Microbial Ecology

Gut microbiota of migrating wild rabbit fish (Siganus guttatus) larvae have low spatial and temporal variability.

--Manuscript Draft--

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Abstract:	We investigated the gut microbiota of rabbit fish larvae at 3 locations in Vietnam (ThuanAn: northern, QuangNam: intermediate, BinhDinh: southern sampling site) over a three-year period. In the wild, the first food for rabbit fish larvae remains unknown, while the juveniles and adults are herbivores, forming schools near the coasts, lagoons and river mouths, and feeding mainly on filamentous algae. This is the first study on the gut microbiota of wild fish larvae and with a large number of individuals analyzed spatially and temporally. The Clostridiales order was the most predominant in the gut, and location-by-location alpha diversity showed significant differences in Chao-1, Hill number 1 and evenness. Analysis of beta diversity indicated that the location, not year, had an effect on the composition of the microbiota. In 2014, the gut microbiota of fish from QuangNam was different from BinhDinh, in 2015, the gut microbiota was different for all locations, and in 2016, ThuanAn was different from the other locations. There was a time-dependent trend in the North-South axis for the gut microbiota, which is considered to be tentative awaiting larger datasets. We found limited variation in the

	gut microbiota geographically and in time, and strong indications for a core microbiome. Five and fifteen OTUs were found in 100 and 99% of the individuals, respectively. This suggests that at this life stage the gut microbiota is under strong selection due to a combination of fish-microbe and microbe-microbe interactions.
Response to Reviewers:	REBUTTAL LETTER Ref.: Ms. No. MECO-D-18-00339R2 Gut microbiota of migrating wild rabbit fish (Siganus guttatus) larvae have low spatial and temporal variability. Microbial Ecology Dear Dr. Karen E. Nelson, Editor-in-ChiefMicrobial Ecology Thank you very much for your response and reviewer's comments on our manuscript. We sincerely apologize for the great time it has taken us to respond to these comments, and hope that a revised version of the manuscript will still be considered by Microbial Ecology. We have modified the paper in response to the reviewer comments. Below we respond to the comments point by point. Reviewers' comments: Reviewer #1: Three of four of the remaining comments that I had were adequately addressed. The 4th one about the possible provision of an OTU Table as supplementary information is not really answered. However, I suppose that since the data was deposited in the ENA archive (and an OTU table was generated for that as is mentioned in line 152) the data can ultimately be found by readers. Response to reviewer: Thank you very much for your comments. Our raw data was deposited in the archive. In addition, an OTU table was uploaded as supplement. We hope the data can ultimately be found by readers. Reviewer #2: SCIENTIFIC COMMENTS 49-53 I think something is missing here. Roeselers et al. looked at zebrafish, but the other studies mentioned looked at a variety of species. I'm actually not sure exactly what is meant did the other studies take a better approach, or did they have (some of) the same problems as the Roeselers paper? Did they conclude there was a core microbiome or not? My best guess is that you mean something like "Several studies have proposed a core microbiome for fish species, beginning with Roeselers et al. (2011) zebrafish study, but studies so far have ignored…[], pooled individuals, and/or used low sample numbers (n=3)[]". (Or just "and", not "and/or", whichever is correct. Response to reviewer: I agree that it was unclear here. I hope we have made it clearer in the revised manuscript (line 50 – 55). Roeselers et al. looked at only one species (zebra fish) and pooled samples. Other studies looked at a variety of species, but had limitations by ignoring the potential spatial and temporal variation, by pooling of individuals and/or by analyzing a limited number of individuals. However, all studies concluded that there was a core microbiome in fish. 332 Does Siganus have the highest known number of OTUs? If so, should say so, or mention whatever the reason is for including this species in particular. Response to reviewer: It is unknown if Siganus have the highest known number of OTUs among fishes. Here we only try to compare the number of OTUs from 2 species of Siganus (Vietnam and Australia). The species from Vietnam (S. guttatus) has higher number of OTUs than the species from Australia (S. fuscescens). We made it clearer in the manuscripts (line 334 – 336). 335 Maybe better "Bacteria belonging to the Verrucomicrobiales and Desulfovibrionales, important orders for seaweed digestion, could be identified to the genus (Akkermansia, up to 17.3%) and family (Desulfovibrionaceae, up to 13.4%) level, respectively". Response to reviewer: Thank you very much for your suggestion. I have incorporated the sentence with some modifications in the manuscript (now line 338 – 340). Use this wording in the manuscript: "Bacteria belonging to the Verrucomicrobiales and Desulfovibrionales, orders that are important for digestion of seaweed, could be identified to the genus (Akkermansia, up to 17.3%) and family (Desulfovibrionaceae, up to 13.4%) level, respectively". 341 Why "compartmentalization" and "across the gut"? That sounds like spatial separation, which may be true, but was not examined here. If you want to suggest this, make it clear it's a hypothesis. Or do you instead mean functional compartmentalization? Response to reviewer: Yes, for sure, we don't have data for being conclusive. However, this is an interesting part of our data. We have rewritten this part to make it only a suggestion, and stated explicitly that this require further studies.

	342 "The abundance of these bacteria showed that fermentation of algal material by, for example, Clostridium spp., is predominately in the marine herbivorous fish." This doesn't make sense as written. Are you proposing that these particular fish may be fermenting algal material, and that Clostridium species are mainly responsible? If so, this would seem to need some references to support it. Response to reviewer: See our response to the comment above. We have added references showing that Clostridium can be involved in fermentation of algae (line 344-346). 391 What do you mean by "gut functionality"? What specific aspects might be measurable in these fish? Response to reviewer: By gut functionality we mainly think about digestion, but also immunology due to release of e.g. glucans and VFA. As this fish is herbivores, the most specific aspects might be the ability to ferment the algal material, for example, by the predominant Clostridium spp. 414 The buffer blanks comment doesn't belong here in this position it might seem to negate the whole study. I would put it at the beginning of the sequencing results, as something that should be kept in mind. There may have been sequences introduced from seawater, or fish surfaces, or during DNA preparation it is hard to completely rule out any of those without proper controls. Response to reviewer: Thank you very much for your comments. I have moved the buffer blanks comment to the beginning of the sequencing results (line 168 – 169). MINOR SUGGESTIONS AND CORRECTIONS 268 Should be "and Burkholderiales". Response to reviewer: I have made the modification in the manuscript. 269 "all but one larva" (singular) Response to reviewer: I have made the modification in the manuscript. 287 Do you mean "developmental signaling"? Response to reviewer: Yes, we meant "developmental signaling". I have made the modification in the manuscript. 326 Should be just "OTUs from Vibrio" (no "The") Response to reviewer: Thank you very much for your comments 328 Should be "normally developing fishes" (or "healthy developing fishes", if that's what you mean not sure) Response to reviewer: I have made the modification to “normally developing fishes” in the manuscript. 328 "in the gut" (not "is") Response to reviewer: I have made the modification in the manuscript. 332 "in" shouldn't be italicized Response to reviewer: I have made the modification in the manuscript. 333 Better "Many bacteria" Response to reviewer: I have made the modification in the manuscript. 339 Should be "of the Clostridium group" Response to reviewer: I have made the modification in the manuscript. 340 No comma needed before "in the gut microbiota" Response to reviewer: I have made the modification in the manuscript. 341 Should be "a compartmentalization" Response to reviewer: I have made the modification in the manuscript. 365 Should be "in terms of" Response to reviewer: I have made the modification in the manuscript. 367 Should be "which abiotic and biotic factors are" (no "that") Response to reviewer: I have made the modification in the manuscript. 382 Should be "were present" Response to reviewer: I have made the modification in the manuscript.

Figure 1

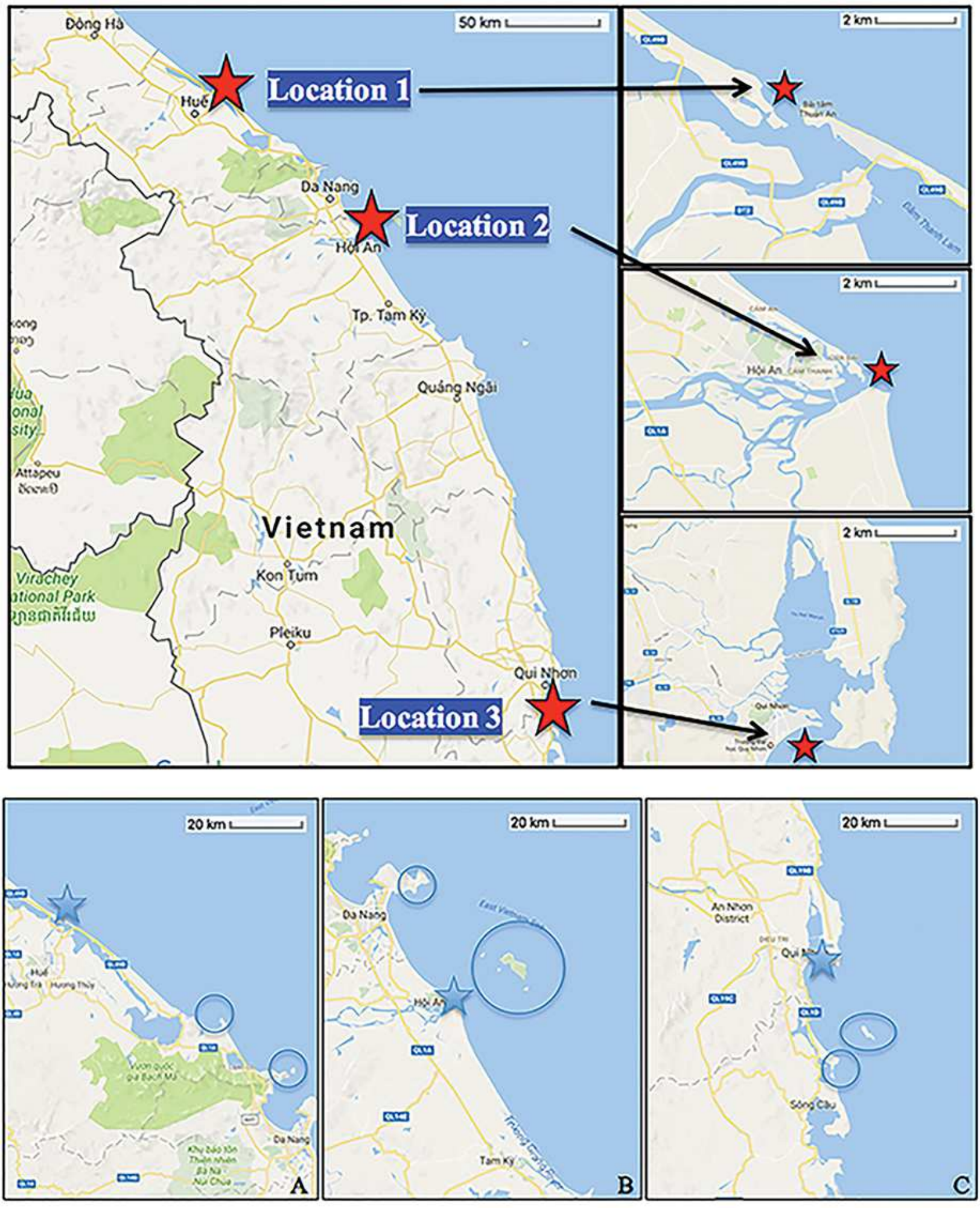
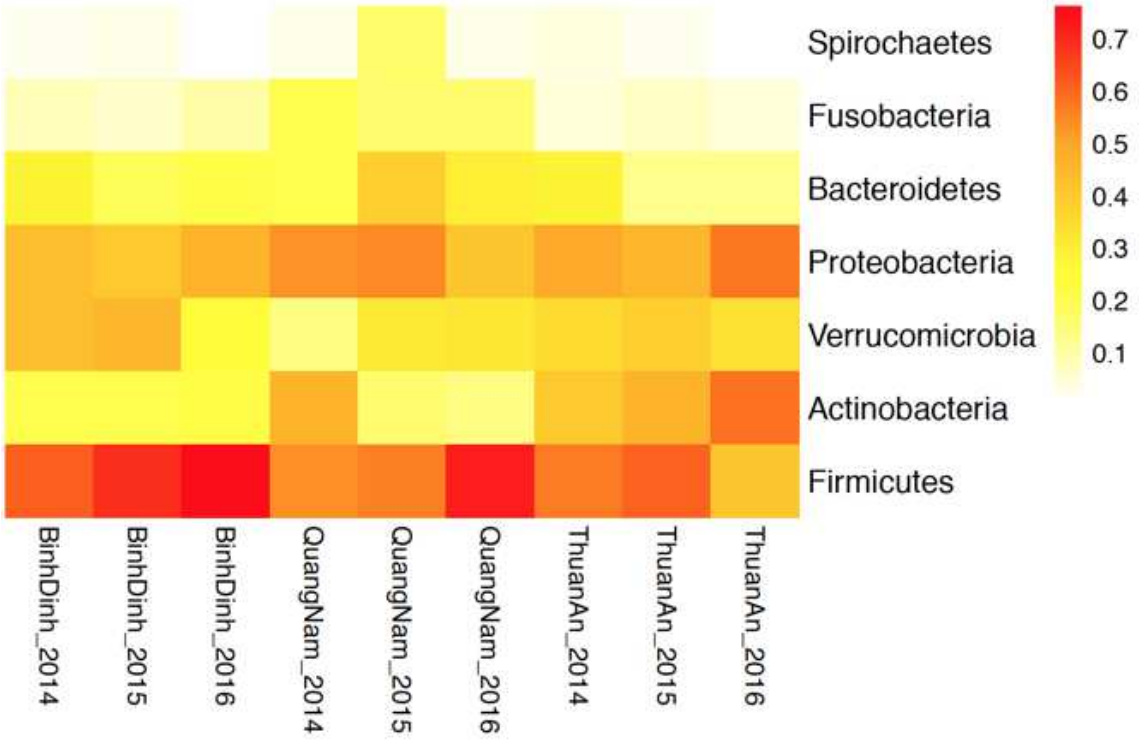

Đông Hà
Huế
Da Nang
Hội An
Tp. Tam Kỳ
Quảng Ngãi
Attapeu
Vietnam
Kon Tum
Pleiku
Qui Nhon
Location 1
Location 2
Location 3
50 km
2 km
2 km
2 km
20 km
20 km
20 km
Da Nang
Hội An
Tam Kỳ
An Nhon District
Sông Cầu
A
B
C

Figure 2


Spirochaetes
Fusobacteria
Bacteroidetes
Proteobacteria
Verrucomicrobia
Actinobacteria
Firmicutes
0.7
0.6
0.5
0.4
0.3
0.2
0.1
BinhDinh_2014
BinhDinh_2015
BinhDinh_2016
QuangNam_2014
QuangNam_2015
QuangNam_2016
ThuanAn_2014
ThuanAn_2015
ThuanAn_2016

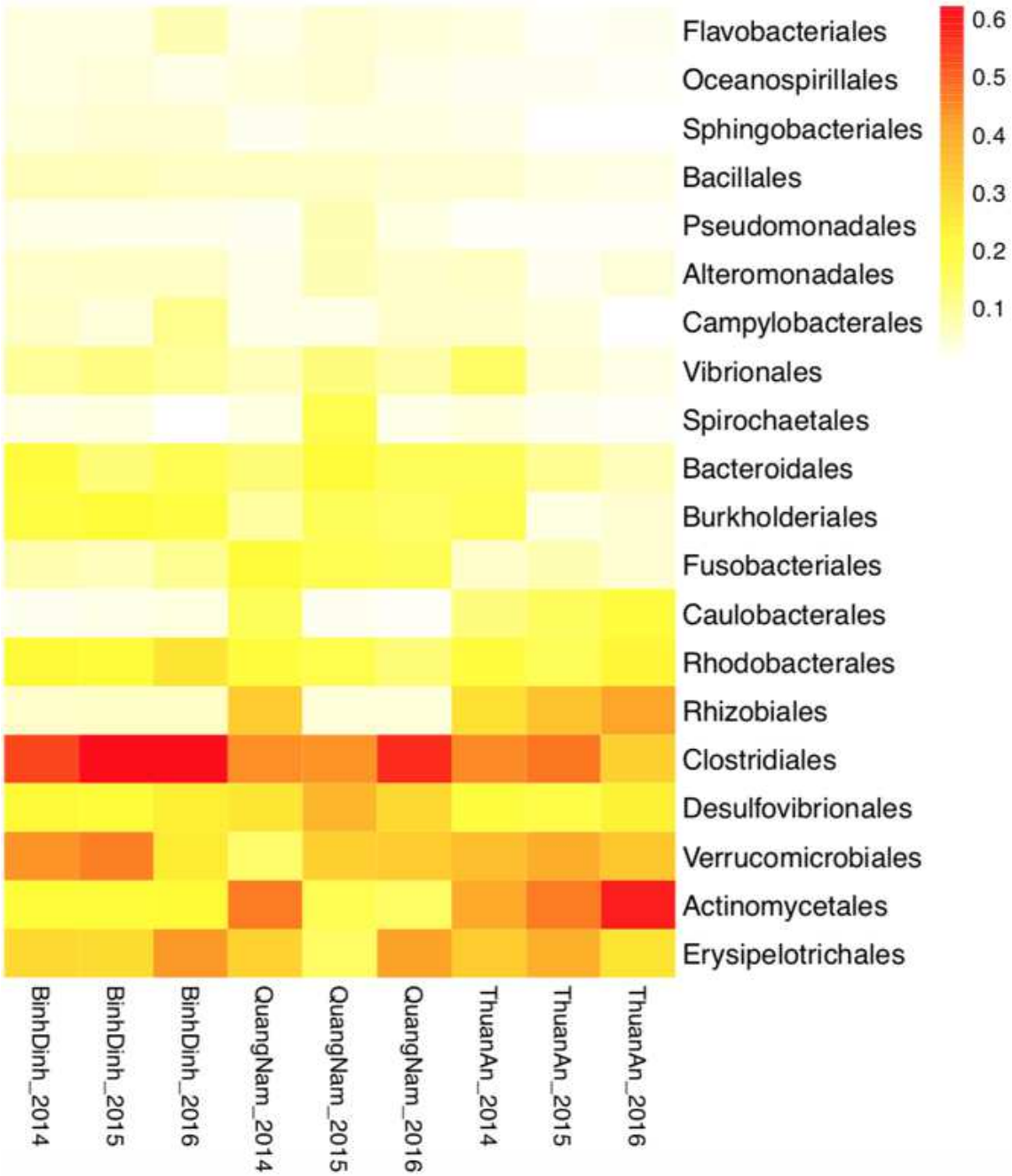
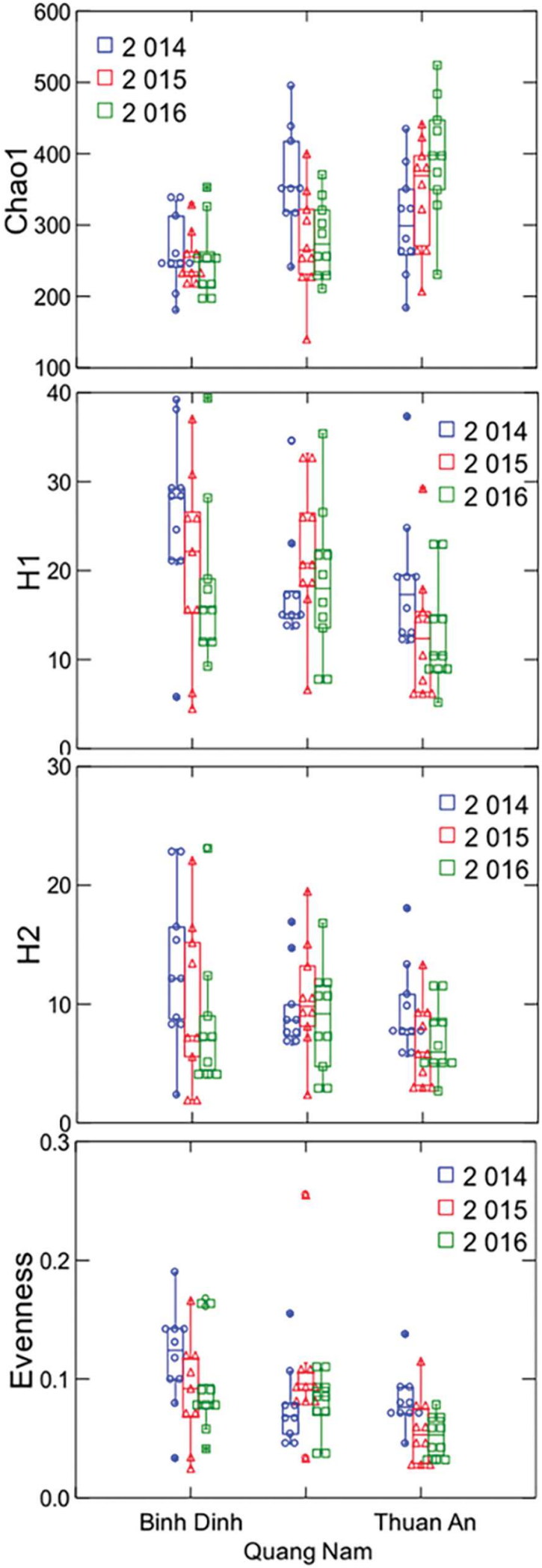
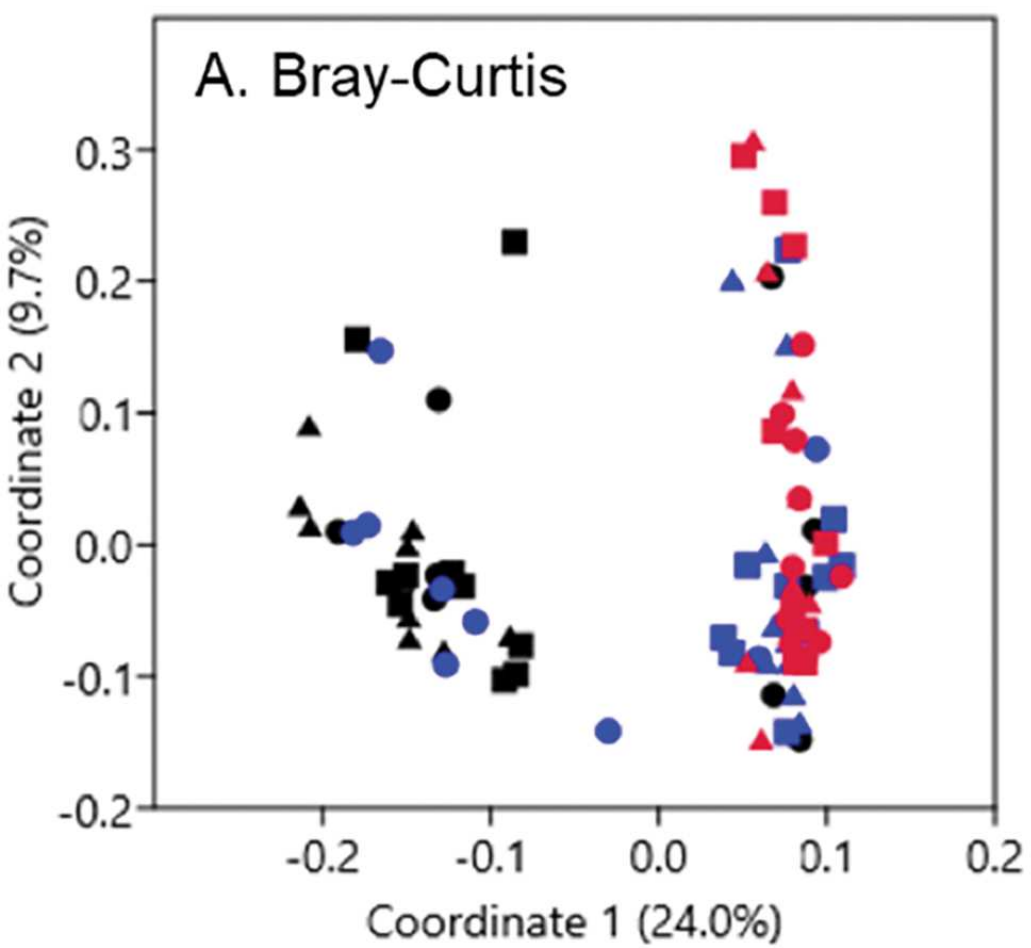

Flavobacteriales
Oceanospirillales
Sphingobacteriales
Bacillales
Pseudomonadales
Alteromonadales
Campylobacterales
Vibrionales
Spirochaetales
Bacteroidales
Burkholderiales
Fusobacteriales
Caulobacterales
Rhodobacterales
Rhizobiales
Clostridiales
Desulfovibrionales
Verrucomicrobiales
Actinomycetales
Erysipelotrichales
0.6
0.5
0.4
0.3
0.2
0.1
BinhDinh_2014
BinhDinh_2015
BinhDinh_2016
QuangNam_2014
QuangNam_2015
QuangNam_2016
ThuanAn_2014
ThuanAn_2015
ThuanAn_2016

Figure 3


Chao1
H1
H2
Evenness
2 014
2 015
2 016
Binh Dinh
Thuan An
Quang Nam


A. Bray-Curtis
Coordinate 2 (9.7%)
Coordinate 1 (24.0%)
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0.2
0.1
0.0
-0.1
-0.2
-0.2
-0.1
0.0
0.1
0.2

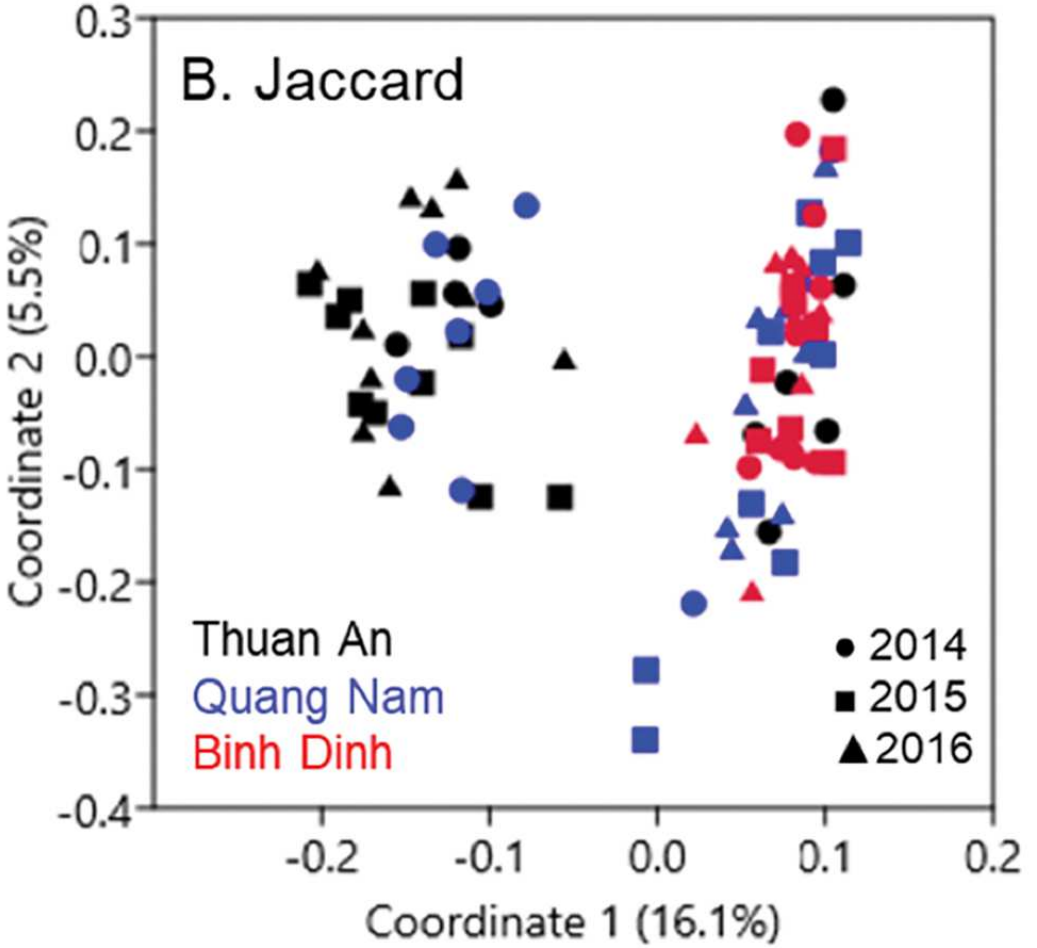
B. Jaccard
Coordinate 2 (5.5%)
Coordinate 1 (16.1%)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.2
-0.1
0.0
0.1
0.2
Thuan An
Quang Nam
Binh Dinh
2014
2015
2016

Figure 5

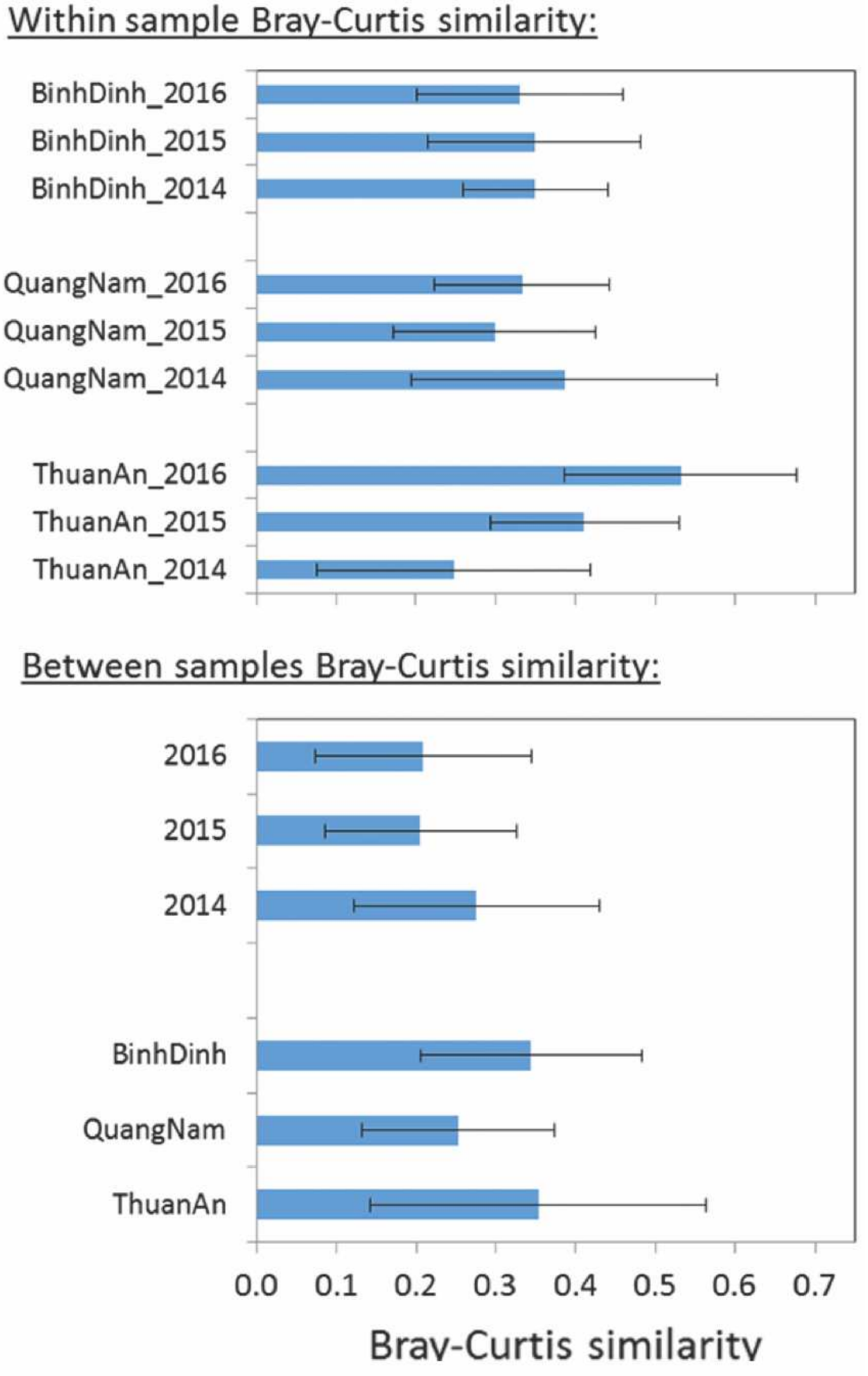

Figure 5

Table

Table 1. Core microbiota in rabbit fish defined as OTUs present in at least 95% of the 87 individuals from ThuanAn, QuangNam and BinhDinh for the years 2014, 2015 and 2016.

ARA ± S.D (%)	CV (%)	% ind. with OTU	% of reads	Cum. %	Phylum	Order
9.65 ± 13.48	139.8	99	9.63	9.6	Firmicutes	Erysipelotrichales
6.51 ± 11.84	181.9	99	5.89	15.5	Verrucomicrobia	Verrucomicrobiales
5.66 ± 6.72	118.7	99	5.79	21.3	Proteobacteria	Desulfovibrionales
6.41 ± 14.58	227.7	95	5.14	26.4	Firmicutes	Clostridiales
4.43 ± 4.88	110.3	100	4.38	30.8	Firmicutes	Clostridiales
3.60 ± 4.75	132.0	98	3.74	34.6	Firmicutes	*unknown*
2.38 ± 3.75	157.5	97	2.56	37.1	Firmicutes	Clostridiales
1.59 ± 1.93	121.9	99	1.68	38.8	Firmicutes	Clostridiales
2.65 ± 5.38	203.1	100	1.62	40.4	Proteobacteria	Rhodobacterales
2.65 ± 4.42	166.8	100	1.61	42.0	Actinobacteria	Actinomycetales
1.08 ± 1.09	100.8	99	1.06	43.1	Firmicutes	Clostridiales
1.14 ± 1.41	123.4	99	1.00	44.1	Bacteroidetes	Bacteroidales
0.85 ± 1.30	152.5	97	0.74	44.8	Firmicutes	Clostridiales
0.86 ± 1.91	223.2	99	0.51	45.3	Proteobacteria	Rhodobacterales
0.42 ± 1.03	244.4	100	0.24	45.6	Proteobacteria	Vibrionales
0.37 ± 0.49	130.8	99	0.21	45.8	Firmicutes	Clostridiales
0.30 ± 0.40	134.1	100	0.18	46.0	Proteobacteria	Burkholderiales
0.14 ± 0.24	175.8	99	0.14	46.1	Proteobacteria	*unknown*
0.14 ± 0.25	178.1	98	0.08	46.2	Actinobacteria	Actinomycetales
0.09 ± 0.12	131.0	99	0.07	46.3	Proteobacteria	Burkholderiales
0.07 ± 0.07	103.7	98	0.05	46.3	Proteobacteria	Burkholderiales

ARA: average relative abundance; CV: coefficient of variance; ind.: individual; cum.: cumulative.

Table 2

Table 2. Summary of the studies on fish gut microbiota using sequencing of amplifications of the 16S-rRNA gene, which had similar gut microbiota at phylum level (*Firmicutes, Proteobacteria, Actinobacteria* and *Verrucomicrobia)* to rabbit fish.

Fish species	Habitats	Feeding habits	References
Whitecheek surgeonfish (*A. nigricans*)	M	H	[42]
Daisy parrotfish (*Chlorurus sordidus*),	M	H	[42]
Bulbnose unicornfish (*Naso tonganus*)	M	H	[42]
Sixbar angelfish (*P. sexstriatus*);	M	H	[42]
black rockcod (*Notothenia coriiceps*)	M	O	[42]
Blunt snout bream (*Megalobrama amblycephala*)	M	O	[43]
Blackfin icefish (*Chaenocephalus aceatus*)	M	C	[42]
Long-snout seahorse (*H. guttulatus*)	M	C	[42]
Two-spot red snapper (*L. bohar*)	M	C	[42]
Sole (*S. senegalensis*)	M	C	[42]
Grass puffer (*Takifugu niphobles*)	M	C	[42]
Rabbit fish	E	H	This study
Grouper (*E. coioides*)	E	C	[42]
Longjaw mudsucker (*Gillichthys mirabilis*)	E	C	[42]
Grass carp (*C. idellus*)	F	H	[43, 45]
Zebra fish (*D. rerio*)	F	O	[42]
Guppy (*Poecilia reticulata*)	F	O	[42]
common carp (*C. carpio*)	F	O	[43, 45]
Silver carp (*H. molitrix*)	F	O	[43, 45]
Bighead carp (*H. nobilis*)	F	O	[43, 45]
Mandarin fish (*Siniperca chuatsi*)	F	O	[43, 45]
Yellowhead catfish (*Pelteobagrus fulvidraco*)	F	C	[14, 42, 43]
Atlantic salmon (*S. salar*)	F	C	[14, 42, 43]

Brown trout (*S. trutta*)	F	C	[14, 42, 43]
Rainbow trout (*O. mykiss*)	F	C	[14, 42, 43]

Habitats: M: marinewater; E: Estuarines; F: Freshwater; Feeding habits: C: carnivores; O: omnivores; H: herbivores).

Supplement Figure 1

Supplementary Table 1

Gut microbiota of migrating wild rabbit fish (*Siganus guttatus*) larvae have low spatial and temporal variability

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Keywords: amplicon sequencing, gut microbiota, core microbiome, rabbit fish, *Siganus guttatus*, wild larvae.

Abstract

We investigated the gut microbiota of rabbit fish larvae at 3 locations in Vietnam (ThuanAn: northern, QuangNam: intermediate, BinhDinh: southern sampling site) over a three-year period. In the wild, the first food for rabbit fish larvae remains unknown, while the juveniles and adults are herbivores, forming schools near the coasts, lagoons and river mouths, and feeding mainly on filamentous algae. This is the first study on the gut microbiota of wild fish larvae and with a large number of individuals analyzed spatially and temporally. The Clostridiales order was the most predominant in the gut, and location-by-location alpha diversity showed significant differences in Chao-1, Hill number 1 and evenness. Analysis of beta diversity indicated that the location, not year, had an effect on the composition of the microbiota. In 2014, the gut microbiota of fish from QuangNam was different from BinhDinh, in 2015, the gut microbiota was different for all locations, and in 2016, ThuanAn was different from the other locations. There was a time-dependent trend in the North-South axis for the gut microbiota, which is considered to be tentative awaiting larger datasets. We found limited variation in the gut microbiota geographically and in time, and strong indications for a core microbiome. Five and fifteen OTUs were found in 100 and 99% of the individuals, respectively. This suggests that at this life stage the gut microbiota is under strong selection due to a combination of fish-microbe and microbe-microbe interactions.

Introduction

During the last years it has been shown that the gut microbiota is essential for normal development and functionality of animals [1, 2]. Previous studies have shown that the composition of the gut microbiota has a crucial function in fish for morphological development, nutrient digestion, immune function and protection from invasive pathogens [3-8]. Data on microbial community (MC) composition in animals are accumulating rapidly, but so far few studies have been published on the MC of wild fish [9]. The microbial composition is affected by the interaction between host nutrition, environment and genetic factors [10], but our knowledge on MC assembly in animals, including fish, is still inadequate [11]. The same is true for variability caused by genetic and geographic distance, and by year-to-year variability.

The concept of "core microbiome" was introduced by Turnbaugh and colleagues [12]. It can be defined as what is common among the gut microbiota of a high fraction of individuals of a species [12] or a core set of microbial species fulfilling the minimal symbiotic functionality [1, 2]. So far, few aquatic animals have been studied to shed light on this concept. Several studies have proposed a core microbiome for fish species, beginning with Roeselers *et al.* (2011) concluding that they had evidence for a core microbiome in zebrafish, but based on one species and pooled samples. Later studies also concluded there was a core microbiome in fish, but these studies had limitations by ignoring potential spatial and temporal variations [1, 2, 13-19], by pooling of individuals, or by analyzing a low number of individuals (n=3) [13, 15, 17, 18, 20]. However, core microbiome primarily makes sense when fish are analyzed at the individual level with a large sample number. The "core microbiome" concept is interesting from a community assembly perspective as it suggests strong selection in the host, independent of the environmental factors such as local MC, temperature and food types. A comprehensive understanding of the gut

microbiota is necessary to explain its function in the overall health status of fish, especially at the larval stage [21-23], and this type of knowledge has implications also for microbial management in larval rearing.

The rabbit fish (*Siganus guttatus*), a native species in Southeast Asia including Central Vietnam, is an important commercial fish in this area [24-27]. So far, the larvae of this species for aquaculture have only been obtained from the wild, and are collected at river mouths in Central Vietnam, mainly in Thua Thien Hue, QuangNam and BinhDinh provinces. The smallest larvae size observed in the river mouths at the collecting points was 14 – 18 mm [24-27].

The aim of this study was to investigate the MC composition of the gut microbiota of migrating rabbit fish from three different locations (Thua Thien Hue, QuangNam and BinhDinh) over a 3-year period (2014 to 2016). We used Illumina 16S rRNA gene amplicon sequencing to characterize the gut microbiota at the individual level using a large sample number (n=10 for 9 samplings), and used these data to analyze alpha and beta diversity geographically (spatially) and temporally, and aiming at evaluating the "core microbiome" concept for wild rabbit fish larvae.

Materials and Methods

Location and sampling procedures

Wild larvae were collected from 3 different river mouths in Central Vietnam. Location 1 (ThuanAn) was in Thua Thien Hue province. Location 2 (QuangNam) was in QuangNam province. Location 3 (BinhDinh) was in BinhDinh province (Fig. 1). The distance from the middle site to the southern and northern sites is 260 and 130 km, respectively. The larvae were collected between 8th and 10th of June in 2014, 2015 and 2016, when the wild larvae first appeared in the river mouths (Fig. 1). Fish larvae were not bar-coded to verify species identity. At the sampling locations the schooling rabbit fish were identified based on appearance characteristics

described by Duray (1998). Water parameters at the sampling points (water temperature, salinity and pH) were measured at 2 meters depth using an electronic device (W-23XD, Horiba, Japan) at 5 different points in the sampling areas. Larvae were collected by fishing net in the morning between 7 – 8 a.m., washed with nuclease free water (Promega, USA), and kept on ice during transport to the laboratory for freeze-drying. The freeze-dried samples were stored at -20 °C until analysis of the gut microbiota. For each sampling year and location, 10 larvae with comparable size were collected for gut microbiota analysis. The gut microbiota analyses were done on single individuals.

Illumina sequencing for gut microbial analysis

The freeze-dried fish samples were hydrated in sodium phosphate buffer prior to extraction. After that, the gut was removed from the fish larvae. The DNA of the gut microbiota was extracted using the FastDNA Spin Kit for Soil (MP Biochemicals, USA), according to the manufacturer's instructions. The DNA concentration in the extract was then normalized to a concentration of 1 ng/µL, and the extracts were sent to LGC Genomics (Berlin, Germany) for Illumina amplicon sequencing with the Miseq platform. The Illumina protocol was written by Kim De Paepe and corrected by Berthold Fartmann (LGC Genomics, Germany). The bacterial 16S rRNA gene was amplified using primers 341F CCTACGGGNGGCWGCAG (forward) and 785R GACTACHVGGGTATCTAAKCC (reverse) [28]. The PCR reaction was carried out in 20 µL volume of MyTaq buffer containing 1.5 units of MyTaq DNA polymerase (Bioline, USA) and 2 µL of BioStabII PCR Enhancer (Sigma, USA). For each DNA sample, both primers carried the same unique 10-nt barcode sequence. The PCR protocol consisted of an initial denaturation step at 96°C for 2 minutes, followed by 20 cycles at 96°C for 15 s, 50°C for 30 s, 70°C for 90 s. Gel electrophoresis was carried out to determine the DNA concentration of the amplicon products of

interest. Up to 48 samples carrying different barcodes were pooled (20 ng DNA of each sample). To remove primer dimers and other by-products, the pooled samples were purified with one volume AMPure XP beads (Agencourt, USA), followed by a MinElute column (Qiagen, The Netherlands) purification step. The purified DNA (100 ng) was used to construct Illumina libraries by means of adaptor ligation, using the Ovation Rapid DR Multiplex System 1-96 (NuGEN, USA). The libraries were pooled, and the size of DNA fragments was determined with gel electrophoresis. The Illumina MiSeq using V3 Chemistry (Illumina) was used for sequencing. The sequencing quality was assessed by including a mock community (in triplicate) in the sequencing run. The mock community is an in-house assembled community that was pooled together from 10 distinct strains based on equal qPCR copies [29]. Three samples (individuals) were excluded from the analysis due to low relative sequencing depth (<7600 sequences, two samples for QuangNam in 2015 and one sample for BinhDinh in 2014).

MC data analysis

Amplicon sequence processing: The mothur software package (1.39.5) was used to process the amplicon sequencing data on a GNU/Linux 3.16.0-46-generic x86_64 system in accordance with the guidelines of Schloss et al. (2009) [30]. Forward and reverse reads were assembled into contigs by a heuristic approach, taking the Phred quality scores into account. Ambiguous contigs or contigs with unsatisfactory overlap were removed, and the remaining sequences were aligned to the Mothur formatted Silva Seed v123 database. Sequences that did not align within the region that was targeted by the primer set or sequences with homopolymer stretches with a length >12 were removed. The sequences were pre-clustered, allowing 1 mismatch for every 100 bp of sequence. Predicted chimeric sequences were removed with UCHIME [31]. The sequences were classified with a naive Bayesian classifier, using the RDP 16S rRNA gene training set, v.14 with

an 85% cut-off for the pseudobootstrap confidence score. Taxa annotated as unknown, Archaea, Chloroplast, Mitochondria, or Eukarya at the kingdom level were excluded. Sequences were binned into operational taxonomic units (OTUs) at a 3% dissimilarity level, as identified by the preceding classification step. A table containing the abundances of the OTUs and their taxonomic assignments was generated.

Analysis of diversity: All statistical analyses of diversity were conducted using the program package PAST, version 3.17 [32], except for ANOVA which was done in SYSTAT (v. 13). Tests of significant difference in larvae length, temperature, salinity and pH between groups of samples were done by one-way ANOVA followed by Tukey-Kramer test for multiple comparison. To calculate alpha diversity the following diversity indices were determined using PAST: Richness (number of OTUs), Chao1, Shannon index, and Simpson index. These indexes were used to calculate Hill numbers of order 1, order 2 and evenness according to Hill [33]. These diversity indices are termed Chao1, H1, H2 and evenness, respectively. Test of significant difference in Chao1 index, Hill numbers order 1 and 2, and evenness between groups of samples was done by two-way and one-way ANOVA followed by Tukey-Kramer test for multiple comparison. Heat maps were generated on different phylogenetic levels (phylum and order), using square root transformations of the biological replicates (R studio version 3.3.1, heat map package) [34].

Beta diversity was analyzed based on similarity measures. Bray-Curtis similarity was used for abundance based data [35] and for presence/absence data we used Jaccard similarity. Tests of significant difference in community structure between groups of samples were done by Nonparametric Multivariate Analysis of Variance (PERMANOVA) using Bray-Curtis and Jaccard as a distance measure [36], and included both one-way and two-way analysis. The Similarity Percentages (SIMPER) analysis [37] was used to determine the contribution from

individual OTUs to the Bray-Curtis dissimilarity among groups of samples of the three locations over three years. Differences were considered significant at $p < 0.05$.

Data deposition: the raw fastq files that were used to create the OTU table and used as a basis for the MC analysis in this paper have been deposited in the European Nucleotide Archive (ENA) database (accession numbers PRJEB21048).

Results

Larval length and abiotic factors

No significant differences were found in the average larval length between datasets (17.1 to 17.9 mm) (ANOVA, n=10 per sample). During the sampling activities, abiotic environmental factors (water temperature, salinity and pH) were measured. At the 3 sampling locations over the 3 years, the water temperature varied from 26.3 to 30.5°C, the water salinity ranged from 27.2 to 28.5 g L^{-1}, and the pH was between 7.5 and 7.8. Significant differences were detected in the temperature and the salinity from three locations over three years ($p<0.05$), and the main tendency was an increase with time. No significant differences were detected for pH. The environmental variables were strongly correlated (Pearson's $r > 0.75$).

Phylogeny of gut microbiota of rabbit fish larvae

In this study, no possible controls (buffer blanks) were added. Hence, this can be considered for future studies.

Firmicutes was the predominant phylum in the gut microbiota of larvae in all samples (35 – 61%), except for ThuanAn in 2016, which was dominated by the Actinobacteria (35%) and Proteobacteria (34%) (Fig. 2). Other dominant OTUs belonged to the phyla Verrucomicrobia (< 22.3%) and Bacterioides (< 16.2%). The predominant order in most sampling locations over the three year period was Clostridiales (10 – 39%). The only exception was in Thua Thien Hue

province in 2016, which was dominated by the Actinomycetales (34%) and Rhizobiales (17%). In addition to Clostridiales, the orders Actinomycetales (16 – 34%), Verrucomicrobiales (10 – 21%), Erysipelotrichales (7 – 18%) and Desulfovibrionales (4 – 14%) were also abundant in all samples. Other orders, such as Rhodobacteriales (< 7%), Bacteroidales (< 4%), Caulobacteriales (< 4%), Fusobacteriales (< 4%), Burkholderiales (< 4%), Spirochaetales (< 3%), Campilobacteriales (< 1%), Pseudomonadales (< 1%) and Flavobacteriales (< 1%) were also detected. The main order of potential pathogens (Vibrionales) was 0.1 – 1.1% of the gut microbiota of larvae (Fig. 2).

At the individual OTU level, only OTUs that were identified at an average relative abundance ≥ 0.1% were considered for further analysis. There were 79 OTUs identified in all samples (0.1% prevalence). The OTUs belonging to the Clostridiales (up to 19.2%), Erysipelotrichaceae (up to 18.1%), Akkermansia (up to 17.3%), Desulfovibrionaceae (up to 13.4%) were found abundantly in the gut microbiota of larvae in all samples. 9 OTUs were found in all samples from Thuan An (across the three sampling years) and Quang Nam (2014), while they were not detected in other samples. These OTUs were Nocardia (10.3 – 23.8%), Aquamicrobium (5.5 – 12.7%), Mycobacterium (4.1 – 10.1%), Brevundimonas (1.4 – 3.8%), Stappia (1.3 – 3.1%), Chelatococcus (0.8 – 1.8%), Phyllobacteriaceae (0.3 – 0.8%), Parvibaculum (0.3 – 0.8%) and Devosia (0.2 – 0.4%) (Supplement Fig 1).

Alpha diversity of the larval gut microbiota

Considerable variation in diversity indices were observed between individuals (Fig. 3). The highest average Chao1 of the gut microbiota was observed in ThuanAn larvae (351 OTUs), which was 17% higher than for fish from QuangNam (299 OTUs) and 37% higher than those from BinhDinh (256 OTUs). By contrast, the Hill order 1 (H1) of the gut microbiota from the ThuanAn

was only 14.7 OTUs, which was 31 and 43% lower than those from QuangNam (19.1 OTUs) and BinhDinh (21.1 OTUs), respectively. Similarly, the evenness in the gut microbiota from the ThuanAn (0.063) was 30 and 60% lower than those from QuangNam (0.082) and BinhDinh (0.101), respectively (Fig. 3). Two-way ANOVA showed no significant effects of sampling year on any alpha-diversity index, but significant effects of sampling location were detected for Chao1, H1 and evenness ($p < 0.005$). Whereas Chao1 increased from south to north, H1, Hill order 2 (H2) and evenness decreased. A significant interaction between sampling year and location was detected for Chao1 ($p = 0.0018$), but not for the other indices. The location-by-location alpha-diversity of the rabbit fish larval gut microbiota showed that there were significant differences in Chao-1 (ANOVA, $p < 0.0001$), H1 ($p = 0.0052$) and evenness ($p = 0.0009$), but not for H2 ($p = 0.1703$). By contrast, there was no significant difference in Chao1, H1, H2 and evenness of the gut microbiota of rabbit fish larvae between years ($p > 0.05$) (Fig. 3).

Beta diversity of the larval gut microbiota

The ordination by Bray-Curtis similarity indicates considerable similarity between samples, except that ThuanAn partly separates from the two other sites (especially 2015 and 2016) and some of the QuangNam samples from 2014 cluster together with ThuanAn samples (Fig. 4A). The pattern is similar when ordination is based on Jaccard similarity (Fig. 4B). This indicates that the separations in the ordination were to a large degree due to changes in the OTU inventory and not only changes in abundance. Typically, the average Bray-Curtis similarity within samples was 0.33 to 0.41 (Fig. 5). The similarity was comparable between sites and years, but with the highest year-to-year variability for ThuanAn. For comparisons between samplings Bray-Curtis was somewhat lower for comparisons of year within site, and approximately 1/3 lower for

220 comparisons within the same year between sites (Fig. 5). These data suggest limited differences
221 in the beta diversity of the gut microbiota of rabbit fish larvae.
222 Two-way PERMANOVA based on Bray-Curtis similarity confirm the observations above, and
223 show a significant effect of location and a significant interaction between sampling year and
224 location (p=0.0001). The last suggests that year-to-year comparisons are different between
225 locations. A two-way PERMANOVA based on Jaccard similarity show very similar results,
226 supporting the conclusion above based on the ordination. For a more detailed analysis of
227 community composition of the gut microbiota, we did further one-way PERMANOVA and
228 pairwise comparisons based on sequential Bonferroni.
229 The results of a year-by-year analysis for each location based on one-way PERMANOVA and
230 Bray-Curtis similarity showed that for 2014 the community composition of the gut microbiota
231 was different from the other two years for ThuanAn ($p = 0.0016$) and QuangNam ($p = 0.0004$),
232 whereas no significant differences between years were observed for BinhDinh ($p = 0.516$). The
233 conclusions are identical when comparisons were made using Jaccard similarity.
234 The results of a comparison of locations for the three different years based on one-way
235 PERMANOVA and Bray-Curtis similarity indicated that the community composition of the gut
236 microbiota of BinhDinh was different from the other two locations ($p = 0.0005$) in 2014. In 2015
237 the gut microbiota was different for all locations ($p = 0.02$), whereas in 2016 the gut microbiota
238 in ThuanAn was different from the two other locations ($p = 0.0001$). When analyses were done
239 based on Jaccard similarity the conclusions were the same. There seems to be a time-dependent
240 trend in the North-South axis for the composition of the gut microbiota: significant differences
241 in 2014 between the southern location BinhDinh and the two northern locations, spatial

differences in 2015 among all locations, and in 2016 between the northern ThuanAn location and those to the south. Extending the available time series could confirm this tentative trend.

A SIMPER analysis based on Bray-Curtis similarity and using the nine samplings as grouping showed that 10 OTUs made up 51.4% of the variance in community composition between groups. These OTUs are Erysipelotrichaceae, Nocardia, Clostridiales, Akkermansia, Desulfovibrionaceae, Aquamicrobium, Verrucomicrobiaceae, Mycobacterium, Bacteria and Lachnospiraceae taxa. The SIMPER analysis based on Bray-Curtis similarity for each location showed that 5 and 9 OTUs made up more than 50% of the differences observed for ThuanAn and QuangNam samples. For these two sites partly the same OTUs contributed to the separation of the 2014 samples from the 2015 and 2016 samples. These OTUs are in decreasing importance Nocardia, Erysipelotrichaceae, Verrucomicrobiaceae, Aquamicrobium, Clostridiales, Akkermansia and Mycobacterium for ThuanAn; Erysipelotrichaceae, Nocardia, Desulfovibrionaceae, Clostridiales, Firmicutes, Bacteria, Akkermansia, Aquamicrobium, Lachnospiraceae and Fusobacterium taxa for QuangNam. Thus, 5 OTUs were the same at the two locations, but with different impact on the variance explained. The SIMPER analysis based on Bray-Curtis similarity for each year showed that 11, 9 and 8 OTUs made up more than 50% of the differences observed in 2014, 2015 and 2016, respectively. All 8 OTUs from the 2016 analysis and 7 out of 9 OTUs from 2015 (except OTUs from Verrucomicrobiaceae and Firmicutes taxa) are included in the 11 OTUs contributing with >50% of the dissimilarity. These OTUs are in decreasing importance Nocardia, Akkermansia, Erysipelotrichaceae, Aquamicrobium, Bacteria, Desulfovibrionaceae, Mycobacterium, Propionibacterium, Clostridiales, Lachnospiraceae and Rhodobacteraceae taxa for the year 2014; Clostridiales, Akkermansia, Nocardia, Erysipelotrichaceae, Verrucomicrobiaceae, Desulfovibrionaceae,

Firmicutes, Aquamicrobium, Lachnospiraceae taxa for the year 2015; Erysipelotrichaceae, Nocardia, Clostridiales, Aquamicrobium, Desulfovibrionaceae, Mycobacterium, Rhodobacteraceae and Akkermansia taxa for the year 2016.

Because of the high degree of similarity in the gut microbiota of rabbit fish on spatial and temporal scales, it is interesting to evaluate if rabbit fish has a core gut microbiota. In terms of prevalence, 5 OTUs were found in all 87 individuals (100%), and these OTUs made up 8.0% of the total number of reads in the whole dataset. These OTUs belong to the orders Clostridiales, Rhodobacterales, Actinomycetales, Vibrionales and Burkholderiales. Fifteen OTUs were present in all but one larva (99%) and these OTUs made up 34.0% of the reads. OTUs of the gut microbiota present in at least 95% (83 individuals) of the 87 individuals included 19 OTUs and these OTUs summed up to 45.8% of the total reads in the dataset (Table 1). These are high cut-off values for a core microbiota. The average percent abundance ± S.D. ranged from 9.65 ± 13.48% (for Erysipelotrichales order) to 0.07 ± 0.07 (for Burkholderiales order). The average percent coefficient of variation (CV) of the core OTUs is 155%. In terms of overall abundance, the six most dominant OTUs of the core community constituted more than 1/3 (34.6%) of the total reads in the dataset. These OTUs belong to the orders Erysipelotrichales, Verrucomicrobiales, Desulfovibrionales, 2 of Clostridiales and one unknown order. The most dominant OTU in the core community (Erysipelotrichales order) constituted almost 10%.

Discussion

The composition of the gut microbiota is influenced by environmental factors and selective factors in the fish, all related to the ecological factors dispersal, drift and selection [38]. The selection in the host depends on host-microbe interactions that depend on *e.g.* species, trophic level of the fish, life stage, and nutrition, and on microbe-microbe interactions in the host [13,

16, 21, 39-46]. However, the relative importance of these factors, including both stochastic and selective aspects, are not clear. The microbiota plays important roles for larvae development, stress handling and disease resistance [47-49], and functional roles in fish physiology include digestive ability, uptake of nutrients, metabolism, development signaling and disease resistance [4, 5]. It is not known whether these functions can be maintained by various configurations of microbiota community structure, or whether some specific key members are required. This question is strongly related to the core microbiota concept. In this study, the wild larvae were collected in 3 different locations every June from 2014 to 2016 in Central Vietnam where the migrating rabbit fish have been found abundantly. This is the first study on rabbit fish gut microbiota analyzed by new high-throughput sequencing methods. This is also the first study in which wild and migrating fish larvae are analyzed both spatially (3 different locations) and temporally (over consecutive 3 years) with a large sample number (n=10 each location each year) at the individual level. This allows a better assessment of the likelihood of the existence of a core microbiome, when compared to all previous studies where fish samples were pooled, or few individuals were analyzed (n=3) (Supplement Table 1). This study is among few studies of the microbiota of wild fish [9], despite the significance of fish in the evolution of vertebrates. Our study revealed three important findings.

First, the phylogeny and alpha diversity analysis showed that the bacteria that were identified in the rabbit fish gut microbiota mainly belonged to the phyla *Firmicutes, Proteobacteria, Actinobacteria* and *Verrucomicrobia*. This is similar to studies on other fish gut microbiota using sequencing of amplifications of the 16S-rRNA gene (Table 2). These studies have been on fish from a variety of habitats, including marine herbivores (whitecheek surgeonfish, daisy parrotfish, bulbnose unicornfish and sixbar angelfish); marine omnivores (black rockcod) [42],

(blunt snout bream) [43]; marine carnivores (blackfin icefish, long-snout seahorse, two-spot red snapper, sole and grass puffer) [42]; estuarine carnivores (grouper and longjaw mudsucker) [42]; freshwater herbivores (grass carp) [43, 45]; freshwater omnivores (zebra fish, guppy) [42], common carp, silver carp, bighead carp and mandarin fish) [43, 45]; freshwater carnivores (rainbow trout, yellowhead catfish, Atlantic salmon and brown trout) [14, 42, 43]. These species had similar gut microbiota at phylum level to rabbit fish. This shows a strong robustness of the gut microbiota of fish at the phylum level (Table 2). The orders Clostridiales and Verrucomicrobiales were predominant in rabbit fish samples. The presence of putative cellulose degrading bacteria, such as *Clostridiales* and *Fusobacteriales*, in the gut, might relate to the fact that these larvae have a herbivorous feeding habit. At the age of sampling (approx. 25 days old) most of the wild larvae have started to consume seaweed [26, 50]. In herbivores, diet is likely one of the strongest modulators of the gut microbiota. A study in mammals reported that the OTU diversity increased from carnivores to omnivores to herbivores [51, 52]. This trend is likely true for fish gut microbiota if we assume that bacterial fermentation has a key function in the conversion of seaweed biomass into short chain fatty acids [6, 45, 53-56]. It has been hypothesized that the presence of bacteria from the orders *Clostridiales* and *Verrucomicrobiales* in the gut microbiota of herbivores is important for seaweed digestion [4, 42, 45, 57]. Hence, an important conclusion is that the composition of the gut microbiota seems to be under strong selection by the food in wild rabbit fish larvae. Within the gut microbiota, the composition of OTUs from potential pathogens was also assessed. OTUs from *Vibrio* were detected in all samples, with up to only 0.24 % abundance of the OTUs in the gut microbiota of the wild larvae. This is similar to the prevalence of *Vibrio spp.* in the gut of other normally developing fishes, *e.g.*, cod larvae [58].

At the individual OTU level, a total of 3028 OTUs were detected in *S. guttatus* in Vietnam, which is higher than the total number of OTUs detected in another species of Siganus genus *(S. fuscescens*) from the Great Barrier Reef (Australia) (1220 OTUs) [62]. Many bacteria, such as the cellulose degrading *Clostridiales* and *Fusobacteriales*, cannot be identified at genus or family level. Bacteria belonging to the Verrucomicrobiales and Desulfovibrionales, important orders for seaweed digestion, could be identified to the genus (Akkermansia, up to 17.3%) and family (Desulfovibrionaceae, up to 13.4%) level, respectively. Overall, the dominance of *Clostridiales*, *Akkermansia* and *Desulfovibrionaceae* suggest a strong selection by food in wild rabbit fish larvae. The presence of the *Clostridium* group (strict anaerobic bacteria), *Desulfovibrio* group (sulfate reducing bacteria) and *Akkermansia* group (mucin degrading bacteria) in the gut microbiota of rabbit fish suggest that the microbial communities of the gut is driven by the nutrition factors. The abundance of these bacteria suggests that fermentation of algal material, by for example *Clostridium spp.*, is predominately in this marine herbivorous fish [68] [69] [70]. These patterns of rabbit fish gut microbiota in Vietnam are in accordance with the gut microbiota of rabbit fish from regions which are rich in sulfated algal polysaccharides such as the Great Barrier Reef (*S. fuscescens*) [62] and the Red Sea [63], and with the gut microbiota of other marine herbivores [64] [65]. This may reflect that the diet of juvenile rabbit fish in Vietnam is dominated by sulfated algal polysaccharides. A verification of this require further studies.

Second, the beta diversity analysis indicated that the location influenced the composition of the microbiota. As mentioned above, the environmental variables were strongly correlated. Consequently, it is not meaningful to use variance partitioning to evaluate how much of the beta diversity could be explained by environmental variables. The Bray-Curtis similarity indicated that ThuanAn partly separates from the two other sites (especially 2015 and 2016).

Understanding the factors modulating the composition of the gut microbiota is important for understanding the development of fish larvae [59]. In this study, the differences in abiotic factors between locations, *e.g.* current direction and temperature, might have an impact on the gut microbiota of rabbit fish in the wild. The flow direction of water currents in Central Vietnam can explain the difference in the gut microbiota between locations. In June, the currents usually flow from BinhDinh to ThuanAn and the currents are partially blocked by the Hai Van pass (peninsular mountains), which are located between ThuanAn and QuangNam [60]. The change of the direction of the current might create differences in the water bodies between the 3 locations, hence affecting the water MC and resulting in the separation of the gut microbiota in ThuanAn from other locations in the south (QuangNam and BinhDinh). Other abiotic factors such as water temperature and identity or quality of the local food could also be the driving factors, alone or in combination. A more extensive monitoring program to characterize the gut microbiota in terms of feeding habits and abiotic factors in combination with an experimental approach, could reveal which abiotic and biotic factors are the main drivers for the composition of the gut microbiota of rabbit fish.

Last, a "core microbiome" conceptualizes the symbiotic functionality of a certain subpopulation of the gut microbiota [1, 2]. The extensive sampling in time, geographic distance and data for individuals at large sample size make the present dataset well-suited for an analysis of the existence of a core microbiota. Moreover, the limited beta diversity observed in our study points to the possible existence of a "core microbiome" in rabbit fish. Interestingly, 5, 15 and 21 OTUs were found in 100, 99 and 95% of the individuals, respectively. The shared number of OTUs in rabbit fish larvae was much higher than those observed from other wild reef fish larvae, *e.g.* wild damselfish larvae sharing 16 OTUs at the high cut-off of 70% prevalence [61]. Furthermore, the

number of shared OTUs in rabbit fish appears to be relatively high compared to the number of shared OTUs in other saltwater species, *e.g.* Atlantic salmon shared only 5 OTUs at 90% prevalence [15], and Atlantic cod shared a core microbiome of 10 OTUs at 80 – 98% prevalence [2]. The presence of a core microbiome was also reported for other herbivorous species such as the blunt snout bream (*Megalobrama amblycephala*) and grass carp (*Ctenopharyngodon idellus*), where only 3 OTUs from the taxa *Clostridium, Citrobacter* and *Leptotrichia* were present in all individuals [45]. At 80% prevalence, only 10 OTUs were found in herbivorous cichlids [17]. The existence of a core microbiome is an indicator of strong selection in the host, either by fish-microbe or microbe-microbe interaction. This contradicts conclusions from other studies suggesting stochastic processes like dispersal and drift to be important [11]. Assuming that the core gut microbiota contribute to gut functionality [21-23], their relative abundance seems relatively unimportant, in view of the large SD across samples. This might be an indication of functional redundancy; this point is speculative and would need to be confirmed by experiments, for instance, by manipulating the core gut microbiota through feed and monitoring its contribution to gut functionality.

The large variance in relative abundance of the core microbiota between individuals is very interesting. So far the core microbiota concept is mainly descriptive, and few studies have focused the functionality related to this concept. The functionality may be related to both microbe-microbe and host-microbe interactions. For microbe-microbe interactions all four high level processes [66] are relevant, but the data best fit selection and homogenizing dispersals as the most important processes. However, with the high growth rates in the digestive tract it is unlikely that dispersal can overrule selection. For rapidly developing young stages of fish it is not realistic to assume that all rabbitfish individuals get similar relative abundance despite the

fact the same OTUs are selected for. For host-microbe interactions the situation is a bit different. First, we have data on relative abundance, whereas absolute abundance is probably what is most important for functionality. This is a general problem with the data we get from amplicon sequencing, that is not much addressed today [67]. Second, for the functionality of a population to take place in a host we may anticipate different types of kinetics dependent on the function. For some there may be a linearity in the response that is correlated to physiology. For others it may be a threshold response with no effect until a critical population is reached, due to *e.g.* quorum sensing. Both types of kinetics allow for considerable variability in relative abundance while maintaining the functionality of the host-microbe interaction. These finding highlight the need to integrate functionality into the core microbiota concept, including as basic questions as whether the core microbiota is driven primarily by microbe-microbe or host-microbe interactions. This is the first study to investigate the fish gut microbiota of migrating fish larvae with an effective sampling strategy for a gut microbiota study, and it gives strong indications of a core gut microbiota in this species and as a consequence limited beta diversity. The significance of the core microbiota for development and health of rabbit fish requires further studies. Data from the present study may facilitate the development of safe and effective methods for manipulating gut microbiota composition to promote the health of rabbit fish for nursery and grow out culture.

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Competing interest

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.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Figure titles/legends

Fig 1 Sampling locations of wild rabbit fish over a three-year period (2014-2016)

Location 1: ThuanAn (Thua Thien Hue province); location 2: QuangNam (QuangNam province); location 3: BinhDinh (BinhDinh province) (Source: https://www.google.com/maps/@15.34538,108.3821484,7.84z). Sampling locations (star) and coral reef breeding ground (circle) of wild rabbit fish. A: ThuanAn; B: QuangNam; C: BinhDinh (Source: A. https://www.google.be/maps/@16.3975183,107.9632426,9.7z?hl=en; B. https://www.google.be/maps/@15.9331892,108.6010506,9.7z?hl=en; C. https://www.google.be/maps/@13.7129966,109.1235103,9.7z?hl=en)

Fig 2 Heat map showing the square root transformed relative abundance of the gut microbiome of the rabbit fish larvae from 3 locations over 3 years

Phylum (upper fig) and order (lower fig) levels. Weighted averages of the replicates are presented

Fig 3 Alpha diversity indices of the rabbit fish larval gut microbiota

Including: Chao1 richness, Hill numbers of order 1 and 2, and Evenness defined as H1/H0.

Fig 4 Principal coordinates analysis (PCoA) ordination for rabbit fish gut microbiota from 3 locations over 3 years

Bray-Curtis (A) and Jaccard (B) similarities. Locations are indicated by colour and years by symbols, see bottom of B

Fig 5 Bray-Curtis similarity of gut microbiota of rabbit fish

Upper figure: within samples Bray-Curtis similarity. Lower figure: between samples Bray-Curtis similarity. Error bars indicate S.D. for 36-45 values for “Within sample” and 81-100 values for “Between samples”

Supplement Fig 1 Heat map showing the square root transformed relative abundance of the gut microbiome of the rabbit fish larvae from 3 locations over 3 years at OTU levels. Weighted averages of the replicates are presented.

REBUTTAL LETTER

Ref.: Ms. No. MECO-D-18-00339R2

Gut microbiota of migrating wild rabbit fish (*Siganus guttatus*) larvae have low spatial and temporal variability.

Microbial Ecology

Dear Dr. Karen E. Nelson,

Editor-in-Chief
Microbial Ecology

Thank you very much for your response and reviewer's comments on our manuscript. We sincerely apologize for the great time it has taken us to respond to these comments, and hope that a revised version of the manuscript will still be considered by Microbial Ecology. We have modified the paper in response to the reviewer comments. Below we respond to the comments point by point.

Reviewers' comments:

Reviewer #1: Three of four of the remaining comments that I had were adequately addressed. The 4th one about the possible provision of an OTU Table as supplementary information is not really answered. However, I suppose that since the data was deposited in the ENA archive (and an OTU table was generated for that as is mentioned in line 152) the data can ultimately be found by readers.

Response to reviewer: Thank you very much for your comments. Our raw data was deposited in the archive. In addition, an OTU table was uploaded as supplement. We hope the data can ultimately be found by readers.

Reviewer #2: SCIENTIFIC COMMENTS

49-53 I think something is missing here. Roeselers et al. looked at zebrafish, but the other studies mentioned looked at a variety of species. I'm actually not sure exactly what is meant did the other studies take a better approach, or did they have (some of) the same problems as the Roeselers paper? Did they conclude there was a core microbiome or not? My best guess is that you mean something like "Several studies have proposed a core microbiome for fish species, beginning with Roeselers et al. (2011) zebrafish study, but studies so far have ignored…[], pooled individuals, and/or used low sample numbers (n=3)[]". (Or just "and", not "and/or", whichever is correct.

Response to reviewer: I agree that it was unclear here. I hope we have made it clearer in the revised manuscript (line 50 – 55). Roeselers et al. looked at only one species (zebra fish) and pooled

samples. Other studies looked at a variety of species, but had limitations by ignoring the potential spatial and temporal variation, by pooling of individuals and/or by analyzing a limited number of individuals. However, all studies concluded that there was a core microbiome in fish.

332 Does Siganus have the highest known number of OTUs? If so, should say so, or mention whatever the reason is for including this species in particular.

Response to reviewer: It is unknown if Siganus have the highest known number of OTUs among fishes. Here we only try to compare the number of OTUs from 2 species of Siganus (Vietnam and Australia). The species from Vietnam (*S. guttatus*) has higher number of OTUs than the species from Australia (*S. fuscescens*). We made it clearer in the manuscripts (line 334 – 336).

335 Maybe better "Bacteria belonging to the Verrucomicrobiales and Desulfovibrionales, important orders for seaweed digestion, could be identified to the genus (Akkermansia, up to 17.3%) and family (Desulfovibrionaceae, up to 13.4%) level, respectively".

Response to reviewer: Thank you very much for your suggestion. I have incorporated the sentence with some modifications in the manuscript (now line 338 – 340).

Use this wording in the manuscript: "Bacteria belonging to the Verrucomicrobiales and Desulfovibrionales, orders that are important for digestion of seaweed, could be identified to the genus (Akkermansia, up to 17.3%) and family (Desulfovibrionaceae, up to 13.4%) level, respectively".

341 Why "compartmentalization" and "across the gut"? That sounds like spatial separation, which may be true, but was not examined here. If you want to suggest this, make it clear it's a hypothesis. Or do you instead mean functional compartmentalization?

Response to reviewer: Yes, for sure, we don't have data for being conclusive. However, this is an interesting part of our data. We have rewritten this part to make it only a suggestion, and stated explicitly that this require further studies.

342 "The abundance of these bacteria showed that fermentation of algal material by, for example, Clostridium spp., is predominately in the marine herbivorous fish." This doesn't make sense as written. Are you proposing that these particular fish may be fermenting algal material, and that Clostridium species are mainly responsible? If so, this would seem to need some references to support it.

Response to reviewer: See our response to the comment above. We have added references showing that Clostridium can be involved in fermentation of algae (line 344-346).

391 What do you mean by "gut functionality"? What specific aspects might be measurable in these fish?

Response to reviewer: By gut functionality we mainly think about digestion, but also immunology due to release of e.g. glucans and VFA. As this fish is herbivores, the most specific aspects might be the ability to ferment the algal material, for example, by the predominant *Clostridium spp.*

414 The buffer blanks comment doesn't belong here in this position it might seem to negate the whole study. I would put it at the beginning of the sequencing results, as something that should be kept in mind. There may have been sequences introduced from seawater, or fish surfaces, or during DNA preparation it is hard to completely rule out any of those without proper controls.

Response to reviewer: Thank you very much for your comments. I have moved the buffer blanks comment to the beginning of the sequencing results (line 168 – 169).

MINOR SUGGESTIONS AND CORRECTIONS

268 Should be "and Burkholderiales".

Response to reviewer: I have made the modification in the manuscript.

269 "all but one larva" (singular)

Response to reviewer: I have made the modification in the manuscript.

287 Do you mean "developmental signaling"?

Response to reviewer: Yes, we meant "developmental signaling". I have made the modification in the manuscript.

326 Should be just "OTUs from Vibrio" (no "The")

Response to reviewer: Thank you very much for your comments

328 Should be "normally developing fishes" (or "healthy developing fishes", if that's what you mean not sure)

Response to reviewer: I have made the modification to “normally developing fishes” in the manuscript.

328 "in the gut" (not "is")

Response to reviewer: I have made the modification in the manuscript.

332 "in" shouldn't be italicized

Response to reviewer: I have made the modification in the manuscript.

333 Better "Many bacteria"

Response to reviewer: I have made the modification in the manuscript.

339 Should be "of the Clostridium group"

Response to reviewer: I have made the modification in the manuscript.

340 No comma needed before "in the gut microbiota"

Response to reviewer: I have made the modification in the manuscript.

341 Should be "a compartmentalization"

Response to reviewer: I have made the modification in the manuscript.

365 Should be "in terms of"

Response to reviewer: I have made the modification in the manuscript.

367 Should be "which abiotic and biotic factors are" (no "that")

Response to reviewer: I have made the modification in the manuscript.

382 Should be "were present"

Response to reviewer: I have made the modification in the manuscript.