Influence of Seaweed Supplements on the Intestinal Bacteria in the Rabbitfish *Siganus Fuscescens*: Evidence for a Core Microbiome

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Abstract

Background

We know very little about natural variation in microbiomes of marine herbivorous fish in the wild or in captivity (aquaculture). Understanding how the consumption of seaweed influences intestinal microbial communities will shed light on how such phytobiotics could enhance the health and productivity of farmed fish. Here we screened the effects of supplementing the diets of mottled rabbitfish (*Siganus fuscescens*), a candidate species for international aquaculture development, with 15 different species of seaweeds and functional supplements currently used in aquaculture, on the bacterial communities that colonised their hindguts.

Results

Remarkably, the second most abundant phylum and the majority (53%) of the bacterial genera were not assigned, highlighting a significant knowledge gap for the field of animal microbiomes. Dietary supplementation increased alpha diversity by up to 23% relative to the control fish. Furthermore, most supplements significantly increased the relative abundance of *Firmicutes,* with similar trends for *Proteobacteria* and consistent decreases in *Bacteroides*. Seaweed supplementation also had important effects at the genus level, including significant increases in *Fusobacterium sp.* in fish fed seaweed - especially the green *Caulerpa taxifolia* – and overall trends for reduced levels of *Arcobacter* sp., a genus that includes fish and human pathogens. When we compared microbiomes in our fish to those from two recently published studies of conspecific populations sampled many thousands of kilometres away, the populations were clearly distinct, however there were 55 ASVs that were shared across the three fish populations, of which 35 were present in 50% of all fish sampled.

Conclusion

The identification of a core microbiome suggests that a host organism relies on certain microbes for key functions and our findings suggest that this candidate aquaculture species has a core microbiome in its hindgut which is robust to dietary manipulations and broad geographical and temporal variation. This insight will help guide future work investigating the functional and mechanistic bases of these relationships, as will improvements in microbial taxonomic resolution. Supplementation with seaweeds did have subtle influences on bacteria in the hindgut of *Siganus fuscescens* which could have important impacts on fish health and should be considered as aquaculture systems are developed for this species.

Background

Our evolving understanding that animals and plants are 'holobionts' with equally important microbial components [1-4] is enhancing our understanding of the ecology of important organisms and our ability to exploit them for food or other applications [4-7]. For example, understanding how microbiomes influence the health and disease resistance of farmed fish could add significant value to the aquaculture industry for which disease is a major cost component, and in many cases a bottleneck to further development [8].

In fish, changes in microbiomes have been linked to ageing [9], nutrient acquisition, immune responses, disease resistance, and general health [see recent reviews; 10, 11, 12]. In turn, many environmental and biological factors can affect the diversity and structure of microbiomes within the gastrointestinal (GI) tracts of fish [13]. Microbiomes in fish GI tracts are highly structured [14] with different parts housing very different microbial communities [15-17]. Fish 'first feeding' is emerging as a critically important step in structuring their GI microbiomes throughout their lifespan [10]. Microbiomes within fish GI tracts are further influenced by trophic level and associated gut morphology [18], with carnivores tending to have lower diversity in their intestinal microflora than herbivores and omnivores [19, 20]. The environment and 'external' microbial communities also influence the composition of microbiomes within fish GI tracts [21, 22], as does fish species [23] and life history stage [24].

We understand less about how diet affects microbiomes within the GI tracts of fish. In wild salmon (*Salma salar*) populations, life history stage and associated changes in habitat and diet type strongly influenced the composition and diversity of microbiomes within the GI tracts, whereas geography had little influence [24]. Similar patterns were observed in Yellowtail Kingfish (*Seriola lalandi*), whose GI microbiomes changed with life history stages, particularly when these developmental changes coincided with changes in diet [25]. Experimental manipulations of diet to reflect seasonal changes in food availability for wild Stickleback (*Gasterosteus aculeatus*) also led to changes in microbiomes within the fish GI track [26]. Understanding how diet can influence the GI microbiomes of commercially important fish species and the follow-on consequences for fish health, growth and disease resistance, would be of great value to the aquaculture industry and provide a pathway towards optimising GI microbiomes to improve the productivity and quality of farmed fish. In general microbiomes from cultivated fish are different to their wild counterparts and influenced by the type of farming [27]. However, the diversity of microbiomes in captive fish tends to be comparable to those in wild stock [28, 29].

Aquaculture recently replaced wild fishers as the main resource of seafood globally [30] and its importance in the provision of protein is likely to increase [31]. One of the greatest challenges for the sustainability of aquaculture is its reliance on fish meal and oil from increasingly depleted wild fisheries [30, 32] and the search for alternative feeds is a very active area of global research [32]. The use of land plant-based ingredients is one alternative [33], however, these novel ingredients which fish rarely encounter naturally, can create novel challenges, such as stunted growth, increased mortality and gut inflammation, especially in highly valuable carnivorous species [33, 34]. Furthermore, dietary supplementation with plant-based materials can reduce the microbial diversity in fish GI microbiomes [35-37]. More recently, the effects of marine and aquatic-derived ingredients (i.e. seaweeds, microalgae and their derivatives) have been investigated and overall have resulted in enhanced microbial diversity within GI tracts and sometimes also enhanced disease resistance [35]. However, dietary changes are not always correlated with changes in the microbiomes of farmed fish species [38] and we know very little about effects of diet on GI microbiomes of non-carnivorous or new candidate aquaculture fish species [35, 39-42]. This is of interest because there is an increasing push for farmers to

move away from carnivorous species (e.g. salmon and Asian sea bass), which require a lot of energy to produce and consequentially have larger carbon footprints [43]. Herbivorous and omnivorous fish are broadly considered more 'future-proof' and sustainable alternatives for the aquaculture industry because of their lower trophic level and ability to consume more varied, sustainable, plant based diets [43] that have considerably smaller carbon footprints.

Currently, a group of fish receiving increased attention for their range-shifting ability and associated impacts on temperate ecosystems ["Tropicalization"; 44] and as a potential aquaculture candidate due to their broad thermal and salinity tolerance, diet (opportunistic omnivore), sustainability and desirable white flesh, is the rabbitfish of the Siganidae family [45-48]. Understanding the microbiome of Siganids and importantly, how particular diets and dietary supplements can affect it (and thus fish production and health) will facilitate the development of this new aquaculture industry. Zhang, et al. [42] recently fed a member of this family, *Siganus canaliculatus*, aquafeed diets supplemented with a green seaweed (*Ulva pertusa*). Despite their very high inclusion rate compared to those reported for seaweed and other functional ingredients fed to higher trophic level fish, the supplementation of seaweed did not alter the diversity of microbiomes within the GI tracts of these fish [49, 50]. Zhang, et al. [42] concluded that there must be a strong core microbiome that is resistant to diet-mediated change.

Here, we investigated the effects of supplementing the diet of another species within this family, the mottled rabbitfish, *Siganus fuscescens*, with eleven different seaweed species and four aquafeed supplements as functional ingredients on the microbial communities of the fish hindgut. Selected seaweeds included members of the red, green and brown taxonomic groups and species that produce a broad range of bioactive, natural compounds (e.g. bromoform in *Asparagopsis taxiformis*, caulerpin in *Caulerpa taxifolia*; and terpenoids in *Sargassum* sp. [e.g. bromoform in *Asparagopsis taxiformis*, caulerpin in *Caulerpa taxifolia*; and terpenoids in *Sargassum* sp; 51, 52, 53]. We hypothesised that supplementation of diets with some of these seaweeds - particularly those with interesting bioactive compounds - at inclusion rates that are realistic in an industrial context, would lead to significant changes to the GI microbiomes in these fish. Recently, several other studies have characterised the GI microbiome of this species and revealed clear differences in microbial communities along the gut axis with more transient and food associated bacteria in the midgut compared to the hindgut, which harboured bacteria more likely to members of the host gut microbiome [15, 17]. In addition to providing a useful baseline, these studies also provided us with an opportunity to compare hindgut microbiomes between distinct populations of the same fish species separated spatially (by thousands of kilometres) and temporally (11-20 months) and assess whether any particular experimental diets influenced similarities between populations [15, 17]. Finally, we assessed whether fish size – as a proxy for age - influenced the diversity or composition of microbiomes across all three datasets.

Results

We sampled a group of wild-caught mottled rabbitfish (*Siganus fuscescens*) after a two week feeding trial where the fish were fed at 3% body weight per day with a control diet (unsupplemented) and diets supplemented at 3% dry weight with 15 supplements (11 seaweed species and 4 aquafeed supplements). The treatments were conducted in triplicate tanks with 3 replicate fish in each and 2 distinct size classes of fish (small and large) per replicate tank to which the treatments and fish were randomly allocated (n = 9 fish/treatment). At the end of the 2 week trial, fish were fasted for 24 h, euthanized and then their entire digestive tract was excised and DNA was extracted from a hindgut section for analysis of bacterial community diversity through sequencing of 16S rRNA genes. Additional *Siganus fuscescens* 16S rRNA gene sequence data was collected from Nielsen, et al. [15] and Jones, et al. [17] for comparative analyses between our captive rabbitfish and wild populations from the east and west coast of Australia. The results of the feed trial screening 15 ingredients are presented in "bacterial community diversity" and "microbiome taxonomic comparison" and the results of the analyses and comparisons between *Siganus fuscescens* populations are presented in "comparison with wild populations" and "microbiome taxonomy".

Bacterial community diversity. In total, we recovered 1,198 ASVs after rarefaction from the hindgut of *Siganus fuscescens* (N = 47) from this experiment. To allow the comparison of both sequencing runs from this trial at the ASV level, the rarefied ASVs abundance agglomerated at the genus level. This left 113 ASVs identified in total all 5 treatment groups (red, green, brown seaweed and aquafeed supplements and control). Out of the 113 ASVs, the hindgut of the fish fed the control and supplemented diets shared 63 ASVs (Fig. 1). The four groups of supplemented diets shared an additional 14 ASVs. The fish fed the five diets incorporating the red seaweeds had the most ASVs in common with those fed the three diets supplemented with green seaweeds (an additional 4 ASVs) and the three diets supplemented with brown seaweeds (3 ASVs) but shared only one unique ASV with the fish fed the four aquafeed supplements. The hindgut microbiome of fish fed green seaweed supplemented diets shared an additional 3 ASVs with that of the fish fed the four aquafeed supplemented diets group while that of the fish fed the three brown seaweed diets shared no additional, unique ASVs with the aquafeed group (Fig. 1). The hindgut of the fish fed the control diet had the lowest number of ASVs (69) while that of the fish fed the five red seaweed supplemented diets had the highest (98 ASVs) followed by those fed the green seaweed diets (95 ASVs), aquafeed supplements diets (93 ASVs) and brown seaweed supplemented diets (87 ASVs).

Despite this substantial variation, no statistical differences were found for the alpha or beta diversity indices, which is due, in part, to the high number of treatments in our screening design and between-individual variation. Nonetheless, there was some notable variation between and within treatments with respect to both the number of observed species and Shannon index (Fig. 2A and B). For example, the observed number of ASVs was highest in the hindgut of fish fed diets supplemented with sodium alginate (a brown seaweed extract), followed by fish fed diets supplemented with the green seaweed *Ulva* and then those supplemented with the red seaweed *Asparagopsis* (Fig. 2A; $F_{1, 15} = 17.67$, $P = 0.28$). The lowest number of observed ASVs were found in the hindgut microbiomes of fish fed diets supplemented with the red seaweed *Kappaphycus*, the green alga *Caulerpa* or the cyanobacterium spirulina (an existing supplement used in the aquaculture industry; Fig. 2A and C). The Shannon index is another measure of alpha diversity which accounts for the species number and the overall evenness of the population. When we compared the Shannon index, most of the fish fed diets supplemented with seaweeds or industry standards had higher mean indices than fish fed the control diet, with the exception of those whose diets were supplemented by the red seaweed *Kappaphycus*, spirulina or Hilyses® (Fig. 2B, $F_{1, 15} = 14.46$, $P = 0.49$). As was observed for observed number of ASVs (above), fish fed with sodium alginate also had the highest Shannon index, followed this time by diets supplemented by the red seaweed *Sarconema* and the green alga *Ulva* (Fig. 2B).

There were also no statistically resolvable differences in alpha diversity among treatment groups, however some interesting patterns emerged (Fig. 2C and D). Overall, supplementing the diets of fish with seaweed or aquafeed supplements, had a positive (albeit statistically non-significant) influence on both the number of observed ASVs (Fig. 2C, $F_{1,4} = 1.33$, $P = 0.85$) and Shannon index (Fig. 2D, $F_{1,4} = 1.56$, $P = 0.81$) of bacterial communities isolated from the hindgut of *S. fuscescens* compared to fish fed the control diet (Fig. 2C and D).

The beta diversity test (PERMANOVA based on Bray-Curtis and unweighted UniFrac measures; P = 0.99 and P = 0.209 respectively) did not show clear differences between the composition of hindgut microbial communities in fish fed different diets compared to the control fish (Fig. 3A and B). There was also no effect of fish size (weight or length) on the microbiome when each size variable was added (independently) as a covariate in separate PERMANOVAs (weight: P = 0.104 and length: P = 0.15 for unweighted UniFrac measure). In fact, more than 50% of all ASVs that contributed to >1% of the identified community were maintained consistently in all fish, regardless of experimental dietary supplementation or size.

Microbiome taxonomic composition. Of the 113 ASVs detected post rarefaction and agglomeration to the genus level, only 17 represented more than 1% of the total ASVs abundance. These were assigned to one unidentified and six known phyla. The unidentified phylum, which was represented by one ASV, had a very high relative abundance in our fish, averaging 15.6% ± 1.1% (mean ± SE) total abundance across all treatments (Fig. 4). The most abundant phylum (*Proteobacteria*) was represented by just two ASVs, one of which could not be identified with any greater taxonomic resolution, but accounted for an average 26.9% ± 2.0% of the total bacterial abundance in our samples. Of the 16 assigned ASVs, eight belonged to the phylum *Firmicutes*, two belonged to the *Proteobacteria*, and another two each to *Bacteroidetes* and *Fusobacteria*. *Proteobacteria* had the highest relative abundance of ASVs in our samples averaging of 28.5% ± 2.1% abundance across all treatments, followed by *Firmicutes* with 20.3% ± 1.4% abundance. *Firmicutes* was the only phylum that differed significantly between the different diets in our screening trial ($F_{1,15} = 3.07$, $P = 0.003$), with the lowest relative abundance observed in the hindgut of fish fed the control diet (9.3% ± 4.0%) compared to an average of 21.1% ± 1.4% for all other treatments (Fig. 4). Fish fed the *Haematococcus* sp. and *Halimeda* sp. diets had the highest relative abundance (28.8% ± 4.2% and 28.5% ± 9.9% respectively) of *Firmicutes* and the average value for the seaweed supplements was 20.5% ± 1.7%. Conversely, the control diets led to an increase in the proportion of bacteria belonging to the phylum *Epsilonbacteraeota* (Fig. 4) although this consistent observation could be resolved statistically.

At the family level, the fish fed the control diet had the lowest relative abundance of *Ruminococcaceae* (3.5% ± 1.6%) compared to supplemented diets, with the highest relative abundance observed in fish fed the calcified green seaweed *Halimeda* sp. (13.0% ± 6.3%; $F_{1,15} = 3.07$, $P = 0.003$). There was also an increase in the relative abundance of bacteria from the *Arcobacteraceae* family in the hindgut of the fish fed the control diet (Fig. S2). Despite the magnitude of these differences, they were not significant when compared to supplemented diets overall or individually.

Although, most of the bacterial genera in our samples were unidentified (53%), we did find some clear differences between the communities in the hindgut of fish fed any of the supplemented diets compared to those fed the control diet at the genus level. For example, although the relative abundance of *Fusobacterium* sp. and *Ruminococcaceae* UCG-014 were low and variable across all fish, including those fed with supplemented diets (average 1.6% ± 0.5% and 1.5% ± 0.4% respectively), they both had extremely low abundance (0.3% ± 0.2%) in the hindgut of fish fed the control diet alone (Fig. S3). This difference was significant for *Fusobacterium* sp. overall ($F_{1,15} = 2.135$, $P = 0.036$,). Although pairwise comparisons were unable to resolve these differences, the largest discrepancy appeared to be between fish fed Hilyses® and control diets (which had 0.1% ± 0.0% and 0.3% ± 0.2% respectively) and those fed *Caulerpa* sp., which had the highest relative abundance this ASV (8.3%± 5.1%). The *Caulerpa* sp. diet also resulted in fish with the highest relative abundance of *Treponema* sp. (9.4% ± 5.2%) compared to an average of 5.6% ± 0.5% across all other treatments (including the control), although this difference was not significant. Furthermore, the genus *Arcobacter* sp. represented on average 2.8% ± 0.8% abundance in the hindgut microbiomes of fish fed supplemented diets (from 0.4% ± 0.2% in *Lobophora* sp. to 9.2% ± 6.8% in *C. taxifolia*), whereas in fish fed the control diet, *Arcobacter* sp. represented 23.6% ± 16.5% of relative abundance (Fig. S3 and Table S2). Despite these large disparities between treatments, our analyses were unable to resolve any statistically significant differences due to our screening of so many different seaweed species.

The genus *Cetobacterium* sp., which represented 5.1% ± 5.1% of the ASVs present in the hindgut of fish fed the control diet, had an average relative abundance of just 1.8% ± 0.4% in the hindgut of the fish fed the supplemented diets. This non-significant trend was consistent, even in the case of fish fed Hilyses®, where the relative abundance of *Cetobacterium* sp. was higher (9.6% ± 9.5%) than all the other treatments (Fig. S3 and Table S2). The genus *Romboutsia* sp. was also in low relative abundance in the hindgut of the control diet fed fish (0.7% ± 0.3%) and on average across all supplemented diet fed fish (1.9% ± 0.4%), however it tended to be detected in higher abundances in the fish fed the red seaweed *Asparagopsis* sp. (5.8% ± 4.7%) and the aquafeed supplement Hilyses® (5.1% ± 2.0%; Table S2).

Comparison with wild populations.

We compared our microbiome data, obtained from fish collected on the subtropical Sunshine Coast in 2018, to those obtained from other hindgut microbiomes in the same fish species from wild-caught individuals in populations located off the Western Australian (WA) coastline (Shark Bay and the Kimberley Coast) and the Great Barrier Reef (GBR; One Tree Island) during 2015 and 2016. In total, we recovered 3,084 ASVs after rarefaction (6290 sequence depth; Fig. S4) from the hindgut of *S. fuscescens* from our combined studies (85 samples in total; with n = 47 fish from our study, n = 16 from Nielsen, et al. [15] and n = 22 from Jones, et al. [17]). In order to compare the three studies, ASVs agglomeration was performed at the genus level. This led to a comparable list of 174 ASVs (101 ASVs from our study on the Sunshine Coast, 99 ASVs from the GBR; Nielsen, et al. [15] and 120 ASVs from WA; Jones, et al. [17]). There were 55 ASVs shared among the hindgut of the mottled rabbitfish that were present in fish from all three studies (Fig. 5). The two studies of wild populations shared an additional 14 ASVs with each other that were not observed in our fish. Our study of microbiomes in the hindgut of fish held under captive conditions for at least four weeks following wild capture did however, share and additional 14 and 9 ASVs with the WA and GBR populations, respectively. The fish from WA had the most unique ASVs (38), followed by the fish from our study and then thirdly, fish from the GBR (21, Fig. 5).

When we compared the different treatment groups in our study (hindgut microbiome of fish fed red, green, brown seaweed, aquafeed supplements and control diets) to the other two studies, there were no significant differences in alpha diversity (neither in the number of observed species:$F_{1,7}$ = 8.82, P = 0.26, nor the Shannon index: $F_{1,7}$ = 7.01, P = 0.42; Fig. 6A and B) and we also detected no significant differences in those parameters when we included all of our diet treatments as one group (number of observed species: $F_{1,3}$ = 7.30, P = 0.06); the Shannon index: $F_{1,3}$ = 85.81, P = 0.12) and compared them to the hindguts of *S. fuscescens* from the other studies (Fig. 6C and D).

Combining all our seaweed treatments into one group, analyses of beta diversity did reveal strong differences between the three studies with respect to relative abundance and composition of hindgut microbial communities in fish (PERMANOVAs based on Bray-Curtis and unweighted UniFrac measures; $F_{1,3}$ = 13.39, P = 0.001and $F_{1,3}$ = 16.34, P = 0.001respectively; Fig. 7). The hindgut microbiome of the three geographically distinct wild mottled rabbitfish populations were also significantly different from each other ($F_{1,2}$ = 14.87, P = 0.001and $F_{1,2}$ = 8.97, P = 0.001 for Bray-Curtis and unweighted UniFrac respectively; Fig. 7A and B). The beta diversity of the two wild populations from Western Australia differed significantly based on Bray-Curtis ($F_{1,1}$ = 2.66, P = 0.009) but not on UniFrac ($F_{1,1}$ = 1.14, P = 0.29). Fish length did not significantly influence beta diversity ($F_{1,1}$ = 1.18, P = 0.26 and $F_{1,1}$ = 1.31, P = 0.2 for Bray-Curtis and unweighted UniFrac respectively).

Microbiome taxonomic composition of the three studies. The three studies were clearly distinguishable from each other with respect to the relative abundance of many differentially abundant ASVs (Fig. 7). The unidentified phylum had a consistently high relative abundance across all populations (10% in GBR samples; 21% in Shark Bay samples; 31% in Kimberley samples and 17% in Sunshine Coast fish). Fish from the GBR had the highest relative abundance of *Proteobacteria* (52.6% ± 2.9%) compared to all the other fish including those in our study (30.9% ± 2.2%) and the fish from WA (26.5% ± 1.9% and 20.6% ± 2.0% for Shark Bay and the Kimberley fish respectively; Fig. 8). The GBR fish were the only fish without ASVs from the *Spirochaetes* family, and they also had the lowest relative abundance of *Fusobacteria* (0.2% ± 0.0%) and *Epsilonbacteraeota* (0.06% ± 0.02%), which across all the other fish represented an average of 3.8% relative abundance (Fig. 8). The four most abundant ASVs in the GBR fish represented 75.2% ± 2.2% of the relative abundance compared to 71.9% ± 2.2% and 61.3% ± 1.9% in Western Australia and the Sunshine Coast fish, respectively.

Fish from Western Australia had more similar hindgut microbiomes to the fish from our feeding trial, particularly those from Shark Bay. For example, the relative abundance of *Fusobacteria* from the Shark Bay fish was comparable to the fish in our study, with (13.6% ± 3.5% and 10.6% ± 0.7% respectively) but lower than in those from the Kimberley (9.6% ± 1.2%) and the GBR (6.1% ± 0.6%; Fig. 7). The fish fed diets supplemented with the green seaweed *Caulerpa* sp., the brown seaweed *Dictyota* sp. and the red seaweeds *Gracilaria* sp. and *Laurencia* sp. had similar abundances of *Fusobacterium* sp. in their hindgut (2.9% ± 1.3% to 8.9% ± 5.4%) to those from Shark Bay (6.8% ± 3.2%), while the other treatment in our study, including Hilyses and *Halimeda* sp. led to much lower (0.1% ± 0.0% and 0.2% ± 0.1% respectively) abundance as was observed in the hindgut of the fish from the GBR (0.15% ± 0.0%) and Kimberley (0.4% ± 0.1%). On the other hand, fish fed the diet supplemented with the red seaweeds *Asparagopsis* sp. (1.1% ± 0.3%) and *Gracilaria* sp. (1.1% ± 0.5%) led to similar hindgut relative abundance of *Ruminococcaceae* UCG-014 to the fish from the Kimberley (1.5% ± 1.1%). Furthermore, the *Bacteroidetes*, which represented 15.0% ± 2.9% of the community in fish from the Kimberley and 16.2% ± 0.8% in fish from our study, but only 4.3% ± 1.3% in Shark Bay fish. The relative abundance of *Ruminococcaceae* UCG-014 was also notably higher in our fish (1.5% ± 0.4%) and those from the Kimberley (1.5% ± 1.1%) than the Shark Bay (0.5% ± 0.2%) and GBR fish (0.04% ± 0.01%; Fig. S5).

One key difference between both of the wild fish studies and ours was the absence or very low relative abundance (0-0.17% ± 0.0%) in the WA and GBR fish of *Spirochaetes*, which had a relative abundance of 5.7% ± 0.5% in our samples (Fig. 8). As for the fish in the feeding trial, the three only 8 out of 17 ASVs were assigned to known genera. Nonetheless, between 12 and 65 ASVs significantly differed in abundance between the hindgut microbiome of the 4 geographically distinct populations of rabbitfish from the 3 studies (Fig. S5 and S6 and Table S3). Compared to the two wild populations (GBR and WA) our fish tended to have increased relative abundances of identified ASVs representing > 1% abundance in the community. These ASVs included *Treponema* sp., *Romboutsia* sp., *Turicibacter* sp., and *Ruminococcaceae* UCG-014, which all consistently and significantly represented greater proportions of the microbial communities in the hindguts of our fish compared to the other populations (Fig. S5 and S6 and Table S4). The hindgut from our fish also had higher *Cetobacterium* sp. compared to those from the Kimberley site and higher *Arcobacter* sp. compared to the GBR fish (Fig. S6 D and F and Table S4), although these differences could not be resolved statistically.

There were some exceptions to this pattern. For example, wild fish hindguts had significantly higher relative abundances of *Akkermansia* sp. and *Tyzzerella* sp. than our fish (Fig. S6 and Table S4). The fish from both sites in WA also had significantly higher relative abundances of *Rikenella* sp. and *Sedimentibacter* sp. than our fish while the GBR population had higher relative abundances of *Terrisporobacter* sp. and *Staphylococcus* sp. than any of the other geographical locations (Fig. S6 and Table S4). The most similar populations were those from the two sites in WA (12 significantly different ASVs; Table S4). Our fish were most similar to those from Shark Bay in WA, which had only 27 ASVs with significantly different relative abundances (Fig. S6B and Table S4). The most distinct populations with respect to hindgut microbiomes were the fish from the GBR, and those from our study, which had significant differences in the relative abundances of 65 ASVs (Fig. S6F; Table S4. Despite this variation, more than one third of the ASVs that contributed > 1% abundance of the bacterial communities within the hindguts of *S. fuscescens* were common across all three populations and the different sites and experimental manipulations within. The core microbiome analysis revealed that out of the 55 shared ASVs between the three studies, 35 of them were present in 50% of all the fish sampled (43 out 86 fish; Table S5). Only seven out of the 35 AVS had assigned genera and included *Fusobacterium* sp., *Romboutsia* sp., *Treponema* sp., *Arcobacter* sp., *Alistipes* sp., *Odoribacter* sp. and *Brenakia* sp. (Table S5).

Discussion

Supplementation of diets with seaweeds and commercially available aquafeed supplements had some subtle effects on the diversity and composition of hindgut microbial communities in the rabbitfish *Siganus fuscescens*, particularly at the genus level, although half of the genera were unknown. The relative

abundance of *Fusobacterium* sp. was enhanced in the hindgut of fish fed diets supplemented with seaweed or other functional ingredients, whereas dietary supplementation reduced the abundance of the genus *Arcobacter* sp. (which was on average five times more abundant in the control fish). Overall, however, the hindgut microbiomes remained remarkably consistent between treatments including the control fish, suggesting either (i) that the dietary supplementations (3%) were insufficient to elicit a strong change in the fish GI microbiomes, or (ii) the existence of a stable, core microbiome in *S. fuscescens*. In this study, all fish sampled shared at least 63 out of a total of 113 ASVs identified in our bioinformatics pipeline. When the sequence data from the current study to those reported in two recently published papers on the hindgut microbiomes in the same fish species, despite some clear differences, 55 out of a total of 174 ASVs were shared and 13 of those represented between 66% and 85% of the total relative abundance. These observations from geographically and temporally distinct populations provide strong evidence for a core microbiome in this omnivorous subtropical fish, which appeared surprisingly stable, despite experimental manipulations of diet in the current empirical work at levels that are known to be able to fundamentally change the outcomes of production and other fish traits [35, 54]. Furthermore, given the number of different seaweed species screened (eleven) alongside other aquafeed products (four) and the very broad differences in both the proximate compositions and natural products chemistry between the various dietary supplements provided, the maintenance of the majority of the GI microbiome in these fish provides unusually comprehensive evidence for a core microbiome in this candidate aquaculture species.

Effects of diet on the hindgut microbiome of S. fuscescens

Some previous studies have observed dramatic effects of experimental diets on the gut microbiomes of farmed fish [25, 27, 29], whereas others have found that microbiomes of fish are relatively stable and do not show much overall change to dietary manipulation [38, 42, 55]. The studies that detected strong changes typically supplemented fish diets with probiotics or other functional feeds for 4–8 weeks experiments, longer than our experiments. However, Wong, et al. [38] fed Rainbow trout experimental diets including grains for a period of 10 months and observed only subtle changes in the fish microbial communities. Lyons, et al. [55] supplemented the diet of rainbow trout but in this case with microalgal meal at a level of 5% and found that, whilst addition increased diversity, the overall structure of microbiomes in the distal guts of the fish were not significantly altered. In another study similar to ours, Zhang, et al. [42] found that supplementing *Siganus canaliculatus* [a color morph of *S. fuscescens*; 56] with 10% *Ulva pertusa* and other additives for a period of 8 weeks did not significantly alter the microbial diversity in the gut content of the fish. They concluded that a strong core microbiome constituted of 86 operational taxonomical units (OTUs) which was shared across all fish regardless of the dietary treatment [42].

Although, all the seaweeds tested in this screening trial seemed to affect the hindgut microbiome composition of the mottled rabbitfish, the lack of impact at a high level was surprising given the breadth of biochemical compounds produced by these taxonomically diverse organisms and their known impacts on microorganisms in nature [57–59] and experiments [60–62]. The microbiome of seaweed varies greatly from its surrounding environment and between seaweed species. One mechanism involved in shaping the microbiome of seaweed is the biochemical composition of the surfaces of seaweed and the metabolites they produce. For example the red seaweed *Delisea pulchra* has been reported to be mainly colonised by Gram negative bacteria and this has been linked to the seaweed's production of N – acyl – homoserine lactones which inhibit the signal pathways in Gram negative bacteria [63].

Seaweed have also been used as prebiotics in animal feeds, including for fish, to induce microbial shifts in the gut of its host towards a more beneficial bacterial assemblage [64–66]. Although, these studies focused on the prebiotic effects of the seaweed complex polysaccharides (e.g. sodium alginate, agar and carrageenan) there is one clear example from land animals that seaweed secondary metabolites can also have strong effects on the gut microbiome of it hots. Feeding ruminants the red seaweed *Asparagopsis taxiformis* quickly (e.g. 3 days) changed the rumen microbial composition leading to drastic reduction of methanogenic *Archaea* and as a result led to reductions in enteric methane emissions [67, 68]. This was the result of supplementing the diets of the ruminants (ovine and bovine) with up to 5% *A. taxiformis* which produces and accumulates halogenated compounds including the anti-methanogenic bromoform [69]. For these reasons, the bioactive and prebiotic potential of seaweeds are receiving increasing levels of attention [70].

Although seaweed are known to be a diverse group (~ 10,000 species) producing a wide range of secondary metabolites with bioactive properties, there is a gap in the literature regarding their potential as dietary supplement to shape intestinal microbiome of animals including fish [71, 72]. Even if the effects of dietary seaweed supplementation on the fish microbiome in our feeding trial were not as pronounced as expected, this study was the first to screen multiple seaweed species including some known for their secondary metabolites (e.g. *Asparagopsis taxiformis*, *Caulerpa taxifolia* and *Laurencia obtusa*). The potential influence of seaweed chemistry was well illustrated by one seaweed species in particular: fish fed diets supplemented with the green alga *Caulerpa taxifolia* more closely resembled those fed the control diet than the other seaweed treatments. In particular the genera *Cetobacterium* sp., *Treponema* sp. and *Fusobacterium* sp. were all enhanced in hindguts of fish that received *C. taxifolia* and this difference was significant for *Fusobacterium* sp.. This seaweed produces many interesting bioactive compounds [73] and its presence on reefs can completely alter sediment microbiomes through chemical modifications of the substrate [see 74 and references therein]. This seaweed is typically avoided by the native herbivorous fish (*Girella tricuspidata*) and invertebrate grazers in Australia [75] and can be toxic to invertebrates forced to consume it in feeding trials [75, 76]. Another seaweed avoided by marine herbivores due to its production and storage of bioactives is the seaweed *A. taxiformis* [77], which also stood out from the other treatments in our trial as it resulted in the highest abundance of *Romboutsia* sp. (2.5 times higher than the other dietary supplementations or control) in the hindgut microbiome of the fish that were fed it.

The *Fusobacterium* genus includes some important human pathogens that have been implicated in diseases such as colorectal cancer [78] and have been identified in GI microbiomes of other commercially important, warm water fish previously [20]. Additionally, although the role of *Fusobacterium* sp. in fish remains largely unknown [20], this genus has demonstrated enzymes for the breakdown of carbohydrates in three fish species [79]. Our observation of them in our fish supports the hypothesis put forward by Larsen et al, (2014) that these may be normal members of microbiomes in the guts of many, diverse fish species. The microbial taxonomic composition of the fish hindgut revealed other bacterial taxa associations typically far removed from fish, seaweed or marine settings. The genus *Arcobacter* sp. is usually associated with water from sewage [80] or farm effluents such as piggeries [81] but can be common in marine invertebrates such as crabs and oysters [82] and includes some intestinal pathogens of both humans and fish [83, 84]. In our study, it was particularly abundant in fish fed the 'control' diets without any supplementation (five times more abundant). This observation suggests that the seaweeds may perform a prebiotic-like role, which enhances the growth of more favourable bacteria, thereby preventing the growth of potential pathogens such as *Arcobacter* sp..

Although our fish were caught near the coast and could have been subjected to farm effluents through river flow during high rainfall events, this ASV was also found - albeit in lower abundances - in the hindgut of the fish on the remote One Tree Island site from the study from Nielsen, et al. [15] and on the two sites from the west coast of Australia from study by Jones, et al. [17], suggesting that it may be a commensal or symbiotic member of the core microbiome, or a widespread, opportunistic pathogen that dietary inclusion of seaweed keeps under control.

The vast majority of host associated intestinal bacteria within any host organism remain unidentified (in the current study and the other two used as comparisons, 53% of the ASVs > 1% abundance could not identified to the genus level) and their functions are largely unknown. For, example despite efforts to increase genera identification, the unknown bacterial genera in the human gut as part of the Human Microbiome Project and the Human Gastrointestinal Bacteria Genome Collection still represent 40% of the recovered genera as of August 2018 [85]. Nonetheless, various intestinal bacteria are known to benefit their host by aiding in digestion, metabolic processes, growth and development, immune responses and resilience to stress and other factors via the production of diverse compounds including short chain fatty acids and vitamins (79). For example, the vitamin B-12 producing *Cetobacterium* spp. was detected in most of the fish in the current study. Although this bacterium is generally associated with freshwater fish such as the grass carp, *Ctenopharyngodon idellus*, its detection could be related to the presence of functional genes associated with protein digestion within the genome of *Cetobacterium* spp. [86], which support host responses to dietary change [87]. Since our fish were wild-caught and, prior to capture, presumably browsed mainly on seaweed, *Cetobacterium* sp. might have been over-represented because these bacteria were supporting their hosts' transition to new diets.

The appearance of *Treponema* sp. in all our fish was another unexpected observation. *Treponema* sp. has been described in the hindgut of *S. fuscescens* previously [15], and was also found in the hindgut of the fish from WA [17], yet is predominantly reported from the GI tracts of pre-industrial, traditional and agrarian human populations including pygmies and Amazonians [88] and other primates, terrestrial mammals and termites [89]. Although of significant interest in human microbiome research because this genus seems to have been eradicated from human GI tracts by unknown processes linked to industrialization, in the context of rabbitfish, this taxon might indicate the presence of a bacterial community using compounds such as xylan, xylose, carboxymethyldcellulose or hemicellulose which are likely present in aquafeed. This bacterium was overrepresented in our samples compared to the wild fish where it was absent (GBR) or present only at minute levels (0.1% and 0.2% for the Shark Bay and Kimberley fish respectively), particularly in those fish fed *C. taxifolia* supplements, to potentially assist the host in obtaining nutrients from these novel types of food, as β-1,3-xylan is a major component in the cell wall of this seaweed.

In the current study, the experimental design (which was optimised to include as many different seaweeds and aquafeed supplements as possible), lacked statistical power in some cases due to the unexpectedly stable microbiome of these fish overall, the lack of genus assignment for half of the recovered ASVs and, the high level of between-individual variation within treatments, thereby limiting the ability to resolve some of the statistically observed differences. Furthermore given the extraordinary ecological breadth of 'fish' (marine, estuarine, freshwater, diadromous lifestyles, carnivorous, omnivorous and herbivorous trophic levels, benthic pelagic, cryptic lifestyles, etc.) and the multiple, complex ways in which their environments (wild [polar, temperate, tropical, shallow, deep, etc.] or aquaculture), and biology (e.g. genetics, life history stage) can affect their gut microbiomes, more research is needed to better understand the general influences of diet on the composition, diversity and function of microbiomes within the GI tracts of fish to enable generalisations about the influence of diet.

Effects of temporal and spatial variation on the hindgut microbiome of S. fuscescens

When comparing the three populations, the hindgut microbiomes from rabbitfish on the GBR seemed more distinct than other populations. Overall, the hindgut microbiome composition of the fish from our study (Sunshine Coast) more closely resembled those from Shark Bay in Western Australia, which is at a similar latitude. Surprisingly our fish were more similar to fish from the tropical Kimberley site than the tropical GBR fish, which were collected from a site much closer than the former.

Potential explanations for these groupings are that all of the seaweed genera fed to our fish have tropical, subtropical or temperate distributions and are common on the eastern coast of Australia, with many also occurring on the west [90]. It is therefore possible that some of the similarities between the wild populations and our seaweed treated fish, may be the result of the fish at those locations feeding on similar diets. Another explanation for the similarities observed between our fish and those from Shark Bay could be similar abiotic and biotic factors, given that the Kimberley and GBR sites are both tropical and the Sunshine Coast and Shark Bay sites are both sub-tropical locations. Furthermore, the fish from our screening trial were collected near shore (< 1 km away), as were the fish from Shark Bay, whereas the fish from the Kimberley site were approximately 25 km from the coast and finally the most distinct hindgut microbiome was found in the fish from One Tree Island on the GBR which is about 70 km from the coast. The impact of rivers, agriculture and other human or land associated impacts may be clearer in nearshore areas, which could explain some of the differences observed here.

Similarly low spatial and temporal variation was observed in the gut microbiomes of larvae from another rabbitfish species, *Siganus guttatus*, across 3 sites separated by up to 390 km across a three year sampling program [91]. The gut microbiota of zebrafish *Danio reiro*, from genetically distinct wild and domesticated populations were strikingly similar despite very different environmental and dietary conditions and the authors speculated that shared intestinal features from the two groups of fish led to the selection of specific bacteria taxa resulting in strongly similar gut microbiome regardless of the origin or domestication status [92].

Because of vastly different biotic and abiotic conditions, along with different sampling times and teams, distance from the coast and a myriad of other possible differences across the three studies, many factors not considered here would likely influence GI microbiomes in the sampled fish so consequentially, the authors from this study will refrain from drawing any broad conclusions regarding specific supplements used in this study, or even broad geographical patterns from these comparisons. However, the overlap between hindgut microbiomes from fish in the three studies against the backdrop of such biological and physical variation, provides further compelling evidence for the existence of a core microbiome in *S. fuscescens*.

Does Siganus fuscescens **have a core microbiome?**

Despite experimental manipulations of diet with a broad and comprehensive list of taxonomically and chemically diverse seaweeds and samples originating from populations in locations separated by up to 4000 km around the Australian coast (the Sunshine Coast in southeast Queensland; present study, Shark Bay and the Kimberley site in Western Australia; Jones, et al. [17], and the Great Barrier Reef; Nielsen, et al. [15]), the mottled rabbitfish in Australia maintained nearly one third of their hind gut microbiota in common, of which 35 ASVs were identified as being part of a core microbiome in this species. Identifying a core microbiome is one of the first steps required to link microbial community structure and diversity to its function and importantly, the role it plays for its host [93]. The existence of a conserved group of bacterial taxa indicates that the functions they provide, persistently throughout multiple populations (and in our case, despite diet manipulation and starvation), could be essential to the development or homeostasis of the host. However, 80% of the identified core ASVs were not identified at the genus level, making it difficult at this stage to speculate on the function they may play. Core microbiomes have been identified in other marine organisms, including corals [94, 95], seaweeds [96] and other cultivated fish [38]. Understanding how these microbial taxa benefit fish could help expedite the development of a new aquaculture industry for Siganids, of which many species including *S. fuscescens*, are being considered for culture in different countries around the world given their flexible dietary nature and tolerance to a broad range of environmental factors [45–48].

Evidence for core microbiomes has been observed in studies of other rabbitfish species [42, 91]. Consistent with our observations, these authors reported both overlap and variation between locations and/or populations and speculated that if the core microbiome related to functionality, rather than taxonomy *per se* then the functional redundancy within the core microbiome could be expected. Functional redundancy has been identified in core microbiomes from other marine organisms, including a seaweed [97], so investigating the functional profiles of these 'core' taxa could be a sensible way to progress towards a better understanding of the roles microbiomes play for their hosts.

Microbiomes and the sustainable development of aquaculture

The existence of a core microbiome would suggest that the members of that microbiome were essential to the normal development or homeostasis of the host (79). Understanding the functions core microbiota play for their hosts will provide valuable insights for the development of sustainable aquaculture practises. For example, understanding the roles of gut microbiomes in herbivorous fish may help to facilitate the transition of carnivorous fish onto more sustainable plant-based diets. It is possible that microbiome manipulations could be applied via microbiome transfer or targeted probiotic strains delivered in the feed. Although, interspecific transfer of microbial communities is a novel area of microbiome research, early results are promising and suggest the possibility of establishing a stable microbiome from one donor species in the GI tract of a recipient host belonging to a completely different species. This has been trialled between humans and pigs and between fish and rats [98]. In one example, microbial community transfer between two rats: an herbivore (*Neotoma albigula)* and an omnivorous laboratory rat, (*Rattus norvegicus*) led to the development in the latter a newly acquired ability to degrade oxalate, a nephrotoxin found in plant materials, which persisted for 9 months after the transplant [99]. Microbiome manipulations such as these have the potential to greatly enhance the sustainability and resilience of the aquaculture industry.

'Designer microbiomes' have been proposed as a potential solution to the global problem of coral bleaching due to thermal stress. van Oppen and Blackall [100] and others have proposed that probiotics (either naturally-occurring or genetically engineered microbiomes) could be delivered to corals to enhance their tolerance to thermal stress. Warming ocean waters are also problematic for aquaculture because of associated reductions in the thermal niches of high latitude fish (e.g. Atlantic salmon *Salmo salar*), thus restricting the availability of places in which they can be farmed, while more temperate and subtropical species tend to be more tolerant to temperature fluctuations [101, 102]. It is possible that designer microbiomes of the future could also address this issue by extending the thermal tolerance of commercially valuable fish species.

A potential barrier to the use of microbiome manipulations for sustainable aquaculture is that a significant proportion of host-associated bacteria have never been identified, in our study and others [15, 17, 103–105]. For example, one ASV from our study could only be taxonomically ascribed with confidence at the Kingdom level, yet it accounted for > 30% of the total abundance of the microbes in some fish populations. Furthermore, this represents a key knowledge gap as functional traits of many of these microbes is almost entirely unknown thus highlighting a priority area for future research into holobionts in ecological or any applied context, including aquaculture.

No clear or consistent effects of fish size on hindgut microbiomes of S. fuscescens

Many researchers have reported on the importance of life history stage in the diversity and structure of gut microbiomes (Egerton et al. 2018). Due to the haphazard nature of our sampling, the fish collected for the feeding trial ranged between 15–21 cm in length and 70 g – 189 g in mass. Fish size can be used as a proxy for fish age, however when fish mass or fish lengths were included as covariates (comparing fish fed different diets and fish from different populations/studies), it did not explain any of the variation in microbial diversity or composition. This could suggest that by the time they are 15 cm + in length, the microbiome of *S. fuscescens* is fairly stable.

Conclusion

The mottled rabbitfish *Siganus fuscescens* has a remarkably stable hindgut microbiome that was only subtly influenced by dietary manipulation with diverse seaweeds and selected commercial products in culture. The results of this study from captive *S. fuscescens*, plus an analysis of the microbiome from three wild *S. fuscescens* populations, indicates that a conserved core microbiome in the hindgut of this species was observable across multiple populations separated by thousands of kilometres. However, the fact that the majority of the bacteria could not be identified is also a reminder that although microbiome studies are increasingly frequent, a wide knowledge gap remains with respect to the identity and function of commensal bacteria within host-associated microbiomes. Nonetheless, we propose that the flexible diet of this opportunistic omnivorous fish [106, 107], its tolerance to a wide range of abiotic [108] and geographic conditions [109, 110] and its specific physiological (e.g. immune system and intestinal environment) pressures on microbial communities, the gut

microbiome of the mottled rabbitfish is relatively stable regardless of the environment or diet. This supports recent reports that *S. fuscescens* poses an important threat as an invasive species moving in to temperate ecosystems ["tropicalisation"; 44]. These observations also highlight an exciting opportunity for using *S. fuscescens* as a candidate species for adaptive and sustainable aquaculture of the future. Further explorations into the functional roles of the core microbiome will help expedite the development of an aquaculture industry for this species, as well as for other *Siganus* species.

Material And Methods

Mottled rabbitfish (*Siganus fuscescens*: 15 cm/70 g to 21 cm/185 g) were captured between February and March 2018 using a drag net (15 m long by 2.1 m deep with a 2.5 cm mesh size) on rocky reefs at Moffat Beach, Queensland Australia (26°47'21.7"S 153°08'36.0"E). This collection was carried out under a "General fisheries permit" (# 195305) issued by the Queensland Department of Agriculture and Fisheries (Fisheries Act 1994). Feeding trials were conducted at the Bribie Island Research Centre (BIRC) on Bribie Island, Queensland, Australia (27°03'15.9"S 153°11'42.9"E). After collection, fish were transferred to BIRC in an oxygenated 500 L tank. The newly captured fish were treated with hydrogen peroxide (200 mg/L for 30 min) to rid them of potential external pathogens and parasites. Following this, the fish were transferred to three 1000 L fibreglass tanks where they were acclimatised and fed the control (unsupplemented 'Native' pellets from Ridley Aquafeeds Ltd) diet for at least two weeks. The Native diet has been formulated for Australian native freshwater fish species and was chosen based on its low protein (38% protein, 10% fat content and 15 MJ/kg gross energy) compared to other commercially available diets. All activities were approved by the animal ethics committee of the University of the Sunshine Coast (AN/S/17/51).

Seaweed and experimental diets

We aimed to screen multiple species of taxonomically and chemically diverse seaweeds for their effects on the hindgut microbiomes of *S. fuscescens.* Eleven species of seaweed (5 red, 3 brown and 3 green species) were evaluated as functional ingredients in feeding trials with *S. fuscescens* (Table S1, hereafter referred to by genus). Four commercially available 'aquafeed' supplements were also evaluated: (i) Hilyses® (MarSyt Inc), a hydrolysed yeast culture derived from the sugarcane fermentation process (and a source of β-glucans), (ii) sodium alginate, the anionic polysaccharide extracted from brown seaweeds, (iii) the cyanobacteria spirulina (high strength organic spirulina, Swiss Wellness Pty Ltd) and (iv) cracked and window refractance encysted (> 95%) dried biomass of the microalga *Haematococcus pluvialis*, which is source of astaxanthin. Together there was a total of 15 supplement treatments in the trial. The proximate composition of each supplement was determined following the recommended methods of the Association of Official Analytical Chemists [111] with the protein estimation using a factor of 5 to multiply the seaweed nitrogen content as recommended by Angell, et al. [112]. The source of each species, their morphological and chemical features of interest of the supplements from the 4 groups (red, green and brown seaweed and aquafeed supplements) are described in Table S1.

For the preparation of the seaweed supplemented diets, fresh seaweeds were rinsed with saltwater (34.5 ppt) to remove sand and biological contaminants. They were then spun in a washing machine (Fisher & Paykel 5.5 kg Quick Smart) on spin cycle (10,000 rpm) for 5 min to remove excess water, frozen at -80 °C, and then lyophilised in a freeze dryer (Thermo Savant model MODULYOD-230) for 3 days at approximately − 44 °C and 206 mbar. Once dried, each seaweed species was vacuum sealed in individual bags with silica desiccant and stored at -20 °C until used. The 'control' (unsupplemented) diets for experimental *S. fuscescens* was produced using the commercial aquafeed 'Native' (Ridley Aquafeeds Ltd). The pellets (1.5 kg in total) were powdered then added to a blender (Hobart A120) with deionised water (30% weight/weight) and combined for approximately 10 min at low speed (agitator rpm of 104) using a dough hook to produce a stiff dough. The dough was extruded through a 4 mm die onto trays which were then placed in a fan-forced oven overnight at 50 °C. Once dried, the feed was packaged in airtight bags and stored at 4 °C until required. All 15 treatment diets (supplemented with seaweed or aquafeed supplement) were made in the same manner but received supplements powdered and sieved through a 300 µm mesh prior to the addition of water during blending.

Experimental design. Due to variation in sizes, fish (N = 144) were sorted in two size classes to ensure an even distribution of sizes across each replicate tank containing three fish: 'small' (ranging from 15 cm/70.5 g to 18 cm/112.1 g) or 'large' (ranging from 18 cm/112.4 g to 21 cm/189.2 g). To ensure that at least one fish in each size class was allocated to each replicate tank, N = 72 fish from each group were randomly allocated into 48 plastic tanks (55 L), with 3 fish $tank^{-1}$. The exact mass and length of each fish was recorded and used as a covariate in analyses assessing the influence of fish diet on microbial community diversity. As processing limitations were forecasted for the end of the trial, the fish were stocked in a staggered manner with one tank per treatment stocked each day over three days to allow for the sampling of one tank per treatment each day over three days at the end of the screening trial.

Fish were fed one of 16 different experimental diets and each treatment diet had three replicate tanks (each containing 3 replicate fish) such that all fish within a tank received the same diet. Therefore 'Tank' was a random factor nested within the fixed factor of 'Diet'. To enable staggered sampling at the end of the experiment and ensure that all fish were exposed to the treatment diets for the same time period (two weeks), one out of three tanks from each dietary treatment was stocked with fish each day, over three days. The *Ulva* dietary treatment included only 2 replicate tanks after the loss of one tank due to water and air supply issues.

Fish were fed by hand at 3% body weight twice a day (10:00 and 15:00) for a period of 14 days. No differences in feed consumption between tanks or treatments were observed as fish in all tanks consumed the total of both morning and afternoon feed allocations in each tank (visual inspection during handfeeding). During the trial, the water temperature was maintained at 27 °C and the pH within the range of 7.9 and 8.1. The system was operated as flow-through, with fresh seawater (34–35 ppt) pumped from approximately 300 m off the beach adjacent to the research station then through a series of 16 spin disk filters (40 µm) and 10 multimedia filters (~ 10–15 µm), after which it received ozone treatment from two 100 gO_3/h generator units (WEDECO OCS-GSO30). The ozone treated seawater was then pumped via ultraviolet filters, providing 80 mJ/cm^2, to two 4 × 2.2 m granular activated carbon vessels for a contact time of > 9 min to remove unwanted by-products from the ozone treatment. Finally, the seawater was pumped to a header tank, which fed directly into a pipe system delivering treated seawater to this experiment. The system was maintained in a temperature and light controlled room kept at 24–26 °C and on a 24L:0D dim central light regime.

Sample collection and preparation. After the feeding trial (14 days), the fish were subjected to a 24 h fasting period. The fish were euthanized in 10 ppt Aqui-S®, then the entire digestive track from each fish was aseptically excised and placed in a Falcon tube (50 ml) before being snap frozen and stored at -80 °C until further processing could occur.

DNA Extraction. To compare the hindgut microbiomes of fish fed different experimental diets, DNA was isolated from the hindgut and digesta of one randomly-selected small and large fish from each tank (except for the *Ulva fasciata* treatment which only had 2 tanks). After the samples thawed, 0.25 g (approximately 0.5 cm length) of hindgut containing digesta was sampled. The rational for choosing to sample the hindgut with digesta was based on results published by Nielsen *et al*. and Jones *et al*. (26, 27). We defined the section of the distal intestine starting 1 cm internally to the anal pore as "hindgut", referred to as such hereafter. Digesta containing hindgut samples were placed directly into isolation buffer in PowerBead tubes from the PowerSoil DNA isolation kit (Mo Bio, San Diego, CA, USA). Microbial DNA was isolated from the hindgut samples following the manufacturer's instructions and then stored at -20 °C.

16S rRNA gene sequencing and bioinformatics

From the isolated DNA, the 16S rRNA gene was amplified the using PCR following previously published methods [113–116]. Briefly, the hypervariable region V3-V4 was targeted using the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') at the Australian Genome Research Facility (AGRF), who then sequenced the amplicons using an Illumina bcl2fastq pipeline version 2.20.0.422 (2 × 300 bp miseq platform). Demultiplexed paired-end reads were assembled by aligning the forward and reverse reads using Quantitative Insights into Microbial Ecology [QIIME2 v2018.8; 117]. To ensure that comparisons were made from sequences assigned in the same hypervariable region (V4) of the comparison studies (below), the raw data from the current study was trimmed using the cutadapt package [118], using the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers as per Yu, et al. [119]. Trimmed sequences were processed and denoised using the DADA2 package [120] and QIIME2 (v2018.8) software, with ASVs tables constructed and aligned against the Silva 16S rRNA 99% reference database [release v132; 121]. Due to practical and budget restraints, the DNA samples were sequenced in two separate runs on the same machine at the same facility (AGRF). Bioinformatical and statistical steps were included to ensure comparability between the two sequencing runs (see below). Raw sequences have been deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) under the bioproject number PRJNA649307.

Approximately 95.1% (398,112) of total reads were quality filtered and retained through this process. Subsequent quality filtering included the removal of singletons, chimeric sequences, mitochondrial DNA, and unassigned or eukaryotic ASVs. This resulted in a total of 1,250 ASVs from 48 samples. Rarefaction to 6,290 counts was performed to account for uneven sequencing depth among samples (Fig. S1). This resulted in the removal of one replicate from the *Laurencia* treatment (4_1_s; 874 counts) and the removal of 52 ASVs no longer present after rarefaction, leaving a total of 1,198 ASVs and 47 samples.

Comparisons with previously published sequences of the hindgut microbiota from wild populations of S. fuscescens

Two recently published papers [15, 17] also characterised microbial communities of in the hindgut of wild-caught individuals of (*S. fuscescens*). With the permission of those authors, we compared the microbiomes of our captive fish (fed experimental diets) to the results obtained from fish caught from wild populations on the east and western coastlines of Australia, respectively. Nielsen, et al. [15] characterised GI microbiomes in wild populations of *S. fuscescens* captured nearby One Tree Island (23°30'27.0"S, 152°05'30.5"E) in the tropical Great Barrier Reef (GBR), whereas Jones, et al. [17] sampled wild fish from two populations in Western Australia (WA), including the subtropical Shark Bay (26°01'47.28"S, 113°33'12.49"E) and the tropical Kimberley region (16°51'14.57"S, 122°10'39.45"E). This gave us two tropical sites (GBR and Kimberley) and two subtropical sites (our study and Shark Bay), from both the east and western coastlines to compare (with the caveat that populations were sampled by different teams at different times). Jones, et al. [17], also collected samples from *S. fuscescens* populations from temperate Western Australian locations, which were not included here due to the absence of comparative samples from Eastern Australia.

Raw sequence data were retrieved from the NCBI Short Read Archive (SRA; Jones, et al. [17]; accession number PRJNA356981 and Nielsen, et al. [15]; accession number PRJNA396430) using the SRA Toolkit software and processed as demultiplexed fastq files. Raw data from both comparison studies (sequenced in the V4 region) were also processed using the cutadapt package [118] to remove respective primer sequences.. From this point, the same bioinformatic pipeline as detailed above was used, with identical denoising, filtering and taxonomic reference database (Silva 16S rRNA gene 99% reference database (release v132) applied. Subsequent quality filtering included the removal of singletons, chimeric sequences, mitochondrial DNA, and unassigned or eukaryotic ASVs. Approximately 95.1% (713,284) of total reads were quality filtered and retained through this process. Subsequent quality filtering included the removal of singletons, chimeric sequences, mitochondrial DNA, and unassigned or eukaryotic ASVs. This resulted in a total of 3,160 ASVs recruited from 86 samples. Rarefaction to 6,290 counts was performed to account for uneven sequencing depth among studies and samples. This resulted in the removal of the same replicate (from the *Laurencia* treatment; (4_1_s; 874 counts) and the removal of 87 ASVs no longer present after rarefaction, leaving a total of 3,073 ASVs and 85 samples (Fig. S4).

Data analysis and statistics. After processing, data were imported into R version 3.6.3 [122] using the package phyloseq [123] for statistical analysis and visualisations. Alpha diversity of microbial communities, Observed ASVs and Shannon-Weaver index (hereafter "Shannon index"), were compared among fish fed different diets and later, between different studies, using Kruskal-Wallis tests. For the rest of the analyses, to allow the comparison of both sequencing runs on a shared number of ASVs, the rarefied ASVs were agglomerated at the genus level. Venn diagrams were used to show the number of shared ASVs among samples and studies and were constructed using the Limma package [124]. Beta diversity was visualised using non-metric multidimensional scaling (nMDS) ordinations and Bray-Curtis and unweighted UniFrac community dissimilarity indices and compared between treatments and fish length as a covariate using PERMANOVA [125]. ASV level differences between each treatments and the control was evaluated using multiple one-way ANOVAs, with square root transformed data to meet the assumptions of homogeneity of variance and improve normality. The 4 geographically distinct rabbitfish populations were analysed using pairwise comparisons of changes in the relative abundances of ASV using Wald tests in the DESeq2 function [126]. Additionally the package *microbiome* [127] was used to identify ASVs that were part of a core microbiome in fish from the 4 geographic populations.

Abbreviations

GI: gastrointestinal; GBR: Great Barrier Reef; WA: Western Australia; BIRC: Bribie Island Research Centre; QIIME: Quantitative Insights into Microbial Ecology; AGRF: Australian Genome Research Facility; NCBI: National Center for Biotechnology Information; rRNA: ribosomal Ribonucleic Acid; DNA: Deoxyribonucleic Acid; ASV: Amplicon Sequence Variant; nMDS: non-metric Multidimensional Scaling; PERMANOVA: Permutational Analysis of Variance; ANOVA: Analysis of Variance; bp: base pair.

Declarations

Ethics approval and consent to participate

This collection of fish from the wild was carried out under a "General fisheries permit" (# 195305) issued by the Queensland Department of Agriculture and Fisheries (Fisheries Act 1994) and all activities were approved by the animal ethics committee of the University of the Sunshine

Coast (AN/S/17/51).

Consent for publication

No applicable

Availability

Raw sequences have been deposited in the NCBI sequence read archive (SRA) under the bioproject number PRJNA649307 at https://www.ncbi.nlm.nih.gov/bioproject/649307.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author's contributions

VT, NP, AC and MR designed the experiment; VT performed the experiment, the laboratory work; VT and JL analysed the data; VT, NP, AC and MR wrote the manuscript. All authors read and approved the final manuscript

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Figures

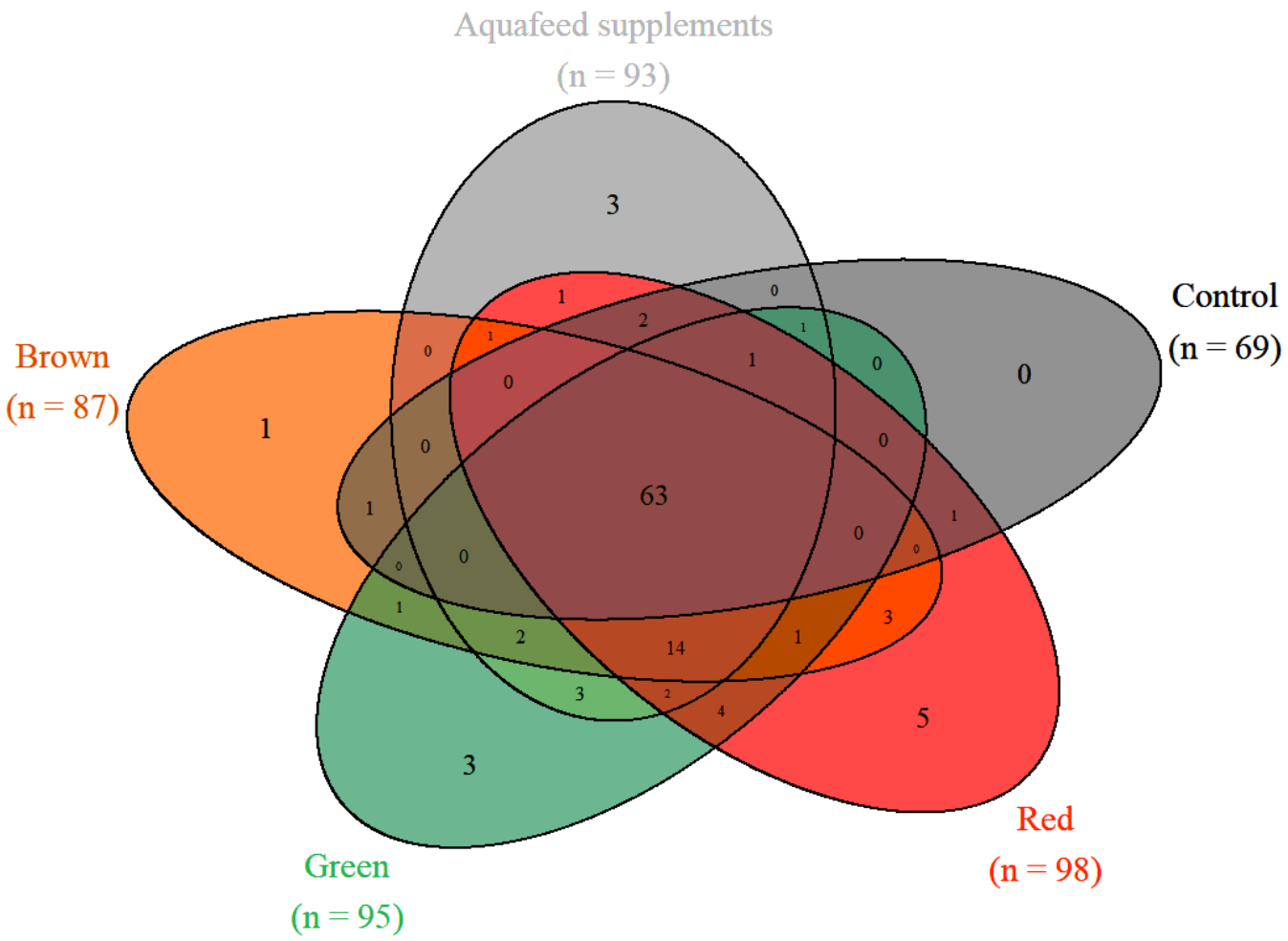

Figure 1

Shared and unique ASVs in the hindgut of the mottled rabbitfish (S. fuscescens) fed the control diet or diets supplemented with reds, greens or browns seaweeds or aquafeed supplements (existing industry dietary additives).

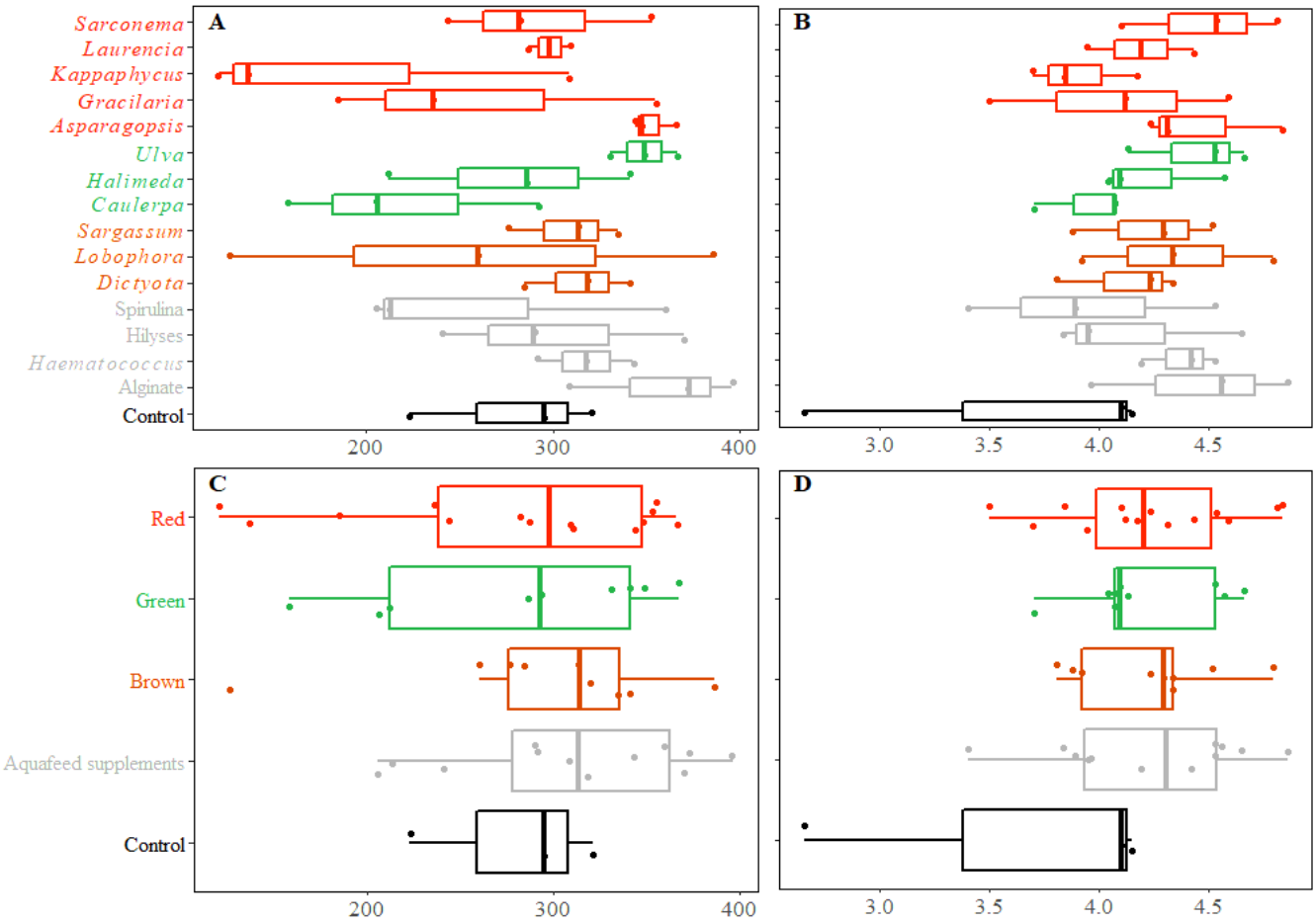

Figure 2

Alpha diversity analysis using species richness (Observed ASVs; A and C) and species diversity (Shannon index; B and D) for all treatments (A and B) and for the different functional groups of seaweeds used in feeding trials (C and D).

Figure 3

nMDS on rarefied ASVs abundance using Bray-Curtis (A) and unweighted UniFrac (B) dissimilarities between the genus-subset hindgut bacterial communities of S. fuscescens fed the supplemented or control diets. Symbol colours correspond to diet treatment type, including brown seaweed (brown symbols), red seaweed (red symbols), green seaweed (green symbols), Aquafeed supplements (grey symbols) and control diets (black symbols).

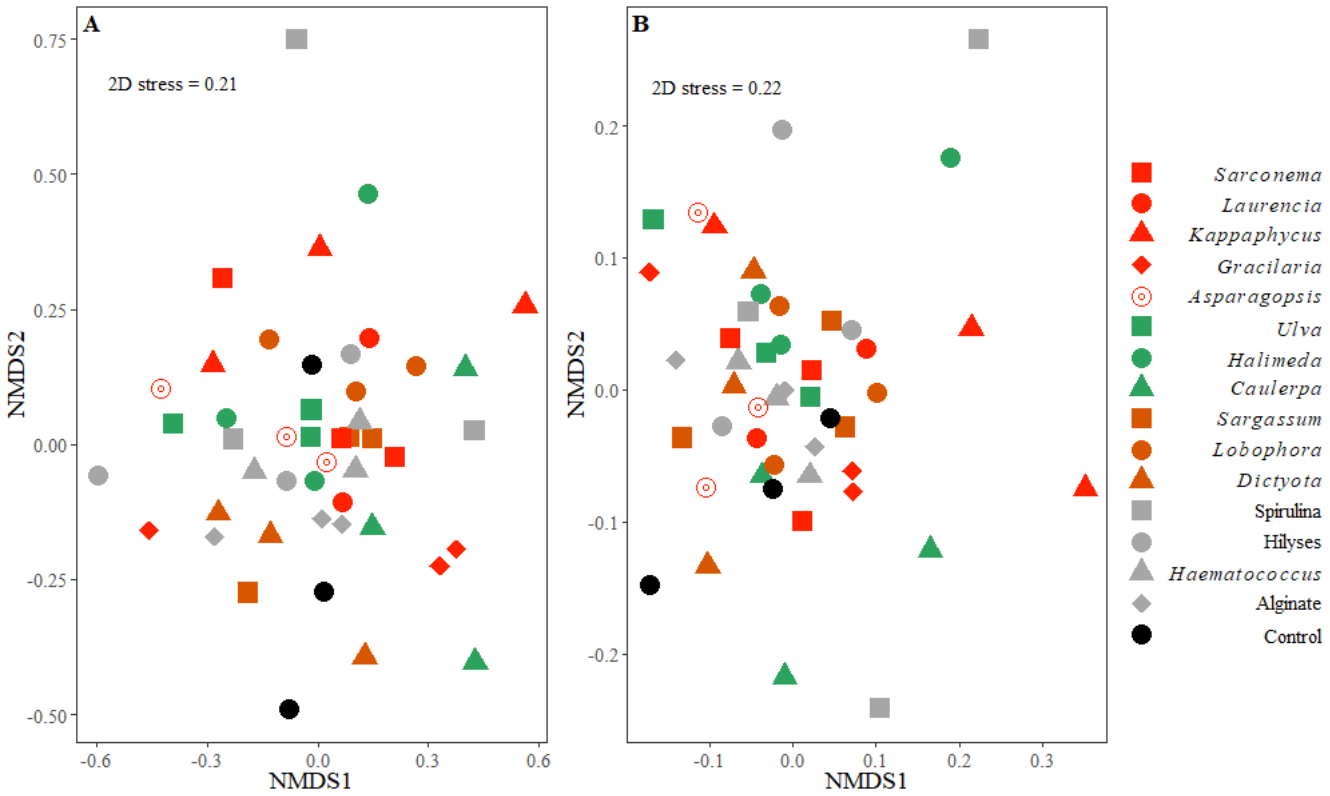

Figure 4

Proportion of phyla contributing to >1% abundance to the microbial communities of the hindgut of S. fuscescens fed control fish pellets or pellets supplemented with seaweeds or aquafeed supplements. On y-axes, red text indicates that diets were supplemented with a species of red seaweed, green text indicates green seaweed and brown text indicates brown seaweed. Aquafeed supplements are indicated in light grey with the control in black.

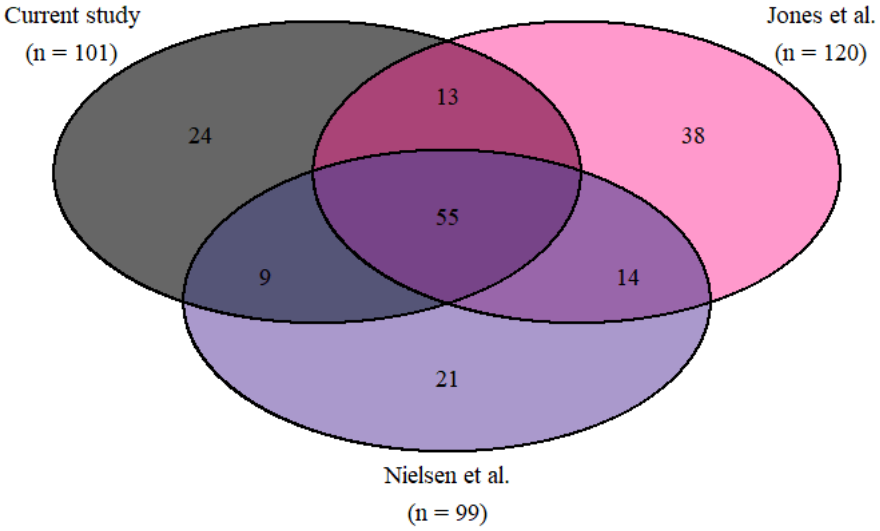

Figure 5

Shared and unique ASVs in the hindgut of the mottled rabbitfish (Siganus fuscescens) from the current study and the two wild S. fuscescens populations.

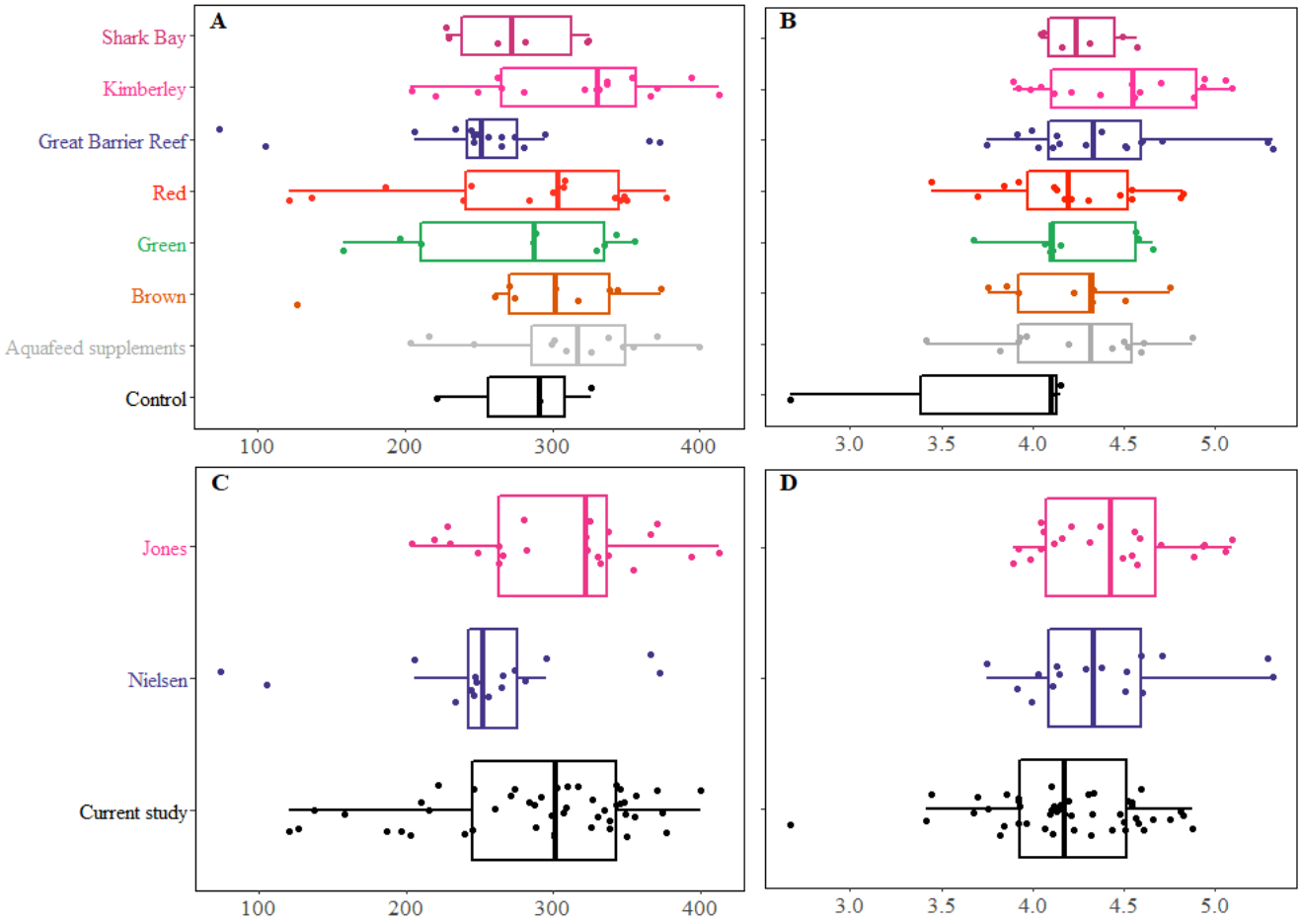

Figure 6

Alpha diversity analysis using species richness (Observed ASVs; A) and species diversity (Shannon index; B) from the three Siganus fuscescens studies. Kruskal-Wallis tests were performed on all treatments.

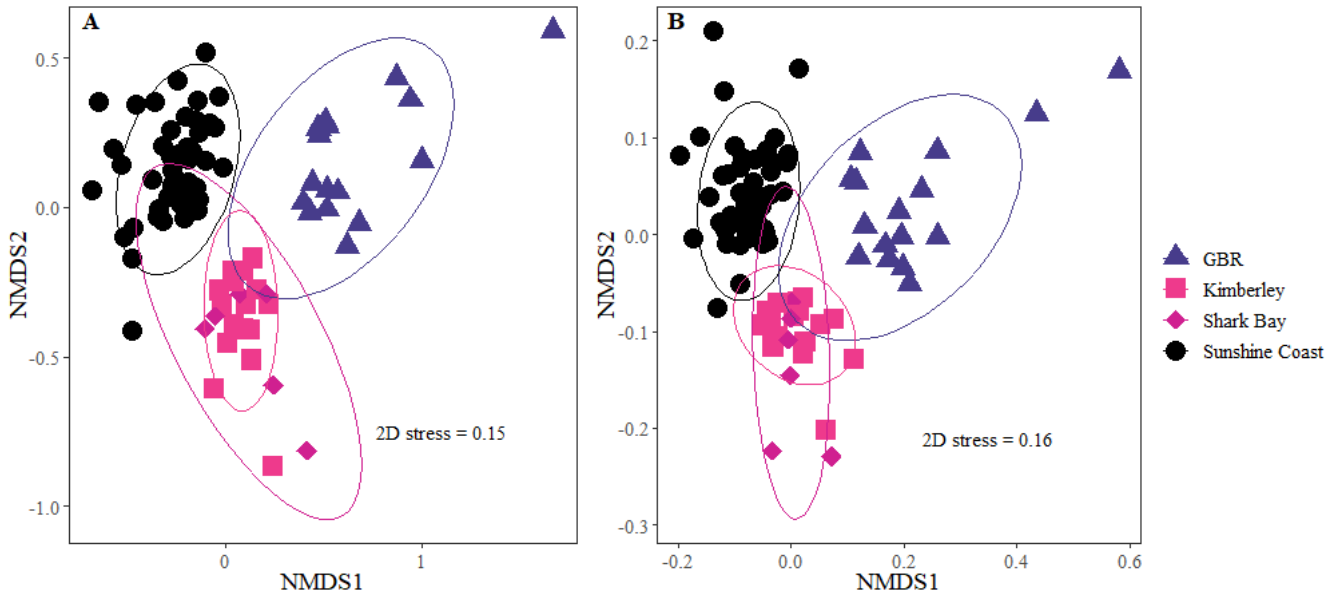

Figure 7

Alpha diversity analysis using species richness (Observed ASVs; A) and species diversity (Shannon index; B) from the three Siganus fuscescens studies. Kruskal-Wallis tests were performed on all treatments.

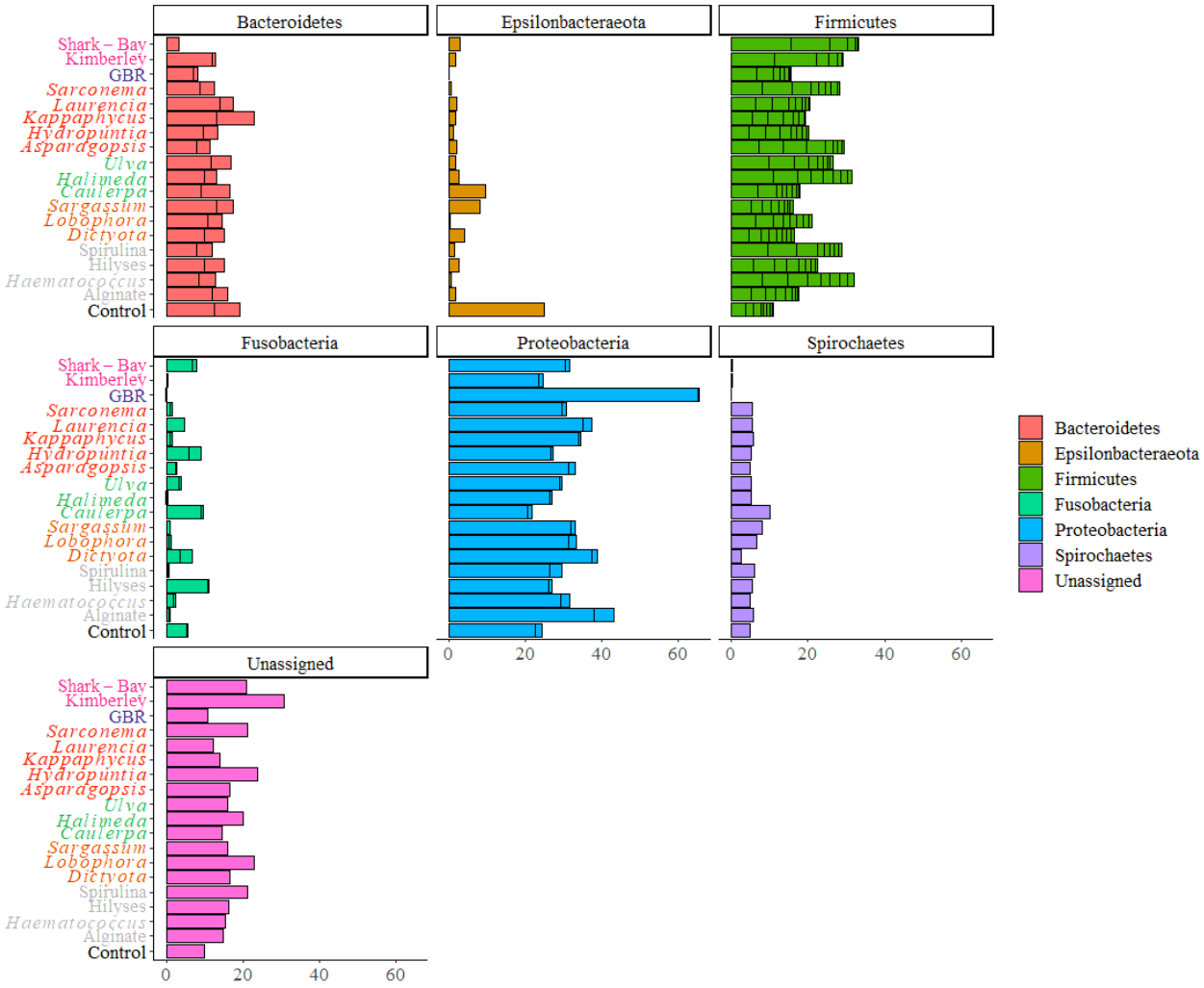

Figure 8

Phyla contributing to >1% abundance to the microbial communities of the hindgut of S. fuscescens of the current study and the two wild population of this fish. On y-axes, red text indicates that diets were supplemented with a species of red seaweed, green text indicates green seaweed and brown text indicates supplementation with brown seaweed. Aquafeed supplements are indicated in light grey with the control in black. The fish from Eastern Australia (GBR) are in blue and those from Western Australia (Shark Bay and Kimberley) are in pink.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarytables.xlsx
- Supplementaryfigures.docx