases by ∼20% during the early embryonic stages and decreased by nearly ∼85% following hatching and through metamorphosis (Carrier and Reitzel, 2019b). From egg to hatching, the early embryonic stages appear to converge taxonomically with the environmental microbiota but then exhibit a host-mediated selection by diverging from this community following the onset of feeding (Carrier and Reitzel, 2019b). In cases where embryonic development includes asexual reproduction (e.g., cloning), the larval clones deviate little from the parent larva by maintaining a high proportion of particular bacteria, including phototropic species (Galac et al., 2016; Carrier et al., 2018b).

The six species of echinoderm larvae with profiled bacterial communities are planktotrophs, and by definition, are required to feed. Five of these species (echinoids: *Strongylocentrotus purpuratus*, *S. droebachiensis*, *Mesocentrotus franciscanus*, and *Lytechinus variegatus*; asteroid: *Acanthaster* sp.) have been differentially fed to test whether community composition varies with food quantity. In each case, bacterial communities are diet-specific with well-fed larvae distinguished from diet-restricted treatments (Carrier and Reitzel, 2018, 2019a; Carrier et al., 2018b). When diet-restriction is prolonged, larval sea urchins compensate by elongating their feeding arms to increase water filtration capacity (Hart and Strathmann, 1994; Miner, 2004; McAlister and Miner, 2018). This change in morphology is correlated with a shift in the composition of the bacterial symbionts, such that larval urchins associate with phenotype-specific microbiota (Carrier and Reitzel, 2018, 2019a; Carrier et al., 2018b).

TABLE 1 | List of echinoderm species noted to associate with bacterial symbionts.

	Species	Type	References
Asteroids	*Acanthaster* sp.	Bacterial community by 16S rRNA profiling	Carrier et al., 2018b
	Luidia sp.	Subcuticle bacteria by electron microscopy	Bosch, 1992
	Patiria miniata	Subcuticle bacteria by electron microscopy	Cameron and Holland, 1983
	Patiriella calcar	Subcuticle bacteria by electron microscopy	Cerra et al., 1997
Crinoidea	N/A	N/A	N/A
Echinoids	*Lytechinus variegatus*	Bacterial community by 16S rRNA profiling	Carrier and Reitzel, 2019a
	Mesocentrotus franciscanus	Bacterial community by 16S rRNA profiling	Carrier and Reitzel, 2018, 2019b
	Strongylocentrotus purpuratus	Bacterial community by 16S rRNA profiling	Carrier and Reitzel, 2018, 2019b
		Subcuticle bacteria by *in situ* hybridization	Schuh et al., 2019
	Strongylocentrotus droebachiensis	Bacterial community by 16S rRNA profiling	Carrier and Reitzel, 2018, 2019b; Carrier et al., 2019
Holothuroidea	N/A	N/A	N/A
Ophiuroidea	*Amphipholis squamata*	Subcuticle bacteria by electron microscopy	Walker and Lesser, 1989

Establishment of a phenotype-specific bacterial community for larvae of the sea urchin *L. variegatus* follows a four-stage succession (Carrier and Reitzel, 2019a). First, larvae across degrees of food availability associated with bacterial communities similar in both composition and structure. Second, different food environments (i.e., algal concentrations) induced diet-specific bacterial communities in both membership and composition. Third, the bacterial communities of diet-restricted larvae associated with similar bacterial communities that are also distinct from that of well-fed larvae, with the latter coinciding with a reduction in community diversity. Lastly, composition and structure are maintained from the prior successional stage and now correlate with the short- and long-arm phenotypes (Carrier and Reitzel, 2019a). This suggests that changes in the larval-associated bacterial community shifts prior to the expression of the environmentally elicited morphological phenotypes and that microbial communities may respond to environmental variation more quickly than morphological changes.

Recent research also suggests that microbial communities differ not only between species but also between populations (e.g., ascidians: Dishaw et al., 2014; seaweed: Marzinelli et al., 2015; fish: Llewellyn et al., 2016; sponges: Marino et al., 2017). Differential feeding of larvae of the echinoid *S. droebachiensis* from three populations in different ocean basins showed parallel responses that resulted in diet-specific bacterial communities (Carrier et al., 2019). Despite each population associating with a diet-specific bacterial community, variation in membership and community composition correlated more strongly with geographic location (Carrier et al., 2019). Moreover, when comparing the taxonomic membership between populations, 20–30% of bacterial taxa were specific to a single location while ∼10% were shared between all three locations (Carrier et al., 2019). Collectively, these data suggest that larvae for a given species associates with a population-specific bacterial community. It is, however, worth noting that this comparison was not performed using common garden culturing, and that it would be expected that these communities are more taxonomically similar but still population-specific, when cultured using identical seawater.

Pathogenic Bacteria

Biological responses to foreign "particles" by echinoderm larvae were first recognized in the late nineteenth century (Metchnikoff, 1891; Tauber, 2003). More recently, echinoderm larvae have been used as a comparative system to define the cellular and molecular mechanisms of immunity when combating pathogenic bacteria. Due to the availability of a genome (Sodergren et al., 2006), the majority of this work has used the echinoid *Strongylocentrotus purpuratus*. The immune response by *S. purpuratus* to pathogenic bacteria was recently reviewed by Buckley and Rast (2017) and Heyland et al. (2018). We refer the reader to these in-depth reviews for the molecular underpinnings of larval immunity, as this section will focus on the ecological components.

From the amoebic disease (*Paramoeba invadens*) of *S. droebachiensis* in Nova Scotia (Scheibling and Stephenson, 1984; Feehan et al., 2013) to the major epizootic that decimated *Diadema antillarum* throughout the Caribbean (Lessios et al., 1984; Lessios, 2016) or, more recently, the densovirus-associated wasting disease of asteroids (Hewson et al., 2014; Harvell et al., 2019), the ecological impacts of disease on echinoderms have been well-documented over the last few decades (Feehan and Scheibling, 2014). Less of this work, however, has focused on embryonic and larval echinoderms. Echinoderm larvae potentially interact with ∼20 million bacteria each day by feeding alone (Carrier et al., 2018a), and a portion of these bacteria may be consumed through bacterivory, be symbionts acquired by horizontal transmission, or pathogenic bacteria that require an immune response.

When faced with variation in food quantity, larval echinoids exhibit a trade-off in the expression of immune and metabolic genes (Carrier et al., 2015, 2018a), such that well-fed larvae upregulate metabolism and suppress immunity. High food availability is also suitable environmental conditions for bacteria to express pathogenic characteristics. If faced with a pathogen in these feeding conditions, food-induced suppression of the immune system may result in a suboptimal physiological response, and larvae may be less able to regulate the associated microbiota. Echinoid larvae may then be at risk of

pathogen-induced disease or exhibiting dysbiosis, both of which are hypothesized to be precursors to larval mortality (Carrier et al., 2018a).

When faced with pathogenic bacteria, echinoid larvae exhibit immune responses, for example, by expressing genes in the interleukin 17 (IL17) complex (Ho et al., 2016; Buckley and Rast, 2017; Buckley et al., 2017). IL17s are known to both serve as a primary barrier to foreign bodies and to regulate the composition of larval-associated microbiota (Buckley and Rast, 2017). For *S. purpuratus*, exposure to the pathogen *V. diazotrophicus* coincides with successive expression of gut epithelial-specific IL17 subtypes to prevent progression of *V. diazotrophicus* and maintain the gut microbiota (Buckley and Rast, 2017; Buckley et al., 2017). When *S. purpuratus* larvae are made nearly germ-free, they become more susceptible to *Vibrio*-induced infections and mortality than counterparts with their native bacteria (Schuh et al., 2019). Pathogens that elicit a response appear to be lineage-specific, as not all *Vibrio* species or strains induce the expression of the IL17s (Buckley and Rast, 2017; Buckley et al., 2017). Such responses by the larval host may contribute to maintaining homeostatic symbioses (e.g., Mortzfeld and Bosch, 2017), but the functional underpinnings for larval echinoderms have yet to be determined (but see Ho et al., 2016; Buckley and Rast, 2017; Buckley et al., 2017).

LARVAL ECHINODERMS IN A CHANGING ENVIRONMENT

Our understanding of echinoderm larvae and their relationship with microbial symbionts has been studied at ambient conditions. Marine invertebrates and their life history stages are, however, encountering a suite of anthropogenic stressors (Byrne et al., 2018) that may disrupt homeostatic symbioses (e.g., Rosenberg et al., 2007). To our knowledge, no studies have focused on how climate-related stressors (e.g., temperature and pH) affect the associated microbiota of echinoderm larvae. Similar to the cnidarian planula (Mortzfeld et al., 2015) and sponge amphiblastula (Webster et al., 2011), we hypothesize that an acclimation response to abiotic or biotic stressors would include shifts in symbiont composition. This, in particular, provides larvae with an opportunity to acquire bacterial symbionts with genes that are novel to the larval hologenome and that may aid in ameliorating physiological stress. Whether at ambient conditions or facing climate-related stressors, the function of larval-associated symbionts as well as if the host benefits from these partnerships remains uncertain.

Determining if and how microbial symbionts contribute to the larval holobiont requires a transition from 16S rRNA profiling to functional studies (Williams and Carrier, 2018). The functional potential and expression profiles of microbial symbionts may be assessed using metagenomics (e.g., Slaby et al., 2017) and metatranscriptomics (e.g., Moitinho-Silva et al., 2014). The impact that particular taxa or the symbiont community have on the larval host may then be assessed by generating microbe-free larvae through an antibiotic treatment or gnotobiotic chambers (Rawls et al., 2004; Bates et al., 2006; Smith et al., 2007; Gloeckner et al., 2013; Leigh et al., 2016; Schuh et al., 2019) and adding back single or a mix of culturable taxa (e.g., Domin et al., 2018). Such techniques can be coupled with established visualization approaches (electron microscopy: Cerra et al., 1997; fluorescent *in situ* hybridization: Schuh et al., 2019) to define the spatial distribution of these symbiont and which tissues they colonize. Lastly, viruses, archaea, and fungi are also functionally important members of host-associated microbial communities (e.g., Webster and Thomas, 2016). Similar molecular and sequencing approaches can and should be used to characterize if and how they interact with the larval host as well as other microbial groups within the microbiome.

CONCLUSION

Our understanding of symbioses between larval echinoderms and microbes has primarily developed in the last few years; yet, in this time we suggest that three primary themes have materialized. First, larval echinoderms associate with subcuticle bacteria that appear to colonize select tissues. Second, the bacterial communities associated with larval echinoderms exhibit compositional shifts that are correlated with several fundamental properties of larval biology and ecology. Third, the echinoderm larval host exhibits strict responses to pathogenic microbiota that may aid in maintaining the symbiont community to avoid dysbiosis.

As echinoderms larvae continue to serve as a fundamental system for understanding development and life history evolution (Love and Strathmann, 2018), this diversity in form and function may act as a strong foundation to understand how and to what extent bacteria and other microbes influence the many dimensions of larval biology (Hammer et al., 2019). In particular, these diverse developmental approaches enable the fields of animal-microbe symbiosis and larval biology to test for unique functional links between host and symbiont during, for example, morphological plasticity and life history transitions. And in a time where it is becoming clear that the bacterial flora cannot be ignored, we must remember that echinoderm larvae have always and will continue to live and evolve in a sea of microbes (Walne, 1956; Zilber-Rosenberg and Rosenberg, 2008; McFall-Ngai et al., 2013; Bordenstein and Theis, 2015).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. Specifically, TC drafted and edited the manuscript and AR edited the manuscript.

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Toward Comprehensive Plant Microbiome Research

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Microbes have driven eco-evolutionary adaptations organizing biodiversity from the origin of life. They are ubiquitous and abundant, facilitating the biochemical processes that make Earth habitable and shape ecosystem structures, functions, and services. Recent studies reveal that commensalistic and beneficial microbes associated with wild and domesticated plants may aid in establishing sustainable agriculture for a changing climate. However, developing microbe-based biotechnologies and ecosystem services requires a thorough understanding of the diversity and complexity of microbial interactions with each other and with higher organisms. We discuss the hot and blind spots in contemporary research on plant microbiomes, and how the latest molecular biological techniques and empirical eco-evolutionary approaches could elevate our perception of microbe–plant interactions through multidisciplinary studies.

Keywords: bacteria, fungi, microbiome, endophytes, pathogens, saprobes, ecology, evolution

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UBIQUITOUS MICROBES IN THE SPOTLIGHT

Recent advances in molecular microbiology have revolutionized the ability of the scientific community to understand and study the diversity and functions of microbes associated with animals and plants, leading to a plethora of related literature. A Web of Science search (January 23, 2020) using "microbiome" or "microbiome and plant" as topic keywords produced 28,733 references published, with 49 and 1,211 of the papers ranked as "hot" and "highly cited." More than 99% of the papers were published after 2010. These studies are largely based on mass sequencing of taxonomic marker genes for bacteria and fungi (community sequencing), and to lesser extent, metagenomes of microbial communities. Currently, studies relying on molecular methodologies dominate plant microbiome literature.

Today we know that microbes are ubiquitous and essential associates of virtually all higher organisms. For example, the microbial cells colonizing the human body appear to be more abundant as our somatic cells and contain overwhelmingly more genes than our human genome (Gilbert et al., 2018). Similarly, microbes have been found in virtually all plants (Partida-Martinez and Heil, 2011), and are known for their immense significance e.g., as plant beneficial mycorrhizae and endophytes. Pathogens such as coffee rust, leaf blight of rubber, Panama disease of banana, chestnut blight, Dutch elm disease, and potato blight have been culprits for great historical convulsions with social impacts (Schumann, 1991). Historically the potato blight, the causal agent of the Irish potato famine causing the death by starvation of one million people and overseas emigration of a further two million people in the mid-1800s (Schumann, 1991), has been particularly influential in the emergence of germ theory and in shaping the conceptual model of disease triangle (Schumann, 1991).

We propose that as microbes associated with their shared host plants comprise multipartite entities, the theories of hologenome evolution (Saikkonen et al., 2006; Zilber-Rosenberg and Rosenberg, 2008; Moran and Sloan, 2015; Vandenkoornhuyse et al., 2015; Theis et al., 2016; Rosenberg and Zilber-Rosenberg, 2018) and the geographic mosaic of coevolution (Thompson, 2005) provide usable frameworks to understand these microbial-plant interactions and their importance for ecological, evolutionary and genetic processes. Similarly to human and animal microbiomes (Gilbert et al., 2018), the majority of plant microbiomes are acquired from the environment or transmitted horizontally between individuals. However, in modular organisms (Chapman, 1981) characterized by somatic embryogenesis, such as most seed plants, many long-lasting microbe-plant interactions involve either vertical or pseudo-vertical transmission from host plant to its sexual and/or vegetative offspring (Wilkinson, 1997; Saikkonen et al., 1998; Cankar et al., 2005). Thus, plant microbiomes provide a particularly fertile ground for ecologists and evolutionary biologists interested in all levels of selection in co-evolutionary processes. The use of metagenomic tools allow us to determine the diversity of microbiomes and the "-omics" approaches (genomics, proteomics and metabolomics) can be used to examine how genomic information is translated into structures and functions in the interactions among plant and its microbial partners (Delmotte et al., 2009; Lundberg et al., 2012; Sessitsch et al., 2012; Hardoim et al., 2015; Agler et al., 2016; Saikkonen et al., 2016; van der Heijden and Hartmann, 2016).

Furthermore, if we take into account that phenotypic selection may treat the plant and its associated microbes individually or in concert as a phenotypic and metagenomic unit, the implications for conceptions of genetics, epigenetics (Jaenisch and Bird, 2003; Zilber-Rosenberg and Rosenberg, 2008; Vannier et al., 2015) and natural selection are profound. The next step toward a better understanding of the diverse roles of microbiomes is to combine metagenomic surveys regarding the composition and function of microbiomes with empirical and theoretical biological approaches including ecology, physiology, genetics and epigenetics, phenotypic evolution, and coevolution of interacting species (Saikkonen et al., 2004, 2016; Prosser et al., 2007; Vandenkoornhuyse et al., 2015; Vannier et al., 2015; Rosenberg and Zilber-Rosenberg, 2016; van Overbeek and Saikkonen, 2016). This requires the acknowledgment and breaking of certain barriers and challenges associated with scientific traditions and management of increasing information overload.

WHY INTERDISCIPLINARY COLLABORATION IS NEEDED

We contend that human perspective often hampers understanding of the complex nature of microbe–plant interactions and their importance to ecosystem functions and services. Conventional wisdom biased by human perceptions might misdirect scientific progress. We outline three constraining human factors, "pitfall of scientific discipline," "the dilemma of classification," and "overly optimistic expectations," which will require multidisciplinary research approaches to be conquered.

Pitfall of scientific discipline, i.e., dogmatic concept formation in scientific discipline, may constrain one's options. Conventional life science disciplines, such as plant physiology, evolutionary ecology or phytopathology, either largely ignore microbes (Holland and Polacco, 1994; Compant et al., 2016; Kauppinen et al., 2016; Saikkonen et al., 2016; van Overbeek and Saikkonen, 2016) or examine microbe–plant interactions from limited, pre-defined perspectives, and largely build on the knowledge and traditions of their own discipline. Such strongly canalized approaches limit our ability to acknowledge the complexity of biological interactions and different levels of regulation and selection (Koskella et al., 2017).

For example, phytopathology examines pathogen–host plant interactions focusing on the ecology and evolution of pathogen–host interactions, and how these interactions might be modulated by microbial community interactions in the pathobiome (Vayssier-Taussat et al., 2014). Although community-level approaches that include other microbial plant associates are increasingly being adopted in plant pathology (Santhanam et al., 2015; Syed Ab Rahman et al., 2018), a significant proportion of phytopathology research is dedicated to epidemiology, disease diagnosis, and management of microbial species of economic or environmental importance alone. This, combined with the long tradition of one microbe–one plant approach in the field, might overemphasize the pathogenic phase of the microbe's lifecycle and divert attention from other relevant aspects of the same microbe–plant interaction, and overlook the impact of whole microbiomes in disease development or repression.

Meanwhile, an increasing number of evolutionary ecology studies reveal that the same microbial species commonly occupy several ecological niches. Thus, the nature of microbe–plant interactions is labile and context-dependent in ecological and evolutionary time rather than always beneficial or disadvantageous to the host (Saikkonen et al., 1998, 2004; Wäli et al., 2013; Rybakova et al., 2016; Selosse et al., 2018). The same microbial species might be labeled a pathogen, parasite, or endophyte, and referred to by different names. For example, a mutation of a single locus may convert a fungal plant pathogen to a non-pathogenic endophytic symbiont (Freeman and Rodriguez, 1993), and the nature of the interaction with the host plant is conditioned on environmental factors (e.g., herbivory or available resources), life-history characters, and genetic combinations of the host and the microbe (Rybakova et al., 2016). Likewise, the bacterial species *Clavibacter michiganensis* is known as a plant pathogenic species, and *C. michiganensis* subspecies are regarded as quarantine organisms. However, an endophytic bacterial strain, *Clavibacter* str Enf12, isolated from subnival alpine plants and classified as *C. michiganensis*, is shown to increase the chilling tolerance of its host plants, and has not been reported to be pathogenic (Ding et al., 2011; Eichenlaub and Gartemann, 2011). Further, the very same microbial species are sometimes named differently in different contexts. As to the naming of microbes, a common endophyte of birch trees, *Fusicladium betulae*, was named *Venturia ditricha* by a forest pathologist. *Venturia* is a teleomorph (the sexual reproductive

stage) and is now its formal name, but the fact is the species is a biotrophic pathogen and is more commonly an asymptomatic anamorphic (an asexual reproductive stage) endophyte than obviously pathogenic (Ahlholm et al., 2002a; Helander et al., 2007). Similarly, some plant mutualistic mycorrhizal fungi are reported as asymptomatic endophytes and organic matter degrading saprotrophs (Weiß et al., 2016; Grelet et al., 2017; Smith et al., 2017; Martino et al., 2018; Schneider-Maunoury et al., 2018). The question is whether we should consider a microbe saprophytic or pathogenic if it inhabits its host plant asymptomatically throughout most of its life cycle—in the case of perennial hosts, perhaps over years—and manifests itself as pathogenic or saprophytic during only a short period of its life.

Similarly to scientific disciplines, the dilemma of classification, i.e., grouping microbes either based on their taxa or functional role, directs and limits researchers' interests to different parts of microbial communities. Consequently, the related scientific theories have largely developed separately (van Overbeek and Saikkonen, 2016). For example, bacteria, fungi, and viruses are usually studied separately, although they coexist and likely often interact with each other (Saikkonen et al., 2004; van Overbeek and Saikkonen, 2016). Furthermore, in ecological and evolutionary literature microbe–plant interactions are generally treated separately from the species–species and multitrophic interactions of higher organisms (e.g., plant–herbivore interactions), although all plant and animal interactions unavoidably involve microbes. We believe that understanding any biological interaction requires taking microbiomes of higher organisms into account, because together the organism and the diverse assemblages of its symbiotic microbes form a holobiont, an extended phenotype and the target of phenotypic selection. This concept of holobiont should include all plant associated microbes whenever experimentally feasible.

Finally, overly optimistic expectations commonly emerge when a scientist assembles information, becomes excited by attractive discoveries, concepts and/or economically profitable application opportunities, and potentially misses fundamental biological background knowledge. Metagenomic and proteomic approaches illuminate the ubiquity, diversity, and importance of microbes, and open our eyes to associated potential opportunities. Evidence shows that the majority of microorganisms detected by metagenomic tools colonize their host plants asymptomatically (Compant et al., 2016; Gopal and Gupta, 2016; Nissinen et al., 2019). These microbial associates of plants are commonly defined as endophytes (Wilson, 1995). Taxonomically, endophytes are diverse; they include archaeal, bacterial, fungal, and protistic microorganisms. Most are considered plant commensals but few, such as the fungal *Epichloë* species commonly inhabiting cool-season grasses, are regarded as mutualistic, especially in high-nutrient agroecosystems (Kauppinen et al., 2016; Saikkonen et al., 2016). Plant mutualistic bacterial endophytes are less documented, but accumulating genomic and metabolomic information on bacterial genomes and modulations in plant metabolism strongly suggests they have a role in plant growth, development, and stress tolerance (Sessitsch et al., 2012; Hardoim et al., 2015; van Overbeek and Saikkonen, 2016; Esmaeel et al., 2018). Inspired by evidence that endophytic microorganisms are involved in denitrification, nitrogen fixation, and greenhouse gas emissions, and that they can affect plant tolerance to abiotic stress, and virtually all types of plant–plant, plant–herbivore or plant–pathogen interactions, an increasing number of scientists have become interested in the potential of endophytic microorganisms in the bioeconomy. Here we propose that this development might have directed the focus on overly ambitious goals and expectations for the following reasons.

The more we look at the basic biology of microbes, the more obvious it becomes that although we could govern endophytic microbes, we cannot fully control them, especially in the agricultural fields and nature. Microbial interactions are known to be labile and context dependent ranging from antagonistic to mutualistic in both ecological and evolutionary time (Saikkonen et al., 1998, 2004; Lopes et al., 2009; Berg et al., 2016; Brader et al., 2017). Microbes have potential to evolve rapidly as response to changing selection forces, and thereby affect nature of their interaction with the host as well as associated community and ecosystem processes (Freeman and Rodriguez, 1993; Saikkonen et al., 2004; terHorst et al., 2014). Thus, mutualistic, commensalistic and antagonistic microbial taxa are inseparable and present in virtually all microbiomes studied. We should aim to understand the microbiome dynamics, and consider microbes as part of the diverse multi-kingdom community (van Elsas et al., 2012; Koch et al., 2018), particularly when utilizing plant mutualistic endophytes in sustainable agriculture and food production.

FUTURE PERSPECTIVES

Recent plant microbiome research is largely focused on describing structure and functions of microbial communities in different plant associated niches, and linking specific microbial taxa to plant performance. Unarguably, these studies have rapidly given us solid understanding of broad taxonomic trends in plant associated microbiota. Integration of metagenomics to proteomics, metabolomics and other omics-approaches is now enabling associating microbiome structural shifts to plant holobiont functioning. However, the mass of data provided by these techniques can easily overwhelm researchers that currently often lack the tools to organize and process the data, resulting in flow of papers cataloging plant and/or microbial functions or even unannotated genes. Here, integration of ecological theories in these studies (as also researcher training) would provide structuring framework, connecting now largely separated microbiome research to other fields of plant-microbe interactions and ecological and evolutionary biology.

Indeed, plant microbiome research is transitioning from descriptive surveys to comprehensive understanding of the plant holobiome in the eco-evolutionary framework. Identification of keystone microbial species and interconnected microbial hubs, as well as their putative functions (Sessitsch et al., 2012; Agler et al., 2016; Saikkonen et al., 2016; van der Heijden and Hartmann, 2016), are the basic pieces required to solve

the eco-evolutionary puzzle, comprised of interacting free-living microbes and holobiome-units in ecosystems.

Easy data acquisition has created the illusion of controllability and the hope of microbiology-based innovations. This has led to the renaissance of microbial research seeking solutions for global issues such as diseases, food security, and sustainable agricultural practices (Duhamel and Vandenkoornhuyse, 2013; Kauppinen et al., 2016; Busby et al., 2017; Finkel et al., 2017). However, the major challenge is that we are still a long way from understanding microbial versatility and how it relates to the ecology and evolution of plant holobionts interacting with each other and their environment. Meeting these challenges requires combining novel molecular and microbiological tools with empirical and theoretical biological approaches including ecology, genetics and epigenetics, phenotypic evolution, and coevolution of interacting species. Otherwise we lack theoretical insights (Prosser et al., 2007), and ignore the complexity and dynamics of microbe–plant interactions in man-made and natural ecosystems.

We propose that to assemble the plant holobiome puzzle, future work should take into account the following premises and presumptions.

First, the majority of plant-microbe studies are either plant or microbe centered. Accordingly, studies are often designed and interpreted with either the plant as the active member which recruits its microbes (Gehring and Whitham, 1994) or the host plant as a background variable (e.g., "plant genotype") or a habitat. Instead, both the plant and associated microbes should be taken into account as active members of the interaction (Saikkonen et al., 1999; Bulgarelli et al., 2012; Dini-Andreote and Raaijmakers, 2018). This requires developing true dialogue, reciprocal understanding, and mutual collaboration between researchers from different life science fields.

Second, we contend that microbe–plant interactions follow similar evolutionary and ecological processes as host–pathogen or host–parasite interactions, and therefore need not to be treated differently (Saikkonen et al., 2004; Cordovez et al., 2019). The prevailing insight is that like other species interactions, even obligate microbe-plant mutualism is based on mutual exploitation rather than reciprocal altruism and benefits to the partners are only rarely symmetric (Thompson, 1994; Maynard Smith and Szathmary, 1995; Doebeli and Knowlton, 1998; Saikkonen et al., 2004). Symbioses between microbes and their host plants may also involve reciprocal manipulation of phenotypes, including morphology and physiology, and lifecycle of partners (Saikkonen et al., 2016). Thus, conflicting selection forces are likely to destabilize them, and the outcome of interactions can change in time and space projecting the ecological surface of a dynamic fitness landscape with adaptive peaks and valleys (Thompson, 1994, 2005; Saikkonen et al., 2004). For example, mutualistic mycorrhizae and *Epichloë* endophytes can become parasitic in the presence of herbivores or hemiparasitic plants especially in resource-limited environments (Ahlholm et al., 2002b; Lehtonen et al., 2005; Saikkonen et al., 2010a; Wäli et al., 2013).

Third, co-occurring plant-associated microbes, irrespective of taxa, are likely to interact with each other. Microbes compete, interact chemically, and/or mediate the host quality to each other. Chemical interplay may include signaling and chemical crosstalk among microbes and their host plant cells (Hamilton et al., 2012; Compant et al., 2016; van Overbeek and Saikkonen, 2016) but can also extend to cover other organisms feeding on the shared host plant as well as associated food webs (Lehtonen et al., 2006; Saikkonen et al., 2006; Saari et al., 2010; Li et al., 2014). It is noteworthy that certain pathways regulating plant responses to heterotrophic organisms, such as salicylic acid and jasmonic acid pathways, have been shown to counteract (Ballaré, 2011; Thaler et al., 2012; Pineda et al., 2013). For instance, chemical crosstalk between mutualistic microbes, biotrophic and necrotrophic pathogens (Bastias et al., 2018), and herbivores may constrain the host plant from reaching optimal pathogen- or herbivore-specific resistance (Ahlholm et al., 2002a), but is likely to be beneficial to overall fitness of the holobiont. Similarly, community-level approaches to bacterial and fungal interactions are needed to understand the importance of the structure and functions of microbial community associated with plants on plant performance as well as on ecosystem functions and services (Dini-Andreote and Raaijmakers, 2018). More knowledge about multispecies coevolution is necessary to fully understand any particular bipartite microbe–plant interactions and how they modulate cascading interactions across trophic layers.

Fourth, the performance of plant-associated organisms such as mutualistic, commensalistic, and antagonistic microbes, as well as herbivores, may also be a response to genetically or epigenetically determined plant traits rather than interconnected associations among them. The role of epigenetics is still poorly understood. In contrast, empirical evidence suggests that for example genetic compatibility can determine endophytic microbe-plant combinations that can be mutualistic or commensalistic depending on environmental conditions (Saikkonen et al., 2010b). Furthermore genetically determined plant traits can determine plant quality to heterotrophic organisms such as microbes and/or herbivores (Fritz and Simms, 1992) and genetic correlations between plant resistance to pathogens and herbivores have been suggested to constrain plants from reaching optimal species-specific resistance (Ahlholm et al., 2002a).

Fifth, microbes are highly diverse and versatile, and the ecological role of microbes is often complex and liable to change. Microbes can occupy several ecological niches that may overlap, and the nature of microbe–plant association is context dependent. The same ecological function can be provided by several different microbes, and individual microbes can be—at least partially—substitutable by others. This calls importance for studies on the microbial mediated key functions in different environments.

The spectrum, complexity, and dynamics of microbe–plant interactions demonstrate that plant microbiomes provide a fertile ground for understanding unit of selection, multitrophic interactions, evolution of life histories, and co-evolutionary processes, and how microbiomes should be taken into account when developing microbe-based biotechnologies and ecosystem services. Two unanswered questions remain: (1) How plants and their associated microbes individually and/or in concert as a phenotypic unit respond to prevailing selection pressures?

(2) What proportion of ecological outcome is determined by genetics, epigenetics, and phenotypic plasticity in the ecologically relevant traits, and thus in the adaptive radiation of holobionts? Present barriers must be dismantled in order to disseminate current knowledge of plant microbiomes and create a more conceptual framework with empirical and theoretical examples, and predictable hypotheses (van Overbeek and Saikkonen, 2016; Frank et al., 2017).

This knowledge is urgently needed to tackle the foremost global challenges of our times: the biodiversity loss, climate change and the increasing demand of food production to meet world's population growth (Springmann et al., 2018; Tollefson, 2019). For example, in the debate on biodiversity loss and endangered species, particular attention should be paid to the importance of abundant keystone species maintaining crucial ecosystem functions that are vital to thriving ecosystems or species that may threaten other species by disrupting ecosystem functions. Microbes are largely ignored in this context although they are essential for processes that make Earth habitable to primary producers and other organisms subsisting on them. Similarly microbes should be taken into account in risk analyses and solutions to aim mitigating climate change as well as in sustainable food production (Duhamel and Vandenkoornhuyse, 2013; Gundel et al., 2013; Kauppinen et al., 2016). For example, recent evidence suggests that microbes can increase carbon sink in terrestrial ecosystems by enhancing carbon uptake into soils and into plants by promoting plant growth (e.g., mycorrhizae, rhizobia and endophytes) or alter the flux of greenhouse gases from the soil to the atmosphere (Clemmensen et al., 2013; Iqbal et al., 2013; Averill et al., 2014). Beneficial microbial plant symbionts have been suggested to have great potential in sustainable agricultural and horticultural practices, and for environmental improvement as well (Duhamel and Vandenkoornhuyse, 2013; Gundel et al., 2013; Kauppinen et al., 2016). These goals might partly be overly ambitious because microbe-plant interactions are complex, labile and context dependent, and thus, we never can fully control plant associated microbiomes in nature. However, some of these ambitious goals can be attained if we invest resources in multidisciplinary collaboration across various fields of expertise, as understanding the plant-microbiome dynamics and factors impacting holobiont ecology and evolution can enable us to utilize the plant microbiomes for sustainable future.

AUTHOR CONTRIBUTIONS

KS, RN, and MH have contributed equally in initializing, innovating, and writing the paper.

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Aphid Facultative Symbionts Aid Recovery of Their Obligate Symbiont and Their Host After Heat Stress

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Environmental conditions affect insect fitness, with many species constrained by specific temperature ranges. Aphids are limited to temperate climates and it is hypothesized that this is partly due to their heat-susceptible obligate nutritional symbiont *Buchnera*. Aphids often carry additional facultative symbionts which can increase the host's fitness after heat stress. Here we used the pea aphid (*Acyrthosiphon pisum*) and three of its facultative endosymbionts (*Candidatus* Regiella insecticola, *Candidatus* Fukatsuia symbiotica (X-type; PAXS), and *Candidatus* Hamiltonella defensa) to investigate how these species respond to heat stress and whether their presence affects the fitness of the host or the obligate symbiont. We exposed aphid lines to a single high temperature event and measured lifetime fecundity and population densities of both obligate and facultative symbionts. Heat shock reduced aphid fecundity, but for aphids infected with two of the facultative symbionts (*Regiella* or *Fukatsuia*), this reduction was less than in uninfected aphids. The population density of *Buchnera* was also reduced after heat shock, and only recovered in aphids infected with *Regiella* or *Fukatsuia* but not in uninfected aphids or those with *Hamiltonella*. Although heat shock initially reduced the densities of two of the facultative symbionts (*Hamiltonella* and *Fukatsuia*), all facultative symbiont densities recovered by adulthood. Two of the facultative symbionts tested therefore aided the recovery of the obligate symbiont and the host, and we discuss possible underlying mechanisms. Our work highlights the beneficial effects of protective symbionts on obligate symbiont recovery after heat stress and how facultative symbionts may affect the wider ecological community.

Keywords: *Acyrthosiphon pisum, Buchnera aphidicola,* facultative symbiont, heat stress, insect symbionts, quantitative PCR, symbiosis

INTRODUCTION

It is well-established that infection with bacterial symbionts can affect an insect host's biology. Reproductive fitness, insect behavior, immune pathway function, and responses to natural enemies may all be influenced by the presence of endosymbionts (Dion et al., 2011; Gerardo and Parker, 2014; Vorburger, 2014; Martinez et al., 2015). By improving the ecological fitness of a host through raising its immunity to natural enemies, or by enhancing its tolerance to environmental stress, a vertically transmitted symbiont increases its own fitness (Oliver et al., 2005; Brownlie and Johnson, 2009).

Rising global temperatures are already affecting insect populations; there is evidence of range shifts (Parmesan and Yohe, 2003), changes in phenology (Walther et al., 2002) and interactions with predators and parasitoids (Harrington et al., 1999; Schmitz and Barton, 2014). For several insect groups infection with various bacterial symbionts has been shown to enhance resistance to temperature stress (Corbin et al., 2017). These effects may be direct symbiont-mediated host protection (Montllor et al., 2002; Neelakanta et al., 2010; Brumin et al., 2011) or indirect effects of temperature on the symbiont itself (Chen et al., 2009; Bordenstein and Bordenstein, 2011).

There are a number of hypotheses for the mechanisms underlying indirect symbiont-mediated protection from heat. Infection with symbionts has been shown to increase the expression of immune system genes (Laughton et al., 2013), and it is hypothesized that this immune response controls the bacteria, restricting growth or location and protecting the host from microbial over-proliferation (Kwong et al., 2017; Maire et al., 2018). It may also bestow temperature tolerance as a by-product. For example, infection of *Rickettsia* in whiteflies leads to the upregulation of stress-response genes in the host and thus increases survival of the insect under heat shock (Brumin et al., 2011); similarly, insects infected with bacterial symbionts often produce more immune cells than those that are uninfected (Schmitz et al., 2012; Weiss et al., 2012; Laughton et al., 2013; Kim et al., 2015). There are close links between insect responses to heat and to infection–many heat shock proteins are chaperones that aid protein production and refolding post-stress, and they may also enhance immune responses (Young et al., 1993).

Instead of indirectly affecting the host's stress or immune responses, symbionts may themselves produce and release heat shock proteins or metabolites that directly protect the host or other microbes that the host depends on (i.e., obligate symbionts) (Burke G. et al., 2010). Obligate symbionts are often a thermal "weak link" and more susceptible to temperature extremes than their hosts (Corbin et al., 2017; Shan et al., 2017; Zhang et al., 2019). Shielding an obligate symbiont from thermal damage would therefore benefit both the host and all of its symbionts. For example, in pea aphids that experience heat shock, the density of the obligate nutritional symbiont *Buchnera aphidicola* is usually reduced, but is maintained at near normal levels in aphids that carry the facultative symbiont *Candidatus* Serratia insecticola (hereafter *Serratia*) (Burke G. et al., 2010). It is also plausible that facultative symbionts might be directly protecting the host by replacing an obligate symbiont that is no longer able to perform its function. When the obligate symbiont *Buchnera* is removed using antibiotics at benign temperatures, *Serratia* in pea aphids moved into the bacteriocytes vacated by *Buchnera* and subsequently allow the stressed aphid to survive and reproduce (Koga et al., 2003, 2007).

Pea aphids, *Acyrthosiphon pisum*, are a model system for understanding how facultative symbionts protect their hosts from thermal stress. They and their obligate symbiont are typically intolerant to heat in laboratory populations (Dixon et al., 1987; Dunbar et al., 2007), but three of their eight potential facultative symbionts (*Serratia*, *Candidatus* Fukatsuia symbiotica and *Candidatus* Hamiltonella defensa; hereafter *Fukatsuia* and *Hamiltonella*, respectively) are known to improve survival or reproduction after heat shock (Montllor et al., 2002; Koga et al., 2003; Russell and Moran, 2006; Heyworth and Ferrari, 2015). The obligate symbiont *Buchnera* synthesizes essential amino acids for the host, which are required for aphids to thrive on their imbalanced diet of plant phloem sap (Douglas, 1998). *Buchnera* has a highly reduced genome (Moran, 1996; Gómez-Valero et al., 2007) and some genotypes are susceptible to high temperatures; under heat stress, just five protective heat shock proteins are deployed (Wilcox et al., 2003) compared to over 75 in its free living relative *Escherichia coli* (Carruthers and Minion, 2009) and during severe heat shock *Buchnera* can be killed.

While the costs and benefits of infection are being explored in a broad spectrum of insect species, relatively little is known about how different facultative symbionts confer increased heat tolerance to their hosts, and how these mechanisms vary depending on symbiont species. Understanding how insects can and will respond to increases in temperature is vital to accurately model current and future populations. We investigate whether three common facultative symbionts of the pea aphid [*Candidatus* Regiella insecticola (hereafter *Regiella*), *Fukatsuia* and *Hamiltonella*] protect the host and how they respond to heat stress themselves. Importantly, we test whether the protection from the effects of heat co-occur with the protection of the obligate symbiont *Buchnera*. We test whether the facultative symbionts directly protect *Buchnera*, allow it to recover after heat stress or protect the host by replacing *Buchnera* and whether this mode of protection is similar for all tested facultative symbionts.

MATERIALS AND METHODS

Aphids and Symbionts

Rapid, asexual reproduction results in clonal lines of aphids that can be kept indefinitely under long-day conditions. This allows the manipulation of facultative symbiont presence through antibiotic curing or artificial infections while maintaining an essentially identical aphid genotype. Two pea aphid genotypes were used for this study, both collected from the UK (**Supplementary Table S1**). Genotype 218 was collected naturally infected with *Fukatsuia* and *Hamiltonella,* and was cured more than a year before use. This was achieved by feeding young aphids with broad bean leaves suspended in a tube of antibiotic solution (0.5% Gentomicin, 1% Ampicillin, 0.5% Cefotaxime in distilled water) over 4 days (McLean et al., 2011). Genotype 200 was collected naturally uninfected, harboring no known facultative symbionts. All aphid lines were screened for *Hamiltonella*, *Regiella*, *Serratia symbiotica*, *Fukatsuia*, *Spiroplasma* sp., *Rickettsia* sp., and *Rickettsiella viridis* following protocols in Tsuchida et al. (2010) and Ferrari et al. (2012) to ensure that they had the appropriate symbiont infection and were not infected with any other known facultative symbionts. The symbiont infections were regularly checked to detect possible contamination. The symbiont-specific PCR primers can be found in **Supplementary Table S2**. The PCR mix comprised 6.25 μl BioMix (Bioline), 0.1 μl (20 μM) of forward and 0.1 μl (20 μM) reverse primer, 5.55 μl distilled water and 1.0 μl sample DNA. The PCR reaction was performed at 94°C for

2 min, followed by 35 cycles of: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. It concluded with 6 min at 72°C and then cooled the sample to 4°C indefinitely. PCR products were run on a 1% agarose gel and the presence of a band confirmed the presence of the symbiont.

Five aphid lines were used in the experiment, two uninfected with facultative symbionts (200 and 218) and the remainder infected singly with one of three facultative symbionts, *Regiella*, *Fukatsuia,* and *Hamiltonella* (**Supplementary Table S1**). *Regiella* was injected into aphid genotype 200, while the other two symbionts were injected singly into genotype 218. These five aphid lines comprise three pairwise comparisons between uninfected and infected aphids, with the uninfected line of 218 used in two comparisons. This design aimed to compare host fitness and symbiont densities within each pair across the same aphid genetic background and thus we conducted no analyses across multiple pairs. These specific isolates of symbiont were chosen because preliminary results indicated that they were likely to provide heat shock protection.

To produce these infections of *Hamiltonella*, *Regiella,* and *Fukatsuia* we used hemolymph injections from infected donor aphids (**Supplementary Table S1**). Hemolymph was extracted from donor aphids under a microscope using glass needles and re-injected into the appropriate aphid line and surviving aphids raised to adulthood. Glass needles were pulled from Kwik-Fil™ borosilicate glass capillaries (1B100-4, World Precision Instruments, 1 mm diameter) using a P-97 Flaming/Brown micropipette Puller (Sutter Instrument Co.). The offspring of the surviving aphids were tested for the successful establishment of the new infection when they were adults, and these aphid lines were retested regularly to ensure the maintenance of the new aphid-symbiont combinations. All injected lines had been maintained in the laboratory for at least a year before being used for experimental assays.

Heat Shock Protocol

This assay was designed to understand symbiont dynamics after aphids have been exposed to heat shock. Aphids were exposed to either a single peak of high temperatures or were maintained at a steady control temperature. The "heat shock" temperature chosen was 38.5°C, which was based on a series of pilot studies (data not shown). Our aim was to find a temperature that had a strong negative effect on fitness, but at which approximately half the individuals in an aphid population still survived. The aim was to explore the phenotypes of the symbionts and not to model natural situations directly. The temperature experienced by aphids near the ground is often considerably higher than meteorological records and depends, for example, on aspect and slope of the site, but it is likely that aphids are exposed to similarly high temperatures in Northern England on hot summer days (Bennie et al., 2008; Suggitt et al., 2011).

To produce age-controlled populations of each of the five lines, groups of young adults were placed into petri dishes (9 cm diameter) that contained a single broad bean (*Vicia faba* var. Sutton Dwarf) leaf, placed in 2% agar. *V. faba* is a host plant that almost all pea aphids perform on (Ferrari et al., 2008). The adults were left to reproduce for 24 h at 20°C, and the offspring were subsequently put onto 2 week old *V. faba* plants in groups of 50 and enclosed in a vented, transparent cage. On the following day (aphid age 24–48 h) the populations were moved into cabinets where they were either exposed to heat stress or 20°C as a control. Temperature cabinets were rigorously checked before and during the experiments to ensure even distribution of heat and the same relative humidity (50%) in both cabinets. Plants containing aphids were also placed in a randomized block pattern within the cabinet to remove any potential effects of uneven heat distribution.

While the control treatment was left at 20°C, the temperature in the heat treatment was increased from 20 to 38.5°C steadily over the course of 2 h, held at 38.5°C for 4 h, and then decreased back to 20°C over a further 2 h. Surviving aphids from both treatments were moved onto fresh 2-week-old plants on the day after heat shock to mitigate any temperature effects on the plant itself. These plants were moved into a different controlled-temperature room (20°C), where aphids from both heat treatments were kept together until being collected for analysis.

Aphids were removed to measure symbiont density at two time points. The first was 24–26 h after the start of the peak heat shock period (when the aphids were 3 days old), and the second 11 days post-heat shock (when the aphids were 14 days old, ∼6 days after an aphid would usually begin reproducing). These two time points were chosen to investigate symbiont densities immediately after stress, and to test if recovery by the onset of reproduction was possible. *Buchnera* densities are known to decrease as aphids age (Simonet et al., 2016) and so this second time point was chosen to be the potential highest density of *Buchnera* during an aphid's development.

The aphids were flash frozen using dry ice and kept at −80°C until DNA extraction. In addition, one surviving apterous individual from each group was placed on a petri dish with a *V. faba* leaf (as above) to measure the number of offspring produced. These dishes were refreshed every 3–4 days to ensure healthy *V. faba* leaves. Offspring counts continued until all aphids had died, measuring total lifetime fecundity. There were 5–6 replicates for fecundity counts and symbiont density for each of the five aphid lines in each treatment (i.e., 10–12 replicates in total for each line), these were performed in two temporal blocks with approximately half the replicates of each treatment in each block.

qPCR Protocol

DNA was extracted from the aphids after samples were defrosted at room temperature. Aphids were homogenized in a 200 μl 5% Chelex solution made in distilled water. Ten microliters of proteinase K (10 mg/ml) was added per sample, and samples were incubated for 6 h at 56°C to facilitate digestion. They were then "boiled" at 100°C for 10 min before being centrifuged at 13,000 rpm for 3 min and the supernatant containing the DNA pipetted into a clean 1.5 ml Eppendorf tube which was stored at −20°C until use. Five aphids per replicate were pooled to generate a sample for the first time point (24–26 h after heat shock) and one aphid for the second time point (11 days later).

Samples were run in duplicate using SYBR® Green reagent on a StepOnePlus™ Real Time PCR machine (Applied Biosystems).

Each reaction consisted of 10 μl FAST SYBR 2× mastermix (Applied Biosystems), 1 μl forward primer (7 μM), 1 μl reverse primer (7 μM), 6 μl nuclease-free water, and 2 μl DNA sample. qPCR primers for *Regiella, Fukatsuia*, *Hamiltonella,* and the aphid housekeeping gene elongation factor-1 alpha (EF-1α) (**Supplementary Table S2**) were tested to ensure high efficiency and similarity between primer sets. Melt curves were performed on each plate to ensure the primers were specific to each target and only bound once. Cycling conditions were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The melt curve involved a further 95°C for 15 s, 60°C for 1 min and then a gradual increase to 95°C over 15 min. Each 96-well qPCR plate was analyzed using StepOne Software v2.2.2 (Applied Biosystems) and Cycle threshold (Ct) values were obtained by comparing each primer sample to a single standard curve of known concentration and using identical threshold and baseline levels for each primer target across plates. Standard curves were created by amplifying positive control samples using PCR, calculating DNA concentrations using a High Sensitivity DNA Assay on a 2100 Bioanalyzer system (Agilent), and then serially diluting the sample 1:10 with distilled water to create a 5-sample curve comprising known concentrations decreasing from 10 pmol/ml. Samples with Ct values over 30 were classed as negative, confirmed by our negative controls. This corresponds to a copy number of <52 per 2 μl sample for all primers, and is below the threshold of detection. Where the difference in Ct values between technical replicates was >1.5, the sample was either rerun or not used in the analysis.

The standard curves were used to calculate the DNA concentration of each sample, and this was converted into copy numbers per 2 μl of DNA extract. To control for aphid size and extraction quality, copy numbers for each sample were presented relative to those of a housekeeping aphid gene as a control, giving a ratio of symbiont copy numbers to aphid copy numbers.

Statistical Analysis

Data were analyzed using the R software v. 3.4.1 (R Core Team, 2018). Since our core question was to test whether the three symbiont species can provide heat shock protection, but not to compare the extent of this protection, the data were analyzed separately for each symbiont species. Thus, in each analysis the infected line was compared with the same uninfected aphid genotype, within and across heat treatments. The data for the uninfected line 218 was therefore used twice, paired with line 218 infected with *Hamiltonella* or *Fukatsuia*. Similarly, we analyzed the two time points separately because symbiont densities change during aphid development, which would complicate the interpretation of the analysis.

Lifetime fecundity of the set of *Regiella* lines was analyzed using a general linear model assuming a normal error distribution. The number of offspring was the response variable, and temporal block, facultative symbiont presence, heat treatment and the interaction between symbiont presence and heat treatment were the explanatory variables. The sets of *Fukatsuia* and *Hamiltonella* lines were analyzed with a non-parametric Kruskal-Wallis test, because the model assumptions of parametric models were not met. This was followed by Wilcoxon tests to identify differences between specific treatments.

The densities of the symbionts were also analyzed with a general linear model assuming a normal error distribution. This was split into six analyses, separate for the lines relating to each symbiont species at each time point, to simplify the interpretation. For *Buchnera* densities, the explanatory variables were again temporal block, facultative symbiont presence and heat treatment as well as the interaction between the latter factors. For the densities of the facultative symbionts, only block and heat treatment were explanatory variables. In most cases model assumptions were met without transforming the data, only the *Buchnera* densities at the first time point in the *Regiella* lines were log-transformed. For *Regiella* densities at the first time point and *Buchnera* densities in the set of *Regiella* lines at the second time point, Kruskal Wallis and Wilcoxon tests were used as described for the fecundity data.

For all general linear models, *post-hoc* tests were only performed when the factor or interaction was significant in the main analysis. This was conducted using the R package "phia" (De Rosario-Martinez, 2015), with Holm's correction for multiple comparisons. All data are available as Supplementary Material (**Data Sheets 2–4**).

RESULTS

Effects of Facultative Symbionts on Fecundity After Heat Shock

We exposed aphids to a short spike of high temperature and measured facultative and obligate symbiont densities and aphid fitness after 1 and 11 days. As expected, heat shock decreased the number of offspring produced in an aphid's lifetime in all three sets of lines [*Regiella* $F_{(1,19)} = 103.23$, $P < 0.001$; *Fukatsuia*: $W = 109$, $P = 0.03$, *Hamiltonella*: $W = 136.5$, $P = 0.001$; **Figure 1**]. However, the extent of this decrease was modified by the presence of *Regiella* and *Fukatsuia* [*Regiella,* symbiont × heat treatment: $F_{(1,19)} = 5.78$, $P = 0.03$; *Fukatsuia*: heat treatment in uninfected lines $W = 36$, $P = 0.004$ and in infected lines $W = 13$, $P = 0.48$]. *Fukatsuia* provided the greatest protection from heat as there was no difference in the fecundity of the infected lines in the control and heat shock treatment, whereas there was a greater reduction in fecundity in the uninfected aphids than in the infected aphids for the *Regiella* lines. In contrast, there was a similar decrease in fecundity for both uninfected and infected *Hamiltonella* lines following heat shock (uninfected: $W = 36$, $P = 0.004$; infected: $W = 34.5$, $P = 0.008$; **Figure 1**). At benign temperatures, two of the symbionts also affected lifetime fecundity: the presence of *Hamiltonella* increased lifetime fecundity ($W = 4$, $P = 0.03$), whereas *Fukatsuia* decreased it ($W = 34$, $P = 0.013$), and there was no difference for *Regiella* (**Figure 1**).

Facultative Symbiont Densities After Heat Shock

We measured the densities of the three facultative symbionts at two time points after exposure to heat, 24–26 h and 11

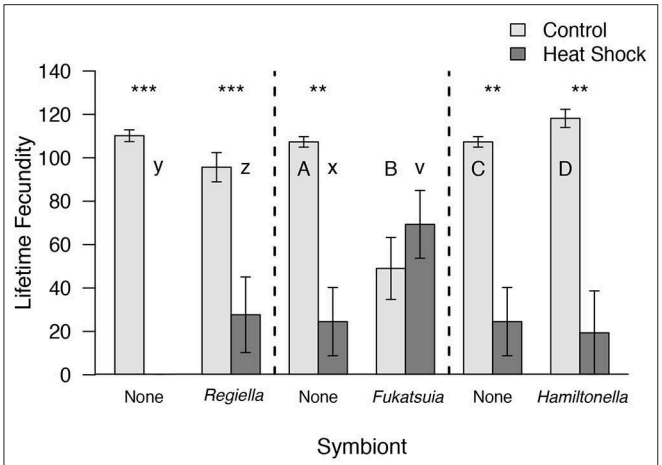

FIGURE 1 | The effect of heat shock and facultative symbiont presence on the number of offspring produced by pea aphids. Aphids from genotype 200 that were uninfected or carrying *Regiella* are compared in the first panel. Aphids from genotype 218 that were uninfected or carrying *Fukatsuia* or *Hamiltonella* are compared separately in the second and third panels, respectively. In all comparisons, there was a significant effect of heat shock compared to controls, but no overall effect of symbiont presence. Means and standard errors are shown. Within each panel separately, upper case letters denote significant differences between aphids with different symbionts within the control treatment and lower case letters denote significant differences in the heat shock treatment. The asterisks show significant differences between heat treatments for aphids of the same symbiont status (**$P < 0.01$, ***$P < 0.001$).

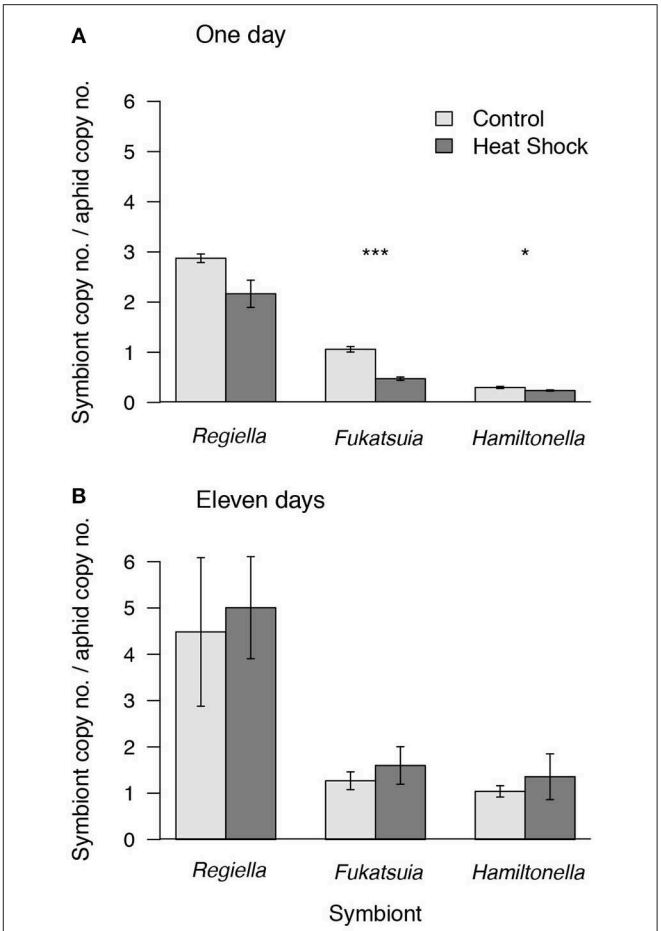

FIGURE 2 | Densities of facultative symbionts in pea aphids after heat shock or in the control treatment **(A)** 24–26 h after the onset of the heat shock and **(B)** 11 days after heat shock. Densities are shown as the copy number of the *gyrB* gene of the facultative symbiont relative to copy number of the aphid gene *EF1-α*. Means and standard errors are shown. Asterisks denote differences between heat treatments for aphids carrying a given symbiont (*$P < 0.05$, ***$P < 0.001$).

days post-heat shock (**Figure 2**). Compared to non-heat shocked controls the densities of two of the symbionts, *Fukatsuia* and *Hamiltonella*, were lower on the day after heat shock [*Fukatsuia*: $F_{(1,8)} = 65.05$, $P < 0.001$, *Hamiltonella*: $F_{(1,8)} = 6.64$, $P = 0.03$], whereas densities of *Regiella* are unaffected ($W = 28$, $p = 0.13$; but note that this is significant in a less conservative parametric test). By the second time point, taken when the aphids were young adults, there was no difference between population densities in heat stressed or control aphids for any of the three facultative symbionts [*Fukatsuia*: $F_{(1,8)} = 0.35$, $P = 0.57$, *Hamiltonella*: $F_{(1,7)} = 0.95$, $P = 0.36$, *Regiella*: $F_{(1,6)} = 0.01$, $P = 0.91$; **Figure 2**], suggesting that heat did not have long-term effects on facultative symbiont populations.

Obligate Symbiont Densities Under Heat Shock

Compared to the control treatment densities of *Buchnera* were decreased on the day after heat shock in each of the three pairs of lines, regardless of facultative symbiont infection [*Regiella* lines: $F_{(1,19)} = 80.36$, $P < 0.001$, *Fukatsuia* lines: $F_{(1,18)} = 70.71$, $P < 0.001$, *Hamiltonella* lines: $F_{(1,18)} = 56.07$, $P < 0.001$; **Figure 3**]. Regardless of treatment, *Buchnera* densities were higher in the lines harboring *Fukatsuia* [$F_{(1,18)} = 33.86$, $P < 0.001$] or *Hamiltonella* [$F_{(1,18)} = 24.52$, $P < 0.001$] compared to uninfected lines, an effect that was not seen in the *Regiella* lines [$F_{(1,19)} = 0.15$, $P = 0.71$]. For the *Fukatsuia* and *Hamiltonella* lines there was also a significant interaction between symbiont presence and heat treatment [*Fukatsuia*: $F_{(1,18)} = 9.57$, $P = 0.006$; *Hamiltonella*: $F_{(1,18)} = 4.80$, $P = 0.04$]: in both cases, *Buchnera* densities in the control treatment were higher in lines with facultative symbionts compared to uninfected aphids but there was no difference between these lines after heat shock. The interaction was not significant for the *Regiella* lines [$F_{(1,19)} = 2.58$, $P = 0.13$] where the extent of the loss of *Buchnera* did not differ between infected and uninfected lines.

At the later time point, when aphids were young adults, heat shock again reduced *Buchnera* densities on average [*Regiella* lines: $W = 105$, $P = 0.004$; *Fukatsuia* lines: $F_{(1,19)} = 6.85$, $P = 0.02$; *Hamiltonella* lines: $F_{(1,17)} = 24.84$, $P < 0.001$]. *Fukatsuia* presence on average also significantly increased the density of *Buchnera* regardless of treatment which was due to high *Buchnera* densities in the heat shocked aphids [$F_{(1,19)} = 7.89$, $P = 0.01$]; a difference that was not found for *Regiella* ($W = 46$, $P = 0.37$) or *Hamiltonella* presence [$F_{(1,17)} = 0.17$, $P = 0.69$]. Importantly, there was a significant interaction between symbiont infection and temperature in the *Fukatsuia* lines [$F_{(1,19)} = 7.55$, $P = 0.01$]

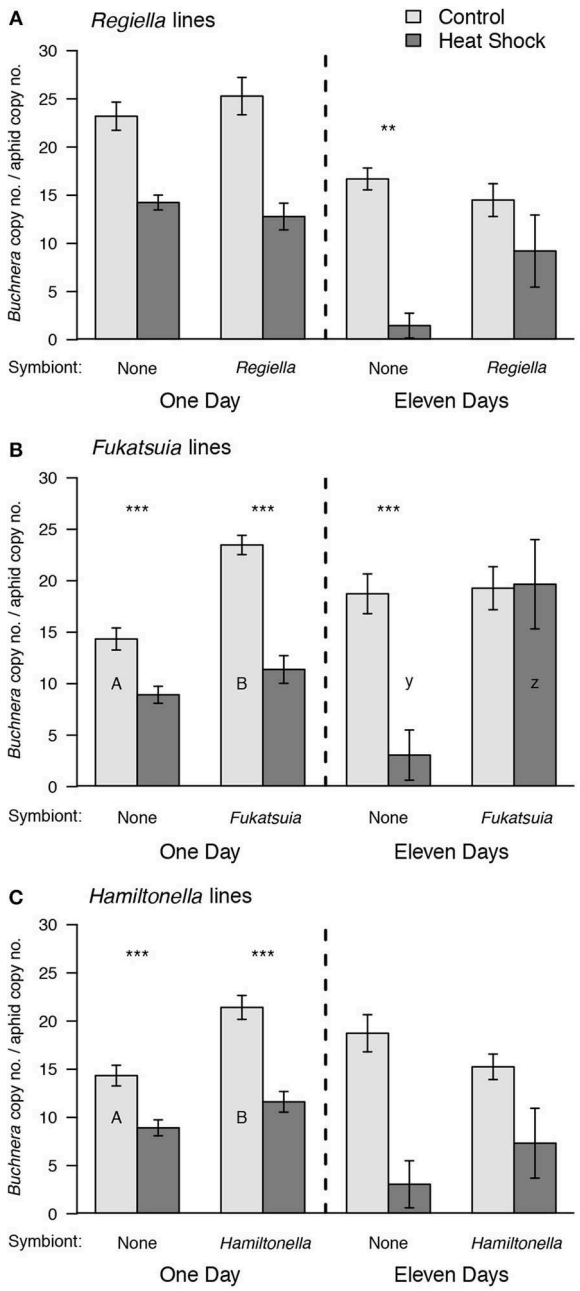

FIGURE 3 | The effect of heat shock and facultative symbiont presence on densities of the primary symbiont *Buchnera* in pea aphids. In each panel *Buchnera* densities are shown 1 or 11 days after heat shock. Uninfected aphids are compared to aphids carrying **(A)** *Regiella*, **(B)** *Fukatsuia,* and **(C)** *Hamiltonella*. The uninfected replicates are the same for the *Fukatsuia* and *Hamiltonella* lines. Means and standard errors are shown. In all cases, there was a significant overall difference between the heat shock and control treatments. There was also a significant overall effect of symbiont presence for the Fukatsuia lines at both time points, and *Hamiltonella* lines 1 day after heat shock. These main effects are not illustrated on the figure. *post-hoc* results from significant interactions are denoted by upper case letters for significant differences between aphids with different symbionts within the control treatment, and lower-case letters denote significant differences in the heat shock treatment. The asterisks show significant *post-hoc* differences between heat treatments for aphids of the same symbiont status (**$P < 0.01$, ***$P < 0.001$).

and an equivalent effect in the *Regiella* lines (heat treatment in uninfected lines: $W = 36$, $P = 0.004$ and in infected lines: $W = 17$, $P = 0.42$) that was not seen for the *Hamiltonella* lines [$F_{(1,17)} = 1.95$, $P = 0.18$]: *Buchnera* densities after heat shock were significantly reduced in uninfected aphids but not when *Fukatsuia* or *Regiella* were present.

DISCUSSION

Our results show that different aphid symbionts can protect the aphid from heat and help the obligate symbiont to recover after heat shock. Infection with *Regiella* and *Fukatsuia* was closely linked to *Buchnera* recovery after heat shock and led to increased production of offspring compared to uninfected controls whereas there was no such protection in aphids infected with *Hamiltonella*. This pattern differs from other studies (Russell and Moran, 2006; Doremus and Oliver, 2017), which found that *Hamiltonella* but not *Regiella* or *Fukatsuia* provided heat protection. As different lines of insects and symbionts were used in these studies, it is likely that these protective effects are dependent on symbiont, host genotype or their interaction and are thus not a universal feature of symbiont infection. The prevalence of heat protection may be overestimated here since we chose genotypes based on preliminary results.

Buchnera densities are closely linked to aphid fitness. Disrupting the obligate symbiosis by removing *Buchnera* leads to large reductions in offspring production and often host death (Koga et al., 2003; Akman Gündüz and Douglas, 2009) Overly high densities of *Buchnera* can also lead to a reduction in fitness (Chong and Moran, 2016), meaning that the relationship between density of the symbiont and number of offspring produced is not directly proportional. However, removal of *Buchnera*, via antibiotics or heat, as also shown in our results, generally leads to aphid sterility in the absence of facultative symbionts (Dunbar et al., 2007; Koga et al., 2007).

A key question that we addressed was whether the facultative symbionts protect *Buchnera* from the effects of heat, and if so whether *Buchnera* is directly protected or its recovery facilitated. The densities of both *Buchnera* and the facultative symbionts were reduced 24 h after heat shock. In aphids carrying *Fukatsuia* or *Regiella*, these densities returned to levels that were similar to those in non-heat shocked controls, thus demonstrating a clear role of the facultative symbionts in the recovery of *Buchnera*. The most parsimonious interpretation of the observed pattern is that the facultative symbionts do not provide immediate protection, although it is possible that the decline in *Buchnera* DNA density occurs due to different processes in aphids with and without facultative symbionts. It is conceivable that *Buchnera* is only truly killed by heat inside the latter and growth is merely arrested in the former and thus some immediate protection occurs.

The patterns of obligate and facultative symbiont densities observed here suggest that a different mechanism underlies the heat protection provided by *Regiella* and *Fukatsuia* compared to that provided by *Serratia* (Montllor et al., 2002; Burke G. et al., 2010). After heat shock, *Serratia* in pea aphids lyse and this coincides with metabolomic changes (Burke G. et al., 2010).

At the same time *Buchnera* densities are maintained at similar levels to those at benign temperatures (Burke G. et al., 2010). In our experiments, in aphids with *Regiella* or *Fukatsuia*, *Buchnera* densities initially decrease. This demonstrates that the protection provided by the facultative symbionts is not instant and suggests that the protection is probably not due to a constitutively activated aphid stress response, but it is still possible that the facultative symbionts prime a stress response by the aphid that helps recovery later on.

The provision of heat-protective compounds, through either lysis or release from a live cell, is also consistent with our observations. Burke G. et al. (2010) explored which metabolites are affected by heat treatment when *Serratia* lyse. They found three metabolic changes linked to the presence of the protective *Serratia* after heat stress, one of which is a decrease in concentration of the antioxidant indole-3-lactate, as well as two other unidentified metabolites (Burke G. et al., 2010). The initial decline of *Fukatsuia* in our study suggest that lysis is likely in this symbiont (and possibly in *Regiella* where the decline is significant using less conservative statistics). However, the protective compounds released during this process appear to act later than in *Serratia* (Burke G. et al., 2010) as *Buchnera* also declines initially. In some cases, facultative symbionts can replace the function of an obligate symbiont (Koga et al., 2003, 2007) and this could be a way of protecting the host from the effects of heat. In our system, both *Buchnera* and the facultative symbionts recover demonstrating that this functional replacement is not a likely mechanism here.

Some strains of *Buchnera* are more resistant to heat than others (Dunbar et al., 2007; Moran and Yun, 2015) and it is possible that the two aphid genotypes here carry different *Buchnera* genotypes. Aphids in laboratory populations often carry *Buchnera* strains with the $ibpA^{12}$ mutant allele that are more sensitive to heat but have higher fitness (Burke, G. R. et al., 2010). We did not sequence *ibpA* in the genotypes used here since we were interested in the effects of facultative symbionts and the *Buchnera* strain is the same within all our comparisons. It is worth noting that the *Fukatsuia* and *Hamiltonella* lines both had the same aphid and *Buchnera* genotype but only *Fukatsuia* protected, suggesting that the *Buchnera* strain does not bias our conclusions. However, in natural populations the absence of facultative symbiont infections is correlated with a higher incidence of this mutation (Burke, G. R. et al., 2010). Aphids thus have two mechanisms which protect *Buchnera* from heat: the absence of the heat-sensitive $ibpA^{12}$ mutant allele and the presence of protective facultative symbionts. Facultative symbionts appear to confer low fitness in the presence of $ibpA^{12}$ and are thus likely selected against in aphids with this mutation (Burke, G. R. et al., 2010). It thus suggests that in natural populations facultative symbionts may only be able to rescue the aphid *Buchnera* from heat in aphids that carry the heat-tolerant $ibpA^{13}$ allele and this may explain the scarcity of $ibpA^{12}$ in natural populations.

As well as the protective effect of *Fukatsuia* and *Regiella*, we observed interesting changes in symbiont densities at benign temperatures. *Fukatsuia* decreased fecundity, as shown previously (Heyworth and Ferrari, 2015; Doremus and Oliver, 2017); the densities of *Buchnera* confirm that this is not due to suppression of the obligate symbiont (Koga et al., 2003), which has been observed for a costly infection by *Rickettsia* in pea aphids (Sakurai et al., 2005). Surprisingly, infection with *Fukatsuia* or *Hamiltonella* leads to an increase of *Buchnera* population levels in younger aphids. This may benefit both the aphid and *Buchnera* when facultative symbionts are present, because additional nutrients may be required. It is possible that either the aphid upregulates *Buchnera* densities or that this strain of *Buchnera* responds to the presence of facultative symbionts by increasing its growth rate. In either case, the density of *Buchnera* cells in infected aphids is comparable to uninfected aphids once the aphids are adult.

The ability of facultative symbionts to protect obligate nutritional symbionts from heat stress has implications for the frequencies and spread of both the microbes and the insects themselves. Many phytophagous insects rely on obligate symbionts to provide essential nutrition, but these are often vulnerable to ecologically stressful situations (Bennett and Moran, 2015; Kikuchi et al., 2016) due to severe genome reduction during coevolution with their hosts (McCutcheon and Moran, 2011). This genome reduction probably led to the heat sensitivity that some facultative symbionts ameliorate, an example of a symbiosis rescuing another symbiosis. It seems improbable that this rescue resulted from close coevolution due to the relatively transient nature of facultative symbiosis infections (Smith et al., 2015).

As we and others (Montllor et al., 2002; Burke G. et al., 2010) have shown, carrying certain isolates of facultative symbionts can protect obligate symbionts from a single, short exposure to heat, but it remains to be investigated whether this protection is also effective under long-term or regular exposure to extreme temperatures. The temperature-dependent fitness effects are likely to alter the frequencies of facultative symbionts in natural populations, but will do so in concert with other abiotic and biotic factors, including the frequencies of heat-tolerant *Buchnera* strains. The symbionts' ability to affect interactions between host and natural enemies is also well-documented (Hrček et al., 2016). These interactions can be affected by a change in temperature, through an effect of temperature on the natural enemy itself (Roux et al., 2010; Nguyen et al., 2013) or on the interaction between host, symbiont and natural enemy (Guay et al., 2009; Jeffs and Lewis, 2013; Heyworth and Ferrari, 2016). In addition, both vertical and horizontal transmission frequencies of symbionts can be affected by temperature (Anbutsu et al., 2008; Osaka et al., 2008; Liu et al., 2019). A combination of temperature-dependent fitness effects and transmission dynamics is therefore a likely reason for the lack of a clear correlation between symbiont mediated benefits seen in laboratory experiments and symbiont frequencies observed in the field (Oliver et al., 2014), and probably contributes to the geographic variation in the composition of facultative symbiont communities (Montllor et al., 2002; Tsuchida et al., 2002; Sepúlveda et al., 2017). There are, however, examples where the patterns based on laboratory experiments are observed: the frequencies of the aphid heat-protective symbiont *Serratia* are high in host populations in the warmer climes of Southern

USA (Chen and Purcell, 1997; Montllor et al., 2002) and more generally in arid compared to temperate regions (Henry et al., 2013). Similarly, land temperature correlates with symbiont prevalence in midges (Morag et al., 2012).

Facultative symbionts alter insect fitness under stressful conditions and can affect not just the host, but also species that the host interacts with directly and indirectly (McLean and Godfray, 2016; Doremus et al., 2018). In extreme cases, hosting a defensive symbiont can lead to cascading extinctions and the collapse of entire communities (Sanders et al., 2016). Our work highlights how the host and its symbiont community is affected by temperature and that this temperature-dependency might result in changes of community interactions under climate change.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

EH, MS, and JF conceived the ideas and designed methodology, collected the data, wrote the manuscript, and gave final approval for publication. EH and JF analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00056/full#supplementary-material

Data Sheet 2 | Densities of the obligate symbiont *Buchnera aphidicola* in the two heat treatments in the presence and absence of facultative symbionts.

Data Sheet 3 | Densities of the facultative symbionts in the two heat treatments.

Data Sheet 4 | Lifetime fecundity of pea aphids in the two heat treatments in the presence and absence of facultative symbionts.

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Fungal Endophytes Enhance the Photoprotective Mechanisms and Photochemical Efficiency in the Antarctic *Colobanthus quitensis* (Kunth) Bartl. Exposed to UV-B Radiation

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Antarctic plants have developed mechanisms to deal with one or more adverse factors which allow them to successfully survive such extreme environment. Certain effective mechanisms to face adverse stress factors can arise from the establishment of functional symbiosis with endophytic fungi. In this work, we explored the role of fungal endophytes on host plant performance under high level of UV-B radiation, a harmful factor known to damage structure and function of cell components. In order to unveil the underlying mechanisms, we characterized the expression of genes associated to UV-B photoreception, accumulation of key flavonoids, and physiological responses of *Colobanthus quitensis* plants with (E+) and without (E−) fungal endophytes, under contrasting levels of UV-B radiation. The deduced proteins of CqUVR8, CqHY5, and CqFLS share the characteristic domains and display high degrees of similarity with other corresponding proteins in plants. Endophyte symbiotic plants showed lower lipid peroxidation and higher photosynthesis efficiency under high UV-B radiation. In comparison with E−, E+ plants showed lower *CqUVR8*, *CqHY5*, and *CqFLS* transcript levels. The content of quercetin, a ROS-scavenger flavonoid, in leaves of E- plants exposed to high UV-B was almost 8-fold higher than that in E+ plants 48 h after treatment. Our results suggest that endophyte fungi minimize cell damage and boost physiological performance in the Antarctic plants increasing the tolerance to UV-B radiation. Fungal endophytes appear as fundamental biological partners for plants to cope with the highly damaging UV-B radiation of Antarctica.

Keywords: UV-B stress, Antarctica, *Colobanthus quitensis*, molecular response, flavonols, fungal endophytes

INTRODUCTION

Although ultraviolet B radiation (UV-B) is a minor component of the solar radiation, it has a disproportionately harmful effect on plants (Jansen et al., 1998). At global level, UV-B radiation (280 to 315 nm) is absorbed by the ozone layer, permitting that a small proportion reaches surface of the Earth. However, the levels of UV-B radiation that reach the earth's surface depends on several factors such as latitude, altitude, season, time of day as well as cloudiness (Seckmeyer et al., 2008). The UV-B radiation affects several aspects of plants such as development, growth, morphology (Beissert and Loser, 2008; Kaling et al., 2015), as well as modulates physiological processes and metabolism (McLeod et al., 2001). At the molecular level, the absorption of UV-B radiation by the leaves is associated with important damage on molecules such as DNA, RNA and proteins with ultimately, have several consequences at physiological level. Thus, plant exposure to high levels of UV-B radiation causes a decrease in the photosynthetic efficiency of photosystem (PS) II (Fv/Fm) due to the degradation of the PSII protein D1 (Caldwell et al., 2007; Shourie et al., 2014). The final consequences at individual level, is a reduction in biomass and reproduction output, while even generating mutations in germinal lines with clear effects on the following generations (Kaling et al., 2015).

The negative effects of UV-B radiation can be prevented in part by accessory pigments such as carotenoids, xanthophylls, and flavonoids, which act in concert as protective screen and regulating the oxidative stress (reactive oxygen species; ROS) (Sequeida et al., 2012). Flavonoids are the most ubiquitous group of natural polyphenols known for their photoprotective and antioxidant role (Henry-Kirk et al., 2018). In particular, the flavonol quercetin has been described as a remarkable ROS scavenger due to its structural properties; thus, it strongly controls oxidative damage (Shourie et al., 2014). Studies in *Arabidopsis thaliana* have shown that the protein encoded by *UV RESISTANT LOCUS 8* (*UVR8*) controls the expression of numerous genes involved in acclimatization and protection against UV-B radiation (Hayes et al., 2017). The genes regulated by UVR8 include precursors of flavonoid biosynthesis, morphogenic regulation of leaves and defense against herbivory (Morales et al., 2015). Recently, great advances have been made in identifying components involved in this specific UV-B signaling pathway (Yang et al., 2018). These components, in addition to UVR8, include constitutive photomorphogenesis 1 (COP1) and the transcription factors bZIP and elongated hypocotyl 5 (HY5). UVR8 accumulates in the nucleus in response to UV-B radiation, where it recognizes *cis*-acting responsive elements present in the promoter region of the gene *HY5*, commanding their expression (Moriconi et al., 2018). COP1 is required for activation of the *HY5* gene stimulated by UV-B (Loyola et al., 2016). In *UVR8*- or *COP1*-defective *A. thaliana* mutants, an increase in sensitivity to UV-B radiation has been described (Ballaré et al., 2011). UVR8 regulates, through a HY5-dependent mechanism, most of the flavonoid biosynthetic genes, namely *CHS, CHI*, and *FLS1* (Müller-Xing et al., 2014), and specifically of flavonols (Ayabe et al., 2010; Martens et al., 2010). These compounds absorb UV-B light in the 280–320 nm range, and their concentration increases in plants exposed to abiotic environmental stress, including the UV-B. Thus, it has been suggested that these flavonols act as protection filters for UV-B radiation in plants (Lois, 1994).

Antarctica is considered one of the most inhospitable environments on earth (Korckzak-Abshire et al., 2011). Despite this, there are two species of vascular plants that have been able to successfully colonize this continent, *Deschampsia antarctica* and *Colobanthus quitensis* (Parnikoza et al., 2011). The Antarctic plant *C. quitensis* is a small dicotyledonous perennial herb that lives from southern Mexico to the Antarctic Maritime (68°42′S) (Moore, 1970; Convey, 1996). Due to its wide latitudinal range of distribution and low connectivity between populations, genetically differentiated and ecologically adapted populations can be distinguished (Acuña-Rodríguez et al., 2017). To cope with the hostile Antarctic environment, these plant species have developed different physiological and biochemical mechanisms among which the accumulation of flavonoids and carotenoids play a role in photoprotection against UV radiation (Pereira et al., 2009). In addition, several works showed that the association of plants with endophytic microorganisms (fungi and bacteria) isolated from Antarctica allow other host plants tolerating harmful environmental conditions (Molina-Montenegro et al., 2016; Gallardo-Cerda et al., 2018; Ramos et al., 2018; Acuña-Rodríguez et al., 2019). Although an increasing number of publications are highlighting the role of symbiotic microorganisms boosting plant growth and defense (Hamilton et al., 2012; Pieterse et al., 2014), seldom studies have clearly linked plant performance under a given stress factor, with the symbiont-mediated changes in gene expression modulating such physiological response (see e.g., Hereme et al., 2020). Occurring plants of *C. quitenis* in Antarctica have been found to host diverse group of fungal endophytes (Rosa et al., 2010). In a previous work with the same species, we recently observed that the endophytic fungi increase total biomass and number of flowers under high UV-B radiation, while modulating the hormonal systems in a complex way (Ramos et al., 2018). Although the expected UV-B mediated activation of defense signaling pathways by the UV-B radiation (Ballaré et al., 2011) was evident in endophyte-free plants, there was not a clear response in endophyte-symbiotic plants. The effect of the UV-B in reducing the levels of the growth hormones (indole-3-acetic acid and abscisic acid) was more pronounced in symbiotic plants than in endophyte-free plants which, counterintuitively, contrasts the observed pattern in biomass and flowers (Ramos et al., 2018).

In order to some shed light on this puzzle, here we present results from an experiment aimed at evaluating the effect of UV-B radiation on the performance of *C. quitensis* plants with and without fungal endophytes. We sought to link the endophyte-mediated effects on the host plant molecular responses to UV-B radiation with that at biochemical and physiology levels. In particular, we assessed the expression of genes involved in the UV-B signal transduction pathway and in the biosynthesis of photoprotective compounds as flavonols. Finally, the flavonol abundance was quantified in plant leaves of *C. quitensis* with and without the presence of Antarctic native endophytic fungi.

MATERIALS AND METHODS

Study Site, Generation of Plant Material and Growth Conditions

Colobanthus quitensis plants were collected at the Polish Antarctic Station "Henryk Arctowski," South Shetland Islands (62°09′S), during the 2016–2017 growing season. We collected 30 plants with ≈150 g of rhizospheric soil (to be used later to prepare the sterile soil mix), selecting individuals that were separated by at least one meter from each other. The plants were transported in plastic containers to the Plant Ecology Laboratory of the University of Talca, Talca, Chile (35°24′S). In the laboratory, the plants were replicated by vegetative propagation to increase the biological material, based on Zuñiga et al. (2009). Briefly, nodal segments from a plant cushion were transplanted 100 mL pots filled with sterile soil mix of sand:soil:peat (4:4:1). The new leaves were developed around 3 months after propagation, prior to UV-B radiation treatment. With reference to Zuñiga et al. (2009), the 6-month-old plants (2–4 cm diameter) were maintained for 30 days in a growth chamber at 4°C, with a photoperiod of 21/3 light/dark (350 μmol photons $m^{-2}s^{-1}$), until the experiment. The plants were irrigated every 3 days with 10 mL of tap water and fertilized with the nutrient solution Phostrogen (0.2 mg L^{-1}).

Because we wanted to evaluate the role of just fungal endophytes on the plants, we subjected the biological material to an antibiotic treatment. All the plants were first treated with the broad-spectrum systemic antibiotic rifampicin (50 μg mL^{-1}) to eliminate bacteria [for details, see Torres-Díaz et al., 2016; Gallardo-Cerda et al., 2018). To evaluate the effectiveness, at the end of the antibiotic treatment, a random sample of tissues from ten plants was taken and cultured on LB agar for bacterial growth. Ten Plates were incubated at 28°C for 3 days. No bacterial colonies were observed growing on the culture media.

In order to obtain plants free of endophytic fungi, half of the clones from each plant were treated with fungicide to obtain plants free of endophytic fungi. Endophytic fungi-free plants (E-) were obtained by applying 2 g L^{-1} of the commercial fungicide Benlate every 3 days [containing Benomyl [methyl 1-[(1-butylamino) carbonyl]-1H-benzimidazol-2-yl] carbamate (DuPont, Wilmington, DE, United States)]. This fungicide treatment has been previously tested on plants of *Colobanthus quitensis* and *Lolium perenne* (Dernoeden and McIntosh, 1991; Torres-Díaz et al., 2016; Hereme et al., 2020) showing very high effectiveness in removing fungi from the whole plant and no apparent phytotoxic effects has been observed. Water was used as control of the fungicide treatment in E+ plants. Two weeks after the fungicide treatment, five E+ and five E− plants were inspected under microscope to verify the presence and/or absence of endophytes; root and shoot tissues were stained with Pelikan blue writing ink (**Supplementary Figure S1**; Hosseini et al., 2017; Oses Pedraza et al., 2018). Only after passing the set criteria (Gallardo-Cerda et al., 2018), fungicide-treated and untreated plants from each group were considered as endophytic (E+) and non-endophytic (E−) clones, respectively. Previously, the five most abundant fungal endophytes isolated from samples obtained from *Colobanthus quitensis* were identified as *Penicillium chrysogenum* (62%), *Penicillium brevicompactum* (27%), *Alternaria* sp. (6%), *Phaeosphaeria* sp. (3%), *and Eupenicillium osmophilum* (2%) (Hereme et al., 2020).

Fungal Endophyte Effect on the Physiological and Biochemical Performance of *C. quitensis* Under UV-B Radiation

All the E+ and E− *C. quitensis* plants were acclimated for 2 days at 4°C prior to UV-B radiation treatment based on Ramos et al. (2018). Briefly, treatment of 46 μmol photons m^{-2} s^{-1} of UV-B radiation (10 μW cm^{-2}), provided by fluorescent tubes (PL-L 36W/01/4P UV-B, Hammond, IN, United States), which are narrowband UV-B tubes with a narrow peak of UV-B at approximately 313 nm was applied (Sager and McFarlane, 1997). Control plants without UV-B radiation treatment, were generated by the presence of normal fluorescent tubes used in the culture chambers with Mylar film (Du-Pont, Wilmington, DE, United States) to absorb the radiation of wavelengths less than 320 nm. The intensity of UV-B radiation was measured with a UV light meter (Sentry ST-513, Taiwan, China). UV-B intensity treatment was considered based on natural conditions from the average UV-B radiation reported in Antarctica (Day et al., 2001; Navarrete-Gallegos et al., 2012). A total of 160 *C. quitensis* plants were randomly assigned to any of the two groups of treatments: I and II) 80 plants with and without endophytes (40 plants E+ and 40 plants E− both without UV-B radiation, respectively) were used as control of UV-B radiation treatment, while III and IV) 80 plants with and without endophytes (40 plants E+ and 40 plants E− 46 μmol photons m^{-2} s^{-1}, respectively) were used for the UV-B radiation treatment. All treatments were performed with a 21/3 light/dark photoperiod. The evaluation of the different parameters on the plants were carried out at the beginning (0), 6, 24, and 48 h since the radiation treatments were imposed. In each time and UV-B intensity, ten plants were removed from each treatment and shoot material was collected, frozen in liquid nitrogen and maintained at −80°C until further analysis.

To assess whether the fungal endophytes increased the tolerance of *C. quitensis* to UV-B radiation, biochemical and physiological parameters were analyzed. All the analytical determinations were performed on shoot tissues from each plant. The oxidative degradation of lipid molecules that leads to high levels of malondialdehyde (MDA) is considered a useful index of general lipid peroxidation (Møller et al., 2007). The level of MDA was determined by the thiobarbituric acid (TBA) reaction method (Heath and Packer, 1968). For this purpose, three biological replicates (single plant at each) were assessed from each condition (time of exposure and presence/absence of endophyte). One milliliter of 20% trichloroacetic acid reagent (TCA) was added to the tissue previously pulverized in liquid nitrogen (0.1 g) and mixed by agitation for 10 mins. The homogenate was centrifuged at 10,000 × *g* for 10 min at room temperature and the supernatant was used for the MDA determination. One milliliter of TCA (20%) and TBA (0.5%) (w/v) was added to 250 μL of the supernatant, which was then incubated at 100°C for 30 min

in a dry bath (Thermolyne 16500, Marshall Scientific) before being chilled on ice. The complex formed by MDA and TBA produces a colorimetric reaction, and absorbance was determined in a spectrophotometer (Unicam model UV/Vis Spectrometer) at 532 and 600 nm. The level of MDA was calculated using an extinction coefficient of 155 mm^{-1} cm^{-1} using the standard equation for weight in grams for each sample: MDA = [((A532 − A600) × 1/155)/g] × 1,000,000.

During the exposure of the plants to UV-B radiation, the photochemical efficiency of PSII was estimated in accordance with Maxwell and Johnson (2000). For this purpose ten plants were assessed for each condition (time of exposure and presence/absence of endophyte) using a pulse-modulated amplitude fluorimeter (FMS 2, Hansatech, Instrument Ltd., Norfolk, United Kingdom). Plant adaptation was carried out for 30 min prior to UV-B treatment using a black box (30 × 20 × 15 cm) to ensure maximum photochemical efficiency based on Torres-Díaz et al. (2016).

Sequence Analysis

Sequences of interest (*UVR8*, *HY5*, and *FLS*) were obtained from an RNAseq transcriptome from *C. quitensis* plants exposed to UV-B radiation (unpublished data). Full-length transcripts (see **Table 1**) were translated using the ExPASy Translate Tool[1]. The amino acid sequences were analyzed by a multi-alignment carried out using ClustalW and BioEdit Sequence Alignment Editor v7.0 software. Phylogenetic tree construction was performed by neighbor-joining (with 1,000 bootstrap replicates) using MEGA X software (Kumar et al., 2018). For each deduced protein, the isoelectric point and molecular mass were predicted using the Compute pI/Mw tool[2].

Relative Expression Analysis of Genes Involved in Response to UV-B Radiation

Total RNA from shoot tissue was extracted with PureLink® Plant RNA Reagent (Invitrogen, United States). DNA traces were removed using the TURBO DNA-free™ kit (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's instructions. Three independent RNA extractions (a single plants at each) were performed from each group of frozen samples. cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer's instructions.

The transcription levels were measured by quantitative PCR (qRT-PCR) using an Mx3000P system (Agilent Technologies, Santa Clara, CA, United States) based on Ramos et al. (2012). All reactions were performed using the Brilliant SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA, United States) according to the procedure described by the manufacturer. For each biological replicate of cDNA (n = 3), qRT-PCR reactions were carried out in triplicates (technical replicates) using 10 μL of Master Mix, 10 μM of each primer, 1 μL of diluted cDNA and nuclease-free water to a final volume of 20 μl. Fluorescence was measured at the end of each amplification cycle. The amplification was followed by a fusion curve analysis with continuous acquisition of fluorescence data during melting at 65–95°C.

TABLE 1 | Nucleotide sequence of primers used in the RT-qPCR, efficiency and genbank accession.

Gene	Primer Forward/reverse	Efficiency (%)	GenBank accession no.
α-TUB	F: TCGGAGGAGGAGATGATG R: GGAAGAGTTGACGGTATGTT	**102**	GCIB01004125.1
B-TUB	F: TCGTCTCCACTTCTTCAT R: GTCCTCATCTTACCTCTG	**106**	GCIB01008463.1
UVR8	F: ACGTGTTAAGCCCTGCTTGT R: CCGGTTCCTAACTGCCCAAA	**105**	MH643782
HY5	F: AATGGAGAGACAGAGACGAGC R: TTTTGAACGTCGTGGAGGTCA	**103**	MH643781
FLS	F: TCAGAGCACGAAGTGGGC R: GCTAGAAGATGGTTTACTG AGGAAG	**101**	MH643780

The raw data were analyzed manually, and the expression was normalized with the genes coding for α-*tubulin* and β-*tubulin*, which were obtained from GenBank[3]. The primers used for qRT-PCR analysis are shown in **Table 1**. For each gene, a standard curve was generated using a serial cDNA dilution, and the resulting PCR efficiency calculations were imported into the analysis of the relative expression data. The Excel macro (Microsoft) GENEX v1.10 (gene expression analysis for iCycler iQ® Real-time PCR detection system, v1.10, 2004; Bio-Rad Laboratories, United States) that is based on the algorithm of Vandesompele et al. (2002) was used to analyze the raw data obtained from the thermocycler.

Determination of Flavonol Contents

The extraction of flavonols was carried out based on a previous report (Ramos et al., 2016). Briefly, leaf samples were pulverized in liquid nitrogen using a mortar. Each biological replicate consisted of frozen shoot tissue (0.1 g) from one plant. All experiments were conducted using three biological replicates. Samples were treated with 1.5 mL of methanol/H_2O (4: 1, v/v) and sonicated for 10 min. The supernatant obtained after centrifugation at 3,000 × g for 10 min was filtered through 0.22 m cellulose disks. The supernatant containing glycosylated flavonols was hydrolyzed with one volume of 2N HCl and subjected to 70°C for 40 min to obtain the aglycones. The aglycones of flavonols were obtained by treatment with an equal volume of ethyl acetate and centrifugation at 15,600 × g for 10 min, which separated the compounds of interest from the aqueous phase. The organic phase was evaporated in a CentriVap® (Labconco., Kansas City, United States) and obtained pellets were dissolved in 0.5 mL dimethylsulfoxide (DMSO). The analysis was performed in an Agilent 1100 series HPLC system provided with a photodiode array detector (DAD) based on Ramos et al. (2016). Compounds were separated on a reverse-phase C18 analytical column (Kromasil 100, 25 cm × 4.6 mm × 5 μm), and data were analyzed with a ChemStation software chromatography system

[1]http://ca.expasy.org

[2]http://web.expasy.org/compute_pi/

[3]https://www.ncbi.nlm.nih.gov

(Hewlett-Packard). Quantifications were carried out between the wavelengths of 280 and 600 nm, monitoring flavonols at 345 nm.

Statistical Analysis

For the determination of significant differences in the levels of MDA, Fv/Fm, relative expression and flavonol content, two-way ANOVA was used with the factors exposure time (h) and presence of endophytic fungi. For all analyses, the assumptions of normality and homogeneity of variances were tested using the Shapiro-Wilks and Bartlett tests, respectively. Subsequently, Tukey's HSD multiple comparisons analysis was performed. Differences were considered significant at $P \leq 0.05$. The analyses were performed with GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

UV-B Radiation Affects Photochemical Performance and Reduce Oxidative Damage of *C. quitensis* Depending on Endophyte Presence

The presence of fungal endophytes improved the tolerance to UV-B radiation by decreasing cell membrane lipid peroxidation. Under high UV-B radiation, the presence of fungal endophytes decreased MDA production (**Figure 1**). In addition, plants without endophytes (E-) showed significantly higher content of thiobarbituric acid reactivity and, thus, cell damage caused by exposure to UV-B radiation (**Table 2**). The accumulation of MDA significantly increased over the course of the experiment (0, 6, 24, and 48 h). This pattern was observed in both control plants and plants exposed to UV-B radiation with and without endophytes (**Figures 1A,B**). However, without symbionts, the values were significantly higher. When examining plants exposed to UV-B radiation, E+ plants showed significantly lower levels of MDA reactivity compared to E- plants (**Table 2**).

The physiological parameter photochemical efficiency of PSII under UV-B radiation was significantly affected by the UV-B treatment, with clear differences between E− and E+ plants (**Table 2**). In UV-B radiation control plants, the values of photochemical efficiencies were similar for both E− and E+ plants. However, E− plants exposed to UV-B radiation displayed a significant reduction in photochemical efficiency compared to both, E- plants under UV-B radiation control and to E+ plants in any of the UV-B situations (**Table 2**). This latter effect was mainly evident after 48 h of UV-B treatment.

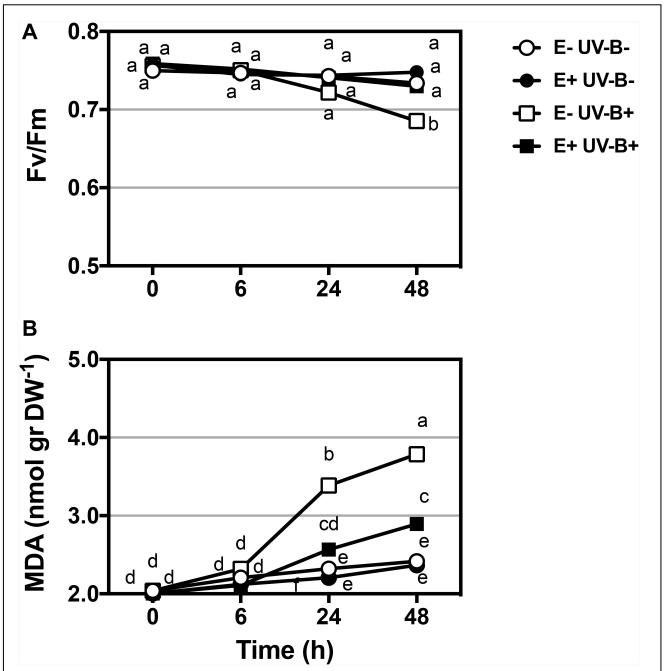

FIGURE 1 | Physiological performance (lipid peroxidation and PSII photochemical efficiency - Fv/Fm) in *Colobanthus quitensis* exposed to UV-B radiation with and without endophytes. PSII photochemical efficiency (Fv/Fm) **(A)** and Malondialdehyde MDA **(B)** variation in the leaves of *Colobanthus quitensis* were evaluated under UV-B radiation. Control (without radiation UV-B radiation) and UV-B (46 μmol m^{-2} s^{-1}) radiation in plants with and without endophytes (E+ and E-) were analyzed. Different letters indicate significant differences ($P < 0.05$; two-way ANOVA). Bars represent means ± SE from three independent experiments.

Identity and Expression Analysis of Signal Transduction and Flavonol Biosynthesis Related Genes

Three sequences encoding UVR8, FLS, and HY5 proteins were identified in a transcriptomic analysis of *C. quitensis* exposed to high UV-B radiation and are now available in GeneBank (**Table 1**). These were highly represented in the RNAseq library (partially annotated) from UV-B-exposed plants and showed strong identity to the UVR8, FLS, and HY5 plant proteins. Multiple alignment analysis and phylogenetic classification of the full-length deduced protein sequences displayed high sequence homology to other UVR8, FLS, and HY5 proteins (**Figure 2**). The deduced UVR8 protein shares 81.3% identity with the reported protein in *Spinacea oleracea* and 79.3% identity with a protein from *Chenopodium quinoa* (**Supplementary Figure S2**). The deduced FLS protein shares 66% identity with the sequence reported in *Nicotiana tabacum* and 65% identity with the one from *Glycine max*. The multiple sequence alignment confirms the presence of the conserved 2-ODD motifs 1 and 2 and also the conserved iron-binding sites (**Supplementary Figure S3**). The deduced HY5 protein shares 79% identity to *S. oleracea* and 72% to the reported *A. thaliana* sequences. Multiple alignment analysis shows that there are conserved domains to the DNA-binding and dimerization domains of the bZIP-class proteins (**Supplementary Figure S4**).

CqUVR8 displays an open reading frame (ORF) of 1,308 bp that encodes a protein of 435 amino acids with a molecular weight (Mw) 46.43 kDa and an isoelectric point (pI) 5.50. In the case of *CqFLS*, an ORF of 1,019 bp encoding a protein of 339 amino acids with a calculated Mw of 38.99 kDa and a pI of 6.68 was observed. *CqHY5* has an ORF of 515 bp, encoding a protein of 171 amino acids with a Mw of 18.54 kDa and a pI of 9.74.

TABLE 2 | Results of factorial two-ways ANOVA evaluating the interactive effects of endophytes presence (E) and different times of treatment for (control: without radiation UV-B radiation, UV-B: 46 μmol m^{-2} s^{-1}) for photochemical performance, measured as maximum quantum yield (Fv/Fm) of photosystem II (PSII) and MDA (mmol mL g FW^{-1}) of *Colobanthus quitensis*.

	Source of variation	df	MS	F	P
Control	**MDA (mmol mL^{-1} g FW^{-1})**				
	Treatment time (T)	4	0.2681	514.6	**P < 0.0001**
	Endophytes (E)	1	0.0898	172.4	**P < 0.0001**
	T × E	4	0.01312	25.19	**P < 0.0001**
	Error	20	0.000521		
	Photochemical efficiency (Fv/Fm)				
	Treatment time (T)	4	0.0009665	14.49	**P < 0.0001**
	Endophytes (E)	1	0.001521	22.8	**P < 0.0001**
	T × E	4	0.0003835	5.748	**P < 0.0004**
	Error	90	0.00006671		
UV-B	**MDA (mmol mL^{-1} g FW^{-1})**				
	Treatment time (T)	4	3.015	1050	**P < 0.0001**
	Endophytes (E)	1	2.696	938.8	**P < 0.0001**
	T × E	4	0.3017	105.1	**P < 0.0001**
	Error	20	0.002872		
	Photochemical efficiency (Fv/Fm)				
	Treatment time (T)	4	0.04178	279.7	**P < 0.0001**
	Endophytes (E)	1	0.03652	244.5	**P < 0.0001**
	T × E	4	0.01273	85.26	**P < 0.0001**
	Error	90	0.0001493		

Significant P values (P < 0.05) are highlighted in bold. df: degrees of freedom, MS: mean squares, F: F-test value, P: probability value.

Expression of *UVR8* and *HY5* in plants exposed to UV-B radiation was significantly affected by the presence of fungal endophytes, exposure time and the interaction between both factors (**Table 3**). The qRT-PCR analysis of *UVR8* showed an increase in expression levels at 24 h compared to time 0, but no significant differences between E− and E+ plants were observed under UV-B radiation control conditions (**Figure 3A**). In plants exposed to UV-B radiation, the expression levels of *UVR8* showed a significant upregulation at 6 h compared to that of the control, with the highest expression at 24 h in E−, which was 2.5-fold higher than the expression observed in E+ plants. As can be observed in **Figure 3** in E+ plants after 24 h compared to the E− plants *UVR8* levels remain low, suggesting that fungal infection protects the plants avoiding the upregulation of this genes (**Figure 3B**).

Analysis of the relative expression levels of *HY5* in control plants showed a significant increase after 24 h compared to 0 h, in both E− and E+ plants, indicating that the fungus did not induce changes in its expression (**Figure 3C**). Under UV-B radiation treatment, *HY5* expression significantly increased at 24 h in E− compared to E+ plants, to then decrease. In addition, no differences were observed in E+ plants during the time-course experiment (**Figure 3D**).

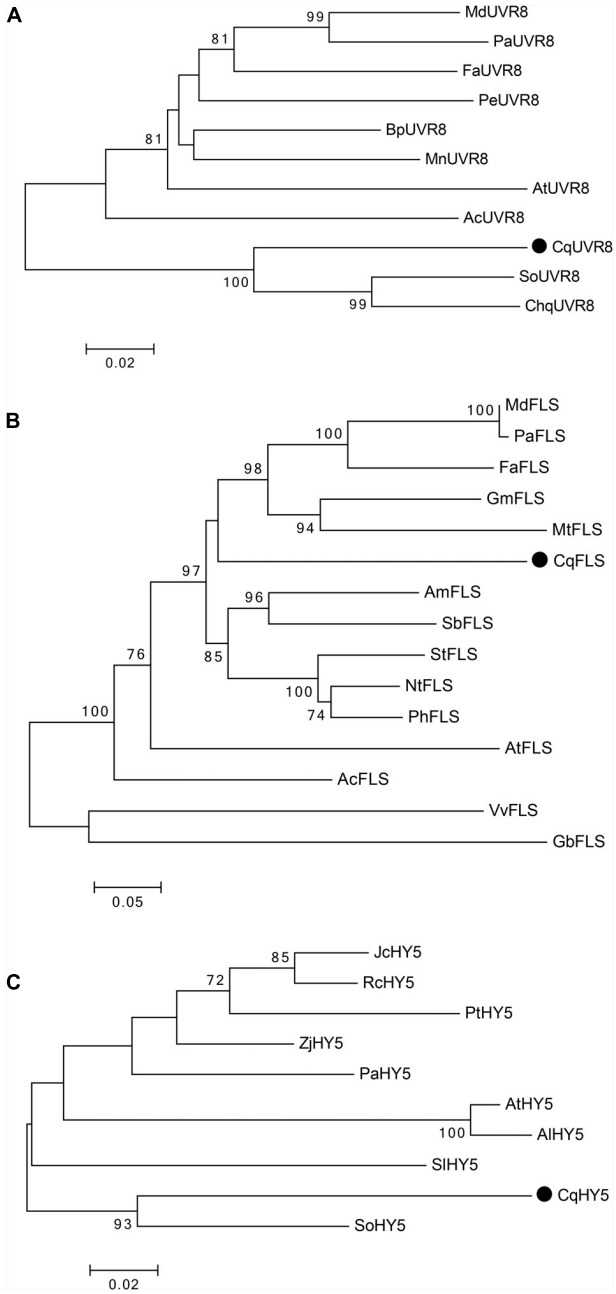

FIGURE 2 | Phylogenetic relationships of the deduced UVR8, FLS, and HY5 proteins from *C. quitensis* with several plant UVR8, FLS and HY5 proteins. **(A)** Phylogenetic analysis was performed using the following plant UVR8 proteins: *Ananas comosus* (XP_020107421), *Arabidopsis thaliana* (AAD43920), *Betula platyphylla* (AHY02156), *Chenopodium quinoa* (XP_021734620), *Fragaria x ananassa* (AOD75227), *Malus domestica* (NP_001315969), *Morus notabilis* (XP_024019180), *Populus euphratica* (AKJ54489), *Spinacea oleracea* (XP_021836012), and *Prunus avium* (XP_021829535). The deduced UVR8 protein of Colobanthus is indicated with a black dot. **(B)** For FLS, the following proteins were used for the phylogenetic analysis: *Glycine max* (NP_001237419), *Allium cepa* (AAT68476), *Vitis vinifera* (BAE75810), *Ginkgo biloba* (ACY00393), *Arabidopsis thaliana* (AAB41504), *Malus domestica* (AAD26261), *Fragaria x ananassa* (AAZ78661), *Nicotiana tabacum* (ABE28017), *Antirrhinum majus* (ABB53382), *Petunia x hybrida*

(Continued)

FIGURE 2 | Continued
(CAA80264), *Solanum tuberosum* (CAA63092), *Prunus avium* (AFO67943), *Medicago truncatula* (AES71332), and *Scutellaria baicalensis* (AHA14501). The deduced FLS protein of Colobanthus is indicated with a black dot. **(C)** For HY5, phylogenetic analysis was performed with the following plant HY5 proteins: *Arabidopsis thaliana* (BAA21116), *Arabidopsis lyrata* (XP_002873502), *Jatropha curcas* (XP_012076602), *Populus trichocarpa* (XP_002308656), *Ziziphus jujuba* (XP_015885857), *Ricinus communis* (XP_002515537), *Spinacea oleracea* (XP_021837612), *Solanum lycopersicum* (NP_001234820), and *Prunus avium* (XP_021827650). The deduced HY5 protein of Colobanthus is indicated with a black dot.

TABLE 3 | Results of factorial ANOVAs evaluating the interactive effects of endophytes presence (E) and different treatment times for (control: without radiation UV-B radiation, UV-B: 46 μmol m^{-2} s^{-1}) for relative level of expression of genes responding to UV-B by qPCR in the leaf of *Colobanthus quitensis*.

	Source of variation	df	MS	F	P
Control	***UVR8***				
	Treatment time (T)	4	42.53	38.37	**P < 0.0001**
	Endophytes (E)	1	6.265	5.653	**P = 0.0275**
	T × E	4	0.086	0.0782	P = 0.9881
	Error	20	1.108		
	HY5				
	Treatment time (T)	4	73.95	69.04	**P < 0.0001**
	Endophytes (E)	1	5.599	5.228	**P = 0.0333**
	T × E	4	2.082	1.944	P = 0.1424
	Error	20	1.071		
	FLS				
	Treatment time (T)	4	6074	273.9	**P < 0.0001**
	Endophytes (E)	1	53.55	2.414	P = 0.1359
	T x E	4	82.65	3.726	**P = 0.0201**
	Error	20	22.18		
UV-B	***UVR8***				
	Treatment time (T)	4	163.8	22.33	**P < 0.0001**
	Endophytes (E)	1	222.4	30.32	**P < 0.0001**
	T × E	4	41.31	5.631	**P < 0.0033**
	Error	20	7.336		
	HY5				
	Treatment time (T)	4	195.2	35.69	**P < 0.0001**
	Endophytes (E)	1	144	26.33	**P < 0.0001**
	T x E	4	111	20.29	**P < 0.0001**
	Error	20	5.469		
	FLS				
	Treatment time (T)	4	5382	76.52	**P < 0.0001**
	Endophytes (E)	1	4246	60.37	**P < 0.0001**
	T × E	4	3986	56.68	**P < 0.0001**
	Error	20	70.33		

Significant P values (P < 0.05) are highlighted in bold. df: degrees of freedom, MS: mean squares, F: F-test value, P: probability value.

Finally, expression levels of *FLS* were analyzed, learning that in the control treatment they were significantly higher at 48 h in E− compared to E+ (**Figure 3E**). In plants exposed to UV-B radiation, a significant upregulation was observed at 24 h compared to the other times of UV exposure in E− compared to E+ plants, but no differences were observed between different times treatment in E+ plants (**Figure 3F**).

Endophytes Affects Flavonols Accumulation in Response to UV-B Radiation

To evaluate changes in flavonol contents, plants exposed to UV-B radiation with and without endophytes were analyzed. In the control and treatment groups, the accumulation of kaempferol remained stable throughout the time course experiment in both E− and E+ plants (**Figures 4A,B**).

Regarding quercetin levels, in the control group, the accumulation remained stable (**Figure 4C**). The plants exposed to UV-B showed a significant increase in the levels of quercetin, specifically at 48 h, which displayed an 8-fold higher content in E- plants (171.72 μg mg FW^{-1}) compared E + (22.37 μg mg FW^{-1}) (**Figure 4D**). Accumulation of quercetin in plants exposed to UV-B radiation was significantly affected by the presence of fungal endophytes, exposure time and the interaction between both factors (**Table 4**).

DISCUSSION

The decrease in stratospheric ozone since the mid-1970s has led to a significant increase in UV-B radiation over Antarctica (Ryan et al., 2009). Such changes add extra stress to the plants present in this region, which has boosted additional interest in elucidating the response mechanisms that could be involved in facilitating the survival of sessile organisms such as plants under these unfavorable conditions. Thus far, most studies focused on the physiological, morphological and reproductive performance effects of UV-B radiation in plants, including *C. quitensis* (Navarrete-Gallegos et al., 2012; Ramos et al., 2018). However, the underlying molecular mechanisms remain poorly understood. In order to improve our knowledge on the underlying molecular mechanisms, we evaluated the UV-B effects on *C. quitensis* at the ecophysiological level. Hereby, we were able to describe some of the molecular mechanisms involved in the response to UV-B stress. At the same time, we assessed how extrinsic biotic factors, such as the presence of fungal endophytes, can modulate the plant response to UV-B stress.

The presented data underline the fact that the presence of a fungal endophyte can positively affect UV-B tolerance in *C. quitensis* plants. Plants that contain the fungal endophytes appear to improve their photochemical efficiency. In addition, we observed changes in gene expression and metabolite production, that can help to explain how this Antarctic plant copes with stress conditions exerted by UV-B radiation.

At the biochemical level, the presence of fungal endophytes improved the tolerance to UV-B radiation by decreasing cell membrane lipid peroxidation, as accounted by MDA production (**Figure 1**). At the molecular level, this study is the first one to evaluate genes that respond to UV-B radiation, and genes associated with the biosynthesis of flavonols in plants of *C. quitensis* modulated by fungal endophytes. It was observed that *UVR8* transcript levels increased earlier than those of *HY5* and *FLS* genes in the leaves of plants exposed to UV-B radiation. Such expression dynamics suggest that UVR8 could control a complex

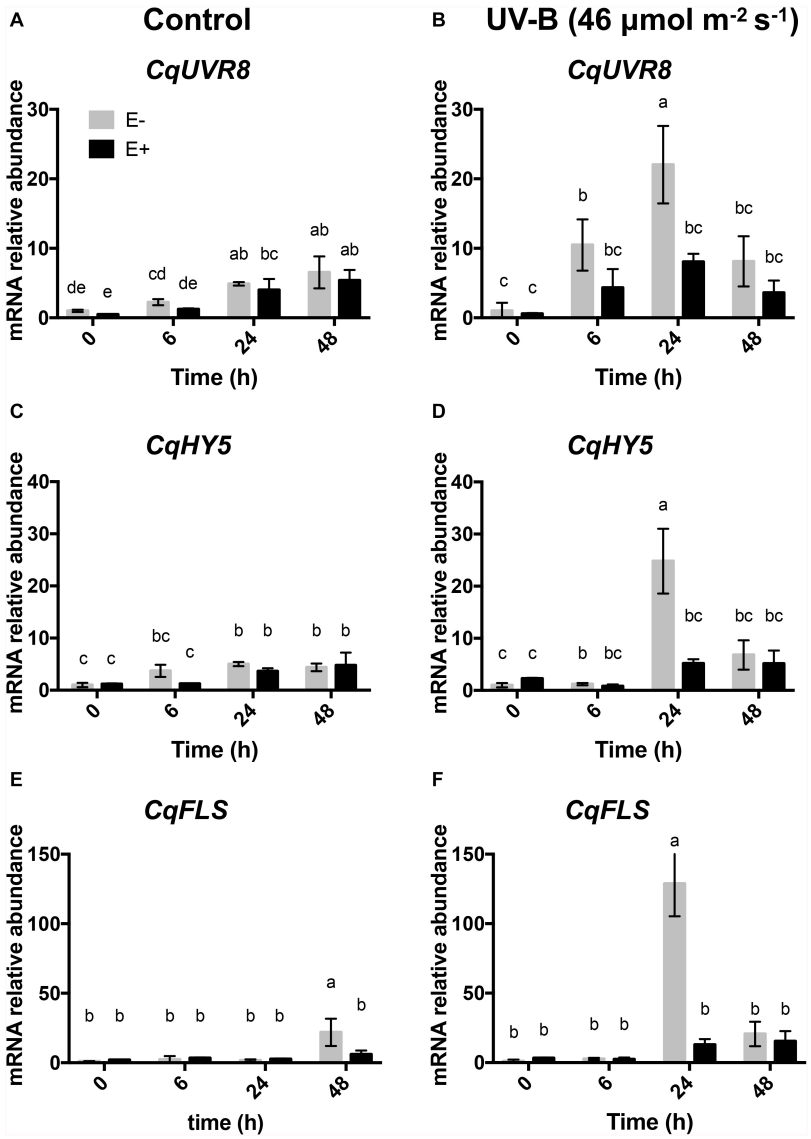

FIGURE 3 | Transcript profile of genes in response to UV-B treatment in the leaves of *C. quitensis*. Transcript levels of *UVR8*, *HY5*, and *FLS* were evaluated in plants exposed to control [without radiation UV-B radiation **(A,C,E)**] and with UV-B [46 (μmol m^{-2} s^{-1} **(B,D,F)**] radiation and with and without endophytes (E+ and E–). For relative transcript levels, normalization was performed against housekeeping genes (α-tubulin and β-tubulin) in all samples. Different letters indicate significant differences ($P < 0.05$; two-way ANOVA). Bars represent the means ± SE of three independent experiments.

genetic program involved in acclimatization and protection against UV-B radiation. In turn, the increase in expression of *HY5* and *FLS* occurred 24 h after the plant exposure to UV-B radiation, triggering the accumulation of flavonols in the aerial tissues.

In *Vitis vinifera* L., variation in the expression of *UVR8, HY5*, and *HYH* has been described, showing a great dependence on the organ, plant developmental stage as well as the type of radiation (Liu et al., 2015). In consequence, it has been proposed that the UV-B response machinery in *V. vinifera* would be mainly triggering the accumulation of flavonols in the fruits through the activation of genes, such as *HY5* and *HYH*, both at high and low levels of UV-B exposure (Loyola et al., 2016). In *A. thaliana,* it has been described that UVR8 controls the expression of several genes involved in acclimatization and protection against UV-B radiation, including those involved in the biosynthesis of flavonoids (phenolic protectants) (reviewed in Ballaré et al., 2011). These metabolites are among the most abundant natural phytochemical compounds with several biological functions that confer tolerance to stress in plants (Henry-Kirk et al., 2018).

In the study by Carrasco-Ríos (2009), it was suggested that high tolerance to UV-B radiation in the Antarctic plants could be attributed to their ability to activate enzymatic and non-enzymatic antioxidant systems, and that secondary metabolites playing an important role in protecting leaves from the highly damaging energy. The increase in the production of secondary metabolites such as polyphenols and flavonoids is considered one

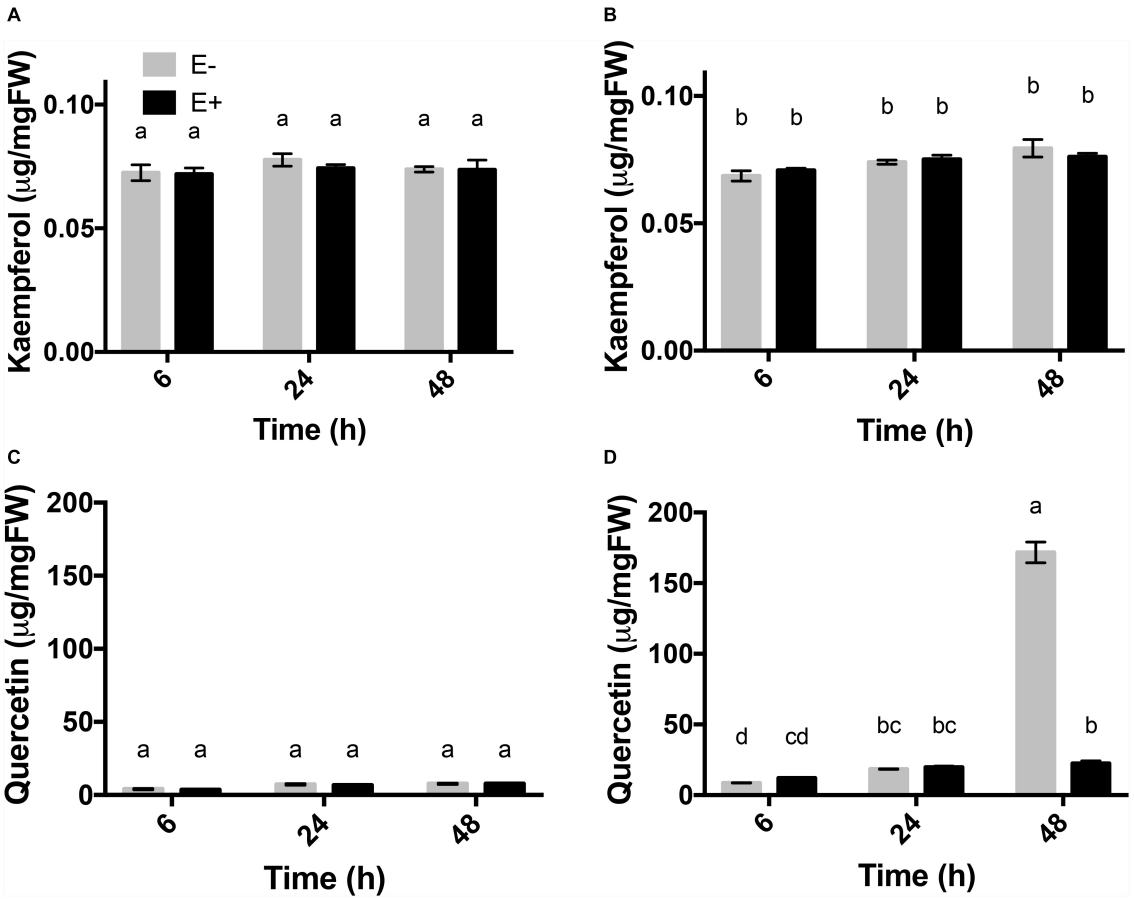

FIGURE 4 | Flavonols accumulation profile in response to UV-B radiation in leaves of *C. quitensis*. Flavonols content (μg/mg fresh weight) of kaempferol **(A,B)** and quercetin **(C,D)** in the leaves of *C. quitensis* obtained at different times of UV-B exposure were analyzed. Plants exposed to control [without radiation UV-B radiation **(A,C)**] and UV-B [46 μmol m^{-2} s^{-1} **(B,D)**] radiation and with and without endophytes (E+ and E-) were evaluated. Different letters indicate significant differences ($P < 0.05$; two-way ANOVA). Bars represent the means ± SE of three independent experiments.

of the key mechanisms of adaptation to UV-B radiation based on its antioxidant role against free radicals (Vasela et al., 2013; Liu et al., 2015). These metabolites accumulate in the epidermal cells of many plant and fruit species and have a radiation absorption capacity between 280 and 360 nm. Consequently, these metabolites alleviate the deleterious effect of UV-B light on different cellular components (Carrasco-Ríos, 2009). Our results suggest that the flavonol quercetin plays a relevant role in the response of endophyte-free plants to UV radiation. This is supported by the observation of an 8-fold increase in the levels of this metabolite in leaves 48 h after the exposure of plants to UV-B radiation. This finding is in agreement with previous data showing, in *Arabidopsis*, an accumulation of flavonols (e.g., saiginol) mainly in floral tissues, correlating with a greater tolerance to UV-B light (Tohge et al., 2016). In *V. vinifera*, it has been described that flavonols, especially the glycosides of quercetin and kaempferol, increase substantially after exposing the plants to UV-B radiation (Carrasco-Ríos, 2009).

To cope with harsh environmental conditions such as those found in Antarctica, plants have evolved their own mechanisms and those ones that arise from the association with beneficial microbial symbionts. Mutualistic interactions between plants and microorganisms, either fungi or bacteria, can confer several benefits to host plants, such as tolerance to drought, salinity, temperature, and heavy metals, that have been reported to positively influence plant persistence and growth (Rodriguez et al., 2004; Hamilton et al., 2012; Acuña-Rodríguez et al., 2020; Pérez-Alonso et al., 2020). Our results suggest that the presence of endophytic fungi has positive effects on the physiological and biochemical performance of *C. quitensis*, likely through the transcriptional modulation of key genes related with the plant response to UV-B radiation. Specifically, under UV-B radiation, the levels of lipid peroxidation were higher, and the Fv/Fm ratio was decreased in the absence of endophytes. Considering that a decrease in the Fv/Fm ratio indicates a reduction in the photochemical efficiency of the PSII and likely damage in the photosynthetic apparatus, our results indicate that endophytic fungi improved the capacities of the plant to respond to high UV-B radiation. The expression of the genes *UVR8, HY5*, and *FLS* was higher in plants without endophytes exposed to UV-B

TABLE 4 | Results of factorial ANOVAs evaluating the interactive effects of endophytes presence (E) and different treatment times for (control: without radiation UV-B radiation, UV-B: 46 μmol m^{-2} s^{-1}) for flavonol content (μg mg fresh $weight^{-1}$) of kaempferol and quercetin in the leaf of *Colobanthus quitensis*.

	Source of variation	df	MS	F	P
Control	**Kaempferol (μg mg FW^{-1})**				
	Treatment time (T)	3	0.00001968	2.080	P = 0.1433
	Endophytes (E)	1	0.00001317	1.392	P = 0.2553
	T × E	3	0.000003084	0.3259	P = 0.8066
	Error	16	0.000009464		
	Quercetin (μg mg FW^{-1})				
	Treatment time (T)	3	569.7	181.5	**P < 0.0001**
	Endophytes (E)	1	57.23	18.24	**P = 0.0006**
	T × E	3	47.47	15.13	**P < 0.0001**
	Error	16	3.138		
UV-B	**Kaempferol (μg mg FW^{-1})**				
	Treatment time (T)	3	0.000612	35.35	**P < 0.0001**
	Endophytes (E)	1	0.000047	2.718	P = 0.1187
	T × E	3	0.000054	3.141	P = 0.0544
	Error	16	0.000017		
	Quercetin (μg mg FW^{-1})				
	Treatment time (T)	3	15741	1992	**P < 0.0001**
	Endophytes (E)	1	2424	306.7	**P < 0.0001**
	T x × E	3	12410	1570	**P < 0.0001**
	Error	16	7.903		

Significant P values (P < 0.05) are highlighted in bold. df: degrees of freedom, MS: mean squares, F: F-test value, P: probability value.

radiation at early times of exposure (24 h), activating the UV-B specific signaling pathway, but this was not observed in endophyte-symbiotic plants. This observation is in line with reports in which endophytes regulate gene expression in plant hosts to increase stress tolerance (Sun et al., 2010; Xu et al., 2017; Dastogeer et al., 2018; Hereme et al., 2020). Thus, fungal endophytes could be involved in a trade-off to increase the physiological fitness, as demonstrated by higher photochemical efficiency, and to improve the biochemical mechanism, as demonstrated by lower cellular damage by lipid peroxidation. The fact that these differences appeared when plants were exposed to the environmental stress, suggests that the plant-endophyte association activates certain molecular mechanisms to improve the host plant fitness is context-dependent. This is in agreement with our previous study in which *C. quitensis* associated with extremophile fungal endophytes and exposed to high UV-B radiation displayed a strong decrease in ABA content in shoot tissues (Ramos et al., 2018). In relation with this observation, ABA has been described to promote the biosynthesis and accumulation of flavonols in *V. vinifera* (Berli et al., 2010, 2011).

It is important to note that plants exposed to UV-B radiation revealed a significant increase in levels of quercetin, specifically at 48 h, with higher levels observed in plants without endophytes compared to those with endophytes (**Figure 4**). This result is interesting, since it could suggest that until 48 h, the accumulation of quercetin could be the product of an intrinsic response of *C. quitensis* against UV-B radiation. These results suggest that in the presence of endophytic fungi, as a product of the ecophysiological state of the plant, the responses are delayed or the increase in flavonols are not necessary because other compensatory mechanisms are triggered. However, new studies considering higher levels of UV-B radiation intensity, increase in the evaluation period and the determination of the relative contribution of each member of the association with respect to quercetin could reveal more information to understand this pattern.

The association of plants with microbial endophytes appears to be key in the plant adaptation to stressful conditions. Studies with different degree of detail, show that not only fungal but also bacterial endophytes can be source of novel metabolites and metabolic functions which, in addition to those of host plants, increase the repertory to respond to the environmental variation (Hamilton et al., 2012; Truyens et al., 2015; Bastías et al., 2017, 2020; Acuña-Rodríguez et al., 2020; Pérez-Alonso et al., 2020). As summary, we had previously shown that endophytic foliar fungi associated with *C. quitensis* under UV-B radiation (5 and 30 μW cm^{-2}), improved the ecophysiological performance of individuals, with higher total biomass and higher number of flowers. Under the same condition, endophyte-symbiotic plants presented lower lipid peroxidation which could be regulated by changes in the hormonal content of salicylic acid, jasmonate, indole-3-acetate and abscisic acid (Ramos et al., 2018).

In this work, we evaluated the effect of fungal endophytes occurring in both above- and below-ground tissues of the plant. Therefore, we cannot rule out that root endophytes have also played a role in modulating the plant tolerance to UV-B radiation. However, we would like to suggest a more relevant role for leaf fungal endophytes. An argument for this is that not only the presence or absence of fungal endophytes are affecting the plant response to UV-B radiation, but it is also expected UV-B to affect the community of microorganisms of the phyllosphere (Jacobs and Sundin, 2001; Santhanam et al., 2017). Therefore, we propose that the modulation of flavonoids in the leaves exposed to sun, results from the positive feedback between the plant-endophyte interaction and the UV-B radiation.

Finally, we were able to link the endophyte presence with UV-B radiation linked gene responses to plant physiological parameters. Although here were separated bacteria from fungi, endophytes are suggested to play part of an integral response of plants to stress factors and should always be considered in future works.

CONCLUSION

The presented results strongly suggest that the functional symbiosis of *C. quitensis* plants with extremophile antarctic endophytic fungi modulate mechanisms in plants to allow them to cope with the stress caused by UV-B radiation. The presence of endophytic fungi improves the physiological performance of

C. quitensis by reducing cellular damage by means of eliciting the metabolic pathways involved in flavonol biosynthesis. At the molecular level, the presence of endophytic fungi in plants caused a late transcriptional response of the components of the UVR8 pathway, which include the genes *UVR8, HY5*, and *FLS*. However, the investigation on the genetic and biochemical basis of symbiotic plant-fungus communication is still in its infancy and the underlying mechanisms are poorly understood (Ramos et al., 2018; Acuña-Rodríguez et al., 2019).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available in GenBank and can be found in the NCBI database under the accession numbers: GCIB01004125.1, GCIB01008463.1, MH643782, MH643781, and MH643780.

AUTHOR CONTRIBUTIONS

AB, PR, and MM-M designed the experiments. AB performed the experiments. AB, MM-M and PR analyzed the data. RH prepared the plant material. AB, RH, SR-L, LL, PG, SP, MM-M and PR wrote the paper and all authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00122/full#supplementary-material

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Stress & Symbiosis: Heads or Tails?

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An increasing number of organisms are subjected to abiotic (e.g., air, water, and soil quality, temperature), but also biotic (e.g., new pathogens) stressors. These stressors may disturb the chemical and physiological homeostasis of living systems, and thus impact their ecology and evolution. Because eukaryotes are often associated with symbionts, these changes do not only impact the host but rather the holobiont, an assemblage of interacting species. Indeed, stressors can modify the symbiotic community composition and functions directly, but also indirectly through their impact on host physiology. Any disruption of the symbiotic homeostasis can then impact the host fitness. On the other side, several symbionts protect their host against various threats, and they may facilitate the adaptation of the holobiont to the new environment by limiting the negative impact of stress on the host. It now remains to clarify if their presence constitutes a driver of adaptation of the host or an obstacle limiting the selection of adaptive traits in the host, and to discuss if symbiosis is always the optimal strategy to cope with stressors. The reciprocal impact between stress and symbiosis can become more complex when stressors are considered in combination, as it occurs in nature. Indeed, synergistic or antagonistic effects may impact the holobiont response, and studies characterizing individual disturbances may not be sufficient. In the current context of climate change and globalized pollution, it is thus crucial to develop integrative approaches to predict how organisms, communities and ecosystems will face combinations of stressors.

Keywords: biotic and abiotic stressors, symbiotic communities, stress-response mechanisms, beneficial symbiosis, adaptation

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INTRODUCTION

Stress is an ambiguous term that causes confusion when attempting to understand organismal responses to environmental change, because it is often used to refer to both the environmental perturbation and the response itself (Schulte, 2014). To gain clarity, we will consider here the *stressor* as any biotic or abiotic environmental factor able to disrupt homeostasis, the *stress* as an actual or potential decline in the fitness of the organism after exposure to a stressor, and the *stress response* as the physiological and behavioral response to the stressor (see review on existing definitions of stress in Schulte, 2014). Depending on the nature of the stressor, its intensity and its occurrence (i.e., single, episodic, or chronic), the organism may adapt -or not- to the environmental stressor through the selection of plastic or genetic stress response mechanisms that

buffer the stressor (Rymer et al., 2016). This ability to persist after such stress is called resilience (Hodgson et al., 2015), but in some cases, the organisms cannot cope with the stressors, which strongly decrease their fitness.

A majority of organisms live in tight relationship with other organisms, and this assemblage is called holobiont. In this case, stressors may affect not only the host but also its symbiotic community (i.e., parasites, commensals, or mutualists), and the relationship between partners. A canonical example is the negative impact of global warming on the coral holobiont, which leads to symbiosis breakdown (Paxton et al., 2013), and reef ecosystem destabilization (Putnam et al., 2017). On the other side, some symbionts are described to protect their host against different kinds of stressors and can play a role in the adaptation of the holobiont to stressful environments (Miransari, 2010; Feldhaar, 2011; Flórez et al., 2015; Shapira, 2016; Hopkins et al., 2017).

Because global changes associated with anthropization impose stressors to many ecosystems, we need to better understand the role of symbiosis in the vulnerability or resilience to stressors. In this review, we will thus focus on acute stressors and directed changes in the environment, such as global change, and their interaction with symbionts across animals and plants. We will first develop some examples on the impact of such biotic and abiotic stressors on holobionts, and on the influence of symbionts on organism resilience (**Figure 1**). We will then discuss different factors that can influence the selection of specific stress response mechanisms, including genetic and phenotypic responses from the host and the symbionts, and debate if the association of organisms with symbionts is the optimal strategy to cope with stressors.

THE VULNERABILITY OF SYMBIOTIC ASSOCIATIONS TO STRESSORS

Symbiotic communities are very dynamic and often change over the host lifespan, in response to changes in host physiological states (life stages, immunity, etc.), but also to environmental perturbations. Many stressors strongly impact relative abundances and shift symbiotic communities of well-established symbioses, thus impacting host physiology, and at a larger scale, ecological communities.

Certain acute stressors can specifically modify the composition of symbiotic communities, as observed in many host species, such as sponges (Blanquer et al., 2016), scleratinian corals (Thurber et al., 2009), mice (Ravussin et al., 2012; Bharwani et al., 2016), plants (Erlacher et al., 2015; Santos-Medellín et al., 2017), or insects (Muturi et al., 2016). When host are colonized by a single symbiont, stressors can also directly impact symbiont density. For instance, a cold shock decreases *Cardinium* density in parasitoid wasps (Doremus et al., 2019). Elevated temperatures can also eliminate symbionts, like the gut bacteria necessary for the survival of the southern green stinkbug *Nezara viridula* (Kikuchi et al., 2016).

The impact of stressors on symbiotic communities can also be indirect, through the modification of the host niche where symbionts reside [e.g., in plants: nutrients, exudates, and signaling molecules (Hartman and Tringe, 2019)]. This is also particularly true for intracellular symbionts, whose density can decrease in response to the effect of stressors on host cell physiology (Moné et al., 2014). For instance, sublethal doses of insecticides (e.g., the neurotoxic molecule thiamethoxan), or pesticides (e.g., the pro-oxidant paraquat), respectively, decrease the density of *Buchnera* obligate symbiont in soybean aphids (Enders and Miller, 2016), and *Wolbachia* facultative endosymbionts in fruit flies (Monnin et al., 2016).

Stressors can also indirectly impact host-associated symbiotic communities through an effect on host immunity. Indeed, the disruption of host immunity can modify the control of symbionts, and/or allow opportunistic symbionts to invade the host (Williams et al., 2016). For instance, *Ostreid herpesvirus* infections cause an immune-compromised state in pacific oysters that leads to a massive opportunistic pathogen proliferation (de Lorgeril et al., 2018), and neonicotinoid pesticides affect honey bee immunity and promote viral pathogen replication (O'Neal et al., 2018). In plants, as the high temperature compromises the salicylic acid pathway that mediates the response against pathogens, climate change may also enhance susceptibility to pathogens (Huot et al., 2017).

Such changes in community are generally characterized at the symbiotic taxonomic level by a rapid decrease in the intra-individual diversity, but stressors can also impact stochastically the microbiome composition and increase the inter-individual diversity (Kandalepas et al., 2015; Rocca et al., 2019). As proposed by the "Anna Karenina principle" applied to the symbiotic community, "all healthy microbiomes are similar but each dysbiotic microbiome is dysbiotic in its own way" (Lesser et al., 2016; Zaneveld et al., 2017). These increases in ß-diversity are notably observed after recovery or directed change in the environment (Silverstein et al., 2015; Ahmed et al., 2019), and could result from a host immune dysregulation or the release of the symbiotic niche by a sensitive symbiont.

Overall, because the symbiotic composition is tightly regulated, any disruption of the symbiotic homeostasis can change the costs and benefits associated with the presence of symbionts. This unbalance can result in a transition along the mutualism-parasitism continuum and impact the associated ecosystem (Kiers et al., 2010). One of the most striking illustrations of the immediate effects of environmental perturbations is the coral bleaching resulting from the impact of heat stress on cnidarian-dinoflagellate symbioses. Indeed, elevated temperatures photo-inhibit dinoflagellate algae and alter the metabolism of both partners. In sub-bleaching conditions, the mutualistic *Symbiodinium* algae then becomes a nutritional parasite, as it overtakes resources from its coral host (Baker et al., 2018). Temperature increase thus induces changes in metabolic profiles and cellular responses associated to bleaching (Hillyer et al., 2016), and the destabilization of the coral ecosystem (Pita et al., 2018).

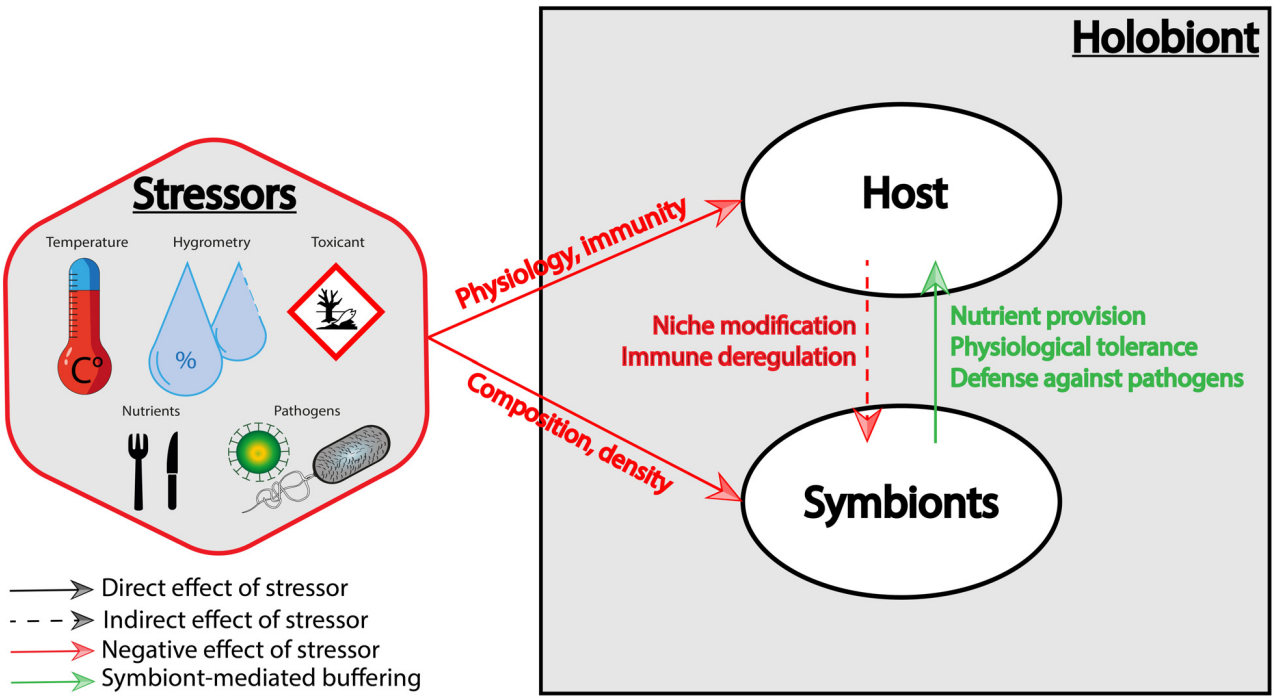

FIGURE 1 | Effects of stressors on the holobiont. Stressors can alter directly (1) the host physiology and immunity and (2) the symbiotic community composition and density. Stressors may also indirectly affect the symbiotic community by altering the host physiology (which represents the symbiotic niche), and the immune state of the host. Conversely, symbionts can buffer stressors via nutrient provision, physiological tolerance, and defense against host natural enemies.

SYMBIOSIS AS AN ADAPTIVE RESPONSE TO STRESSORS

We have shown above that stressors can negatively impact symbiotic partners, but symbionts can also participate in the buffering of stressors, in providing to their host a resistance or a tolerance mechanism, which that reduces the fitness costs associated to stressors (Miransari, 2010; Clay, 2014; Shapira, 2016; Lemoine et al., 2020). These two mechanisms are involved in the resilience to stressors, through the withstanding of a stressor that is normally unfavorable. Classically defined in immunology, resistance limits the parasite burden while tolerance limits the physiological impact of the parasite burden without decreasing its amount (Råberg et al., 2009), but these definitions can be broadened to any kind of stressor. The following is a non-exhaustive series of examples that suggest the driver effect of symbiosis in host adaptation to stressors.

A first series concerns symbionts that limit perturbations associated with seasonal abiotic variations, such as drought, salinity, or heat stress. For instance, endophytic fungi can promote plant growth and survival under such abiotic stress (Azad and Kaminskyj, 2016), potentially *via* the elicitation of induced systemic tolerance (Qin et al., 2016). Arbuscular mycorrhiza can promote plant tolerance to salinity and water stress by an adjustment of osmolytes (e.g., carbohydrates and electrolytes) in plant roots (Miransari, 2010). The endosymbiont *Buchnera* confers thermal tolerance to aphids through the expression of a small heat-shock protein (Dunbar et al., 2007), and tyrosine-supplementing symbionts protect beetles from desiccation through the production of a thicker cuticle (Vigneron et al., 2014; Anbutsu et al., 2017; Engl et al., 2018). Also, the proportion of heat-tolerant algal or bacterial symbionts increases in reefs that are severely affected by climate change (Baker et al., 2004), which promotes carbohydrate metabolism, nitrogen fixation, iron scavenging, and protein folding (Ziegler et al., 2017). Seasonal abiotic variations can also limit resources, and certain symbionts can benefit hosts experiencing a sporadic nutritional stress. For instance, arbuscular mycorrhiza improve Phosphorus and Nitrogen fixation in plants from impoverished soils (Johnson et al., 2010), and fly gut microbiota (*Lactobacillus plantarum* in particular) promotes larval growth upon food deprivation by modulating hormonal signals (Storelli et al., 2011).

In addition to abiotic stressors, various symbionts can protect their host against biotic stressors: their natural enemies. Because of the abundant literature in this field, we will focus on insect symbioses, and highlight a few canonical examples [but see extensive reviews such as Flórez et al. (2015) in animals and Pieterse et al. (2014) in plants]. Symbionts can protect their host in different ways. The most documented way is when defensive symbionts produce specific toxins targeting the competitor. For instance, the APSE bacteriophage from *Hamiltonella defensa* protects aphids against parasitoid wasp attacks (Oliver et al., 2009; Martinez et al., 2014). The maternally inherited bacterium *Spiroplasma* limits the sterilization of *Drosophila neotestacea* flies by parasitic nematodes, through the production of a toxin

(Jaenike et al., 2010; Hamilton et al., 2016). *Streptomyces* bacteria are released from the antennal reservoirs of beewolves, coat the brood cell, and secrete antimicrobial peptides that protect the immature wasp against opportunistic fungi from the soil (Kaltenpoth et al., 2005; Koehler et al., 2013). Symbionts can also turn the insect food into compounds that are toxic against many pathogens. This is the case of phenolic compounds converted by locust gut symbionts from plant secondary metabolites (Dillon and Charnley, 2002), or organic acids produced by honeybee gut symbionts from lactic acid fermentation (Olofsson et al., 2016). Such organic products decrease pH, and can modify the host niche in a way that limits pathogen survival (Palmer-Young et al., 2019). Additionally, symbionts can prime host immunity, as it has been reported with *Wolbachia* in the mosquito *Aedes aegypti* (Moreira et al., 2009). Finally, they can exploit the same limited resource as the parasite (Gerardo and Parker, 2014), and enter in competition with it. For instance *Wolbachia*, which blocks viral replication of positive-strand RNA viruses in *Drosophila* and mosquitoes (Teixeira et al., 2008; Bian et al., 2010), possibly exploits cholesterol necessary for viral replication (Caragata et al., 2013).

To summarize, symbionts constitute a "pool of genes" potentially involved in several physiological functions and can be acquired rapidly by the host. Such symbionts could thus be part of an adaptive response to environmental changes, and eventually get to fixation if biotic and abiotic constraints are stable and impose a strong selective pressure, and if the benefit of carrying symbionts is higher than its cost.

SYMBIOSIS AND ADAPTATION: ALWAYS AN OPTIMAL STRATEGY?

As presented above, the presence of symbionts can limit the fitness costs associated with a stressful condition (Latef et al., 2016; Shapira, 2016; Corbin et al., 2017; Hopkins et al., 2017) through an impact on various physiological functions (Oliver et al., 2003; Webster and Reusch, 2017; Cheng et al., 2019). However, various host mechanisms can also be involved in the buffering of stressors (examples for immune responses: Palmer and Traylor-Knowles, 2012; Lamiable and Imler, 2014; Obbard and Dudas, 2014; Kenney et al., 2017; Miller et al., 2017). The question that arises is thus whether the association with symbionts is always the optimal strategy, that is, the more efficient one to buffer the stress at a reduced cost. In other words, does symbiosis drive or limit host adaptation to a new, stressful environment?

First imagine that the stressor is frequent, such as a directed change in the environment. In that case, the selection for genetic, heritable mechanisms that counteract stressors should be strong. It can include specific host genes, such as resistance genes against particular stressors. For instance, high affinity transporter genes are selected in plants under scarce Nitrogen availability (Kiba and Krapp, 2016). It can also include the selection of specific symbionts such as *Regiella insecticola* in pea aphids, whose frequency follows a gradient throughout Japan and is associated with changes in temperature, precipitation and host plant (Tsuchida et al., 2002). Both mechanisms can also be selected for: adaptation of *Platygyra* corals to high temperatures mirrors the fixation of host alleles limiting oxidative stress together with the selection of zooxanthellae variants that are photosynthetically thermotolerant (Howells et al., 2016). On a broader evolutionary time scale and in the case of stable changes, symbiont acquisition can play an important role in ecological adaptation and innovation (Wernegreen and Wheeler, 2009; Douglas, 2015; Sudakaran et al., 2017). An extreme case is the evolution of co-dependency between insects feeding on unbalanced diets (e.g., phloem, blood) and their nutritional vertically-transmitted symbionts (Moran et al., 2003; Douglas, 2009): *Buchnera* or *Serratia* in aphids (Shigenobu and Wilson, 2011; Monnin et al., 2020); *Wigglesworthia*, *Wolbachia* and *Rickettsia* in tsetse flies, bed bugs, and ticks, respectively (Rio et al., 2016; Duron et al., 2018).

Let's now imagine a case where the stressor(s) fluctuate(s), in term of intensity or temporality. We would expect that plastic mechanisms should be favored in that situation. Indeed, host resistance mechanisms may be costly when constitutively expressed, unless compensation mechanisms are selected for, in the absence of stressor (Kliot and Ghanim, 2012; but see Ffrench-Constant and Bass, 2017). Presence of symbionts may also be costly in the absence of stressor, notably when the response against stressor is mediated by a high symbiotic density that consumes nutrients and uses the cellular machinery (Martinez et al., 2015; Hopkins et al., 2017). This is particularly true for vertically-transmitted symbionts, whose modulation by the host in the absence of stressor is limited. For instance, the presence of *Hamiltonella defensa*, an endosymbiont that efficiently protects aphids against parasitoid attacks but is highly costly in its absence (Polin et al., 2014), might not be an optimal defense strategy (Hopkins et al., 2017). In addition, the presence of symbionts can negatively impact the selection of host genes that buffer stressors. Indeed, Metcalf and Koskella (2019) predict from a theoretical point of view that defensive symbionts (or more generally microbiomes that favor resistance or tolerance against a pathogen) can decrease the selection for host immune mechanisms and drive the loss of immunity, especially when the cost of host immunity is high. The potential of symbionts to be a possible obstacle to the selection of host adaptive responses has been tested in fruit flies, where *Wolbachia* can protect the host against RNA viruses (Teixeira et al., 2008). After evolution in a context of viral infection, the presence of *Wolbachia* in flies reduced the strength of selection for the host resistance gene *Pastrel* (Martinez et al., 2016); strength that was increased again after the removal of *Wolbachia* (Faria et al., 2018). Alternatively, plastic mechanisms, such as programming of host gene expression, chromatin remodeling (e.g., histone modifications, chromatin compartmentalization) have been observed during many fluctuating biotic and abiotic stress responses in insects, mammals (Vihervaara et al., 2018), plants (Lämke and Bäurle, 2017), and marine organisms (Eirin-Lopez and Putnam, 2019). Also, a rapid change in the composition of the host-associated symbiotic community, notably when symbionts are horizontally acquired from the environment, can constitute a tremendous resource of biological

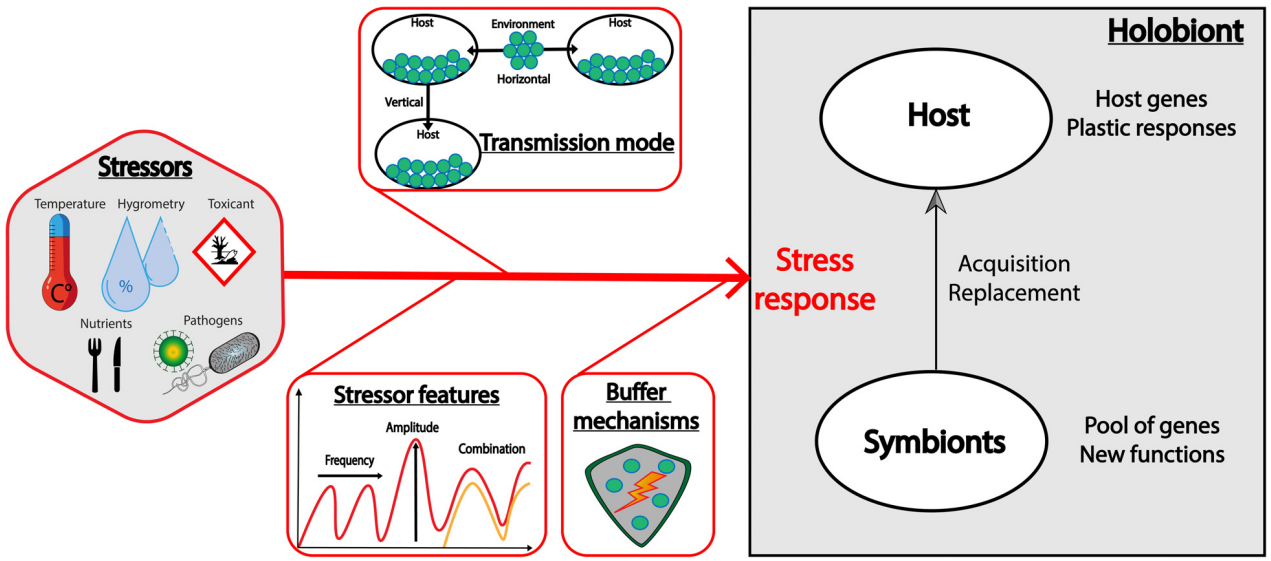

FIGURE 2 | Factors that can influence the holobiont stress response. The holobiont response to stressors is difficult to predict as many factors can be under selection. This includes host resistance genes and plastic mechanisms, but also acquisition of symbionts that can constitute a pool of genes with new functions. We describe here some key factors that can preferentially select for the host or the symbionts to adapt to stressors: (1) the features of the stressor, such as its frequency or amplitude, but also its combination with another stressor that can lead to an additive, synergistic or antagonist interaction, (2) the transmission mode of the symbionts, and (3) the specificity and the efficiency of the given buffering mechanism, and the net balance between its cost and its benefit.

functions that can be involved in the control of stressors. For instance, the composition and functional profile of the microbiome of the coral *Acropora hyacinthus* quickly shifts in response to a transient heat stress (Ziegler et al., 2017). These composition changes can result from the selection of fittest symbionts in this new stressful environment (Suggett et al., 2017), but can also result from host partner choice within a "biological market" of horizontally-transmitted symbionts (Werner et al., 2014). This partner choice has been exemplified in symbioses between plants and arbuscular mycorrhizal fungi, where fungi provide nutrients such as Nitrogen and Phosphorus to plants in exchange of carbohydrates. Depending on the resource abundance and fluctuations, plants can select for specific strains of fungi that offer the most soil-limited nutrients (Werner and Kiers, 2015).

To summarize (**Figure 2**), the two extreme situations above suggest that the frequency of stressors can select for different degrees of plasticity for the buffering mechanism. Both host and symbionts can carry this stress response mechanism, but in case of symbiosis, the transmission mode plays a central role in the versatility of the response. Indeed, horizontally-transmitted symbionts should be favored in a fluctuant environment, as they constitute a flexible microbial pool in the environment from which certain variants can be selected for, while vertically-transmitted symbionts should be favored in a constant environment, as they are durably associated with their host. Whether the stressor is frequent or fluctuant, the acquisition of a symbiont by a host (when possible) is an efficient way to acquire simultaneously a pool of genes potentially encoding for functions that the host does not necessarily possess. However, when host and symbionts express genes with redundant functions, the presence of symbionts might not always be the optimal strategy, as it can be more costly than the expression of specific host genes, and/or can limit the evolution of the host.

PERSPECTIVES: HOW CAN WE INTEGRATE SYMBIOSIS IN OUR UNDERSTANDING OF BIOLOGICAL RESPONSE TO GLOBAL CHANGES?

Because stressors can impact symbiotic associations and, reciprocally, symbiosis can impact the response of organisms to stressors, it is important to consider symbiosis when we study the response of organisms in a context of global change. But global change means that stressors occur in combination rather than alone (Crain et al., 2008; Munns, 2011; Goulson et al., 2015; Van Dam et al., 2015), and this strongly complicates the framework developed above: the combination of stressors can vary depending on the timing of each stressor, the frequency and intensity of the exposure, and the spatial distribution of all individual stressors; the effect of several stressors can be additive, synergistic or antagonistic (i.e., interactions leading to a equal, greater or a lower effect than the sum of the stresses, respectively); and symbiotic composition and function can vary differentially in response to each stressor. In insects for instance, the reciprocal link between symbiosis and stressors is critical to consider, as the genome reduction of obligate nutritional symbionts makes them particularly sensitive to heat. In the case of a temperature increase, the reduction of symbiont density could decrease the buffering effect against another stress (e.g., infection, nutritional

stress). In case of obligate mutualism, the loss of symbionts could indirectly lead to the extinction of the insect species, except if symbiont switching/complementation occurs (Wernegreen, 2013; Kikuchi et al., 2016; Corbin et al., 2017; Renoz et al., 2019).

Considering as many stressors as possible, together with their dynamics (e.g., constant/fluctuant, simultaneous/sequential), is crucial to fit to ecologically relevant situations (Gunderson et al., 2016), but very difficult to investigate. Still, quantifying the relative effect of each stressor remains determinant to pinpoint the main disruptive factors, and to focus studies on these few stressors. A characterization of their effects on tissues, individuals and ecosystems can then help to define actions that will limit their impacts (Anthony et al., 2015; Foucart, 2019). To reach this goal, a few mathematical models have been developed to integrate multiple stressors (Liess et al., 2016), to explore tipping points under combined stressors including parasitic pressure (bee decline: Henry et al., 2017), or to predict the evolution of species dynamics (coral bleaching: Cunning et al., 2017). However, their development requires an extensive knowledge on molecular, physiological, and ecological mechanisms associated with symbiosis to define appropriate formulation and parameterization (Widder et al., 2016). For instance, knowing the mechanism of action of two stressors on both partners can help in determining if they are conserved or pleiotropic (Sewelam et al., 2016; Jacob et al., 2017), and thus to predict a potential synergy or antagonism (Kaunisto et al., 2016). There is thus an urgent need to develop integrative studies to better understand the molecular mechanisms involved in response to stressors, the link between structure and function of symbiotic communities, the dialogue between host and symbionts, and the influence of symbiotic functions on more global ecological processes. This integrative approach can combine methodologies characterizing multiple organization levels such as genomics, transcriptomics, proteomics, metabolomics, and ecological network analyses (e.g., Bissett et al., 2013; Colgan et al., 2017; Larrainzar and Wienkoop, 2017; Meena et al., 2017; Rodriguez et al., 2019; Weis, 2019).

Finally, as the acquisition of new symbionts or the modification of symbiotic communities can play a major role in the adaptation to stressors, studying symbiosis might be of particular interest to study the adaptive potential and the resilience of organisms to stressors. For that purpose, experimental evolution constitutes a great tool to study the direct and evolved response to stressors, as well as the evolution of the symbiotic association under a controlled stressful environment (Hoang et al., 2016; Erkosar et al., 2017). Developing experimental and theoretical evolutionary studies can thus help to pinpoint molecular mechanisms at play when evolutionary forces act and to validate predictive mathematical models.

AUTHOR CONTRIBUTIONS

All authors made an intellectual contribution to conceive the mini-review. In addition, NK supervised its writing, illustration, and editing. AB contributed sections and illustrations. All authors edited the manuscript and approved the submitted version.

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Coral Bleaching Susceptibility Is Predictive of Subsequent Mortality Within but Not Between Coral Species

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Marine heat waves instigated by anthropogenic climate change are causing increasingly frequent and severe coral bleaching events that often lead to widespread coral mortality. While community-wide increases in coral mortality following bleaching events have been documented on reefs around the world, the ecological consequences for conspecific individual colonies exhibiting contrasting phenotypes during heat stress (e.g., bleached vs. not bleached) are not well understood. Here we describe the ecological outcomes of the two dominant reef-building coral species in Kāne'ohe Bay, Hawai'i, *Montipora capitata* and *Porites compressa,* by monitoring the fates of individuals that exhibited either a bleaching susceptible phenotype (bleached) or resistant phenotype (non-bleached) following the second of two consecutive coral bleaching events in Hawai'i in 2015. Conspecific pairs of adjacent bleaching susceptible vs. resistant corals were tagged on patch reefs in two regions of Kāne'ohe Bay with different seawater residence times and terrestrial influence. The ecological consequences (symbiont recovery and mortality) were monitored for 2 years following the peak of the bleaching event. Bleaching susceptible corals suffered higher partial mortality than bleaching resistant corals of the same species in the first 6 months following heat stress. Surprisingly, *P. compressa* had greater resilience following bleaching (faster pigment recovery and lower post-bleaching mortality) than *M. capitata,* despite having less resistance to bleaching (higher bleaching prevalence and severity). These differences indicate that bleaching susceptibility of a species is not always a good predictor of mortality following a bleaching event. By tracking the fate of individual colonies of resistant and susceptible phenotypes, contrasting ecological consequences of heat stress were revealed that were undetectable at the population level. Furthermore, this approach revealed individuals that underwent particularly rapid recovery from mortality, including some colonies over a meter in diameter that recovered all live tissue cover from >60% partial mortality within just 1 year. These coral pairs (44 pairs of each species) continue to be maintained and monitored in the field, serving as a "living library" for future investigations on the ecology and physiology of coral bleaching.

Keywords: *Montipora capitata*, *Porites compressa*, coral bleaching, mortality, symbiosis, thermal stress, ecology

INTRODUCTION

Ocean warming due to anthropogenic climate change has caused an increase in the frequency and severity of coral bleaching, a visually striking stress response where the coral host expels its endosymbiotic algae (dinoflagellates of the family Symbiodiniaceae) thus revealing the white coral skeleton beneath the translucent animal tissue (Gates et al., 1992; Putnam et al., 2017). In this nutritional symbiosis, tropical corals are obligate partners that require the algae for the majority of their energy (Muscatine and Porter, 1977; Muller-Parker et al., 2015). Because of coral dependence on this partnership, prolonged heat waves that cause sustained coral bleaching can lead to depletion of the host's energy supply and reserves (Grottoli et al., 2004; Rodrigues and Grottoli, 2007; Imbs and Yakovleva, 2012; Wall et al., 2019). This stress can elicit a variety of sublethal effects, including declines in growth and reproduction (Ward et al., 2000; Baird and Marshall, 2002; Baker et al., 2008; Hughes et al., 2019a), and at worst can result in widespread coral mortality (Loya et al., 2001; McClanahan, 2004; Baker et al., 2008; Eakin et al., 2010; Hughes et al., 2017; Sully et al., 2019). The loss of live coral cover is often followed by rapid erosion of the structural framework of the reef (Couch et al., 2017; Hughes et al., 2018b; Fordyce et al., 2019; Leggat et al., 2019), reducing habitat complexity (Magel et al., 2019) and negatively impacting the diversity of the broader reef community (e.g., fish (Pratchett et al., 2011; Darling et al., 2017; Richardson et al., 2018). Coral mortality following bleaching can also alter the structure of the coral community itself, as bleaching-susceptible species are lost from the community (Loya et al., 2001; McClanahan, 2004; Baker et al., 2008; Bahr et al., 2017; Hughes et al., 2017) while stress-tolerant species remain (Edmunds, 2018; Hughes et al., 2018b) and "weedy" genera that are better suited for rapid recovery following bleaching become dominant (Darling et al., 2012; Edmunds, 2018). These changes in community composition alter the ecological function of the reef (Alvarez-Filip et al., 2013), which alongside the structural degradation following bleaching leads to declines in ecosystem goods and services ranging from fisheries production to coastal protection and tourism (Costanza et al., 2014).

The susceptibility of a reef system to coral bleaching during a marine heat wave depends on a combination of physical and biological factors (Swain et al., 2016). Differences in the local microenvironment (e.g., flow, turbidity, surface reflectance, internal waves, etc.) can ameliorate or exacerbate the magnitude of heat stress and lead to differential bleaching and mortality on nearby reefs (Anthony et al., 2007; Wyatt et al., 2019). Local temperature dynamics also can influence coral bleaching by altering the physiological tolerance of coral populations to heat stress. For example, corals exposed to high diel temperature variability often have higher heat tolerance and greater resistance to bleaching than nearby conspecifics in more stable regimes (Putnam and Edmunds, 2011; Palumbi et al., 2014; Schoepf et al., 2015, 2019; Barshis et al., 2018). These data indicate that environmental history of individuals and populations plays a significant role in determining coral responses to stress (Hughes et al., 2019b), and is likely driven by a combination of both acclimatization (Bay et al., 2013; Bay and Palumbi, 2015; Kenkel and Matz, 2016) and adaptation (Barshis et al., 2013, 2018; Palumbi et al., 2014; Matz et al., 2018). On a global scale, there is recent evidence that the temperature threshold for coral bleaching has risen in correspondence with global warming, suggesting widespread acclimatization and/or adaptation (Coles et al., 2018; DeCarlo et al., 2019). Alternatively, differences in the composition of coral communities between reefs can also influence bleaching extent due to species-specific differences in thermal tolerance (Rowan et al., 1997; Berkelmans and Van Oppen, 2006; Sampayo et al., 2008; Swain et al., 2016; Edmunds, 2018). Indeed, the global loss of many heat sensitive coral species from reef communities (e.g., Loya et al., 2001; McClanahan, 2004; Hughes et al., 2018b; Kim et al., 2019), may be a contributing factor in rising bleaching thresholds. Similarly, intraspecific diversity may also contribute to changes in the bleaching threshold if tolerant genotypes persist in populations and sensitive genotypes are lost. While this would promote reef resistance to bleaching, the ecological consequences of corresponding reductions in genetic diversity remain to be seen.

The effect of intraspecific diversity in bleaching susceptibility (i.e., presence of individuals with contrasting bleached vs. not bleached phenotypes) on downstream ecological outcomes is not well understood, despite an abundance of studies concluding that interspecific differences in bleaching susceptibility are predictive of mortality such that species resistant to bleaching have lower mortality than susceptible species (Loya et al., 2001; McClanahan, 2004; Baker et al., 2008; Bahr et al., 2017; Hughes et al., 2017). Kāneʻohe Bay, Hawaiʻi, located on the northeast coast of Oʻahu, is an opportune system for investigating this question of how intraspecific variability in coral responses to coral bleaching events driven by climate change influence coral survival. The two dominant reef-building coral species in the bay, *Montipora capitata* and *Porites compressa,* both exhibit differences in heat stress responses within and between species during bleaching (Grottoli et al., 2006; Cunning et al., 2016; Wall et al., 2019). Differences in symbiont associations and nutritional plasticity are hypothesized to influence bleaching resistance (ability to withstand heat stress without bleaching) and resilience (ability to recover from bleaching) between these two species (Grottoli et al., 2006; Cunning et al., 2016; Wall et al., 2019), while within *M. capitata* the species of symbionts associated with different individual colonies contributes to differences in heat tolerance (Cunning et al., 2016). However, the influence of intraspecific variation in bleaching susceptibility on ecological outcomes following bleaching events remains unexplored. Understanding how intraspecific variation influences differential outcomes following heat stress is critical for understanding how the increasing frequency and severity of coral bleaching events will impact the function of these important ecosystems.

To better understand how individual colony responses to heat stress can influence ecological outcomes following a coral bleaching event, we conducted a 2-year monitoring study to characterize the recovery dynamics of bleaching susceptible and bleaching resistant coral colonies of *M. capitata* and *P. compressa* following a regional bleaching event in Kāneʻohe Bay in 2015. Hawaiʻi experienced anomalously high seawater temperatures in late summer (August–September) of 2015, resulting in

widespread coral bleaching throughout the region (Bahr et al., 2017). This was the first consecutive coral bleaching event ever observed on Hawaiian reefs, occurring 1 year following a regional bleaching event in 2014 (Bahr et al., 2015). A total of 22 conspecific pairs of adjacent corals with contrasting bleaching phenotypes (bleaching susceptible or bleaching resistant) of each species were tagged and georeferenced at each of two reefs in the bay with different environmental conditions. Coral pigmentation recovery and mortality were monitored periodically over the following 2 years to examine how bleaching influences the ecological trajectories within and between species. These colonies are visited on a semiannual basis to maintain visibility of the tags for the purpose of establishing these corals as a living library archived *in situ* for use in future research on coral bleaching mechanisms, thermal physiology, and climate change resilience.

MATERIALS AND METHODS

Site Selection and Characterization

This study was initiated during the peak of the 2015 coral bleaching event in Kāneʻohe Bay, Oʻahu. The lagoon of Kāneʻohe Bay consists of a shallow network of fringing and patch reefs protected by a barrier reef (Bahr et al., 2015), which restricts seawater exchange with the open ocean leading to reefs within the bay with high and low seawater residence times (Lowe et al., 2009). Two patch reefs dominated by the reef-building corals *Montipora capitata* and *Porites compressa* from two different regions of the bay were selected for this study: (1) Inner Lagoon region: patch reef 4 (PR4; 21.4339°N, 157.7984°W), an inshore reef with relatively high terrestrial influence (e.g., sedimentation, fresh water and nutrient run-off) and long (>30 days) seawater residence time (Lowe et al., 2009) and (2) Outer Lagoon region: patch reef 13 (PR13; 21.4515°N, 157.7966°W), a seaward reef with less terrestrial influence and short (<24 h) seawater residence time (Lowe et al., 2009; **Figures 1A,B**). Temperature loggers (Onset U22 Hobo; 0.21°C accuracy; 0.02°C resolution) were deployed on the benthos of each reef at 2–3 m depth on September 24, 2015, and seawater temperature was recorded in 15-minute intervals. The loggers were cross calibrated in an aquarium prior to deployment. Temperature data were collected through October 6, 2016 at the Inner Lagoon reef, and through July 20, 2016 at the Outer Lagoon reef. Because these loggers were not deployed until after the peak of the heatwave, data from nearby monitoring stations within each region were used to determine if there were any differences in cumulative heat stress between the two regions. These data were collected from PR12, located ∼100 m from PR13 in Outer Lagoon (**Figure 1D** and **Table 1**; data courtesy of the Division of Aquatic Resources of the State of Hawai'i[1]), and PR1 (Coconut Island; data from PACIOOS[2]), in the same Inner Lagoon flow regime as PR4. Overlapping time series data showed seawater temperatures were similar and highly correlated within each lagoon region (**Supplementary Figure S1**). Degree heating weeks (DHW) were calculated for the Inner and Outer lagoon reefs from the PR1 and PR12 data, respectively, according to the methods described by Liu et al. (2013) with minor modification. Specifically, DHW were calculated as the hourly accumulation of temperatures ≥1°C above the local mean monthly maximum seawater temperature (MMM) in Kāneʻohe Bay (28.0°C; Jury and Toonen, 2019), which is ∼1°C higher than the MMM of the reefs of the Main Hawaiian Islands (Jokiel and Brown, 2004; Jury and Toonen, 2019).

Characterization of Benthic Community Composition and Coral Bleaching Responses

Surveys were performed to assess benthic community composition and coral bleaching responses during the 2015 bleaching event, and again after 1.5, 4, 10, and 18–19 months of recovery. Four surveys were conducted at each site for each time point along the reef slope in parallel to the reef crest at a depth of 2 m (± 1 m). Each survey was carried out along a 40 m transect laid parallel to the reef crest. A photoquadrat (0.33 m^2) was placed on the benthos and images were taken every 2 m along the transect using an underwater camera (Canon PowerShot or Olympus TG5). Images were analyzed using Coral Point Count with Excel extensions (CPCe)[3] (100 points per image). Corals were identified to species, and bleaching severity was recorded. Bleaching severity of the coral tissue under each point was assessed visually, and categorized as either white (severely bleached), pale (moderately bleached), or fully pigmented (not bleached). Bleaching prevalence was calculated as the proportion of live coral affected by severe to moderate bleaching (i.e., cumulative proportion of white or pale out of total). All other organisms were classified into functional groups (turf algae, crustose coralline algae (CCA), macroalgae, sediment and sand, sponges).

Monitoring of Bleaching Resistant and Susceptible Corals

Individual coral colonies of *M. capitata* and *P. compressa* with contrasting bleaching phenotypes were identified at the peak of the bleaching event from late September through the first week of November 2015. Bleaching resistant corals were defined as those that remained pigmented during the bleaching event, while bleaching susceptible corals were defined as those that appeared completely white. All corals were located along the edge of the reef crest (approximately 1 m depth) and down the reef slope (up to approximately 3 m depth). A plastic cattle tag with a unique identification number was attached to each colony, then the colony was photographed and its GPS location was recorded. All tagged colonies were conspecific pairs of colonies with contrasting bleaching phenotypes that were located adjacent to one another on the reef (e.g., **Figure 2**). A total of 22 pairs (44 colonies) of each species were tagged at each of two reefs (PR4 in the Inner Lagoon and PR13 in the Outer Lagoon; **Supplementary Data S1**). This paired design eliminated

[1]Division of Aquatic Resources, State of Hawaii: DLNR.aquatics@hawaii.gov; http://dlnr.hawaii.gov/dar/

[2]http://www.pacioos.hawaii.edu/weather/obs-mokuoloe/

[3]https://cnso.nova.edu/cpce/index.html

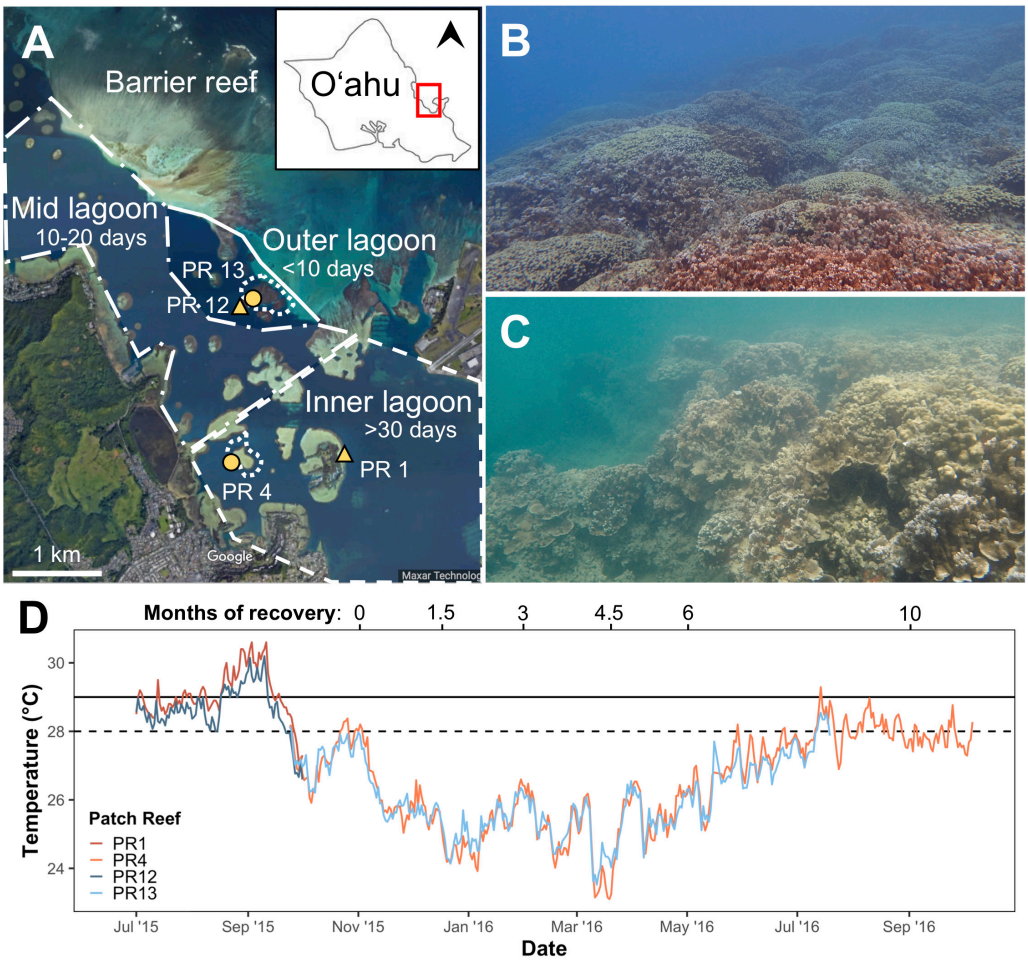

FIGURE 1 | (A) Map of the southern region of Kāne'ohe Bay, O'ahu. Inset shows the island of O'ahu, with north indicated by the arrowhead and the red square indicating the southern region of Kāne'ohe Bay enlarged in detail. Distinct hydrodynamic regimes within the lagoon are indicated by the polygons: the dashed line surrounds the Inner Lagoon region where seawater residence times are >30 days; the dash-dot line surrounds the Mid Lagoon region where seawater residence times are 10–20 days; the solid line surrounds the Outer Lagoon region where seawater residence times are <10 days (from Lowe et al., 2009). Yellow symbols indicate locations of *in situ* temperature loggers. Representative images of each reef are shown for **(B)** Outer lagoon (PR13) and **(C)** Inner lagoon (PR4). **(D)** Maximum daily temperatures at each reef. Dashed horizontal line indicates local MMM; solid horizontal line indicates coral bleaching threshold (MMM+1).

TABLE 1 | Summary of seawater hydrodynamics and temperature conditions within the Inner and Outer lagoon regions in Kāne'ohe Bay, Hawai'i during the peak of the heat stress event and the initial recovery period.

	Region:	**Inner Lagoon**		**Outer Lagoon**		**References**
	Patch Reef:	**PR 1 (Coconut Island)**	**PR 4**	**PR 12**	**PR 13**	
Physical regime	Dominant physical force	Tide	Tide	Wave	Wave	Lowe et al., 2009; Bahr et al., 2017
	Seawater residence time	>30 days	>30 days	1–5 days	<1 day	Lowe et al., 2009
Peak heat stress (July 1 - Oct 1, 2015)	Mean maximum daily temperature (°C)	**29.08***	ND	**28.64***	ND	**Figure 1**
	Mean minimum daily temperature (°C)	**28.44***	ND	**27.94***	ND	**Figure 1**
	Degree heating weeks (DHW)	5.84	ND	1.77	ND	**Figure 1**
Initial recovery (Nov 1, 2015 - Feb 1, 2016)	Mean maximum daily temperature (°C)	25.60	25.59	25.35	25.62	**Figure 1**
	Mean minimum daily temperature (°C)	25.01	24.80	24.75	24.93	**Figure 1**

Bold numbers with asterisks indicate statistical significance ($p < 0.03$; Wilcoxon rank sum test).

the potential for differences in the local microenvironment experienced by one phenotype but not the other (e.g., light intensity, flow) to confound interpretation of differences in their bleaching susceptibility. This paired design is also powerful because it can distinguish between mortality related to heat stress (both phenotypes) and mortality related to symbiont loss (bleached phenotypes). Bleaching recovery (where applicable) and partial mortality of tagged individuals were monitored every ∼6 weeks for the first 6 months following the bleaching event, then once every 6 months up to 24 months following the bleaching event. At each time point, colonies were photographed and visual observations of their bleaching status were recorded on a three-point scale (as in Guest et al., 2016; Coles et al., 2018; Ritson-Williams and Gates, 2020). Corals were given a color score based on their visual color: (1) white (>80% of colony white with no visible pigmentation); (2) pale (>10% colony affected by pigment loss); or (3) fully pigmented (<10% colony with any pale coloration). Partial mortality was quantified in 20% intervals (0–100%) from the photographs. The mean color scores and partial mortality for each species were calculated for each bleaching phenotype and site at each time point. For colonies with a missing observation, mortality scores were estimated using the mortality score of the previous time point. This is a conservative estimate of mortality (i.e., minimum possible mortality at that time point), and in most cases mortality scores did not change surrounding the missing observation. Missing observations of color scores were never interpolated across the time series due to the possibility for rapid loss or recovery of pigmentation.

Statistical Analyses

Statistical analyses were conducted in R and RStudio v. 3.6.1 (R Core Team, 2019). Seawater temperature means were compared using Wilcoxon rank sum tests. Linear mixed effects models (*lme4* package, Bates et al., 2015) were used to analyze four different response variables from the benthic survey time series: (1) proportion of live coral cover, (2) prevalence of bleached coral cover [proportion of coral cover showing symptoms of bleaching (white or pale)], (3) prevalence of severely bleached coral cover (white only), and (4) bleaching severity (proportion of symptomatic live coral exhibiting severe bleaching). We hypothesized that corals with different bleaching phenotypes would recover at different rates and that these rates would differ between reefs, and therefore our models reflect *a priori* hypotheses. Survey depth was included as a random intercept. For coral cover, lagoon and time point were the main effects. For coral bleaching prevalence and severity, we used a full factorial design with coral species, lagoon, and time point as the main effects. To test hypotheses about the differential recovery of individually monitored bleaching susceptible versus bleaching resistant corals, we used linear mixed effects models to examine how (1) mean color score and (2) mean tissue loss in each species differed over time, lagoon, and bleaching phenotype as fixed effects (**Tables 2**, **3**). *Montipora capitata* and *P. compressa* were analyzed separately, with coral identification number as a random effect to account for repeated measures. For models in which the three-way interaction between lagoon, bleaching phenotype, and time point was significant, we further analyzed the effect of lagoon and bleaching phenotype and their interaction with separate linear mixed models subset at each time point. Because not every colony within a pair was found at every time point, we tested for the effects of intermittent partial pairs by analyzing a subset of the dataset that included only data from pairs where both partners were found at a time point ("pairs" models). We tested the effect of lagoon and bleaching phenotype and their interaction on the differences in pigmentation and mortality scores using the models described above, with the addition of the random effect of pair identification number to test for spatial heterogeneity within lagoons. For all analyses, *p*-values were calculated using Type II analysis of variance (ANOVA) with Satterthwaite's approximation method. Tukey honest significant difference *post hoc* tests (R package emmeans, Lenth, 2019) were used to test for significant pairwise differences in main effects. Results are presented with *p*-values specifying the main effects of bleaching phenotype (P), recovery month (M), and lagoon (L).

RESULTS

Temperature Dynamics Throughout Bleaching and Recovery

Midday seawater temperatures exceeded the local bleaching threshold of 29.0°C throughout Kāneʻohe Bay in September 2015 (**Figure 1**; Bahr et al., 2017). Daily highs met or exceeded 29°C for a total of 46 days at the Inner Lagoon reef (PR1) versus 24 days at the Outer Lagoon reef (PR12; **Figure 1D**). The average maximum daily seawater temperature during the heatwave was 29.08°C in the Inner Lagoon, which was on average 0.44°C warmer than the Outer Lagoon (28.64°C, $p < 0.01$, Wilcoxon test; **Table 1**). The average minimum daily seawater temperature was also higher at the Inner Lagoon than the Outer Lagoon during this time (+0.50°C, $p < 0.01$, Wilcoxon test; **Table 1**), while the daily temperature range did not differ significantly between the two sites ($p = 0.118$, Wilcoxon test). This resulted in higher accumulated heat stress at Inner Lagoon relative to the Outer Lagoon, with a total of 5.84 degree heating weeks (DHW) at Inner Lagoon versus 1.77 DHW at the Outer Lagoon (**Table 1**). In contrast, during the first 3 months following the heatwave, the average daily maximum and minimum seawater temperatures were not significantly different between the two regions (**Figure 1D** and **Table 1**).

Benthic Community Composition

Coral cover was significantly different between the Inner and Outer Lagoon reefs ($p_L < 0.01$, **Supplementary Table S1**), but did not change significantly over the course of the 2-year bleaching recovery period ($p_{L:M} = 0.1$, **Figure 3** and **Supplementary Table S1**. At the initiation of this study, coral cover at PR13 in the Outer Lagoon was 80 ± 4.2% (**Figure 3B**), and at PR4 in the Inner Lagoon was 52 ± 6.0% (**Figure 3A**). Community composition of each reef is reported below as the range from min to max across the entire time series. The coral community of the Outer Lagoon reef was dominated by *P. compressa* throughout the time series (62–77% of live coral cover) relative to *M. capitata* (23–38% of live coral cover). The relative abundance of each

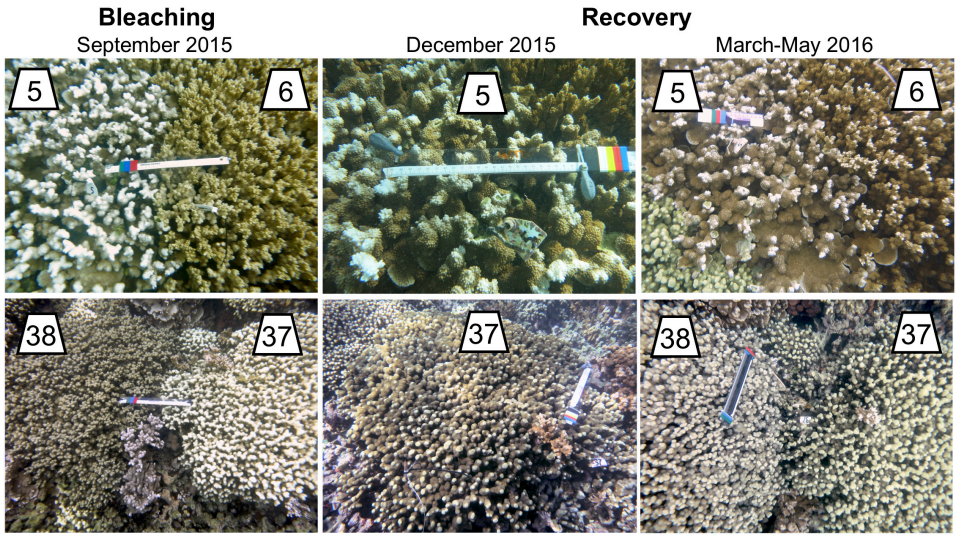

FIGURE 2 | Representative images of tagged bleached and non-bleached corals. Top row: *M. capitata* pairs 5 and 6; Bottom row: *P. compressa* pairs 37 and 38. Left column shows pairs during the bleaching event (Sept. 2015). Center column shows the bleaching susceptible colony of the pair after 1.5 months of recovery (Dec. 2015). Right column shows the same pairs after 3–6 months of recovery [Mar. 2016 (colonies 5 and 6); May 2016 (colonies 37 and 38)].

species at the Inner Lagoon reef was 54–63% for *P. compressa* vs. 37–46% for *M. capitata*. Turf algae were the second most abundant functional group at both reefs, comprising 6–10% of the benthos at the Outer Lagoon reef and 25–40% at the Inner Lagoon reef. Sediments were also common along the benthos at the Inner Lagoon reef (8.5–21%; **Figure 3A**), but were found in low abundance at the Outer Lagoon reef (<1%; **Figure 3B**). Both reefs had a low abundance (<5%) of macroalgae, crustose coralline algae (CCA) and sponges. The Inner Lagoon reef had an abundance of small filter feeders (e.g., boring oysters and sponges) that were not readily apparent on photoquadrat images but were observed by divers/snorkelers during surveys. This reef also typically had low visibility (<5 m; **Figure 1C**), whereas the Outer Lagoon reef tended to have higher visibility (∼20–30 m, personal observations; **Figure 1B**).

Initial Coral Bleaching Prevalence and Severity Differed Between Species and Reefs

Coral tagging and benthic surveys were initiated during the peak extent of bleaching in Kāneʻohe Bay in late September through October 2015 (Bahr et al., 2017). There were two significant interactions for bleaching prevalence: lagoon and recovery time ($p_{L:M} < 0.01$) and species and recovery time ($p_{S:M} < 0.01$; **Supplementary Table S2**). Additionally, there was a significant three-way interaction of lagoon, species and recovery time for both the prevalence of severely bleached tissue ($p_{L:S:M} < 0.01$; **Supplementary Table S3**) and bleaching severity (the proportion of completely bleached tissue out of all affected corals; $p_{S:L:M} < 0.01$; **Supplementary Table S4**). At peak bleaching, there was a higher prevalence of coral bleaching (proportion of live coral that was symptomatic, either white or pale) at the Inner Lagoon reef (79%) than the Outer Lagoon reef (44%; *post hoc* $p < 0.01$). Separated by species, initial coral bleaching prevalence was 69 ± 3% at the Inner Lagoon reef vs. 39 ± 12% at the Outer Lagoon reef for *M. capitata*, and 87 ± 7% at the Inner Lagoon reef vs. 45 ± 5% at the Outer Lagoon reef for *P. compressa* (**Figure 4**). Within reefs, the prevalence of severely bleached (white) tissue was lower for *M. capitata* (26 ± 6% of all live tissue) than for *P. compressa* (71 ± 10% of all live tissue) at the Inner Lagoon reef (*post hoc* $p < 0.01$; **Supplementary Figure S2**), whereas there was no significant difference in severe bleaching prevalence between species at the Outer Lagoon reef (*post hoc* $p > 0.05$; **Figure 4**). Bleaching severity (the proportion of symptomatic tissue that was completely bleached) was higher at the Inner Lagoon reef for *P. compressa* (80 ± 5%) than for *M. capitata* (37 ± 7%, **Figure 4**; *post hoc* $p < 0.01$). Bleaching severity for *P. compressa* was substantially lower at the Outer Lagoon reef (29 ± 10%; *post hoc* $p < 0.01$) than the Inner Lagoon reef (80 ± 5%), whereas bleaching severity did not differ significantly between sites for *M. capitata* (33 ± 5% at the Outer Lagoon reef vs. 37 ± 7% at the Inner Lagoon reef; *post hoc* $p < 0.05$).

Decline in Coral Bleaching Prevalence Differs Between Species During Recovery

Bleaching prevalence rapidly decreased for *P. compressa* at both reefs, with a 97% decrease in bleaching prevalence observed after 1.5 months of recovery at the Outer Lagoon reef to <2% overall prevalence, and an 89% decrease at the Inner Lagoon reef to <10% prevalence (**Figure 4**). In contrast, bleaching prevalence in *M. capitata* declined more slowly, with a mean decrease of 67% at the Inner Lagoon reef versus only a 9% decrease at the Outer Lagoon reef after 1.5 months. At that point, <3% of *M. capitata* remained fully bleached at either site,

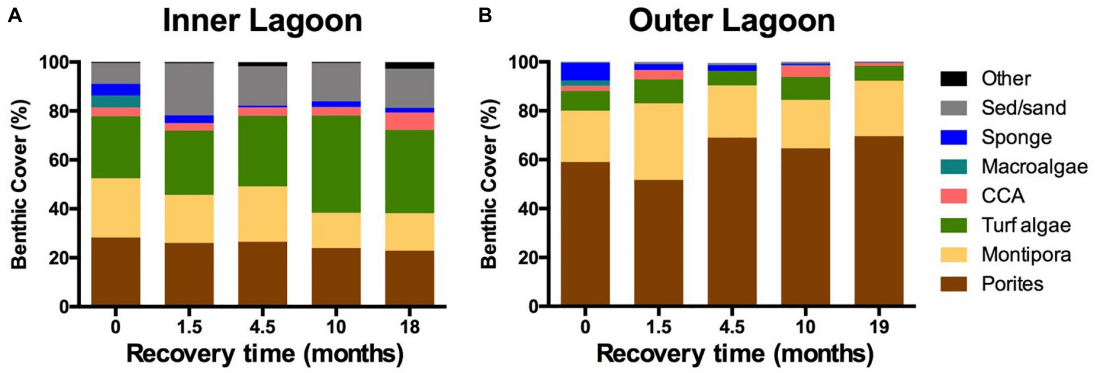

FIGURE 3 | Benthic community composition from the peak of the coral bleaching event (Oct. 2015) and after 1.5, 4.5, 10, and 18–19 months of recovery for **(A)** the Inner lagoon (PR4) and **(B)** the Outer lagoon (PR13) in Kāne'ohe Bay, O'ahu. Data represent the means of four replicate transects.

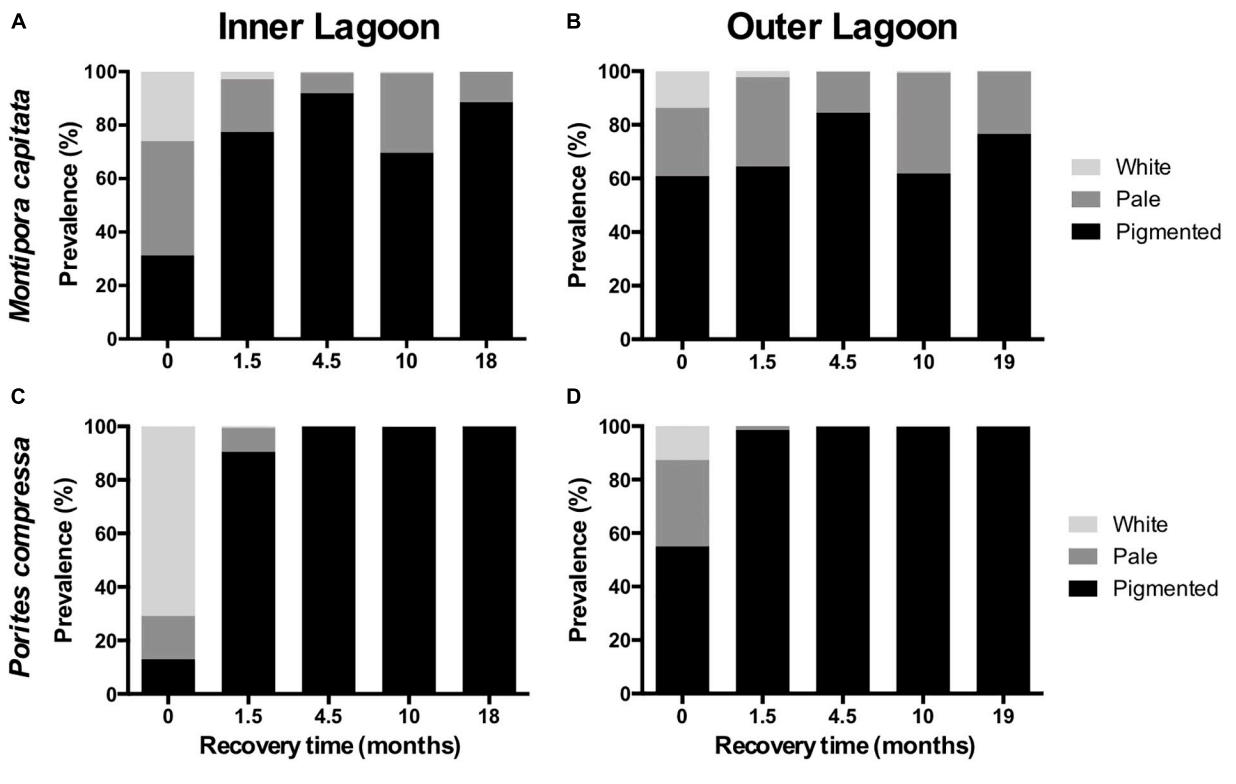

FIGURE 4 | Prevalence of coral bleaching from the peak of the bleaching event (Oct. 2015) and after 1.5, 4.5, 10, and 18–19 months of recovery for *Montipora capitata* **(A,B)** and *Porites compressa* **(C,D)** at the Inner lagoon (left column) and Outer lagoon (right column). Data represent the means of four replicate transects.

and 20–33% remained pale. In contrast to peak bleaching, coral bleaching prevalence was significantly higher for *M. capitata* than *P. compressa* after 1.5 months of recovery (**Figure 4**; *post hoc* $p = 0.02$) due to its slower recovery from bleaching. By 4.5 months of recovery, bleaching prevalence no longer differed between species (**Figure 4**). Interestingly, after several months of declining prevalence, the seasonal peak in water temperatures in September 2016 (Month 10; **Figure 1D**) corresponded with an increase in the prevalence of pale *M. capitata* (*post hoc* $p = 0.03$; **Figures 4A,B**). *Porites compressa* pigmentation was not affected by this seasonal warming, and bleaching prevalence remained below 2% from 1.5 months of recovery onward (**Figures 4C,D**).

Pigmentation Recovery Dynamics of Individual Coral Colonies Following Heat Stress

Bleaching resistant corals remained pigmented throughout the entire 24-month recovery period for both species, whereas bleached corals exhibited increasing pigmentation over the

first 3 months of recovery for both species (**Figures 2**, **5A–D**). This resulted in a significant interaction of bleaching phenotype and time point on coral pigmentation for both *M. capitata* and *P. compressa* ($p_{P:M} < 0.01$, **Table 2**). Additionally, there was a significant three-way interaction of bleaching phenotype, lagoon and month ($p_{P:L:M} < 0.01$, **Table 2**), and so we investigated how phenotype differed by lagoon at each time point. This analysis indicated that bleaching recovery times differed between *M. capitata* and *P. compressa*. For example, bleaching susceptible colonies of *P. compressa* remained distinguishable from resistant colonies 1.5 months post peak bleaching ($p_P < 0.01$, **Supplementary Table S5**), but recovered full visual pigmentation by 3 months at both reefs ($p_{P:L} > 0.05$; **Supplementary Table S5**; **Figures 5C,D**). In contrast, recovery was slower for *M. capitata*. Only 31% of bleaching susceptible *M. capitata* fully recovered normal pigmentation at the Inner Lagoon reef after 3 months, while no individuals had fully recovered at the Outer Lagoon reef at that time (**Figures 5A,B**). The phenotype effect differed by lagoon between three and six months of recovery ($p_{P:L} < 0.05$, **Supplementary Table S5**), such that there was a larger difference between phenotypes at the Outer Lagoon than the Inner Lagoon. Full pigmentation recovery for bleaching susceptible *M. capitata* colonies took as long as 24 months at the Inner Lagoon reef, going from 25% of individuals with full pigmentation at 18 months to 94% at 24 months (**Figure 5A**). At the Outer Lagoon reef, most bleaching susceptible *M. capitata* colonies (68%) remained pale for the entire 24-month duration of this study (**Figure 5B**).

Because both partners of each pair were not located at every time point, models were run on a subset of the data where any coral colony whose pair was not found at a time point was excluded ("pairs" models) in order to test for effects of spatial location on the reef on recovery dynamics. Here, the main effects were the same as for the "full" models. For *P. compressa*, the statistical patterns were identical to the three-way (phenotype:lagoon:month) and individual time point "full" models (**Supplementary Table S6**). For *M. capitata*, the statistical patterns were identical for the three-way model across time, although there were a few differences at individual time points. Specifically, the interaction of lagoon and bleaching phenotype that was significant in the "full" models at months 3–6, was not significant in the "pairs" models (**Supplementary Table S6**), indicating that differences in color score between adjacent bleached and unbleached corals were equal between lagoons. This indicates that there was no effect of local environment driving differences in bleaching recovery.

Colony Mortality Following Bleaching Differs Between Bleaching Phenotypes

Partial mortality increased significantly for both species over time following the bleaching event, the extent of which differed between bleaching phenotypes (*M. capitata*: $p_{P:M} < 0.01$, *P. compressa* $p_{P:M} = 0.03$; **Table 3** and **Figure 5**) and site ($p_{P:M:L} = 0.02$; **Table 2**). For each species, bleaching susceptible colonies had significantly more partial mortality than bleaching resistant colonies across both sites during the first year of recovery ($p_P < 0.01$, **Supplementary Table S7**), and most partial mortality of bleaching susceptible colonies occurred in the first 6 months following the heat stress (**Figures 5E–H**). At three months of recovery, partial mortality of bleaching susceptible and resistant phenotypes differed significantly more at the Inner Lagoon for *M. capitata*, and at the Outer Lagoon for *P. compressa* ($p_{L:P} = 0.01$; **Supplementary Table S7**). Overall, partial mortality was <20% in the first year following heat stress for both phenotypes of both species at the Outer Lagoon reef and for both phenotypes of *P. compressa* at the Inner Lagoon reef (**Figures 5F–H**). In contrast, mean partial mortality for *M. capitata* at the Inner Lagoon reef was 60% for bleaching susceptible colonies vs. 32% for bleaching resistant colonies at this time (**Figure 5E**). Mean partial mortality remained significantly higher for *M. capitata* at the Inner Lagoon reef than the Outer Lagoon reef at every time point across the 24-month recovery for both phenotypes ($p_L \leq 0.01$; **Supplementary Table S7**). Similarly, mean partial mortality for *P. compressa* was also higher at the Inner Lagoon reef than the Outer Lagoon reef regardless of bleaching phenotype after 24 months ($p_L < 0.01$; **Supplementary Table S7**).

While bleaching resistant corals showed lower partial mortality in the first year following the heat stress than bleaching sensitive corals, bleaching resistant corals showed increasing tissue loss across the second year of recovery. This resulted in no significant differences in mean partial mortality between phenotypes for either species at either site by 24 months (**Supplementary Table S7**). The one exception to the pattern that drove this convergence was for *P. compressa* at the Outer Lagoon reef, where bleaching sensitive *P. compressa* actually showed an average decline in partial mortality (**Figure 5H**). The most dramatic example of colony regrowth was *P. compressa* Tag no. 225, which suffered ∼60% mortality in the first 2 months following bleaching but then regrew to nearly 100% live tissue within one year following the heatwave. Only five colonies (one *P. compressa* and four *M. capitata*) experienced full mortality during the 2-year recovery period, and all were located at the Inner Lagoon reef. The statistical patterns did not differ when analyzing partial mortality scores from the "pairs" models, suggesting that spatial location on the reef did not greatly influence the difference in partial mortality between phenotypes (**Supplementary Table S8**).

DISCUSSION

Bleaching Susceptibility of a Species Is Not Predictive of Mortality

Porites compressa experienced higher bleaching prevalence and severity (i.e., lower bleaching resistance) than *Montipora capitata* at the Inner Lagoon reef, and yet bleaching susceptible individuals of *P. compressa* experienced significantly less partial mortality (i.e., greater bleaching resilience) than bleaching susceptible individuals of *M. capitata* at this same site. These results are in contrast to the common pattern of greater bleaching prevalence of a species leading to greater mortality (Baird and Marshall, 2002; Jones, 2008; Hughes et al., 2018b), and may reflect

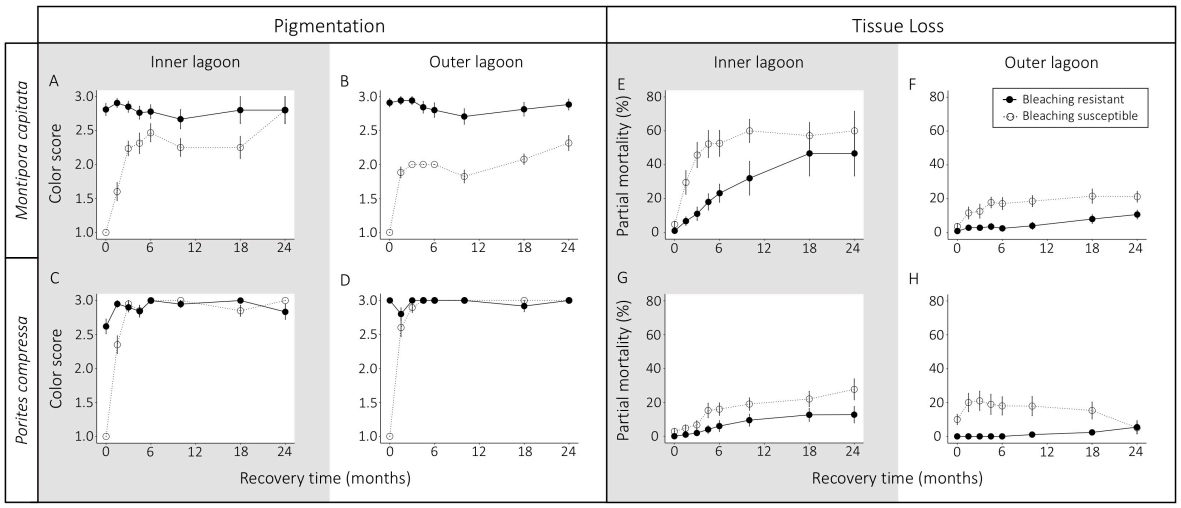

FIGURE 5 | Average color score **(A–D)** and partial mortality **(E–H)** of bleaching susceptible versus bleaching resistant colonies of *Montipora capitata* **(A,B,E,F)** and *Porites compressa* **(C,D,G,H)** at the Inner Lagoon **(A,C,E,G)** and Outer Lagoon **(B,D,F,H)** from the peak of the coral bleaching event (September–October 2015) through the following 24 months of recovery. Solid lines indicate bleaching resistant colonies; dashed lines indicate bleaching susceptible colonies. Color scores: 1, white; 2, pale; 3, pigmented. Error bars indicate SEM.

TABLE 2 | Type II Analysis of Variance Table with Satterthwaite approximation for the effects of bleaching phenotype, lagoon, and recovery time across the course of the study, on pigmentation recovery of bleaching susceptible and resistant colonies for *Montipora capitata* and *Porites compressa* at Inner and Outer lagoons (Color Score∼Phenotype × Lagoon × Month+(1|Coral Tag ID).

		SumSq	df (num, den)	*F*-value	*p*-value
Montipora capitata	Phenotype	32.43	1, 81.43	320.48	**<0.01**
	Lagoon	0.15	1, 84.01	1.46	0.23
	Month	23.54	7, 416.77	33.23	**<0.01**
	Phenotype:Lagoon	0.48	1, 84.16	4.74	**0.03**
	Phenotype:Month	28.07	7, 416.97	39.63	**<0.01**
	Lagoon:Month	1.84	7, 418.65	2.59	**0.01**
	Lagoon:Phenotype:Month	1.86	7, 418.69	2.62	**0.01**
Porites compressa	Phenotype	11.29	1, 76.98	167.58	**<0.01**
	Lagoon	0.56	1, 77.86	8.25	**0.01**
	Month	77.68	7, 483.42	164.71	**<0.01**
	Phenotype:Lagoon	0.01	1, 77.87	0.13	0.72
	Phenotype:Month	56.08	7, 483.44	118.90	**<0.01**
	Lagoon:Month	0.64	7, 484.54	1.35	0.23
	Lagoon:Phenotype:Month	1.96	7, 484.13	4.15	**<0.01**

Bold indicates statistical significance ($p < 0.05$).

two different species-specific heat stress response strategies. In the case of *M. capitata,* this species resisted bleaching to a greater extent than *P. compressa*, but individuals that bleached had lower resilience following bleaching (slower recovery and higher mortality). *P. compressa,* on the other hand, was more susceptible to bleaching but had greater resilience following bleaching (faster recovery and lower mortality) than *M. capitata*. The relatively lower resilience (higher partial mortality) of *M. capitata* than *P. compressa* following bleaching observed here was somewhat surprising because *M. capitata* typically replaces lost biomass following bleaching faster than *P. compressa* (Grottoli et al., 2006; Rodrigues and Grottoli, 2007; Wall et al., 2019), and was thus expected to have lower partial mortality. Faster biomass recovery in *M. capitata* occurs despite the observation that this species typically recovers pigmentation (chlorophyll *a*) slower than *P. compressa* (Grottoli et al., 2006; Rodrigues and Grottoli, 2007; Wall et al., 2019), though not always (Wall et al., 2019). Interestingly, while bleached *M. capitata* recovered pigmentation faster than *P. compressa* following the 2014 coral bleaching event (Wall et al., 2019), the reverse was observed following the 2015 event (**Figure 5**). This suggests that coral recovery rates following heat stress may depend on the frequency of stress events. As our observations describe the responses of these corals to the second of two bleaching events to occur within the span of one year, the discrepancies between the outcomes we observed and those of previous studies could be due in part

TABLE 3 | Type II Analysis of Variance Table with Satterthwaite approximation for the effects of bleaching phenotype, lagoon, and recovery time across the course of the study, on partial mortality of bleaching susceptible and resistant colonies for *Montipora capitata* and *Porites compressa* at Inner and Outer lagoons (Partial Mortality~Phenotype × Lagoon × Month+(1|Coral Tag ID).

		SumSq	df (num, den)	*F* value	*p*-value
Montipora capitata	Phenotype	2868	1, 84.64	22.15	**<0.01**
	Lagoon	5129	1, 84.74	39.61	**<0.01**
	Month	39812	7, 433.07	43.93	**<0.01**
	Phenotype:Lagoon	515	1, 84.90	3.98	**<0.05**
	Phenotype:Month	5986	7, 433.20	6.60	**<0.01**
	Lagoon:Month	18490	7, 433.34	20.40	**<0.01**
	Lagoon:Month:Phenotype	2176	7, 433.42	2.40	**0.02**
Porites compressa	Phenotype	1132.3	1, 77.94	16.80	**<0.01**
	Lagoon	2.5	1, 77.94	0.04	0.85
	Month	7181.9	7, 493.77	15.22	**<0.01**
	Phenotype:Lagoon	139.8	1, 77.94	2.07	0.15
	Phenotype:Month	1084.6	7, 493.78	2.30	**0.03**
	Lagoon:Month	5423.3	7, 493.78	11.49	**<0.01**
	Site:B2015:month	1112.8	7, 493.78	2.36	**0.02**

Bold indicates statistical significance ($p < 0.05$).

to the recurrent nature of the stress. This pattern has also been observed in other reef systems, where annual repeat bleaching events turn some Caribbean coral species from winners into losers (Grottoli et al., 2014).

Ecological Outcomes Differ Between Bleaching Susceptible and Resistant Phenotypes

Intraspecific differences in bleaching susceptibility had a significant influence on the ecological outcomes of populations of both coral species in the months following heat stress. For both *M. capitata* and *P. compressa*, bleaching susceptible individuals suffered higher partial mortality than bleaching resistant individuals located on the same reef. This resulted in significant losses of live coral cover from the reef, which despite not being detected in reef-wide surveys, likely has a significant impact on the ecological function of each reef. From an evolutionary perspective, these differences in partial mortality are likely to negatively impact reproductive success, as reproductive output is positively correlated with colony size in colonial coral species (Hall and Hughes, 1996) and thus corals suffering tissue loss will likely have lower fecundity in subsequent spawning seasons than they would have had. Differential partial mortality between susceptible and resistant phenotypes therefore likely influences individual fitness and thus the genetic composition of offspring released in subsequent reproductive events. Further exacerbating this loss, corals that have recently undergone bleaching have a lower likelihood of reproducing at all relative to bleaching resistant conspecifics, and those that do manage to reproduce tend to release fewer and less provisioned gametes (Ward et al., 2000; Fisch et al., 2019). Together these factors will likely limit the evolutionary success of bleaching susceptible genotypes and the ecological resilience of the reef by reducing the recruitment pool and live coral cover (Fisch et al., 2019; Hughes et al., 2019a). However, the low frequency of complete colony mortality during this bleaching event indicates the adult gene pool has remained mostly intact, maintaining the genetic diversity of the current population. Intraspecific differences in coral mortality also affect the ecological landscape of coral symbionts. In *M. capitata,* for example, bleaching resistant phenotypes tend to be dominated by *Durusdinium glynii*, whereas bleaching susceptible individuals are mostly dominated by *Cladocopium* sp. (Cunning et al., 2016), and lower partial mortality of *D. glynii*-dominated individuals would likely increase the relative abundance of *D. glynii* in the community. As symbiont transmission occurs vertically in this species (Padilla-Gamiño et al., 2012), this would also increase the proportion of larvae inheriting *D. glynii,* potentially altering the composition of the symbiont community for generations. Furthermore, as heat stress events increase in frequency and severity (Hughes et al., 2018a), the relative growth benefits associated with hosting thermally sensitive symbionts like *Cladocopium* spp. at non-stressful temperatures may fail to make up for the higher costs of these associations during heat stress, altering the tradeoffs of the association (Cunning et al., 2015). Mechanisms driving differential stress responses in *P. compressa* are less well understood, particularly as symbiont variation in this species is limited to a single species of *Cladocopium* (ITS2 clade C15; LaJeunesse et al., 2004). Differential bleaching in this species is thus likely driven by differences in host physiology (Baird et al., 2009), the composition of the microbiome (Morrow et al., 2018), or a combination thereof.

Coral Bleaching and Recovery Dynamics Differed Between Inner and Outer Lagoon Reefs

Montipora capitata and *P. compressa* both experienced higher bleaching prevalence and severity at the Inner Lagoon reef

than the Outer Lagoon reef, which corresponded with higher partial mortality for *M. capitata*. These results are reflective of coral mortality patterns from bay-wide surveys, which also observed higher cumulative coral mortality on reefs in the Inner Lagoon region (Bahr et al., 2017). Higher rates of bleaching and mortality within the Inner Lagoon were likely due to the higher accumulated heat stress at this site. However, additional environmental factors were likely involved because bleaching resistant *M. capitata* also had higher mortality at the Inner Lagoon reef relative to either phenotype at the Outer Lagoon reef. These data suggest that differences in coral bleaching and mortality between the two reefs were likely due to a combination of environmental factors, with worse outcomes within the Inner Lagoon potentially driven by the lower flow rates, longer seawater residence times (>30 days; Lowe et al., 2009), and closer proximity to land and associated freshwater runoff relative to the Outer Lagoon reef. Low flow environments can limit nutrient and waste exchange in the coral boundary layer, suppressing coral metabolism (Mass et al., 2010; Putnam et al., 2017). In addition, higher freshwater input (Bahr et al., 2015) and associated allochthonous inorganic nutrients (Stimson, 2015), which can lead to greater rates of bleaching (Wiedenmann et al., 2013; Vega Thurber et al., 2014), may have exacerbated coral stress and bleaching during the heat wave, limiting coral recovery and exacerbating tissue loss in the Inner Lagoon relative to the Outer Lagoon. Indeed, pigmentation recovery rates were slower within the Inner Lagoon in both the 2015 (this study) and 2014 bleaching events (Cunning et al., 2016; Wall et al., 2019). However, we cannot disentangle the role of nutrients versus differences in temperature and light regimes in driving heat stress responses in this study.

Coral Recovery Requires More Time Between Bleaching Events

Consecutive annual bleaching events have become a feature of coral reefs in Hawaii and around the world (Grottoli et al., 2014; Bahr et al., 2017; Hughes et al., 2018a; Raymundo et al., 2019). For example, corals in Hawaii had ∼10 months to recover from the effects of the 2014 heatwave before a second heatwave hit in 2015. This short recovery period is likely one reason the 2015 heatwave caused the highest cumulative coral mortality ever observed in Hawaii following a bleaching event (Bahr et al., 2017). Short intervals between heat waves such as these can lead to higher coral mortality following the second event because they may prevent individual corals from fully recovering energetically from the first heat stress prior to exposure to the second (e.g., Sale et al., 2019), and thus are likely to make individuals less resistant to bleaching and mortality in the second event. This short time interval also likely limits acclimatization, and indeed many colonies in Hawaii that bleached in 2014 tended to bleach again in 2015 (Ritson-Williams and Gates, 2020), suggesting that these corals had not acclimatized to higher temperatures. Longer recovery periods are also important for regrowth of tissue lost during a heatwave, and our data showed that tissue lost due to partial mortality was generally not replaced by live coral cover in the 2 years following the bleaching event. This indicates that a predominance of corals in Kāneʻohe Bay require longer recovery intervals to replace lost coral cover following bleaching-related mortality. Furthermore, our data indicate that symbiont recovery rates may take longer following repeat bleaching events, as *M. capitata* recovered visual pigmentation and symbiont abundance in a span of 1–3 months in previous bleaching events (Jokiel and Brown, 2004; Cunning et al., 2016; Wall et al., 2019; Ritson-Williams and Gates, 2020) and recovers in 8 months or less in mesocosm experiments (Rodrigues and Grottoli, 2007), while in 2015 we found that it took as much as 2 years for many bleached *M. capitata* individuals to fully recover pigmentation. In addition, repetitive bleaching events may influence the differential success of the major reef building species in Kāneʻohe Bay. We found that *P. compressa* recovered pigmentation faster than *M. capitata* and had a lower rate of tissue loss. This was similar to patterns of pigmentation recovery and mortality in these same species at other patch reefs in Kāneʻohe Bay during both the 2014 and 2015 bleaching events (Ritson-Williams and Gates, 2020), and suggests that *P. compressa* may gain ecological advantage if bleaching events become more common. Encouragingly, both the 2014 and 2015 events led to low rates of complete colony mortality, with <3% of corals suffering 100% mortality (*M. capitata* and *P. compressa;* this study) versus <2% of *M. capitata* in 2014 (Cunning et al., 2015). Also encouraging, there were individuals that underwent a complete recovery of live tissue cover following significant (>50%) partial mortality, suggesting that at least a few individuals, albeit a minority, are highly resilient and that given a few years of recovery between stress events can rapidly replace lost tissue when mortality is incomplete.

Individual Tracking Uncovers Low Levels of Partial Mortality Following Heat Stress

Both bleaching susceptible and bleaching resistant individuals suffered partial mortality in the months following the bleaching event, indicating that although resistant individuals did not visually bleach, all corals were negatively affected by heat stress. Interestingly, while bleaching-susceptible colonies of both species suffered an average of ∼20% tissue loss in the first 6 months following the bleaching event, these losses did not manifest as significant changes in live coral cover. This discrepancy could be due in part to the low mortality of bleaching resistant phenotypes masking the higher mortality of bleaching-susceptible individuals in community wide surveys. In addition, the high variance of photoquadrat surveys makes it difficult to detect small changes in benthic cover (Jokiel et al., 2015). Photoquadrat surveys are also commonly used to quantify recently dead coral cover during or following a bleaching event, however, this method cannot discern whether the coral that died had in fact bleached, whereas individual colony data revealed the consequences of heat stress for both bleaching phenotypes. While these losses in live coral cover were not detected at the community level, the significant partial mortality observed at the colony level has a negative impact on the ecology and evolution of coral communities as described above. Loss of susceptible individuals may also lower the genotypic richness of the population, which in corals can correlate with lower reproductive success

(Baums et al., 2013). That said, the lack of a significant decline in coral cover at these two reefs is encouraging for reef recovery from this bleaching event.

Living Library: Coral Pairs Are a Resource for Future Research and Restoration

By identifying individual coral colonies with distinct heat stress responses and tracking them over a multi-year period, we have generated and continue to maintain a live geo-referenced biological archive in the field as a resource for research on the effects of environmental history and thermal stress responses on the ecology and physiology of reef-building corals. This resource will be particularly valuable in light of predicted increases in the frequency and severity of coral bleaching events, as it allows for prospective sampling of bleaching susceptible vs. resistant phenotypes before, during, and following a bleaching event. This experimental system allows researchers to address questions of coral acclimatization and adaptation to changing oceans, such as examining whether individual coral responses predict tolerance to future stress. In addition, it allows researchers to identify if individual coral responses to subsequent events change relative to the outcomes observed in the current event (e.g., acclimatization, loss of resistance and/or resilience), potentially altering which species within the community are considered the ecological winners. Finally, fully recovered corals of known contrasting heat tolerances can be used to investigate the interaction of temperature tolerance with coral responses to other stressors, including but not limited to ocean acidification, eutrophication, and pathogens, helping to identify potential tradeoffs of heat tolerance in corals and the importance of phenotypic and genetic diversity in coral reef resilience (Stachowicz et al., 2007; Ladd et al., 2017). This will be useful for informing coral restoration strategies, particularly in light of the movement toward human assisted evolution (e.g., assisted gene flow), as it allows assessment of the potential tradeoffs of propagating heat tolerant genotypes (Van Oppen et al., 2015, 2017). From a management perspective, bleaching resistant individuals have the potential to serve as a resource for reef managers interested in propagating climate change resilient genotypes on damaged or degraded coral reefs (Van Oppen et al., 2017). Comparing bleaching responses within species demonstrates the importance of understanding individual colony susceptibility to heat stress and trajectories of recovery in the face of ongoing climate change. These questions are important for understanding the persistence of coral reefs into the future.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study and R scripts can be found in Zenodo at doi: 10.5281/zenodo.3862838. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

KB and RG designed the study. EL, JD, and JH helped to collect the data. KB, SM, AP, TI, and AH collected and analyzed the data. KB and SM wrote the manuscript. All authors edited and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00178/full#supplementary-material

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Exploring the Diversity and Metabolic Profiles of Bacterial Communities Associated With Antarctic Sponges (Terra Nova Bay, Ross Sea)

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Complex cell-to-cell interactions (including the production of antibiotics and the quorum sensing phenomenon) occur between benthic marine organisms and bacteria, leading to the establishment of synergistic interactions, especially in extreme and harsh environments, such as Antarctica. Despite this, current data concerning the composition, host- and site-relatedness, and biotechnological values of the bacterial community associated with Antarctic sponges are limited to few works, resulting in a still fragmented and incomplete knowledge. In this context, a total of 11 sponge species (belonging to Demospongiae and Hexactinellida) from the Terra Nova Bay area (Ross Sea) were explored for the associated bacterial diversity by the ION Torrent sequencing. An additional predictive functional analysis on 16S rRNA gene data was performed to unravel metabolic and biotechnological potentials of the associated bacterial communities. Data obtained highlighted the predominance of Proteobacteria, mainly affiliated to Alpha- and Gammaproteobacteria. Retrieved phyla were similarly distributed across samples, with dissimilarities encountered for the sponge *Haliclona* (*Rhizoniera*) *dancoi* (Topsent, 1901). Functional prediction results suggested that the associated bacterial community may be involved in the biosynthesis of antibiotics, quorum sensing, and degradation of aromatic compounds.

Keywords: bacterial diversity, NGS, predictive metabolic profiling, sponge-bacteria association, Antarctica

INTRODUCTION

Symbiosis is one of the most fascinating phenomena occurring in nature, bringing relevant implications from ecological, evolutionistic, and bioprospecting perspectives (Li et al., 2014). Marine sponges are the most ancient multicellular invertebrates, peculiar representatives of the benthic communities, and very sensitive indicator organisms (Glasl et al., 2019). Symbiotic

relationships involving marine sponges possess a great evolutionary and ecological significance. These peculiar interactions are affected by a diverse set of environmental parameters and require the establishment of complex signal communication systems and fine regulating processes between the host and its symbionts, as well as within the symbiotic community itself (Mohamed et al., 2008; Mangano et al., 2009, 2018). Several studies have contributed to the prokaryotic diversity characterization of sponge-associated assemblages. They have been mainly aimed at establishing the taxonomical specificity of sponge *core* microbiome and/or the possible vertical or horizontal transmission processes involved in the acquisition and maintenance of ancient symbionts (Taylor et al., 2007; Webster et al., 2010). However, greater efforts are available for the exploration of sponges inhabiting more accessible study areas, mainly tropical and temperate habitats, while studies performed in polar regions remain very scant (Cárdenas et al., 2014; Lo Giudice and Rizzo, 2018; Lo Giudice et al., 2019a).

Even though sponges dominate vast areas of the Antarctic shelves, the sponge–bacteria association has been seldom explored (Savoca et al., 2019 and references therein). Recently, interesting evolutionary and ecological insights have been pointed out, and they highlight the great versatility of Antarctic sponges as hosts of a wide range of microorganisms, with Proteobacteria and Bacteroidetes dominance within the whole prokaryotic community (Webster et al., 2004; Rodríguez-Marconi et al., 2015; Cárdenas et al., 2018). Rodríguez-Marconi et al. (2015) also emphasized differences occurring in the taxonomical structure of sponge-associated bacterial assemblages in Antarctic and tropical-temperate regions, by suggesting strong peculiarity and specificity for Antarctic sponges. Indeed, in contrast with some studies reporting the high stability of sponge-associated bacterial assemblages (Cárdenas et al., 2014; Erwin et al., 2015), others detected important shifts under thermal stress (Lemoine et al., 2007; Webster et al., 2008; Fan et al., 2013) or under a combination of stressful factors (Pineda et al., 2016, 2017). Beside culture-independent methods, the cultivable-based approach should not be neglected, as a complementary tool to the molecular approach, gaining useful information in an applicative purview and a better comprehension of metabolic and physiological processes, also in Antarctic sponges (Papaleo et al., 2012; Savoca et al., 2019).

Contrary to bacterial diversity, the metabolic functional aspects of prokaryotic symbionts of Antarctic sponges (from the Antarctic Peninsula) have been only recently explored by Steinert et al. (2019), highlighting the possible role of symbionts in the biosynthesis of secondary metabolites alongside their involvement in the biodegradation of xenobiotics. The predicted functional evaluation of sponge-associated bacterial communities is a new challenge of sponge symbioses, especially in extreme environments whose peculiar conditions might be responsible for the development of unique adaptation strategies. This approach could be a time-reducing tool useful to support the discovery of novel metabolic mechanisms of remarkable interest in a bioprospecting outlook (Sipkema, 2016; Steinert et al., 2019).

In line with these considerations, this work was aimed at expanding our current knowledge on the taxonomic composition of the whole bacterial communities associated with Antarctic sponges, by coupling these new information with a predictive profiling approach applied on 16S rRNA data. The study contributes to provide interesting insights on the diversity, biotechnological potential, and ecological role of bacterial sponge symbionts in the Terra Nova Bay area, one of the most studied in terms of sponge biodiversity in the Ross Sea (Ghiglione et al., 2018 and references therein).

MATERIALS AND METHODS

Sampling Area

Sampling activities were carried out in the Terra Nova Bay area (hereafter TNB). TNB is often ice-free during polar summer months and span for about 64 km along the coast of Victoria Land, being comprised between Cape Washington and the Drygalski Ice Tongue. It represents an important site for long-term research on the marine ecology of benthic communities (e.g., Piazza et al., 2019, 2020), supporting rich and complex sponge and anthozoan communities, alongside loosely structured and low diversity assemblages, often coexisting in mosaics (e.g., Sarà et al., 1992; Cattaneo-Vietti et al., 1997; Gambi et al., 1997; Cerrano et al., 2009).

Collection of Sponge Specimens

Sponge specimens (n = 12) were collected from four sites (namely, A, B, C, and D) at Terra Nova Bay (Ross Sea, Antarctica) during the XXIX Italian Expedition to Antarctica. Sampling depths ranged between 30 and 271 m (**Table 1**). Sponge specimen collection (by dredge, SCUBA diver, or remotely operated underwater vehicle, ROV) was authorized by the PRNA project, conformably to the Antarctic Treaty legislation and the SCAR Code of Conduct for the Use of Animals for Scientific Purposes in Antarctica (ATCM XXXIV, 2011). To minimize the risk of environmental contamination (especially in the case of specimens collected by dredging), dissected specimens were rinsed with 0.2 μm filtered seawater (Mangano et al., 2009; Steinert et al., 2019). Briefly, organisms were immediately washed at least three times with filter-sterilized natural seawater to remove transient and loosely attached bacteria and/or debris. Specimens were then placed into individual sterile plastic bags containing filter-sterilized natural seawater and transported directly to the laboratory at 4°C for microbiological processing (within 2 h after sampling). Fragments of each specimen were then preserved in 70% ethanol for taxonomic identification and at –20°C for DNA extraction. Sponge fragments and glass slides of spicules used for morphological identification are curated at the Italian National Antarctic Museum (MNA, Section of Genoa, Italy). The MNA voucher codes of the specimens here studied are reported in **Table 1**.

Sponge Taxonomy

Skeletal architecture was examined by light microscopy. Hand-cut sections of the ectosome and choanosome were made following Hooper (2000). Spicule complement was analyzed according to Rützler (1978). Taxonomic decisions are in

TABLE 1 | Sample identifiers, sponge identification, sampling site and depth, and coordinates.

Site	Sample ID	Sponge taxonomy	MNA code	Sampling method	Sampling depth (m)	Coordinates
A	THB2	*Microxina sarai* Calcinai & Pansini, 2000	MNA11773	SCUBA	30	74°40,635′ S-164° 03,925′E
	THB19	*Tedania* (*Tedaniopsis*) *oxeata* Topsent, 1916	MNA11788	ROV	250	74°40,599′ S-164°04,001′E
B	THB6	*Hemigellius pilosus* (Kirkpatrick, 1907)	MNA12274	dredge	40	74°41,705′ S-164°04,882′E
	THB7	*Hemigellius pilosus* (Kirkpatrick, 1907)	MNA12275	dredge	60	74°41,705′ S-164°04,882′E
	THB8	*Haliclona* (*Rhizoniera*) *dancoi* (Topsent, 1901)	MNA11779	dredge	40	74°41,705′ S-164°04,882′E
	THB9	*Mycale* (*Oxymycale*) *acerata* Kirkpatrick, 1907	MNA11780	dredge	60	74°41,705′ S-164°04,882′E
C	THB10	*Mycale* (*Aegogropila*) sp.	MNA11777	dredge	40	74°41,400′ S-164°06,109′E
	THB11	*Myxodoryx hanitschi* (Kirkpatrick, 1907)	MNA11781	dredge	40	74°41,400′ S-164°06,109′E
D	THB15	*Rossella villosa* Burton, 1929	MNA11784	dredge	271	74°43,152′ S-164°12,484′E
	THB16	*Cinachyra antarctica* (Carter, 1872)	MNA11785	dredge	271	74°43,152′ S-164°12,484′E
	THB17	*Lissodendoryx* (*Ectyodoryx*) *ramilobosa* (Topsent, 1916)	MNA11786	dredge	260	74°43,170′ S-164°12,764′E
	THB18	*Isodictya erinacea* (Topsent, 1916)	MNA11787	dredge	260	74°43,170′ S-164°12,764′E

agreement with the Systema Porifera (Hooper and van Soest, 2002), the revision of demosponge classification of Morrow and Cárdenas (2015), and the World Porifera Database (WPD) (Van Soest et al., 2018).

Bacterial Community Diversity and Composition

DNA Extraction

Portions of sponge tissues (between 1 and 1.5 g) were reduced to pulp by using sterile pestles. Obtained pulps were used for DNA extraction by employing the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, United States) according to the manufacturer's instructions. DNA concentration and purity were quantified by using a NanoDrop ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies, United States).

Amplification of 16S rRNA Genes by ION Torrent Sequencing

Extracted DNA was used to amplify the V1–V2 region of 16S rRNA gene of Bacteria (primers 27f 5′-AGAGTTTGATCCTGGCTCAG-3′ and 338 5′-GCT GCC TCC CGT AGG AGT-3′). PCR amplification was carried out under the conditions described by Conte et al. (2018). Briefly, in order to reduce bias in massive sequencing, a two-step PCR protocol was applied: the first step consisted of a conventional PCR, and then amplicons were used as a template for the second PCR with barcoded primers for Ion Torrent sequencing. PCR products were purified using the AgencourtAMPure XP kit (Beckman Coulter, Inc.), according to the manufacturer's instructions, and then quantified using the Qubit dsDNA HS Assay Kit with Qubit Fluorometer 2.0 (Invitrogen, Thermo Fisher Scientific). Each purified product (20 ng) was pooled for emulsion PCR with Ion PGM Template OT2 400 Kit. Sequencing was performed on an Ion Torrent Personal Genome Machine™, using the Ion PGM Sequencing 400 Kit and the Ion 314™ chip (all Ion Torrent reagents by Thermo Fischer Scientific) following the manufacturer's protocols. All steps during amplification and sequencing were checked using negative controls. Ion Torrent sequence data obtained from this study have been registered as an NCBI BioProject **PRJNA594262**.

Post-run Analysis

At the quality check and trimming steps, read portions with a Phred quality score less than 20 per base and four or more consecutive low-quality base calls were removed using Trimmomatic (sliding window 4:20) (Bolger et al., 2014). Then, denoising was carried out with DADA2 with the denoise-single command (using –p-trim-left 20 –p-trunc-len 0 –i-demultiplexed-seqs). Quality trimming resulted in approximately 338,091 high-quality reads for 12 samples with an average of 28,174 reads (65%) per sample. All samples were included in the downstream quantitative analyses. Sequences were taxonomically classified in QIIME2 (version 2019.4) (Bolyen et al., 2019) by SILVA reference files (SILVA release 132 full-length sequences and taxonomy references) using classify-consensus-blast. Sequences were clustered into OTUs and taxonomically assigned at 97% identity. The resulting OTU table was normalized to the lowest number of reads among the samples (24,980) for the downstream diversity and quantitative analyses.

Predictive Functional Profiling

Metagenomes from 16S data were predicted by QIIME2 with the PICRUSt2 tool (version 2.1.2), which uses evolutionary modeling and a reference genome database (Douglas et al., 2019). The Hidden state prediction method with the mp (Maximum Parsimony) approach was used. To specify how distantly a sequence needs to be placed in the reference phylogeny, before it is excluded, the command –p-max-nsti (cutoff 2) was applied. The accuracy of metagenome predictions was tested trough the Nearest Sequenced Taxon Index (NSTI). The accuracy prediction is related to the presence of closely representative bacterial genomes. The lower values reveal a closer mean relationship. The data obtained by PICRUSt2 were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017).

Statistical Analyses

Shannon diversity index (*H'*) for each sample was calculated by QIIME2 software based on the total clustered OTU number.

Bacterial community structure and predicted expressed pathways were normalized by Trimmed Mean of M-values (TTM) normalization using R package HTSFilter (Bioconductor 3.10) (Rau et al., 2013). The abundance of all retrieved OTUs was clustered in a heat map using R pheatmap package (v 1.0.12). Abundance results were used to perform statistical analyses using PRIMER v6 for Windows (PRIMER-E Ltd, Plymouth, United Kingdom). Data were analyzed for eventual differences/similarities among the bacterial communities, by considering the sampling sites (A to D) as factors. The Bray–Curtis similarity matrices were used to perform the cluster analysis. Non-metric multidimensional scaling analysis (nMDS) was computed on Bray–Curtis similarity matrices obtained from OTU relative abundances after a log(x+1) transformation.

RESULTS

Sponge Taxonomy

We collected in total 12 sponge specimens and identified 11 species belonging to two classes: Demospongiae (10) and Hexactinellida (1). Demospongiae were represented by three orders: Haplosclerida [i.e., *Hemigellius pilosus* (Kirkpatrick, 1907), *Microxina sarai* Calcinai & Pansini, 2000, and *Haliclona* (*Rhizoniera*) *dancoi* (Topsent, 1901)], Poecilosclerida (i.e., *Isodictya erinacea* (Topsent, 1916), *Lissodendoryx* (*Ectyodoryx*) *ramilobosa* (Topsent, 1916), *Mycale* spp., *Myxodoryx hanitschi* (Kirkpatrick, 1907), and *Tedania* (*Tedaniopsis*) *oxeata* Topsent, 1916), and Tetractinellida [i.e., *Cinachyra antarctica* (Carter, 1872)]; instead, the class Hexactinellida was represented by the sole Lyssacinosida order (i.e., *Rossella villosa* Burton, 1929) (**Table 1**).

Diversity of Sponge-Associated Bacterial Communities

Analyzed bacterial sequences were resolved in a total of 177 OTUs. On average, 40 distinct OTUs were found *per* sample, with the lowest value (21 OTUs) that was observed for the sponges THB6 and THB7, both classified as *H. pilosus* (**Table 2**). The nMDS analysis computed on data from the relative abundances of retrieved OTUs is shown in **Figure 1**. Most sponge samples clustered in a larger group, which included a subcluster composed of *H.* (*R.*) *dancoi*_THB8 and *R. villosa*_THB15. The sponges *H. pilosus*_THB6 and THB7 grouped together, as well as *L.* (*E.*) *ramibolosa*_THB17 that clustered separately from the rest.

Overall, OTUs were distributed in 16 bacterial phyla, differently distributed among samples (**Figure 2** and **Table 2**). Proteobacteria dominated in all samples (range 57.3–90.8% of the total community) and were particularly abundant within the bacterial community associated with *L.* (*E.*) *ramilobosa*_THB17 (90.8% of sequences for this sponge). Minor contributes were provided by Bacteroidetes, Actinobacteria, and Firmicutes, accounting on average for 7.3, 7.1, and 6.5% of the total community, respectively. Bacteroidetes were mainly represented in *C. antarctica*_THB16 (9.6%), while Actinobacteria presented a higher relative abundance in *M. sarai*_THB2 (12.2%). Firmicutes resulted considerably abundant in *C. antarctica*_THB16 (14.8%), whereas they ranged from 0.3 to 7.2% in all other sponge samples. The relative abundances of the remaining phyla (i.e., Acidobacteria, BRC1, Cyanobacteria, Deinococcus-Thermus, Fusobacteria, Nitrospinae, Patescibacteria, Planctomycetes, Spirochaetes, Tenericutes), among which there were representatives of some typical sponge-associated bacteria, were mainly less than ≤1% each, and they were generally not shared among all sponge specimens (**Table 2**). Within Proteobacteria, Alpha- and Gammaproteobacteria were well represented. Alphaproteobacteria were particularly abundant within the bacterial community associated with *H. pilosus* (samples THB6 and THB7) and *L.* (*E.*) *ramilobosa*_THB 17. Gammaproteobacteria mainly dominated the bacterial communities associated with *Mycale* (*A.*) sp._THB10 and *R. villosa*_THB15. Cyanobacteria were quite absent in all sponge samples.

The 70% of the total high-quality bacterial sequences were classified at genus level, by resulting in a total of 157 genera (from 0.01 to 38.2% of total sequences) (heatmaps in **Figure 3**). In line with their marked dominance, a high number of retrieved genera were affiliated with Alpha- and Gammaproteobacteria. In detail, among Alphaproteobacteria, a total of 46 genera were detected, with the predominance of *Methylobacterium* (up to 38.2%) that occurred at high percentages in almost all samples (exceptions were *H. pilosus*_THB6 and THB7). The genus *Sphingomonas* showed a similar distribution among samples, with the highest relative abundance (15.9%) in *H.* (*R.*) *dancoi*_THB8. Conversely, the genera *Thalassobius* and *Amylibacter* resulted dominant in *H. pilosus*_THB6 and THB7 (19.9 and 7.2% in THB6, respectively; 16.3 and 6.3% in THB7, respectively). The genus *Bradyrhizobium* was exclusively detected in *M. hanitschi*_THB11 (20.5% of total sequences). Among Gammaproteobacteria, the genus *Methylobacter* was retrieved in *L.* (*E.*) *ramilobosa*_THB17 (9%), while *Erwinia* representatives were detected at a high percentage (on average 7.1%) in almost all samples (exceptions were *H. pilosus*_THB6 and THB7). All the remaining alpha- and gammaproteobacterial genera occurred at percentages <3%. With respect to Bacteroidetes, the genus *Prevotella* was particularly abundant (up to 12.1%) and ubiquitous, while *Polaribacter* members were observed almost only in *M. hanitschi*_THB11 (4.0%). With respect to Gram-positives, among Actinobacteria, the genus *Cutibacterium* occurred in all samples, ranging from 0.3 and 7.8%, while *Rhodococcus* members were present only in *H.* (*R.*) *dancoi*_THB8 (3.7%). Finally, a total of 23 genera were detected among Firmicutes, each occurring at <2% in all samples. Exception was the genus *Paenibacillus*, which was well represented in *C. antarctica*_THB16 and *T.* (*T.*) *oxeata*_THB19 (17.1 and 4%, respectively). The bacterial community composition (with respect to both phyla and genera) is also shown as interactive Krona charts in **Supplementary Material S1**: i.e., SiteA.html, SiteB.html, SiteC.html, SiteD.html (Krona charts are in HTML format and need to use a web browser to be opened).

Shannon diversity values (ranging from 3.1 and 5.5) suggested a very different bacterial community composition among samples (**Table 3**). The cluster analysis of the total retrieved genera in all samples pointed out a larger group of samples

TABLE 2 | Relative abundance values (%) of bacterial phyla retrieved in sponge samples.

Phylum	SITE A		SITE B				SITE C			SITE D		
	M. sarai **THB2**	***T.* (*T.*) *oxeata*** **THB19**	***H. pilosus*** **THB6**	***H. pilosus*** **THB7**	***H.* (*R.*) *dancoi*** **THB8**	***M.* (*O.*) *acerata*** **THB9**	***Mycale* (*A.*) sp.** **THB10**	***M. hanitschi*** **THB11**	***R. villosa*** **THB15**	***C. antarctica*** **THB16**	***L.* (*E.*) *ramilobosa*** **THB17**	***I. erinacea*** **THB18**
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0
Actinobacteria	12.2	3.3	0.4	0.0	4.9	4.2	2.1	2.5	7.7	2.0	2.2	2.6
BRC1	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteroidetes	5.2	4.1	0.6	0.1	4.4	2.3	4.5	5.5	5.0	9.6	1.5	2.9
Cyanobacteria	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5
Deinococcus-Thermus	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0
Firmicutes	4.1	7.2	0.3	0.3	2.0	2.4	1.2	1.9	6.0	14.8	0.9	3.5
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Nitrospinae	1.1	0.0	0.0	0.1	0.8	0.0	0.0	0.0	0.0	0.4	0.0	0.0
Patescibacteria	0.4	0.0	0.3	0.6	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.5	2.9
Alphaproteobacteria	13.7	31.5	76.9	68.8	29.6	25.6	22.2	19.7	13.6	18.3	55.5	9.8
Deltaproteobacteria	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.4	0.0	0.0
Gammaproteobacteria	25.9	37.7	7.3	3.4	40.1	40.0	57.0	28.4	48.0	33.6	33.6	37.1
Proteobacteria unclassified	18.5	12.4	2.2	8.0	15.3	13.6	9.1	25.2	10.0	8.9	1.7	10.4
Spirochaetes	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1

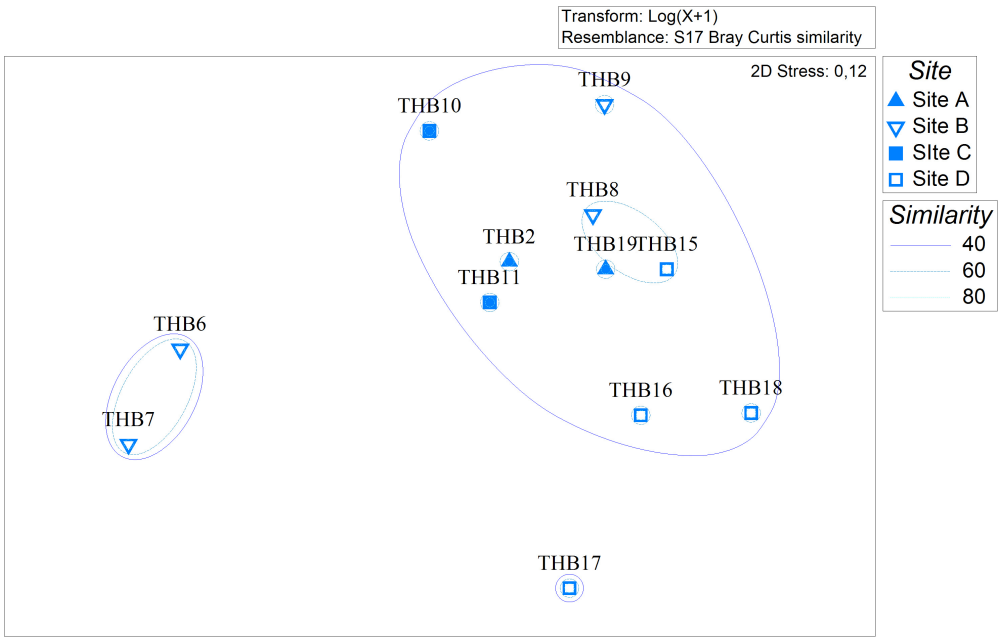

FIGURE 1 | Non-metric multidimensional scaling analysis (nMDS) computed on Bray-Curtis similarity values obtained for relative abundances of bacterial OTUs retrieved in sponge samples.

composed of *Mycale* sp._THB10, *T.* (*T.*) *oxeata*_THB19, *H.* (*R.*) *dancoi*_THB8, *M.* (*O.*) *acerata*_THB9, *R. villosa*_THB15, and *I. erinacea*_THB18. On the other side, samples *H. pilosus*_THB6 and THB7 clearly separated from the rest, whereas *L.* (*E.*) *ramibolosa*_THB17, *C. antarctica*_THB16, *M. sarai*_THB2, and *M. hanitschi*_THB11 were more related to the larger group, even if they appeared separated one to each other. Instead, the analysis generated from results of all genera retrieved within Proteobacteria showed a larger group constituted of *M. sarai*_THB2, *M.* (*O.*) *acerata*_THB9, *R. villosa*_THB15, *C. antarctica*_THB16, *L.* (*E.*) *ramibolosa*_THB17 and *I. erinacea*_THB18. *H. pilosus*_THB6, and THB7 clearly separated from the rest, whereas *H.* (*R.*) *dancoi*_THB8, *Mycale* (*A.*) sp._THB10, *M. hanitschi*_THB11, and *T.* (*T.*) *oxeata*_THB19 generated a second group of samples (**Figure 3A**). The clustering obtained from all genera not related to Proteobacteria (i.e., Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Fusobacterial, Nitrospinae, Planctomycete, Spirochaetes, and Tenericutes) underlined a different situation in which *C. antarctica*_THB16 was completely separated from the other samples. Two main groups were observed. The first one was composed by *M. hanitschi*_THB11, *L.* (*E.*) *ramibolosa*_THB17, *H. pilosus*_THB6, and THB7 (these latter were closely related). The second group was composed by *M. sarai*_THB2, *R. villosa*_THB15, *H.* (*R.*) *dancoi*_THB8, *M.* (*O.*) *acerata*_THB9, *Mycale* (*A.*) sp._THB10, *I. erinacea*_THB18, and *T.* (*T.*) *oxeata*_THB19 (**Figure 3B**).

Predictive Functional Profiling

A total of 5,523 predicted KEGG Orthologs (KOs; i.e., sets of homologous sequences) were present in the analyzed samples. Overall, 752 KEGG pathways resulting in 70 different molecular functions were found. Results relative to biological processes (>2%) predicted for the whole bacterial community associated with sponges are reported in **Figure 4**. In detail, the "biosynthesis of secondary metabolites" was the most represented KEGG pathway (49.9%), followed by the ATP-binding cassette transporters (ABC transporters; one of the largest and possibly one of the oldest gene families) and the "two-component system" (TCS), consisting of two proteins in most bacteria, a sensor histidine kinase and a response regulator involved in chemical signal transmission and reception (22.8 and 14.1%, respectively). The "biosynthesis of antibiotics" (4.5%), "quorum sensing" (3.2%), "methane metabolism" (3.2%), and "degradation of aromatic compounds" (2%) pathways were less represented. A detailed list of the biological processes, with the corresponding molecular functions, exhibited by the individual sponge-associated bacterial communities, is reported in **Table 4** and detailed below.

Antibiotic Biosynthesis

Myo-inositol-1(or 4)-monophosphatase (KO01093) and glucose-1-phosphate thymidylyltransferase (KO00973) molecular functions, both associated with streptomycin biosynthesis, were observed in all samples (range 3.4–6.4% and 2.4–3.4%, respectively) (**Supplementary Figure S1**). The former was particularly evident in *H. pilosus*_THB6 and THB7 (5.9 and 6.4%, respectively) and *M. hanitschi*_THB11 (3.9%). Other pathways retrieved in all samples were correlated with the staurosporine biosynthesis (i.e., glutamate 5-kinase, KO00931; UTP-glucose-1-phosphate uridylyltransferase, KO00963; range 2.5–3.4 and 2.1–3.4%, respectively).

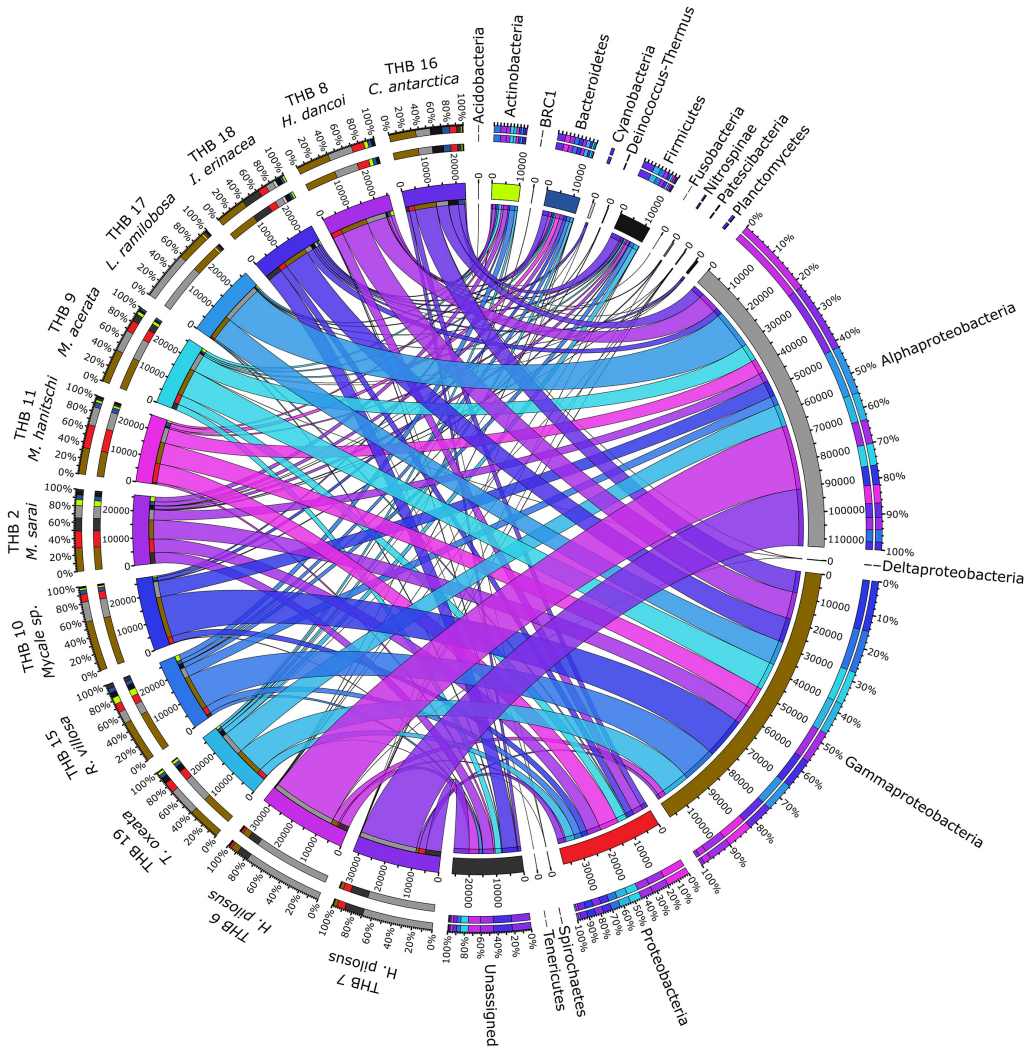

FIGURE 2 | The figure shows the connection among main phyla found in association with the different analyzed sponges. The sponges analyzed are shown on the right of the image, with the color scale from blue to purple (the colors of the ribbons inside the figure). Main phyla found in different colors are shown on the left (colors from top to bottom: orange, yellow, sea blue, silver, gray, black, light green, dark blue, blue, ash, light gray, teal, brown, red, dark green, light green and dark gray). Ribbon size in the figure codifies cell value associated with Sponge/phyla segment pair. Finally, the histograms present under the different sponge species report the percentage distribution of the different phyla inside the sponge.

Degradation Pathways

The potential ability to degrade aromatic compounds was highlighted by the occurrence of two specific functions (i.e., 3-phenylpropionate/trans-cinnamate dioxygenase ferredoxin reductase component, KO00529, and 4-carboxymuconolactone decarboxylase, K01607; range 1.01–2.8 and 1.6–3.5%, respectively) that are related to the degradation of toluene, benzaldehyde, benzyl alcohol, benzoate, and other water-insoluble aromatic hydrocarbons (**Supplementary Figure S2**).

Methane Metabolism

Molecular functions related to methane metabolism (**Supplementary Figure S3**) were also observed in all samples. Among the most abundant processes, there were K00123 (formate dehydrogenase major subunit) and K00831 (phosphoserine aminotransferase) (ranged between 2.2–5.3 and 1.6–3.3%, respectively). They are involved in the oxidation of carbon compounds, such as methanol and methane. Otherwise, the KO14083 (trimethylamine-corrinoid protein co-methyltransferase), involved in methanogenesis from trimethylamine, was mainly abundant in the bacterial community from *C. antartica*_THB16 and *L.* (*E.*) *ramilobosa*_THB17 (6.4 and 5.4%, respectively).

Quorum Sensing

Molecular functions associated with the quorum sensing phenomenon (**Supplementary Figure S4**) were generally retrieved at very high percentages. Among the most abundant processes, a number of KOs were associated with sensing

FIGURE 3 | Heatmaps showing the genera distribution and clustering based on relative abundances detected in sponge samples. Color blocks represent the relative abundance of genera. More dark indicates a higher relative abundance. **(A)** Proteobacterial genera. **(B)** Genera belonging to other phyla or classes.

proteins, such as Qrr, LuxQ, LuxO, LuxU, CqsS, CAI-1, LitR/HapR, LuxP, AI-2, LuxN/AinR, and AI- (e.g., branched-chain amino acid transport system permease protein, K01998; peptide/nickel transport system ATP-binding protein, K02032; preprotein translocase subunit SecA, K03070; signal recognition particle subunit SRP54; K03106; range 5.7–9.3, 8.5–14.9, 2.2–3.4, and 2.2–3.4%, respectively). The acyl homoserine lactone synthase (KO13060) was mainly expressed by the bacterial communities associated with *H. pilosus*_THB6 and THB7 (1.4 and 1.3%, respectively).

DISCUSSION

Previous microbiological studies on sponges from the Terra Nova Bay (Ross Sea) area were mainly focused on the phylogenetic affiliation, on the ecological roles and biotechnological potential of the cultivable bacterial fraction (e.g., Mangano et al., 2009, 2014, 2018; Papaleo et al., 2012; Caruso et al., 2018; Savoca et al., 2019), and, at a lesser extent, on the diversity and ecology of associated microeukaryotes, specifically diatoms (e.g., Cerrano et al., 2000, 2004a,b; Totti et al., 2005) and, in even

TABLE 3 | Results of the next-generation sequencing analysis and retrieved diversity associated with Antarctic sponge samples.

Site	Sponge species_specimen	Total reads (n°)	Good-quality reads (n°)	OTU (n°)	Shannon *H'*	Evenness
A	*Microxina sarai*_THB2	42805	28600	50	5.101	0.827
	Tedania (*Tedaniopsis*) *oxeata*_THB19	39427	27755	42	4.186	0.721
B	*Hemigellius pilosus*_THB6	46541	32849	21	3.114	0.629
	*Hemigellius pilosus*_THB7	40125	32287	21	3.082	0.641
	Haliclona (*Rhizoniera*) *dancoi*_THB8	46617	26060	49	3.961	0.705
	Mycale (*Oxymycale*) *acerata*_THB9	45348	26640	42	4.593	0.765
C	*Mycale* (*Aegogropila*) sp._THB10	44374	27858	24	3.664	0.720
	*Myxodoryx hanitschi*_THB11	41321	29249	35	4.524	0.779
	*Rossella villosa*_THB15	41333	28169	52	4.926	0.812
D	*Cinachyra antarctica*_THB16	44423	24980	58	5.462	0.841
	Lissodendoryx (*Ectyodoryx*) *ramilobosa*_THB17	44908	27849	33	3.499	0.641
	*Isodictya erinacea*_THB18	43915	25795	48	4.686	0.800

more rare cases, on the associated macrofauna (e.g., Schiaparelli et al., 2003). In this context, we aimed at exploring more in depth the diversity and predictive functional profiles of the whole bacterial communities associated with 11 Antarctic sponge species, within the classes Demospongiae and Hexactinellida, commonly found in the Terra Nova Bay. In particular, in consideration of the growing interest in Antarctic resources, the predictive analysis on the bacterial metabolic potentials was performed to evaluate the potential exploitability in biotechnological fields of bacteria associated with Antarctic sponges. Data obtained in this study add further elements to the current knowledge, which still remains rather scarce and fragmentary (also in terms of biogeography), on the Antarctic sponges–bacteria association (Webster et al., 2004; Rodríguez-Marconi et al., 2015; Cárdenas et al., 2018). It is noteworthy that Antarctica must be preserved and, also by virtue of the best intentions, great care must be taken toward it and its living and non-living resources. In this regard, in order to keep sampling as less invasive as possible, sponge specimens belonging to different species (sufficiently representative for an explorative purpose) were collected from four different sites within the sampling area.

Phylogenetic Affiliation of Sponge-Associated Bacterial Communities

Taxonomic affiliation identified a diversified bacterial communities hosted by the analyzed sponges (overall, 177 distinct OTUs were retrieved), with the number of OTUs hosted *per* species that ranged between 21 (*H. pilosus*_THB6 and THB7) and 58 (*C. antarctica*_THB16). Such numbers of bacterial OTUs are lower than those reported by other authors for Antarctic sponges. In fact, Steinert et al. (2019) and Rodríguez-Marconi et al. (2015) yielded average values of 142 and 1,601 (this latter including Bacteria and Archaea) distinct OTUs *per* sponge sample, respectively. However, in line with previous results by Cárdenas et al. (2018) and Rodríguez-Marconi et al. (2015), a high diversity value (H' values >3) was calculated for our samples. These two aspects taken in consideration together suggested a rather uniform distribution of OTUs among the analyzed sponge samples.

Overall, a strong dominance of Proteobacteria, with considerable abundance of both Alpha- and Gammaproteobacteria, characterized the communities of sponge-associated bacteria. Bacteroidetes, Actinobacteria, and Firmicutes were less represented than Proteobacteria, even if they were retrieved in almost all samples. Finally, members of other phyla (i.e., Acidobacteria, Nitrospinae, and Cyanobacteria) were sporadically detected in a smaller number of sponges. Such findings are in line with previous investigations (e.g., Webster et al., 2004; Savoca et al., 2019; Steinert et al., 2019) reporting a high abundance of associated Proteobacteria (in addition to Actinobacteria and Bacteroidetes), including cultivable members, in cold-water sponges, and with general observations on marine sponges worldwide (Hentschel et al., 2012; Thomas et al., 2016; Moitinho-Silva et al., 2017). The bacterial communities associated with the analyzed sponges were affiliated to 157 different known genera, which differed in both number and frequency among sponges. In particular, Proteobacteria genera strongly contributed to the diversification of sponges at the genus level. The bacterial communities associated with Antarctic sponges analyzed in this study included members involved in methane biochemical processes, such as *Methylobacterium* and *Methylobacter* (among Alpha- and Gammaproteobacteria, respectively). Some *Methylobacterium* affiliates are able to grow on one-carbon compounds (such as methanol, methylamine, formaldehyde, and formate). They are frequently found in association with plants (Green, 2006), but recently *Methylobacterium* sequences were also retrieved in sponges from temperate environments (Najafi et al., 2018). *Methylobacter* is a methanotroph frequently detected in numerous freshwater and marine habitats (Smith et al., 2018). Interestingly, in line with Steinert et al. (2019), the predictive analysis (see below) highlighted the presence of pathways involved in the methane metabolism. Bacterial members involved in the nitrogen cycle, i.e., *Bradyrhizobium* spp., were also found, even if only in *M. hanitschi*_THB11. Members in such genus are well-known typical symbionts of leguminous plant, but they were recently detected also in the microbiome of sponges from the Persian

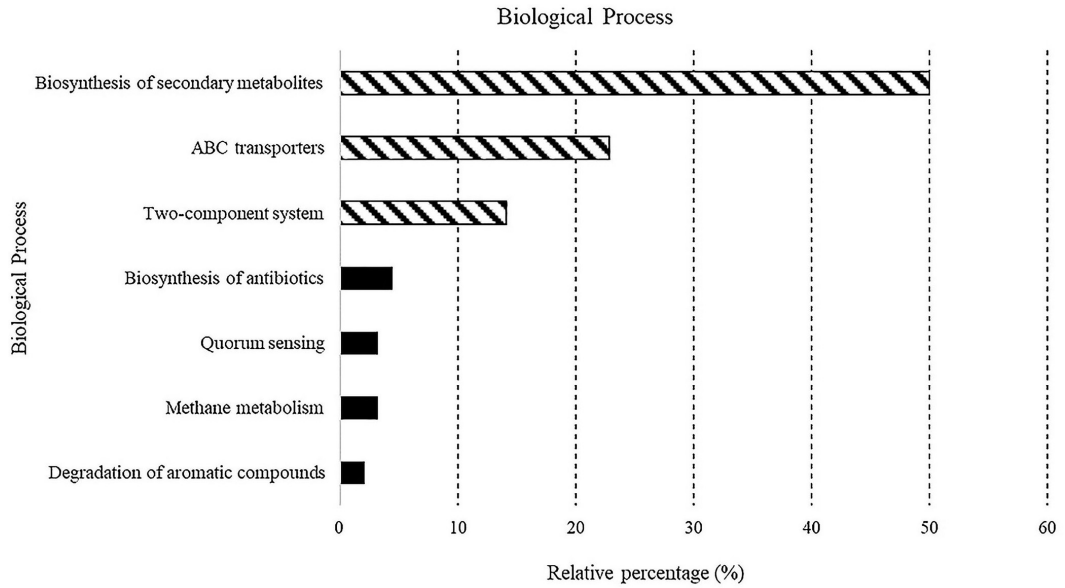

FIGURE 4 | Predicted biological processes expressed as percentages in all samples.

Gulf (Najafi et al., 2018). Among Alphaproteobacteria, the genus *Sphingomonas* was present at similar percentages in almost all sponges. *Sphingomonas* members are frequently found in marine matrices and play important ecological roles in the marine environment (Cavicchioli et al., 1999). Its occurrence was already reported in association with the Antarctic marine sponges *Haliclona virens* (Topsent, 1908), *Tedania* sp., and *Lissodendoryx* (*Ectyodoryx*) *nobilis* (Ridley and Dendy, 1886) from the Terra Nova Bay area (i.e., the Adelie Cove) (Savoca et al., 2019). Finally, among Bacteroidetes, we observed the occurrence of *Polaribacter* members, especially in *M. hanitschi*_THB11. Such cold-adapted copiotrophic bacteria are frequently found in marine environments of polar regions, also in association with *Myxodoryx hanitschi* from Antarctica (Webster et al., 2004; Savoca et al., 2019).

Results obtained for sponges collected from the same site highlighted that the bacterial communities associated with *M. sarai*_THB2 and *T.* (*T.*) *oxeata*_THB19 from site A (OTU-sharing 88.7%), and *H. pilosus*_THB6 and THB7 from site B were similar at the OTU level (OTU-sharing 55.5%). Interestingly, the specimens THB6 and THB7, both affiliated to *H. pilosus*, showed a high similarity in terms of genera, with a sharing percentage of 98% mainly due to the relative abundances of the genera *Acinetobacter* and *Enterobacter*, and hosted bacterial communities different from those observed for the other sponges. Such differences may be mainly dependent on the lower abundances of Gammaproteobacteria (below 10% of total sequences) and the higher abundances of Alphaproteobacteria (approx. 70%) than other sponge samples. The cultivable community associated with *H. pilosus* from the Terra Nova Bay was previously characterized by Mangano et al. (2014), who reported on the predominance of Gammaproteobacteria. Such discrepancy is probably dependent on the r-strategy adopted by members of this phylogenetic group that, being able to rapidly grow on nutrient-rich media (such as marine agar) and successfully competing under heterotrophic conditions, are generally well represented in culture collection (Lo Giudice et al., 2012). Further, if compared with the other samples, members of the genera *Thalassobius*, *Amylibacter*, *Roseovarius*, and *Sulphitobacter* were better represented within the bacterial communities of *H. pilosus*_THB6 and THB7, whereas other genera (such as *Cutibacterium*, *Sphingomonas*, and *Erwinia*) were absent or less represented. In particular, *Thalassobius* spp. are chemoorganotrophic marine bacteria showing complex ionic requirements to grow (Pujalte et al., 2018).

The similarity observed at the OTU (29.4%) and genus (47.7%) levels for *Mycale* (*O.*) *acerata*_THB9 and *Mycale* (*A.*) sp._THB10, collected from two different sites (site B and site C, respectively), mainly derived from the relative abundances of Bacteroidetes and *Chryseobacterium* spp., suggesting a possible influence of sponge genus features in shaping the associated bacterial community. In this regard, the microbiota associated with *Mycale* spp. was previously characterized by Webster et al. (2004) and Cárdenas et al. (2018) using culture-independent techniques. In particular, Cárdenas et al. (2018) explored the microbial communities associated with the *Mycale* (*Aegogroipila*) *magellanica* and *Mycale* (*Oxymycale*) *acerata* sampled from distant areas. Results revealed a high similarity (total OTU-sharing, 74%) in the community composition, with the dominance of Proteobacteria and Bacteroidetes. Our *Mycale* spp. [i.e., *M.* (*O.*) *acerata*_THB9 and *Mycale* (*A.*) sp._THB10] shared the 29.5 and 47.7% of OTUs and genera, respectively. The predominance of Proteobacteria observed by Cárdenas et al. (2018) in *Mycale* spp. sponges was confirmed by the present study. Conversely, Bacteroidetes were less represented,

TABLE 4 | KEGG pathway stored by biological process and molecular function with relative predicted enrichment value in all samples.

Ko terms	*Biological process*	*Molecular function*	*M. sarai* THB2	*T. oxeata* THB19	*H. pilosus* THB6	*H. pilosus* THB7	*H. dancoi* THB8	*M. acerata* THB9	*Mycale* sp. THB10	*M hanitschi* THB11	*R. villosa* THB13	*C. antarctica* THB16	*L. ramilobosa* THB17	*I. erinacea* THB18
K08295		2-aminobenzoate-CoA ligase												
K00177		2-oxoglutarate ferredoxin oxidoreductase subunit gamma												
K00955		bifunctional enzyme CysN/CysC												
K01060		cephalosporin-C deacetylase												
K05555		cyclase												
K01697		cystathionine beta-synthase												
K17217		cystathionine gamma-lyase / homocysteine desulfhydrase												
K00200		formylmethanofuran dehydrogenase subunit A												
K00973		glucose-1-phosphate thymidylyltransferase												
K00931		glutamate 5-kinase												
K00865		glicerate 2-kinase												
K01655		homocitrate synthase												
K05375	*Biosynthesis of antibiotics*	MbtH protein												
K05551		minimal PKS ketosynthase (KS/KS alpha)												
K00010		myo-inositol 2-dehydrogenase / D-chiro-inositol 1-dehydrogenase												
K01092		myo-inositol-1(or 4)-monophosphatase												
K01443		N-acetylglucosamine-6-phosphate deacetylase												
K01434		penicillin amidase												
K09459		phosphonopyruvate decarboxylase												
K00831		phosphoserine aminotransferase												
K00958		sulfate adenylyltransferase												
K00956		sulfate adenylyltransferase subunit 1												
K00957		sulfate adenylyltransferase subunit 2												
K06998		trans-2,3-dihydro-3-hydroxyanthranilate isomerase												
K00963		UTP--glucose-1-phosphate uridylyltransferase												
K00208		enoyl-[acyl-carrier protein] reductase I												
K05712		3-(3-hydroxy-phenyl)propionate hydroxylase												
K14727		3-oxoadipate enol-lactonase / 4-carboxymuconolactone decarboxylase												
K05710		3-phenylpropionate/trans-cinnamate dioxygenase ferredoxin component												
K00529		3-phenylpropionate/trans-cinnamate dioxygenase ferredoxin reductase component												
K01607		4-carboxymuconolactone decarboxylase												
K04105		4-hydroxybenzoate-CoA ligase												
K00483		4-hydroxyphenylacetate 3-monooxygenase												
K00151	*Degradation of aromatic compounds*	5-carboxymethyl-2-hydroxymuconic-semialdehyde dehydrogenase												
K06912		alpha-ketoglutarate-dependent 2,4-dichlorophenoxyacetate dioxygenase												
K07104		catechol 2,3-dioxygenase												
K03379		cyclohexanone monooxygenase												
K00484		flavin reductase (NADH)												
K14519		NADP-dependent aldehyde dehydrogenase												
K00481		p-hydroxybenzoate 3-monooxygenase												
K00480		salicylate hydroxylase												

(Continued)

TABLE 4 | Continued

Ko terms	*Biological process*	*Molecular function*	*M. sarai* **THB2**	*T. oxeata* **THB19**	*H. pilosus* **THB6**	*H. pilosus* **THB7**	*H. dancoi* **THB8**	*M. acerata* **THB9**	*Mycale* sp. **THB10**	*M hanitschi* **THB11**	*R. villosa* **THB13**	*C. antarctica* **THB16**	*L. ramilobosa* **THB17**	*I. erinacea* **THB18**
K00122		formate dehydrogenase												
K00123		formate dehydrogenase major subunit												
K00124		formate dehydrogenase iron-sulfur subunit												
K00126		formate dehydrogenase subunit delta												
K00127		formate dehydrogenase subunit gamma												
K00831	*Methane metabolism*	phosphoserine aminotransferase												
K00863		triose/dihydroxyacetone kinase / FAD-AMP lyase (cyclizing)												
K00925		acetate kinase												
K01070		S-formylglutathione hydrolase												
K01499		methenyltetrahydromethanopterin cyclohydrolase												
K14083		trimethylamine---corrinoid protein Co-methyltransferase												
K13060		acyl homoserine lactone synthase												
K10556		AI-2 transport system permease protein												
K10555		AI-2 transport system substrate-binding protein												
K01998		branched-chain amino acid transport system permease protein												
K01999		branched-chain amino acid transport system substrate-binding protein												
K10914		CRP/FNR family transcriptional regulator, cyclic AMP receptor protein												
K09823		Fur family transcriptional regulator, zinc uptake regulator												
K18306		membrane fusion protein, multidrug efflux system												
K18307	*Quorum sensing*	multidrug efflux pump												
K02032		peptide/nickel transport system ATP-binding protein												
K03070		preprotein translocase subunit SecA												
K03076		preprotein translocase subunit SecY												
K02054		putative spermidine/putrescine transport system permease protein												
K03106		signal recognition particle subunit SRP54												
K07173		S-ribosylhomocysteine lyase												
K06998		trans-2,3-dihydro-3-hydroxyanthranilate isomerase												
K07666		two-component system, OmpR family, response regulator QseB												
K07645		two-component system, OmpR family, sensor histidine kinase QseC												

whereas an increased presence of Firmicutes was observed. Differently from Cárdenas et al. (2018), Gammaproteobacteria were more abundant than Alphaproteobacteria in *M.* (*O.*) *acerata*_THB9.

Predictive Functional Profiling

To date, the functional analysis of Antarctic sponge-associated prokaryotic communities has been rarely performed (Steinert et al., 2019). It should be noted that findings on the functional patterns are predictive hypotheses and they do not necessary reflect the real microbial processes inside the sponges. However, this approach provides more insight into the potential role that microbes play in this critical host association. From a functional viewpoint, it was very interesting that a very high percentage of predicted pathways was associated with the biosynthesis of secondary metabolites. This finding might be related to the production of bioactive compounds playing a role in the inhibition of microorganisms, which could act as competitors for essential nutrients. The nature of these bioactive compounds is still unknown, but some of them might be volatile organic compounds (VOCs), as previously suggested for bacteria associated with *Haliclonissa verrucosa* Burton, 1932, *Lissodendoryx* (*E.*) *nobilis*, and *Anoxycalyx* (*Scolymastra*) *joubini* (Topsent, 1916) from the Terra Nova Bay (Romoli et al., 2011, 2014; Papaleo et al., 2012, 2013). Such results highlighted the important role of sponge/benthic invertebrate symbiotic bacteria, also from polar areas, in a bioprospecting scenario as producers of bioactive natural products (Lo Giudice and Rizzo, 2018; Rizzo and Lo Giudice, 2018). Several pathways involved in the production of antibiotics were predicted in this study. Among them, molecular functions correlated with the biosynthesis of streptomycin (expressed at particularly high extent by the bacterial communities associated with *H. pilosus*_THB6 and THB7 and *M. hanitschi*_THB11) and staurosporine. In particular, molecular processes at the basis of streptomycin biosynthesis were also evidenced by Steinert et al. (2019), who suggested that the presence of such predicted pathways might indicate a host-specific antimicrobial activity in the interested sponges.

The second highly predicted processes were the two-component systems and ABC transporter. This finding is not surprising since bacteria inhabiting such harsh environments should sense the presence of nutrients, even at low concentrations, to growth and proliferate under extreme conditions.

Quorum sensing was firstly reported in the present study as a prediction for bacterial communities associated with Antarctic sponges. Cell-to-cell communication occurs within the dense bacterial communities associated with sponges (Taylor et al., 2004). Quorum sensing is among environmental sensing systems involved in regulating the bacterial symbiotic colonization of metazoan organisms, including population density, social activities, and physiological processes (such as the formation of biofilm for bacterial adhesion to surfaces; Mohamed et al., 2008). Our results reinforced previous observations by Mangano et al. (2018) on 211 cultivable Gram-negative bacteria isolated from Terra Nova Bay sponges (i.e., *Lissodendoryx* (*Ectyodoryx*) *nobilis*, *Phorbas glaberrimus*, and *Myxodoryx hanitschi*) and able to produce *N*-Acyl homoserine lactones (AHLs), signal molecules involved in the QS mechanism. The AHL producer mainly belonged to bacterial genera that are known to be involved in surface colonization by biofilm synthesis.

Interestingly, biological processes related to the degradation of xenobiotics (such as toluene, benzaldehyde, benzyl alcohol, benzoate, and other hydrophobic compounds), particularly aromatic substances, were also predicted. Such compounds are well-known environmental contaminants, which could accumulate in marine filter-feeding organism tissues, thus probably stimulating the development of specific bacterial symbiotic communities with the ability to utilize them as a source of carbon and energy (Mangano et al., 2018; Rizzo and Lo Giudice, 2018; Lo Giudice et al., 2019b). The occurrence of molecular pathways involved in pollutant degradation represents a starting step for exploring more in depth the possible implications in bioremediation processes of Antarctic sponge-associated bacteria. Further insights are necessary in order to understand if the predicted degradation pathway may be an indication of the local pollution level and/or if such capacities are included in the detoxifying system owned by prokaryotes within the sponge-holobiont, as previously suggested (Steinert et al., 2019 and therein references).

CONCLUDING REMARKS

In conclusion, the application of a combined approach, which included the analysis of both diversity and predictive functional metabolic profile of Antarctic sponge-associated bacterial communities, furnished novel and interesting insights on the symbiotic communities hosted by benthic filter-feeding invertebrates living in polar areas, and allowed individuating a number of their putative ecological roles and biotechnological potentialities. In terms of diversity, our Antarctic sponges seemed to be attributable to low microbial abundance (LMA) sponges, which commonly host bacterial communities dominated by few taxonomic groups (mainly Proteobacteria and Bacteroidetes). This assumption is strengthened, as it was previously reported for sponges of Antarctic areas different from the Terra Nova Bay, by the absence of the *Poribacter* group, which are instead normally associated with high microbial abundance (HMA) sponges. Furthermore, our results confirm the so far documented absence of such taxon in the Antarctic environments. Further investigation should be performed to establish at which extent the surrounding environment (e.g., seawater and sediment) influences the composition of the bacterial communities associated with Antarctic sponges, as well as the processes involved in the selection of true symbiotic (instead of potentially transient) bacteria. At this regard, more research efforts should be addressed to the quorum sensing phenomenon, which is probably strictly involved in the adhesion (and the subsequent colonization) of symbiotic bacteria to the sponge surfaces through the formation of biofilm. Finally, on the basis of the predictive metabolic profiling, the involvement of associated bacteria in possible host defensive strategies (including the biodegradation of toxic compounds and the biosynthesis

of metabolites inhibiting sponge pathogens or biofouling) is not to be excluded.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI BioProject, NCBI Accession No. PRJNA594262.

AUTHOR CONTRIBUTIONS

AL and RF designed and conceived the study. LM, MP, and SS collected samples during the XXIX Italian Expedition to Antarctica. MB and GC classified the sponge samples. MP and CR undertook the laboratory work and sequencing, and generated data. AP-G and MP performed bioinformatics analysis. AL, MP, CR, and MA analyzed and interpreted the data and wrote the first draft of the manuscript. All authors contributed to manuscript corrections and improved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00268/full#supplementary-material

MATERIAL S1 | Supplementary Krona charts: *SiteA.html*, Kronachart relative to the bacterial community composition in samples THB2 (*M. sarai*) and THB19 [*T.* (*T.*) *oxeata*]; *SiteB.html*, Kronachart relative to the bacterial community composition in samples THB6, THB7 (both *H. pilosus*), THB8 [*H.* (*R.*) *dancoi*] and THB9 [*M.* (*O.*) *acerata*]; *SiteC.html*; Kronachart relative to the bacterial community composition in samples THB10 (*Mycale* (*A.*) sp.) and THB11 (*M. hanitschi*); *SiteD.html*, Kronachart relative to the bacterial community composition in samples THB15 (*R. villosa*), THB16 (*C. antarctica*), THB17 (*L.* (*E.*) *ramilobosa*) and THB18 (*I. erinacea*).

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Effects of Climate Change Stressors on the Prokaryotic Communities of the Antarctic Sponge *Isodictya kerguelenensis*

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Microbial symbionts of marine sponges play important roles for the hosts and also for their ecosystems. The unique tolerance of marine sponges to a wide diversity of microbial symbionts allows them to acquire a wide variety of "evolutionary solutions" to environmental challenges. Ice scour is one of the main forces structuring Antarctic benthic communities, and its effect is expected to increase as further warming is projected for the Western Antarctic Peninsula (WAP). The interaction of these physical drivers may have a significant impact, shaping the microbiome of Antarctic sponges under current and future scenarios of climate change. The aim of this research was to assess how stressors, such as warming and injuries produced by ice scour, affect the microbiome of the marine Antarctic sponge *Isodictya kerguelenensis* under current and predicted scenarios. Individuals of *I. kerguelenensis* were sampled in shallow waters (10 m) off the coast of Doumer Island, Palmer Archipelago, WAP. In order to mimic the effect of tissue damage produced by ice scour, tissue samples were taken at days 0 (T0d) and 15 (T15d) from individuals placed in a control (0.5°C) and two temperature treatments (3 and 6°C). Our analysis of 16S libraries from the V4–V5 region revealed two phyla of archaea and 22 of bacteria. Proteobacteria and Bacteroidetes were the most representative in terms of both number of operational taxonomic units (OTUs) and sequence abundances. The analysis at the OTU level shows a significant interactive effect of injury and temperature. Principal coordinate analysis (PCoA) shows a clear group of uninjured sponges and three other groups of injured sponges according to temperature. Our results also show a group of OTUs that were only present in injured sponges and are potential markers of sponge damage. Our study suggests that the disturbance produced by icebergs may have a direct impact on the sponge microbiome. Future climate change scenarios with warming and increases in iceberg impacts may lead to prokaryotic symbiont disruption on sponge species, potentially having cascading effects for the host and the functional roles they play in the Antarctic ecosystem; however, the potential effects of this disruption are to be further studied.

Keywords: microbiome, Porifera, benthic communities, tissue injury, heat-stress, ice scour disturbance, sponge health

INTRODUCTION

The phylum Porifera, known as sponges, is a highly important metazoan, playing roles in three main areas: (a) impacts on the substrate (provoking bio-erosion, building and stabilizing reefs, and consolidating and regenerating the benthos substrate), (b) bentho-pelagic coupling (interfering in carbon, silicon, oxygen, and nitrogen cycles and coupling energy from the pelagic to the benthic ecosystem), and (c) sponge associations with other organisms (facilitating primary production, being directly implicated in secondary production, and providing a complex microhabitat from bacteria and archaea to other macro-invertebrates and fish) (Bell, 2008).

Sponges are the earliest diverged metazoan group still extant (van Soest et al., 2012) and continue to survive in vast numbers of marine and freshwater habitats, adapting to drastic changes in environmental factors and competing biota (Müller, 2003). According to van Soest et al. (2012), the simple body organization of sponges and relative plasticity of the cellular elements, coupled with a unique tolerance toward symbiotic microorganisms, allows for a great diversity of "evolutionary solutions" to environmental challenges. Antarctic sponges are no exception; they are important members of benthic communities and host diverse prokaryotic communities with a remarkable host specificity of the microbiome at spatial (Rodríguez-Marconi et al., 2015; Cárdenas et al., 2018a; Steinert et al., 2019) and temporal scales (Cárdenas et al., 2019). However, the number of studied species is still very limited (see Lo Giudice et al., 2019 for review).

The Western Antarctic Peninsula (WAP) is one of the areas of the planet experiencing some of the most significant environmental changes (Stenni et al., 2017; Znój et al., 2017; Siegert et al., 2019). The increase in seawater temperature constitutes a major threat to Antarctic ecosystems and recent reports have shown that some areas in the WAP seem to be more exposed to environmental variation than others, suggesting organisms inhabiting different zones might show different responses depending on the habitats where they occur (Cárdenas et al., 2018b). In addition, ice scour is considered to be a major force shaping the ecological characteristics of the Antarctic benthos in shallow waters (Gutt and Starmans, 2001; Brown et al., 2004; Barnes, 2017; Barnes et al., 2018; Lo Giudice et al., 2019; Morley et al., 2019). Disturbance produced by icebergs can affect the seabed and associated benthic communities (Gutt, 2001; Cook et al., 2005). In some areas of the WAP, up to a third of the substrate in shallow waters is disturbed by icebergs within a year (Barnes, 2017). In this regard, some research has assessed the responses of benthic organisms to damage, such as that produced by ice scour. Research on other Antarctic invertebrates has shown the existence of differences in recovering from shell damage and shell thickness in the Antarctic bivalve *Laternula elliptica* depending on the level of exposure to ice scour (Harper et al., 2012). In addition, a study on the transcriptomic response of shell damage in the same species (Sleight et al., 2015) revealed that different important molecular pathways were affected, switching from energy production to biomineralization during the shell repair process. Similar studies on the effect of ice scour on the ecology/physiology of other sessile Antarctic organisms, such as sponges, are yet to be conducted.

In order to improve predictions regarding the impact of climate change on Antarctic biota, it is necessary to study the synergistic effects of multiple environmental stressors because responses to a single stressor tested individually may not be the same as when they are tested at the same time (Bell and Carballo, 2017). However, only a few studies on the combined effects of pH and temperature in Antarctic invertebrates have been conducted (Ericson et al., 2012; Suckling et al., 2015; Schram et al., 2016). This is explained by difficulties associated with working in the Antarctic and the availability of facilities that are able to undertake complex laboratory experiments (Kennicutt et al., 2016).

The increase of sea-surface temperature can have negative impacts on some sponge species by negatively affecting growth and survival, producing high levels of tissue necrosis and bleaching (Bennett et al., 2017). Recent studies have reported mass mortality events due to warming in sub-Arctic sponges (Ereskovsky et al., 2019). In contrast, other sponge species are more resistant to thermal stress (Guzman and Conaco, 2016; González-Aravena et al., 2019). Several studies have also tested the effect of warming on the sponge-associated microbial community, showing contrasting results. Although some studies have demonstrated the ability of some sponge species to remain stable with no effect on their microbiome (Simister et al., 2012; Pineda et al., 2016; Cárdenas et al., 2019), in others, warming produced changes and even disruption of the microbiome, affecting sponge health (Lemoine et al., 2007; Luter et al., 2012b; Fan et al., 2013). In addition, other studies (e.g., Lesser et al., 2016) reported that, although warming produced no change in the microbiome composition in the tropical sponge *Xestospongia muta*, the combined stress of warming and pH decrease changed the microbiome composition. In contrast, the microbiome of the bioeroding sponge *Cliona orientalis* remained stable up to a 4°C increase, but a community shift occurred at a further 6°C increase, and near 32°C, the microbe composition changed significantly (Ramsby et al., 2018).

The effect of physical disturbance on the sponge microbiome remains unstudied. Studies from tropical corals have reported changes in some bacterial groups after injuries and differences between diseased and healthy sponges (Meyer et al., 2016; Shirur et al., 2016). A study on the Great Barrier Reef sponge *Rhopaloeides odorabile* reported that sublethal temperature stress did not produce bacterial alteration; however, a bacterial shift was observed in necrotic individuals, revealing microbiome shifts when lesions are visible (Simister et al., 2012). Regarding unhealthy and dying sponges, considerable microbiome alteration has been revealed with shifts in the abundance of some taxa (Blanquer et al., 2016; Luter et al., 2017; Belikov et al., 2019). In light of previous research from other latitudes, microbiome composition can be a good marker of holobiont health. In this regard, despite an increase of Antarctic sponge microbiome information, few studies have provided insights on holobiont health that take into account all environmental threats with which the sponge holobiont must cope at the present and in future conditions in Antarctica. In the case of the Antarctic

sponge *Isodictya* sp., a previous transcriptomic study suggested that increased temperature could cause stress at the limits of the organism's capacities (González-Aravena et al., 2019).

Because climate change projections suggest warming will induce further ice shelf collapse, glacier retreat, and reduction of sea ice, ice scour is expected to have increased effects on benthic organisms as more icebergs will be able to float freely in open water, reaching shallow areas (Barnes et al., 2018; Morley et al., 2019). For this reason, we tested if the interaction of increased temperature and physical injuries produced by ice scour could impact the stability of the microbiome of the Antarctic sponge *Isodictya kerguelenensis* (Ridley and Dendy, 1886), which is a common sponge in shallow areas around the Palmer Archipelago, WAP.

MATERIALS AND METHODS

Sponge Collection and Experimental Design

Eighteen small individuals of the Antarctic sponge *Isodictya kerguelenensis* (∼6–8 cm) were collected by SCUBA divers at 10 m depth in Cape Kemp, off Yelcho Research Station, Doumer Island, Palmer Archipelago, WAP (64°51′S 63°35′W), in January 2018 (**Figure 1A**). Samples were collected by hand and placed in plastic bags inside buckets for transportation underwater. Sponge individuals were then transported to the laboratory facility at Yelcho Research Station (INACH), where they were maintained in 140 L fiberglass tanks with unfiltered seawater at approximately 0.5°C for a week to allow acclimation to laboratory conditions. Water was changed daily, and circulation within each tank was provided by submersible aquarium pumps.

Experimental tanks were covered with two layers of 1-mm mesh shade cloth (fiberglass 50% neutral density screen) in order to represent light levels occurring *in situ*. After the acclimation period, three individuals were randomly allocated to one of three different experimental tanks (**Figure 1B**). The experimental design consisted of three treatments: control (0.5°C) and two heat-stress treatments at 3°C and 6°C, and these same sponges were cut to mimic the effect of ice scour (n = 3 per treatment).

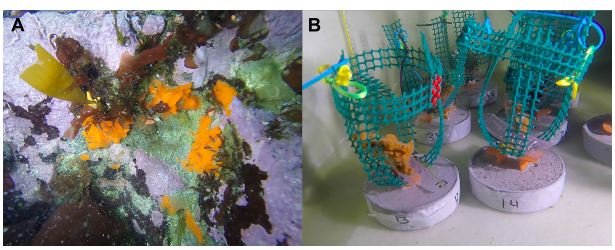

FIGURE 1 | The Antarctic sponge *Isodictya kerguelenensis*. **(A)** Photographed *in situ* at 10 m depth in Cape Kemp off Yelcho Research Station, Doumer Island, WAP (64°51′S 63°35′W) in January 2017. **(B)** *Isodictya kerguelenensis* individuals maintained under experimental conditions in holding tanks at Yelcho Research Station at Doumer Island, WAP.

In order to assess the effect of increased temperature on the prokaryotic communities of *I. kerguelenensis*, a small piece of tissue (∼0.3 g) from each individual was obtained at day 0 (T0, before injury) and after 15 days (T15, after injury) for each temperature condition (0.5, 3, and 6°C). Sponges in the control tank were also sampled to assess for effects of tissue injuries by cutting a piece (∼0.3 g) to mimic the effect produced by ice scour on sponges. Individuals were maintained in closely controlled aquarium conditions to assess the impact of temperature increase and tissue injuries as well as the potential interactions of tissue damage with increased temperature. Previous work in the study area has shown that Antarctic sponges suffer injuries by ice scour (Cárdenas et al., 2019). Ice scour can produce partial loss of pieces of the body, or in other cases, individuals are removed entirely (Barnes, 2017). Here, we mimic sublethal effects of ice scour (partial tissue loss) to determine if even smaller injuries can provoke shifts in the prokaryotic community. A total of 18 samples were obtained at the end of the experiment, but one individual in the 3°C condition was another cryptic species. This individual was removed from the analysis, and for that reason, the 3°C (days 0 and 15) condition has only two individuals. Identification of the material used in the experiment was assisted by sponge taxonomy experts from the Museu Nacional UFRJ, Brazil.

Seawater was warmed and maintained at a set temperature with SOBO aquarium heaters (500 watt), and seawater was replaced every day from the natural *Isodictya kerguelenensis* environment. Temperatures used in the heat-stress treatments corresponded to unusual summer peaks recorded in shallow waters at Doumer Island (Cárdenas et al., 2018b) and the predicted temperature under the high-end 2080 IPCC scenario (IPCC, 2014), respectively.

DNA Extraction and Sequencing

Genomic DNA from sponge tissue for 16S rRNA gene amplicon sequencing was extracted using the Precellys© Evolution homogenizer with a Cryolys cooling unit (Bertin Technologies, Montigny-Le-Bretonneux, France) and a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extractions were performed using both internal and external sponge tissue in order to obtain the whole bacterial community. DNA concentrations were measured using a Nanoquant spectrophotometer (Tecan, Switzerland). Ten microliters of DNA sample were sent to Dalhousie University CGEB-IMR[1] for V4–V5 rRNA gene library preparation and sequencing. Primers used correspond to 517f GTGYCAGCMGCCGCGGTAA and 926r CCGYCAATTYMTTTRAGTTT (Walters et al., 2016). Samples were multiplexed using a dual indexing approach and sequenced using an Illumina Miseq with paired-end 300 + 300 bp reads. All PCR procedures, primers, and Illumina sequencing details were as described in Comeau et al. (2017). The cDNA sequences of 16 libraries were deposited at NCBI as a BioProject with accession

[1]http://cgeb-imr.ca/

ID PRJNA595145 with the exception of two libraries corresponding to the same individual at day 0 (before injury) and day 15 (after injury) at 3°C. These libraries were not included in the posterior analysis because this sponge individual, in fact, corresponds to a cryptic related species (González-Aravena et al., 2019).

Data Processing and Analysis

Microbiome data was analyzed with the QIIME2 pipeline (Bolyen et al., 2018), using available plugins from the QIIME2 website[2]. Briefly, 16 paired-end libraries in fastq compressed files (.gz) were imported to perform merge reads from R1 and R2 libraries per sample, trimming primers and low-quality nucleotides at the end of the sequences, denoising, removing chimeras, and clustering sequences. This procedure produces three files: one with cluster sequences in a FASTA file and a second file with a table of cluster abundances. The third file contains the overall stats. Clustered and filtered sequences were then affiliated using the SILVA132 database with a 99% identity for seven levels of taxonomy. Before statistical analyses, clusters affiliated as chloroplasts and mitochondria were removed.

A rooted phylogenetic tree was created to be used for statistical diversity analyses. A core metric plugin of Qiime2 was applied to operational taxonomic unit (OTU) abundance data for each sample to obtain values for observed OTUs, Chao1, Shannon, and Simpson diversity indexes. Prokaryotic community compositions at the order level were plotted with a bar plot graphic with the most frequent prokaryotic orders. A heat map plot was created according to the OTUs' relative abundance. A principal coordinate analysis (PCoA) was then performed to visualize the differences between samples using a Bray-Curtis distance matrix of beta diversity using the diversity core metric plugin. In order to determine the existence of OTUs present in all samples from all treatments, we examined the core OTUs that were present before and after stress exposure, selecting only OTUs present in all 16 samples from the whole OTU list. Differences in the beta diversity of the prokaryotic community between treatments at the order and OTU levels were tested with PERMANOVA analyses based on Bray-Curtis distance matrices. PERMANOVA tests also were performed (treated as a univariate measure) to test for differences in univariate measures of diversity (Sobs, Chao1, Shannon, and Simpson) among experimental treatments. Tests were performed based on Euclidean distance matrices, and Monte Carlo tests were used when the number of unique permutations was low as recommended by Anderson et al. (2008). We used PERMANOVA for univariate analyses as it allows for two-factor designs, considers an interaction term, and does not assume a normal distribution of errors. A similarity percentage analysis (SIMPER) was also performed for detecting the OTUs contributing most to the dissimilarity between experimental conditions. PERMANOVA and SIMPER analyses were performed using Primer v7 (Anderson et al., 2008).

[2]https://docs.qiime2.org/2019.4/plugins/available/

RESULTS

General Patterns in the Prokaryotic Community

Analysis of the prokaryotic community of 16 samples of *Isodictya kerguelenensis* yielded 1,905,120 paired-end reads, from which 1,555,032 reads (81.62%) were retained after filtering and denoising. Then, 1,371,028 reads (71.97%) were assembled in 685,514 contigs of which 515,163 (75.15%) were retained after chimera, chloroplast, and mitochondrial sequence removal.

A total of 648 OTUs were obtained, from which only one had no taxonomic affiliation (**Supplementary File 1**). The affiliation process detected one archaea phyla (Thaumarchaeota), having only two OTUs with low abundance, and 22 bacterial phyla; Proteobacteria and Bacteroidetes were the most represented for both OTUs and sequence abundance (**Table 1**). Other well-represented phyla for OTU frequency were Planctomycetes, Firmicutes, and Verrucomicrobia with more than 10 OTUs each. Regarding sequence frequency, other phyla that were well represented were Fusobacteria and Epsilonbacteraeota with more than 1000 sequences each.

The rarefaction curves revealed that samples from treatment 3C–15d showed the highest number of observed OTUs, which was very distant from the other conditions (**Supplementary Figure S1**).

TABLE 1 | OTU and sequence frequencies by phylum from samples of *Isodictya kerguelenensis* maintained under experimental conditions.

Phylum	Number of OTUs	Relative abundance
Thaumarchaeota	2	182
Acidobacteria	6	55
Actinobacteria	8	179
Bacteroidetes	211	138,348
Chloroflexi	2	20
Dadabacteria	1	9
Epsilonbacteraeota	5	1840
Fibrobacteres	1	38
Firmicutes	14	89
Fusobacteria	3	5877
Kiritimatiellaeota	2	17
Latescibacteria	2	8
Lentisphaerae	3	57
Margulisbacteria	1	20
Nitrospinae	5	55
Nitrospirae	1	48
Omnitrophicaeota	3	43
Patescibacteria	2	4
Planctomycetes	47	842
Proteobacteria	313	366,845
Sphirochaetes	2	77
Tenericutes	1	2
Verrucomicrobia	11	464
Undetermined Bacteria	1	44
Total	647	515,163

Stress Effects on Prokaryotic Diversity

The effects of the experimental treatment on diversity indexes was variable (**Supplementary File 2**). The observed and estimated (Chao1) number of OTUs, Shannon, and Simpson were affected by injury over time (**Figure 2**), whereas temperature did not have an effect. However, in most cases, a significant interaction between injury and temperature was recorded (**Supplementary File 2**).

Stress Effects on Prokaryotic Community and Structure

The PCoA showed a clear group separation between samples from T0d and T15d indicating an effect of physical damage over time, suggesting that tissue damage rather than temperature drove observed differences (**Figure 3**). Axis 1 explains 55.09% of the variation, separating the T0 control group from the other three groups. Axis 2 explains 14.03% of the differences, separating the injured sponges according to temperature and showing a moderate effect of temperature on microbiome composition. A significant effect of temperature (PERMANOVA $F = 2.8349$, $R^2 = 0.15$, $p = 0.0005$) and injury (PERMANOVA $F = 13.965$, $R^2 = 0.37$, $p = 0.0001$) and the interaction between both factors was also significant (PERMANOVA $F = 2.9865$, $R^2 = 0.16$, $p = 0.0003$). The microbiota of the uninjured sponges (T0d) was highly dominated by an unaffiliated order of Gammaproteobacteria (66.71%) compared with samples from injured sponges (T15d; **Figure 4**), where most showed low relative abundance values (1.58%). In contrast, an increase in relative abundance of Altermonadales (34.92%) was recorded in injured sponges compared with uninjured sponges (4.44%; **Figure 4**).

The core community comprised 13 OTUs (**Supplementary File 3**), and all of them were present among the 31 most abundant OTUs (see **Supplementary Table S1**). From these OTUs, 10 belonged to Proteobacteria and three to Bacteroidia. Proteobacterial OTUs belonged mainly to Alteromonadales, Rhodobacterales, and Oceanospirillales, whereas Bacteroidial OTUs belonged to Flavobacteriales.

The SIMPER analysis revealed that 40 OTUs contributed to 50% of the differences between the T0 uninjured and T15d injured samples (**Supplementary File 4**). From these, seven OTUs represented 25% of the differences between the two conditions, belonging to the 31 most abundant OTUs (**Supplementary Table S1**). From these OTUs, four were found in the core microbiome (**Supplementary File 3**), showing that the abundance of some core OTUs was influenced by injuries. Although OTU 631 was highly represented in healthy sponges, OTU 175 was present almost uniquely in injured individuals.

The heat map of the most representative OTUs in terms of relative abundance reveals clear differences in the abundance of several OTUs between uninjured and injured sponges

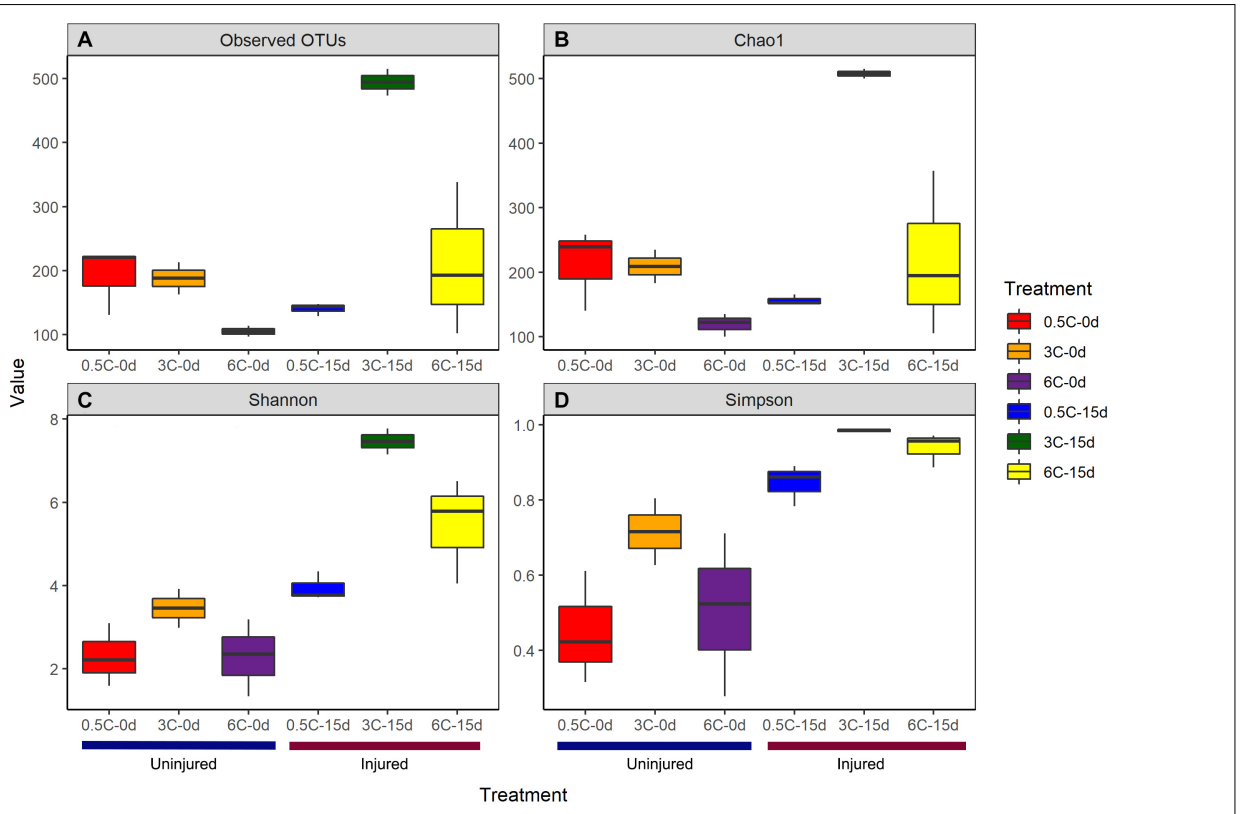

FIGURE 2 | (A) Observed OTU richness (Sobs) **(B)** Estimated OTU richness (Chao1) **(C)** Simpson **(D)** Shannon diversity indices of the prokaryotic communities associated with specimens of *Isodictya kerguelenensis* exposed to different experimental conditions.

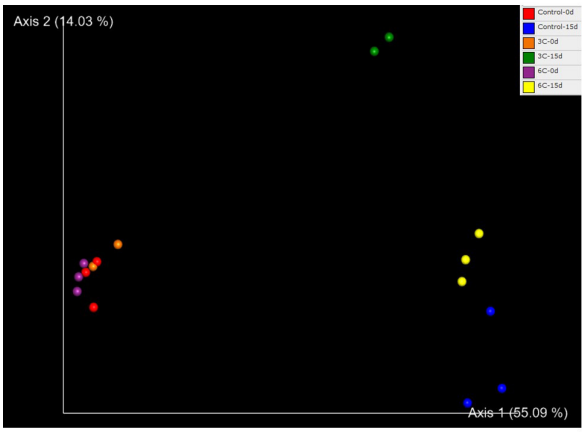

FIGURE 3 | Principal coordinate analysis of prokaryotic OTUs associated with *Isodictya kerguelenensis* exposed to different experimental conditions.

(**Figure 5**). Among these OTUs, seven OTUs were also some the most discriminant, explaining differences between treatments according to the SIMPER analysis (**Supplementary File 4**). Overall, the shift in abundance shown in the heat map is highly concordant with the SIMPER analysis; the gammaproteobacterial OTU631 showed the highest abundance in uninjured sponges, reaching 83.25%, whereas its abundance was greatly reduced to 3.80% in injured sponges. Inversely, another gammaproteobacterial OTU (OTU466, Gammaproteobacteria, Alteromonadales, *Colwellia* genus) showed considerably higher abundance in injured sponges (48.60%) compared with uninjured sponges (2.22%). Another OTU revealed by the SIMPER analysis that showed marked differences in relative abundance between uninjured and injured sponges (as seen in the heat map) is the OTU175, which belongs to *Flavobacteriaceae* (genus *Pseudofulvibacter*).

DISCUSSION

The present study has characterized a prokaryotic community shift in the common Antarctic sponge *Isodictya kerguelenensis* after being experimentally exposed to tissue injury and warming. To our knowledge, this is the first study that aims to mimic the sublethal effects of ice scour, which is one of the main forces affecting Antarctic benthic communities by damaging and removing organisms from the seabed. As was observed in a previous study at Doumer Island (Cárdenas et al., 2019), sponges and other organisms are exposed to frequent disturbance produced by ice scour, especially in summer. The current study assessed the effect of small tissue damage and its potential effects on the prokaryotic community associated with a common

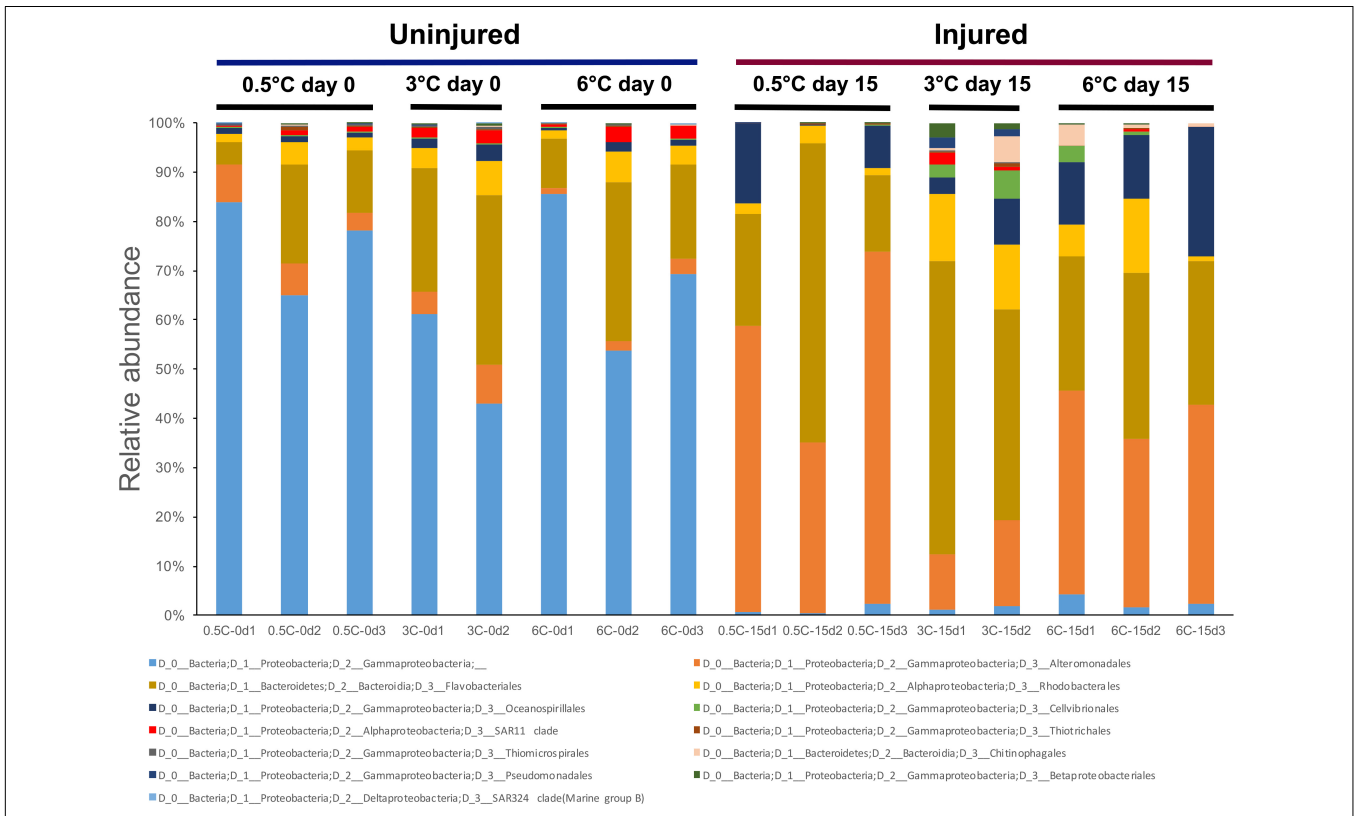

FIGURE 4 | Relative abundance of prokaryotic groups at the order level for *Isodictya kerguelenensis* individuals exposed to increased temperature and physical injuries.

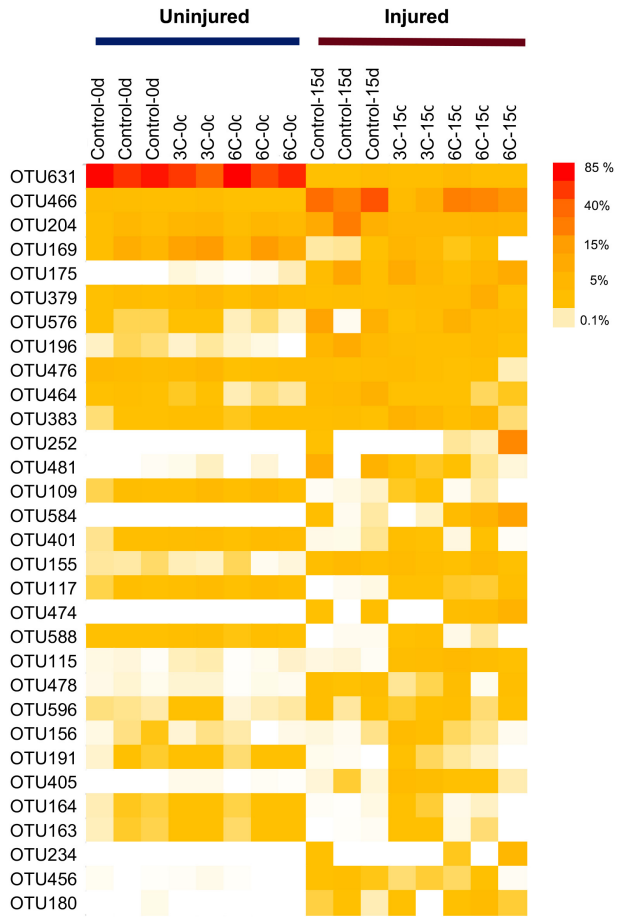

FIGURE 5 | Heat map of the 31 most abundant prokaryotic OTUs associated with *Isodictya kerguelenensis* individuals and exposed to different experimental conditions.

Antarctic sponge. Our experimental study suggests that tissue injuries similar to those produced by ice scour, a common physical stressor in polar environments, may alter the sponge-associated prokaryotic communities. Current evidence suggests that, on average, almost 30% of the substrate in shallow waters in some areas of the WAP is affected by icebergs each year (Barnes, 2017), and this is expected to increase in the future as a consequence of climate change, further affecting sponges (Morley et al., 2019). This is the first study evaluating the combined effects of acute heat stress and tissue injury in an Antarctic sponge, providing valuable information about microbiome composition shifts and potential microbial markers of healthy and injured specimens of *I. kerguelenensis*.

The healthy sponge holobiont is considered an ecosystem that is in a state of dynamic equilibrium, but the strength and outcome of the interactions among the members of the holobiont may be affected by perturbations that challenge the healthy equilibrium. This equilibrium is a dynamic ecosystem that is characterized by its resistance and resilience (Pita et al., 2018). This study explored these characteristics using an Antarctic sponge exposed to disturbance. Our results suggest that tissue injuries on *Isodictya* individuals are enough to provoke a prokaryotic shift, suggesting that tissue injuries, such as those provoked by iceberg scour, can alter the dynamic equilibrium of the sponge–prokaryotes relationship.

Changes in seawater temperature have produced significant changes in sponge abundance, diseases, and mass mortality from various latitudes (Bell et al., 2015; Blanquer et al., 2016; Luter et al., 2017; Belikov et al., 2019). A recent study reported a mass mortality event in sub-Arctic sponge communities particularly affecting the sponge species *Isodictya palmata* and *Halichondria sitiens* (Ereskovsky et al., 2019); despite observing signs of illness, no further analyses were carried out.

Recent evidence suggests that, in general, sponges and their microbiome are able to cope with environmental perturbations (Gantt et al., 2017; Glasl et al., 2018) and show remarkable seasonal stability, confirming a host-specific stable association between sponges and their prokaryotic consortiums (Erwin et al., 2012; Cárdenas et al., 2018a, 2019). However, in some cases, warming can disrupt the balance of the sponge microbiome. For instance, Lesser et al. (2016) showed a significant effect of increased seawater temperature and ocean acidification on the microbiome of the tropical sponge *X. muta*. Similar effects have been reported for other organisms, such as mussels, when exposed to a high temperature for 3 days, producing a proliferation of opportunistic bacteria, which may promote susceptibility to disease (Li et al., 2018).

A previous study on *Isodictya* sp. monitored *in situ* during a 24-month period on Doumer Island reports high stability of the microbiome even after being exposed to abnormal summer temperatures (reaching 3°C; Cárdenas et al., 2019). In addition, a transcriptomic analysis of the effects of heat stress under laboratory conditions reports a response of *Isodictya* sp. at the limits of its capacity when exposed to acute heat stress (3 and 5°C; González-Aravena et al., 2019). Our experimental study further increases our knowledge of the responses of this species to environmental variation. Although we do not have clear evidence of the effect on prokaryotic composition of acute thermal stress exposure, a significant change occurred with injury induction. Interestingly, the OTUs responsible for the main differences between injured and uninjured sponges belong to *Colwelliaceae* and *Flavobacteriaceae*, being more abundant in injured sponges. The relative abundance of OTU466 (*Colwelliaceae*) increased from 1.31% in the uninjured sponges to 24.79% in the injured ones. For *Flavobacteriaceae* OTUs, the relative abundance of OTU204 increased from 3.72 to 9.14% in injured sponges, whereas OTU175 increased from 0.03 to 5.62% in injured sponges, and finally, OTU196 increased from 0.10 to 3.12%. Members of these families are related to sponge and coral diseases (Simister et al., 2012; Fan et al., 2013; Gajigan et al., 2017). *Colwelliaceae* OTUs have been reported as disease-related in necrotic and stressed sponges (Simister et al., 2012; Fan et al., 2013), whereas OTUs belonging to *Flavobacteriaceae* have been associated with diseases and stress in coral species (Gajigan et al., 2017). This suggests that tissue injuries can disrupt the holobiont balance, which may further affect their roles, potentially cascading to the benthic ecosystem; however, the

roles of the symbionts and potential cascading effects are yet to be studied. Cárdenas et al. (2019) reported high stability in the microbiome of sponges after repeated sampling of the same individuals over three consecutive summers, suggesting that some sponge individuals might show some degree of resilience to increased temperature. In this regard, resilience of sponges to changes in environmental factors, such as temperature, has been extensively documented in experimental studies from other latitudes (Erwin et al., 2012; Luter et al., 2012a; Cárdenas et al., 2014; Turon et al., 2019). Previous studies highlight the capacity of Antarctic sponges to cope with environmental change as a potential key factor in their success (Morley et al., 2016; Cárdenas et al., 2019). Although our results provide the first insights into the responses of sponges to stressors, such as tissue damage and temperature, at present it is unknown if Antarctic sponges would cope in the long term with such types of stress.

The composition and diversity of sponge microbiomes is directly correlated with environmental conditions as has been shown between pristine and polluted zones (Turon et al., 2019); hence, the sponge microbiome can play an important role as an indicator of significant environmental disturbance. Changes in the sponge microbiome may even be related to mass mortalities, beginning with early disruption of the microbiome balance at the OTU level with a potential decline of sponge fitness and resistance to infections (Blanquer et al., 2016). For instance, Blanquer et al. (2016) found that bacterial diversity increased significantly in diseased tissues, which is in accordance with our results, and an increase in Shannon and Simpson diversity indices were recorded in injured sponges. In the case of our study, the alpha diversity increased after physical injuries with the apparition of particular OTUs of Alteromonadales, Flavobacteriales, and Oceanospirillales orders only in injured sponges potentially being opportunistic bacteria.

Although overall results of this study suggest a microbiome disruption by the interaction of tissue injuries and heat stress at the microbiome assembly level, it was not possible to assess the effect of these stressors over a longer period of time or to assess the existence of potential resilience. This has to be acknowledged; however, it is difficult to perform long-term experiments due to logistics constraints associated with work in Antarctica, which limits the duration of the experiments; in many cases, access to facilities is limited to a short period of time during the summer months. Future metagenomics and functional features analyses of the microbiome of *I. kerguelenensis* exposed to heat stress and tissue injury will be necessary to elucidate in more detail how prokaryotic functions are affected by these stressors and the potential resilience of its microbiome over a longer period of time.

CONCLUSION

Our study assessed for the first time the effects of combined climate change stressors on the microbiome of an Antarctic sponge. The results suggest that disturbance produced by icebergs may have a direct impact on the microbiome of the Antarctic sponge *I. kerguelenensis* with further potential effects as temperature increases. Future climate change scenarios for the WAP, including warming and increases in ice scour, may play a critical role shaping sponge symbiosis on this and other species. The potential effects of the disruption of the holobiont balance and its wider effects on the ecosystem are yet to be understood.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI BioProject, NCBI Accession No. PRJNA595145.

AUTHOR CONTRIBUTIONS

CC conceived the experimental design. MO carried out the experimental assays. RR carried out the sequences analyses. RR, CC, and AF carried out the statistical analyses. RR, CC, and MG-A wrote the manuscript with contribution of AF. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00262/full#supplementary-material

FIGURE S1 | Rarefaction curves of the observed number of OTUs from *Isodictya kerguelenensis* at different sequencing depth by each experimental condition.

TABLE S1 | Taxonomy of the 31 most abundant OTUs (as shown in **Figure 5**) in samples of *Isodictya kerguelenensis* maintained under experimental conditions.

FILE S1 | Overall OTUs obtained with their sequence abundances.

FILE S2 | Permanova results testing the effect of treatment temperature and injuries on prokaryotic diversity indexes.

FILE S3 | Number of sequences of core OTUs in samples of *Isodictya kerguelensis* maintained under experimental conditions.

FILE S4 | Similarity percentages analysis (SIMPER) revealing the most important OTUs explaining dissimilarities in sponge microbiomes between experimental treatments.

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Presence and Genetic Identity of Symbiodiniaceae in the Bioeroding Sponge Genera *Cliona* and *Spheciospongia* (Clionaidae) in the Spermonde Archipelago (SW Sulawesi), Indonesia

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Members of the family Symbiodiniaceae form symbiotic relationships with several metazoan groups on coral reefs, most notably scleractinian corals. However, despite their importance to the health of coral reefs, their relationship with other host organisms such as bioeroding sponges (Clionaidae) is still relatively understudied. In this study we investigate the presence and identity of Symbiodiniaceae in Clionaidae species in Indonesia and evaluate findings related to the evolution and ecology of the host-symbiont relationship. Clionaidae were collected throughout the Spermonde Archipelago in Indonesia. Morphological and molecular techniques were used to identify the sponge host (28S ribosomal DNA) and their Symbiodiniaceae symbionts (ITS2). Seven Clionaidae species were found, of which four species contained Symbiodiniaceae. *Cliona* aff. *orientalis*, *Cliona thomasi* and *Spheciospongia maeandrina* were host to *Cladocopium*, while *Spheciospongia digitata* contained *Durusdinium* and *Freudenthalidium*. In the remaining species: *Cliona* sp., *Cliona utricularis* and *Spheciospongia trincomaliensis* no evidence of the presence of Symbiodiniaceae was found. Our results provide the first record of Symbiodiniaceae in the sponge genus *Spheciospongia*. Additionally, we provide the first findings of *Freudenthalidium* and first molecular evidence of *Durusdinium* in bioeroding sponges. Our results indicate coevolution between *Spheciospongia digitata*, *Spheciospongia maeandrina* and their symbionts. We discuss that the diversity of Symbiodiniaceae within bioeroding sponges is likely far greater than currently reported in literature. Considering the threat bioeroding sponges can pose to the health of coral reefs, it is crucial to understand

Symbiodiniaceae diversity within Clionaidae and their effect on the functioning of Clionaidae species. We propose that the identity of the symbiont species is mostly related to the host species, but we did observe a potential case of environmental adaptation related to environmental stressors.

Keywords: zooxanthellae, symbiosis, Porifera, *Cladocopium*, *Durusdinium*, *Freudenthalidium*

INTRODUCTION

Coral reefs are known as biodiversity hotspots (Roberts et al., 2002; Bellwood et al., 2004). While coral reefs occupy less than 0.1% of the world ocean surface area, they provide shelter to at least 25% of all marine species (Spalding and Grenfell, 1997). Corals rely heavily on organic carbon provided through their symbiosis with zooxanthellae (dinoflagellates belonging to the family Symbiodiniaceae) (Trench, 1993, 1997; Rowan, 1998; Baker, 2003). Symbiodiniaceae can provide up to 95% of the energy required by the host coral to survive through photosynthesis (Muscatine, 1990). In return, the host provides the Symbiodiniaceae with protection and inorganic nutrients (Muscatine and Porter, 1977).

The symbiotic relationship between scleractinian corals and Symbiodiniaceae has been extensively studied (Marshall and Schuttenberg, 2006; Baker et al., 2008). Elevated sea surface temperatures (SST) can lead to the Symbiodiniaceae being expelled by their host in a process called "bleaching" (Brown, 1997). The bleached corals are devoid of nearly their entire energy budget, leading to reduced growth and eventually mortality (Baker et al., 2008). If the SST decrease in a short period of time, the symbionts may eventually return to the host, after which the host can recover. However, the predicted increase in frequency and intensity of bleaching events poses a big threat to the survival of corals (Brown, 1997; Baker et al., 2008).

While bleaching is considered a threat to coral reefs, it is also a mechanism for corals to adapt to certain changes. Studies have shown that symbiont communities within the host can shift after bleaching events (Baker, 2001; Jones et al., 2008; Kemp et al., 2014). A shift in symbiont community toward more thermo-tolerant Symbiodiniaceae types can increase the fitness of the holobiont, making it more resilient to stressors and future bleaching events (Baker, 2001; Mieog et al., 2009).

Until recently, the family Symbiodiniaceae contained a single genus, *Symbiodinium* Freudenthal (1962), which was divided into a number of clades (A to I) and numerous subclades (Baker, 2003; Pochon and Gates, 2010; Pochon et al., 2014). "*Symbiodinium* types" were used to differentiate species within the genus, although these were not formally described. A taxonomic revision proposed that with the consideration of molecular, morphological, physiological, and ecological data, the divergent *Symbiodinium* "clades" represent fifteen lineages equivalent to genera and provided formal descriptions for seven new genera (LaJeunesse et al., 2018). Recently, two more lineages have been described, resulting in nine formally described Symbiodiniaceae genera (Nitschke et al., 2020). Genera within Symbiodiniaceae differ in ecological properties. An example is the Symbiodiniaceae genus *Durusdinium*, which is known for its thermal tolerance in hosts (Stat and Gates, 2011).

Most studies on host-Symbiodiniaceae relationships focus on reef building scleractinian corals (Cnidaria). However, other organisms besides scleractinian corals, such as Foraminifera, Mollusca and Porifera are also known to associate with Symbiodiniaceae. Research on the host-symbiont relationships of these taxa and Symbiodiniaceae is still fairly limited. Molecular research on Symbiodiniaceae-sponge relationships is limited to a small number of papers (e.g., Schönberg and Loh, 2005; Granados et al., 2008; Hill et al., 2011; Ramsby et al., 2017). Interestingly, sponges associating with Symbiodiniaceae show a certain putative hardiness to stressors (Schönberg and Suwa, 2007). Therefore, studying host-symbiont relationships in sponges can contribute significantly to a better understanding of mechanisms supporting stress resistance in coral reefs. Presumably, sponges are better hosts to Symbiodiniaceae than scleractinian corals, because sponge hosts can provide better protection to the Symbiodiniaceae by moving their symbionts inside their tissue (Schönberg and Suwa, 2007). Associations between sponges and Symbiodiniaceae have only been discovered in a limited number of species, mainly within the order Clionaida and specifically the family Clionaidae. The majority of species belonging to Clionaidae are known to aggressively attack live corals or excavate in dead substrate (Schönberg and Wilkinson, 2001). Symbiodiniaceae are known to enhance the boring and growth rates of these sponges (Rosell and Uriz, 1992; Hill, 1996; Schönberg, 2006; Schönberg et al., 2017).

The family Clionaidae contain ten genera, all of which have bioeroding or excavating abilities. Within the Clionaidae, the genus *Cliona* is the most speciose group. *Cliona* species can be very abundant on coral reefs and therefore have been the main focus of research into the relationship between Symbiodiniaceae and sponges. The genus *Cliona* harbors more than 80 accepted species in which the published literature only detected eight associations with *Cliona* and Symbiodiniaceae so far (van Soest et al., 2020). The first association between a marine sponge, *Cliona viridis* (Schmidt, 1862), and Symbiodiniaceae was observed by Sarà and Liaci (1964) from the Mediterranean Sea. Subsequent studies have shown that in the Caribbean region, the species *C. aprica* (Pang 1973); *C. caribbaea* (Carter 1882); *C. tenuis* (Zea and Weil 2003); *C. lativicola* (Pang 1973); and *C. varians* (Duchassaing and Michelotti, 1864) were discovered to contain the Symbiodiniaceae genera *Symbiodinium*, *Breviolum,* and *Gerakladium* (Granados et al., 2008; Hill et al., 2011). Additionally, in the Indo-Pacific region *C. orientalis* (Thiele 1900); *C. caesia* (Schönberg, 2000) and *C. jullieni* (Topsent 1891), have been discovered to contain *Symbiodinium*, *Cladocopium* and *Gerakladium* (Schönberg and Loh, 2005; Hill et al., 2011;

Ramsby et al., 2017; **Table 1**). For many Clionaidae it is, thus, still unknown whether species harbor Symbiodiniaceae, and what their identity is.

In this study, we investigated the presence and identity of Symbiodiniaceae in Clionaidae in Indonesia. First, we identified Clionaidae species in the Spermonde Archipelago and recorded their distribution. Secondly, we examined all collected Clionaidae for the presence and identity of the dominant Symbiodiniaceae type using molecular techniques. Additionally, we evaluated findings related to the evolution and ecology of the host-symbiont relationship between Symbiodiniaceae and Clionaidae.

MATERIALS AND METHODS

Site Description

This research was conducted in the Spermonde archipelago, located just off the coast to the city of Makassar, Sulawesi, Indonesia. The archipelago consists of 2800 km^2 surface area and contains 160 different reefs (de Voogd et al., 2006). The area is located on a continental shelf, therefore, there is a steady increase in depth and oceanic influences along the shore-offshore gradient. Several other components also influence the environmental factors of the archipelago, including the influx of two major rivers, the Jeneberang and the Tallo, that emerge in the archipelago, resulting in freshwater run-off and anthropogenic disturbances from the city of Makassar (de Voogd et al., 2006). A map of the study site and sixteen sampling locations can be found in **Supplementary Figure 1**.

Sample Collection

The fieldwork was carried out from April to May 2018. Sponges belonging to the family Clionaidae were visually identified and collected by scuba diving and snorkeling. In total, 75 samples were used for molecular analysis. Sponge samples were collected by removing sponge tissue from the substrate using a knife or hammer and chisel. Samples were immediately preserved in microtubes filled with 96% ethanol and after fieldwork these were deposited in the sponge collection of the Naturalis Biodiversity Center, The Netherlands as RMNH.POR.11772-11800 and RMNH.POR.12367-12436 (**Supplementary Table 2**).

The seawater temperatures of the shallow reef flat were recorded using an ONSET Hobo MX2202 data loggers at four sampling sites (Lumulumu, Samalona, Barang Barangan, and Karangan). The temperature loggers were placed at a central location on the reef flat during sponge sampling for three to four hours, measuring the temperature of the reef flat every minute.

Species Identification

Sponges were identified using classical morphological characters and molecular 28S ribosomal DNA (28S rDNA) analysis. For classical morphological characters, skeletal slides of the choanosome and the ectosome were made by cutting sections of the sponge by hand. Spicule slides were prepared by dissolving a small piece of sponge tissue (choanosome and ectosome) in household bleach (sodium hypochlorite <3%). Dissolved sponge tissue was rinsed with distilled water and 96% ethanol. Spicule sizes were measured using a Leica DM5500 B light microscope. Spicules were further analyzed using a JEOL JSM 6480 LV Scanning Electron Microscope. Ectosomal and choanosomal tissue were investigated for the presence of Symbiodiniaceae in the Clionaidae sponges. Photographs of cross sections were made, investigated and photographed with a Leica DFC320 camera on the Leica DM5500 B light microscope.

For molecular analyses, DNA was extracted using Qiagen Blood & Tissue kit, following the manufacturers protocol for spin column extractions. Both choanosome and ectosome of the sponge has been subsampled to include any Symbiodiniaceae present in the ectosome. Sponge 28S rDNA was amplified using the primers 28S-C2-fwd (5'-GAAAAGAACTTTGRARAGAGAGT-3') and 28S-D2-rev (5'-TCCGTGTTTCAAGACGGG-3') (Chombard et al., 1998) as used in the sponge barcoding project (Wörheide et al., 2007). Dominant Symbiodiniaceae types were identified using the ITS2 marker using SYM_VAR_5.8S (5'-GAATTGCAGAACTCCGTGAACC-3') (Hume et al., 2015) and SYM_VAR_REV (5'-CGGGTTCWCTTGTYTGACTTCATGC-3') (Hume et al., 2013, 2018).

Sponge 28S rDNA region was amplified in a 25 μl reaction containing 15.8 μl distilled water (sterile milliQ); 2.5 μl 10× Qiagen Coral Load PCR buffer; 1.0 μl 25 mM Qiagen $MgCl_2$; 1.0 μl 10 mg/ml Life BSA; 1.0 μl 10 pMol/μl forward primer; 1.0 μl 10 pMol/μl reverse primer; 1.0 μl 2.5 mM dNTP; 0.25 μl 5 U/μl Qiagen Taq and 1.5 μl 0.05–0.5 ng/μl DNA template. The following amplification parameters were used: initial denature of 96°C for 3 min, followed by 40 cycles of 96°C for 30 sec, 60°C for 45 sec and 72°C for 1 min and a final extension of 72°C for 10 min. Symbiodiniaceae ITS2 region was amplified in a 25 μl reaction containing 9.0 μl distilled water (sterile milliQ); 12.5 μl Taqman environmental master mix 2.0; 1.0 μl 10 pMol/μl forward primer; 1.0 μl 10 pMol/μl reverse primer and 1.5 μl 0.05–0.5 ng/μl DNA template. The following amplification parameters were used: initial denature of 95°C for 10 min, followed by 38 cycles of 95°C for 15 sec, 56°C for 30 sec and 72°C for 40 sec and a final extension of 72°C for 5 min. PCR product yield was checked using Invitrogen E-gels 96 2% agarose. PCR products were sent to Baseclear B.V. in The Netherlands for forward and reverse sanger sequencing using the same primers used for PCR amplification. All successful ITS2 PCR amplifications provided a genetic sequence.

Sequences were analyzed and edited in Geneious 8.1.8[1]. Reads were assembled using a *De Novo* Assemble. Contigs were stripped of primer sequences and analyzed for reverse complements and quality. Sequences were aligned using MAFFT v7.017 (Katoh et al., 2002). A phylogenetic tree was constructed using MrBayes (Huelsenbeck et al., 2001). A GTR substitution model with "invgamma" rate variation was used, setting the chain length to 11,00,000 with a subsampling frequency of 1,000 and a burn-in length of 1,00,000. A maximum likelihood (ML) tree was created using PhyML for bootstrap support values using

[1]https://www.geneious.com

TABLE 1 | Overview of Symbiodiniaceae genera found in Porifera using molecular techniques.

Host	Symbiodiniaceae											Geographic location	References
	Present										Not found		
	Symbiodinium	*Breviolum*	*Cladocopium*	*Durusdinium*	*Effrenium*	*Fugacium*	*Freudenthalidium*	*Gerakladium*	*Halluxium*	*Clade I*			
Cervicornia cuspidifera								X				United States (Florida Keys)	Hill et al., 2011
Cliona aprica	X	X										Colombia (Caribbean Sea)	Granados et al., 2008
Cliona caesia			X									Australia, Japan	Hill et al., 2011
Cliona caribbaea	X							X				Belize, United States (Florida Keys), Colombia (Caribbean Sea)	Granados et al., 2008; Hill et al., 2011
Cliona jullieni	X											New Caledonia	Hill et al., 2011
Cliona laticavicola	X	X										Colombia (Caribbean Sea)	Granados et al., 2008
Cliona orientalis								X				Australia	Schönberg and Loh, 2005; Hill et al., 2011; Ramsby et al., 2017
Cliona* aff. *orientalis			**X**									Indonesia (SW Sulawesi)	**This study**
***Cliona* sp.**											**X**	Indonesia (SW Sulawesi)	**This study**
Cliona tenuis	X											Caribbean Sea	Granados et al., 2008
Cliona thomasi			**X**									Indonesia (SW Sulawesi)	**This study**
Cliona tumula								X				United States (Florida Keys)	Ramsby et al., 2017
Cliona utricularis											**X**	Indonesia (SW Sulawesi)	**This study**
Cliona varians		X						X				United States (Florida Keys), Colombia (Caribbean Sea)	Granados et al., 2008; Hill et al., 2011; Ramsby et al., 2017
Spheciospongia digitata				**X**			**X**					Indonesia (SW Sulawesi)	**This study**
Spheciospongia maeandrina			**X**									Indonesia (SW Sulawesi)	**This study**
Spheciospongia trincomaliensis											**X**	Indonesia (SW Sulawesi)	**This study**

the GTR substitution model with an estimated proportion of invariable sites and gamma distribution parameter. The number of bootstraps was set to 1,000 (Guindon et al., 2010). All sequences were deposited in GenBank under the accession numbers MT756867-MT756897 and MT756898-MT756972.

Symbiodiniaceae ITS2 sequences were identified using the custom Symbiodiniaceae ITS2 database created by Arif et al. (2014). Extracted ITS2 sequences were blasted using a Megablast in Geneious to the custom database. For the results an *E*-value cutoff of 1E-100 was used. The blast results have been included in **Supplementary Table 3**.

RESULTS

Clionaidae Species and Distribution

Seven different species belonging to the family Clionaidae were observed in the present study and identified as: *Cliona* aff. o*rientalis, Cliona thomasi* (Mote et al., 2019), *Cliona utricularis* (Calcinai et al. 2005), a so far unidentified *Cliona* sp., *Spheciospongia digitata* (Dendy, 1887), *Spheciospongia maeandrina* (Dendy, 1887) and *Spheciospongia trincomaliensis* (Ridley, 1884) (**Supplementary Figure 4**).

Spheciospongia digitata and *S. maeandrina* were initially identified as a single species by morphological characters. The genetic analyses of 28S rDNA, however, showed a clear distinction between the two species (**Figure 1**). The *Cliona* species *C. thomasi* and *Cliona* aff. *orientalis* were also morphologically identical, but 28 rDNA analyses showed several different species in a single clade (**Figure 1**). Morphological descriptions of the collected species are provided in **Supplementary Figure 4**.

Two species (*S. maeandrina* and *S. digitata*) were exclusively found on the shallow reef flats (<3 m depth) and occurred sympatrically on three of the twelve reefs on which the species was encountered (Barang Lompo, Lumulumu, and Langai). The remaining Clionaidae species were constrained to the deeper reef slopes (between three- and twenty-meters depth). *Spheciospongia* species were very common and present on every sampling site, whereas in total only a couple of *Cliona* samples were collected throughout the entire Spermonde Archipelago.

The water temperature on the reef flats ranged between a minimum of 30.5°C and a maximum of 32.7°C. *Spheciospongia maeandrina* and *S. digitata* were very abundant on the reef flats and thrived very close to the shore in between the seagrass. Despite the high water temperatures, no bleaching of the sponges has been observed.

Symbiodiniaceae Diversity in Clionaidae

The morphological examination of the Symbiodiniaceae in the thick sections of the sponges show Symbiodiniaceae incorporated in the tissue of *Cliona* aff. *orientalis*, *C. thomasi*, *S. digitata*, and *S. maeandrina* (**Figure 2**). No Symbiodiniaceae have been found in the thick sections of *Spheciospongia trincomaliensis*, *Cliona* sp., and *C. utricularis*.

From 31 of the 75 Clionaid specimens Symbiodiniaceae ITS2 sequences were obtained (PCR gel included in **Supplementary Table 5**). Three different Symbiodiniaceae ITS2 types were identified (**Supplementary Table 3**). *Cladocopium* (C1*) was the most abundant symbiont and was detected in twenty samples, *Durusdinium* (D8) was detected nine times and *Freudenthalidium* (F3.1) was only detected two times. A Symbiodiniaceae ITS2 phylogenetic tree can be found in **Supplementary Figure 6**. Four of the seven collected Clionaidae species contained Symbiodiniaceae. *Cliona* aff. *orientalis*, *C. thomasi* and *S. maeandrina* featured exclusively *Cladocopium* symbionts (**Figure 1**; blue circles). *Spheciospongia digitata* hosted both *Durusdinium* and *Freudenthalidium* (**Figure 1**; red and yellow circles, respectively). In *Spheciospongia trincomaliensis*, *Cliona* sp. and *C. utricularis* no Symbiodiniaceae were found. An overview of Symbiodiniaceae symbionts that have been detected by molecular techniques in Clionaid species is provided in **Table 1**.

DISCUSSION

In this study we added to the currently existing knowledge on symbiotic relationships between Symbiodiniaceae and bioeroding sponges from the Clionaidae family in the Indo-Pacific region. We studied seven Clionaid species, of which six had not been investigated yet for presence and molecular identity of symbiotic Symbiodiniaceae. For the first time, we have recorded symbiotic relationships between Symbiodiniaceae and the sponge genus *Spheciospongia*. The shallow-water sponge species *S. digitata* and *S. maeandrina* contained three different Symbiodiniaceae genera (*Cladocopium*, *Durusdinium*, and *Freudenthalidium*). For *C. thomasi*, it was known that the species contains Symbiodiniaceae, but their identity was still unknown (Mote et al., 2019). We determined their molecular identity as *Cladocopium* sp. We also identified *Cladocopium* symbionts in *Cliona* aff. *orientalis*.

We have provided the first record of *Freudenthalidium* in sponge hosts. *Freudenthalidium* was originally described as subclade Fr3 by Pawlowski et al. (2001) and formally described as a genus by Nitschke et al. (2020). *Freudenthalidium* is found across the tropical Indo-Pacific region and is known from free-living species and species associating with benthic foraminifera from the genera *Marginopora, Amphisorus*, and *Sorites* and in the intertidal anthozoans from the genus *Anthopleura* (Nitschke et al., 2020).

Furthermore, we have provided the first evidence of *Durusdinium* directly extracted from a sponge host using molecular techniques. The only known record of *Durusdinium* from a sponge is from the non-Clionaid sponge *Haliclona koremella* (Carlos et al., 1999) from Palau. The symbiont type in *H. koremella* was determined by incubating a drop of body fluid and identifying Symbiodiniaceae isolates from the enrichment culture. As noted by the authors, it is unsure whether the isolate was a symbiont within *H. koremella* or a free-living strain. Furthermore, with the exception of *H. koremella*, symbiotic relationships of Symbiodiniaceae with sponges are constrained within Clionaidae. These considerations make the presence of *Durusdinium* within *H. koremella* uncertain. Furthermore, recently collected samples of this sponge do not show any

FIGURE 1 | Phylogenetic tree resulting from the Bayesian analysis of 28S rDNA from all available Indo-Pacific *Cliona* and *Spheciospongia* species. Node support values indicate posterior probability values and PhyML bootstrap values, respectively. Sequences derived in this study are displayed in bold and if the sample contained Symbiodiniaceae, the identity is given as a a colored dot behind the sequence.

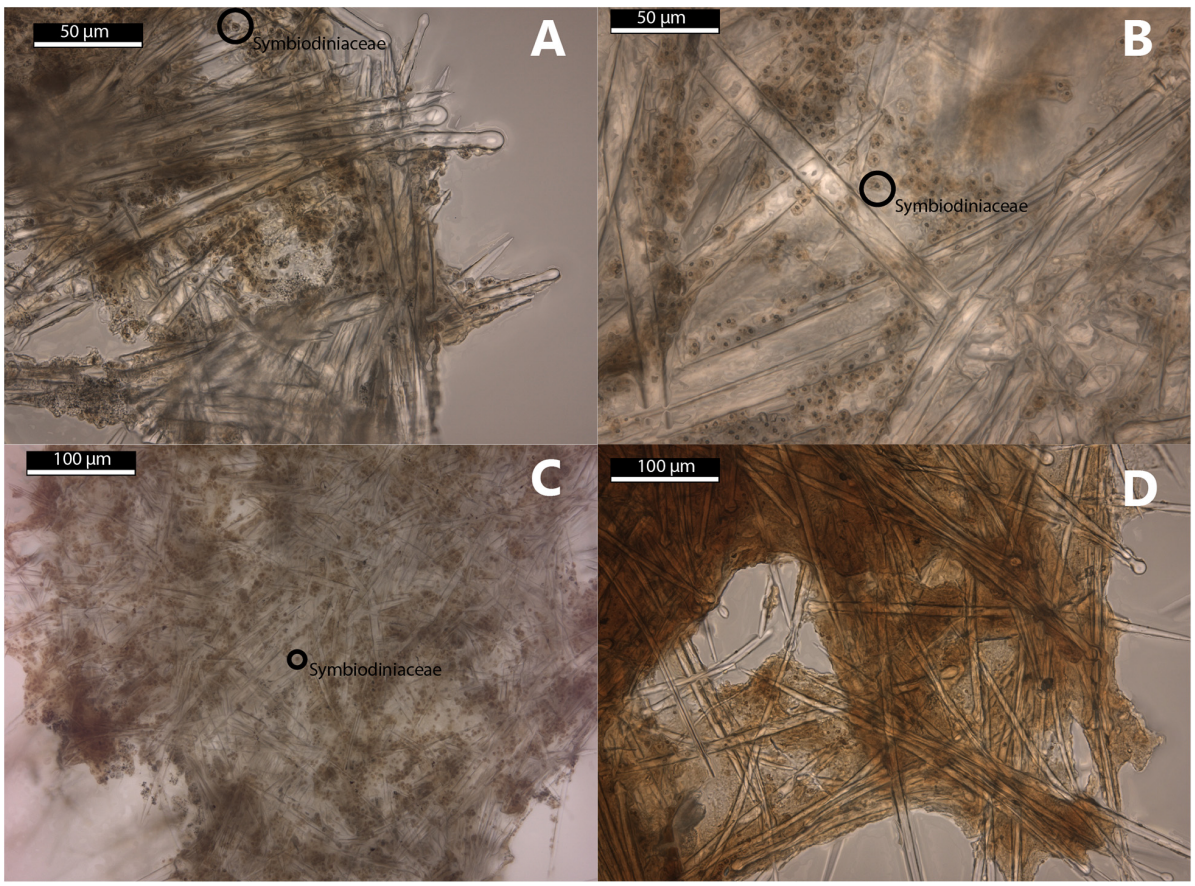

FIGURE 2 | Thick sections of selected Clionaidae species collected in this study. **(A–C)** Symbiodiniaceae present in ectosomal tissue of the sponge host. **(A)** *Cliona thomasi* (RMNH.POR.11777) **(B)** *Spheciospongia maeandrina* (RMNH.POR.11795) **(C)** *Spheciospongia digitata* (RMNH.POR.11784) **(D)** *Cliona utricularis* (RMNH.POR.11778), no Symbiodiniaceae observed.

evidence of Symbiodiniaceae (LaJeunesse et al., 2018). With the results of this study we can confidently conclude that *Durusdinium* is cohabiting with sponges.

In situ observations provided interesting insights into the evolution and ecology of the host-symbiont relationship between Symbiodiniaceae and Clionaidae. Three out of the four studied species hosting Symbiodiniaceae contained the same *Cladocopium* symbiont and a single species (*S. digitata*) contained two different symbionts. Numerous factors could be related to the symbiont identity within each sponge species. Firstly, the fact that *S. digitata* and *S. maeandrina* contained their own symbiont could suggest that host identity would be the most influential factor in symbiont identity. Most of the *S. digitata* samples featured the same *Durusdinium* symbiont type, indicating that *Durusdinium* is the preferred symbiont type for *S. digitata*. Secondly, our data suggests that the environment is another important factor to consider in symbiont identity. Two *S. digitata* samples, found on the reefs of Lae Lae, featured *Freudenthalidium* symbionts. The reefs surrounding this island are in very close proximity to the city of Makassar and thus highly impacted by anthropogenic disturbances and discharge from the major rivers, of which the effects were visually observed as highly turbid waters (Renema and Troelstra, 2001; de Voogd et al., 2006). This could be an example of hosts adapting to the environment by taking in symbionts more adapted to the stressful environment.

Another ecological observation was that both *S. digitata* and *S. maeandrina* were able to maintain their Symbiodiniaceae while living in very high temperature conditions. On the reef flats temperatures up to nearly 33°C were observed. The ability of Clionaidae and their Symbiodiniaceae symbionts to persist on the reef flats underlines the putative hardiness of these sponges in regard to stress tolerance compared to corals (Schönberg and Suwa, 2007).

The final observation in the relationship between symbiont and host, is that *S. maeandrina* and *S. digitata*, while morphologically nearly identical and living sympatrically, contained different symbionts. This indicates coevolution between host and symbiont. Examples of coevolution or evolutionary relationships between host and symbionts within a marine ecosystem are quite common (Hill et al., 2011; van der Meij, 2015; Thomas et al., 2016; O'Brien et al., 2019). More research into the evolution of Symbiodiniaceae and their hosts could potentially provide useful insights in the

resilience of certain host groups to environmental changes and might explain the difference in stress tolerance of certain hosts compared to others.

When including the results of this study to existing literature, many of the major Symbiodiniaceae genera (*Symbiodinium*, *Breviolum*, *Cladocopium*, *Durusdinium*, *Freudenthalidium*, and *Gerakladium*) have been detected in sponge hosts. The lack of available data on the presence and molecular identity of Symbiodiniaceae in bioeroding sponges is likely the result of limited research on Symbiodiniaceae in sponges. The fact that most Symbiodiniaceae types are found in coral hosts is most likely the result of a sampling bias, as most Symbiodiniaceae research is targeted at corals. The diversity of Symbiodiniaceae within clionaid sponges and other invertebrate hosts could prove just as great compared to coral hosts. Therefore, more Clionaidae hosts need to be examined for symbiotic relationships with Symbiodiniaceae.

Investigating the symbiotic assemblages in bioeroding sponges and the way they are affected by environmental factors such as depth, temperature and disturbances is crucial from a conservation perspective. With global climate change, the abundance of bioeroding sponges, and thus bioerosion, on reefs is expected to increase, further threatening coral reefs (Fang et al., 2013). As bioeroding sponges play an important role in bioerosion on reefs, and the growth and boring rates of Clionaidae are enhanced by Symbiodiniaceae (Rosell and Uriz, 1992; Hill, 1996; Schönberg, 2006; Schönberg et al., 2017), it is crucial to understand how the relationship between Clionaidae and Symbiodiniaceae is affected by environmental changes in the future. There are some projections that bioeroding sponges will not be able to cope with the projected ocean warming (Ramsby et al., 2018), but our study indicates otherwise. The thriving sponge-symbiont communities of the reef flat environments in the Spermonde Archipelago highlight a very high thermal tolerance of some bioeroding sponges. It is important to investigate the functional role of the identity of the symbiont in order to fully comprehend how bioeroding sponges will cope with environmental changes and furthermore, to understand the threat of bioeroding sponges to coral reefs in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the NCBI GenBank, the article, and the **Supplementary Material**. The NCBI GenBank accession numbers can be found in the article and **Supplementary Material**.

AUTHOR CONTRIBUTIONS

NW and NV developed the research questions, designed the experimental design, collected the field data, and wrote the first draft of the manuscript. NW and EE analyzed the data. NW created the graphics. All authors contributed to the manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.595452/full#supplementary-material

Supplementary Figure 1 | Map of sampling locations within the Spermonde Archipelago, Makassar, Indonesia. Original map is created from modified NASA Landsat ETM + imagery by Plass-Johnson et al. (2016).

Supplementary Table 2 | Datasheet of the samples used in this study, including collection data, RMNH voucher data and GenBank accession numbers for extracted host and symbiont molecular sequences.

Supplementary Table 3 | Megablast results for Symbiodiniaceae ITS2 sequences.

Supplementary Figure 4 | Photographs and morphological descriptions of the Clionaidae species encountered in this study.

Supplementary Table 5 | PCR gel results ITS2 amplification Symbiodiniaceae.

Supplementary Figure 6 | Phylogenetic tree Symbiodiniaceae ITS2 sequences.

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The Metabolic Response to Infection With *Wolbachia* Implicates the Insulin/Insulin-Like-Growth Factor and Hypoxia Signaling Pathways in *Drosophila melanogaster*

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The endosymbiotic bacteria, *Wolbachia*, are best known for their ability to manipulate insect-host reproduction systems that enhance their vertical transmission within host populations. Increasingly, *Wolbachia* have been shown to depend on their hosts' metabolism for survival and in turn provision metabolites to their host. *Wolbachia* depends completely on the host for iron and as such iron has been speculated to be a fundamental aspect of *Wolbachia*-host interplay. However, the mechanisms by which dietary iron levels, *Wolbachia*, and its host interact remain to be elucidated. To understand the metabolic dependence of *Wolbachia* on its host, the possibility of metabolic provisioning and extraction, and the interplay with available dietary iron, we have used NMR-based metabolomics and compared metabolite profiles of *Wolbachia*-infected and uninfected *Drosophila melanogaster* flies raised on varying levels of dietary iron. We observed marked metabolite differences in the affected metabolite pathways between *Wolbachia*-infected and uninfected *Drosophila*, which were dependent on the dietary iron levels. Excess iron led to lipid accumulation, whereas iron deficiency led to changes in carbohydrate levels. This represents a major metabolic shift triggered by alterations in iron levels. Lipids, some amino acids, carboxylic acids, and nucleosides were the major metabolites altered by infection. The metabolic response to infection showed a reprogramming of the mitochondrial metabolism in the host. Based on these observations, we developed a physiological model which postulates that the host's insulin/insulin-like-growth factor pathway is depressed and the hypoxia signaling pathway is activated upon *Wolbachia* infection. This reprogramming leads to predominantly non-oxidative metabolism in the host, whereas *Wolbachia* maintains oxidative metabolism. Our data also support earlier predictions of the extraction of alanine from the host while provisioning riboflavin and ATP to the host.

Keywords: *Wolbachia*, *Drosophila melanogaster*, iron, metabolomics, NMR, insulin/insulin-like growth factor signaling (IIS) pathway, hypoxia

1. INTRODUCTION

Wolbachia pipientis, a maternally-inherited endosymbiont of invertebrates are best known for their ability to manipulate insect-host reproduction systems that enhance their transmission within host populations. Increasingly *Wolbachia*, have been shown to affect other insect-host life history traits including reproduction, lifespan, behavior, protection against viral infections or in limited examples, metabolic provisioning and extraction.

Metabolic provisioning and extraction by *Wolbachia* has been well-characterized in strains that infect filarial nematodes, where they establish obligate mutalistic associations (Hoerauf et al., 1999; Langworthy et al., 2000; Foster et al., 2005; Darby et al., 2012; Godel et al., 2012; Gill et al., 2014). Genome sequencing of both the nematode and *Wolbachia* symbionts have shown that both partners are dependent upon the other to complete several metabolic pathways. For example, the filarial nematode *Brugia malayi* lacks complete biosynthetic pathways for riboflavin, heme, or flavin adenine dinucleotide and nucleotides, while its *Wolbachia* symbiont *w*Bm encodes complete biosynthetic pathways (Foster et al., 2005). Conversely, *w*Bm lacks complete biosynthetic pathways for a range of metobolites including biotin, coenzyme A, folate, lipoic acid, nicotinamide adenine dinucleotide, pyridoxal phosphate and ubiquinone, which it acquires from its nematode host (Foster et al., 2005). Therefore, as an obligate endosymbiont, *Wolbachia* is highly dependent on host-derived metabolites for survival, despite its potential to provision metabolites (Wu et al., 2004; Jiménez et al., 2019; Newton and Rice, 2020). Nevertheless, direct experimental evidence for metabolic provisioning and extraction by *Wolbachia* in insect hosts has been limited. The clearest example is that observed in the blood feeding bedbug *Cimex lectularius* which is provided riboflavin (Moriyama et al., 2015) and biotin (Nikoh et al., 2014) by *Wolbachia*. Removal of *Wolbachia* reduces growth rates and fecundity, but these fitness traits could be restored when riboflavin was supplemented (Moriyama et al., 2015).

Recent analysis of genome-scale metabolic models predict that most *Wolbachia* strains are dependent upon their host for several metabolites. For instance, *w*Mel, a strain that naturally infects *Drosophila melanogaster*, is predicted to depend on its host for alanine, glycine and serine metabolism, as well as for biosynthesis of lipopolysaccharides, antibiotic precursors, and biotin (Jiménez et al., 2019; Newton and Rice, 2020). Moreover, *Wolbachia* completely depends on the host for iron (Gill et al., 2014; Jiménez et al., 2019). As iron levels are relatively low in the natural environment of *Drosophila* (Brownlie et al., 2009), both *Wolbachia* and the host compete for dietary iron (Newton and Rice, 2020). Thus, iron has been speculated to be a fundamental aspect of the interplay between *Wolbachia* and the host (Gill et al., 2014). Studies in wasps and flies have shown that *Wolbachia* directly influences host expression of two major iron-regulating proteins, ferritin and transferritin, in direct response to dietary iron (Kremer et al., 2009; Gill et al., 2014). In a related study, we showed that *w*Mel provide mild or strong fecundity advantages to infected female *D. melanogaster* flies when subjected to diets with low or high iron, respectively, corresponding to iron-deficiency or -overload (Brownlie et al., 2009). However, the mechanisms by which these advantages were conferred and how iron levels in diet and *Wolbachia* interact are unknown.

Here we have used for the first time NMR-based metabolomics to characterize *Wolbachia*-host interactions. We compare metabolite profiles of *Wolbachia*-infected and uninfected *D. melanogaster* raised on varying levels of dietary iron to understand (1) the metabolic dependence of *Wolbachia* on its host, (2) the possibility of metabolic provisioning and extraction, and (3) the interplay with available dietary iron. We show that there are strong interactions between the levels of dietary iron and *Wolbachia* infection in *Drosophila*. High levels of dietary iron affect mostly lipid metabolism, whereas low dietary iron predominantly impinges on carbohydrate metabolism. Furthermore, we show that lipids, some amino acids, carboxylic acids, and nucleosides are all affected in *Wolbachia*-infected flies. Based on these observations, we have developed a physiological model that explains the mechanism behind these metabolic changes. We propose that a reprogramming of the mitochondrial metabolism occurs in *Wolbachia*-infected hosts, facilitated by the insulin/insulin-like growth factor and hypoxia signaling pathways. This reprogramming leads to predominately non-oxidative metabolism in the host, whereas *Wolbachia* maintains oxidative metabolism. Our work also relates how these underlying biochemical and regulatory pathways are involved in metabolic interactions between the symbiont and host.

2. MATERIALS AND METHODS

2.1. Insects

The *Drosophila melanogaster* strain BNE was derived from field-caught female flies from Brisbane, Australia, and is described in detail elsewhere (Brownlie et al., 2009; Yamada et al., 2011). Two genetically paired fly lines, one infected by *w*Mel the other *Wolbachia*-free, were maintained at ∼25°C on a 12/12 h light/dark schedule throughout the study. Tetracycline treatments were performed as described previously (Hoffmann et al., 1986) to generate a genetically identical fly line that lacked the *Wolbachia* infection. To reconstitute gut flora standardized methods were used (Chrostek et al., 2013) and all experiments were conducted at a minimum of seven generations post tetracycline treatment. To minimize genetic drift between these fly lines, approximately every 10 generations reciprocal crosses (BNE-*w*Mel Female × BNE Tet male; BNE Tet female × BNE-*w*Mel Male) were performed using 1-week-old adults. Fly lines were reared on three types of diets.

Cornmeal fly diet was made from yellow corn meal medium (Sigma). As described in detail elsewhere (Brownlie et al., 2009), the amount of available dietary iron was reduced by substituting the water that was used to make up the medium with an aqueous extract of black tea (*Camellia sinensis*)—which reduces iron availability by chelating free iron—or increased by the addition of a $FeCl_3$ solution to the cornmeal fly diet to a final concentration of 10 mM.

TABLE 1 | Experimental groups and sample numbers.

Diet	***Wolbachia*-infected** (W)	**Uninfected controls** (T)
Low iron diet (L)	WL, $n = 17$	TL, $n = 16$
Standard iron diet (S)	WS, $n = 19$	TS, $n = 20$
High iron diet (H)	WH, $n = 22$	TH, $n = 17$

2.2. Elemental Analysis Content and Estimate of *Wolbachia* Infection Density

The total content of nine biologically relevant metals (manganese, iron, cobalt, nickel, copper, zinc, cadmium, lead, and arsenic) present in flies reared on each of the food types (listed above), was determined using inductively coupled plasma mass spectrometry (ICP-MS) at the Advanced Center for Isotope Research Excellence at The University of Queensland. The only metal responsive to diet was iron. Pools of ten 5-day old flies were used for each analysis (*Wolbachia*-infected and uninfected controls) and replicated 15 times. Comparisons of the average total iron levels across diet and infection status were made using two-way ANOVA (GraphPad Prism).

Wolbachia infection density in 5-day old flies reared on each food type were estimated using an established relative qPCR assay that compares the abundance of the single-copy *Wolbachia* ankyrin repeat gene *WD0550* to that of the single-copy *D. melanogaster* gene *Act88F* (McMeniman et al., 2008). Briefly, ten adult flies reared on each food type were collected and genomic DNA isolated using a QIAGEN DNeasy Blood and Tissue Kit according to manufacturer instructions (QIAGEN, Doncaster, VIC) and target genes amplified using SYBR-Green pre-mix (Qiagen). Comparisons of the average relative density of *Wolbachia* were made using one-way ANOVA (GraphPad Prism).

2.3. Sample Preparation

A total of 20 five-day old mated female flies per sample were collected from each combination of strain (*Wolbachia*-infected, or uninfected controls) and diet (low-, standard-, or high-iron), snap frozen in liquid nitrogen and stored at −80°C. **Table 1** lists the number of samples for each of these six groups. The frozen flies from each sample were funneled into pre-labeled microfuge tubes containing 400 μl of ice-cold acetonitrile (50%, v/v) and homogenized using silica beads in a tissue-lyser (QIAGEN) system. Samples were then centrifuged at 14,000×g for 10 min at 4°C. The supernatant was transferred to another set of pre-labeled and pre-weighed microfuge tubes, and stored at −80°C until lyophilized.

The freeze-dried extracts were thawed and dissolved in 185 μL of 200 mM sodium phosphate buffer, pH 7.4, 10 μL 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate-d_6 (DSS) in D_2O as chemical shift reference, and 5 μL 5 mM difluorotrimethylsilanylphosphonic acid (DFTMP) in D_2O as internal pH indicator, yielding a final sample volume of 200 μL with final concentrations of 250 μM DSS, 125 μM DFTMP, and 7.5% D_2O. Samples were transferred into 3-mm NMR tubes for measurement. For sample numbers and group IDs see **Table 1**.

2.4. NMR Spectroscopy

^{1}H NMR spectra were recorded on a Bruker AV900 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a ^{1}H frequency of 900.13 MHz and equipped with a 5 mm self-shielded z-gradient triple resonance probe. For each sample a 1D NOESY spectrum was acquired at 298 K with the *noesypr1d* pulse sequence [(RD) – 90° – t1 – 90° – τ_m – 90° – acq] (Bruker Biospin pulse program library). The transmitter frequency was set to the frequency of the water signal, and water suppression was achieved by continuous wave irradiation during both the relaxation delay of 3.0 s and the mixing time (τ_m) of 100 ms. After 16 dummy scans, 256 transients were collected into 32,768 data points using a spectral width of 14 ppm, leading to a total experiment time of 18.9 min per spectrum. Samples were manually changed after each 1D spectrum, and spectra were recorded in four different datasets. All spectra were processed using TOPSPIN version 3.5 (Bruker Biospin, Rheinstetten, Germany). The free induction decays (FIDs) were multiplied by a sine bell window function shifted by $\pi/2$ along the direct dimension before Fourier transformation, manual phase and baseline correction. The resulting spectra were referenced to the DSS signal at $\delta = 0$ ppm.

The assignment of peaks to specific metabolites (**Supplementary Table 1**) was performed with Chenomx NMR Suite, version 8.4 (Chenomx Inc., Edmonton, Canada) as well as by comparing spectra with the online databases Human Metabolome Database and Biological Magnetic Resonance Bank (Wishart et al., 2018, Ulrich et al., 2007). In addition, the assignment of metabolites to peaks in the 1D loadings plots of the multivariate statistics (see below), was aided by calculating the covariance matrix (STOCSY) (Cloarec et al., 2005a) at full spectral resolution—either over all NMR spectra, or only over the groups of spectra that were part of the respective multivariate model. This analysis also uncovered covariation between different individual metabolites.

Assignments were confirmed by 2-dimensional (2D) ^{1}H–^{13}C heteronuclear single quantum coherence (^{13}C–HSQC) spectra measured on selected samples. The 2D NMR spectra were acquired on a Bruker Avance 900 spectrometer operating at a ^{1}H frequency of 900.13 MHz, equipped with a 5 mm self-shielded z-gradient triple resonance cryogenic probe. In all 2D spectra, the ^{1}H carrier frequency was positioned on the water resonance. The ^{13}C–HSQC and experiments were performed with spectral widths of 14.03 ppm in the ^{1}H dimension. The spectral width in the ^{13}C dimension were 110 or 120 ppm, and the ^{13}C carrier frequency was set at 45 or 50 ppm, respectively. A total of at least 128 increments with 128 transients were recorded into 4,096 data points in the direct dimension, and a relaxation delay of 1.1 s was used. GARP decoupling of the ^{13}C channel was used during the acquisition time. The spectra were multiplied by a squared sine bell window function shifted by $\pi/2$ along the direct and indirect dimensions before two-dimensional Fourier transformation.

2.5. Data Pre-processing

To correct for pH and ionic strength-dependent shift variations of individual NMR signals, the ^{1}H NMR spectra were aligned in specific segments with the icoshift algorithm (Savorani et al.,

2010) in MATLAB (MathWorks, Massachusetts, U.S.A) and then data-reduced with an in-house MATLAB script to consecutive integral regions of 0.001 ppm width ("buckets"), covering the range of δ = 10.0–0.25 ppm. The chemical shift region at δ = 5.10–4.67 ppm was excluded to eliminate the effects of imperfect water suppression. For each spectrum, the resulting integral regions were normalized to the total intensity of the spectrum to correct for inter-sample differences in weight and dilution. Subsequently, the bucketed data matrices were imported into the SIMCA 16 software package (Sartorius Stedim AB, Umeå, Sweden) for multivariate statistical analysis.

2.6. Multivariate Statistical Analysis

The data matrix with the bucketed 1D spectra was Pareto scaled for the subsequent analysis. An initial principal components analysis (PCA) was performed to determine whether there were any sample outliers and to investigate inherent differences in the samples (Hotelling, 1933). Nine samples were considerably outside the tolerance range of three standard deviations of the average score values in at least two latent components. Three additional samples were outside the 99% Hotelling's T2 limit. All twelve samples were removed as outliers in the subsequent analysis.

To maximize the distinction between classes, a supervised orthogonal partial least-squares (OPLS) analysis was performed on all remaining spectra (**M1**) (Trygg and Wold, 2002). To characterize global differences as a result of infection or diet, three OPLS models were fitted, one comparing all infected to all uninfected samples, irrespective of diet (**M2**), and one model each comparing all low- vs. standard-iron and standard- vs. high-iron diet samples, respectively (**M3**, **M4**), irrespective of infection status. Finally, to compare systematic differences between pairs of sample groups in more detail, a series of pairwise OPLS models was subsequently fitted (**M5**-**M13**). Supervised multivariate methods need additional input data about the class membership of individual samples, which are provided in the form of a Y–table, against which the PLS or OPLS algorithm performs a regression of the X-matrix. In our study the group identity of the six sample groups (0–5) was used as the Y–table for **M1**, and **M2**-**M13** used the *Wolbachia* infection status (0 or 1) or the type of diet (0, 1, or 2), as appropriate.

In SIMCA, the number of OPLS components (A) for all models was optimized by cross validation. R^2X, R^2Y, and Q^2 were used to evaluate model quality. R^2X and R^2Y are the fraction of the sum of squares explained by the latent components in the model, representing the variance of the X and Y variables, respectively, and Q^2 is the predictive ability parameter of the model, which is estimated by cross validation. All OPLS models were validated by CV-ANOVA (Eriksson et al., 2008). The figures of merit of all multivariate models are listed in **Supplementary Table 2**.

Scores plots and loadings plots were used to interpret the various OPLS models. Spectral features representing elevated metabolite levels were identified from bivariate 1D loadings plots, in which loadings coefficients p were plotted against the chemical shift values of their respective variables, and the correlation-scaled loadings coefficients $|p(corr)|$ were superimposed on the loadings plot as a heatmap color scale (Cloarec et al., 2005b). These plots contain the same information as a traditional S-plot, which indicates which variables influence the model with high reliability and are of relevance in the search for significantly altered metabolites.

To obtain a list of significantly changing metabolites (**Supplementary Table 3**), we used a combination of the variable importance in projection (VIP) score, $|p|$, and $|p(corr)|$ (Galindo-Prieto et al., 2014). Generally, variables with VIP scores greater than 1 are considered significant (Eriksson et al., 2013). This criterion was converted into cutoffs for $|p|$ and $|p(corr)|$ by inspecting the S-plots for variables with VIP values of $>$ 1. For all diet pairwise comparisons (Models **M8**-**M13**), cutoffs of $|p| \geq 0.015$ and $|p(corr)| \geq 0.6$ were defined. For all *Wolbachia v.* uninfected pairwise comparisons (Models **M5**-**M7**) a cutoff for $|p| \geq 0.015$ and $|p(corr)| \geq 0.45$ was defined. The respective cutoffs for the global comparisons (Models **M2**-**M5**) were $|p| \geq 0.015$, and $|p(corr)| \geq 0.5$ for the diet comparisons (Models **M4**-**M5**) and $|p(corr)| \geq 0.4$ for the *Wolbachia v.* uninfected comparison (Model **M2**).

2.7. Univariate Statistical Analysis

The list of metabolites observed to change significantly in the multivariate analysis (**Supplementary Table 4**) was then further explored using univariate statistical analysis. Only metabolite signals with minimal peak overlap were used in this analysis (**Supplementary Table 5**). Metabolite signal intensity in each spectrum was calculated by peak integration of the spectral region(s) containing signals of the respective metabolite with an in-house MATLAB script on the non-normalized full resolution spectra. Subsequently, the signal intensities from each spectrum were averaged for each of the six experimental groups. The resulting average signal intensities for each metabolite are listed in **Supplementary Tables 6**, **7**. In both tables, average signal intensities lower than the limit of quantification (LOQ) for the respective metabolite were labeled "$<LOQ$" and omitted from further analysis. The LOQ for each metabolite was determined as follows. The noise level for each spectrum was calculated as the standard deviation of the baseline noise in the region between $\delta = 11 - 10$ ppm. The noise levels for all spectra were then averaged to obtain the mean baseline noise level. This value is the error that is contributed by the baseline noise to the intensity of each data point of a spectrum. To obtain the total error contributed by the baseline noise to the integrated intensity of a metabolite, the average baseline noise level was multiplied by the number of data points in the integration regions for the particular metabolite (**Supplementary Table 5**). The LOQ was then conservatively defined as 5 times the resulting value, i.e., the threshold at which the baseline noise contributes more than 20% error to the intensity integral of a metabolite.

For each pairwise comparison the relative fold change in signal intensities was calculated as the ratio of the averaged signal intensities between the two groups (**Supplementary Table 8**). To determine significantly changing metabolites across each of the nine pairwise comparisons, the signal intensities of each metabolite (**Supplementary Tables 6**, **7**) were subjected to Mann–Whitney U-tests. The Benjamini–Hochberg method

for multiple testing correction was then applied with a false discovery rate of 0.05 on the obtained p values. Metabolite intensities with a p value less than the calculated critical value, Q, were deemed significantly altered in the respective comparison, and the Benjamini–Hochberg corrected p values are listed in **Supplementary Table 9**.

3. RESULTS

3.1. Iron Content and *Wolbachia* Density Analysis

Genetically paired lines of *Drosophila melanogaster* (BNE) that differed by their *Wolbachia* (*w*Mel) infection status were reared on one of three diets: a standard fly diet that contained black tea extract reducing the available dietary iron, standard fly diet, or a standard fly diet supplemented with additional iron (**Table 1**). The total content of iron, manganese, cobalt, nickel, copper, zinc, cadmium, lead and arsenic was determined using mass spectrometry in 5-day old flies. Only the total iron content was affected (**Supplementary Figure 1**), as had been observed previously (Brownlie et al., 2009). Furthermore, there were no significant differences in iron levels when comparing uninfected controls and *Wolbachia*-infected insects. To determine if altering dietary iron influenced *Wolbachia* densities within 5-day old flies, a standard quantitative-PCR assay was used (McMeniman et al., 2008). *Wolbachia* densities were equivalent in hosts reared on all diets (**Supplementary Figure 2**). Taken together, these two results mean that any metabolic changes we observe across the three different diets are directly related to the iron content in the insect.

3.2. Global Analysis

Multivariate statistical analysis (MVSA) was used to investigate whether there are any systematic differences between the metabolite profiles of the six experimental groups of insects. After identifying 12 outliers in a preliminary PCA, an OPLS model of all remaining samples (model **M1**) showed definitive differences in the metabolite profile between all six experimental groups, and that all groups can be clearly distinguished from each other in the 3D scores plot (**Figure 1**). Note that the relationship between the six experimental groups to each other is unconstrained in the OPLS model, and thus their spatial arrangement and the distances between the centers of the six sample groups (**Table 2**) reflect the biological effects that distinguish the groups from each other. Strikingly, the spatial arrangement of the six groups with respect to each other in the 3D scores plot (**Figure 1**) is such that none of the first three latent components (t[1], t[2], or t[3]) align directly with the two factors of the study—*Wolbachia* infection, or dietary iron levels. In fact, five latent components are needed to fully describe the spatial arrangement of all groups with respect to each other. This arrangement of the sample groups, and especially the fact that the six groups are arranged in a non-planar manner shows immediately that (a) there are prominent interactions between *Wolbachia* infection and dietary iron levels, and (b) the effects of dietary iron are non-linear.

The Euclidean distances between the six groups (**Table 2**) highlight that the effect of dietary iron levels is more pronounced in the *Wolbachia*-infected insects than in the uninfected controls. This bears witness to *Wolbachia*'s dependency on the host for iron supply. The biological effect of *Wolbachia* infection is highly variable across the three different dietary conditions. It is the smallest in the context of the standard-iron diet. In the context of the high-iron diet the effect of infection is among the largest biological effects in the study. This highlights that the effects of infection are highly dependent on the diet context and that there are intricate interactions between infection and diet, which will complicate attempts to look at the two effects independently.

3.3. Overarching Effects of *Wolbachia* Infection or Diet

In a next step, we nevertheless attempted to characterize any overarching effects of *Wolbachia* infection or dietary iron levels in the study. We constructed three OPLS models that looked at each effect independently. Two models investigated the effects of diet, irrespective of infection (standard- vs. high-iron and low- vs. standard-iron diet), and the third model characterized the effects of infection, irrespective of diet. Clear group differences were noted in the scores plots of all three OPLS models (**Supplementary Figure 3**). In the corresponding loadings plots (**Supplementary Figure 4**), more dramatic global effects were seen for dietary iron levels than for *Wolbachia* infection.

Looking at the effect of diet first, the differences between the low- and standard-iron diet (**Supplementary Figure 4B**, model **M4**) are characterized by increased levels of complex carbohydrates in the standard-iron diet, and decreased levels of proline, glycine, and propionate. The differences between the standard- and the high-iron diet (**Supplementary Figure 4A**, model **M3**) are more severe, with the most noticeable changes being an increase in the levels of both saturated and unsaturated lipids, as well as increased levels of trehalose and tyrosine, and a decrease in the levels of maltose and complex carbohydrates, next to decreasing levels of inosine, guanosine, ortho-phosphocholine, choline, and dimethylamine.

Overall, the differences in dietary iron levels are predominantly reflected in alterations in carbohydrate and amino acid metabolism when comparing low- and standard-iron diets, but are dominated by lipids between standard- and high-iron diets. These overarching effects indicate that a metabolic switch occurs between carbohydrate and lipid metabolism when insects are subjected to iron deficiency vs. iron overload.

Interestingly, the levels of trehalose and lipids are strongly correlated with each other in this study. Thus, in a one-dimensional STOCSY plot the covariance between the anomeric signal of trehalose and all lipid signals is about 0.68, nearly as much as the covariance with the rest of the trehalose signals (0.7–0.8), which are heavily overlapped in the NMR spectra, leading to their correlation to the anomeric proton signal being statistically diluted by contributions from other metabolites. Maltose and complex carbohydrates show a biphasic non-linear behavior with respect to diet, as levels of maltose and complex carbohydrate are highest in the standard-iron diet and lower in both the low- and high-iron diet.

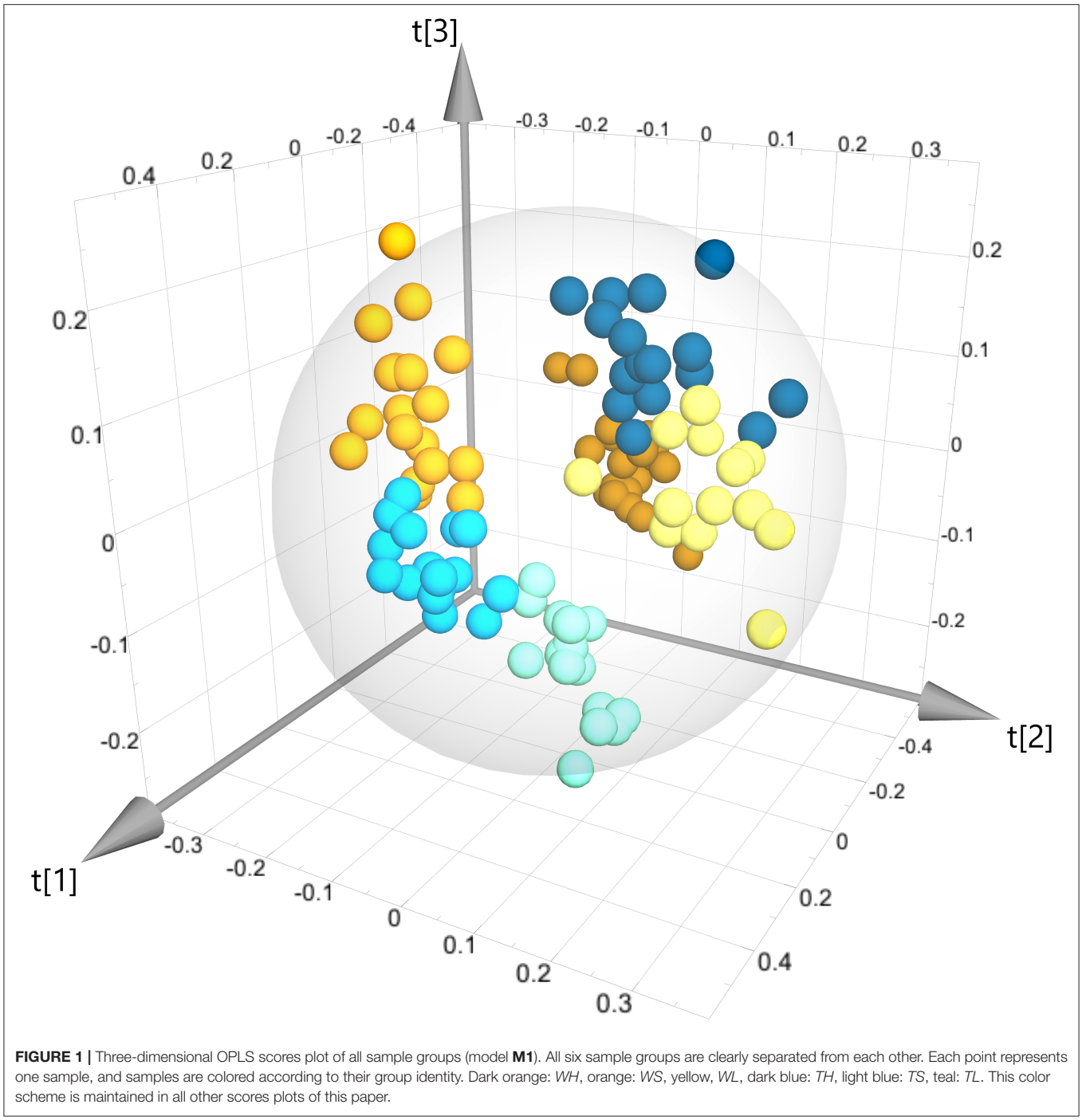

FIGURE 1 | Three-dimensional OPLS scores plot of all sample groups (model **M1**). All six sample groups are clearly separated from each other. Each point represents one sample, and samples are colored according to their group identity. Dark orange: *WH*, orange: *WS*, yellow, *WL*, dark blue: *TH*, light blue: *TS*, teal: *TL*. This color scheme is maintained in all other scores plots of this paper.

The overarching effects of *Wolbachia* infection were less pronounced. The metabolites that pass the significance filter (**Supplementary Figure 4C**, model **M2**) are generally only weakly correlated with the infection status of the insects—a stark contrast to the pronounced effects associated with dietary iron. Decreased levels of several nucleosides, glycerophosphocholine, asparagine and choline were observed in infected insects together with an increase in trehalose. These weak overarching effects of infection arise due to the strong interactions between *Wolbachia* infection and dietary iron, and thus no highly correlated overarching metabolite changes were observed when comparing all infected insects to all uninfected controls—a picture also reflected in the Euclidean distances of the global OPLS model.

3.4. Detailed Analysis of Infection and Diet Effects

Due to the intrinsic interplay between *Wolbachia* infection and dietary iron, it was necessary to examine the detailed

TABLE 2 | Euclidean distances between sample groups in model M1.

Comparison		Euclidean distance
Wolbachia v. uninfected (W – T):	H	0.479
	S	0.202
	L	0.345
Wolbachia-infected (W):	S – H	0.592
	L – S	0.422
	L – H	0.583
Uninfected control (T):	S – H	0.414
	L – S	0.257
	L – H	0.356

*The OPLS model **M1** contains all six sample groups in the study without any constraints for their relative relationship, and as such the Euclidean distances between groups are a reflection of the biological effects that distinguish the groups from each other. The Euclidean distances were calculated between the center of each sample group.*

effects of infection in the context of the three different diets, and at the differences between the three diets in the context of *Wolbachia*-infected insects and uninfected controls. Nine pairwise OPLS models (**M5**-**M13**) were subsequently fitted to investigate the effects of diet on infection status and vice versa (**Supplementary Figure 5**). Metabolites significantly changing in these nine pairwise models are listed in **Supplementary Table 4**. To complement the metabolite alterations identified in these multivariate models, univariate statistical analysis (UVSA) was also undertaken to analyse these metabolite changes semi-quantitatively and to calculate fold-changes and *p*-values for the significance of the fold-changes (**Supplementary Tables 5**–**9**).

3.4.1. Effects of Infection

As anticipated by the analysis so far, the metabolic effects of *Wolbachia* infection are indeed dramatically different in the context of the three iron diets (**Figure 2**). In the low-iron-diet (**Figure 2C**, model **M7**), *Wolbachia* infection leads to decreased levels of β-alanine, complex carbohydrates, and nucleosides such as inosine, IMP, guanosine and uridine. Conversely, *Wolbachia*-infected flies had increased levels of propionate, citrate, o-phosphocholine, tyrosine and adenosine.

Strikingly, the metabolic effects of infection in the context of the standard-iron diet are almost solely dominated by a decrease in proline levels (**Figure 2B**, model **M6**), which overshadows other less pronounced decreases in the levels of uridine, guanosine, DMA, furmarate, o-phosphocholine, and N-acetylated metabolites. Only lipids were noted to increase in infected flies. The fact that proline is dominating the bivariate loadings plots, suggests that this amino acid is a key part of the response to infection under standard iron-conditions. This observation also single-handedly explains why these sample groups are the two groups most similar to each other, as also demonstrated by their Euclidean distance in the global OPLS.

The metabolic consequences of infection are the most pronounced in the high-iron diet (**Figure 2A**, model **M5**), with the most notable effect being decreased levels in choline, GPC, glycine and formate, next to less pronounced decreases in inosine, IMP and asparagine. Levels of trehalose, pyruvate, acetate, lactate, lipids and 2-hydroxyisobutyrate were all increased in *Wolbachia*-infected flies. The changes in pyruvate, acetate and lactate indicate alterations to glycolysis and anaerobic cellular energy metabolism.

Because the metabolic effects of infection are highly dependent on the three different dietary iron levels, interpretation of the underlying pathways that are affected by infection requires careful consideration of the diet context.

3.4.2. Diet Effects in Uninfected Controls

In uninfected control insects, the differences between low- and standard-iron diet are relatively mild with none of the metabolites reaching high $|p(corr)|$ values (**Figure 3B**, model **M9**). The most pronounced changes are increases in the levels of glucose, and o-phosphocholine in the standard-iron diet, and decreased levels of glycine, the branched-chain amino acids, proline, and lipids. These minor metabolite changes are likely indicating that *Drosophila* possesses robust mechanisms for adaptation to sub-optimal dietary iron levels.

In contrast, the metabolic differences between the standard- and high-iron diets (**Figure 3A**, model M8) are much more pronounced, and dominated by highly correlated increases in the levels of both saturated and unsaturated lipids, as well as trehalose in the high-iron diet. Levels of nucleosides, including adenosine, guanosine and uridine, are decreased in the high-iron diet, as are the levels of complex carbohydrates, o-phosphocholine, DMA, and alanine. The changes to fatty acids and carbohydrates suggest regulatory pathways that link carbohydrate and lipid metabolism upon iron overload.

The direct comparison of low- and high-iron diet (**Figure 3C**, model **M10**) shows essentially a superposition of the low- to standard- and standard- to high-iron diet comparisons, with a few alterations. The differences are also dominated by an increase in lipid levels, but this is not as pronounced as between standard- and high-iron diet, because lipid levels decreased between the low-and standard-iron diets. In addition, citrate levels are increased in the high-iron diet, whereas levels of guanosine and inosine are decreased, as are the levels of DMA, alanine and β-alanine.

In several NMR spectra, broad signals of lipids overlap with sharper signals of other metabolites, specifically lactate and proline. This offsets the peak loadings values [p and $p(corr)$] of those metabolites and means proper deconvolution is required to accurately quantify these metabolites. Nevertheless, because our bucket table has a resolution of 0.001 ppm, we were able to determine accurately that in the uninfected controls the levels of both lactate and proline decrease in the high-iron diet when compared to the standard- and low-iron diets (**Figures 3A,C** and **Supplementary Figures 6A–D**), despite the overlap from the broad signal of the lipid methyl and methylene protons, respectively.

In summary, the metabolic effects of dietary iron in the insect are more dramatic for iron overload, in contrast to the milder effects of iron-deficiency. This is perhaps unsurprising as flies in the wild have equivalent total iron levels to that of flies reared on

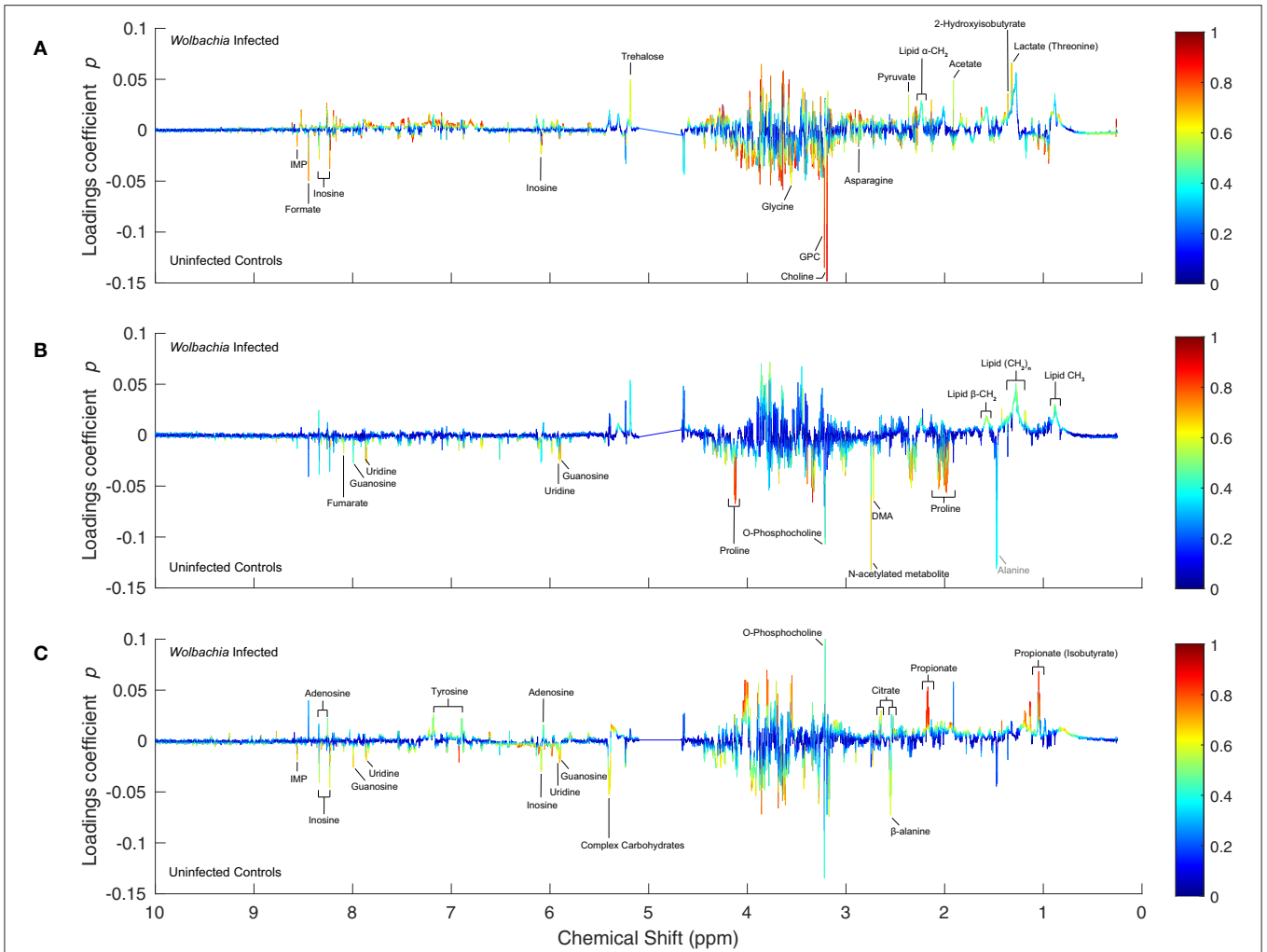

FIGURE 2 | One-dimensional S-plots of the pairwise OPLS models comparing *Wolbachia*-infected insects with the uninfected controls. **(A–C)** For all panels, the loadings coefficients *p* were plotted against the chemical shift of the respective variables in the NMR spectrum, and the correlation-scaled loadings coefficients |*p*(*corr*)| were superimposed on the loadings plot as a heatmap color scale. The significantly altered metabolites ($|p| \geq 0.015$ & $|p(corr)| \geq 0.45$) are annotated in black in each panel: **(A)** High-iron diet (model **M5**), **(B)** Standard-iron diet (model **M6**), and **(C)** Low-iron diet (model **M7**). Gray annotations: metabolites referred to in the discussion.

low-iron conditions (Brownlie et al., 2009), and thus presumably have adapted to these conditions.

3.4.3. Diet Effects in *Wolbachia*-Infected Insects

In *Wolbachia*-infected *Drosophila*, the differences between low- and standard-iron diet (**Figure 4B**, model **M12**) comprise only a few metabolites, but their concentration changes are pronounced and highly correlated. The standard-iron diet is characterized by increased levels of maltose and complex carbohydrates compared to the low-iron diet, and decreased levels of proline, glycine and propionate. The prominent changes to maltose and complex oligosaccharides indicate the involvement of carbohydrate metabolism in the response to iron deficiency.

The differences between the standard- and high-iron diet (**Figure 4A**, model **M11**) comprise more metabolites, but not all metabolite changes are as highly correlated as between the low- and standard-iron diet. In the high-iron diet levels of tyrosine, propionate, and lipids are elevated, whereas the levels of complex carbohydrates, glycine, inosine, and choline are decreased. This metabolic profile difference is strikingly similar to that of the uninfected controls, meaning the regulatory mechanism upon iron overload are common to both groups and independent of infection status.

The comparison between low- and high-iron diet (**Figure 4C**, model **M13**) is dominated by increased levels of trehalose and complex carbohydrates in the high-iron diet, next to elevated levels of 2-hydroxyisobutyrate. Levels of glycine, asparagine, choline, GPC, propionate, DMA, ethanol, and inosine are decreased.

Similarly to the analysis of overlapping lipid and proline signals in the uninfected controls, we were able to determine that in the *Wolbachia*-infected insects proline levels are increased in the high-iron diet when compared to the

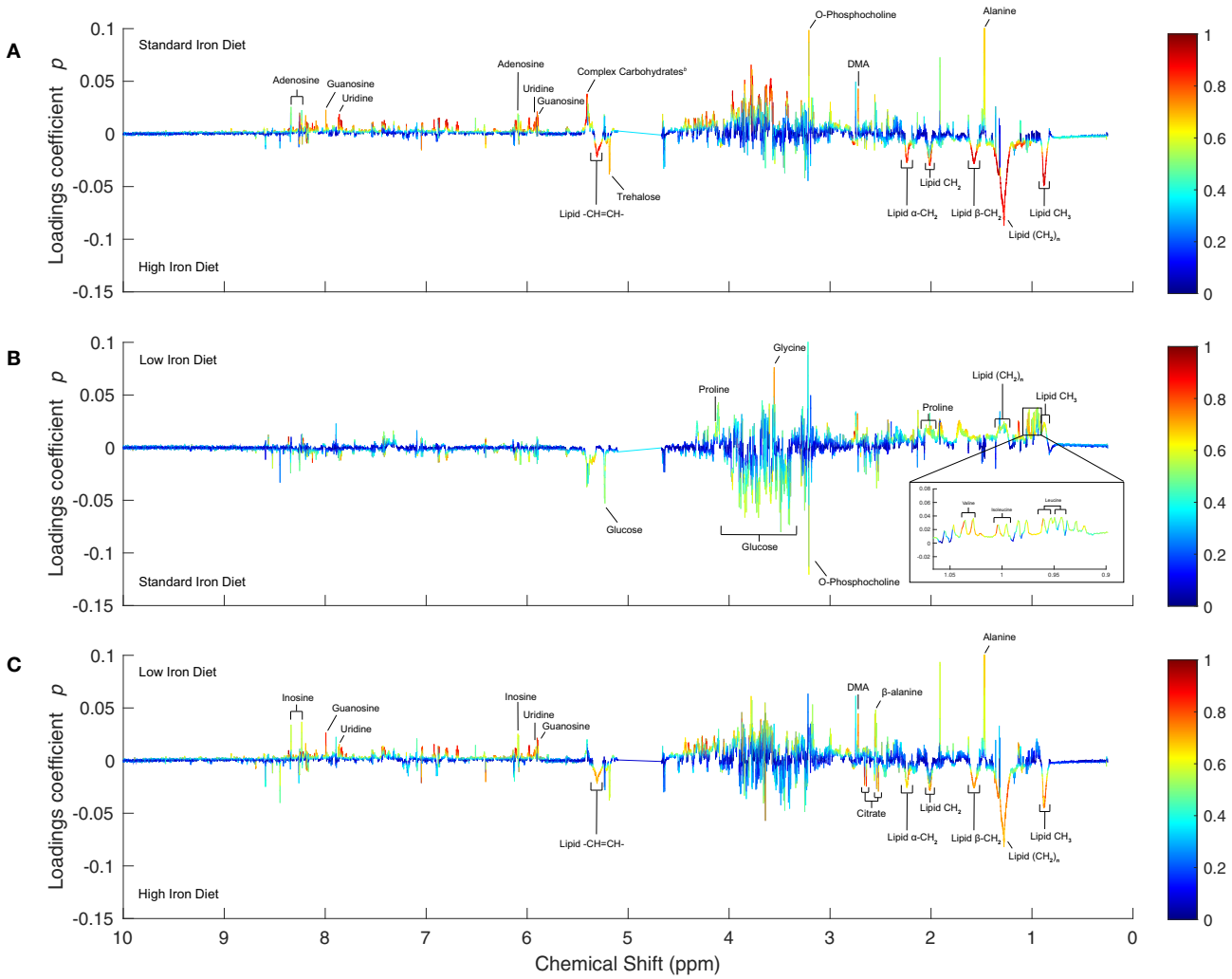

FIGURE 3 | OPLS 1D S-Plots of the pairwise diet comparisons for uninfected controls. **(A–C)** The 1D S-plots are analogous to **Figure 2**, but show the diet comparisons for uninfected controls. The significantly altered metabolites ($|p| \geq 0.015$ & $|p(corr)| \geq 0.6$) are annotated in each panel: **(A)** High- *v.* standard-iron diet (model **M8**), **(B)** Standard- *v.* low-iron diet (model **M9**), and **(C)** High- *v.* low-iron diet (model **M10**).

standard-iron diet (**Figure 4A** and **Supplementary Figure 6E**), but decreased when compared to the low-iron diet (**Figure 4C** and **Supplementary Figure 6F**)—in accordance with the result showing strongly increased proline levels in the low- over the standard-iron diet (**Figure 4B**).

Overall, the effects of dietary iron are strongly pronounced in the *Wolbachia*-infected insects for both high and low levels of dietary iron, when compared to the uninfected insects.

4. DISCUSSION

4.1. Global and Dietary Iron Effects

In this study we have characterized the metabolic changes as a result of *Wolbachia* infection in *Drosophila melanogaster* under different levels of dietary iron. The Euclidean distances between experimental groups in the global analysis already provide some insight into the relative biological effects. For example, the metabolic differences between infected insects and uninfected controls under standard iron conditions was the smallest distance in the whole study. This highlights that *Wolbachia* places only a minimal burden on *Drosophila* under standard-iron conditions, and reflects *Wolbachia*'s adaptation as a mutualistic symbiont. Furthermore, the standard diet is nutritionally rich, and as such masks any obvious *Wolbachia*-host effects. In contrast, the high-iron diet is substantially different to the standard- and low-iron diets in both the uninfected controls and the *Wolbachia*-infected insects. This reflects the impost that iron levels in excess of the natural environment have on both groups. Of further interest are the distances between dietary conditions, which are always larger in the *Wolbachia*-infected groups when compared to the respective distance in the uninfected controls. This is especially evident under conditions of iron deficiency, as the distance between low- and standard-iron conditions in *Wolbachia*-infected insects

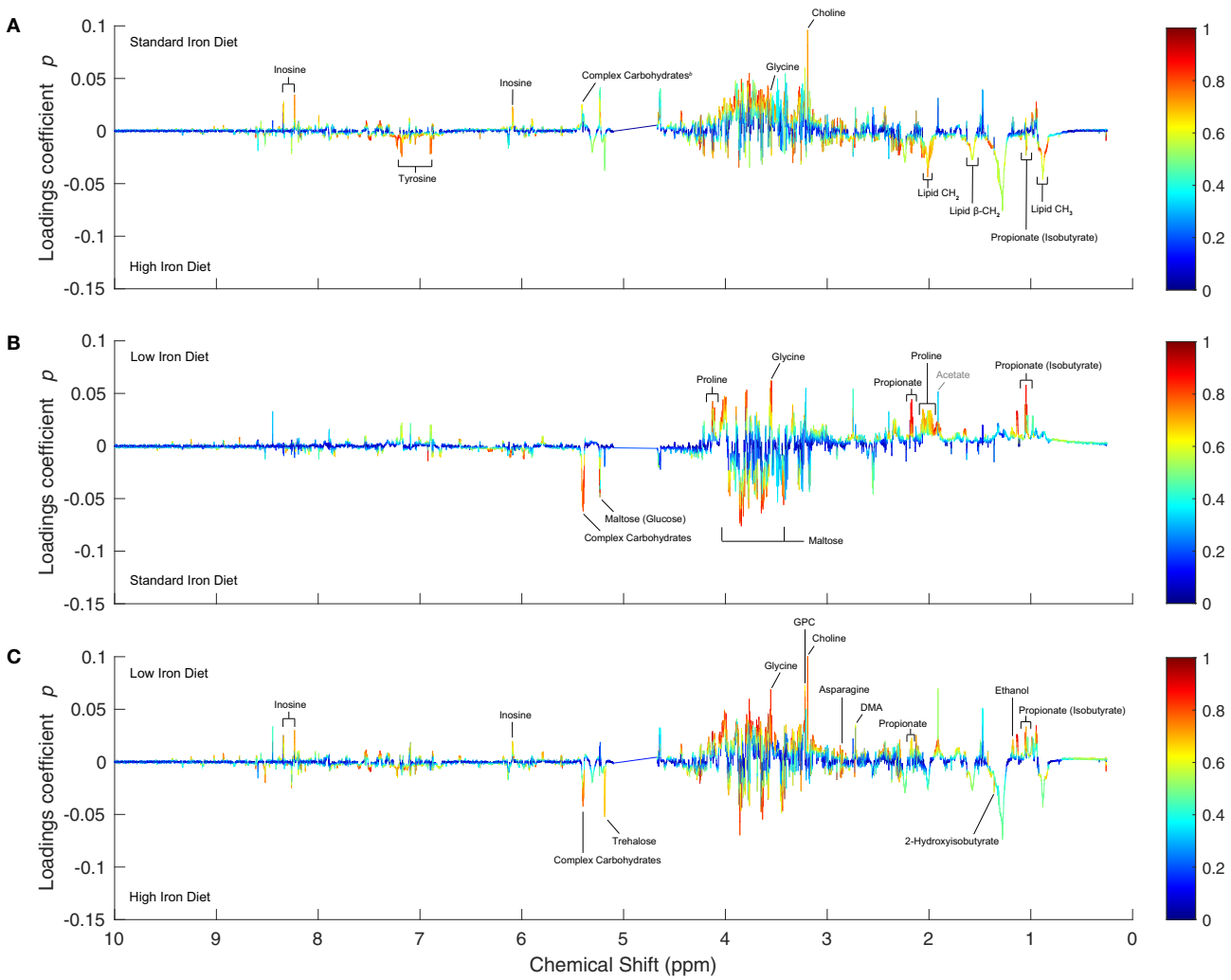

FIGURE 4 | OPLS 1D S-Plots of the pairwise diet comparisons for *Wolbachia*-infected samples. **(A–C)** The 1D S-plots are analogous to **Figure 2**, but show the diet comparisons for *Wolbachia*-infected samples. The significantly altered metabolites ($|p| \geq 0.015$ & $|p(corr)| \geq 0.6$) are annotated in black in each panel: **(A)** High- *v.* standard-iron diet (model **M11**), **(B)** Standard- *v.* low-iron diet (model **M12**), and **(C)** High- *v.* low-iron diet (model **M13**). Gray annotations: metabolites referred to in the discussion.

is almost twice the corresponding distance for the uninfected controls. This bears witness to *Wolbachia*'s dependency on the host for iron and the extra demands it makes on the host for iron supply.

The Euclidean distances are a result of marked metabolite profile differences between the six groups of insects in this study. In both *Wolbachia*-infected insects and uninfected controls, differences in dietary iron levels lead predominantly to alterations in carbohydrate and amino acid metabolism, between low- and standard-iron diets, but are dominated by lipids between standard- and high-iron diets. This indicates that a metabolic shift occurs between carbohydrate metabolism and fatty acid metabolism when comparing low- and high-iron levels, highlighting fundamental changes in metabolism. Interestingly, this metabolic shift is slightly different in *Wolbachia*-infected insects and uninfected controls. Across the board, the changes in carbohydrates and lipids are accompanied by slightly different sets of other metabolites. In addition, under iron deficiency the changes in carbohydrate metabolism are dominated by changes in glucose levels in the uninfected controls, whereas in the *Wolbachia*-infected insects levels of maltose and complex carbohydrates are affected.

When considering the biological and regulatory mechanisms that are likely to underpin the observed metabolic changes, it is worthwhile to keep in mind that metabolic effects that occur in both *Wolbachia*-infected insects and uninfected controls are likely to result from regulatory mechanisms in *Drosophila* itself. Examples of this are the strongly increased lipid levels and decreased levels of complex carbohydrates in the high-iron diet which are observed in both *Wolbachia*-infected insects and uninfected controls. Our observation is confirmed by other studies who have also observed lipid

accumulation under high-iron conditions in a variety of organisms, including *Drosophila* (Navarro et al., 2010; Wang et al., 2016; Rockfield et al., 2018) and humans (Ahmed et al., 2012). Unsurprisingly, lipid and carbohydrate metabolism are intrinsically interlinked with the energy metabolism and mitochondrial function of the cell. A potential mechanism for this link between different parts of the metabolism is the outer mitochondrial membrane protein mitoNEET, which modulates mitochondrial function and iron content (Ferecatu et al., 2014). Upon iron excess, mitoNEET is upregulated leading to lipid uptake and storage within the host as well as to downregulated β-oxidation, and thus to increased adipose tissue mass (Kusminski et al., 2012). In addition, upregulated mitoNEET increases the rate of glycolysis (Kusminski et al., 2012; Wang et al., 2017), which could explain the lower levels of complex carbohydrates observed in our study. Furthermore, upregulated mitoNEET inhibits iron transport into the mitochondrial matrix, thus limiting both oxidative phosphorylation and ROS damage.

Under iron-deficiency conditions one might expect to see metabolic adaptations that lead to conservation of iron, iron scavenging, and a move to or reliance on metabolic pathways that do not require iron, such as glycolysis. Indeed, alterations to carbohydrate metabolism are among the most prominent metabolic changes we observed in both infected and uninfected insects under the low-iron diet. Interestingly, mitoNEET could also be involved in these metabolic alterations. Recently, a glycogen branching enzyme was established to be a key regulator of iron homeostasis under iron-depleted conditions in *Drosophila*. This enzyme controls cellular iron homeostasis via binding to the Iron-regulatory protein 1A (IRP1A) and mitoNEET (Huynh et al., 2019). This link between cellular iron homeostasis and glycogen metabolism may explain why carbohydrates change significantly upon iron deficiency. Additionally, the interaction between the glycogen-branching enzyme, mitoNEET, and IRP1A leads to the downregulation of iron-intensive processes as well as increasing the levels of bio-available iron for the cell (Huynh et al., 2019).

4.2. Effects of *Wolbachia* Infection

There are marked differences in the affected metabolic pathways between *Wolbachia*-infected insects and controls, which are also dependent on the iron levels in the diet. Indeed, when comparing the metabolite profiles of infected insects and uninfected controls, the major metabolites affected are carboxylic acids, nuclosides, cholines, and amino acids in the low- and high-iron diets. In contrast, in the standard-iron diet the major metabolic effect of infection is a decrease in proline levels. Taken together, these observations mean that the detected metabolic differences between the six study groups are driven by an interaction of both the *Wolbachia* symbiont and the iron levels inside the host cells. This highlights that signaling pathways and/or mechanisms that regulate iron homeostasis in the cell play a pivotal role in the interaction between *Wolbachia* and the host.

Earlier studies have shown that *Wolbachia* affects iron homeostasis of the host by regulating the expression of ferritin (Kremer et al., 2009; Gill et al., 2014). Iron homeostasis and ferritin expression are regulated in multiple organisms including *D. melanogaster* via the insulin/insulin-like-growth factor signaling (IIS) pathway and the hypoxia signaling pathway (Ackerman and Gems, 2012; Nässel et al., 2015; Altintas et al., 2016). Based on these studies and our results, we postulate that *Wolbachia* affects both of these pathways in the host. Previous studies have predicted that *Wolbachia* competes for iron as well as oxygen with the mitochondria of the host cell to run its own oxidative phosphorylation, leading to host cells becoming hypoxic in the presence of *Wolbachia* (Kremer et al., 2009; Gill et al., 2014; Dutra et al., 2017). Hypoxia depresses the IIS pathway (Wong et al., 2014; Texada et al., 2019; Barretto et al., 2020) and upregulates the hypoxia signaling pathway, as shown in **Figure 5**. The hypoxia signaling pathway, mediated by hypoxia-inducible factor 1 (HIF-1) (Mylonis et al., 2019), represses ferritin expression, leading to increased bioavaliability of iron in the cell (Kremer et al., 2009). A previous study has indeed shown reduced ferritin expression in *Wolbachia*-infected host cells (Kremer et al., 2009). *Wolbachia* competes for the intracellular iron to maintain oxidative phosphorylation, which will lead to the production of reactive-oxygen species (ROS) by the symbiont (Gill et al., 2014). In addition, the increased iron bioavailability in the host cells leads to secondary ROS production (Gill et al., 2014). In support of this interpretation, we have previously shown that ROS are being induced as a result of *Wolbachia* infection even in co-evolved hosts such as *Drosophila* (Wong et al., 2015). These metabolic effects observed here are likely to be driven by *Wolbachia*, modified by the context of the dietary iron levels, rather than being a primarily iron-driven response.

Interestingly, the ATP generated by *Wolbachia* for its own purposes can potentially be provided to the host (Darby et al., 2012; Gill et al., 2014). In accordance with that hypothesis we observed during *Wolbachia* infection at low-iron conditions a significant increase of adenosine levels, which are likely to be a proxy for the whole pool of adenosine, AMP, ADP, and ATP, due to our extraction procedure. The increase in adenosine levels we observed is arguably from the symbiont, because recent studies noted that *Wolbachia* infection in *Drosophila* yields significantly reduced ATP levels in the host (Carneiro Dutra et al., 2020, Carneiro Dutra et al., 2020).

On the other hand, depressing the IIS pathway leads to the induction of mechanisms that will modulate the increased ROS production induced by *Wolbachia* infection (Van Heemst, 2010). This aligns with an earlier hypothesis (Zug and Hammerstein, 2015) that in native hosts *Wolbachia* infection is associated with both increased ROS production and increased mechanisms of redox homeostasis and ROS protection. Firstly, the hypoxia-induced reduction of the IIS pathway leads via phosphoinositide 3-kinase (PI3K)/Akt signaling to an activation of the *daf-16*/FoxO transcription factor (Barretto et al., 2020), which mitigates oxidative stress resistance through regulating antioxidants (Mattila and Hietakangas, 2017) and increasing ferritin expression (Ackerman and Gems, 2012). The latter effect is likely to provide some counterbalance to the ferritin repression triggered by hypoxia and HIF-1. Secondly, proline catabolism is promoted, which regulates both ROS production and homeostasis (Zarse et al., 2012; Tang and Pang, 2016). In

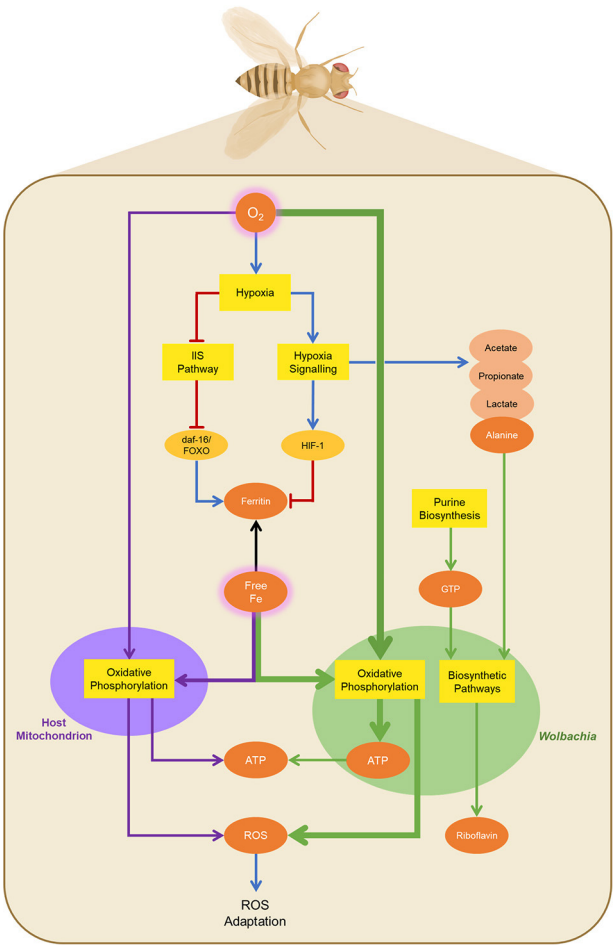

FIGURE 5 | General regulatory and metabolic pathways involved in *Wolbachia* infection. The presence of *Wolbachia* (green oval) inside *Drosophila* cells (beige rectangle) leads to competition for oxygen, causing hypoxia in the host cells. Hypoxia leads to the downregulation of the insulin/insulin-like growth factor signaling (IIS) pathway and upregulation of the hypoxia signaling pathway, and subsequently to reprogramming of mitochondrial metabolism (purple oval). The hypoxia signaling pathway leads to increased bioavailability of free iron in the host cells, as iron sequestration by ferritin is reduced. Iron is redirected to *Wolbachia*, which relies on the host for iron supply, leading to ROS production as a consequence of oxidative phosphorylation in the symbiont (green arrows). ATP produced by *Wolbachia* via this route can be exported to the host. The increased bioavailability of iron also leads to ROS production in *Drosophila* (purple arrows). Both pathways of ROS production lead to the subsequent activation of ROS adaptation mechanisms. In contrast to the oxidative metabolism inside *Wolbachia*, the hypoxic metabolism in *Drosophila* leads to the production of end products, such as alanine, lactate, acetate, and propionate, of which alanine is provided to *Wolbachia* as a precursor for several biosynthetic pathways, as is GTP. *Wolbachia* also synthesizes riboflavin, which is provided to the host. Black Arrows: general metabolite flow. Purple and green arrows: metabolite flow to/from host mitochondria and *Wolbachia*, respectively. Blue arrows: Activatory regulation. Red arrows: Inhibitory regulation. Yellow boxes: biochemical modules. Beige ovals: general metabolites. Brown ovals: metabolites involved in provisioning/extraction with *Wolbachia*.

agreement with this interpretation, we observed significantly reduced proline levels in *Wolbachia* infected insects on the standard iron diet. However, this *Wolbachia*-related reduction in proline levels is diet dependent, as significant proline accumulation is promoted upon iron deficiency (Kitajima et al., 2003). Likewise, we observe in our data less pronounced changes in proline levels in low-iron conditions.

Reprogramming of lipid metabolism occurs under HIF-1 and insulin signaling. Upregulation of HIF-1 enhances lipogenesis through modulation of proteins involved in fatty acid uptake, synthesis, storage and usage, and diminishes fatty acid catabolism (Mylonis et al., 2019). Similarly, the disruption of the IIS pathway can also induce lipogenesis via the *daf-16*/FoxO transcription factors (Perez and Van Gilst, 2008). Subsequently, the fatty acids are converted into triacylglycerols and stored in lipid droplets (Mylonis et al., 2019). An increase in localized lipid droplets has indeed been noted as a result of *Wolbachia* infection (Geoghegan et al., 2017). Similarly, we observed increased lipid levels in *Wolbachia*-infected insects compared to the uninfected controls under standard and high-iron diet conditions.

When oxygen is sparse, HIF-1 upregulation leads to inhibition of the pyruvate dehydrogenase complex decreasing the flow of pyruvate into the TCA cycle. In uninfected *Drosophila*, this causes an accumulation of lactate, acetate and alanine as anaerobic end-products (Feala et al., 2007). *Wolbachia* extracts alanine from the host as precursor of several other metabolites, as it cannot synthesize alanine itself (Jiménez et al., 2019), thus redirecting metabolic flux away from lactate and acetate. Consistent with this interpretation, we observed reduced levels of alanine, acetate and lactate in *Wolbachia*-infected individuals under standard-iron conditions. Keeping host cells in a hypoxic state that favors the production of an essential metabolite for *Wolbachia* is a strategy that is advantageous for the bacterium.

Wolbachia is able to synthesize riboflavin and provision it to the host (Jiménez et al., 2019). Riboflavin supplementation significantly contributes to prolonged lifespan, growth and fecundity in both *Cimex lectularius* and *Drosophila melanogaster* (Moriyama et al., 2015; Zou et al., 2017)—effects that are similar to *Wolbachia* infection. In several bacterial species there is evidence for increased riboflavin biosynthesis overcoming iron-restrictive conditions (as reviewed in Sepúlveda Cisternas et al., 2018). The riboflavin biosynthesis pathway starts with GTP, which in turn is formed through the purine biosynthesis pathway from IMP. Thus, increased riboflavin production after *Wolbachia* infection would be associated with a decrease of IMP and GTP levels compared to the uninfected controls. Indeed, during *Wolbachia* infection in iron-deficient conditions we observed a significant reduction of both inosine/IMP and guanosine—supporting the hypothesis of riboflavin provisioning by *Wolbachia*.

Wolbachia infection under low-iron conditions leads to a significant increase of propionate levels with a smaller and correlated increase in acetate levels. This could arise from two potential metabolic pathways. The first possibility is malate dismutation, an ATP generating pathway known in helminths, and nematodes, including *C. elegans* in which the second half of the TCA cycle runs backwards under low-oxygen conditions, leading to the production of both propionate and acetate (Müller et al., 2012; Stairs et al., 2015). Although the malate dismutation pathway has not been actively studied in

either *Drosophila* or *Wolbachia* yet, both organisms possess the majority of enzymes required for this pathway, including some enzymes whose presence only seems to make sense if the whole malate dismutation pathway is present. In agreement with this hypothesis, *Drosophila* are strongly hypoxia and anoxia tolerant (Krishnan et al., 1997; O'Farrell, 2001; Haddad, 2006). Moreover, depressing the IIS pathway in *C. elegans* leads to high propionate levels (Fuchs et al., 2010). Thus, malate dismutation is a possible contender for the observed propionate production. Secondly, *Drosophila* possess a direct metabolic pathway for production of propionate from pyruvate (Kanehisa and Goto, 2000), and thus the production of acetate and propionate could be a simple follow-on from anaerobic glycolysis. In both of these scenarios, the production of propionate and acetate is more likely to be an outcome of *Drosophila* metabolism relying on anaerobic pathways to compensate and accommodate the reduced availability of oxygen induced by *Wolbachia* infection.

It is interesting to note that the metabolic alterations as a consequence of *Wolbachia* infection described here are characteristic of metabolic reprogramming, rather than an immune response. A study by Wong et al. confirms this observation as they noted no measurably increased expression of immune genes in *w*Mel-infected *Drosophila* (Wong et al., 2011). In other words, there is no priming of the immune system by *w*Mel. In addition, we see no evidence for a loss of lipogenesis and glycogenesis in our study, which are metabolic markers for an immune response (Clark et al., 2013; Davoodi et al., 2019). However, we do observe an effect of diet on these pathways irrespective of infection status. Thus, the observed metabolic changes are a result of host-symbiont-environment interactions, rather than an immune response by the host. The above findings also fit with the general hypothesis by Zug and Hammerstein who postulated that strains of *Wolbachia* that have coevolved with their respective hosts do not stimulate an immune response (Zug and Hammerstein, 2015).

Previous studies that have compared host and *Wolbachia* genomes have predicted biochemical and physiological interactions unique to host and symbiont (Min and Benzer, 1997; Foster et al., 2005; Gill et al., 2014; Kosmidis et al., 2014; Newton and Rice, 2020). Indeed, in other host-*Wolbachia* pairs interactions range from facultative parasitism to obligate mutualism (Gill et al., 2014). Nevertheless, iron has been repeatedly shown to be of crucial importance for the interaction between *Wolbachia* and host (Gill et al., 2014), and thus the regulatory pathways implicated here are likely to have broader ramifications for *Wolbachia*-host pairings in general, or potentially even other endosymbiont-host interactions.

In summary, the presence of *Wolbachia* depresses the IIS cascade in *D. melanogaster* whilst inducing the hypoxia signaling pathway. This in turn causes both ROS production and ROS adaptations, as well as other metabolic changes, that steer metabolism away from oxygen-intensive pathways and enable metabolite extraction by the symbiont and metabolite provisioning to the host (**Figure 5**). Finally, this metabolic reprogramming is likely to be driven by *Wolbachia*, and modified by the dietary iron levels.

Earlier work has suggested that *Wolbachia* increases insulin signaling (Ikeya et al., 2009) rather than the downregulation inferred by our own work. However, these results were omitting important wild-type or *Wolbachia*-infected controls when assessing direct activation levels of Akt and FoxO, two of the key proteins in the IIS pathway. Furthermore, the authors' observation of an accumulation of triacylglycerides in the absence of *Wolbachia* is not supported by the experimental observations made by us—that lipid levels increase in the presence of *Wolbachia*. Increased IIS signaling in *Wolbachia*-infected *Drosophila* was also inferred by a recent study (Carneiro Dutra et al., 2020). However, that study used different strains of *Wolbachia* and *Drosophila* than those used by us, and reared flies on a carbohydrate-rich diet. Given the specificity outlined above of the metabolic interactions between strain, host and diet, any differences in results and interpretation are not surprising.

Our study has three limitations. Firstly, *Wolbachia* are known to establish high infection densities in the fat-body and adult brain of *Drosophila* (Albertson et al., 2013)—two tissues that are also known to be interlinked by the IIS pathway, supporting the likely involvement of this pathway in *Wolbachia* infection (Carneiro Dutra et al., 2020). However, because our experimental setup used the entire insect for NMR analysis, we cannot resolve any tissue-specific metabolic changes. Secondly, for the same reason, we can not directly distinguish metabolite changes associated with *Wolbachia* vs. the host. Lastly, we can only infer the regulatory pathways in our model from the metabolic changes observed here. Other proteins or signaling pathways also regulate iron, e.g., transferrin or iron-regulating proteins (IRPs) (Tang and Zhou, 2013; Cronin et al., 2019), but the pathways inferred here are supported by previous experimental observations (Kremer et al., 2009; Gill et al., 2014; Wong et al., 2014; Dutra et al., 2017; Texada et al., 2019; Barretto et al., 2020) and are thus the most probable explanation for our results. Future experiments aimed at direct manipulation of the IIS and hypoxia pathways will be able to confirm their involvement.

4.3. Future Directions

Apart from their direct manipulation as suggested above, the involvement of both the IIS and hypoxia signaling pathways as a consequence of *Wolbachia* infection opens up several exciting avenues for further discovery. Given the strain-host specificity discussed above, it would be interesting to apply the same NMR techniques to explore the metabolomics of different *Wolbachia* strain/host pairings, such as facultative *Wolbachia* strains that infect filarial nematodes, *w*Mel that have been established in novel insect hosts, or *Wolbachia* strains (e.g., *w*Au) that impose no obvious effect on host reproduction (Hoffmann et al., 1996; Cao et al., 2019). Moreover, extending such investigations to pairings of *Wolbachia* and *Aedes aegypti* would be interesting, given the potential of the former in limiting the spread of tropical diseases carried by the latter (McGraw and O'Neill, 2013; O'Neill et al., 2018; Ryan et al., 2019). Furthermore, due to the strong involvement of lipid metabolism, a complementary lipidomics study would allow for greater metabolome coverage in that area of metabolism. If some

of the metabolic effects are indeed localized to certain tissues, then performing tissue-specific metabolomics experiments is an obvious further step. Such experiments could also shed light on which of the metabolic changes are derived from *Wolbachia* metabolism and which are due to insect metabolism, given the preferential localization of *Wolbachia* in specific tissues. In addition, the present analyses are a static snapshot of *Wolbachia* infection, and as such changes in metabolite levels do not directly provide information about changes in metabolic fluxes, as the relationship between metabolic fluxes and metabolite levels is complex. Future fluxomics experiments are well-placed to resolve this point. In this context, it would also be intriguing to further explore the hypothesis that *Wolbachia* produces riboflavin for the *Drosophila* host cells, by designing fluxomics experiments that target specifically the fate of riboflavin. The metabolic interactions between *Wolbachia* and *Drosophila* also lend themselves to exploration via genome-scale modeling using available genome-scale metabolic models for both organisms (Coquin et al., 2008; Jiménez et al., 2019; Schönborn et al., 2019).

5. CONCLUSIONS

Here we have shown that in *D. melanogaster* infected with *Wolbachia wMel*, an endosymbiont with an oxidative metabolism, host metabolism is characterized by a hypoxic response. We have deduced the involvement of both the insulin/insulin-like growth factor and hypoxia signaling pathways in this reprogramming of mitochondrial metabolism. The downstream signaling cascades of both of these pathways support previous studies on *Wolbachia* infection in several insects. Furthermore, we show that the effects of *Wolbachia* infection are highly dependent on dietary iron levels in the host. The burden of iron overload is evident on both infected and uninfected *Drosophila*. In contrast, iron deficiency conditions lead to extra demands of *Wolbachia* on the host. Our results also support the hypothesis of metabolite extraction and provisioning between *Wolbachia* and the host, specifically the consumption of alanine by *Wolbachia* and the export of riboflavin and ATP. Lastly, our analysis has put forward a potential mechanism on how *Wolbachia* maintains infection, as both the metabolic reprogramming of mitochondrial metabolism and metabolic provisioning and extraction enables *Wolbachia* to survive inside the host cells.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The raw NMR data for this study have been deposited in the MetaboLights database (Haug et al., 2020) under accession number MTBLS2090 (http://www.ebi.ac.uk/metabolights/MTBLS2090).

AUTHOR CONTRIBUTIONS

JB and SO'N: experimental design. JB: insects. HS, GP, and JB: sample preparation. HS and GP: NMR spectroscopy. HS and LH: NMR data processing. DC-R, LH, NM, and HS: data analysis. DC-R, HS, and JB: biological interpretation. DC-R, LH, HS, JB, GP, NM, and SO'N: manuscript preparation, review and editing. HS, JB, and SO'N: supervision and funding resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.623561/full#supplementary-material

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