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The microbiome of the pelagic tunicate *Dolioletta gegenbauri*: A potential link between the grazing and microbial food web

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Abstract

Bloom-forming gelatinous zooplankton occur circumglobally and significantly influence the structure of pelagic marine food webs and biogeochemical cycling through interactions with microbial communities. During bloom conditions especially, gelatinous zooplankton are keystone taxa that help determine the fate of primary production, nutrient remineralization, and carbon export. Using the pelagic tunicate *Dolioletta gegenbauri* as a model system for gelatinous zooplankton, we carried out a laboratory-based feeding experiment to investigate the potential ecosystem impacts of doliolid gut microbiomes and microbial communities associated with doliolid faecal pellets and the surrounding seawater. Metabarcoding targeting Bacteria and Archaea 16S rRNA genes/Archaea) and qPCR approaches were used to characterize microbiome assemblages. Comparison between sample types revealed distinct patterns in microbial diversity and biomass that were replicable across experiments. These observations support the hypothesis that through their presence and trophic activity, doliolids influence the structure of pelagic food webs and biogeochemical cycling in subtropical continental shelf systems where tunicate blooms are common. Bacteria associated with starved doliolids (representative of the resident gut microbiome) possessed distinct low-biomass and low-diversity microbial assemblages, suggesting that the doliolid microbiome is optimized to support a detrital trophic mode. Bacterial genera *Pseudoalteromomas* and *Shimia* were the most abundant potential core microbiome taxa, similar to patterns observed in other marine invertebrates. Exploratory bioinformatic analyses of predicted functional genes suggest that doliolids, via their interactions with bacterial communities, may affect important biogeochemical processes including nitrogen, sulphur, and organic matter cycling.

KEYWORDS
16S rRNA, amplicon sequence variants – ASVs, *Dolioletta gegenbauri*, doliolid, faecal pellets, microbiome, South Atlantic bight – SAB

1 | INTRODUCTION

Marine gelatinous zooplankton, including the mucus-feeding pelagic tunicates (appendicularia, pyrosomes, salps, and doliolids), occur circumglobally and play a central role in marine planktonic food webs (Alldredge & Madin, 1982; Conley et al., 2018; Frischer et al., 2021). In pelagic marine ecosystems, microbial processes and the fate of primary production in the water column may be influenced by frequent and widespread blooms of gelatinous zooplankton. Because their life histories typically involve alternating sexual and asexual stages, pelagic tunicates are capable of rapid reproduction that can lead to bloom formation and that probably significantly influence the structure of pelagic marine food webs and biogeochemical cycling (Deibel, 1998; Frischer et al., 2021; Walters, Lamboley, et al., 2019). Filter-feeding pelagic tunicates within the order Doliolida (including the species *Dolioletta gegenbauri*) frequently form massive blooms on most of the world's continental shelfs, where such blooms can exceed 1000 zooids/m^3 and extend for hundreds of kilometres (Boero et al., 2008; Deibel & Lowen, 2011; Frischer et al., 2021; Greer et al., in review). Salps, a sister group to doliolids, also form massive blooms but generally in productive ocean environments including the Southern Ocean (Décima et al., in review; Perissinotto & Pakhomov, 1998; Smetacek et al., 2004; Stukel et al., 2021). These pelagic tunicate blooms can decouple grazing linkages between the mesozooplankton and higher trophic levels through depletion of microbial populations, competition for prey, and direct feeding of nekton early life stages (Boero et al., 2008; Sullivan & Kremer, 2011). Consequently, in some scenarios the sinking of gelatinous biomass results in rapid and permanent vertical export of carbon to the deep ocean (Lebrato et al., 2019; Richardson, 2019). Our understanding of the ecological role of these fragile species is limited, however, primarily due to methodological challenges that limit the ability to observe, sample, and culture them (Walters, Gibson, et al., 2019). Thus, there is a pressing need for more targeted field and experimental studies that elucidate the linkages between the grazing (gelatinous zooplankton) and microbial food webs, providing a more comprehensive understanding of marine pelagic ecosystem functioning, biogeochemical cycling, and carbon export.

While it is increasingly recognized that blooms of large gelatinous organisms (e.g., jellyfish) influence marine microbial assemblages by releasing large amounts of mucus (Condon et al., 2011; Hao et al., 2019; Lebrato et al., 2019), less is known about the interaction between smaller gelatinous zooplankton species and microbial processes. The link between small gelatinous zooplankton (e.g., doliolids) and pelagic microbial communities, however, is potentially more fundamental to ecosystem functioning due to their much higher abundances (Greer et al., 2021; Takahashi et al., 2015; Walters, Lamboley, et al., 2019) and their potential to shunt a considerable fraction of pelagic water column productivity to the microbial food web via the production of microbially-labile faecal pellets. Based on laboratory-derived estimates of doliolid clearance rates (reviewed by Deibel, 1998) and considering the range of observed zooid abundances, doliolids have the potential to remove almost 100% of shelf water column primary production. Doliolids, because they are prolific producers of faecal material that contain high quantities of minimally-degraded organic matter and are relatively buoyant and slow sinking (Paffenhöfer & Köster, 2005; Patonai et al., 2011; Pomeroy & Deibel, 1980) may have a particularly large impact on microbial loop processes in the surface ocean. In contrast, copepods produce membrane-bound, dense, and fast-sinking faecal pellets (Alldredge & Madin, 1982; Köster et al., 2011). The slow sinking rates of doliolid faecal pellets probably allows them to linger in surface water where they can be rapidly colonized and remineralized in surface water rather than be exported to depth. Taken together, doliolids have the potential to significantly influence shelf carbon cycling and pelagic–benthic coupling across the world's oceans (Deibel, 1985; Ishak et al., 2020).

As with all pelagic tunicates, doliolids are efficient filter feeders and capable of clearing large volumes of water in both low- and high-food concentration environments (Lucas & Dawson, 2014). Based on anatomical considerations, laboratory-based experimental studies, and inferences from field observations, doliolids are capable of ingesting particles over a wide size range from less than a micron (bacteria) to 100 s of microns (large diatoms, copepod eggs, nauplii) but optimally between 1–50 μm (Deibel, 1985; Tebeau & Madin, 1994). Historically, because of their feeding mechanism, doliolids were assumed to be passive grazers that nonselectively capture particles they encounter (Crocker et al., 1991; Vargas & Madin, 2004), although some prey items such as diatoms and metazoans are known to be poorly assimilated (Frischer et al., 2021; Paffenhöfer & Köster, 2005). More recently, however, using molecular gut content analysis and stable isotope tools applied to cultured and wild-caught doliolids, it has been recognized that doliolids are capable of selective feeding and that a significant portion of their diet is probably derived from microbial-processed detrital material (Frischer et al., 2021; Walters, Lamboley, et al., 2019). Recent studies have also suggested that reingestion of faecal pellets is an important component of the doliolid diet, especially when phytoplankton abundance is low (Köster & Paffenhöfer, 2017). Thus, current evidence implies that doliolids may function primarily as detritivores by consuming microbial-enriched particles, acting as a significant driver of shelf microbial processes by feeding on the detrital pool to which they also contribute.

What is the role of the doliolid gut microbiome in faecal pellet processing and acquisition of carbon/nutrients from detrital food sources? A recent analysis of >1000 specimens across 21 animal phyla supports the idea that marine invertebrates generally have low-diversity microbiome assemblages that are distinct from surrounding seawater, but many host-associated bacteria may be generalist taxa able to associate with a diversity of host species (and thus marine invertebrates may lack a signature of true phylosymbiosis; [Boscaro et al., 2022]). In general, the functional significance of marine invertebrate microbiomes is not yet well

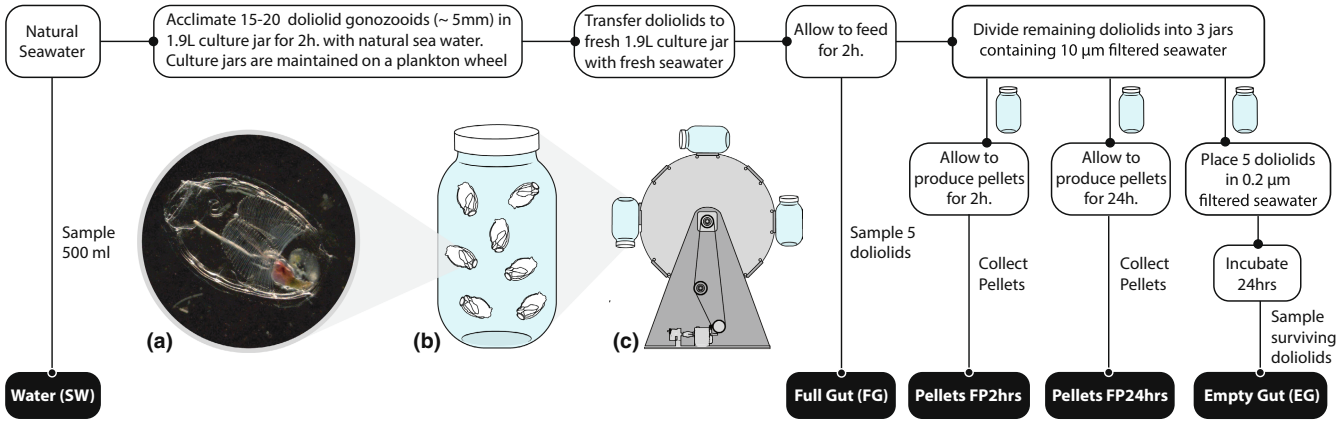

FIGURE 1 Schematic of experimental design used to produce the samples analysed in this study. Experimental procedures (clear boxes) and samples collected (dark boxes) are highlighted. Inset: (a) micrograph of *Dolioletta gegenbauri* gonozooid. (b) Culture jar (1.9 L) used to acclimate and feed doliolids. (c) Plankton wheel used to incubate doliolids and produce faecal pellet samples

understood, and host-associated community assembly may be based on community gene content and preservation of functional redundancy as opposed to selection for specific microbial clades or species (Burke et al., 2011; Louca et al., 2018). Investigations of zooplankton microbiomes have been relatively rare to date, and such studies have primarily focused on taxonomic profiling of host-associated communities. Copepod microbiomes contain diverse and abundant bacterial communities (Datta et al., 2018; De Corte et al., 2018; Scavotto et al., 2015; Shoemaker et al., 2019; Tang et al., 2019) that differ significantly from bacterial communities associated with the surrounding seawater. Similarly, in the few studies that have examined microbiomes associated with gelatinous zooplankton, host microbiomes differed from the surrounding water; however, unlike copepods, the diversity of microbiomes associated with gelatinous zooplankton appears to be very low (Daniels & Breitbart, 2012; Jaspers et al., 2020; Tinta et al., 2019; Viver et al., 2017).

In this study, we expand on previous work elucidating linkages between doliolid feeding ecology and continental shelf microbial processes. Here, we specifically investigate the potential role of the doliolid gut microbiomes and faecal-pellet associated microbial communities in pelagic biogeochemical cycling and nutrient remineralization, focusing on the following two hypotheses: (1) doliolids have a low diversity, low biomass microbiome optimized for faecal pellet packaging and acquisition of carbon/nutrition from detrital feeding, and (2) doliolids predictably restructure particle-associated microbial communities in the continental shelf, impacting both assemblage structure and microbial functional roles. By characterizing microbiomes associated with doliolids that had been feeding in freshly collected seawater containing natural microbial communities and comparing microbiomes associated with the (1) feeding water, (2) faecal pellets, and (3) the animals, interactions between doliolids and microbial communities are inferred. In addition, characterization of starved doliolids provides the first description of the host-associated doliolid gut microbiome.

2 | MATERIALS AND METHODS

2.1 | Collection and culture of doliolids

Laboratory cultures of *D. gegenbauri* were initiated and maintained as previously described (Walters, Lamboley, et al., 2019). Briefly, cultures were maintained in filtered (~0.45 µm) seawater in 3.9 L glass jars on a slowly rotating plankton wheel at ~20°C and supplemented daily with an algal mixture consisting of *Isochrysis galbana*, *Rhodomonas* sp. and the diatom *Thalassiosira weissflogii*. Algal concentrations were maintained at ~80 µg C/L. *Dolioletta gegenbauri* zooids were collected using a conical net with a 0.5 m opening, 202 µm mesh net, and a 4 L aquarium cod end from the mid-shelf region (25–40 m isobath) of the South Atlantic Bight (SAB) between 29.6–31.2° N and 80.1–80.3° W. The net was towed from a drifting ship by slowly lowering and raising the net through the depth of the water column. The culture was initiated in May 2015 and maintained until the experiments were completed in April 2016. Over the course of this period, the culture was supplemented twice with freshly collected *D. gegenbauri* zooids in August and December 2015.

2.2 | Doliolid feeding experiments

Figure 1 provides an overview of the design of the feeding experiments. Independent feeding experiments were conducted in January 2016 (Exp1), March 2016 (Exp2), and April 2016 (Exp3). Briefly, seawater containing natural microbial communities was collected from the same area in the SAB where doliolids were collected prior to each experiment. Prior to use, water was stored in 20 L carboys in the dark at 20°C. Water used in Experiment 1 was collected 1 month prior to the experiment on 2 December 2015, water used in Experiment 2 was collected 2 days prior to the experiment on 16 March 2016, and water used in Experiment 3 was collected 8 days prior to the experiment. Water was collected

from near the bottom of the water column where particle concentrations were highest. Over the period that water was collected, near bottom water temperature ranged from 17.0–22.6°C, salinity ranged from 34.2–35.8 PSU, and chlorophyll-*a* concentration ranged from 1.04–1.14 μg/L. Experiments were initiated by acclimating 15–20 *D. gegenbauri* zooids from the culture in a 1.9 L culture jar containing fresh seawater and allowed to feed for 2 h while rotating on a plankton wheel. Following the acclimation period, the zooids were transferred to a clean 1.9 L jar containing fresh seawater and allowed to feed while on the plankton wheel for an additional 2 h after which five zooids were sampled. Based on the gut residence times and estimated clearance rates (Gibson & Paffenhöfer, 2000), it is expected that doliolids would have cleared 250–700 ml (13%–35%) of the feeding vessel volume during the 2 h incubation. Following the feeding period, the remaining zooids were divided into three clean 1.9 L culture jars and returned to the plankton wheel. Two of the jars contained 10 μm filtered water depleted of algal and detrital particles but would be expected to have retained natural bacterial communities. Faecal pellet samples were collected from these jars after 2 and 24 h, respectively. To produce starved "Empty Gut" samples to characterize a core gut microbiome, an additional five zooids were incubated for 24 h in a third 1.9 L jar containing 0.2 μm filtered seawater. An initial series of three pilot experiments conducted in 2012 and following the procedures described above generated the materials used to estimate bacterial concentrations in starved (EG), aged faecal pellets (FP24h), and seawater (SW) by quantitative PCR (qPCR).

Over the course of each experiment four types of samples were collected for 16S metabarcoding characterization (Figure 1). Seawater (SW) containing natural microbial communities was sampled at the start of the 2 h feeding period of experiment. Prior to filtration, the water was prefiltered through a 63 μm sieve to remove any larger aggregates and metazoans. Triplicate 500 ml samples were gently filtered onto 25 mm 0.2 μm Supor filters (PALL Life Sciences). Filters were placed in sterile 2 ml cryovials and stored at −80°C until DNA was extracted. Following the 2 h feeding period, five zooids with full guts (FG) were sampled to characterize microbial communities associated with both the doliolid and the prey that was consumed. These samples were expected to be dominated by fresh faecal material. Following 2 h of incubation in 10 μm filtered seawater faecal pellets were collected (FP2h). This material was considered freshly released and representative of initial colonization by natural bacterial communities. Following 24 h of incubation in 10 μm filtered seawater a second set of faecal pellet samples were collected (FP24h). This material was considered aged faecal material and representative of material with a developed mature microbial community. It was not possible to recover intact pellets after 24 h as they were highly degraded and had either broken-up completely or dissolved.

Following collection of *D. gegenbauri* zooid samples, zooids were stabilized prior to DNA extraction as previously described (Walters, Lamboley, et al., 2019). Briefly, zooids were anaesthetized in 0.2 μm filtered seawater containing 0.4% MS-222 (3-aminobenzoic acid ethyl ester; Alfa Aesar). After rinsing each zooid three times in fresh 0.2 μm filtered seawater containing MS-222 they were transferred to 2 ml tubes containing extraction ATL buffer from the DNeasy Blood and Tissue kit (Qiagen). Faecal pellets were collected by centrifugation (500*g* for 5 min) after they were rinsed three times in 0.2 μm filtered seawater. Pellets were transferred to 2 ml tubes containing extraction ATL buffer. All samples were stored at 4°C until DNA was extracted, usually within 24–48 h after initial collection.

2.3 | DNA extraction, PCR, and Illumina sequencing

Genomic DNA from doliolid zooids was extracted and purified using the Qiagen DNeasy Blood and Tissue kit. DNA from water samples was purified using the Qiagen PowerWater kit (Qiagen). Manufacturer's instructions were followed for both kits. Purified DNA extracts were quantified using a Qubit 2.0 fluorometer with the dsDNA HS assay reagents (ThermoFisher Scientific). Yields ranged from 40–254 ng (0.20–1.3 ng/μl) DNA per gonozooid and 0.26–0.49 ng/μl DNA per 100 ml of water. DNA samples were stored at −20°C until further analysis.

DNA extracts were used to amplify (in triplicate) the V4 region of the 16S rRNA gene using the primers 515F (Parada et al., 2016) and 806R (Apprill et al., 2015). Dual-index primer constructs were designed by modifying the Earth Microbiome Project (EMP) Illumina amplicon protocol as described in Schuelke et al. (2018). All primer constructs and oligo sequences have been made available on FigShare (https://doi.org/10.6084/m9.figshare.5701090). Amplification of the 16S rRNA gene was performed following the EMP protocols (Caporaso et al., 2012). PCR reactions contained 1 μl of DNA template, 0.5 μl of each primer (10 μM), 10 μl of Platinum Hot Start PCR Master Mix (2x) (Thermo Fisher), and 13 μl of molecular-grade water. Positive (ZymoBIOMICS Microbial Community Standard; Zymo Research) and negative (molecular-grade water, HyClone HyPure Water, GE, Healthcare Life Sciences) controls were included in all PCRs. Thermocycling profile included the following steps: 94°C for 3 min; 94°C for 45 s, 50°C for 60 s and 72°C for 90 s for 35 cycles; and 72°C for 10 min. Amplification success was evaluated with gel electrophoresis (agar 1%). Additional details on PCR conditions and purification are also provided in Schuelke et al. (2018).

Sample DNA concentrations were measured using a Qubit 3.0 fluorometer with the dsDNA HS (high sensitivity) assay kit (Thermo Fisher Scientific) and normalized prior to pooling. The DNA library was subjected to a final magnetic bead cleanup step, followed by size selection (300–700 bp range) on a BluePippin (Sage Science) to remove any nontargeted DNA. A bioanalyser trace was run on the size-selected pool as a quality control measure, and the library sequenced on the Illumina MiSeq Platform (2 x 300-bp paired-end run) at the UC Davis Genomics Core Facility (Schuelke et al., 2018).

All wet laboratory protocols and downstream bioinformatics scripts used in this study have been deposited on GitHub (https://github.com/BikLab/doliolids).

2.4 | Quantitative polymerase chain reaction (qPCR) assay

Bacterial abundances associated with EG, FP24h, and SW sample types were estimated by real-time qPCR on a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories) using 932F and 1062R universal 16S rRNA targeted primers (Allen et al., 2005). qPCR reactions were performed in 20 µl reactions containing a final concentration of 1x SsoFast EvaGreen Supermix (Bio-Rad Laboratories), 0.3 µmol of each primer, and 1 µl genomic DNA per reaction. Quantitative standard curves were generated from a six-order of magnitude serial dilution of plasmid DNA (pDNA) containing a cloned copy of the target 16S rRNA gene (*E. coli*) ranging from 10^1 to 10^7 target gene copies per reaction. qPCR cycling conditions included the following steps: 95°C for 30 s followed by 45 cycles of denaturation (95°C, 5 s) and annealing/extension (54.7°C, 5 s). After cycling, product melt temperatures were evaluated from 60 to 95°C at 0.5°C increments for 5 s each. Samples, standards, and no-template controls were assayed in triplicate. rRNA gene copy numbers were normalized to volume. Volumes of doliolids and faecal pellets were estimated from microscopically determined size estimates assuming a barrel and spherical shape, respectively.

2.5 | Bioinformatics and statistical analyses

Raw Illumina data were demultiplexed using a custom script for handling dual-index barcodes and then analysed in QIIME2 version 2020.11 (Bolyen et al., 2019). Primer and adapter sequences were trimmed (error rate of 0.1 and reads discarded when lacking adapter/primer) using the cutadapt plugin (Martin, 2011). Denoising was based on optimal parameters (forward and reverse reads truncated at 237 and 253 bp, respectively; median PHRED score of ≥30). Amplicon sequence variants (ASVs) were estimated using the high-resolution single-nucleotide difference DADA2 method (Callahan et al., 2016) based on default parameters and consensus chimera checking parameters. Taxonomy assignments of ASVs were obtained with the BLAST+ consensus taxonomy classifier (minimum confidence value of 0.8; [Camacho et al., 2009]) and the SILVA 138 SSURef NR99 release (Quast et al., 2013).

Our final data set consisted of 115 samples, and except for PCR negative controls, all had high sequence depth (>2000 reads, Table S1, Appendix S1). Preliminary analyses revealed that ASVs found in PCR controls were not shared by experimental samples (Figure S1, Appendix S2). Still, the R package decontam (prevalence; threshold of 0.5) was used to assess the levels of contamination in the data set (Davis et al., 2018). Only four ASVs were identified as contaminants, which were removed prior to analyses assessing patterns of microbial community variation among sample types.

Diversity estimates including Observed, Shannon H′ (Log_2), Inverted Simpson (D), and Pielou's Evenness (J') were extracted from the filtered ASV table using the package phyloseq (McMurdie & Holmes, 2013), and compared among sample types for each experiment separately. Data normality was assessed using Shapiro–Wilk's method, and Kruskal–Wallis (K–W) tests were used to assess differences among sample types with the package FSA version 0.8.24 in R version 4.1.2 (R Core Team, 2021). The Mann–Whitney U test with adjustments for *p*-value (BH method; [Benjamini & Hochberg, 1995]) was used for pairwise comparisons (Zar, 2010).

To visualize the similarity of microbial communities among sample types, a similarity matrix based on Bray–Curtis similarity and ASV-transformed abundances standardized by total and square root transformed values was constructed. Ordination was done by non-parametric multidimensional scaling (nMDS) and Goodness-of-fit given by the stress value (Clarke, 1993). The permutational analysis of variance (PERMANOVA) was used to test for significance among sample types (Anderson et al., 2008).

Differential abundance analyses were performed using the R package ALDEx2 (version 1.12.0) (Fernandes et al., 2013, 2014). ASV counts were transformed using the centred-log ratio method for a compositionally coherent inference and estimates. Significant differences ($p < .05$) among sample types were assessed through K–W tests at each taxonomic rank. False discovery rates (FDRs) were estimated using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). A heatmap (taxa: rows; samples: columns) depicting the variation among sample types of taxa differentially abundant was produced. PICRUSt2 was used to predict potential gene functions from microbial community profiles associated with sample types (Douglas et al., 2020). Differential abundance analyses on the matrices of predicted gene functions were performed and visualized as described above. Additionally, predicted gene functions were organized into distinct metabolic pathways following Yilmaz et al. (2015). In this study, all visualizations were produced with ggplot2 version 3.1.1 (Wickham, 2016) using R version 4.1.2 (R Core Team, 2021).

3 | RESULTS

3.1 | Estimates of bacterial abundance

Bacterial biomass, as inferred from 16S rRNA gene copy abundance, was assessed by qPCR and normalized to sample volume (Figure 2). Bacterial cell numbers were not estimated from rRNA copy numbers because rRNA copy number is known to vary based on cell type and activity (Klappenbach et al., 2001). The abundance of bacterial rRNA gene copies in FP24h was at least two orders of magnitude higher than that found in SW ($8.0 \pm 7.1 \times 10^5$ vs.

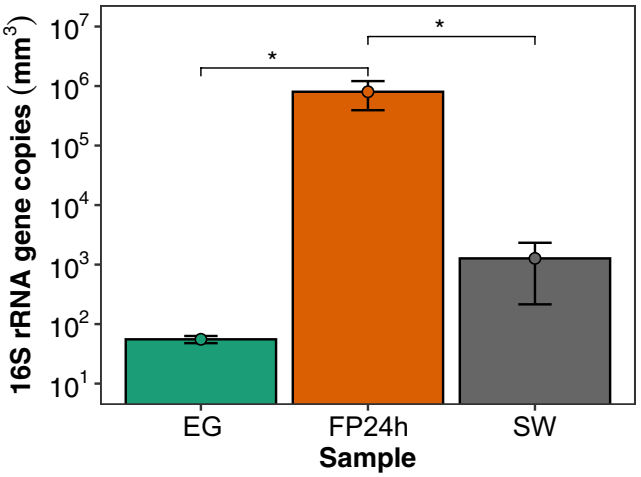

FIGURE 2 Abundance (mean ± SE) of bacterial communities associated with EG, FP24h, and SW samples. Bacterial abundance was inferred from 16S rRNA gene copy abundance quantified by quantitative PCR (qPCR) and normalized to sample volume. For significant differences ($p < .05$) in pairwise comparisons among sample types, the reader is referred to Table S2

$1.3 \pm 1.8 \times 10^3$ copies mm^{-3}) indicating that faecal pellets support the rapid proliferation of bacterial communities. In contrast, the abundance of 16S rRNA copies was an order of magnitude lower in EG samples compared to the water column on a per-volume basis ($0.05 \pm 0.01 \times 10^3$ copies mm^{-3}).

3.2 | Microbial α-diversity estimates across sample types

Overall, the mean number of reads and ASVs were similar across experiments (Exp1: 32,274 and 139, Exp2: 38,518 and 144, Exp1: 27,833 and 145; Figure 3). Lowest diversity estimates were observed in EG samples, except in Experiment 1 where the lowest diversity observed was in the FG samples. Conversely, α-diversity estimates were generally highest in SW samples followed by either FP2h or FP24h. A summary of comparisons across sample types for the number of reads and different diversity estimates is provided in Figure 3.

Significant differences ($p < .05$, K–W analysis) among sample types were detected for all the diversity indices. Pairwise comparisons revealed that EG samples often had significantly lower values for all α-diversity indices when compared to the other sample types. Alternatively, equivalent α-diversity levels were observed ($p > .05$) in the SW, FP2h, and FP24h samples (Figure 3; Table S2, Appendix S1).

Only 20%–30% of ASVs (30–40) were shared among all sample types. In Experiments 1 and 3, the highest number of shared ASVs was observed between FG and FP2h/FP24h samples (26 and 79 ASVs, respectively), whereas in Experiment 2, the highest number (55 ASVs) was observed between FG and FP24h samples (Figure S2, Appendix S2). The FG samples contained the most unique ASVs (221–566), especially in Experiments 2 and 3, whereas FP2h and FP24h often had the lowest number of ASVs (Figure S2, Appendix S2).

At the phylum level, microbial communities associated with the different sample types were dominated by four (out of 37 phyla) major groups including Proteobacteria (up to 95%), Cyanobacteria (up to 59%), Planctomycetota (up to 38%), and Bacteroidota (up to 16%; Figure 4a; Figure S3, Appendix S2).Whereas Proteobacteria was highly abundant in all experiments and sample types, especially in the SW and EG samples, Cyanobacteria and Planctomycetota tended to be more abundant in FG and faecal pellets samples (Figure 4a). Interestingly, of the Proteobacteria, the EG and aged FP (FP24h) samples were generally dominated by representatives of the Enterobacterales while the other sample types were dominated by Pseudomonadales or Rhodobacterales (Figure 4b). The presence of SAR11 (Alphaproteobacteria) was primarily observed in SW samples (9%–38%). Of these, representatives of SAR11 Clade 1a were most common. Moreover, SW samples were often characterized by many low abundant taxa classified as "Others" (Figure 4; Figure S4, Appendix S2).

3.3 | Potential evidence for a core microbiome in the doliolid gut

To explore the presence of a "core microbiome" in the doliolid gut, we analysed EG samples in greater detail. A total of 29 ASVs were exclusively found in EG samples with nine often recovered as ≥50% in all three feeding experiments (Figure 5). These ASVs may be representative of a doliolid core microbiome. Among these potential core taxa, the genera *Pseudoalteromomas* and *Shimia* were the most abundant. Other potential core microbiome members include the genera *Pelagibaca* and *Alteromonas*. In addition to being abundant in the EG samples, these taxa were recovered in all EG samples (Table S4, Appendix S1). Although EG doliolids harboured a considerable number of unique ASVs (148–250), less than 50 of these were shared with other sample types (Figure S2, Appendix S2). Together, these findings support the idea that EG doliolids have a distinct microbial community.

3.4 | Microbial β-diversity patterns and distinct groupings by sample type

Regardless of the experiment, microbial communities were always structured by sample type (Figure 6). The least diverse EG samples clearly separated from the other sample types. Although significantly different, FG, FP2h, and FP24h tended to be close in nMDS bidimensional space, whereas SW samples also differed from all other sample types (Figure 6).

PERMANOVA analysis confirmed that the associated microbial community significantly differed among sample types. Highest similarity values were always found between FP2h and FP24h sample types (52.7%–54.8%) and lowest between SW and FG (10.2%) or between SW and EG (2.9%–9.1%) sample types. In other words, faecal

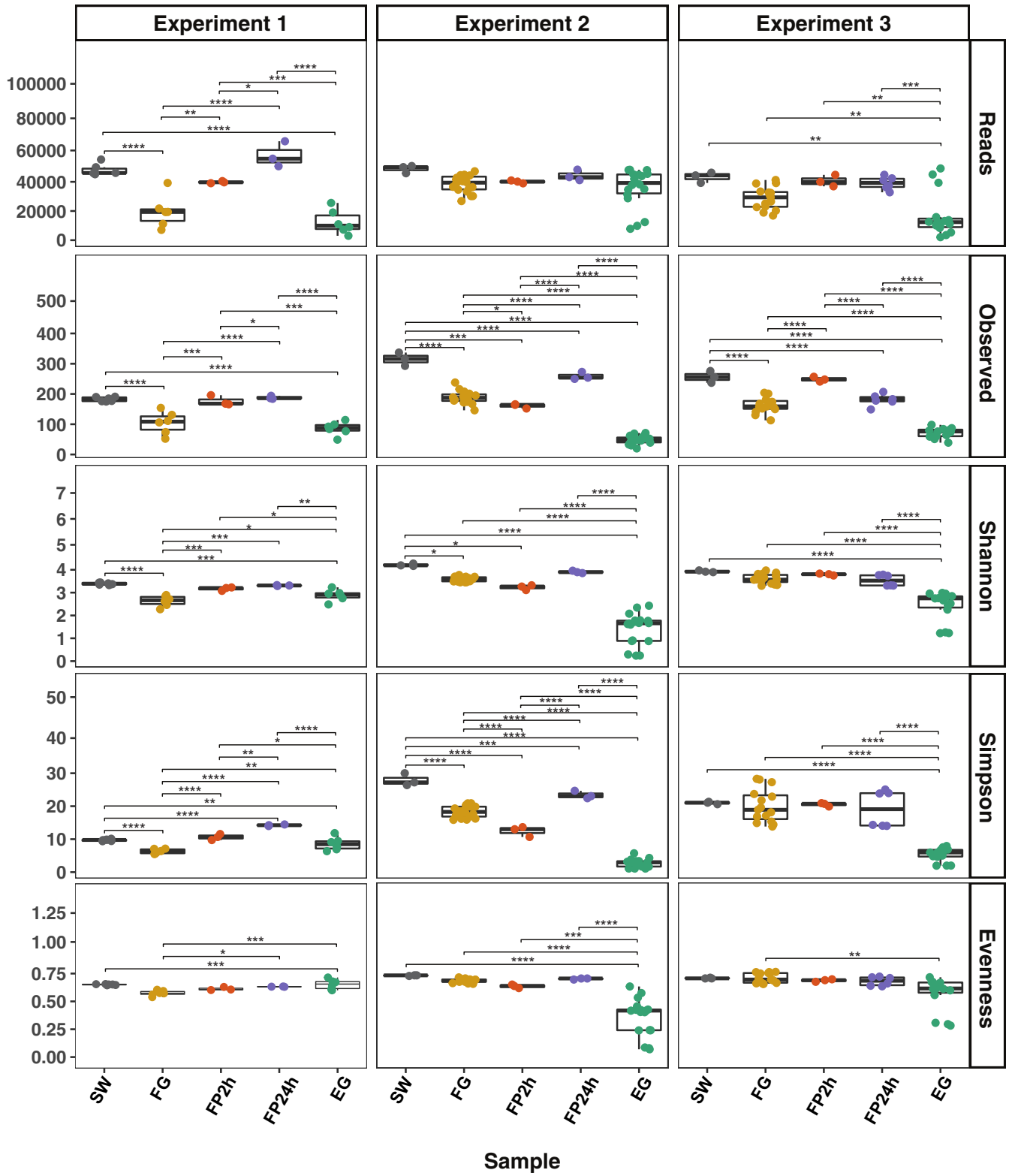

FIGURE 3 Univariate descriptors (mean ± SE) for microbial communities associated with doliolids (FG and EG), faecal pellets (FP2h and FP24h), and seawater (SW) samples in different feeding experiments (1–3). Observed diversity refers to the number of unique ASVs. Only significant differences ($p < .05$) among sample types are shown (see Table S3 for additional details)

pellet microbial communities tend to always resemble each other, whereas the doliolid gut microbiome exhibits a unique and distinct microbial community structure compared to seawater. The analysis of average similarity also indicated that the microbial communities associated with FG and SW samples were more similar to those in the FP2h or FP24h samples. These values, however, were often higher between FG and faecal pellets than between SW and faecal pellets (Table S5, Appendix S1).

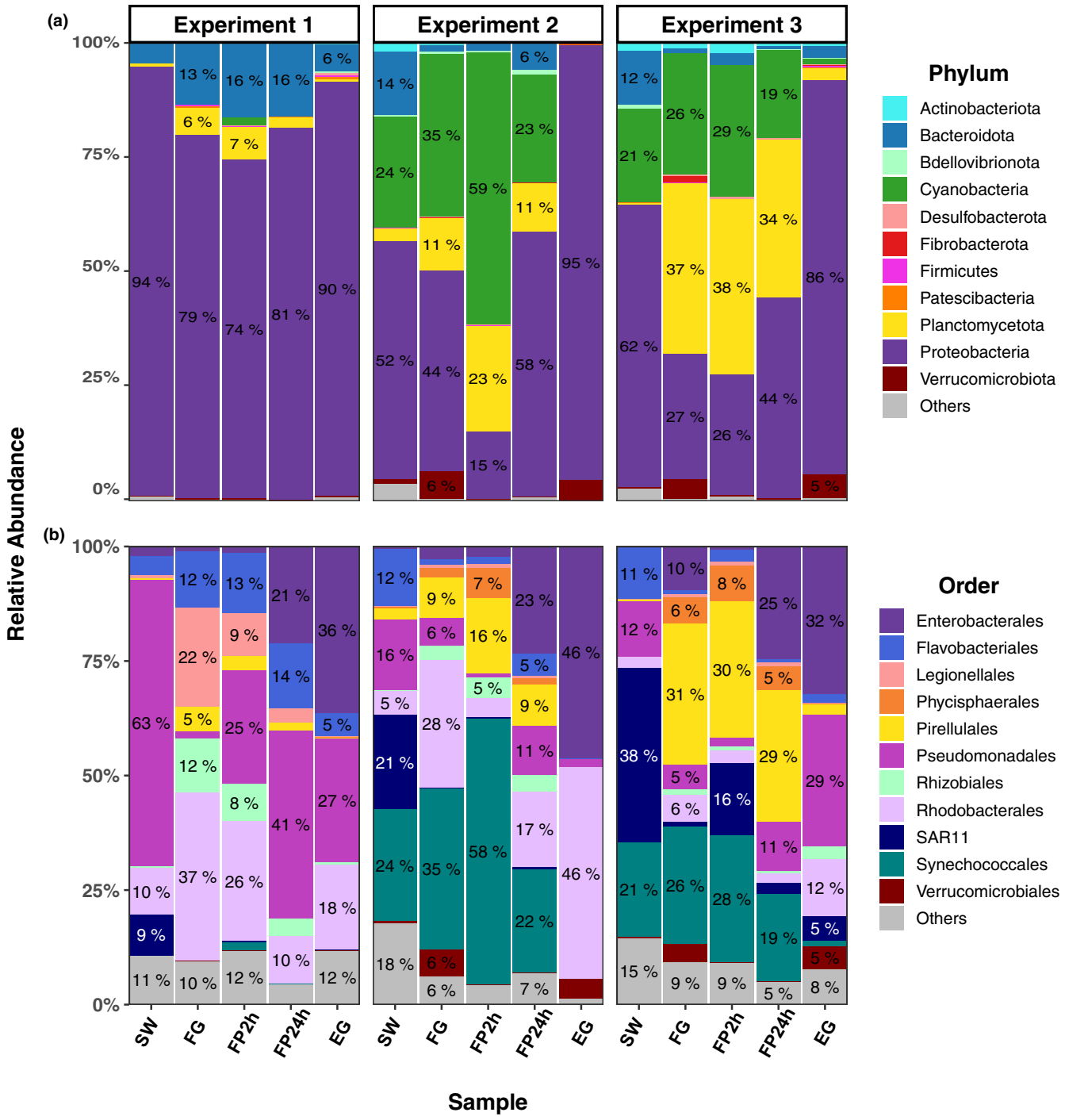

FIGURE 4 Microbial community composition associated with doliolids (FG and EG), faecal pellets (FP2h and FP24h), and seawater (SW) samples in different feeding experiments (1–3). Taxonomy of the 11 most abundant bacterial phyla (a) and orders (b) are given. Low abundance taxa were grouped into the "others" category. Relative abundance of taxa contributing to ≥5% is displayed in the barplots

3.5 | Differential abundance analysis of microbial taxa

Among bacterial phyla, Planctomycetota was the most differentially abundant taxa across sample types in all three experiments and it was consistently more abundant in FP2h, FP24h, and FG samples. The SAR324 MG-B group was important in SW samples in all three experiments (ranked in 5, 10, and 12 in abundance, respectively), in addition to *Marinimicrobia* (ranked 15 and 16 in abundance) and *Bacteroidota* (ranked 8 and 6 in abundance, respectively). Conversely, *Acidobacteriota* was differentially abundant and more commonly found in EG and FG samples. *Cyanobacteria* was differentially abundant in all three experiments (ranked 4, 2, and 2 in abundance, respectively) and particularly important in the FP2h, FP24h, and FG samples (Figure S5, Appendix S2).

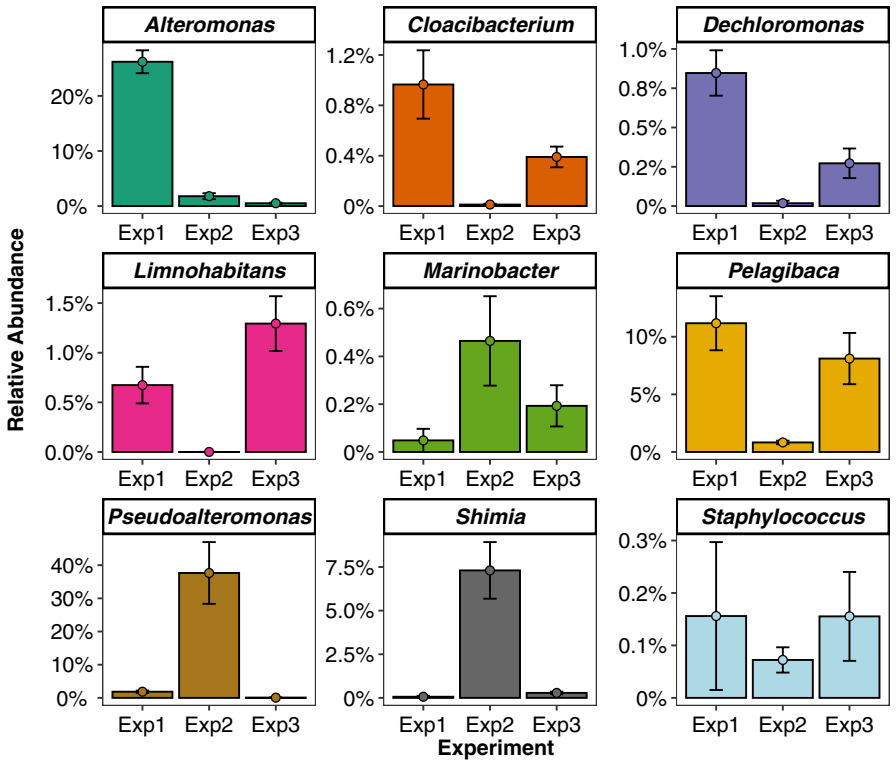

FIGURE 5 Abundance (mean ± SE) of nine frequently recovered bacterial taxa (abundance ≥50%) in EG samples across different feeding experiments. Additional information, including from other taxa often recovered in EG samples, is provided in Table S4

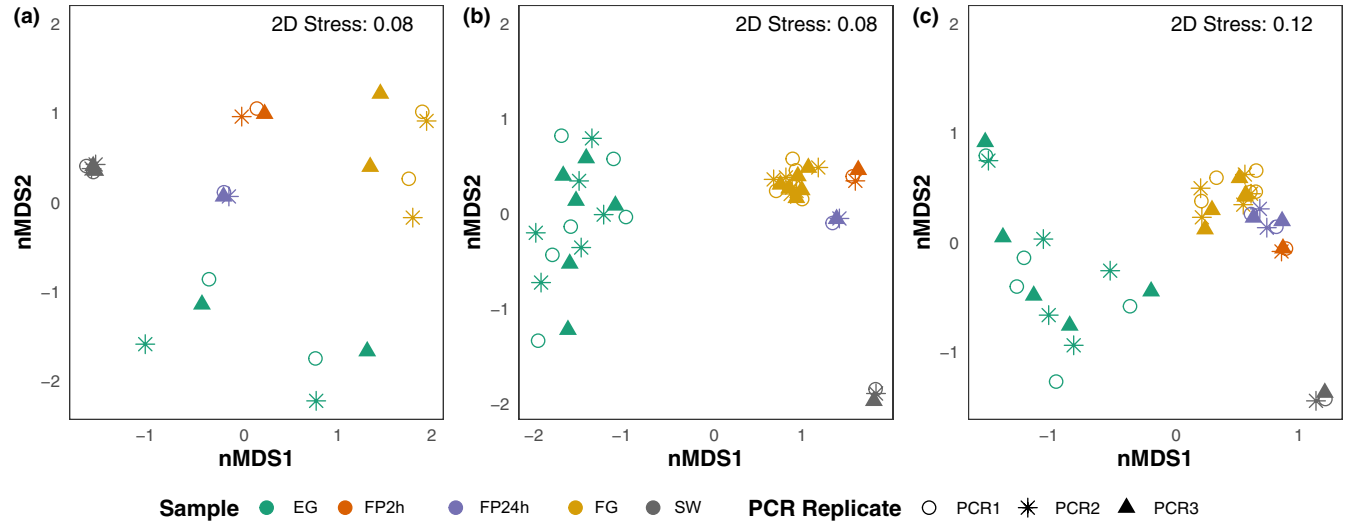

FIGURE 6 Microbial community structure according to experiment (a) Experiment 1, (b) Experiment 2, (c) Experiment 3) and sample types: doliolids (FG and EG), faecal pellets (FP2h and FP24h), and seawater (SW). The nMDS ordination is based on the Bray–Curtis similarity constructed from the relative abundance of ASVs (square root transformed). Control samples and ASVs determined to be contaminants were removed from the analysis (see methods for additional details; Figure S1, Appendix S2)

At the genus level, sample types including FP2h, FP24h, and to a lesser extent FG, displayed similar patterns of differentially abundant taxa (Figure S6, Appendix S2). For example, an uncharacterized Pirellulaceae family member was most important in these three sample types. In the EG sample *Alteromonas* was differentially abundant whereas *Cyanobium* PCC-6307 and *Blastopirellula* were relatively important across all sample types, except in EG doliolids. Variation of differentially abundant taxa across sample types and experiments was also observed (Appendix S1, Table S6).

3.6 | Predicted functional genes and metabolic pathways

The most abundant predicted metabolic functions were consistently recovered across sample types and experiments and reflected the core functions of microbial cellular machinery. For example, a DNA polymerase (EC:2.7.7.7), was recovered with higher predicted abundance (always >1%) in SW, EG, and FG samples and a DNA helicase (EC:3.6.4.12) in FP2h. Uronate dehydrogenase (EC:1.1.1.203) was also important in all sample types, except EG doliolids, whereas

tryptophan 2,3-dioxygenase (EC:1.13.11.11) was more important in EG and SW samples. Differentially abundant predicted functions also supported similarities among FP2h, FP24h, FG, and to a lesser extent SW sample types (EC:2.7.1.31 - Glycerate 3-kinase, EC:2.3.1.31 - Homoserine O-acetyltransferase). A summary of the most differentially abundant predicted gene functions for each experiment is provided in Figure S7 (see Table S7, Appendix S1 for additional details).

Similar to the separation observed in the nMDS analysis of the composition of doliolid associated microbial communities (Figure 6) sample types were also separated by predicted functional genes (Figure S8, Appendix S2). The predicted functions/genes recovered in the *PICRUSt2* analysis, and differently abundant among sample types, were also grouped into key metabolic processes in the marine environment including nitrogen, carbon, and sulphur cycling (Figure 7). The contribution of individual predicted functions/genes to the overall abundance was often low (RA<0.1%) and varied according to the feeding experiment. Nevertheless, key genes including those involved in the nitrogen (Ammonification: *ureC*, Denitrification: *narG*, N reduction: *nasA* and *nirB*) and sulphur (sulphate reduction: *sat*, *met3*) cycling were predicted to contribute to the functional potential found in the different sample types. The major contributor to a specific predicted function also varied by experiment. For example, the major contribution to *narG* (EC:1.7.99.4) involved in denitrification came from SW, FG, and EG for experiments 1, 2, and 3, respectively. A complete list of the predicted functions/genes and their contribution to specific metabolic processes are provided in Appendix S1 (Table S8).

4 | DISCUSSION

Due to their intimate relationship with microbial-rich particles, gelatinous filter-feeding zooplankton are probably critical mediators between the grazing food web and microbially-mediated biogeochemical cycling (Frischer et al., 2021). Despite the likely importance of small gelatinous zooplankton species as a conduit of energy and matter into the microbial loop rather than into the grazing food web, the relationship between microbial loop processes and taxa such as doliolids has not been carefully investigated. In this study we focused on characterizing doliolid-associated microbiomes and the ecosystem components (seawater and faecal pellets) that they directly interact with. Characterization of doliolid microbial communities using a 16S rRNA targeted metabarcoding approach indicates that at the highest taxonomic levels, doliolid-associated bacterial communities are characteristic of marine bacterioplankton communities around the globe (Coutinho et al., 2021; Schauer et al., 2003; Silva et al., 2017; Yilmaz et al., 2015). Doliolid associated bacterial communities were dominated by Gammaproteobacteria (avg 35.1 ± 23.5%), Alphaproteobacteria (avg 26.6 ± 14.5%),

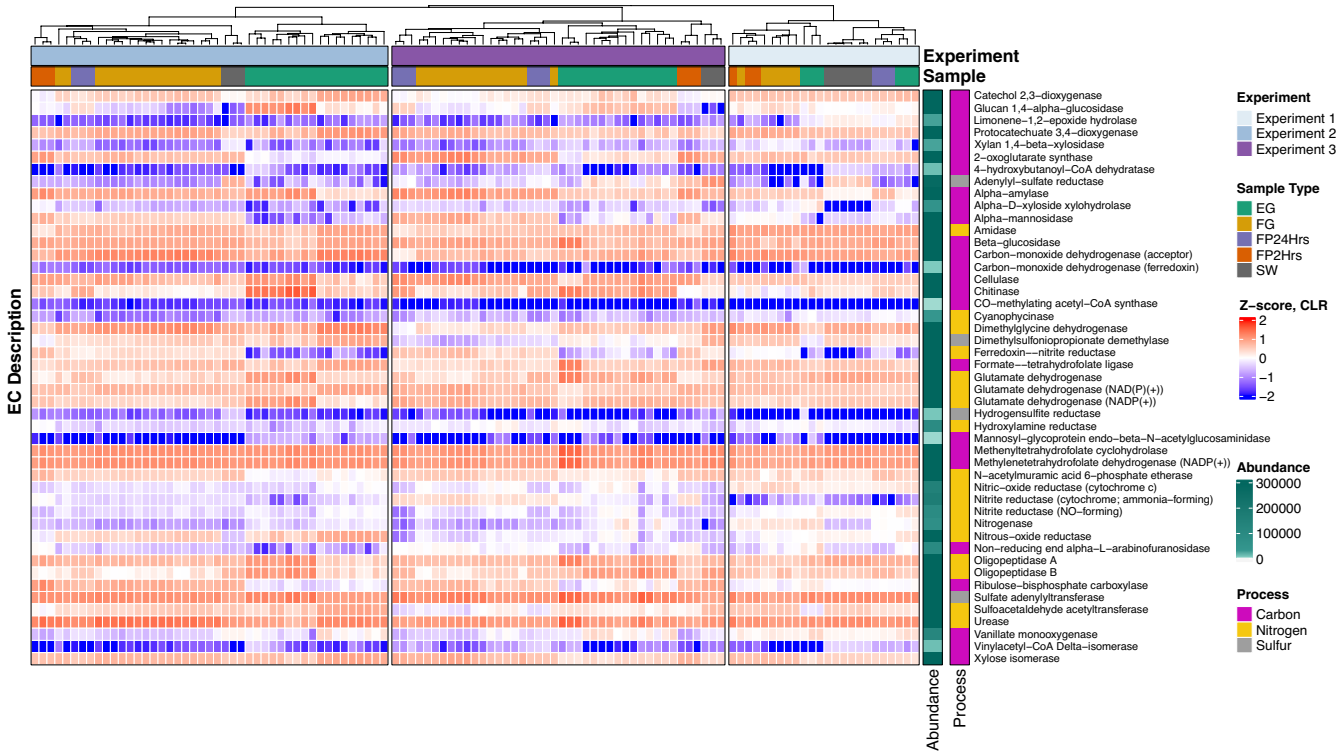

FIGURE 7 Heatmap of differentially abundant predicted functions associated with carbon, nitrogen, and sulphur cycling across sample types (EG: Empty gut, FG: Full gut, FP24h: Faecal pellet 24 h, FP2h: Faecal pellet 2 h, SW: Seawater) and experiments. The abundance of different functions/genes was transformed using the centred-log ratio (CLR). Warm colours indicate high abundance whereas cold colours indicate low abundance. A complete list of functions/genes differentially abundant, including their description, for each experiment and across sample types is provided in Table S7

Cyanobacteria (avg 16.3 ± 17.3%), Planctomycetes (avg 9.5 ± 11.7%), Bacteroidia (avg 6.3 ± 6.1%), and Phycisphaerae (avg 1.9 ± 2.9%) (Figures 4, 5, 7; Figure S3, Appendix S2). Comparison between sample types in our experimental approach, however, revealed distinct patterns in diversity and biomass.

4.1 | Doliolids have a low-diversity microbiome distinct from surrounding seawater and faecal pellets

Bacterial communities associated with the empty guts of starved doliolids (EG), presumably representative of the doliolid gut microbiome, possessed the lowest volume normalized biomass (log 1.7 ± 0.1 16S rRNA copies mm^{-3}; Figure 2) and lowest α-diversity ($H' = 2.24 \pm 0.75$; Figure 3) of all the sample types examined in this study. In the three independent experiments conducted, the number of ASVs that were unique to EG samples averaged 13.5 ± 2.2% of the total ASVs observed among all the samples and support the hypothesis that doliolids possess a unique but low diversity, low biomass microbiome, similar to previous descriptions of the microbiomes of marine gelatinous species (Berger et al., 2021; Daley et al., 2016; Daniels & Breitbart, 2012; Kos Kramar et al., 2019; Thompson et al., 2021). This observation is consistent with the hypothesis that the doliolid gut is a highly specialized environment optimized for detritivory, and further supports the emerging understanding that small gelatinous zooplankton such as doliolids fill a trophic niche as functional detritivores (Frischer et al., 2021). Among the potential core taxa, representatives of the Gammaproteobacteria class in the genus *Pseudoalteromomas* and the Alphaproteobacteria class in the genus *Shimia* were the most abundant taxa recovered. Both these groups of bacteria are typically associated with the digestion of marine autotrophic biomass in marine invertebrates, including gelatinous species (Choi & Cho, 2006; Thomé & Rivera-Ortega, 2022). Interestingly, marine *Pseudoalteromomas* species are often reported to be associated with marine eukaryotes (especially microbial metazoan taxa; [Boscaro et al., 2022]) and are known to exhibit a range of antibacterial, bacteriolytic, agarolytic and algicidal activities (Bosi et al., 2017; Holmström & Kjelleberg, 1999). Their presence in the doliolid microbiome may help explain the low bacterial biomass and diversity observed in the EG doliolids. Furthermore, these core microbiome taxa are consistent with observations of host-associated bacterial clades identified across diverse marine invertebrates (Boscaro et al., 2022), suggesting that gelatinous zooplankton taxa have similar microbiome assemblages to other pelagic and benthic invertebrate taxa found in marine habitats worldwide.

In contrast to the doliolid gut microbiome, microbial communities in seawater in which the doliolids had been contained exhibited the highest α-diversity ($H' = 3.84 \pm 0.4$; Figure 3) and intermediate biomass (log 2.72 ± 0.7 16S rRNA copies mm^{-3}; Figure 2). Seawater bacterial communities were characterized by relatively high proportions of well-known oligotrophic members of the SAR11 clade, particularly representatives of SAR11 clade 1a. This group of bacteria are typical oligotrophic members of ocean bacterioplankton including in the SAB (Giovannoni, 2017; Lu et al., 2015). In addition to bacterial taxa that were common in all doliolid sample types, seawater microbial assemblages were also characterized by a large number of low abundance (relative abundance <5%) bacterial taxa. These contrasting α-diversity patterns between doliolid-associated and seawater microbial assemblages are consistent with findings from other gelatinous zooplankton groups such as pyrosomes (Berger et al., 2021).

Bacterial communities associated with full gut animals and faecal pellets exhibited intermediate α-diversity ($H' = 3.27–3.58$) and high biomass (log 16S rRNA copies = 2.5–5.7). These observations are consistent with selection for copiotrophic microbial communities in faecal material capable of utilizing nutrient rich prey particles concentrated by the doliolid feeding (Gasol & Kirchman, 2018). Full gut animals are presumably characterized by imported microbes (as the biomass of empty gut animals is so low), suggesting that digestion of prey particles is initiated in the concentrated environment of the doliolid gut but completed following faecal pellet egestion – a scenario where additional microbial communities are recruited from bacterial communities present in the surrounding seawater.

Microeukaryotic components of faecal pellet and doliolid gut communities were not explored in this study but are likely to also be present and contribute to the processing of consumed and egested materials. Pomeroy and Deibel (1980) reported observing that fresh *D. gegenbauri* pellets contained dense bacterial populations followed by protistan communities that persisted for up to 4 days by consuming bacteria. Previous studies investigating the diet of *D. gegenbauri* in the SAB that utilized 18S rRNA targeted molecular gut content analysis approaches reported that wild caught *D. gegenbauri* gut samples contained diverse protist communities (Frischer et al., 2021; Walters, Lamboley, et al., 2019). It could not be determined in these studies whether these organisms were consumed as prey or recruited as symbionts. Future work should also investigate the potential contributions of eukaryotic microbiome taxa to faecal pellet processing and nutrient remineralization in diverse gelatinous zooplankton taxa.

Although there was considerable variability between experiments in terms of the overall composition of microbial communities (Figure 3), the observed microbial assemblages were consistently structured by sample type across experiments (Figure 6). In all three experiments, bacterial communities associated with each sample type were more similar to themselves than bacterial assemblages recovered from other sample groups. Empty gut samples clustered together separately from full gut and faecal pellet-associated communities, and seawater-associated communities. Despite differences in bacterial communities present in the seawater in each of the three independent feeding experiments, the relationships in similarity between communities associated with the different sample types remained consistent, supporting the hypothesis that the presence of doliolids and the environments and particles that they produce predictably influence the structure of microbial communities. Notably, these findings were consistent even when starting seawater communities contained distinct microbial assemblages (distinct clustering of seawater microbes from Experiment 1 vs. Experiments 2 and

3), suggesting that doliolid feeding activities and pellet production are likely to produce similar impacts on nutrient remineralization and biogeochemical cycling regardless of local habitat factors in pelagic ecosystems worldwide.

4.2 | Ecological significance of doliolid microbial interactions: Implications for biogeochemical cycling in marine pelagic ecosystems

Comparisons between bacterial communities associated with seawater, feeding doliolids, and faecal materials derived from these experimental studies suggests that through their presence and trophic activity, small gelatinous zooplankton such as doliolids influence the structure of pelagic food webs and biogeochemical cycling in subtropical continental shelf systems where filter-feeding tunicate blooms are common. The presence of both copiotrophic bacteria and typical oceanic oligotrophic bacteria in seawater samples suggests that the activities of doliolids enrich oceanic microbial communities with copiotrophic bacteria, supporting the hypothesis that doliolid blooms, similar to jellyfish blooms, would be expected to intensify system heterotrophy, reduce the efficiency of the biological pump, and limit trophic transfer to the grazing food web (Condon et al., 2011; Tinta et al., 2021).

The presence of doliolids may also influence biogeochemical cycling. In this study, the functional potential of microbial communities associated with the different doliolid sample types was predicted, providing initial insights into how doliolids and their faecal pellets may impact the ocean microbial loop and metabolic processes. Although functional predictions based on 16S rRNA sequencing have significant limitations (Sun et al., 2020), with growing databases and experimental validation, the reliability of this approach has been rapidly improving (Douglas et al., 2020). Importantly, these gene predictions provide a basis for generating hypotheses related to the functional potential of microbial communities associated with gelatinous zooplankton and doliolid faecal pellets that will be useful for guiding future research utilizing more targeted -Omics approaches including transcriptomic studies (de Voogd et al., 2015; Suárez-Moo et al., 2020; Yilmaz et al., 2015).

The predicted functional analysis conducted in this study identified a large number of genes related to ecologically significant metabolic processes. Those that were detected at high relative abundances (<0.1) and were related to important pelagic biogeochemical cycling processes are discussed here. Key genes involved in the nitrogen cycle (Kuypers et al., 2018) and sulphur cycle (Tang, 2020) were detected as being over-represented in doliolid-associated samples. For example, genes involved in nitrogen reduction (*nir*B) and denitrification (*nir*S, *nir*K, and *nos*Z) were predicted to be present in faecal pellets (especially FP2h) in all three experiments. Nitrogen fixation (*nif*H) was predicted to be associated with EG doliolids and faecal pellets suggesting that doliolids may enhance the N content of ingested detrital particles. Interestingly, Scavotto et al. (2015) reported *nifH* in seawater particles and full gut copepods leading the authors to conclude that copepods may acquire the bacteria capable of N_2 fixation through feeding. The proportion of potential bacteria carrying *nifH* in SW samples, however, was low in this study suggesting that doliolids may acquire *nifH* differently than copepods. The presence of genes involved in sulphur cycling including *dmd*A involved in the metabolism of dimethylsulphoniopropionate was also predicted to be elevated in abundance in EG doliolid, faecal pellets and seawater suggesting the involvement of doliolids in key sulphur cycling processes. In ctenophores, Daniels and Breitbart (2012) associated this functional potential with high abundance of the bacterial genus *Marinomonas*. Various members of the Marinobacter group including *Marinomonas* were identified in this study as a potential member of the doliolid core microbiome (Figure 5).

Predicted functions to degrade organic compounds including chitin were relatively abundant in doliolid materials compared to seawater samples. Chitinase (EC:3.2.1.14) encoding genes, for example, were detected at high relative abundances in FP24h and EG samples (Figure S7, Appendix S2). Similarly, De Corte et al. (2018), using a metagenomic approach, speculated on the potential of zooplankton-associated bacterial communities in metabolizing chitin and other complex organic molecules based on the presence of chitinase encoding genes. Chitin degradation in copepods (Scavotto et al., 2015) and jellyfish (Kos Kramar et al., 2019) have been associated with bacterial communities dominated by *Vibrio* spp. In this study, the proportion of Vibrionaceae including *Vibrio*, although relatively low and mostly restricted to Experiment 3, was primarily found in FG and EG samples. Chitinases have also been reported to be produced by *Pseudoalteromomas* ssp. (Cottrell et al., 2000), a taxon extremely abundant in EG samples of Experiment 2. In fact, predicted abundances of potential bacteria that may produce chitinases were highest in EG samples from Experiment 2. These community-level predictions of microbial function provide an important stepping stone between microbiome taxonomic profiles in marine invertebrates (Boscaro et al., 2022) and future experimental approaches designed to test functional hypotheses regarding the role of gelatinous zooplankton in pelagic marine ecosystems worldwide.

5 | CONCLUSIONS

Our results suggest that host-associated microbial communities in small gelatinous zooplankton taxa play important roles in the biology and ecology of groups such as doliolids, influencing pelagic marine bacterioplankton communities and the processes they mediate. Because doliolids and other filter-feeding pelagic tunicates are capable of forming massive blooms in some of the most productive regions of the ocean, the ecological implications of doliolid-microbe interactions are likely to be significant. While many physical, chemical, and biological factors influence the composition and stability of host-associated microbiomes, our analysis of starved doliolids suggest that some microbial taxa

are probably part of a resident low-biomass doliolid microbiome, and these core taxa are consistent with host-associated bacteria identified across diverse marine invertebrate clades (Boscaro et al., 2022). To our knowledge, this is the first study to characterize the bacterial communities associated with doliolids and their faecal pellets using a 16S rRNA metabarcoding approach. 16S rRNA metabarcoding distinguished bacterial communities associated with the different sample types, providing new insights into the interactions between doliolids and microbial communities in productive subtropical continental shelf systems. Comparison of microbial communities found in seawater to doliolid-associated communities support the hypothesis that doliolid blooms would be expected to intensify system heterotrophy, reduce the efficiency of the biological pump, and limit trophic transfer to the grazing food web. Exploratory bioinformatic analyses of predicted functional genes suggest that gelatinous zooplankton groups such as doliolids, via their interactions with bacterial communities, may affect important biogeochemical processes including nitrogen, sulphur, and organic matter cycling.

AUTHOR CONTRIBUTIONS

Tiago J. Pereira, Tina L. Walters, Holly M. Bik, and Marc E. Frischer designed and conceived the study, contributed to data analysis, and wrote the manuscript. Tina L. Walters and Marc E. Frischer performed feeding experiments and performed DNA extractions from all sample types. Hisham M. El-Shaffey conducted qPCR analysis and provided intellectual contributions to the manuscript. Tiago J. Pereira completed 16S rRNA PCR amplification, prepared libraries for Illumina sequencing, processed raw sequence data analysis, and executed bioinformatic workflows. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw Illumina reads generated in this study have been submitted to the NCBI Sequence Read Archive (BioProject PRJNA863883 and SRA accession SUB11852035). Primer constructs, QIIME mapping files, and final ASV tables have been deposited in FigShare (https://doi.org/10.6084/m9.figshare.5701090). All scripts used for processing and analysing the data are available on GitHub (https://github.com/BikLab/doliolids).

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data was also submitted to Dryad: https://doi.org/10.5061/dryad.kd51c5b8s.

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