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Diversity and stability of coral endolithic microbial communities at a naturally high pCO_2 reef

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Abstract

The health and functioning of reef-building corals is dependent on a balanced association with prokaryotic and eukaryotic microbes. The coral skeleton harbours numerous endolithic microbes, but their diversity, ecological roles and responses to environmental stress, including ocean acidification (OA), are not well characterized. This study tests whether pH affects the diversity and structure of prokaryotic and eukaryotic algal communities associated with skeletons of *Porites* spp. using targeted amplicon (16S rRNA gene, UPA and *tuf*A) sequencing. We found that the composition of endolithic communities in the massive coral *Porites* spp. inhabiting a naturally high pCO_2 reef (avg. pCO_2 811 μatm) is not significantly different from corals inhabiting reference sites (avg. pCO_2 357 μatm), suggesting that these microbiomes are less disturbed by OA than previously thought. Possible explanations may be that the endolithic microhabitat is highly homeostatic or that the endolithic micro-organisms are well adapted to a wide pH range. Some of the microbial taxa identified include nitrogen-fixing bacteria (*Rhizobiales* and cyanobacteria), algicidal bacteria in the phylum *Bacteroidetes*, symbiotic bacteria in the family *Endozoicomoniaceae*, and endolithic green algae, considered the major microbial agent of reef bioerosion. Additionally, we test whether host species has an effect on the endolithic community structure. We show that the endolithic community of massive *Porites* spp. is substantially different and more diverse than that found in skeletons of the branching species *Seriatopora hystrix* and *Pocillopora damicornis*. This study reveals highly diverse and structured microbial communities in *Porites* spp. skeletons that are possibly resilient to OA.

KEYWORDS
coral skeletons, limestone-boring, microbiome, multimarker metabarcoding, ocean acidification, *Ostreobium*

1 | INTRODUCTION

Ocean acidification (OA) is predicted to threaten the persistence of coral reefs by affecting the balance between constructive forces (calcification and growth of reef builders) and destructive forces (bioerosion and carbonate dissolution) (Tribollet, 2008a; Andersson & Gledhill, 2013). Acidification lowers the saturation state of calcium carbonate ($CaCO_3$), making it more difficult for calcifying organisms, such as stony corals, to build their skeletons (Orr, Fabry, Aumont, Bopp, & Doney, 2005; Hofmann et al., 2010). OA has been shown to slow down calcification and cause structural deformities in juvenile corals (Crook, Cohen, Rebolledo-Vieyra, Hernandez, & Paytan,

2013; Foster, Falter, McCulloch, & Clode, 2016). Some studies, however, indicate that corals are able to regulate the pH at the tissue–skeleton interface, where calcification takes place, mitigating the potential consequences of OA on the calcification process (McCulloch, Falter, Trotter, & Montagna, 2012; Venn et al., 2013; Georgiou, Falter, Trotter, Kline, & Holcomb, 2015). Rates of biological dissolution of $CaCO_3$ (bioerosion) tend to increase under low pH conditions, mostly due to an increase in biomass of the boring organisms living inside coral skeletons (Manzello et al., 2008; Tribollet, Godinot, Atkinson, & Langdon, 2009; Crook et al., 2013; Fang et al., 2013; Reyes-Nivia, Diaz-Pulido, Kline, Guldberg, & Dove, 2013; Enochs, Manzello, Tribollet, Valentino, & Kolodziej, 2016), potentially resulting in a shift from a net reef accretion condition to one of net erosion (Andersson & Gledhill, 2013).

The skeletons of live and dead corals harbour bacteria, fungi, sponges and an abundant population of limestone-boring algae, all having important roles in the reef's $CaCO_3$ budget (Le Campion-Alsumard, Golubic, & Hutchings, 1995; Tribollet, 2008a; Verbruggen & Tribollet, 2011). For example, the green alga *Ostreobium* can be responsible for 70%–90% of carbonate dissolution within dead corals, eroding as much as 1 kg of reef carbonate per m^2 per year (Tribollet, 2008b; Grange, Rybarczyk, & Tribollet, 2015). Green algal biomass in live coral skeletons exceeds *Symbiodinium* biomass in coral tissues by about 16 times (Odum & Odum, 1955), making the limestone attractive to grazers and further increasing bioerosion (Chazottes, Campion-Alsumard, & Peyrot-Clausade, 1995; Clements, German, Piché, Tribollet, & Choat, 2016). However, endolithic algae also protect corals from high light stress (Yamazaki, Nakamura, & Yamasaki, 2008) and provide vital nutrients to corals, potentially extending the time they can survive without *Symbiodinium* during bleaching events (Schlichter, Zscharnack, & Krisch, 1995; Fine & Loya, 2002). Endolithic algae have exceptionally high levels of cryptic diversity (Marcelino & Verbruggen, 2016; Sauvage, Schmidt, Suda, & Fredericq, 2016; Del Campo, Pombert, Slapeta, Larkum, & Keeling, 2017), and although it is known that their biomass increases substantially upon acidification and warming (Tribollet et al., 2009; Reyes-Nivia et al., 2013), it is not known which of the cryptic species increase in relative abundance.

The endolithic community, along with the coral host and its other symbionts, constitutes the coral holobiont (Rohwer, Seguritan, Azam, & Knowlton, 2002). The responses of the coral microbiome, including both prokaryotic and eukaryotic members, to acidification has gained attention as we continue to uncover vital roles played by micro-organisms in holobiont health and resilience (Bourne et al., 2009; Sharp & Ritchie, 2012; Krediet, Ritchie, Paul, & Teplitski, 2013; Blackall, Wilson, & van Oppen, 2015; Bourne, Morrow, & Webster, 2016). Because the ocean pH naturally changes throughout seasons, along depth gradients, with productivity and other biological factors, marine micro-organisms may have the physiological plasticity required to cope with the predicted levels of OA over the next 100 years (Joint, Doney, & Karl, 2011). This notion is supported by several studies showing stable coral prokaryotic community when shifted from ambient to high CO_2 partial pressure (pCO_2) and therefore reduced sea water pH conditions (Meron et al., 2012; Webster, Negri, Botte, Laffy, & Flores, 2016; Zhou, Yuan, Cai, Zhang, & Tian, 2016). However, other studies have demonstrated that a reduced sea water pH can lead to the loss of *Symbiodinium* (coral bleaching) and trigger shifts from a healthy microbiome composition to a microbial community typically associated with diseased corals (Anthony, Kline, Diaz-Pulido, Dove, & Hoegh-Guldberg, 2008; Vega Thurber, Willner-Hall, Rodriguez-Mueller, Desnues, & Edwards, 2009; Meron et al., 2011; Webster, Negri, Flores, Humphrey, & Soo, 2013; Morrow, Bourne, Humphrey, Botte, & Laffy, 2015). These different responses of a coral's microbiome to reduced sea water pH may reflect differences in resilience across coral species to acidification or different experimental setups used in the various studies.

Reefs at the Milne Bay Provence of Papua New Guinea (PNG) are in close proximity to volcanic seeps (expelling ~99% pure CO_2) and constitute a good model system to study the impacts of acidification in situ on the microbial community associated with corals. Both coral species composition and the prokaryotic microbial community associated with coral tissue and mucus differ between high pCO_2 seep sites and nearby reference sites with ambient pCO_2 (Fabricius, Langdon, Uthicke, Humphrey, & Noonan, 2011; Morrow et al., 2015). However, little is known about the coral endolithic communities and how these may change under various sea water pH conditions. Previous studies have screened 16S rRNA gene clone libraries and demonstrated contrasting results, with significant effects of OA community composition within the skeleton in an experimental system (Meron et al., 2011) but no significant changes in corals transplanted to a natural CO_2 seep site (Meron et al., 2012). One limitation with the 16S rRNA gene marker is that it underestimates the diversity of eukaryotic algae (Marcelino & Verbruggen, 2016), and as a consequence, the major microbial agents of bioerosion have been overlooked.

Here, we use high-throughput amplicon sequencing to investigate the effects of OA on the diversity and structure of endolithic microbial communities of corals inhabiting a high pCO_2 site in PNG. Our goals are to (1) test whether the community composition of prokaryotes and photosynthetic eukaryotes (assessed with the 16S rRNA gene, universal plastid amplicon [UPA] and *tuf*A markers) within the skeletons of massive colonies of *Porites* spp. differs between high pCO_2 sites and nearby reference sites where pCO_2 is not affected by the volcanic seeps; (2) compare the endolithic communities of *Porites* spp. with those of two branching coral species (*Seriatopora hystrix* and *Pocillopora damicornis*) to investigate whether the microbiome in coral skeletons varies among host species; and (3) describe the endolithic community diversity found in corals of PNG and discuss the potential functional roles of this microbiome under OA.

2 | METHODS

2.1 | Field sites and sampling

Samples of massive *Porites* spp. (n = 24, six per site and month) were collected in April and November 2014 at two high pCO_2 (seep) and

reference sites within the D'Entrecasteaux Islands, Milne Bay Province, PNG. High pCO_2 samples were collected at Illi Illi (Upa-U) Seep (09.82425S, 150.81789E) and Dobu Seep (09.73646S, 150.86894E), and at nearby reference sites (ambient pCO_2) not exposed to elevated pCO_2 conditions (Illi Illi Reference, 09.82806S, 150.82028E and Dobu Reference, 09.75211S, 150.85410E) (Fabricius et al., 2011; Uthicke, Momigliano, & Fabricius, 2013). High pCO_2 and reference sites were ~500 m and ~3 km apart from one another at Illi Illi and Dobu, respectively. Samples of the branching corals *Seriatopora hystrix* (n = 3 at each site) and *Pocillopora damicornis* (n = 3 at each site) were only collected in April 2014 at Illi Illi seep and reference sites (same as above). Sea water carbonate chemistry varies in response to bubble activity and water motion at the seep sites; thus, at Illi Illi seep, corals experience a pH range (defined here as the 5th and 95th percentiles) of 7.28–8.01 (avg. pCO_2 624 μatm) and at Dobu seep a pH range of 7.08–7.99 (avg. pCO_2 998 μatm). At Illi Illi reference site the pH ranges from 7.91 to 8.09 (avg. pCO_2 346 μatm) and at Dobu reference site the pH ranges from 7.91 to 8.10 (avg. pCO_2 368 μatm) (Fabricius, De'ath, Noonan, & Uthicke, 2014), which is within the range of future predictions for the year 2100 (Moss, Edmonds, Hibbard, Manning, & Rose, 2010).

Coral fragments were collected using bone cutters or a hammer and chisel and placed into individual sections within a plastic tackle box, which allowed for water flow while underwater. After returning to the boat, samples were immediately placed into flowing sea water sourced directly from the collection site. Large pieces of *Porites* spp. were chipped into smaller fragments, rinsed thoroughly with sterile 0.02 μm filtered sea water and placed in 50 ml Falcon tubes with RNAlater (Ambion). Samples were kept in a cooler with ice until returned to the laboratories at the Australian Institute of Marine Science (AIMS) where they were processed.

Fragments were removed from RNAlater and soaked in 0.2 μm filtered calcium and magnesium-free sea water for ~10 min at room temperature (CMFSW; 0.45 M NaCl, 10 mM KCl, 7 mM Na_2SO_4, 0.5 mM $NaHCO_3$ and milli-Q water; (Esteves, Amer, Nguyen, & Thomas, 2016)) to aid in the initial removal of tissues from the skeleton. Tissues were removed into the CMFSW using an air gun fitted with a sterile tip. Skeletons with tissues removed were placed back into the original RNAlater collection buffer and stored at −80°C until shipment to the University of Melbourne where DNA was isolated from the endolithic community.

2.2 | DNA isolation, library preparation and sequencing

Total DNA was isolated from coral skeletons using the Wizard Genomic DNA Purification Kit (Promega). The manufacturer's protocol for plant DNA was followed, with the exception of an extended 3 hr incubation step with the first extraction buffer to allow the DNA to leak out from the limestone into the solution. Amplified DNA products for library preparation were obtained with a two-step process described by Marcelino and Verbruggen (2016). During the first PCR step, three metabarcoding markers were amplified: the 16S rRNA gene (Klindworth et al., 2013); the UPA, which is a fragment of the 23S rRNA gene (Presting, 2006; Sherwood & Presting, 2007) and the elongation factor Tu (*tuf*A), which targets green algae (Ulvophyceae) (Fama, Wysor, Kooistra, & Zuccarello, 2002; Marcelino & Verbruggen, 2016). During the second PCR step, barcodes and Illumina adapters were attached to both 3′ and 5′ ends of the amplicons. One negative control was performed with each amplification (six in total, one per marker and per amplification step) and sequenced with the library, even though no DNA was detected in any negative control during quantification. Two mock "blank" extractions were also performed along with the samples DNA isolation, processed through the amplification process and sequenced to further control for possible contamination. DNA isolation and PCR preparation were carried out inside a dedicated dead-air box (PCR workstation) sterilized with UV light for 15 min prior to each use. Libraries were quantified using the Quant-It PicoGreen reagent (Invitrogen) and pooled with other samples of another project. The library was sequenced using the Illumina MiSeq platform (2 × 300 bp paired end reads) at the Centre for Translational Pathology, University of Melbourne. Further details about the primers and library preparation are provided in Appendix S1.

2.3 | Data processing

The MiSeq run yielded one file containing all amplicons per sample, which were demultiplexed based on the primer sequences. The 3′ ends of reads were trimmed to improve consensus quality; forward and reverse reads were merged using FLASH (Magoc & Salzberg, 2011), and sequences having average quality scores smaller than 35 or lengths shorter than a threshold (350 bp for 16S rRNA gene, 320 bp for UPA and 400 bp for the *tuf*A) were filtered out using PRINSEQ (Schmieder & Edwards, 2011). To verify that the data lost during quality control did not affect the results, we also performed the analyses with a less stringent quality filtering (Appendix S1). Sequences were clustered into operational taxonomic units (OTUs) using UPARSE (Edgar, 2013). A similarity threshold of 98% was set for the *tuf*A marker, a threshold near species level for this marker (Sauvage et al., 2016). For the other markers, the default threshold of 97% was used. The 16S rRNA gene OTUs were aligned with PYNAST (Caporaso, Biitinger et al., 2010) while the UPA and *tuf*A data sets were aligned with MAFFT (Katoh, Misawa, Kumar, & Miyata, 2002). A taxonomy affinity was assigned to the OTUs using the Naïve Bayesian Classifier (RDP) implemented in QIIME v.1.9.1 (Wang, Garrity, Tiedje, & Cole, 2007; Caporaso, Kuczynski, Stombaugh, Bittinger, & Bushman, 2010). The GREENGENES v.13.8 data set (DeSantis, Hugenholtz, Larsen, Rojas, & Brodie, 2006) was used to classify the 16S rRNA gene sequences, and custom-made reference data sets (described and available in Marcelino & Verbruggen, 2017) were used for *tuf*A and UPA. The resulting OTU table went through a filtering process to remove OTUs found in the negative controls (3 OTUs in the 16S rRNA gene, 5 OTUs in the UPA and 2 OTUs in the *tuf*A data sets) and rare OTUs (i.e., OTUs with less than five reads across all samples and OTUs from samples where they are present with

two or less reads). OTUs were also filtered based on their taxonomic classification to focus on the taxonomic groups that each marker best characterizes: chloroplast sequences were excluded from the 16S rRNA gene data set, and bacterial sequences were excluded from the *tuf*A data set. Further details about the data processing pipeline are provided in the Appendix S1.

2.4 | Statistical analysis

There were no significant differences related to the time of collection (Appendix S1); therefore, all *Porites* spp. samples ($n = 24$) were used to investigate the effects of pCO_2 in endolithic communities associated with this coral genus. Rarefaction curves of the number of observed OTUs per number of reads were constructed by randomly subsampling the reads in QIIME, allowing to set a threshold for each marker where the curve reaches saturation (i.e., a plateau in the rarefaction curve), which was 2,200 reads in the 16S rRNA gene, 1,400 in the *tuf*A and 7,000 in the UPA data set (Fig. S1). Samples with sequencing depths lower than these thresholds were excluded, resulting in 20 samples in the 16S rRNA gene, and 22 samples in the UPA and *tuf*A data sets (Table S1). Alpha diversity indices (Chao1 and observed OTUs) were calculated using QIIME (Caporaso, Kuczynski, et al., 2010). The relative abundance of individual OTUs and taxonomic groups (i.e., OTUs grouped at phylum level for bacteria and genus level for algae) between sites was tested for significant differences with a Kruskal–Wallis test (for OTUs) and ANOVA (for taxon groups) using QIIME (Caporaso, Kuczynski, et al., 2010).

To further investigate the distribution of green algal lineages, a maximum-likelihood phylogeny was built with the green algal *tuf*A OTUs together with reference sequences (from GenBank) using a GTR+gamma model of sequence evolution in RAXML v.8.2.6 (Stamatakis, 2006). OTUs present in less than three samples were excluded, and their relative abundances were normalized with cumulative sum scaling (Paulson, Stine, Bravo, & Pop, 2013) and visualized alongside a phylogenetic tree using the R package phytools (Revell, 2012).

Potential differences in community composition between high pCO_2 and reference sites (beta-diversity) were investigated with a combination of statistical methods. Principal coordinate analysis (PCoA) on weighted UniFrac distance matrices was performed using QIIME (Caporaso, Kuczynski, et al., 2010; Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011) and the results visualized using the GGPLOT2 package in R (Wickham, 2009). A multivariate generalized linear model (MGLM) was used to investigate potential differences in community composition between high pCO_2 and reference sites. The MGLM was computed using the MVABUND R package (Wang, Naumann, Wright, & Warton, 2012), considering a negative binomial distribution. The null hypothesis of no difference among sites was statistically tested with analysis of deviance using 999 bootstrap interactions (R scripts provided in Appendix S2). To verify that the results are not a consequence of PCR bias, PCoA and MGLM analyses were also performed with a distance matrix based on Sørensen similarity, which is a presence/absence index (Appendix S1).

The number of samples of the branching species (*S. hystrix* and *P. damicornis*) did not allow statistical analyses to test for differences between high pCO_2 and reference sites, although it did permit a comparison of the endolithic communities associated with the different coral hosts (Table S1). To investigate the community structure related to host species, a rarefaction threshold of 707 reads for the 16S rRNA gene, 713 for the *tuf*A and 3257 for the UPA marker was used, allowing the inclusion of a larger number of samples in the analysis. Samples with sequencing depths lower than these thresholds were excluded, resulting in 35 samples in the 16S rRNA gene and UPA data sets and 27 in the *tuf*A data sets (Table S1). Alpha diversity, Kruskal–Wallis test (for OTUs), ANOVA (for taxon groups), PCoA and MGLM were performed on this data set as previously described, but here, samples from different pCO_2 conditions from conspecific host species were combined to investigate the community structure purely associated with coral host species.

3 | RESULTS

3.1 | Effects of pCO_2 conditions on the *Porites* spp. endolithic microbiome

A total of 6,584,274 sequence reads were recovered for the samples analysed here, 4,405,336 belonging to *Porites* spp. samples (ca. 25% of these reads belonged to a marker that is not analysed in this manuscript). After stringent pre- and post-OTU-clustering and filtering, a total of 119,367 (16S rRNA gene), 109,948 (*tuf*A) and 393,816 (UPA) reads remained in the analysed *Porites* spp. data set. A less stringent quality filtering which resulted in inclusion of more sequence reads did not alter the community patterns (see Appendix S1). The alpha diversity statistics, including Chao1 and observed OTUs, indicated that the species richness of the endolithic communities associated with *Porites* spp. was not significantly different between high pCO_2 and reference sites (Table 1). Although the relative abundance of some microbial taxa differed between high pCO_2 and reference sites (Figure 1, see also Fig. S2 for a sample-based representation), the differences were not statistically significant (Bonferroni-corrected p-values = 1), neither at the OTU level (Table S2) nor at higher taxonomic levels (Table S3). Accordingly, PCoA and MGLM did not reveal any significant pattern between sites with all three markers (Figure 2).

We further investigated whether any of the different phylogenetic lineages in the endolithic algal communities differed in abundance at high pCO_2 and reference sites. A phylogenetic heatmap of relative abundances (Fig. S3) indicated that phylogenetic relatedness among green algae is not correlated with different abundances in high pCO_2 or reference sites. Notably, three algal OTUs were present in either high pCO_2 or reference sites, but not in both (Fig. S3).

3.2 | Taxonomic profiling of the *Porites* spp. endolithic community

The microbial community observed in the skeletons of *Porites* spp. was highly diverse and variable among samples within pCO_2

TABLE 1 Diversity indices based on the microbiome of *Porites* spp. skeletons from reference and high pCO_2 sites and standard deviations (±)

				Chao1			Obs. OTUS		
	N	Seqs	OTUs	Reference	high pCO_2	*p*-Value	Reference	high pCO_2	*p*-Value
16S	20	2,200	890	141.2 ± 52.4	148.5 ± 45.8	.79	133.6 ± 49.9	140.7 ± 44.8	.77
*tuf*A	22	1,400	59	7.8 ± 3.3	7.4 ± 2.8	.78	7.7 ± 3.2	7.3 ± 2.8	.74
UPA	22	7,000	164	21.6 ± 7.1	24.4 ± 5.4	.33	20.6 ± 6.7	23.2 ± 5.1	.33

Chao1 and number of observed operational taxonomic units (OTUs) between high pCO_2 and reference sites were compared with a two-sample *t* test, *p*-values ≤ .05 suggest significant differences between sites, but were not observed.
N, number of samples after rarefaction; Seqs, rarefaction threshold; OTUs, number of OTUs retrieved in each dataset after quality filtering.

conditions. Prokaryotic members of the microbiome (observed in the 16S rRNA gene data set) accounted for most of the species diversity (Table 1), and no bacterial OTUs were present in all *Porites* spp. samples. The most abundant phylum recovered was *Proteobacteria*, followed by *Bacteroidetes* and Archaea (Figure 1a, Fig. S2). The relative abundance of the nitrogen-fixing order *Rhizobiales* (Alphaproteobacteria) in all *Porites* spp. samples was 9.1% ± 4% standard deviation (hereafter ± only), and the phylum of green sulphur bacteria, *Chlorobi*, was 0.4% ± 1%. Members of the *Bacteroidetes* were twice as abundant at high pCO_2 sites (12.1% ± 10% in reference vs. 23.9% ± 17% in high pCO_2 sites), mostly due to a higher abundance within the classes *Cytophagia* (4.8% ± 7% vs. 10.4% ± 9%), *Flavobacteria* (2.2% ± 2% vs. 3.7% ± 3%) and *Saprospirae* (2.4% ± 5% vs. 9.4% ± 15%). We also observed a lower abundance of the Archaeal class *Parvarchaea* in the high pCO_2 site (1.6% ± 0.9% vs. 11.6% ± 12% in high pCO_2 and reference sites, respectively). A representation of the relative abundances of bacterial phyla on a sample-by-sample basis can be found in Fig. S2. These differences were, however, not statistically significant based on ANOVA and Kruskal–Wallis tests (Bonferroni-corrected *p*-values = 1, Tables S2 and S3).

The *tuf*A data set (Figure 1b) suggested that the algal community was dominated (64.7% ± 36%) by lineages of the Ostreobiaceae (Chlorophyta, Bryopsidales). Although no *tuf*A OTUs were found to be omnipresent in *Porites* spp., siphonous green algae (order Bryopsidales) were present in all samples analysed here, indicating that they are ubiquitous members of the coral skeleton core microbiome. *Ostreobium* clade #1 showed the highest relative abundance (33% ± 40%), followed by *Ostreobium* clades #4, #3 and #2. While the relative abundance of clade #1 was similar between sites, the relative abundance of the other *Ostreobium* clades varied substantially, but not statistically significantly, between reference and high pCO_2 sites. A high abundance of an unclassified group of OTUs belonging to the green algal order Bryopsidales was also observed, particularly in the reference site (Figure 1b).

The prevalence of green algal lineages in the skeletons of *Porites* spp. was also suggested by the UPA data set (Figure 1c), which shows that 86.8% ± 15% of the reads belong to the green algal order Bryopsidales. There are no UPA reference sequences for *Ostreobium* clades #1 and #2; therefore, possible sequences of these clades might have been classified as clades #3, #4 or "unclassified Bryopsidales" by the RDP classifier, which may explain the differences in the abundances of *Ostreobium* clades between the *tuf*A and UPA data sets. One UPA OTU was found across all *Porites* spp. samples (OTU_3). This OTU was only classified at the kingdom level as a eukaryote (Table S2) and showed little similarity to known red algae and Stramenopiles species (BLASTN *e*-values ≤ 4e-79 but Identity ≤79%). Differences in the relative abundances between reference and high pCO_2 sites were minimal (Figure 1c, Fig. S2).

3.3 | Endolithic communities across different coral host species

The prokaryotic endolithic communities of *Seriatopora hystrix* and *Pocillopora damicornis* were significantly less rich than those found in *Porites* spp., as indicated by Chao1 and observed OTUs indices (*p*-value = .003, Table 2). A significant difference was detected in the relative abundances of certain OTUs belonging to the *Endozoicimonaceae* family between coral species, with the highest abundance in *P. damicornis* (Kruskal–Wallis, *p*-values < .0002, Table S4). The *Porites* spp. samples had a higher relative abundance of an OTU related to the order *Rhizobiales* (genus *Afifella*), and *P. damicornis* showed a significantly (*p*-value = .03) higher abundance of an OTU related to the phylum *Bacteroidetes* (order *Cytophagales*; Table S4). At higher taxonomic levels, the relative abundance of the phyla *Planctomycetes*, *Bacteroidetes* and the bacterial phylum OD1 was significantly different among coral hosts (ANOVA, *p*-values = .007 and .01 respectively, Figure 3a, Fig. S2, Table S5). Principal coordinate analysis showed evident differences in the prokaryotic microbiome associated with different coral hosts: *Porites* spp. samples clustered together, clearly separated from the two branching species (Figure 4a). The MGLM analysis confirmed a significant difference between the prokaryotic communities of different coral hosts (Figure 4a).

The alpha diversity of green algae (i.e., Chao 1 and observed OTUs within the *tuf*A data set) was significantly different between *S. hystrix* and *Porites* spp., but no significant differences were observed within the taxonomically broader spectrum of eukaryotic algae amplified with the UPA marker (Table 2). The Kruskal–Wallis test suggested no significant difference in the relative abundances of particular OTUs between host species (neither within the *tuf*A nor within the UPA data set), at least when corrected (Bonferroni) *p*-values were taken into consideration (Table S4). After rarefying

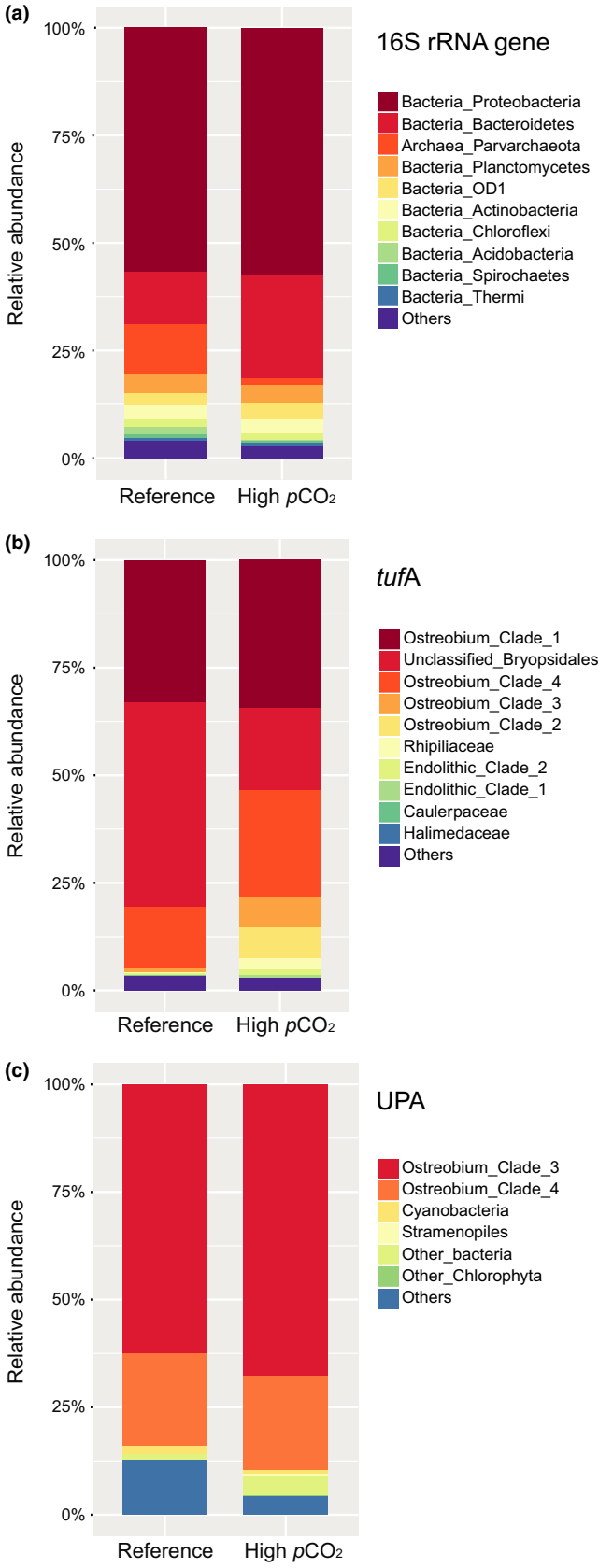

FIGURE 1 Relative abundances of the most common microbial taxa in coral skeletons of *Porites* spp. from high pCO_2 and reference sites. Results were based on all samples from each site, averaging the relative abundances at the taxonomic level displayed in the legend; (a) biodiversity survey targeting prokaryotes based on the 16S rRNA gene; (b) survey of the eukaryotic green algal members of the microbiome based on the *tuf*A marker; (c) biodiversity survey using the universal plastid amplicon [Color figure can be viewed at wileyonlinelibrary.com]

the sequences, the *tuf*A data set was reduced to a single *P. damicornis* sample (Table S1); therefore, we could not test for differences in alpha diversity or relative abundances in the *P. damicornis* community compared to other host corals. We observed a significantly different relative abundance of *Ostreobium* spp. (order Bryopsidales) among coral hosts within the UPA data set (ANOVA, *p*-value = .000002), but not in the *tuf*A data set (*p*-values > .05, Figures 3b,c, Fig. S2, Table S5). *Seriatopora hystrix* had a high and variable (83% ± 57%) relative abundance of endolithic lineages related to the macroalga *Halimeda* spp. within the *tuf*A data set, while this group constituted a minimal fraction (0.04% ± 0.2%) of the endolithic community of *Porites* spp. (Figure 3b). No pattern was observed within the PCoA plot of the community composition using the *tuf*A marker (Figure 4b). The UPA marker, which comprised more samples of the branching species, showed that *Porites* spp. samples cluster together and away from *S. hystrix* and *P. damicornis*, although two outliers of branching samples cluster with *Porites* spp. (Figure 4c). The MGLM analysis suggested no significant differences between the algal communities of different coral hosts (Figure 4b,c), except when a presence–absence distance matrix was used (Appendix S1, Fig. S4).

4 | DISCUSSION

Our results show that the prokaryotic and eukaryotic members of the microbiome in the skeletons of *Porites* spp. are highly diverse but indistinguishable between corals inhabiting naturally high pCO_2 reefs and ambient conditions. Ocean acidification is predicted to affect the coral reef $CaCO_3$ budget and its biological associations (Meron et al., 2011; Andersson & Gledhill, 2013; Morrow et al., 2015), and depending on the experiment, endolithic communities were shown to either exacerbate or buffer the effects of these environmental changes (Fine & Loya, 2002; Tribollet et al., 2009; Reyes-Nivia et al., 2013). Our results suggest that the composition of endolithic communities, at least in *Porites* spp., is virtually unaffected by the surrounding high pCO_2 water from a natural volcanic seep and therefore less likely to be disturbed by OA than we previously thought. Although homogeneous between high pCO_2 and reference sites, we show that the endolithic community is highly diverse and structured among coral host species.

4.1 | A stable microbiome

The mechanisms influencing the structure of the endolithic microbiome (regardless of variable pCO_2 conditions) are currently unknown. We

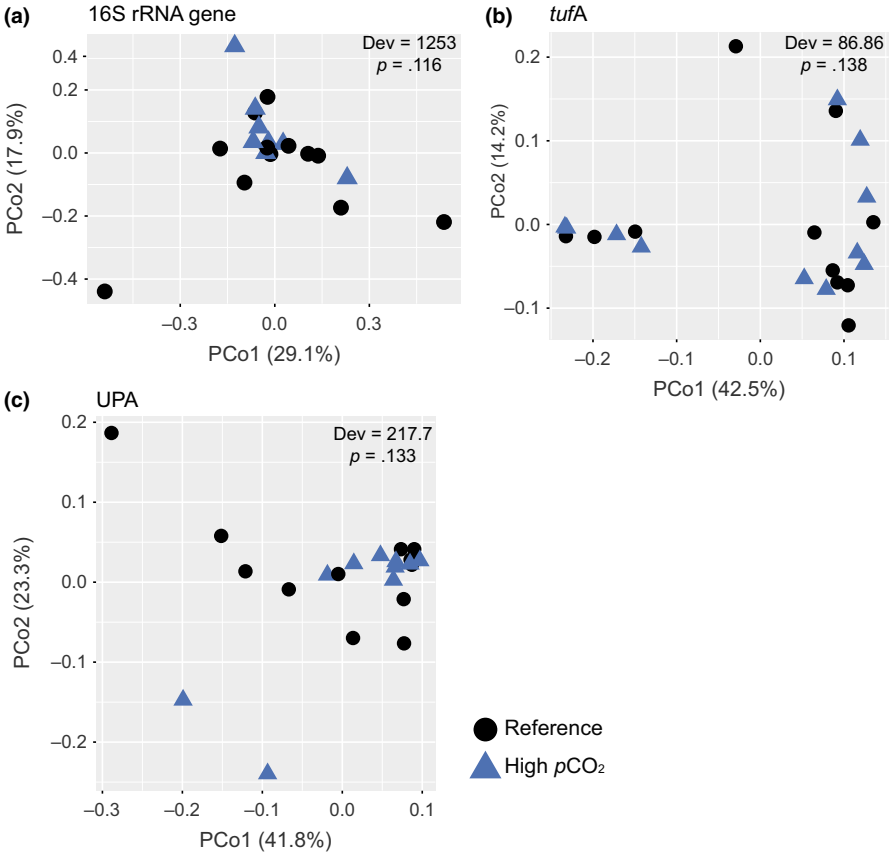

FIGURE 2 Principal coordinate analysis of microbial communities present in limestone skeletons of *Porites* spp. from high pCO_2 and reference sites. The analyses were based on weighted UniFrac distance matrices calculated with operational taxonomic units (OTU)-level abundances for each metabarcoding marker: (a) prokaryotic 16S rRNA gene marker; (b) eukaryotic green algae *tuf*A marker; (c) universal plastid amplicon marker. The results of the MGLM analysis (Deviance and *p*-value) are shown [Color figure can be viewed at wileyonlinelibrary.com]

raise here two hypotheses that may explain our results. The "stable habitat" hypothesis assumes that the endolithic environment is highly homeostatic so that pH is maintained inside the skeletons regardless of external changes in the surrounding water. The "tolerant endolith" hypothesis is based on the notion that endolithic micro-organisms have a wide pH tolerance range, potentially wider than the microbes associated with the tissues and mucus.

The first hypothesis is supported by the ability of some corals to upregulate the pH at the tissue–skeleton interface, which allows them to calcify and grow even under high pCO_2 (McCulloch et al., 2012; Venn et al., 2013; Georgiou et al., 2015). The pH within coral cells remains relatively constant throughout the day (7.05–7.46 units), likely due to membrane transporters that extrude the excess of by-products of photosynthesis and respiration to maintain a stable intracellular pH (Laurent, Tambutte, Tambutte, Allemand, & Venn, 2013). This process may indirectly create a stable microhabitat within the coral skeleton that is protected from shifting pH in the surrounding sea water (see also Shashar, Banaszak, Lesser, Amrami, & Gan, 1997). The observation that radioactivity in sea water impacted corals' living tissue but did not reach their endolithic zone (Odum & Odum, 1955) supports this notion.

One problem with the stable habitat hypothesis is that the pH in the skeletons of *Porites* (*compressa*) can vary daily from 7.7 to 8.5 pH units, mostly due to the by-products of respiration and photosynthesis of the coral and *Symbiodinium* that are exported to the skeleton (Shashar & Stambler, 1992). This daily variation is well above the projections of OA for the near future, which predicts a pH drop of 0.4 units by 2,100, and up to 0.7 units by 2,300 (Raven et al., 2005; Hoegh-Guldberg, Mumby, Hooten, Steneck, & Greenfield, 2007). A reasonable counterargument is that the direction of the movement (pH reduction due to OA) may be more important than the daily variation within skeletons—for example, micro-organisms living under 7.7–8.5 pH units not necessarily withstand a sea water pH shift from 7.8 to 7.6 pH units. The resilience of at least some microbes might also be related to their boring mechanism, which involves a sophisticated control of intracellular pH (associated with calcium pumps and protons counter-transport) in endolithic cyanobacteria (Garcia-Pichel, 2006; Garcia-Pichel, Ramirez-Reinat, & Gao, 2010). Therefore, our second hypothesis that organisms exposed to daily pH fluctuations within the skeleton are adapted to cope with a wide range of pCO_2 conditions may be more accurate. Experimental work and genomic data of endolithic organisms will help to test the tolerant endolith and the stable habitat hypotheses. For example, specialization to the low light experienced in the endolithic habitat has been observed in the plastid genome of *Ostreobium quekettii* (Marcelino, Cremen, Jackson, Larkum, & Verbruggen, 2016) and the presumed pH tolerance may also be reflected in the genomes of endolithic organisms.

The lack of discernible differences in the endolithic community composition between high pCO_2 and reference corals observed here is in agreement with the results of an experiment where *Balanophyllia europaea* and *Cladocora caespitose* corals were transplanted to a naturally high pCO_2 area (Meron et al., 2012). In an aquarium-based experiment conducted over a shorter time, the bacterial community composition present in the tissue, skeleton and mucus of *Acropora eurystoma* were found to be affected by high pCO_2, but further

TABLE 2 Comparison of endolithic community diversity indices between *Porites* spp., *Seriatopora hystrix* and *Pocillopora damicornis* corals

Group 1	Group 2	Group 1 mean	Group 1 std	Group 2 mean	Group 2 std	*t* stat	*p*-value
16S rRNA gene Chao1							
S. hystrix	*P. damicornis*	29.087	29.494	21.514	15.705	0.507	1.000
S. hystrix	*Porites* spp.	29.087	29.494	125.296	49.889	−4.363	.003*
P. damicornis	*Porites* spp.	21.514	15.705	125.296	49.889	−4.854	.003*
16S rRNA gene observed OTUs							
S. hystrix	*P. damicornis*	21.767	19.758	19.083	12.331	0.258	1.000
S. hystrix	*Porites* spp.	21.767	19.758	101.274	37.282	−4.865	.003*
P. damicornis	*Porites* spp.	19.083	12.331	101.274	37.282	−5.138	.003*
*tuf*A Chao1							
S. hystrix	*P. damicornis*	2.033	0.858	3.450	0.000	1.168	1.000
S. hystrix	*Porites* spp.	2.033	0.858	9.606	3.849	−3.264	.006*
P. damicornis[a]	*Porites* spp.	3.450	0.000	9.606	3.849	-	-
*tuf*A observed OTUs							
S. hystrix	*P. damicornis*	1.933	0.736	3.000	0.000	1.024	1.000
S. hystrix	*Porites* spp.	1.933	0.736	9.330	3.623	−3.388	.003*
P. damicornis[a]	*Porites* spp.	3.000	0.000	9.330	3.623	-	-
UPA Chao 1							
S. hystrix	*P. damicornis*	19.353	14.158	13.386	16.038	0.624	1.000
S. hystrix	*Porites* spp.	19.353	14.158	21.732	6.187	−0.591	1.000
P. damicornis	*Porites* spp.	13.386	16.038	21.732	6.187	−1.921	.162
UPA observed OTUs							
S. hystrix	*P. damicornis*	18.100	12.882	12.567	14.816	0.630	1.000
S. hystrix	*Porites* spp.	18.100	12.882	19.539	5.572	−0.394	1.000
P. damicornis	*Porites* spp.	12.567	14.816	19.539	5.572	−1.754	.327

Chao1 and number of observed OTUs were compared with a two-sample *t* test, *p*-values ≤ .05 indicate significant differences between host species and are marked with an asterisk.

Std, Standard deviation.

[a]The *tuf*A data set has only one *P. damicornis* sample (after rarefaction); therefore, the significance cannot be calculated. See Table S1 for number of samples.

analysis using clone libraries suggested that only the prokaryotic communities of the mucus and tissue, not the skeleton, were affected by low pH (Meron et al., 2011). These different observations might be associated with the different time spans and experimental setups of the two studies, and it is likely that the microbial community associated with different coral taxa has different responses to acidification. The resilience of endolithic algae to acidification has also been observed: the net photosynthesis and respiration of algae growing at the surface of dead coral blocks was severely impacted upon exposure to high pCO_2 treatments, while the endolithic flora was unaffected (Tribollet, Atkinson, & Langdon, 2006). Studies have demonstrated that endolithic algae actually benefit from low pH and tend to increase in biomass under high pCO_2 conditions (Tribollet et al., 2009; Reyes-Nivia et al., 2013; Enochs et al., 2016).

The observation that high pCO_2 did not impact the endolithic community of *Porites* spp. does not necessarily imply that coral holobionts are immune to OA. It is possible that the methods used here are not sufficiently powerful to detect the effects of high pCO_2 on endolithic microbial communities. However, the fact that we detected significant differences among coral hosts even though the sampling size for the branching corals was smaller, indicates that our methods and sampling design are adequate and it is unlikely that differences among high pCO_2 and reference sites were present but went undetected. It is possible though that high pCO_2 impacts the endolithic communities of other coral species that were not examined. It is interesting to note that massive *Porites* spp. dominate the reef near volcanic seeps while the presence of branching species (e.g., *Acropora* spp.) was largely reduced (Fabricius et al., 2011). Our analyses are restricted to the volcanic seeps of Milne Bay, which have relatively small areas under high pCO_2 and are surrounded by ambient sea water. Further studies at additional sites impacted by high pCO_2 and across a wider range of coral species are required to evaluate the results in our study across the broader ecological context of effects of OA on coral microbiomes.

4.2 | Diversity and potential functional roles of the endolithic microbiome

Bacteria related to *Endozoicomonas* spp. (class Gammaproteobacteria) are predicted to have a key role in the coral holobiont. These

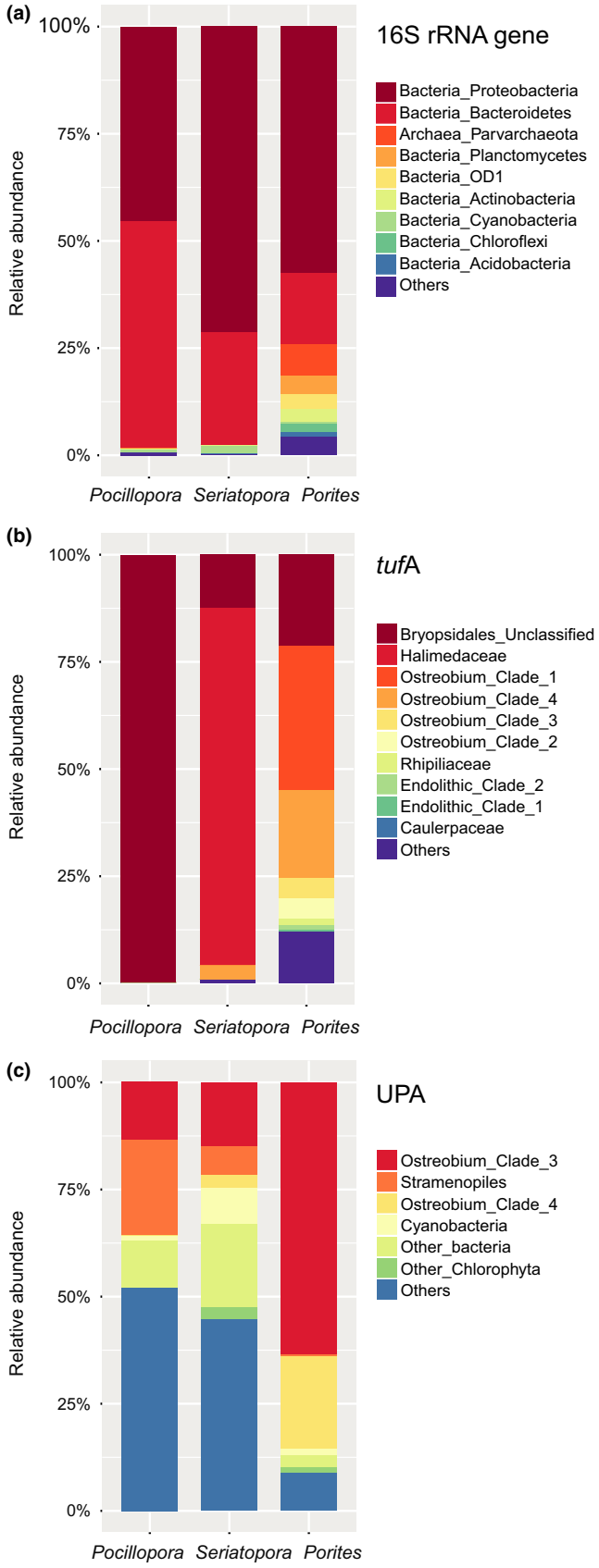

FIGURE 3 Relative abundances of the most common microbial taxa in coral skeletons of *Pocillopora damicornis*, *Seriatopora hystrix* and *Porites* spp. Results were based on all samples from each coral species, averaging the relative abundances at the taxonomic level displayed in the legend. (a) Biodiversity survey targeting prokaryotes based on the 16S rRNA gene; (b) survey of the eukaryotic green algal members of the microbiome based on the *tuf*A marker; (c) biodiversity survey using the universal plastid amplicon [Color figure can be viewed at wileyonlinelibrary.com]

bacteria have been shown to be endosymbionts, forming aggregations within coral tissues (Neave et al., 2017), potentially contributing to nutrient cycling and structuring of the microbiome through the production of quorum-sensing signalling metabolites and antimicrobial compounds (Meyer, Paul, & Teplitski, 2014; Morrow et al., 2015 and references therein). The relative abundance of *Endozoicomoniaceae* within coral tissues appears to be sensitive to high pCO_2 (Morrow et al., 2015; Webster et al., 2016), but in the skeletons of *Porites* spp. analysed here, they did not differ significantly between samples from different pCO_2 conditions (Table S2). We observed a significantly higher relative abundance of two *Endozoicomoniaceae* OTUs in the skeletons of *Pocillopora damicornis* when compared to the other two coral species, possibly reflecting stable associations of *Endozoicomoniaceae* species with this coral host (see Neave et al., 2017). Although some of the sequences retrieved here may derive from other parts of the coral, other studies have also detected members of *Endozoicomonaceae* in the endolithic community (Williams, Brown, Putchim, & Sweet, 2015; Marcelino & Verbruggen, 2016; see also Ainsworth, Krause, Bridge, Torda, & Raina, 2015).

Bacteria in the phylum *Bacteroidetes* are often associated with coral disease and have been shown to increase in relative abundance under reduced pH (Vega Thurber et al., 2009). The average relative abundance of this group doubled in endolithic communities from high pCO_2 sites, but this difference was not significant likely due to the high level of variation in community composition among colonies within sites. This increase was mostly due to a higher abundance of the classes *Saprospirae*, *Flavobacteria* and *Cytophagia*, which contain most of the known marine algicide bacteria (Furusawa, Yoshikawa, Yasuda, & Sakata, 2003; Mayali & Azam, 2004; Zozaya-Valdes, Egan, & Thomas, 2015). It is plausible that a higher relative abundance of these micro-organisms is associated with an increase in endolithic algal biomass under high pCO_2 (see Reyes-Nivia et al., 2013; Johnson, Comeau, Lantz, & Smith, 2017). Rather than compromising coral health, these bacteria may control excessive endolithic algal growth and may help to maintain a stable community composition under OA.

Micro-organisms involved in nitrogen cycling may be fundamental to coral resilience to OA and climate change (Rädecker, Meyer, Bednarz, Cardini, & Wild, 2014; Santos, Carmo, Duarte, Dini-Andreote, & Castro, 2014; Radecker, Pogoreutz, Voolstra, Wiedenmann, & Wild, 2015). We found a diverse community of nitrogen-fixing (diazotrophic) micro-organisms inhabiting coral skeletons. The majority (in terms of relative abundances) belonged to the order *Rhizobiales*, a group that appears to form stable symbiotic associations with corals (Lema, Willis, & Bourne, 2014). Green sulphur (also diazotrophic) bacteria in the phylum *Chlorobi*, previously documented as prevalent members of the endolithic community in the coral *Isopora* (Yang, Lee, Huang, Tseng, & Chiang, 2016), were found at low

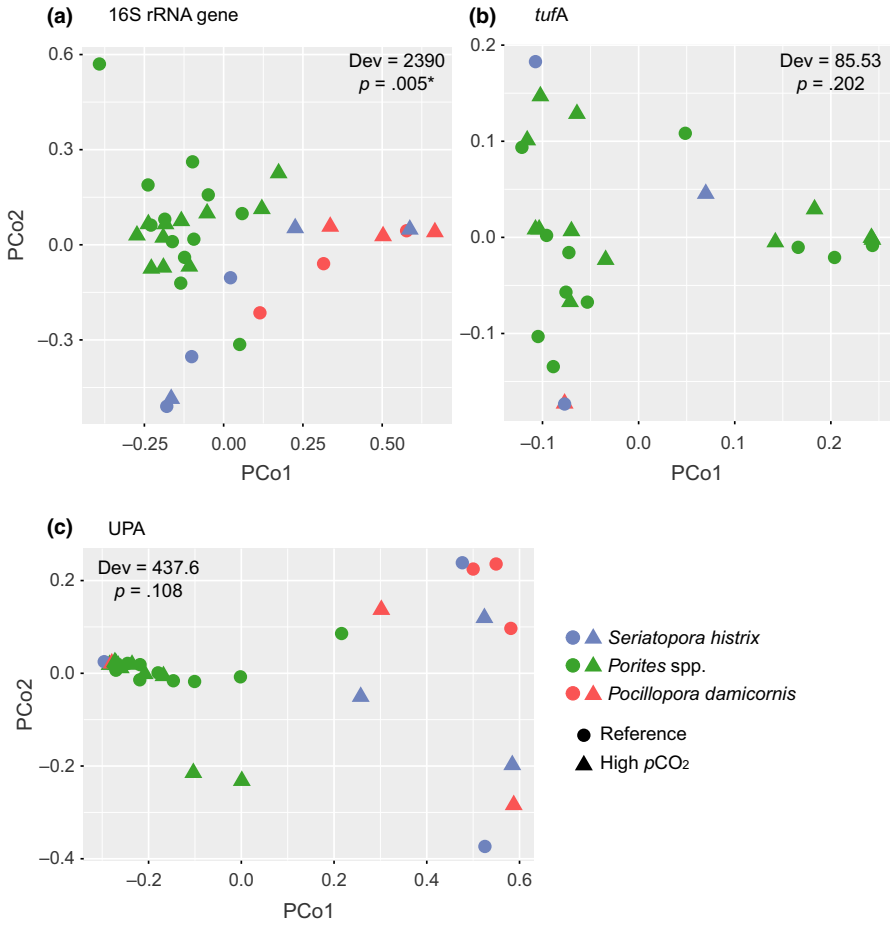

FIGURE 4 Principal coordinate analysis of microbial communities present in limestone skeletons of three coral host species collected in high pCO_2 and reference sites. The analyses were based on weighted UniFrac distance matrices calculated with operational taxonomic units (OTU)-level abundances for each metabarcoding marker: (a) prokaryotic 16S rRNA gene marker; (b) eukaryotic green algae *tuf*A marker; (c) universal plastid amplicon marker. The results of the MGLM analysis (Deviance and *p*-value) are shown [Colour figure can be viewed at wileyonlinelibrary.com]

relative abundances in the samples analysed here and in a previous study (Marcelino & Verbruggen, 2016). Cyanobacterial OTUs captured with the UPA marker, while not abundant, were very diverse and mostly unclassified at lower taxonomic levels. Cyanobacteria have been shown to fix nitrogen in coral tissues (Lesser, Mazel, Gorbunov, & Falkowski, 2004; Radecker et al., 2015) and can be responsible for a large fraction of the nitrogen fixation observed in their skeletons (Crossland & Barnes, 1976; Davey, Holmes, & Johnstone, 2007).

Endolithic algal biomass has been shown to increase under high pCO_2, as phototrophic organisms benefit from the increased availability of carbon dioxide for photosynthesis (Tribollet et al., 2009; Reyes-Nivia et al., 2013). Indeed, we observed a higher relative abundance of all *Ostreobium* clades in *Porites* spp. samples from high pCO_2 sites, but the variability among replicates (i.e., *Porites* spp. samples within sites) was also high, making it difficult to draw conclusions about whether *Ostreobium* spp. are competitively superior to other endolithic algal lineages under OA. Whether the increase in algal biomass is a threat to corals under OA depends on whether the associated bioerosion levels will exceed reef accretion (calcification). Besides increasing bioerosion, endolithic algae growing excessively can penetrate the coral living tissue, possibly increasing their susceptibility to infections (Peters, 1984; Fine, Roff, Ainsworth, & Hoegh-Guldberg, 2006). An increase in endolithic algae may also be beneficial to the coral by providing them with vital nutrients, which is especially important during coral bleaching events (Schlichter et al., 1995; Fine & Loya, 2002).

The possibility that the endolithic microbiome contributes to the resilience of corals under future OA conditions deserves further attention. Massive *Porites* spp. may be considered more competitive under OA than branching species based on their prevalence at naturally high pCO_2 sites (Fabricius et al., 2011). We visibly observed that our massive *Porites* spp. samples had higher colonization with endolithic algae compared to the branching species. The photosynthetic activity of *Symbiodinium* plays an important role in maintaining pH homeostasis within corals (Gibbin, Putnam, Davy, & Gates, 2014), and it is possible that endolithic algae provide a similar service within the skeleton. The biomass of endolithic algae may exceed that of *Symbiodinium* by 16-fold (Odum & Odum, 1955) and can contribute significantly to the buffering capacity of the holobiont (see Yamazaki et al., 2008; Reyes-Nivia, Diaz-Pulido, & Dove, 2014). It is noteworthy that several functionally important micro-organisms (e.g., *Endozoicomoniaceae* and *Bacteroidetes*) often found in coral tissues and mucus also occur in coral skeletons (Sweet, Croquer, & Bythell, 2010; Ainsworth et al., 2015; Marcelino & Verbruggen, 2016; Williams et al., 2015; this study). It is possible that some of these micro-organisms were initially associated with polyp tissues, but remained after removing the tissues from the skeleton with pressurized air, especially in *Porites* spp. which is a perforate coral with tissues that penetrate the skeleton, or that they penetrated the coral

skeleton when the samples were placed in the storage buffer. It is noteworthy however that multiple studies have found tissue-associated microbes within coral skeletons and that the majority of OTUs commonly found in healthy corals have also been found in bare coral skeleton, but not in sea water or in a diseased coral tissue (Fernando, Wang, Sparling, Garcia, & Francini-Filho, 2015). It is possible therefore that the coral skeleton serves as a reservoir for the microbiome and provides a source of beneficial bacteria to coral tissues, analogous to the human appendix which functions as a safe house for symbiotic microbes that repopulate the intestine following acute illness (Randal Bollinger, Barbas, Bush, Lin, & Parker, 2007). Acute environmental stress can disrupt symbiotic relationships among hosts and symbionts (see Hawkins, Bradley, & Davy, 2013), and a stable endolithic community may assist in the recovery of the coral microbiome after environmental (and/or physiological) conditions stabilize.

4.3 | Different host species harbour distinct endolithic communities

The endolithic communities of the branching corals *Seriatopora hystrix*, *P. damicornis* and the massive *Porites* spp. contain significantly different relative abundances of functionally important members of the microbiome (including species of *Endozoicimonaceae* and *Bacteroidetes*) and appear to separate based on morphology or taxonomy (as both branching species belong to the family *Pocilloporidae*). First, the two branching species harbour a reduced diversity of bacteria and algae. The low relative abundance of *Ostreobium* spp. in the endolithic communities of branching species is surprising, considering the generally ubiquitous nature of this alga in coral skeletons (Odum & Odum, 1955; Tribollet, 2008a; Gutner-Hoch & Fine, 2011). Instead of *Ostreobium* spp., the coral *S. hystrix* has a high relative abundance of OTUs related to a macroalga (*Halimeda* spp.), which has only recently been reported in coral skeletons. It is possible that *Halimeda* spp. occur in the coral skeleton in the form of rhizoids that have penetrated the limestone, or most likely, as an unknown microscopic and endolithic life stage of two *Halimeda* species (*H. discoidea* and *H. micronesica*) that are commonly present in metabarcoding studies of endolithic communities (Marcelino & Verbruggen, 2016; Sauvage et al., 2016; this study).

The observed differences in endolithic community composition among coral hosts may be a result of specialization to particular host traits or reflect co-evolution between coral hosts and endolithic species. The living tissue of *Porites* (*lobata*) is about five times thicker, penetrates the skeleton and contains a higher density of *Symbiodinium* than the living tissue of *P. damicornis* and *S. hystrix* (Yost et al., 2013). Tissue thickness would influence the amount of light that penetrates and reflects within the inner parts of the skeleton and may influence the composition of the endolithic community. Branching coral species also tend to grow faster than massive corals (Gates & Ainsworth, 2011), and the branch tips collected in this study may have a younger population of endoliths in comparison to more mature sections of the colony base (a pattern reported in Pica et al., 2016). Future studies would benefit from examining the microbiome associated with different areas of the colony and possible specialization to skeletal features. Alternatively (and not mutually exclusively), endolithic lineages might form stable community assemblies that have co-evolved with the coral host, or the host species have some control over the composition of the endolithic community by selecting beneficial taxa. Mutualistic relationships between corals and their endolithic associates have been suggested in several studies (Odum & Odum, 1955; Schlichter et al., 1995; Schlichter, Kampmann, & Conrady, 1997; Fine & Loya, 2002; Försterra & Häussermann, 2008; Titlyanov, Kiyashko, Titlyanova, & Yakovleva, 2009), and future research would benefit from characterizing possible co-evolutionary processes among coral species and endolithic micro-organisms.

5 | CONCLUSIONS

This study reports a diverse microbiome within the skeletons of *Porites* spp. and demonstrates that little discernible patterns exist in this microbiome across ambient and naturally high pCO_2 environments. We show that the endolithic community shares several functionally important microbes with the coral tissue layer. Environmental stress can induce corals to lose their symbiotic micro-organisms, and a diverse endolithic microbial community might serve as a reservoir to recolonize the microbiome in the coral tissue after the re-establishment of their physiological equilibrium. We found functionally important members in the endolithic community, including members in the *Endozoicimonaceae* and *Bacteroidetes*, forming distinct associations with the different host coral families, an observation consistent with the endolithic reservoir proposition. The diversity and community structure observed in this study form the baseline for future studies aiming to investigate the roles of endolithic micro-organisms in enabling corals to endure climate change.

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DATA ACCESSIBILITY

DNA sequences have been deposited in NCBI's Sequence Read Archive (SRA) under the Accession IDs SAMN07251731–SAMN07251770.

AUTHOR CONTRIBUTIONS

K.M.M. and D.G.B. conceived and conducted the sampling. V.R.M. performed DNA extractions, library preparation, analyses and drafted the manuscript. M.v.O., K.M.M., D.G.B. and H.V. reviewed the analyses and contributed to writing.

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