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The effect of grazing on the microbiome of two commercially important agarophytes, *Gracilaria firma* and *G. salicornia* (Gracilariaceae, Rhodophyta)

Teck-Toh Tan[1,2] · Sze-Looi Song[1,3] · Sze-Wan Poong[1] · Georgia M. Ward[4] · Juliet Brodie[4] · Phaik-Eem Lim[1]

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Abstract

Grazing, which leads to losses in biomass and drastic declines in total crop production, is one of the main concerns in seaweed aquaculture. This is also thought to affect the composition of the associated bacterial communities which are believed to play a crucial role in determining the host's health and development. Apart from morphological impairment, studying changes in the prokaryotic microbiome composition and predicted functional responses to grazing will allow us to understand the underlying effects of grazing on the seaweed host. This study is the first report of the effect of grazing on the prokaryotic microbiome of two economically important agarophytes, *Gracilaria firma* and *Gracilaria salicornia*, by high-throughput sequencing targeting the V3-V4 variable region of the 16S rRNA gene. The results indicated that for *G. firma*, the microbiome composition of tissues grazed by marine herbivores had significantly more agarolytic bacteria *Marinagarivorans* sp. and *Algisphaera* sp. than in ungrazed tissues. The predictive functional metagenomics for this species revealed that grazing escalated the pathway activities related to nucleotide degradation, aromatic compound degradation and aerobic sugar metabolism, while pathways associated with cell wall synthesis, aerobic respiration, vitamin biosynthesis and amino acid biosynthesis were reduced. However, for *G. salicornia*, the bacterial communities were not significantly affected by grazing. Nevertheless, pathways relating to anaerobic respiration and amino acid, coenzyme and vitamin B-6 biosynthesis in this species were predicted to be more active in grazed tissues, whereas the microbiome of ungrazed tissues had higher activities in bacteriochlorophyll *a*, fatty acid, secondary metabolite and heme biosynthesis.

Keywords 16S metagenomics · Gracilarioid red algae · Grazers · Microbiota · Predicted functional pathways · Seaweed aquaculture

Introduction

Species of *Gracilaria* are widely cultivated in Asian countries, including China, Indonesia, the Philippines and South Korea, as well as across the continent of South America in Chile, Peru and Argentina, as the principal resource for the agar industry (Kim et al. 2017). In Malaysia, small-scale farms of *Gracilaria manilaensis* Yamamoto & Trono (Yamamoto and Trono 1994) and *Gracilaria firma* C.F.Chang & B.-M.Xia (Chang and Xia 1976) have been established at the West Coast of Peninsular Malaysia since the mid-1990s (Phang 1998; Prud'homme van Reine and Trono 2001; Phang et al. 2019). *Gracilaria*, due to its high yields of good-quality agar with high gel strength, contributed nearly 80% of total agar production in the global market in the year 2009 (Santelices 2014). Turnover from *Gracilaria* aquaculture has also been maximized by utilizing the residue from agar extraction which

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✉ Phaik-Eem Lim
phaikeem@um.edu.my

[1] Institute of Ocean and Earth Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia

[2] Institute for Advanced Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia

[3] China-Asean College of Marine Sciences, Xiamen University Malaysia, 43900 Sepang, Selangor, Malaysia

[4] Department of Life Sciences, Natural History Museum, London SW7 5BD, UK

is integrated into papermaking (Pei et al. 2013), biofuel production (Amanullah et al. 2013; Kumar et al. 2013) and biofertilizers (Kumar et al. 2013). *Gracilaria* has also been grown in polyculture with fish or shellfish (Chopin et al. 2001) for bioremediation and harvested for human consumption (Norziah and Ching 2000) due to its high nutrient content. Owing to *Gracilaria*'s wide range of applications, its annual production has increased markedly and amounted to 4.14 million tonnes in the year 2016 (FAO 2018).

Seaweeds harbour a rich diversity of microorganisms which symbiotically influence the development and physiology of their hosts (Egan et al. 2013; Brodie et al. 2016). The seaweed provides the microbial colonizers with a habitat and organic carbon. In return, these naturally associated microbes assist in the life cycle of the host and protect it from environmental stresses, as well as modulating the host's interaction with incoming foulers, grazers and epiphytes (Wahl et al. 2012). This beneficial interaction has been demonstrated in *Gracilaria dura* (C.Agardh) J.Agardh where new buds were induced and regenerated as a result of the presence of associated epiphytic and endophytic bacterial which can fix nitrogen and produce indole-3-acetic acid (IAA) (Singh et al. 2011). In another example, host-specific bacterial associations observed in *Gracilaria vermiculophylla* (Ohmi) Papenfuss (now regarded as *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N.Norris et Fredericq) prevented the subsequent colonization by eukaryotic larvae and displayed antibacterial activities (Lachnit et al. 2011). However, microbes can also negatively affect the seaweed by causing diseases (Minich et al. 2018). For instance, an agarolytic strain of *Vibrio* sp. and non-agarolytic *Vibrio alginolyticus* isolated from a rotten thallus of *Gracilaria verrucosa* (Hudson) Papenfuss were tentatively identified as opportunistic pathogens and thought to infect algae suffering from some physiological stress (Beleneva and Zhukova 2006).

The causative agents of many observed syndromes in *Gracilaria* spp., including rotten thallus, cell wall degradation, "white tip syndrome" and "brown point syndrome", remain unidentified and uncharacterized due to the limitations of conventional bacterial culture techniques (Egan et al. 2014). High-throughput sequencing technologies provide new approaches to evaluating the host's health by examining the microbial communities associated with host tissues. Hitherto, various approaches have been applied to study the algal-bacterial associations for *Gracilaria* spp., such as culturing methods which rely on morphological and biochemical tests, used for *G. verrucosa* (Beleneva and Zhukova 2006) and *G. dura* (Singh et al. 2011); denaturing gradient gel electrophoresis (DGGE) and Sanger sequencing of 16S rRNA gene clones, used for *G. vermiculophylla* (Lachnit et al. 2011); and whole genome sequencing, used for *Gracilaria firma* (Ho et al. 2018). However, the microbiota associated with *Gracilaria* spp. are largely unexplored using targeted next-generation sequencing methods, particularly endophytic associations. In addition, existing studies only provide basic information about the taxonomic composition of the bacterial communities.

Grazers are herbivores that naturally feed on seaweed and are regarded as pests in seaweed aquaculture (San 2012; Santelices 2014). They are classified as either mesograzers or micrograzers according to their body size (Cruz-Rivera and Friedlander 2011). Mesograzers include fish, amphipods, crabs and isopods, while micrograzers consist of polychaetes, crustaceans and gastropods ranging from a few millimetres to several centimetres long. Their grazing behaviour causes a loss of biomass and reduces the quality of seaweeds, leading to a tremendous decline in total production and revenue (Cruz-Rivera and Friedlander 2011). In some cases, grazers are recruited as natural predators to control epiphyte infestations in seaweed farms (Sumi and Scheibling 2005; Nejrup et al. 2012). Nonetheless, their beneficial effect is offset by their high densities which consume the host seaweed when epiphytes are depleted (Cruz-Rivera and Friedlander 2011). Apart from morphological damage, the impacts of grazing on seaweeds are largely understudied. Investigation of seaweed bacterial community profiles associated with grazing may help to infer potential consequences to the host. To the best of our knowledge to date, there has been no report on the microbiome of grazed seaweed.

This study was carried out to investigate the effects of marine herbivores grazing on the prokaryotic microbiome structure and predicted functional metagenomes of non-endophytic attached bacteria associated with *G. firma* and *Gracilaria salicornia* (C.Agardh) E.Y.Dawson (Dawson 1954). Grazing potentially alters the microbiome and may affect some of the normal microbiome functions associated with the host. The outcome of this study is important for seaweed aquaculture management strategies.

Materials and methods

Sample collection and DNA extraction

Gracilaria firma and *Gracilaria salicornia* are indigenous organisms in Malaysia which are not endangered or protected by the law. No permits were required to study these specimens. Five fronds each of *G. firma* and *G. salicornia* with both grazed (Fig. 1) and ungrazed tissues were collected from a mangrove swamp in Morib, Selangor, Malaysia (2° 45′ 24.827″ N, 101° 26′ 22.724″ E), during a spring tide on 20 February 2019. Specimens were transported on the same day to the laboratory under cool and moist conditions. From each species, five samples each of grazed and ungrazed tissue measuring approximately 2 cm in length were excised, rinsed with sterile artificial seawater followed by sterile distilled water and

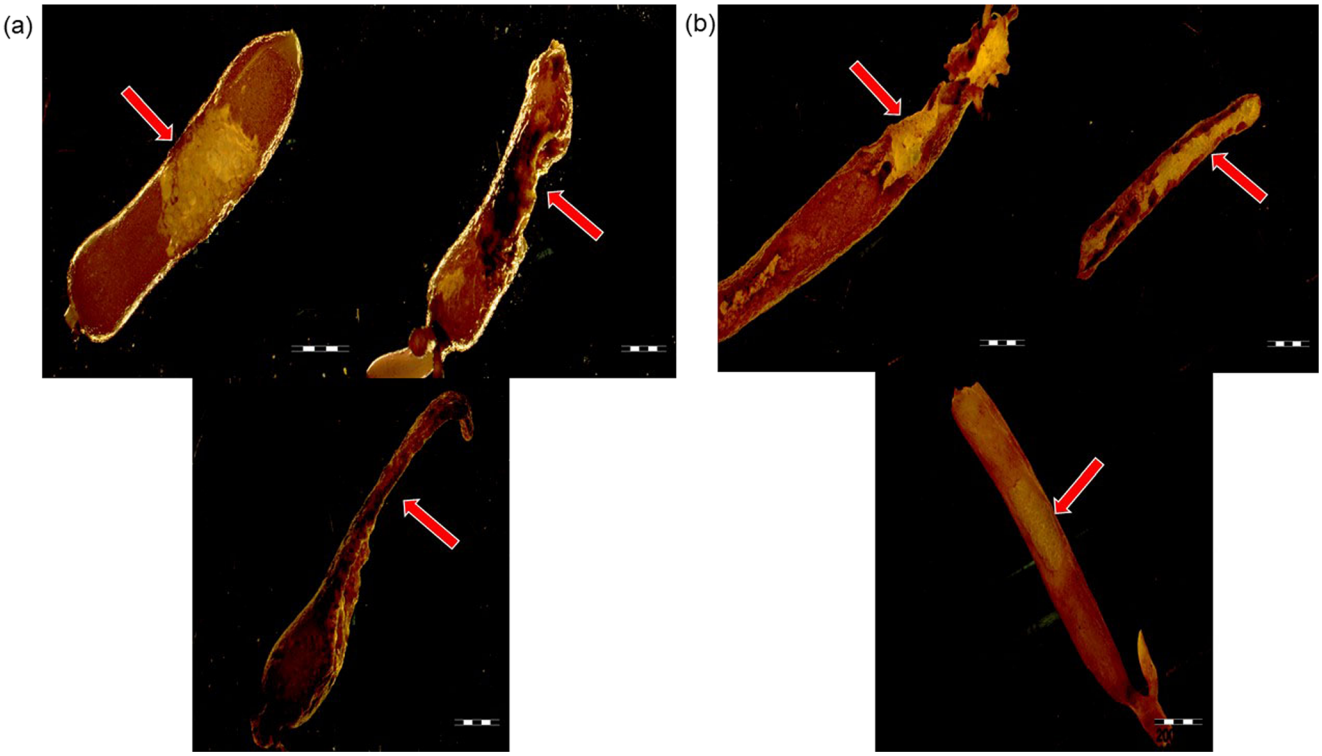

Fig. 1 Grazed tissues of a *G. salicornia* and b *G. firma* under × 6.3 magnification and phase contrast. Red arrows indicate parts that were grazed. Scale bar: 2000 μm.

70% ethanol for 15 s to remove mud, nematodes and epiphytes. DNA was extracted using the NucleoSpin Plant II kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions with some modifications, namely the addition of Proteinase K (ThermoFisher Scientific, USA) with final concentration of 1 mg mL^{-1} into suspension containing Buffer PL2 and RNase A in the cell lysis step. The suspension was sonicated for 20 min at 65 °C and 40 kHz frequency to disrupt the seaweed cell wall and cellular membrane of the associated endophytic bacteria, subsequently liberating bacterial cells. Prior to the neutralization step, the suspension was incubated for additional 2 to 3 h at 65 °C to enhance the DNA yields.

Targeted 16S rRNA amplicon sequencing

16S V3 and V4 amplicons were generated from the extracted genomic DNA by polymerase chain reaction (PCR) using KAPA HiFi HotStart ReadyMix PCR Kit (KAPA BioSystems, USA) and primer pairs MiSeq341F (5′-TCGT CGGCAGCGTCAGATGTGTATAAGAGACAGCCTAC GGGNGGCWGCAG-3′) and MiSeq805R (5′-GTCT CGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC-3′) (Klindworth et al. 2013). This primer pair was predicted to cover 86.8% bacteria and 0.6% archaea based on TestPrime in silico evaluation (www.arb-silva.de/search/testprime) with respect to SILVA 16S rRNA non-redundant reference dataset (SSURef 132NR). PCR conditions and reaction composition followed the standard Illumina 16S metagenomic sequencing library preparation protocol (Illumina Part #15044223 Rev. B). The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, USA) before ligation of indices and sequencing adapters using the Nextera XT Index Kit V2 (Illumina, USA). Following a final clean-up using Agencourt AMPure XP beads, libraries were quantified on a 2100 Bioanalyzer using a High Sensitivity DNA Analysis Kit (Agilent Technologies, USA). Libraries were sequenced on an Illumina MiSeq using a v3 600 cycle (2 × 300 bp) reagent kit (Illumina, USA).

Microbial taxonomic analysis

The adapter sequences were removed from demultiplexed raw sequences using Scythe (v0.994) (https://github.com/vsbuffalo/scythe) and Sickle programme (Joshi and Fass 2011). The sequence reads were imported into the Quantitative Insights into Microbial Ecology 2 (Qiime2 v. 2019.4) (Bolyen et al. 2019) package for complete microbiome analysis. Qiime2 is an extensive platform that wraps multiple bioinformatics software and statistical tools for analyzing microbial amplicon sequence data. The sequence reads were trimmed according to the quality of demultiplexed sequence reads (forward: trimmed left at 5th

sequence base and truncated at position 283rd; reverse: trimmed left at 5th sequence base and truncated at position 268th), denoised, merged, filtered from chimeric sequences and dereplicated using DADA2 software ("default parameter") (Callahan et al. 2016) (Table S1).

The representative sequences for each amplicon sequence variant (ASV) were taxonomically annotated using a pretrained naive Bayes machine-learning classifier (Pedregosa et al. 2011; Bokulich et al. 2018) that was trained to differentiate taxa present in 99% SILVA 132 reference set trimmed to V3-V4 hypervariable region (corresponding to MiSeq341F and MiSeq805R primer pairs). This recovered 110 sequences (4.54%) and 29 sequences (1.20%) assigned to chloroplast and mitochondria respectively, which were filtered out from further analyses.

An alpha rarefaction plot based upon "observed OTUs" metric on dataset rarefied to median sequence counts per sample (approximately 7140) was used to assess the diversity coverage and determine a reasonable downsampling depth based on the plateau state achieved by all samples (Fig. S1). One thousand six hundred was chosen as downsampling depth based on the plateau state achieved by all samples, except for one ungrazed *Gracilaria firma* sample (cfF1). This outlier sample was removed from further analysis, leaving four replicates for ungrazed *G. firma*.

Alpha diversity (microbial diversity within individual samples) was analysed using observed OTUs metric (qualitative approach to measure community richness) and Pielou's evenness (a metric to measure community evenness) (Pielou 1966). Kruskal-Wallis pairwise test (Kruskal and Wallis 1952) was applied to statistically compare the community richness between sample groups. p value was corrected using Benjamini & Hochberg's false discovery rate (B&H FDR) multiple testing adjustment (Benjamini and Hochberg 1995).

Beta diversity (microbial community dissimilarity between groups of samples) was analysed qualitatively based upon Jaccard distance metric (present or absent of feature) (Jaccard 1908) and quantitatively based upon Bray-Curtis distance metric (abundance or read count data) (Bray and Curtis 1957). A principal coordinate analysis (PCoA) plot (Halko et al. 2011; Vázquez-Baeza et al. 2013) was constructed based upon Jaccard distance and Bray-Curtis distance metrics to display the bacterial community dissimilarity between sample groups. Permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was performed to statistically determine the differences between group pairs based on Jaccard and Bray-Curtis distance metrics. Since significant difference might reflect large differences between sample groups or large variances within a group, a test for homogeneity of multivariate dispersions (PERMDISP) (Anderson 2006) was applied to rule out differences due to high degree of dispersion. Therefore, only the sample group with B&H FDR (Benjamini and Hochberg 1995) corrected p value < 0.05 in PERMANOVA and BH-FDR adjusted p value > 0.05 in PERMDISP tests are recognized as truly different.

For bacterial taxonomic profiles, differential abundance tests were carried out with analysis of composition of microbiomes (ANCOM) (Mandal et al. 2015) to statistically determine the abundance differences of taxa across sample groups. Low abundance ASVs (< 0.01%) were filtered out before the analysis to provide better resolution. ANCOM operates on the assumption of less than 25% of the features are changing between groups. In ANCOM, W statistic is the number of ANCOM subhypotheses that have passed for each individual taxon, indicating that the ratios of that taxon's relative abundance to the relative abundance of W other taxa were detected to be significantly different (FDR-adjusted $p < 0.05$).

Predictive functional metagenomics analysis

ASVs produced by DADA2 and filtered to remove chloroplast, mitochondria and sample "cfF1" affiliated sequences were analysed using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Observed States). PICRUSt2 is a wraparound workflow which places ASVs into a reference phylogeny using HMMER (www.hmmer.org) and EPA-NG (Barbera et al. 2018), followed by the castor R package (Louca and Doebeli 2018) to predict gene family abundances using hidden-state prediction. A cut-off nearest-sequenced taxon index (NSTI) value of 0.15 was employed to remove unreliable predictions, as recommended by Langille et al. (2013). Metagenomes were then predicted based on study sequence abundances normalized by predicted 16S copy numbers and predicted gene family abundances. Pathway abundances were inferred based on the predicted sample functional profiles that can be linked to reactions within pathways using a modified version of MinPath (Ye and Doak 2009).

The predicted pathway abundances were then analysed in STAMP (statistical analysis of taxonomic and functional profiles) (Parks et al. 2014). For each species, grazed and ungrazed conditions were statistically compared using a two-sided Welch's t test (White et al. 2009). Pathways with a p value < 0.05 and an effect size of > 0.1 were considered as statistically and potentially biologically significant. The metabolic functions of these pathways were then referenced against the MetaCyc database (Caspi et al. 2018).

Results

16S amplicon sequence variant output

Following the DADA2 quality control workflow, the number of representative ASVs retained per sample varied between 20 (sample cfF1) and 441 (sample grSfF1) (Table S1). The number of ASVs varied among samples within the same host

species (e.g. 20–183 in ungrazed *G. firma*; 61–229 in ungrazed *G. salicornia*) and across different host species (e.g. mean of 180 features in grazed *G. firma* versus mean of 256 features in grazed *G. salicornia*).

Bacterial diversity

For alpha diversity, all sample groups had similar community richness and evenness distribution (Fig. S2). None was identified to be significantly different from each other (Kruskal-Wallis pairwise test, $p \geq 0.05$) (Tables S2 and S3).

For beta diversity, the structure of microbiome communities for each sample group was dissimilar to each other both qualitatively and quantitatively, as observed in the distinct clustering of each sample group in the Jaccard and Bray-Curtis distance-based PCoA plots (Fig. 2). PERMANOVA and PERMDISP pairwise statistical tests further attested that the bacterial composition of ungrazed *G. firma* is significantly different (PERMANOVA: $p \leq 0.05$, 999 permutations; PERMDISP: $p \geq 0.05$, 999 permutations) to the ungrazed *G. salicornia* based upon Jaccard distance and Bray-Curtis distance metrics (Figs. S3 and S4; Table S4). There were significant differences (PERMANOVA: $p \leq 0.05$, 999 permutations; PERMDISP: $p \geq 0.05$, 999 permutations) between the bacterial community composition of ungrazed *G. firma* and its grazed counterpart both qualitatively and quantitatively. In contrast, the bacterial community composition (qualitative and quantitative) of ungrazed *G. salicornia* was not significantly varied (PERMANOVA: $p \leq 0.05$, 999 permutations; PERMDISP: $p \leq 0.05$, 999 permutations) from its grazed counterparts.

Taxonomic analysis

High-throughput sequencing of V3-V4 domain of bacterial 16S rRNA gene of 19 agarophyte samples revealed the presence of 9 bacterial phyla (all assignable to known phyla; Fig. 3), comprising 15 classes (all assignable to known classes), 46 orders (44 identified; 2 unidentified), 63 families (57 identified; 6 unidentified), 106 genera (87 identified; 19 unidentified) and 137 species (78 identified; 59 unidentified). Analysis of our dataset indicates that approximately 34% of the bacterial community are unculturable bacteria and 43% are unidentified at species level (Table S8).

At the phylum level, the bacterial composition for all investigated sample groups was dominated by the Proteobacteria (Fig. 3; Table S5). The phylum Actinobacteria was present (ANCOM $W = 3$) in ungrazed *G. salicornia* but absent in ungrazed *G. firma*. Comparison of grazed and ungrazed *G. firma* detected no significant difference in abundance of any bacterial phylum. Bacterial phyla associated with grazed *G. salicornia* were identified to be significantly lower in abundance for the phyla Proteobacteria (1.23-fold, $W = 3$) and Planctomycetes (1.64-fold, $W = 2$), but showed significantly higher abundance of the phyla Acidobacteria ($W = 2$) and Verrucomicrobia ($W = 2$), as compared to its ungrazed counterpart.

At the species level, out of 122 bacterial species shared between ungrazed tissues of *G. firma* and *G. salicornia*, 19 species were detected to be significantly different (Fig. 4a; Table S6). In general, *Filomicrobium* sp. ($W = 32$), *Erythrobacter vulgaris* ($W = 13$), an unclassified Hyphomonadaceae ($W = 8$) and an uncultured alpha proteobacterium ($W = 5$) were significantly higher in abundance in ungrazed *G. firma*, whereas unclassified Alphaproteobacteria ($W = 23$), uncultured Rhizobiaceae ($W = 5$) and uncultured Hyphomonadaceae ($W = 5$) were significantly more abundant in ungrazed *G. salicornia*.

Of grazed *G. firma*, relative to its ungrazed counterpart, 9 out of 123 bacterial species were detected to be significantly different (Fig. 4b; Table S7), in which uncultured *Marinagarivorans* sp. ($W = 21$), *Algisphaera* sp. ($W = 5$) and

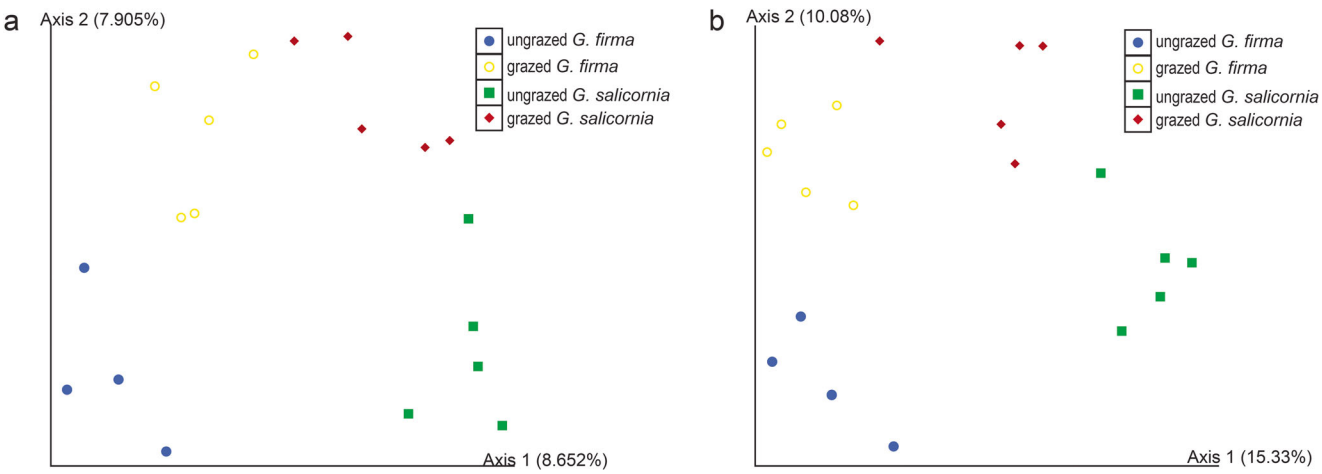

Fig. 2 Comparison of bacterial diversity across sample groups by principal coordinate analysis (PCoA) based on **a** Jaccard distance and **b** Bray-Curtis distance metrics

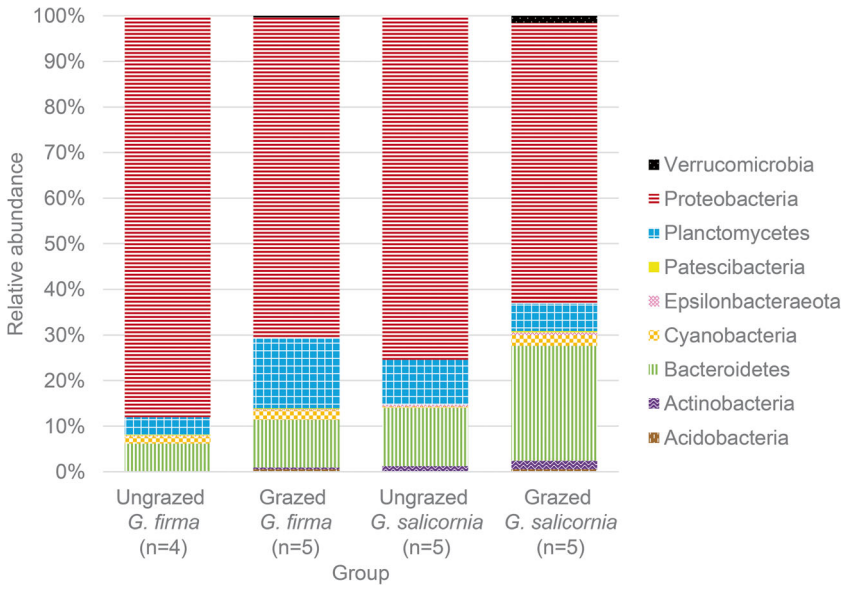

Fig. 3 Taxonomic composition of bacterial phyla in grazed and ungrazed tissues of both *G. firma* and *G. salicornia*

Planctomycetales ($W = 2$) were significantly higher in abundance in grazed *G. firma*, whereas *Maribacter* sp. B1 ($W = 6$), unclassified species of Hyphomonadaceae ($W = 5$) and *Erythrobacter vulgaris* ($W = 2$) were significantly higher in abundance in ungrazed *G. firma*. On the other hand, no bacterial species were identified to be significantly different between grazed *G. salicornia* and its ungrazed counterpart.

Predictive functional analysis

Predictive functional metagenomics using PICRUSt2 and subsequent statistical analysis using STAMP showed a number of differences between the ungrazed tissues of two *Gracilaria* species (Fig. S5; Table S9) as well as between ungrazed and grazed conditions for each species (Fig. 5; Table S9).

Comparison of ungrazed tissues of *G. firma* and *G. salicornia* resulted in the identification of 12 metabolic pathways for which statistically and potentially biologically significant differences could be observed between groups (Fig. S5; Table S9). Pathways associated with amino acid degradation, vitamin B-9 biosynthesis (6-hydroxymethyl-dihydropterin diphosphate biosynthesis), carbon metabolism (ethylmalonyl-CoA pathway) and protein synthesis (formaldehyde assimilation) were predicted as being more active in *G. firma*, while those associated with fatty acid biosynthesis, glucose fermentation, secondary metabolite biosynthesis, bacteriochlorophyll biosynthesis and biosynthesis of heme, which functions as a regulatory molecule in protein synthesis pathways, were more active in *G. salicornia*.

The tissues of *G. firma* that had been grazed by herbivores had higher predicted activity of nucleotide degradation/recycling (guanosine nucleotides degradation III, purine ribonucleoside degradation, adenosine nucleotide degradation II and superpathway of purine deoxyribonucleoside degradation) and aromatic compound degradation comparing to ungrazed tissues. Ungrazed *G. firma* tissues were predicted to have associated microbial communities more active in pathways associated with cell wall synthesis, aerobic respiration, vitamin/cofactor biosynthesis, proteinogenic amino acid biosynthesis and phospholipid biosynthesis (Fig. 5; Table S9).

Ungrazed tissues of *G. salicornia* showed higher predicted activity of fatty acid synthesis pathways, bacteriochlorophyll biosynthesis and biosynthesis of heme, in relation to its grazed tissues which were predicted to be more active in bacterial pathways related to anaerobic respiration, amino acid synthesis, vitamin B-6 biosynthesis and coenzyme biosynthesis (Fig. 5; Table S9).

Discussion

For the first time, we have demonstrated that the composition of the bacterial communities of *G. firma* and *G. salicornia* differs in ungrazed and grazed individuals of these two economically important red algae. Furthermore, the composition of the microbiome is different between the two species with some bacterial species confined to one or other species (Fig. 4a; Table S6). These results add to a growing body of knowledge of the variability of host-associated microbial communities in general (Burke et al. 2011b; Brodie et al. 2015).

The high proportion of unclassified bacterial taxa in 16S short-target amplicon-based microbiome study, i.e. 43% of unidentified bacteria at the species level, is another typical finding in similar studies (Miranda et al. 2013; Brodie et al. 2015) and partially reflects the scarcity of seaweed-bacterial studies until relatively recently (Hollants et al. 2013). The results are also almost certainly related to the limitations and

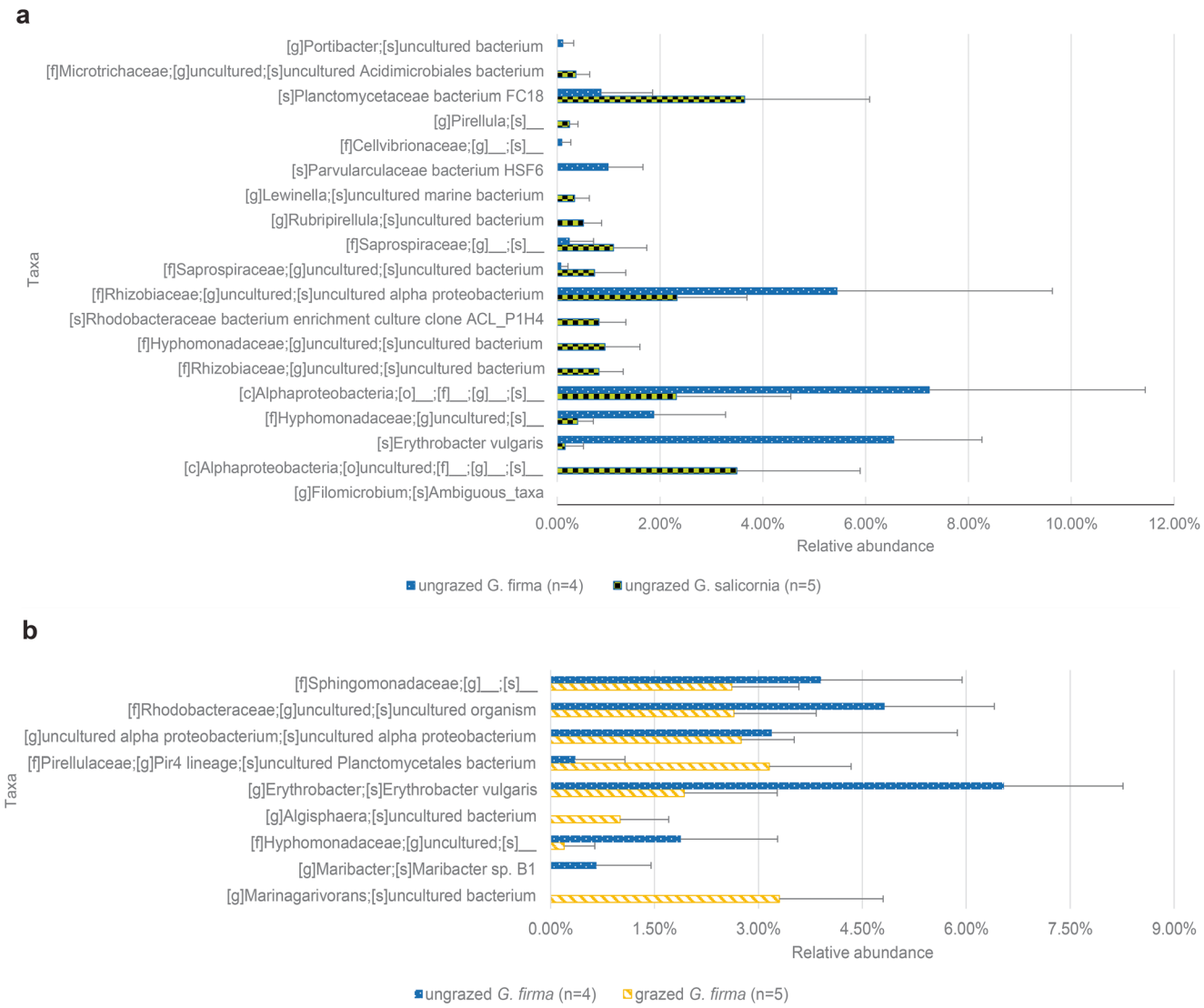

Fig. 4 Bacterial communities significantly different in abundance between **a** ungrazed tissues of *G. firma* and *G. salicornia* and **b** grazed *G. firma* to its ungrazed counterpart, as determined by analysis of composition of microbiomes (ANCOM)

biases of available databases. A high proportion of reads identified as uncultured bacteria is expected in metagenomics—34% of uncultured bacterium at species level in this study—as it is estimated that less than 1% of microorganisms observable in nature are culturable by using conventional techniques (Amann et al. 1995; Philip et al. 1998). As such, many bacterial taxa associated with both *Gracilaria* species and conditions (ungrazed and grazed) remain uncharacterized. Given the taxonomic limitation observed, the predicted effect and interaction of associated bacteria on algal host was derived mainly from the type strain or the closest affiliated taxonomic rank and incorporated with predictive functional metagenomics. The high proportion of unclassified and uncultured bacteria in *G. firma* and *G. salicornia* may imply that marine *Gracilaria* represent a potential source for discovery of novel bacteria and commercially important biological products, such as antibiotics and enzymes (Das et al. 2006).

Physiological and biochemical properties of substrata may predetermine the composition of associated microbes (Beleneva and Zhukova 2006; Bondoso et al. 2017). The agar content of *G. firma* (12–29% dry weight) was reported to be higher than *G. salicornia* (9–14% dry weight) (Phang et al. 1996; Lee et al. 2017). The difference in polysaccharide content may explain the specificity of microbial consortia to the algal host, although it could be that we are observing functionally equivalent, but distinctive OTUs, a concept supported by the studies of Burke et al. (2011a) and Miranda et al. (2013).

This can be observed in the presence of *Portibacter* sp., which consists of agarolytic members (McIlroy and Nielsen 2014), in ungrazed *G. firma* ($W = 3$), but not in ungrazed *G. salicornia* (Fig. 4; Table S6). Nevertheless, both species of *Gracilaria* hosted a core microbiome consisting of the phyla Proteobacteria, Planctomycetes and Bacteroidetes (Fig. 3;

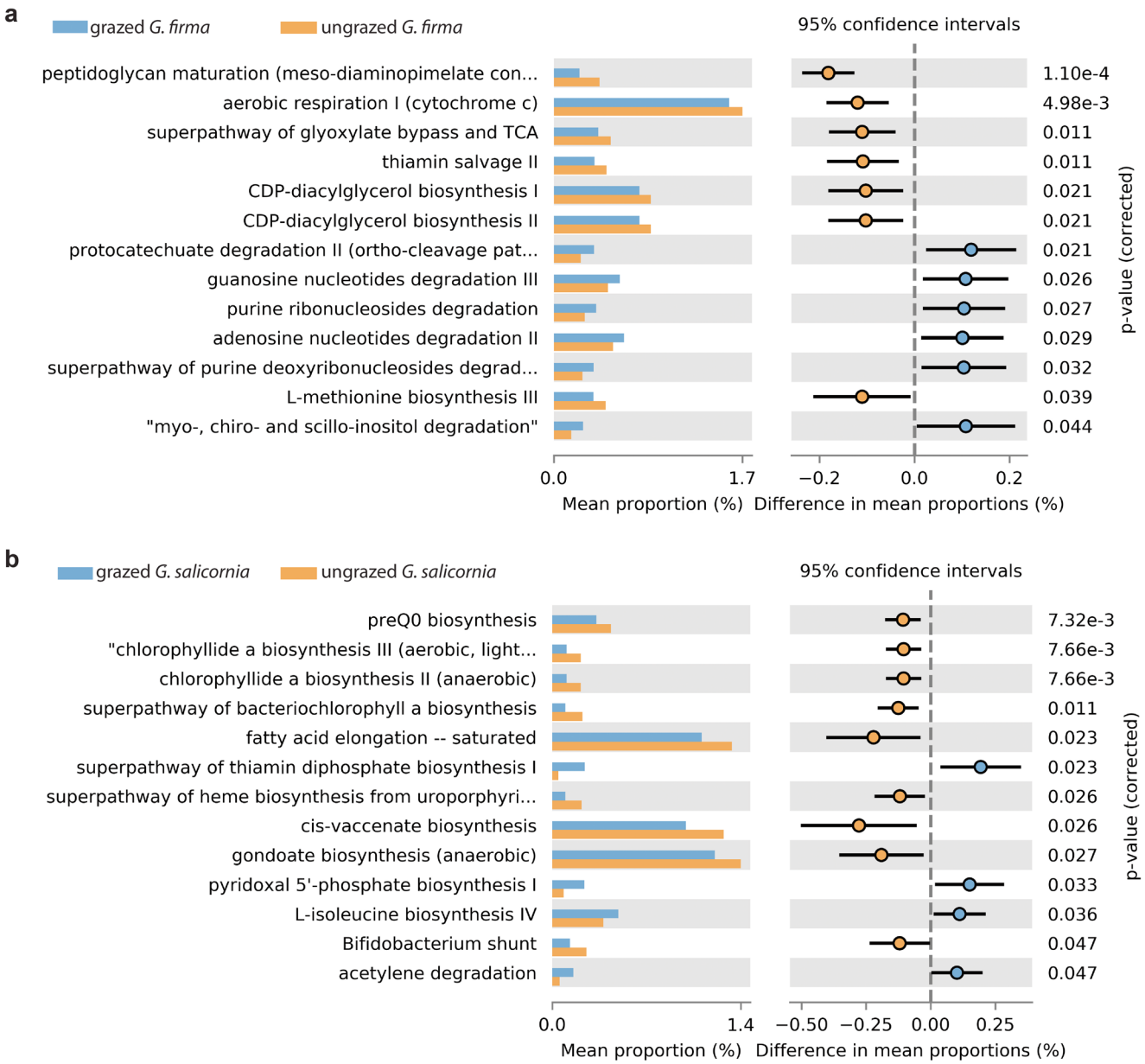

Fig. 5 Statistical comparison of predicted pathway abundances between ungrazed and grazed tissues of **a** *G. firma* and **b** *G. salicornia* as tested by statistical analysis of taxonomic and functional profiles (STAMP)

Table S5). This result is in agreement with a whole genome study on *G. firma* (Ho et al. 2018) and a study on the epiphytic communities of *G. vermiculophylla* analysed by DGGE and 16S cloning approaches (Lachnit et al. 2011), in which the phyla Proteobacteria and Bacteroidetes were prevalent. However, a comparison of the microbiome composition of *G. firma* at genus level in our study (Table S8) with that of Ho et al. (2018) shows that only 4.7% of bacterial genera (*Pseudoalteromonas* sp., *Vibrio* sp., *Arcobacter* sp., *Robiginitalea* sp. and *Blastopirellula* sp.) were shared in both studies. This might be attributed to the differences in sample collection and processing, sequencing approach, variation in reference database and replicate inadequacy of the previous study (Minich et al. 2018) or another example of functional equivalence.

Generally, polysaccharides are the main component of macroalgal cell walls (Imran et al. 2017). These polysaccharides are the substrate for various polysaccharide-degrading enzymes produced by agarolytic bacteria, which utilize polysaccharides as a carbon and energy source and facilitate the biogeochemical cycle. Notwithstanding, polysaccharides play an important role in protecting the host from pathogens and predators (Imran et al. 2017). Therefore, the presence of agar-degrading bacteria *Marinagarivorans* sp. and *Algisphaera* sp. in grazed *G. firma* but not in its ungrazed counterpart (Fig. 4b; Table S7) might be detrimental to the host. Despite the

possible deleterious impact of grazing on the algal host, the grazed *G. firma* can potentially be utilized as a bioresource for production of agar-derived biofuel due to its readily available reservoir of agarolytic bacteria (Kwak et al. 2012). Moreover, polysaccharide-degrading bacteria isolated from grazed *G. firma* can potentially be applied in the degradation of algal waste (Satomi and Fujii 2014; Imran et al. 2017).

Grazing may perturb the plant-bacteria symbiosis, resulting in developmental failure in the plant host. The reduced abundance of an uncultured bacterial species belonging to the family Rhodobacteraceae in grazed *G. firma* compared to its ungrazed counterpart (Fig. 4b; Table S7) may be an example of this. Some members of Rhodobacteraceae are known to play a vital role as plant symbionts, such as synthesis of B_1 and B_{12} vitamins (Minich et al. 2018), growth factors (Dogs et al. 2017), siderophores that enhance algal growth under iron deficiency, deterrents for pathogens and metabolism of algal osmolytes (Dogs et al. 2017). This observation corresponds to the lower predicted bacterial activity of vitamin biosynthesis (thiamin salvage) in grazed *G. firma* in relative to its ungrazed counterpart (Fig. 5). In addition, the reduced abundance of an unclassified species belonging to the family Hyphomonadaceae in the microbiome of grazed *G. firma* relative to its ungrazed counterpart (Fig. 4b; Table S7) may affect the development of the plant, as some members within the family Hyphomonadaceae are known to induce normal morphogenesis in red algae (Fukui et al. 2014).

From the predicted functional analysis, four pathways associated with nucleotide and nucleoside degradation were enriched significantly in grazed material of *G. firma* (Fig. 5; Table S9). In this pathway, nucleotides and nucleosides are metabolized to provide nutrients, energy and basic building blocks such as carbohydrates, proteins, lipids and nucleic acids to the bacteria (Caspi et al. 2018). We hypothesize that the dissolved organic matter was significantly decreased in grazed algal tissues; hence, the associated bacterial residents required an alternative source to survive.

The results of this study may be beneficial to the in situ cultivation of *Gracilaria*, including "rope farming" and "bottom stacking" as well as wild stock collection. Since disease management strategies and a posteriori containment are challenging and ineffectually implemented, good cultivation practices regulated by preventative measures should be applied. Simple mechanical devices such as micro-grid size fences and cages can be recruited to physically exclude the grazers (Cruz-Rivera and Friedlander 2011). Additionally, the floating culture techniques may mitigate the grazing problem on seaweed crops (San 2012; Santelices 2014). The introduction of algal species that are preferentially targeted by grazers may protect the crop alga against predation (Cruz-Rivera and Friedlander 2011). Results from this study are primarily observations and predictions which need to be linked with bacterial transcriptomics and metabolomic profiles to verify the findings. As a comparative study, future exploration of the effect of grazing on the microbiota of off-site cultivation systems, such as pond farming and land-based or raceway tank farming, is needed to provide a comprehensive perspective into challenges in the aquaculture of *Gracilaria*.

In summary, this study reports for the first time the distinct composition of bacterial communities across two species of *Gracilaria*, and the communities' predicted functional response to grazing. Although marine herbivores grazing on both *G. firma* and *G. salicornia* caused no significant effect on the richness of associated bacterial community, the bacterial community structure associated with *G. firma* was significantly altered upon grazing. Grazing may be detrimental to *G. firma* due to the enrichment of polysaccharide-degrading bacteria and the ensuing impact of microbiome dysbiosis, where the normal symbiotic relationship between host and associated microbes is disrupted and results in unfavourable morphogenesis in the algal host. Despite the significant loss in production, grazed *G. firma* is a potential candidate for use in biofuel production and waste management. In contrast, grazing had no significant effect on the microbiome structure of *G. salicornia*. Nonetheless, the predicted functional profiles exhibited significant variation of bacterial pathways between the grazed and ungrazed tissues of *G. salicornia*. These findings identify *Gracilaria* as a potential source of novel bacteria and point to the continuing need for basic bacterial taxonomic work.

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