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Distinct microbiota composition and fermentation products indicate functional compartmentalization in the hindgut of a marine herbivorous fish

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Abstract

Many marine herbivorous fishes harbour diverse microbial communities in the hindgut that can play important roles in host health and nutrition. *Kyphosus sydneyanus* is a temperate marine herbivorous fish that feeds predominantly on brown seaweeds. We employed 16S rRNA gene amplicon sequencing and gas chromatography to characterize microbial communities and their metabolites in different hindgut regions of six *K. sydneyanus*. Measurements were confined to three distal sections of the intestine, labelled III, IV and V from anterior to posterior. A total of 625 operational taxonomic units from 20 phyla and 123 genera were obtained. Bacteroidota, Firmicutes and Proteobacteria were the major phyla in mean relative abundance, which varied along the gut. Firmicutes (76%) was the most dominant group in section III, whereas Bacteroidota (69.3%) dominated section V. Total short-chain fatty acid (SCFA) concentration was highest in sections IV and V, confirming active fermentation in these two most distal sections. The abundance of Bacteroidota correlated with propionate concentration in section V, while Firmicutes positively correlated with formate in sections III and IV. Acetate levels were highest in sections IV and V, which correlated with abundance of Bacteroidota. Despite differences in gut microbial community composition, SCFA profiles were consistent between individual fish in the different hindgut regions of *K. sydneyanus*, although proportions of SCFAs differed among gut sections. These findings demonstrate functional compartmentalization of the hindgut microbial community, highlighting the need for regional sampling when interpreting overall microbiome function. These results support previous work suggesting that hindgut microbiota in marine herbivorous fish are important to nutrition in some host species by converting dietary carbohydrates into metabolically useful SCFAs.

KEYWORDS
16S rRNA, algae, digestion, microbiota, short-chain fatty acids

1 | INTRODUCTION

Most terrestrial vertebrate herbivores rely on gastrointestinal symbioses with microorganisms to digest the refractory carbohydrates in plant material (Cummings & Macfarlane, 1997; Flint et al., 2012; Greene et al., 2020; Van Soest, 1994). Similarly, some marine herbivorous fishes derive nutrition from macroalgae through symbioses with gut microorganisms that convert otherwise indigestible algal carbohydrates to metabolically-useful short-chain fatty acids (SCFAs) (Clements et al., 1994, 2014; Mountfort et al.,2002). Earlier studies had revealed a positive correlation between the concentration of SCFAs and microbial density in the gut of temperate marine herbivorous fishes including kyphosids (Fidopiastis et al., 2005; Rimmer & Wiebe, 1987), aplodactylids and labrids (Clements, 1991; Clements et al., 1994). Microbiota-derived SCFAs are absorbed and utilized by the fish for energy and lipid synthesis (Clements et al., 1994; Mountfort et al., 2002), so in these fishes the microbiome contributes directly to host nutrition. Importantly, levels of SCFAs, and thus the contributions of gut microbes to host nutrition, vary considerably among taxa of marine herbivorous fish, reflecting differences in diet, gut anatomy and gut throughput rates (Clements et al., 2014, 2017; Clements & Choat, 1995; Crossman et al., 2005). However, even in nonhindgut fermenting, carnivorous fish such as zebrafish, SCFAs have a beneficial effect on food intake and growth (Zhang et al., 2020).

Our understanding of the functional significance of gut microbiota is limited for most species of fishes (Clements et al., 2014; Ngugi et al., 2017; Perry et al., 2020), with most studies to date focusing on microbial community composition only. Hindgut communities in marine herbivorous fishes are much more similar to the gut microbiota of terrestrial mammals than to populations of microbes found in the immediate environment (Scott et al., 2020; Sullam et al., 2012), especially in the prevalence of the bacterial phyla Firmicutes and Bacteroidota (Miyake et al., 2015; Smriga et al., 2010; Sullam et al., 2012). Such studies are starting to reveal relationships between microbiota composition and diet, with intestinal microbial communities of algal feeding fishes differing from those in hosts that feed on cyanobacteria, microalgae and detritus (Clements et al., 2017; Miyake et al., 2015; Scott et al., 2020; Smriga et al., 2010). Species that feed on detritus and microalgae typically display low levels of SCFAs in the gut, and have higher proportions of branched-chain SCFAs indicating that peptides and amino acids are major fermentation substrates rather than carbohydrates (Clements et al., 2017).

It is widely recognized that the gastrointestinal tracts of monogastric animals are divided into functionally distinct compartments (Donaldson et al., 2016; Stevens & Hume, 1998). For example, in humans the colon is the major site of fermentation (Cummings & Macfarlane, 1997), with each of the ascending, transverse and descending regions providing a distinct environment for microbial populations (Macfarlane & Macfarlane, 1997). Similarly, in fish the environment for intestinal microbes varies along the length of the intestinal tract with different regions associated with variation in microbial populations (Moran et al., 2005; Ye et al., 2014; Zhou et al., 2009). SCFA concentrations of both marine and freshwater fishes also vary considerably along the gut, with highest levels typically in the most distal gut sections (Clements et al., 1994, 2014; Clements & Choat, 1995; Dacey et al., 1994). Unfortunately, most fish microbiota censuses analyse the entire homogenized contents of the gastrointestinal tract, thus blurring details of community structure and functional differences (cf. Miyake et al., 2015; Scott et al., 2020).

The highest levels of SCFAs measured in fish to date are in the chubs, family Kyphosidae (Clements & Choat, 1997; Clements et al., 2017). These fishes display very high rates of fermentation in the hindgut (Mountfort et al., 2002), and display adaptation of metabolic pathways that involve metabolites associated with hindgut fermentation (Willmott et al., 2005). Kyphosid fishes typically feed on macroalgae (Choat et al., 2002; Clements & Choat, 1997; Dell et al., 2020; Moran & Clements, 2002), and are distributed globally in both tropical and extratropical waters, extending into temperate waters in Australasia (Knudsen et al., 2019). To date, our understanding of their gut microbiota composition has been limited to relatively few sequences obtained from 16S rRNA gene clone libraries (Clements et al., 2007; Fidopiastis et al., 2005; Moran et al., 2005).

Here, we examine the relationship between microbial community composition and the levels of fermentation products in three regions of the hindgut of *Kyphosus sydneyanus* ($N = 6$), a marine herbivorous fish found in warm temperate waters of Australia and New Zealand. Adult *K. sydneyanus* are exclusively herbivorous and feed mainly on phaeophyte algae with lesser amounts of chlorophyte and rhodophyte algae (Moran & Clements, 2002). This species has a hindgut chamber that is separated from the proximal intestine by a sphincter (Mountfort et al., 2002; Rimmer & Wiebe, 1987) (Figure 1), and SCFA concentration in this and the preceding intestine are exceptionally high for fish (Clements & Choat, 1997; Mountfort et al., 2002; Rimmer & Wiebe, 1987). We hypothesize that: (i) microbial community composition will display regional variation along the gut, especially between the hindgut chamber and the preceding intestine; (ii) intestinal communities will be dominated by the carbohydrate-fermenting phyla Firmicutes and Bacteroidota, as in other herbivorous vertebrates; (iii) levels of total SCFA and the ratio of acetate:propionate:butyrate in different gut regions will be more consistent among individual fish than community composition, reflecting the lack of vertical transmission in the symbiosis; and (iv) relative abundance of Bacteroidota will correlate positively with the SCFA propionate, as in other gut systems (Aguirre et al., 2016; den Besten et al., 2013; Louis & Flint, 2017; Mi et al., 2018; Salonen et al., 2014).

2 | MATERIALS AND METHODS

2.1 | Capture of fish and sample collection

Six adult individuals (410–530 mm standard length) of *K. sydneyanus* were collected by underwater spear from Nelson Island near Great Barrier Island in the Hauraki Gulf, New Zealand (36°.165'S;

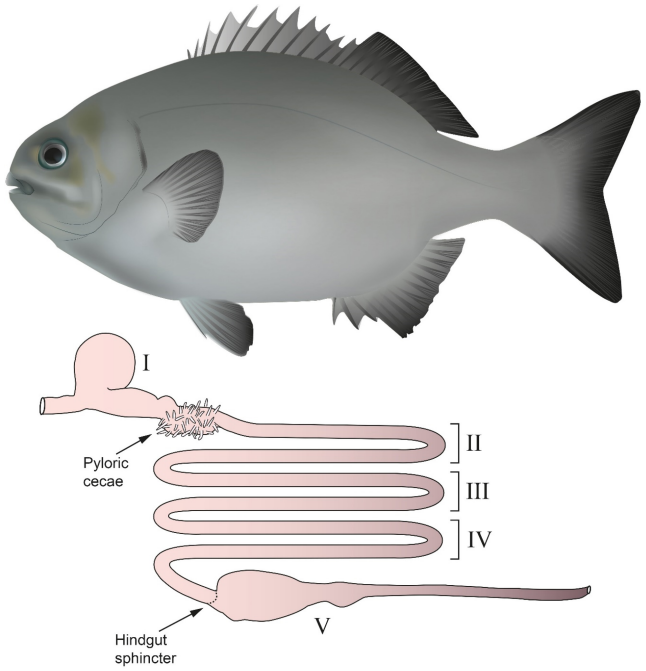

FIGURE 1 Schematic diagram showing the gut anatomy and designation of gut segments in *K. sydneyanus*. The stomach was defined as section I and the hindgut chamber as section V. The intervening intestinal tract was divided into three sections of equal length designated sections II, III and IV

175.301'E) in May 2018. Fish collection was conducted under University of Auckland Animal Ethics approval AEC-001949. Fish were removed immediately from the water and processed aboard the University of Auckland research vessel RV Hawere. The gut was removed and divided into five segments, numbered I–V, as described previously (Johnson & Clements, 2021). The samples used for the total microbiota counts were three adult *K. sydneyanus* collected by underwater spear from Little Barrier Island in the Hauraki Gulf, New Zealand (36°.182'S,175°.119'E) in August 2008. These fish were collected under University of Auckland Animal Ethics approval AEC/03/2006/R456.

For DNA extractions, gut content samples of gut sections III, IV and V from each individual fish were collected in 2 ml microcentrifuge tubes and frozen immediately. For targeted analyses of organic acids, gut contents were filtered through cheese cloth and the filtrate centrifuged at 10,000*g* for 10 min. The supernatant was placed in 1.5 ml microcentrifuge tubes. All samples were kept at −20°C on the research vessel and then stored at −80°C in the laboratory until processed.

To examine the role of allochthonous organisms in microbiome community assembly we collected ten plants of *Ecklonia radiata*, the main dietary alga of *K. sydneyanus* in the study location (Moran & Clements, 2002), from 2–4 m depth in Katherine Bay, Great Barrier Island in April, 2021. Several secondary laminae were subsampled from each plant, rinsed with seawater to remove loosely associated epibionts, and laminae were placed in tubes and frozen in liquid nitrogen on the research vessel. All samples were stored at −80˚C in the laboratory until processed.

2.2 | DNA extraction

DNA was isolated from the gut contents of *K. sydneyanus* using a DNeasy PowerSoil Kit (Qiagen) with the following modifications. Each sample was thawed, inverted a few times and approximately 250 µl was transferred onto an ice-cold 30 mm petri dish with a sterile inoculating loop. Each sample was then mixed with 1,250 µl of DNA/RNA Shield (Zymo Research) and homogenized using a 1 ml syringe. The slurry was transferred into a bead-beating tube from the kit and then placed on an ice-cold 24 adapter, which was inserted in a Qiagen TissueLyser II. Bead beating was performed for 2 min at 25Hz in two shaking steps with a rotation of the adapter after the first minute. The remaining steps were performed following manufacturer's instructions. After the last step, the DNA was treated with 2 µl of RNase A/T1 mixture (2 mg/ml of RNaseA and 5,000 U/ml of RNase T1) and incubated at 37°C for 20 min. Removal of proteins and DNA precipitation was performed following standard techniques (Sambrook & Russell, 2001).

DNA was isolated from the *E. radiata* samples by first grinding the seaweed tissue in liquid nitrogen into a fine powder using a mortar and pestle. 0.25 g of the seaweed homogenate was then used for DNA extraction using the DNeasy PowerSoil Kit (Qiagen).

All DNA samples were evaluated spectrophotometrically with a Nanodrop N60 (IMPLEN) using the A260/280 OD ratio for protein and 260/230 for organic contaminants. The concentration of DNA was measured using a Qubit High Sensitivity Assay on a Qubit fluorimeter 2.0 (Thermo Fisher Scientific). DNA integrity was evaluated by agarose gel electrophoresis.

2.3 | Marker gene amplification and sequence analysis

Amplification and data analysis of the 16S rRNA gene amplicon (V3–V4 region), which targets both bacteria and archaea, followed Lee et al. (2016). Separate rounds of PCR and sequencing were also conducted for the detection of Archaea (specifically targeting the taxon) and Fungi. The primers used for Archaea were Arch516F (5'GYCAGCCGCCGCGGTAAHACCVGC 3') (Takai & Horikoshi, 2000) and Ar9r (5' CCCGCCAATTCCTTTAAGTTTC 3') (Jurgens et al., 1997), and for Fungi the internal transcribed spacer (ITS) region between the 18S and 5.8S rRNA genes was amplified using the primers ITS1 forward (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS2 reverse (5' GCTGCGTTCTTCATCGATGC 3') (White et al., 1990). Sequencing was performed on an Illumina MiSeq platform (Illumina) with the 600 cycle V3 chemistry (300 bp paired-end reads) at Auckland University of Technology, New Zealand and GENEWIZ Shuzhou, China.

2.4 | Data processing

USEARCH v8.0.1623 (Edgar, 2013) was used for quality control and processing the 5,028,572 raw paired-end sequencing reads. Paired-end reads were merged by the USEARCH merge_fastq command, and the merged reads with lengths outside 200–500 bp range or exceeding six homopolymers were removed by Mothur v1.36.1 (Schloss et al., 2009). Q score filtering was used to remove reads with maximum expected error >1 and singletons were removed. The processed sequences were clustered at 97% similarity using the USEARCH *cluster_otus* command. A representative sequence of each bacterial operational taxonomic unit (OTU) was classified using the naïve Bayesian classifier implemented in the R package DADA2 (Callahan et al., 2016) with SILVA release 138.1 (2020) (Quast et al., 2012) as the reference database.

Absolute abundances were estimated by multiplying the relative abundance values by total microbial cell density determined by direct counts. Gut content samples of gut sections III, IV and V from three *K. sydneyanus* individuals were fixed in PBS-buffered 1% formaldehyde in the field. When the samples were returned to the laboratory, the gut contents were pelleted, and the supernatant removed. The pellet was resuspended in PBS-buffered 50% ethanol and stored at 4°C. For staining, the samples were diluted in 10 mM Tris, 1 mM EDTA, pH 8 and stained with 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Each sample was filtered and bacterial cells were counted using epifluorescence microscopy (Olympus BX61).

Fungal ITS sequences were compared with the UNITE.8.0.2018-11-18 reference database (Nilsson et al., 2019) using the RDP classifier.

All sequence data generated by this study have been submitted to the EMBL European Nucleotide Archive (ENA) under BioProject PRJEB34792.

2.5 | Quantification of organic acids

The following organic acids were quantified with gas chromatography (GC) using a method modified from Richardson et al. (1989): formate, the SCFAs acetate, propionate, butyrate and valerate, the branched SCFAs isobutyrate and isovalerate, the medium-chain carboxylic acids hexanoate and heptanoate, the alpha-hydroxy acid lactate and the dicarboxylic acid succinate.

Gut content supernatant (100 μl) was transferred to a 2 ml microcentrifuge tube and mixed with 400 μl of 0.01 M phosphate buffered saline containing 6.25 mM 2-ethylbutyric acid (an internal standard). The solution was acidified by adding 250 μl concentrated hydrochloric acid, followed by 1,000 μl diethyl ether. After vortexing for 10 s, the sample was centrifuged at 10,000*g* for 5 min (4°C). The diethyl ether phase was transferred to a clean 2 ml Eppendorf tube and stored at −80°C until analysis on GC.

A 100 μl aliquot of the diethyl ether phase was placed in a capped GC vial. The sample was derivatized with 20 μl N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) in a water bath at 80°C for 20 min then left at room temperature for 48 h to allow complete derivatization before analysis on GC. Standards containing 5 mM 2-ethylbutyric acid were prepared in parallel with the samples.

Samples were analyzed with a Shimadzu capillary GC system (GC-2010 Plus, Tokyo, Japan) equipped with a flame ionization detector (Fidopiastis et al., 2005) and fitted with a Restek column (SH-Rtx-1, 30 m × 0.25 mm ID × 0.25 μm) (Shimadzu). The carrier gas was helium with a total flow rate of 21.2 ml/min and pressure of 131.2 kPa. The make-up gas was nitrogen. The temperature programme began at 70°C increasing at 6°C/min to 115°C, with a final increase at 60°C/min to 300°C, and holding for 3 min. The flow control mode was set to linear velocity; 37.5 cm/s. The injector and detector temperatures were 260°C and 310°C, respectively. Samples were injected (1 μl) with a split injection (split ratio: 10:1). The GC instrument was controlled and data processed using Shimadzu GC Work Station LabSolutions Version 5.3. Acquired data were expressed as μmol organic acid/ml supernatant.

2.6 | Statistical analysis and bioinformatics

Graphing and statistical analyses were conducted using the Graphpad Prism Program (version 8.0) and R (R Core Team, 2013). The package phyloseq (McMurdie & Holmes, 2013) was used for data handling and community analyses, ggplot2 (Wickham, 2016) for general plotting, vegan (Oksanen et al., 2018) for ecological analyses and ComplexHeatmap (Gu et al., 2016) for generating the hierarchical clustered heatmap. Differential abundance analysis of the microbiome data was conducted using ANCOM-BC (Lin & Peddada, 2020). Sparse partial least squares regression (sPLS) was conducted using R package mixOmics v6.18.0 (Rohart et al., 2017). The OTU abundance table was rarefied to lowest minimum count (30,010) and then agglomerated at the genus level. The genus-level groups were filtered to remove lower count taxa (contributing <5% counts to the data set). The data was transformed using centered log ratio transformation (CLR). SPLS regression was conducted using the spls function in regression mode and validation was conducted using the perf function, with 10 Mfold validation and 100 repeats. First two components were chosen for visualization, using the cim function to generate a correlation heatmap, based on the Q2.total values from the validation process with the 0.0975 threshold as recommended by the authors of mixOmics. Explained variance of sPLS components was calculated and the structure of the variables (OTUs and SCFAs) was individually visualized.

Results are presented as means ± standard error.

3 | RESULTS

3.1 | Richness and diversity of *K. sydneyanus* intestine microbial communities

3.1.1 | Bacteria

From the 18 gut content samples, we detected 2,518,236 bacterial sequences. A total of 75 reads were unclassified (i.e., not identified

as bacteria) and were removed. A further 328 reads were not classified under any bacterial phylum and were excluded from visualization or analyses. Excluding one section IV sample that yielded few reads, the remaining 17 samples had read depths ranging from 30,989 to 310,681 with a mean read depth of 138,035. The most abundant OTUs (150 out of the total 625 OTUs) account for ~95% of the reads (Figure S1).

Richness and diversity of OTUs representing bacterial communities in the three gut sections of *K. sydneyanus* were estimated using the Chao1 and Shannon indices, respectively (Figure 2). Highest median OTU richness was in gut section III and highest median OTU diversity was in gut section V, although these differences between gut sections in alpha diversity were not significant (Table 1).

3.1.2 | Archaea

The V3–V4 16S rRNA gene primers (see Methods) used here were designed to target both bacteria and archaea (Klindworth et al., 2013) but no archaeal sequences were recovered. An archaeal primer set yielded 2,349,210 sequences from the 18 samples but 97.7% of these total reads were not from archaeal taxa. Only six Euryarchaeotal OTUs were detected, two of which were *Halococcus* (one specifically *Halococcus hamelinensis*).

3.1.3 | Fungi

The analysis of the fungal ITS1 region recovered a total of 161,126 sequences from the 18 samples. Of these, eight samples had read counts below 300 (Figure S2) and were removed from rarefaction analyses (Figure S3) and calculations of diversity indices. Section III samples had roughly 1,000 reads and a Chao1 value of 100, whereas section V samples had 1,000–10,000 reads but Chao1 (richness) did not increase substantially (Figure S4).

3.2 | Microbial community composition

The classified bacterial sequences belonged to 20 phyla, among which Actinobacteriota, Bacteroidota, Cyanobacteria, Firmicutes, Proteobacteria, Spirochaetota and Verrucomicrobiota were the most dominant (Figure S5). Firmicutes were significantly higher in section III, while Bacteroidota dominated section V. Proteobacteria were abundant in some section III and IV samples, but this variation among gut sections was not significant (Figure S5 and Table 2). Results from an analysis of the same data at the family level are shown in Figure S6, with finer details provided in supplementary information (Tables S1A–C).

Section III was dominated by Firmicutes which represented 47–96% of OTUs in the six fish. While some of these Firmicutes could not

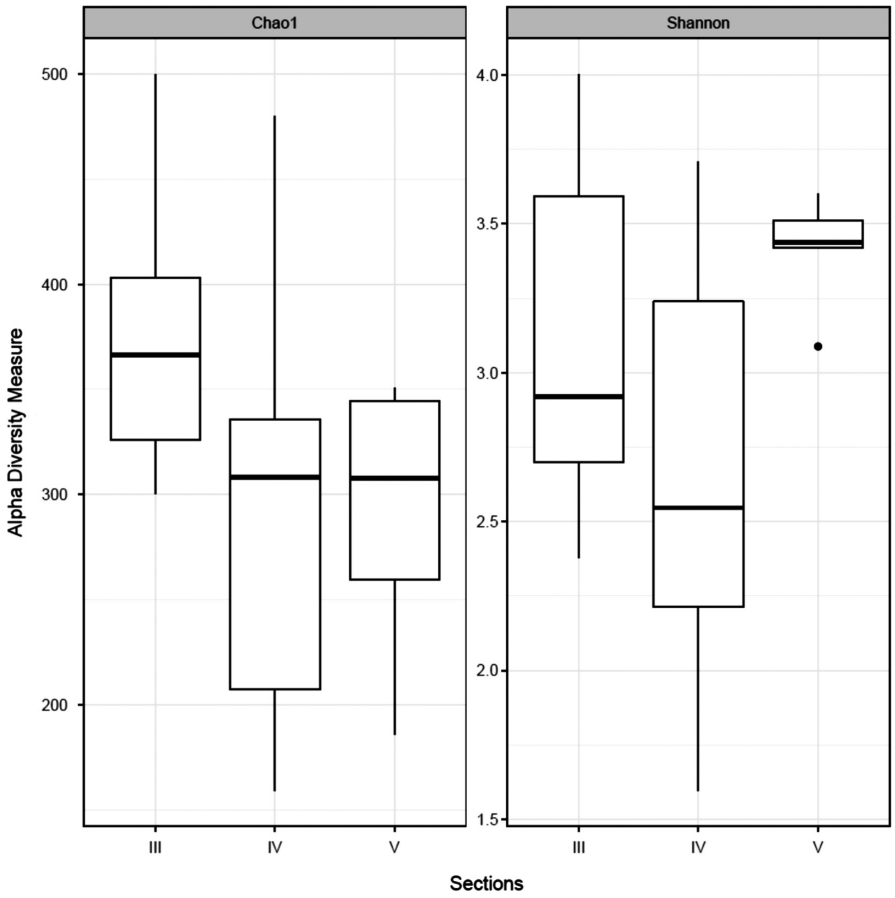

FIGURE 2 Alpha diversity indices (Shannon and Chao1) for microbial communities in the three sections of the hindgut of *K. sydneyanus*. Median values and interquartile ranges are indicated in the boxplots. $N = 6$ in sections III and V, and 5 in section IV. ANOVA test statistics between the sections are shown in Table 1

TABLE 1 Chao1 richness and Shannon diversity in gut microbial communities in gut sections of *K. sydneyanus* compared using ANOVA

	Chao1 richness			Shannon diversity		
	Df	F	*p*-value	Df	F	*p*-value
Gut region	2	1.311	.303	2	1.876	.192
Residuals	13			13		

Note: $N = 6$ in sections III and V, and 5 in section IV.

be classified beyond order, the majority were distributed between the families *Lachnospiraceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae and Acholeplasmataceae*. Of particular note, the genus *Erysipelatoclostridium* (family *Erysipelatoclostridiaceae*) represented ≥10% in four fish. Proteobacteria were present at >20% in two fish and Bacteroidota did not exceed 5% in section III of any fish.

OTUs recovered from section IV differed markedly from the OTUs recovered from section III in the same individual, despite the lack of any anatomical feature delineating these two regions of the intestinal tract. In the five fish analyzed, Bacteroidota and Firmicutes were present at 4%–57% and 32%–89%, respectively. The candidate genus *DMI* (family *Acholeplasmataceae*) was present at >10% in all five fish. *Erysipelatoclostridiaceae*, *Oscillospiraceae and Lachnospiraceae* were present at >10% in one fish. Proteobacteria were exceptionally high (52%) in one fish and they were almost entirely comprised of members of the family *Vibrionaceae*.

Generally, the microbiota composition of the hindgut chamber (section V) differed from sequences recovered from sections III and IV. The family *Rikenellaceae* (Bacteriodota phylum) dominated, comprising 66%–74% of OTUs in all fish. *Alistepes* was the predominant genus present, the rest being *Rikenella* (Table S1C). Firmicutes were much less common, comprising only 14%–24% of OTUs recovered from the six fish. Verrucomicrobiota were present in all six fish at 2%–15% of OTUs.

Ordination based on principal coordinate analysis of Bray-Curtis dissimilarities of the bacterial communities from the 17 gut samples are shown in Figure 3. Gut section accounted for roughly half (49%) of the variation and was significant in structuring the gut microbial communities (PERMANOVA, $F(2,14) = 7.3039$, $R^2 = 0.51062$, $p < .001$) (Figure 3). All gut sections were significantly different from one another (pairwise PERMANOVA contrast, *all* $p < .05$, see Data set S1).

Similarity, richness, and diversity of the bacterial communities in the hindgut of *K. sydneyanus* are presented in a hierarchically clustered heatmap (Figure 4). Again, this clearly shows the abundance of Bacteroidota in section V. The heatmap also reveals pairs of co-occurring taxa, which suggests metabolic cooperativity. To investigate further how the different taxa differ between different gut sections we performed differential abundance analysis of the microbiome data to complement Figure 4 (Data set S2). Those genera listed as TRUE under diff_abund differed significantly ($p < .05$) in abundance between at least two gut sections. Note that there are more genera in this list than the heatmap since the heatmap can only display a limited number of taxa. Only those genera >0.5% mean relative abundance were shown.

Basidiomycota and Ascomycota were the most prevalent fungal phyla, with Basidiomycota comprising 44%–97% of total OTUs and Agaricomycetes was the most dominant class (Figure S7). There was substantial difference in fungal composition at the class level across the different sections of the hindgut (Figure S7).

To test for the presence of allochthonous organisms in the gut microbiome of *K. sydneyanus* the intestinal communities were compared with the microbial assemblage associated with the main dietary alga, *E. radiata*. The algal and hindgut communities differed considerably in taxonomic composition, as the *E. radiata* communities were dominated by Proteobacteria and Planctomycetota, with much lower abundances and different taxa of Bacteroidota and Verrucomicrobiota than seen in the hindgut microbiota (Figure S8). Bray-Curtis distances of the communities (rarefied to even depth) revealed significantly distinct clustering between fish gut and *E. radiata* communities (PERMANOVA, $F(1) = 14.453$, $R^2 = 0.36633$, $p < .001$) (Figure 5).

Absolute abundances of the 44 most abundant genera were calculated from sequencing data using direct counts of bacteria (Figure S9). In brief, relative abundance of each taxon at genus level was measured by sequencing and these numbers were multiplied by the mean total cell counts of bacteria present in gut sections III, IV and V of three *K. sydneyanus* individuals as estimated by DAPI staining and epifluorescence microscopy. The mean total cell counts in sections III, IV and V were 5.03×10^9, 2.18×10^{10} and 3.25×10^{11} cells/ml, respectively.

We could see no outstanding patterns that indicate some taxa were present in constant numbers, in fact, the variability of the absolute abundances of the various taxa were almost identical to that of the compositional data (Figure S9).

TABLE 2 Relative abundance of dominant bacterial phyla in gut sections of *K. sydneyanus* compared by Welch's ANOVA ($p < .05$)

Bacterial Phyla	Section III	Section IV	Section V	*p*-value
Bacteroidota	2.77 ± 0.82	17.95 ± 8.58	69.33 ± 1.83	<.05
Firmicutes	76.59 ± 8.24	50.59 ± 13.44	19.14 ± 1.69	<.05
Proteobacteria	11.43 ± 4.77	11.45 ± 8.20	3.86 ± 0.82	.2911

Note: Data presented as mean ± SE. $N = 6$ in sections III and V, and 5 in section IV.

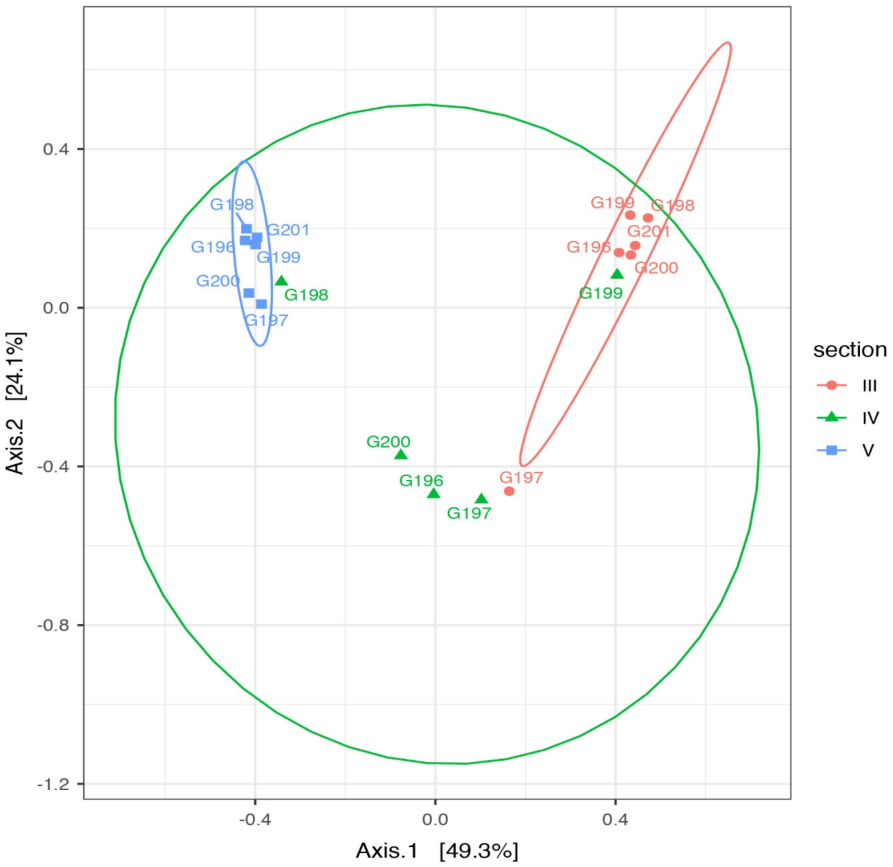

FIGURE 3 Principal coordinates analysis of the Bray-Curtis dissimilarities of microbial community composition among the samples in the three gut sections of *K. sydneyanus*. Percentages indicate the relative contribution of the two principal coordinates. (PERMANOVA, $F(2,14) = 7.3039$, $R^2 = 0.51062$, $p < .001$, based on 999 permutations, pairwise PERMANOVA between all *sections* $p < .05$, see Data set S1). $N = 6$ in sections III and V, and 5 in section IV

3.3 | Microbial fermentation products in the *K. sydneyanus* hindgut

Organic acids that are products of microbial fermentation in anoxic environments were targeted for analysis. The concentrations of four of the organic acids assayed varied between different sections of the gut (Table 3). Levels of acetate were significantly higher in section IV and section V than in section III, and propionate was highest in section V (Table 3). Conversely, formate concentration was greater in sections III and IV than section V (Table 3). Butyrate concentration did not differ among the gut sections (Table 3). The ratio of acetate:propionate:butyrate was similar between individual fish in different gut sections although the proportions differed between gut sections (Figure S10). The concentrations of seven other organic acids assayed were below the level of detection: valerate (<0.1 mM), hexanoate (<0.1 mM), heptanoate (<0.1 mM), isobutyrate (<0.3 mM), isovalerate (<0.1 mM), lactate (<0.25 mM), and succinate (<0.3 mM). The highest total concentration of all measured acids was found in section IV, while the highest sum of the SCFAs (acetate, propionate and butyrate concentrations) was in section V.

sPLS regression was conducted to reveal the correlation between the predominant taxa and organic acid concentrations in sections III, IV and V (Figure 6a). The majority of the Bacteroidota taxa correlated positively with acetate with dgA-11 gut group (*Rikenellaceae* family) showing the strongest positive correlation. *Rickettsiales* (Proteobacteria) also showed strong positive correlation with acetate. Some other taxa such as Firmicutes member *Butyricicoccaceae* (unknown genus), Verrucomicrobiota members *Puniceicoccaceae* (unknown genus) and *Victivallaceae* (unknown genus) also displayed positive correlation with acetate.

Several Bacteroidota taxa such as genera *Alistepes*, *Rikenella* and dgA-11 gut group (family *Rikenellaceae)*, and unknown genera of families *Porphyromonadaceae*, *Muribaculaceae*, *Barnesiellaceae* and *Paludibacteraceae* displayed positive correlations with propionate. Verrucomicrobiota taxa such as *Akkermansiaceae* (genus Akkermansia), *Puniceicoccaceae* (genus unknown) and *Victivallaceae* (unknown genus) also displayed positive correlation with propionate. Some other taxa that exhibited strong positive correlation with propionate are *Ruminococcaceae* (genus *Faecalibacterium*), *Desulfovibrionaceae* (genus *Desulfovibrio*) and Gastranaerophilales (unknown family). Only OTUs affiliated with the phylum Firmicutes showed a positive correlation with formate (Figure 6a).

Explained variance between the sPLS components were calculated. The variance explained by genera was 67% on component 1 and 10% on component 2, and the variance explained by SCFAs was 50% on component 1 and 42% on component 2 (Figure 6b and c).

The correlation matrix used to draw the clustered image map (heatmap) in Figure 6 is presented in Data set S3.

4 | DISCUSSION

The present study builds on earlier work describing the microbial hindgut community of *K. sydneyanus* (Clements et al., 2007; Moran

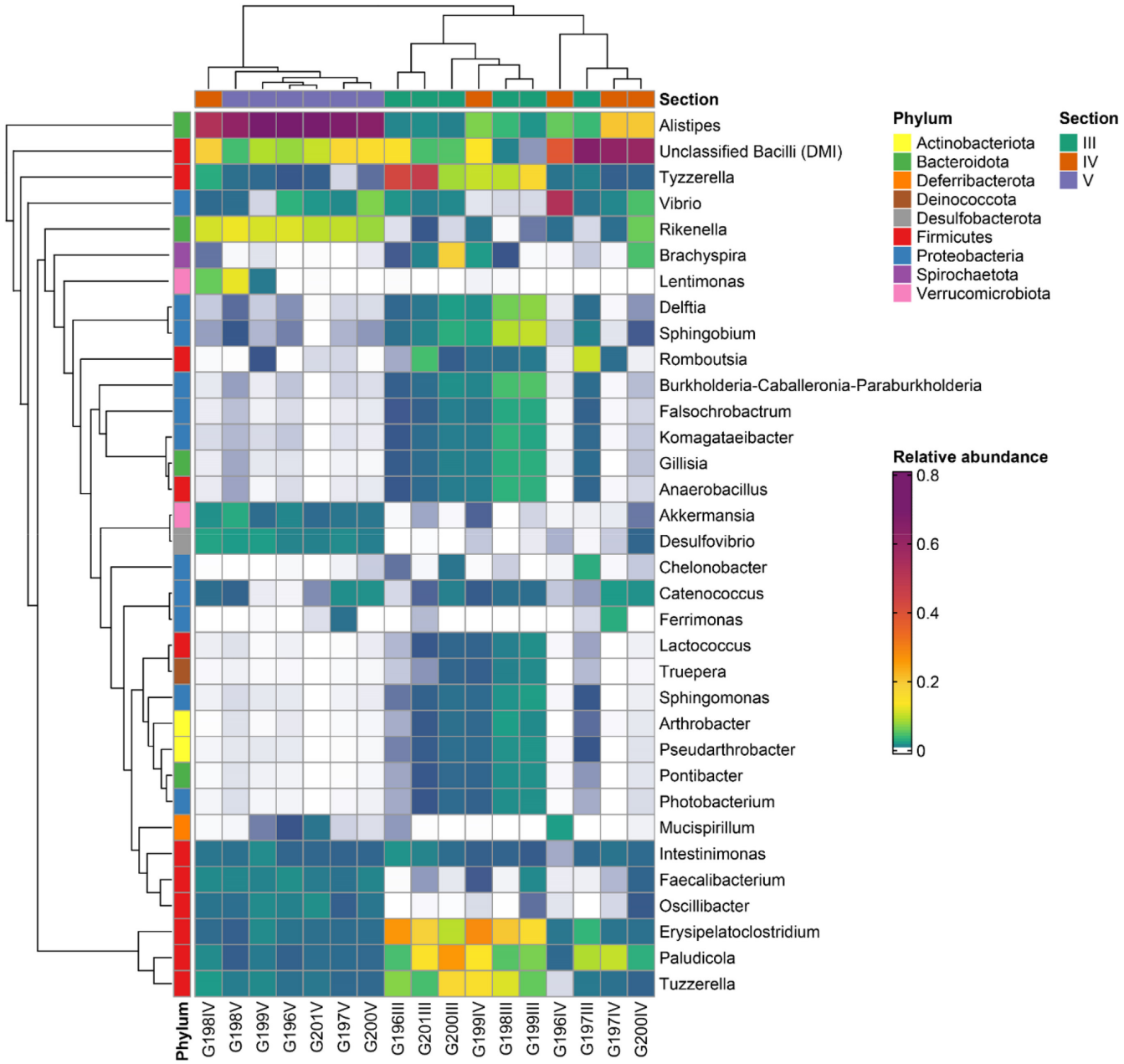

FIGURE 4 Heat map showing the relative abundance of bacterial genera that are >0.5% mean relative abundance across the fish hindgut samples. Genera are listed to the right. Sample codes are listed at the bottom. The dendrograms were produced by hierarchical clustering

et al., 2005). We again sampled free-living animals collected from their natural environment and analysed gut contents from specific regions of the alimentary tract since microbial communities are known to vary along the gut of vertebrates (Donaldson et al., 2016), including fish (Jones et al., 2018; Parata et al., 2020). Sample variation due to season, temperature, location and food source was minimized by collecting all fish on the same day from the same site at Great Barrier Island, Hauraki Gulf. We used a high-throughput Illumina sequencing approach for a deeper analysis of bacterial diversity and included archaeal and fungal populations. Microbiota OTU community structure differed strikingly between the three distal gut regions sampled and the microbiota of the main dietary alga *E. radiata*, consistent with our first hypothesis. Gut segments III to V all displayed a distinct profile of organic acids produced by fermentation. It is likely that these differences in OTU composition and fermentation products are linked to the progressive breakdown of constituents of the seaweed diet along the gut. A few co-occurring taxa exhibited remarkably similar relative abundance patterns in all fish and the patterns were similar across all three gut sections. This suggests metabolic interactions or cooperativity (Yang et al., 2019), which will be examined in future metagenomic and bacterial genome-based metabolic reconstructions.

The phylum Firmicutes was commonly the dominant bacterial group in the proximal (section III) hindgut of *K. sydneyanus*. We note that comparisons of OTU community composition between studies are generally problematical due to differences in methodology,

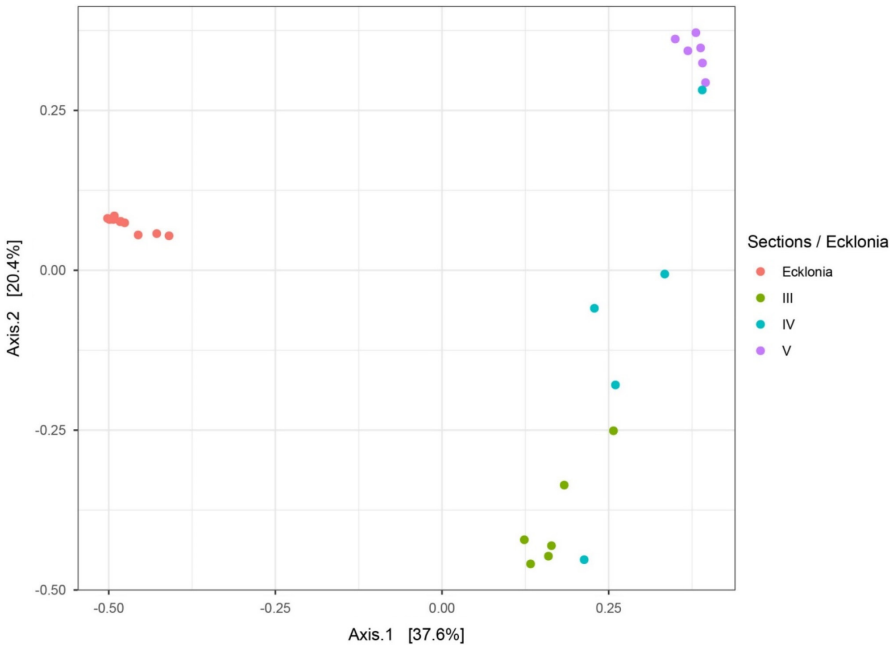

FIGURE 5 Principal coordinate analysis (PCA) of the Bray-Curtis dissimilarities of microbial community composition among the samples in the three gut sections of *K. sydneyanus* and the *E. radiata* samples. Percentages indicate the relative contribution of the two principal coordinates. The two types of communities (gut, including all sections and *E. radiata*) showed significant differences (PERMANOVA, F(1) = 14.453, $R^2 = 0.36633$ $p < .001$, based on 999 permutations)

TABLE 3 SCFA and formate concentration in gut sections of *K. sydneyanus* compared by Welch's ANOVA ($p < .05$)

SCFA (mM)	Section III	Section IV	Section V	*p*-value
Formate	18.15 ± 4.87	23.38 ± 4.63	3.05 ± 1.04	<.05
Acetate	17.12 ± 4.01	45.08 ± 3.18	49.20 ± 4.01	<.05
Propionate	0.98 ± 0.21	2.28 ± 0.68	7.85 ± 0.89	<.05
Butyrate	1.25 ± 0.25	1.77 ± 0.33	1.02 ± 0.02	.13
Total SCFA (Acetate, propionate and butyrate)	19.35 ± 4.31	49.13 ± 2.62	58.07 ± 4.62	<.05

Note: Data presented as mean ± SE. $N = 6$ for all gut section.

especially in relation to sampling design and taxonomic assignment. Despite these issues, bacteria belonging to the phylum Firmicutes have been frequently identified as a dominant bacterial group in the gut of other hindgut-fermenting herbivorous fish including rabbitfish (Jones et al., 2018; Wu et al., 2020), surgeonfish (Miyake et al., 2015) and odacine butterfish (Clements et al., 2007). Bacteroidota consistently dominated the distal (section V or hindgut chamber) hindgut of *K. sydneyanus*, as in the gut of the surgeonfish *Acanthurus nigricans* (Smriga et al., 2010). Proteobacteria, which along with Bacteroidota and Firmicutes, may contribute to 90% of the intestinal microbiota of some fish species (Ghanbari et al., 2015), were generally not present in large numbers in the hindgut of *K. sydneyanus*.

We now turn to axial variation in OTU community composition along the gut. Section III samples varied among fish in composition, but the four families *Lachnospiraceae*, *Oscillospiraceae*, *Ruminococcaceae*, and *Erysipelatoclostridiaceae* comprised 45%–87% of the sequences from five of the six study fish. These families are abundant in other hindgut fermenting herbivorous vertebrates including marine turtles (Campos et al., 2018), lemurs (Greene et al., 2020) and manatees (Suzuki et al., 2019), as well as humans (Flint et al., 2012). Members of these families are known for their ability to catabolise refractory plant polysaccharides (Biddle et al., 2013; Brulc et al., 2009).

The dominant bacterial phylum recovered from section IV varied between individual fish with Firmicutes, Bacteroidota or Proteobacteria seen as most abundant. The class Bacilli (phylum Firmicutes) accounted for 16%–54% of the sequences of all the five study fish. Most of the OTUs within the class Bacilli belonged to the order Acholeplasmatales. It should be noted that Acholeplasmatales was formerly included in the Phylum Tenericutes until they were reclassified by GTDB into a Bacilli clade of Firmicutes (Parks et al., 2018). Tenericutes have previously been reported in low to moderate abundance from the guts of herbivorous rabbitfish (Jones et al., 2018; Zhang et al., 2018), and can be abundant in the guts of carnivorous species including king mackerel (Givens et al., 2015) and Atlantic salmon (Llewellyn et al., 2016). When comparing samples taken from sections III and IV from the same individual, the composition differed substantially at all taxonomic levels up to and including phylum level. In previous studies, it was shown that bacterial density is approximately three times higher in section IV compared to section III in *K. sydneyanus* (Rimmer & Wiebe, 1987). Bacterial density values can be used to calculate the absolute abundance of microbial taxa from relative abundance data (Props et al., 2017) in addition to several other methods (Barlow et al., 2020; Jian et al., 2020). Absolute abundance data can be useful to explore if the cell numbers of any taxon remains constant throughout the

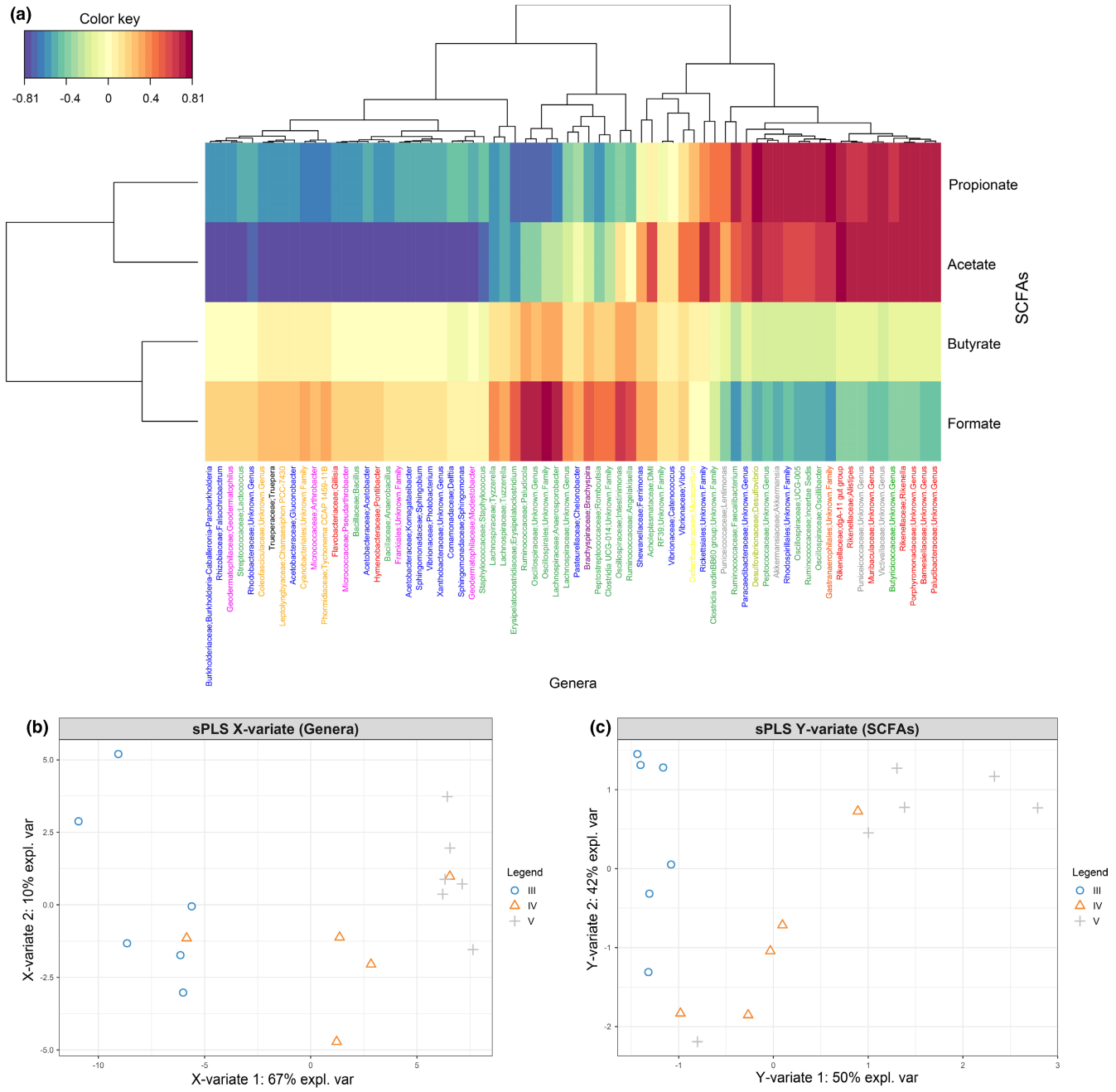

FIGURE 6 (a) Clustered image map (heatmap) showing correlations from sPLS between concentrations of four abundant organic acids and 70 most abundant genus-level groups, with each of these taxa representing >5% of total reads in the data set, excluding chloroplasts and mitochondria. The colour intensity indicates Pearson-like correlations (González et al., 2012). The rows and columns were arranged via hierarchical clustering. The genus-level groups were labelled by (family name); (genus name) where available. When both genus and family names were unknown, the order name was used. The taxa assigned to different phyla were colour coded: green, Firmicutes; red, Bacteroidota; blue, Proteobacteria; pink, Actinobacteriota; grey, Verrucomicrobiota; orange, Cyanobacteria; purple, Spirochaetota; yellow, Deferribacterota; pear, Desulphobacterota and black, Deinococcota. (b) and (c) Sample plots showing the explained variance between the sPLS components. Explained variance for the components are shown in axis labels

gastrointestinal tract in spite of different relative abundance values. Our absolute abundance data estimated using direct counts of DAPI-stained cells showed that the distribution patterns of the relative and absolute abundances of dominant genera were very similar (Figure S9). Together these data indicate that different bacterial taxa inhabit different regions of the fish intestinal tract despite the lack of any anatomical delineation (cf. sections IV and V, which are separated by the hindgut sphincter).

The microbiota of section V differed considerably from that in sections III and IV but showed the most similarity in phylum-level bacterial composition from one fish to another (Figure S5). Bacteroidota represented 64%–77% of the microbiota, and mostly belonged to

the genus *Alistepes* of the family *Rikenellaceae*. Bacteroidota are characterized by metabolic flexibility (Johnson et al., 2017) with the capacity to hydrolyse host mucin and plant polysaccharides, including the seaweed polysaccharides alginate and laminarin (Salyers et al., 1977; Tailford et al., 2015). *Rikenellaceae* is a newly established family containing only a few known genera (Graf, 2014) and is not yet well described. Members of the *Rikenellaceae* family have the genomic potential to produce butyrate, and in this way may play a beneficial role in colon health in humans (Hamer et al., 2008; Lai et al., 2018; Vital et al., 2014). However, in *K. sydneyanus* we found no correlation between abundance of *Rikenellaceae* and butyrate (Figure 6a). *Alistepes* is a new genus in the family *Rikenellaceae* (Rautio et al., 2003) and has been found in the gastrointestinal tract of both healthy and diseased human subjects (Parker et al., 2020).

Verrucomicrobiota were more minor components of section V. The mucin-degrading genus *Akkermansia* from this phylum has been previously reported from other herbivorous fish including rabbitfish (Le et al., 2020; Nielsen et al., 2017) and surgeonfish (Miyake et al., 2015), and is abundant in the gut microbiota of healthy Chilean human subjects (Derrien et al., 2008; Fujio-Vejar et al., 2017).

Also present in small relative numbers in the gut were the bacterial phyla Actinobacteriota, Cyanobacteria, and Spirochaetota. Cyanobacteria in section III were probably ingested epiphytes on the macroalgal diet (Egan et al., 2013), and not part of the gut microbiota. However, the sequences recovered in section V are probably residents as they are related to members of a deeply divergent and diverse Cyanobacterial lineage found in gut environments (Soo et al., 2014). Spirochaetota were previously reported as an abundant component of the hindgut microbiota in *K. sydneyanus* based on microscope observations (Rimmer & Wiebe, 1987).

We explored fungal and archaeal diversity in *K. sydneyanus* because these groups often play a role in carbon and energy cycling in gut ecosystems and yet are seldom examined in fish microbiota studies. The low read counts in our fungal ITS analyses probably represent low fungal biomass, or possibly the presence of taxa resistant to DNA extraction and amplification. The two dominant fungal phyla identified were the Basidiomycota and the Ascomycota (Figure S7), both of which have been reported from the gut of other fish (Romero et al., 2014). Agaricomycetes was the most dominant class found in *K. sydneyanus* (Figure S7). *Rhodotorula*, a genus of Basidiomycota, are red yeast commonly detected in the gut of both marine and freshwater fish (Andlid et al., 1995; Newman et al., 1972; Romero et al., 2014). The low abundance of archaeal sequences recovered from our samples is consistent with the very low levels of methanogenesis in the gut of *K. sydneyanus* (Mountfort et al., 2002) and the negative effects of seaweed on methanogenesis in in vitro ruminal fermentations (Maia et al., 2016). The negative effects of seaweed on methanogenesis in cow rumen have been attributed to the presence of naturally synthesized halogenated compounds such as bromoform found in seaweeds (Kinley et al., 2020; Roque et al., 2019, 2021). Dietary algae of *K. sydneyanus* such as *E. radiata and Gigartina macrocarpa* (Moran & Clements, 2002) also contain similar compounds (Carpenter & Liss, 2000; Mihaila, 2020; Nightingale et al., 1995), and this might contribute to the relative paucity of Archaea generally and methanogens specifically in these hindgut communities. However, we cannot rule out primer incompatibility with some potential symbiotic methanogens such as Methanoplasmatales (Paul et al., 2012).

The concentrations of SCFAs reported here in the different gut sections of *K. sydneyanus* are comparable to those previously reported (Clements & Choat, 1997; Mountfort et al., 2002). The significantly higher level of total SCFAs in sections IV and V (Table 3) suggest that these sections are important sites of fermentation and generation of energy for host fish. The ratios of SCFAs observed support our third hypothesis, that is, that SCFAs would be more consistent across different fish than microbial community composition. Acetate was detected in lowest concentrations in section III samples, higher concentrations in section IV, and highest in section V. This pattern probably reflects the overall fermentation activity as algal substrates are degraded and become available for fermentation by the microbiota. Propionate was 3–10-fold higher in section V compared to IV, probably due to fermentation by the Bacteroidota that predominate section V (Macfarlane & Macfarlane, 2003). Bacteroidota are major propionate producers in gut of humans (Aguirre et al., 2016; Salonen et al., 2014). Propionate is the principal gluconeogenic substrate in ruminants and a source of amino acid-carbon skeletons for the host. Butyrate did not vary significantly between gut sections, and is primarily a metabolic end product of Firmicutes (Macfarlane & Macfarlane, 2003). The increase in propionate and stable concentration of butyrate along the gut suggests that butyrate-producing bacteria were relatively consistent in density and metabolism, whilst the density and/or metabolism of propionate-producing bacteria increased. This disproportionate increase in propionate producers would yield an apparent decrease in relative abundance of butyrate producers. Formate was high in sections III and IV of the intestinal tract, and dropped off dramatically in section V, suggesting a major shift in carbohydrate fermentation pattern and carbon use (Kim & Gadd, 2019) and/or a change in formate metabolism by bacteria or uptake by the fish intestinal tract.

The concentration of the organic acids detected in the three gut sections only partially represents production from seaweed fermentation by bacteria, and also represents a net reflection of the processes of production, metabolism to other products, absorption into the hepatic portal system and blood, transport from more anterior regions, and transport to more distal regions. For example, SCFAs are absorbed across the gut wall and also may be metabolized by gut microbiota (Flint et al., 2012; Kim & Gadd, 2019; Mountfort et al., 2002). Valerate, hexanoate, heptanoate, isobutyrate, isovalerate, lactate, and succinate were not detected in our SCFA analysis but this does not necessarily mean they were not produced. Lactate and succinate are typically only produced and accumulate in elevated quantities in response to rapid fermentation of readily degradable carbohydrates (Wolfe, 2005). The most abundant carbohydrate present in the diet of *K. sydneyanus* is the sugar alcohol mannitol (Mountfort et al., 2002; White et al., 2010), which cannot be metabolized by vertebrates. However, mannitol is efficiently fermented by bacteria such as *Clostridium butyricum* especially in the presence of glucose

or acetate (Heyndrickx et al., 1989). The β-1,3-glucan laminarin, the storage polysaccharide of brown algae, is slowly catabolized by gut microbes due to the presence of unusual β-1,6-glucose linkages and mannitol residues (Michel et al., 1996). At least some of the propionate in the distal gut is probably produced by Bacteroidota from a succinate intermediate to yield more ATP from poorly fermentable substrates (den Besten et al., 2013). Overall, the fermentation profiles in *K. sydneyanus* closely resemble those produced by human faecal fermentation of seaweed (Mathieu et al., 2018; Michel et al., 1996). Conversely, the much lower concentrations of SCFAs and different fermentation profiles found in parrotfishes and detritivorous surgeonfishes indicate that these fishes do not receive substantial nutritional benefit from fermentation of carbohydrates (Clements et al., 2017; cf. Scott et al., 2020). Feeding behaviour can be useful in determining what is ingested by fish, but microbial processing of this material must be considered to understand the overall nutritional ecology of the species. The position of microbial residents in the gut, the densities of microbial populations and the length of time ingested material is held within the intestinal tract all influence the role of the microbiota in the breakdown of refractory material and the generation of fermentation products. Although some of these features are difficult to assess, fermentation products can be used for direct inference of microbial processes by quantifying abundant metabolic by-products. Future studies should consider these factors when exploring microbiota function in marine herbivorous fishes.

Correlations between SCFA concentrations and predominant bacterial taxa in sections III, IV and V of the gut of *K. sydneyanus* indicate the most likely contributors to fermentation profile in different parts of the gut (Figure 6a). Proteobacteria (genera in Rickettsiales) and Bacteroidota (*Rikenellaceae*, dgA-11 gut group) were positively correlated with acetate. *Rikenellaceae* which are abundant in section V displayed a strong positive correlation with propionate consistent with our fourth hypothesis, thus linking higher propionate concentrations with greater Bacteroidota numbers. Verrucomicrobiota (genus *Akkermansia*) also positively correlated with propionate. The human intestinal mucin degrading bacterium *Akkermansia muciniphila* has been well documented as a propionate producer (Ottman et al., 2017). Only Ruminococcaceae genera displayed a positive correlation with formate (Figure 6a).

In conclusion, our data provide a comprehensive and detailed description of gut microbiota composition in a kyphosid species and demonstrate significant compartmentalization among gut sections in terms of both bacterial community structure and fermentation product profile. While many 16S rRNA gene based studies of fish microbiota have been published, few have provided information about microbial processes and function within different regions of the gut. Relative to other herbivorous fish species, some of the highest levels of SCFAs have been observed in the hindgut of *K. sydneyanus*. Not all marine herbivorous fishes process refractory polysaccharides and ferment the sugars and sugar alcohols released from consumed algae. Some of these fishes, notably parrotfishes and detritivorous surgeonfishes, feed on protein-rich material that is passed rapidly through the gut in a manner inconsistent with considerable hindgut fermentation. Field observations of fish feeding behaviour provide a framework for understanding the potential role of a fish in an ecological context but these observations must be interpreted in the context of microbial fermentation to determine the nutritional ecology of that fish. Collectively, these microbial and organic acid data support our second hypothesis, that intestinal communities of *K. sydneyanus* were dominated by carbohydrate-fermenting, SCFA-producing Firmicutes and Bacteroidota.

In *K. sydneyanus*, microbial fermentation of simple carbohydrates occurs at substantial rates and symbiotic associations are important. This is remarkable considering the challenges faced by an herbivore that feeds primarily on phaeophyte algae. The simple carbohydrates and complex polysaccharides of this diet are largely unavailable to the host fish without the microbial conversion of these substances to nutritionally accessible compounds. Microbiota community composition of particular gut segments differed markedly between individual fish, whereas fermentation pattern, based on amounts of organic acids recovered from a gut segment, were fairly consistent. This suggests that the metabolic functions that are associated with seaweed degradation, and are critical to providing host nutrition, can be performed by different microbial communities. Although Firmicutes taxa in section III were present at intermediate levels in most individual fish, and genera from the family *Rikenellaceae* were consistently present in section V, our data did not support the concept of a "core" microbiota common to all individual host fish. We would expect to see even less support for the concept of a core microbiota when fish caught at different locations in different seasons are analysed. Future studies will involve characterization of the various undescribed bacteria detected in this study using culture-based methods, metagenomics and metatranscriptomics approaches. These will allow us to compare the functional genomic capacity of these organisms across wild fish communities.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

Bikiran Pardesi, Anthony M. Roberton and Kendall D. Clements planned and designed the study. Svetlana Boycheva and Bikiran Pardesi prepared the samples for sequencing. Kevin C. Lee and Bikiran Pardesi performed the bioinformatics and statistical analyses. Esther R. Angert and Kendall D. Clements provided advice on analyses and interpretation of results. William Lindsey White, Kendall D. Clements, Bikiran Pardesi and Anthony M. Roberton performed the fieldwork and sampling. Bikiran Pardesi, Anthony M. Roberton, Esther R. Angert and Kendall D. Clements wrote the manuscript and all authors contributed to final preparation.

DATA AVAILABILITY STATEMENT

All raw sequence data generated by this study have been submitted to the EMBL European Nucleotide Archive (ENA) under BioProject PRJEB34792. Sample metadata are also stored in the SRA (PRJEB34792) using the GSC MIxS host associated package ERC000013.

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