Sources of variation in community composition of the hindgut microbiota in two tropical Kyphosus species

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Abstract Gut microbiota play a fundamental role in the nutrition of many vertebrate herbivores through foregut and hindgut fermentation of plant carbohydrates. Some species of marine herbivorous fishes contain moderate to high levels of short-chain fatty acids in the hindgut, indicating the importance of hindgut fermentation. Herbivorous fish hindgut microbiota are diverse and can vary with geographic location, but data on the scale of geographic variation involving a few km of separation are limited. Here, we used the 16S rRNA gene to describe community composition of the gut microbiota of the herbivorous species Kyphosus vaigiensis and K. cinerascens collected in the vicinity of Lizard Island, northern Great Barrier Reef, Australia, in 2011 and 2017. Microbiota community structure differed between posterior hindgut sections, host species, sampling years and two mid-shelf and outer reef locations approximately 20 km apart. Hindgut bacterial community composition varied remarkably between mid-shelf and outer reef locations, and among individual fish on the mid-shelf reef. In both fish species, the most abundant phyla were Pseudomonadota, Bacillota and Bacteroidota, followed by Spirochaetota, Thermodesulfobacteriota and Verrucomicrobiota. There were no clear differences between the host species in terms of the relative abundance and composition of bacterial genera in outer reef samples. In contrast, the dominant genera differed between mid-shelf samples of K. cinerascens and K. vaigiensis, being Endozoicomonas-like (Pseudomonadota) and Brevinema (Spirochaetota), respectively. Endozoicomonas are emerging as important symbionts in many marine hosts worldwide and are thought to be important in the coral sulphur cycle. Differences in microbiota composition were not associated with variation in fish condition, suggesting that the different microbial taxa perform equivalent functional roles.

Keywords Kyphosidae · Herbivory · Microbiome · ASVs · Coral reef · Endozoicomonas

Introduction

Gut-associated bacteria play fundamental roles in metazoan physiology (Lee and Hase 2014) and nutrition (Stevens and Hume 1998), such as contributing to the digestion of plants in many herbivores (Choat and Clements 1998; Greene et al. 2020). Algal carbohydrates represent a major source of potential energy for marine herbivorous fishes, which different fish taxa exploit to varying degrees (Krogdahl et al. 2005; Clements et al. 2014, 2017). For example, some taxa including mugilids, scarine parrotfish and some grazing acanthurids employ mechanical trituration in a pharyngeal mill or gizzard-like stomach and depend mainly on rapid gut throughput and digestion of protein, lipid and soluble carbohydrate (Clements et al. 2014). Other marine herbivorous
fish taxa, e.g. kyphosids, odacine labrids, aplodactylids and nasine acanthurids, have lengthier gut retention times and rely on gastrointestinal fermentation to salvage energy from refractory carbohydrate (Choat and Clements 1998; Mountfort et al. 2002). Given the algae-rich diet of the latter group of fish taxa, carbohydrate fermentation represents a key process in the marine environment (Choat and Clements 1998). Short-chain fatty acid (SCFA) profiles from the gut of species of the families Acanthuridae, Siganidae, Pomacanthidae and Kyphosidae suggest that the anaerobic microbial populations inhabiting the intestine of these species play a significant role in the nutritional ecology of the host (Clements and Choat 1995; Clements et al. 2009). These fish species contain the highest level of SCFAs in posterior gut sections, also defined as sections IV and V of the intestine (Clements and Choat 1995, 1997), indicating that hindgut sections have the highest levels of microbial fermentation.

Fish gut microbial communities can be highly diverse (Wang et al. 2018) and differ among fish species in taxonomic composition (Sullam et al. 2012). Three bacterial phyla, Pseudomonadota, Fusobacteriota and Bacterioidota (formerly Proteobacteria, Fusobacteria and Firmicutes, respectively), generally dominate the gut microbiota of fish (Tarnecki et al. 2017), with Vibrionales tending to be the most abundant order in many marine fish taxa (Sullam et al. 2012). Trophic level is also known to play a role in shaping the gut microbiota (Sullam et al. 2012). Although there are remarkable differences among trophic levels in fish gut community composition, common microbial taxa are present in carnivores, omnivores and zooplanktivores (Egerton et al. 2018). For example, the family Vibrionaceae, and the genera Aeromonas and Pseudomonas, are frequently reported in fish gut microbiome studies (Egerton et al. 2018), regardless of fish trophic level.

Dietary composition and geographic location influence microbial community composition in these fish gut communities (Tarnecki et al. 2017; Parata et al. 2020). In general, four processes, namely environmental selection, historical contingency, ecological drift and dispersal, interact with each other in different ways to influence microbial community assembly in the host gut (Costello et al. 2012). These processes can all lead to geographic variation in gut microbial community composition (Sullam et al. 2012; Taranaki et al. 2017; Jones et al. 2018; Senghor et al. 2018; Arroyo et al. 2019). Despite our increasing knowledge of fish gut microbiota, we still lack information on the influence of geographic location on gut community composition, and whether such variation influences community function, such as digestion of foods including seaweed and its constituent carbohydrates.

The family Kyphosidae has a worldwide tropical marine distribution and also occurs in temperate Australasia (Knudsen et al. 2019). Herbivorous Kyphosus spp. display high concentrations of SCFA in the hindgut (Clements and Choat 1995, 1997; Choat and Clements 1998; Clements et al. 2017), indicating that gut microbial communities play an important role in nutrient assimilation from seaweeds. The tropical Kyphosus cinerasces and K. vaigiensis display some of the highest levels of carbohydrate fermentation products among other coral reef associated herbivorous fishes and related omnivorous and planktivorous taxa (Clements et al. 2017). However, levels of SCFA do not differ between the posterior gut segments IV and V in K. vaigiensis, whereas in K. cinerasces section V has the highest levels of SCFA in the gut (Clements and Choat 1995, 1997). Most Kyphosus species have specialized gut anatomy associated with microbial fermentation (Clements and Choat 1997; Johnson and Clements 2021); however, the high level of SCFAs is not restricted to distinct hindgut structures (i.e. hindgut chamber) (Clements and Choat, 1997). In K. vaigiensis, the terminal part of the intestine (section V) forms a hindgut chamber or caecal pouch (Rimmer and Wibe 1987); this feature is absent in K. cinerasces (Clements 1997). The two species also exhibit differences in diet. Previous studies conducted at Lizard Island, Australia, show that Kyphosus vaigiensis has a diet dominated by phaeophytes, while K. cinerasces consumes a high proportion of rhodophytes (Clements and Choat 1997; Choat et al. 2002).

The present study used 16S rRNA gene sequences to characterize hindgut microbial community composition in K. cinerasces and K. vaigiensis collected in 2011 and 2017 from two locations in the vicinity of Lizard Island, Great Barrier Reef, Australia. We sought to determine the differences in community composition between: (1) posterior hindgut sections IV and V, (2) the two Kyphosus species, and (3) mid-shelf reef and outer reef locations separated by approximately 20 kms.

Material and methods

Sample collection

Adult specimens of K. cinerasces (n = 10) and K. vaigiensis (n = 8) were collected by spear gun while snorkelling from the northern Great Barrier Reef, Australia (14 40 S, 145 28 E). Fish were collected from two locations, the Lizard Island complex (mid-shelf reef) and the adjacent outer reefs (Fig. 1 and Table 1) during March 2011 and December 2017 with some crossover between years and locations (Supplementary Table 1). Fish were transported on ice to the laboratory at the Lizard Island Research Station (LIRS). Fish length and weight were recorded for each specimen, and intestinal content was collected from posterior gut sections IV and V following Choat et al. (2002). Gut content (digested and partially digested material in the lumen) samples were fixed
Fig. 1 Study location for sample collection on the northern Great Barrier Reef, Australia. Blue-dashed circle indicates mid-shelf reef collection area; red-dashed circle indicates outer reef collection area.

Table 1 Details of fish species, geographic location, number of samples and IDs. Fish collected in 2011 have labels starting with M, while fish collected in 2017 have a G.

| Hindgut section | Kyphosus cinerascens (n = 10) | Kyphosus vaigiensis (n = 8) |
|-----------------|--------------------------------|------------------------------|
|                 | IV                             | V                            | IV                             | V                     |
| Mid-shelf reef IDs | (n = 4); G160IV, G161IV, G179IV, G184IV | (n = 5); G157IV, G158IV, G159IV, G162IV, M241IV | (n = 5); G157V, G158V, G159V, G162V, M241V |
| Outer Reef IDs   | (n = 5); G135IV, G138IV, M96IV, M97V, M103IV, M235IV | (n = 5); G138V, M96V, M97V, M103V, M235V | (n = 2); M90IV, M104IV | (n = 3); M90V, M104V, M106V |
| Total per section | 9 section IV | 8 section V | 7 section IV | 8 section V |
in 80% ethanol and shipped to the University of Auckland, New Zealand, for further analysis in PC1 laboratories following standard, clean laboratory procedures.

DNA extraction

Sufficient DNA for sequencing was obtained from 16 samples each of gut sections IV and V (see Table 1). Prior to DNA extraction, the 80% ethanol-fixed samples were pelleted for three minutes at 20,800 × g (Eppendorf 5804 centrifuge, Hamburg, Germany) and supernatant was removed. DNA extractions were carried out according to the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) manufacturer’s instructions.

PCR amplification and sequencing

The hypervariable V3-V4 region of the 16S rRNA gene was amplified from the hindgut samples. For the 2011 samples, PCR amplification was performed using the following Illumina tagged and barcoded primers: 341F (5′-CCTACG GGNGGCWGCAG-3′) forward (Herlemann et al. 2011) and 806R (5′-GACTACHVGGGTWTCTAAT-3′) reverse (Caporaso et al. 2011). For the 2017 samples, the forward primer was the same, while the reverse primer was changed to 785R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al. 2011). The primer set in 2017 was changed as consistent of an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 50 °C for 45 s, 68 °C for 90 s, and a final extension of 10 min at 68 °C. For the 2017 samples, the denaturation step was 3 min at 95 °C, followed by 25 cycles of 95 °C for 30 s 55 °C for 30 s, 72 °C for 30 s, and a final extension of 5 min at 72 °C. For the 2011 samples, the resulting amplicons were cleaned from the hindgut samples. For the 2017 samples, the forward primer was the same, while the reverse primer was changed to 785R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al. 2011). The primer set in 2017 was changed as consistent of an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 50 °C for 45 s, 68 °C for 90 s, and a final extension of 10 min at 68 °C. For the 2017 samples, the denaturation step was 3 min at 95 °C, followed by 25 cycles of 95 °C for 30 s 55 °C for 30 s, 72 °C for 30 s, and a final extension of 5 min at 72 °C. For the 2011 samples, the resulting amplicons were cleaned from the hindgut samples.

Fish and microbial community analysis

The length–weight relationship (LWR) for each fish species was calculated following Ricker (1973). QGIS 3.2 Bonn (QGIS Development Team, 2018) was used to differentiate the fish collection locations. Hindgut microbial community and alpha diversity indices, observed ASVs, Chao1, Shannon (Shannon and Weaver 1949) and Pielou’s evenness (Pielou 1969), were plotted using R v4.0.1 with Rstudio software version 1.2.1335 and R packages ggplot2 v3.3.5 and vegan v2.5–7. Bacterial community beta diversity was plotted with nonmetric multidimensional scaling (NMDS) of weighted UniFrac distances (Lozupone and Knight 2005). Statistical analysis of alpha diversity indices and NMDS was performed in R using analysis of variance (ANOVA) and permutational multivariate analysis of variance (PERMANOVA, through adonis2), respectively.

Endozoicomonas phylogenetic tree

A phylogenetic tree was constructed using the 18 ASV Endozoicomonas-like sequences and three Actinobacter ASV sequences (used as an outgroup to root the tree) recovered from this study and 14 reference sequences of full length 16S rRNA recovered from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Taxonomy of ASVs
was annotated based on the SILVA SSU Ref NR99 v138 database (Quast et al. 2013). Sequences were aligned with MUSCLE using Geneious (v2020.05, https://www.geneious.com). 16S full-length amplicons were trimmed to increase the quality of the alignment with the regions V3-V4 of the ASVs. A maximum-likelihood phylogenetic tree was built using FastTree v2.1.10-gimkl-2018b (Price et al. 2009, 2010). Finally, the tree was visualized using iTOL (Letunic and Bork 2021).

### Results

#### Hindgut microbiota composition

Based on 16S rRNA gene sequence data, the relatively dominant phyla, on average, across most fish were Pseudomonadota, Bacillota and Bacteroidota, followed by less abundant phyla that included Spirochaetes, Thermodesulfobacteriota and Verrucomicrobiota (Fig. 2). The dominant bacterial taxa in *Kyphosus* species differed between the two geographic areas and gut sections (Fig. 2). In the mid-shelf reef, Pseudomonadota were the most abundant phylum in fish hindgut sections IV (72.8 ± 7.7%, mean ± SE) and V

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**Fig. 2** From left to right: photographs of *Kyphosus* study species; illustrations of their main dietary components: *Laurencia majuscula*, *Sphacelaria tribuloides* and *Cladophora rugulosa* for *Kyphosus cinerascens* and *Udotea argentea* and *Turbinaria ornata* for *K. vaigiensis* (following Choat et al. 2002); gastrointestinal tract morphology and sampling regions for the two study species; and mean ASVs relative abundances for sections IV and V from mid-shelf and outer reef samples of each fish species.
(45.7 ± 13.4%, mean ± SE) of K. cinerascens, and in section V (56.3 ± 7.1%, mean ± SE) of K. vaigiensis. In contrast, in the outer reef, Bacillota were highly abundant in the communities of both sections IV (38.3 ± 5.1%, mean ± SE) and V (51.7 ± 7.7%, mean ± SE) of K. vaigiensis and in section IV (52.8 ± 12.4%, mean ± SE) of K. cinerascens. While Thermodesulfobacteriota were not the most abundant members of the hindgut communities, they comprised up to 5% of some communities. They tended to be more abundant in section IV than V of both fish species, regardless of location fish were collected, whereas Spirochaetes exhibited the greatest difference between fish species, as they tended to be more abundant in K. vaigiensis from the mid-shelf reef, particularly in section IV (40.7 ± 16.7%, mean ± SE).

In K. cinerascens from mid-shelf reef Endozoicomonas-like sequences (Pseudomonadota) were numerically dominant in section IV of all fish studied, where they comprised over 50% of each community (all 2017 samples), and up to 85.2% in sample G184IV (Fig. 3). They were also very abundant in the corresponding section V communities (also 2017 samples), but strikingly less abundant overall in the outer reef samples of the same fish species, regardless of year sampled (2011 or 2017). Gut communities of K. cinerascens from the outer reef exhibited no clear trends in microbial community composition and varied considerably between individual fish. For example, the most abundant genera were Macelibacteroides in section IV sample M96IV (53.6%), and Enterovibrio in section V sample M235V (78.9%), yet their relative abundance was very low in the other samples from the same gut sections (Fig. 3).

Remarkably, the Endozoicomonas-like genus was also very abundant (61.5%) in one individual of K. vaigiensis (section V, mid-shelf reef). The gut microbial community was highly distinct between K. vaigiensis collected at mid-shelf reef (section V) and outer reef (sections IV and V samples) locations, and the prevalent gut taxa were Oscillospiraceae and Enterovibrio (Fig. 3). Other prevalent taxa associated with K. vaigiensis were Acinetobacter and Pseudomonas. While not possible to compare differences in sampling location in K. vaigiensis without considering the potential for temporal effects (all but one fish from the mid-shelf reef were from 2017 and both fish from the outer reef were from 2011), we found the genus Brevinema (Spirochaetes) dominated or were prominent in certain groups of samples and not others. In particular, Brevinema dominated section IV gut communities of K. vaigiensis from the

![Fig. 3 Relative abundance of the 25 most abundant bacterial genera in gut sections IV and V of K. cinerascens and K. vaigiensis from mid-shelf and outer reef samples. Data is based on 16S rRNA gene ASVs, and relative abundances are summed to genus level or the nearest higher rank. Labels with M = samples collected in 2011; those with G = 2017.](https://example.com/fig3.png)
mid-shelf reef, but were largely absent from section V samples from the same fish species and year (2017). Brevinema were also notable community members in six out of seven 2011 samples in sections IV and V (all out reef and one of two mid-shelf reef samples), indicating considerable variation across gut sections and time or space.

One possible reason for the difference in microbial composition between fish collected from mid-shelf reef and outer reef sites across two different sampling years could be variation in fish condition, dysbiosis or an aberrant gut microbiota in some of the fish in one of the two locations. However, our data show that mid-shelf reef and outer reef fish all fell on the same length–weight line \( R^2 = 0.9479 \) for *K. cinerascens* and \( R^2 = 0.9423 \) for *K. vaigiensis*, indicating that all fish were in similar condition.

**Alpha and beta diversity analysis**

Weighted UniFrac beta diversity was used to analyse the differences in microbial community structure between hindgut sections, fish species, sampling year and geographic location. Findings indicate that no one factor alone explained all variation, and factors contributing to variation differed between posterior gut sections in the study fish. Again, geographic location (PERMANOVA, \( p \)-value < 0.05), sampling years (PERMANOVA, \( p \)-value < 0.01) and fish (PERMANOVA, \( p \)-value < 0.001) appeared to influence the gut microbiota of section IV in the two fish species, wherein the outer reef samples (which are a mix of 2011 and 2017 and fish species) clustered together, while mid-shelf samples from both fish species formed a discrete cluster (all 2017, Fig. 5). In contrast, community structure in hindgut section V (Fig. 5) was significantly influenced by fish species (PERMANOVA, \( p \)-value < 0.01).

To evaluate alpha diversity, we assessed a range of metrics based on 16S rRNA gene ASVs. Diversity indices (Shannon and Pielou) and richness estimates (Chao1, ASVs richness) of combined sections IV and V showed no significant difference between location, time or fish species (Fig. 6a), as also confirmed using ANOVA (\( p \)-value > 0.1). Despite sampling different locations and years, microbial community alpha diversity of combined gut sections appeared to be broadly similar (based on ASVs, Fig. 6a). However, when considering genus-level data, where sections IV and V were treated separately (Fig. 3), there is a discernible difference in alpha diversity among samples, particularly in section IV. We therefore also calculated alpha diversity metrics (richness and Shannon index) for each gut section separately based

**Fig. 4** Relationship between length and weight of *Kyphosus* specimens. *K. vaigiensis* shown in orange and *K. cinerascens* in black. Circles represent mid-shelf specimens, and triangles represent outer reef specimens. The length–weight relationship is based on the equation \( \log(W) = \log a + b \log(L) \) (a is the intercept and b is the slope of the equation)
on ASVs (Fig. 6b). These confirmed statistically significant differences in alpha diversity between fish species at each location in section IV across sampling years, but not section V (Fig. 6b, Shannon index). Overall, alpha diversity results revealed similar richness between communities when sections were combined (Fig. 6a), although this was largely due to equivalent community diversity in section V among fish from the two locations. Microbial communities in hindgut section IV were highly distinct due to low alpha diversity in fish from the mid-shelf reef (Fig. 3).

Endozoicomonas phylogenetic tree

To examine the relationship of fish-associated Endozoicomonas-like sequences to previously described Endozoicomonas, we built a phylogenetic tree with reference sequences of Endozoicomonas from GenBank. The Endozoicomonas-like ASV sequences displayed high similarity and formed a distinct clade to that containing reference sequences of Endozoicomonas (Fig. 7). Accordingly, we designated these Kyphosus-associated ASVs as Endozoicomonas-like throughout our study.

Discussion

Microbiota community composition in this study differed considerably between posterior hindgut sections, fish species, two mid-shelf and outer reef locations approximately 20 km apart, and sampling years. The dominant phyla in both fish species were Pseudomonadota, Bacillota and Bacteroidota, followed by Spirochaetota, Thermodesulfobacteriota and Verrucomicrobiota. No single microbial genus was dominant across individual fish of the same species in the microbial composition of outer reef samples. Conversely, the dominant genera differed between mid-shelf samples of K. cinerascens and K. vaigiensis, being Endozoicomonas (Pseudomonadota) and Brevinema (Spirochaetota), respectively. Bacillota and/or Pseudomonadota (formerly Firmicutes and Proteobacteria) taxa are often the dominant groups in herbivorous fishes such as surgeonfish (Miyake et al. 2015;
and butterflyfish (Clever et al. 2020). Bacillota are associated with high-fibre diets and SCFA production in humans (Nobel et al. 2018), and were associated with carbohydride production in surgeonfish (Ngugi et al. 2017). Bacillota were the most abundant taxon in the temperate fish species Kyphosus sydneyanus, Odax pullus and Aplodactylus arctidens (Clements et al. 2007), all of which have diets dominated by macroalgae and moderate to high levels of SCFA in the hindgut (Mountfort et al. 2002). Although K. vaigiensis and K. cinerascens both contain high levels of SCFA in the hindgut (Clements et al. 2017), in our present study Bacillota were only dominant in fish collected from outer reef sites.

Spirochaetes were also a notable phylum distinguishing the gut communities in K. vaigiensis from those in K. cinerascens, particularly in section IV from mid-shelf reef. Sparagon et al. (2022) examined the gut microbiota of five Kyphosus individuals collected in Hawaii, and based on their supplementary photo of their Kyphosus specimens we were able to identify three individuals (fish 4, 5 and 8) as K. vaigiensis and one individual (fish 6) as K. cinerascens. The gut microbiota of some of the samples from these fish species reported by Sparagon and colleagues were in some cases similar to the gut microbiome of K. vaigiensis and K. cinerascens in our study (i.e. the presence of the Spirochaete genus Brevinema and the Bacteroidota genus Alistipes). Spirochaetes are often reported as pathogenic in humans (Lee and Hampson. 1994); however, they can play a beneficial role in termites and in other terrestrial herbivores through acetogenesis and dinitrogen fixation (Ohkuma et al. 2015; Tokuda et al. 2018; Blankenchip et al. 2018). Spirochaetes have also been suggested to play an important ecological role in herbivorous fish (Clements et al. 2009; Parata et al. 2020).

A number of studies collectively show that the gut microbial community structure is distinct between species of tropical herbivorous fish (Smriga et al. 2010; Miyake et al. 2015; Jones et al. 2018; Scott et al. 2020). For example, according to Scott et al. 2020, the most abundant phyla in the gut microbiota of the herbivorous fish Acanthurus coeruleus, Acanthusus tractus, Scarus taeniopterus. Sparisoma aurofrenatum and Sparisoma viride were Proteobacteria, Firmicutes and Planctomycetes (now known as Pseudomonadota, Bacillota and Planctomycetota, respectively; Oren and Garrity 2021). While species composition was relatively consistent between these fish species, the relative abundance of each microbial taxonomic group differed strongly (e.g. Sp. aurofrenatum harboured a less diverse intestinal microbiome than Sc. Taeniopterus or A. tractus) (Scott et al. 2020). This indicates considerable functional diversity within coral reef fish, as also suggested by our results (i.e. we found different bacterial taxa within individual fish of the same
species, suggesting that different microbial taxa can perform equivalent functional roles). Microbial communities in faecal samples of *Acanthurus nigricans* were dominated by Pseudomonadota (non-Vibrionaceae), Bacteroidota (formerly Bacteroidetes), and Bacillota, while those in *Chlorurus sordidus* (now *C. spilurus*) and *Lutjanus bohar* were dominated by Pseudomonadota-Vibrionaceae (Smriga et al. 2010). These observations are consistent with findings here, with an overall high abundance of Pseudomonadota, Bacillota and Bacteroidetes in *K. cinerascens* and *K. vaigiensis* gut microbial communities. Additionally, we found that the relative abundances of microbial taxa in *K. cinerascens* and *K. vaigiensis* were influenced by different factors, including gut sections, host fish species, geographic locations and sampling years. Similarly, Jones et al. (2018) reported that latitudinal gradient influences the gut microbiota of the rabbitfish *Siganus fuscescens* collected from over 2000 km on the coast of Western Australia.

Variation in community composition of gut microbiota could drive host niche differentiation (Greene et al. 2020). For example, the gut microbiomes in some Madagascar lemurs can vary depending on host diet, gut morphology and geographic location (Greene et al. 2020). Similarly, in our study the dominant genera, *Endozoicomonas*-like and *Brevinema*, varied depending on host fish species, hindgut section and location or time (Fig. 3). Both *Endozoicomonas* and *Brevinema* were the most abundant groups in butterflyfish on Caribbean coral reefs (Clever et al. 2020), and *Brevinema* was one of the most prevalent genera in surgeonfish from the Great Barrier Reef (Parata et al. 2020).

*Endozoicomonas* are emerging as diverse and important symbionts in many marine hosts worldwide (Neave et al. 2016) and were one of the most abundant genera in planktivorous damselfish and cardinalfish collected from Lizard Island (Parris et al. 2016), where the present study was conducted. *Endozoicomonas* species have been suggested to play a role in the coral sulphur cycle via dimethyl sulfoxoniopropionate (DMSP) metabolism to produce dimethyl sulphide (DMS) (Tandon et al. 2020). DMS is also produced in significant quantities by red algae (Dacey et al. 1994), a major dietary component of *K. cinerascens* (Choat et al. 2002). We examined the phylogenetic relationship of the *Endozoicomonas*-like sequences in our data set to reference *Endozoicomonas* sequences from other marine taxa to
explore patterns of host-symbiont association. The *Endozoicomonas*-like ASVs formed a distinct clade to that containing the reference *Endozoicomonas* sequences, indicating that our ASV sequences comprise a novel *K. cinerascens*-associated clade that is closely related to *Endozoicomonas* found in other marine animals.

Differences in populations of gut symbionts of marine herbivorous fish have been detected previously between mid-shelf and outer shelf reef locations on the northern Great Barrier Reef (Arroyo et al. 2019), showing that it is possible that geographic comparisons could be confounded by temporal factors. The Lizard Island Complex and nearby outer reefs were affected by two major cyclones in 2014 and 2015, and a mass bleaching event in 2016 (Madin et al. 2018; Zawada et al. 2019). Our 2011 samples were collected prior to these disturbances, while the 2017 sampling was collected one year after the bleaching event. It is possible that these disturbances influenced community assembly in our gut communities, either through historical contingency, ecological drift or dispersal (Costello et al. 2012), and so we explored this in more detail.

We observed a strong difference in community composition at genus level between sections IV and V in *K. vaigiensis* from mid-shelf reef (Fig. 3). This could be due to the presence in this species of a hindgut chamber (Clements and Choat 1997). Specimens of *Kyphosus cinerascens* lack a hindgut chamber, and share broadly similar bacterial taxa between sections IV and V, particularly on the mid-shelf reefs, such as the genus *Endozoicomonas*-like. This may indicate that the presence of a hindgut chamber that is separated from the proximal intestine by a sphincter increases total microbiota diversity. Community composition also differed markedly between the two host species. This could be due to a diet dominated by green (*Udotea argentea*) and brown (*Turbinaria ornata*) algae in *K. vaigiensis* (Choat et al. 2002) (Fig. 2), and by a mixture of red (*Laurencia majuscula*), brown (*Sphacelaria tribuloides*) and green (*Cladophora rugulosa*) algae in *K. cinerascens* (Choat et al. 2002) (Fig. 2). Gut morphology and diet have a strong influence on microbial community composition in animals (Ley et al. 2008), especially herbivores (Greene et al. 2020; Escalas et al. 2021). In addition, we observed variation among individual fish in their gut microbiota for both fish species and gut sections, especially in *K. cinerascens* from the outer reefs and in *K. vaigiensis* from mid-shelf reefs (Fig. 3). Although individual conspecific fish displayed variation in gut microbial communities, these gut communities should be consistent in overall metabolic function, as both fish species have consistently high levels of SCFA in the hindgut (Clements and Choat 1995, 1997).

The importance of gut microbiota to nutrition in kyphosid fishes (Mountfort et al. 2002; Clements et al. 2014) raised the possibility that highly variant gut communities may influence fish condition and health. We tested this by examining the relationship between length and weight for all of our sampled fish (Fig. 4). Fish that are nutritionally compromised would be expected to weigh less for a given length than fish with a healthy gut microbiota. However, we detected no difference in length/weight between either sampling locations or sampling years. This suggests that the considerable variation in gut microbiome community composition did not influence community function, and that community assembly is labile and driven by factors other than microbiota phylogeny.

This study suggests that spatial variation, even over scales of only tens of kilometres, can influence gut community composition in herbivorous fishes. There is a significant difference in the hindgut microbial community composition in *K. cinerascens* between mid-shelf and outer-shelf reefs, as seen in *Naso tongsan* over the same spatial scales (Arroyo et al. 2019), while marked variation in *K. vaigiensis* may be attributed to either spatial or temporal factors, similarly to the variation in microbial composition observed in *S. fuscescens* with latitudinal changes in Jones et al. (2018). We also observed considerable interspecific variation in the hindgut microbiota of fish living in the same geographic area at the same time, i.e. outer-shelf reef for *K. cinerascens* and mid-shelf reef for *K. vaigiensis*. Our data also suggest that the *Endozoicomonas*-like genus, which dominated communities in mid-shelf samples of *K. cinerascens*, most likely makes an important contribution to metabolic function and immune defence, as *Endozoicomonas* does in corals and other invertebrates (Neave et al. 2016; Tandon et al. 2020; Jensen et al. 2021).

Future work should examine the level of spatial and temporal variation in gut community composition in other herbivorous fishes over larger spatial scales, while taking into account the influence of potential spatial and/or temporal variation in diet. Our study suggests that while there must be strong environmental selection for functionality in these gut communities, this is not strictly tied to particular bacterial taxa, and the effects of variation in many factors including diet and location, and the interaction of different processes including historical contingency, random sampling and dispersal may also influence community assembly (Costello et al. 2012).

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**Data availability** All raw reads associated with this work have been deposited at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/) under BioProject ID: PRJNA783643.

**Declarations**

**Conflicts of interest** The authors declare that they have no conflicts of interest.

**Ethics approval** Fish collection was covered by James Cook University Animal Ethics Committee approvals A1641 and A2237.

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**References**

Arroyo FA, Pawlowske TE, Choat JH, Clements KD, Angert ER (2019) Recombination contributes to population diversification in the polyploid intestinal symbiont *Epulopiscium* sp type B. ISME J 13:1084–1097

Blankenchip CL, Michels DE, Braker HE, Goffredi SK (2018) Diet breadth and exploitation of exotic plants shift the core microbiome of *Cephaloleia* a group of tropical herbivorous beetles. PeerJ 6:e4793

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttenhower CA, Caporaso JG (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:1–17

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Meth 13:581–583

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci 108(Supplement 1):4516–4522

Choat JH, Clements KD (1998) Vertebrate herbivores in marine and terrestrial environments: a nutritional ecology perspective. Annu Rev Ecol Syst 29:375–403

Choat JH, Clements KD, Robbins W (2002) The trophic status of herbivorous fishes on coral reefs. Mar Biol 140:613–623

Clements KD, Choat JH (1995) Fermentation in tropical marine herbivorous fishes. Physiol Zool 68:355–378

Clements KD, Choat JH (1997) Comparison of herbivory in the closely-related marine fish genera *Girella* and *Kyphosus*. Mar Biol 127:579–586

Clements KD, Pasch IB, Moran D, Turner SJ (2007) Clostridia dominate 16S rRNA gene libraries prepared from the hindgut of temperate marine herbivorous fishes. Mar Biol 150:1431–1440

Clements KD, Raubenheimer D, Choat JH (2009) Nutritional ecology of marine herbivorous fishes: ten years on. Funct Ecol 23:79–92

Clements KD, Angert ER, Montgomery WL, Choat H (2014) Intestinal microbiota in fishes: what’s known and what’s not. Mol Ecol 23:1891–1898

Clements KD, German DP, Piché J, Tribollet A, Choat JH (2017) Integrating ecological roles and trophic diversification on coral reefs: multiple lines of evidence identify parrotfishes as microphages. Biol J Linn Soc Lond 120:729–751

Clements KD (1997) Fermentation and Gastrointestinal microorganisms in fishes. In: Mackie R.I., White B.A. (eds) Gastrointestinal Microbiology. Chapman & Hall Microbiology Series. Springer, Boston pp 156–198

Clever F, Sourisse JM, Preziosi RF, Eisaen JA, Guerra ECR, Scott JJ, Wilkins LG, Altiere AH, McMillan WO, Leray M (2020) The gut microbiome stability of a butterflyfish is disrupted on severely degraded Caribbean coral reefs. bioRxiv

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJ, Relman DA (2012) The application of ecological theory toward an understanding of the human microbiome. Science 336:1255–1262

Dacey JWH, King GM, Lobel PS (1994) Herbivory by reef fishes and the production of dimethylsulfide and acrylic acid. Mar Ecol Prog Ser 112:67–74

Egerton S, Culloty S, Whooley J, Stanton C, Ross RP (2018) The gut microbiota of marine fish. Front Microbiol 9:873

Escalas A, Auguet JC, Avouac A, Seguin R, Gradel A, Borrossi L, Villéger S (2021) Ecological specialization within a carnivorous fish family is supported by a herbivorous microbiome shaped by a combination of gut traits and specific diet. Front Mar Sci 8:91

Greene LK, Williams CV, Junge RE, Mahefarisoa KL, Rajaonarivelos T, Rakotondrainibe H, O’Connell TM, Drea CM (2020) A role for gut microbiota in host niche differentiation. ISME J 14:1675–1687

Herlemann DRP, Labrenz M, Juergens K, Bertilsson S, Wanek JJ, Anderrson AF (2011) Transition in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J 5:1571–1579

Jensen S, Frank JA, Arntzen MO, Duperron S, Vaaje-Kolstad G, Hovland M (2021) Endozoicomonas symbiont in gills of *Acesta* clam encodes genes for essential nutrients and polysaccharide degradation. FEMS Microbiol Ecol 97:fiab070

Johnson KS, Clements KD (2021) Histology and ultrastructure of the gastrointestinal tract in four temperate marine herbivorous fishes. J Morphol 283:16–34

Jones J, DiBattista JD, Stat M, Bunce M, Boyce MC, Fairclough DV, Travers MJ, Huggett MJ (2018) The microbiome of the gastrointestinal tract of a range-shifting marine herbivorous fish. Front Microbiol 9:2000

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glickner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:e1–e1

Knudsen SW, Choat JH, Clements KD (2019) The herbivorous fish family Kyphosidae (*Telescopium* *Perciformes*) represents a recent radiation from higher latitudes. J Biogeogr 46:2067–2080

Krogdahl Å, Hembre GI, Mommersen TP (2005) Carbohydrates in fish muscle and *Acesta* clam encodes genes for essential nutrients and polysaccharide degradation. FEMS Microbiol Ecol 97:fiab070

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