Gut microbiota in homologous Chinese soft-shell turtle (*Pelodiscus sinensis*) under different habitats

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Abstract

Background

Chinese soft-shell turtle (*Pelodiscus sinensis*) is an important commercial species for its high nutritive and medicinal value, but it has been a vulnerable species due to habitat loss. In this study, homologous juvenile turtles were allocated to lake, pond and paddy field to investigate the habitat effects on turtles.

Results

The growth, morphology and gut microbial communities were monitored during the 4 months cultural period. It showed higher growth rate of turtles in paddy field and pond. The appearance, visceral coefficients, gut morphology and microbial communities in turtles were distinct among different habitats, which was mainly attributed to food abundance and living space. The diversity of gut microbial community was obviously higher in turtles from paddy field than pond and lake. Significant differences on dominant phyla, genera and species were found among initial samples and subsequent samples from different habitats. Firmicutes was the most abundant phylum in turtle gut sampled from greenhouse at initial and Proteobacteria was the most abundant phylum after cultivated in different habitats. The functional predictions indicated that both habitat type and sampling time had significant effects on metabolic pathways, especially amino acid and carbohydrate metabolism.

Conclusion

The microbial abundance was different among the habitats and varied within cultural periods. Gut microbes in turtles from paddy field presented high diversity and richness. Rice-turtle coculture was a potential ecological and economic farming mode for its high production and quality of both turtles and rice, which would play important roles in wild turtle protection, food security and paddy field environment improvement.

Background

Owing to the so-called high nutrition and/or better taste, wild animals are popular in many countries or areas, which has accelerated the recession of wild resources. However, consumption of wild animals may carry highly pathogenic viruses and bacterium that posing potential threat on humans. Wildlife conservation has become impending in decades. Chinese soft-shell turtle (*Pelodiscus sinensis*, hereinafter referred to as turtle) is an important commercial aquatic species in southeast Asia including China, which is commonly considered as tonic food for their high nutritive and medicinal value[1, 2]. It has been a vulnerable species due to habitat loss and immoderate fishing, thus being listed as "vulnerable" on the IUCN Red List at 2000 (retrieved at 2018)[3]. Turtles live in water and have been traditionally cultured in lakes, rivers or reservoirs in low stocking density. Nowadays, several captive modes have been promoted to satisfy the market demand, therein hothouse and artificial ponds are commonly selected as turtle aquaculture sites for high production[4]. The intensive cultivation can significantly increase the production of commercial turtles meanwhile shorten culturing period, however it also accompanies with problems such as high risk of disease, defective appearance and low quality. Furthermore, the high energy consumption rearing patterns are not conducive to agricultural sustainable development and have aroused increasing public concerns[4, 5]. Intensive breeding is inclined to cause diseases as bacterial infection and esoenteritis due to crowed living space, superfluous but simplex feeds[6–8]. Consequently, antimicrobial resistant bacteria and/or antiseptic medicines have been abused[9, 10], thus leading to more serious environmental and health problems that affecting both the quality of product and animal welfare[11–13]. Probiotics are developed for cultured animals as immunopotentiator[14, 15], but the positive effects were limited and temporaryand more healthy culture mode should be applied for high quality products[16, 17].

Turtles from different habitats generally show obvious differences on appearance, morphology, texture properties, chemical composition and flavor substances contents[5, 18]. The physiological disorders are always associated with remarkable changes of gut microbial communities. Gut microbiota is a protective barrier for organisms to prevent pathogen invasion[19], which is always affected by both internal and external environment[20, 21]. The diversity and variation of gut microbial communities have been considered as indicators of health status for gut and host[22]. In recent years, coculture of rice and aquatic animals as rice-fish, rice-crayfish and rice-turtle have been rapidly developed in southeastern Asia regions, especially in south China[23]. The paddy fields can

provide capacious space, shelters and natural food for cultured animals. Cultured animals are able to prey on pest, meanwhile, activities of cultured animals could loosen the soil and provide organic fertilizer for paddies thus significantly decrease the utilization of chemical fertilizers and pesticides[24, 25]. Therefore, the coculture mode has been considered as an economical and ecological culture mode in rice-growing regions.

Host genetics, diets and ambient environmental conditions could affect the composition of the complex gut microbiota[26, 27]. It is difficult to fully disclose the diversity and dynamics of gut microbiota and identify keystone species for specific functions[28]. In the present study, the homologous juvenile turtles in similar genotype and early life conditions were allocated to different habitats to investigate the difference in growth and morphology and analyze the diversity and variation of gut microbial communities within cultural periods. Efforts were also made to find some functional microbes or representative communities as bio-markers to evaluate the physiological status of turtles in different habitats.

Results

Turtle growth and morphology under different habitats

The mortality was negligible both in paddy fields and ponds during the experiment period. However, only a small number of turtles were caught from lake at 60d and no marked turtles were recaptured at 120d, resulting in incomplete statistics on mortality and growth for turtles in lake. We selected a small sample size for turtle resource protection purpose and the difficult in sample collection from nature lake, and the differences on growth, physiology and gut microbiota were distinct among different groups. The body weight of turtles in paddy fields and ponds were obviously higher than those in lake ($p<0.05$) and the divergence occurred in the early days. The growth rates of turtles were 0.76%/d, 0.68%/d and 0.40%/d for habitats of paddy fields, ponds and lake in first 60d. It was 0.72%/d and 0.62%/d for turtles in paddy fields and ponds during the whole 120d. The hepato-smatic index and clumpy fat index were highest in ponds, secondly in paddy fields and lowest in lake ($p<0.05$). The gut-smatic index on weight (DSI_W) for turtles from pond was significantly higher than lake and paddy field ($p<0.05$). Inversely, the gut-smatic index on length (DSI_L) was higher for turtles from paddy field and lake compared to pond. Measured values are presented as mean ± standard deviation, the different superscript letters in same row indicated significant difference($p<0.05$)(Table 1).

There was no obvious trauma for most of turtles from lake except occasional leeches parasitic on calipash. Meanwhile, more bruises or scars were observed for the turtles from ponds than paddy fields. The appearance such as color of carapace and plastron were different among turtles in different habitats. The carapace of turtles cultured in ponds presented bottle green, but the individuals from paddy fields presented bottle green with slight golden yellow, which were similar with turtles from lake. There was no significant difference on main somatotype index ($p>0.05$), but the calipash lateral width was relative higher for turtles from ponds than paddy fields (Table 1).

Composition and diversity of turtle gut microbiota

The grouping details for samples from different habitats, cultured days and intestinal segment were listed in Table 2. The gut samples A total of 1 723 158 valid bacterial 16S rRNA gene reads were obtined and 4 901 OTUs were identified from all samples. The observed total OTUs varied in 64~822. The total number of OTUs was significantly less in initial groups IF and IL, and more in groups F1F and F1L from paddy fields at 60d. The number was 17~48 on OTUs more than 0.01% of total OTUs (Table S1). Significant differences were found in OTU composition among groups(Fig S1). Guts sampled at 120d had few unique OTUs, both in former and later part. The alpha diversity was calculated according to the composition and relative abundance of OTUs. Generally, the alpha diversity indices of microbes in later gut were higher than those in former gut. Besides, it was obviously lower in initial turtle guts from hothouse ($p<0.05$), whereas obviously higher in turtle gut sampled from paddy fields than ponds and lake (Fig.1). The species and number of OTUs varied significantly at 60d, different from that relatively harmonious at 120d. The microbial abundance was higher in samples from paddy fields than lake and ponds during the experiment. The microbial community presented relatively high similarity in guts sampled at same time. The PCA (Principal Component Analysis) showed high microbial community similarity in guts from the same individual or group, and significant discrepancy in samples from different habitats and sampling time (Fig.2). Generally, both sampling time and living habitats affected the variation of gut microbial communities.

Dominant microbes

The recognized microbes belonged to 27 phyla, 59 classes, 97 orders, 151 families, 219 genera from all the samples based on GreenGene. The phylum and genus level were emphasized in analysis. Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria were the most dominant phyla, accounting for more than 95% of the total bacteria in all samples. Firmicutes was the most abundant phylum in turtle gut sampled from hothouse at initial, while Proteobacteria was the most abundant phylum after cultivated in different habitats then followed by Bacteroidetes. Firmicutes and Fusobacteria commonly existed at 60d but rarely present at 120d in all the three habitats (**Fig.3a**). Additionally, the unidentified bacteria were more in lake compared to paddy fields and ponds.

There was significant difference on dominant genera among initial samples and subsequent samples from different habitats. The dominant genera in initial samples were an unclassified genus belonging to Bacteroidales, *Romboutsia*, *Cetobacterium*, *Weissella*, *Lactococcus*, *Lactobacillus*, *Clostridium*, *Edwardsiella*, *Plesiomonas*, and *Sarcina*. As for samples from the mentioned three habitats, the dominant genera were *Cetobacterium*, *Chryseobacterium*,*Clostridium*, *Epulopiscium*, *Flavobacterium*, *Helicobacter*, *Pseudomonas*, *Stenotrophomonas* and another unclassified genus belonging to Xanthomonadaceae. The abundance of dominant genera varied with different habitats, sampling time and gut location. For turtles sampled from paddy fields, the most dominant genus in foregut sampling at 60d was *Clostridium* and in hindgut was *Cetobacterium*, while at 120d, the most dominant genus was *Stenotrophomonas* both in former and later gut. For turtles sampled from pond, the most dominant genus at 60d was *Flavobacterium* and *Cetobacterium* in former and later gut, while at 120d, the most dominant genus was also *Stenotrophomonas*. For turtles sampled from lake, the most dominant genus at 60d was *Flavobacterium* and *Cetobacterium* in former and later gut, respectively (**Fig.3b**).

The dominant species in different gut location was also distinct. In foregut, the dominant species were *Weissella cibaria*, *Enterococcus durans*, *Lactobacillus sakei*, *Lactococcus lactis*, *Lactococcus garvieae*, *Sarcina sp.* and *Pseudomonas sp.*, whereas in hindgut, *Clostridium sensu stricto*, *Romboutsia sp.*, *Weissella cibaria*, *Escherichia coli*, *Plesiomonas shigelloides*, *Edwardsiella tarda*, *Paeniclostridium sp.*, *Cetobacterium sp.*, *Terrisporobacter sp.* and other two unclassified species belonging to Bacteroidales were abundant.

Microbial community in different habitats and sampling time

The microbial community was relatively complex at 60d, especially in former gut. At 60d, the species of microbes were significantly more in field, following by pond and lake. There were 140 common species (8.2%) in former gut from the three different habitats (**Fig.4a**), thereinto, *Flavobacterium* sp., *Pseudomonas* sp., *Chryseobacterium* sp. and two species belonging to Xanthomonadaceae were relatively abundant. *Cetobacterium somerae* was more abundant in paddy field than pond and lake. For later gut, there were 205 common species (8.1%) in the three different habitats (**Fig.4b**). Among these, one specie belonging to Bacteroidaceae was abundant in all habitats. *Cetobacterium somerae*, *Epulopiscium* sp., *Pseudomonas* sp., *Stenotrophomonas* sp. and *Flavobacterium* sp. were more abundant in paddy field and lake than in pond, while *Clostridium* sp. and *Epulopiscium* sp. were relatively abundant in pond. Meanwhile, *Chryseobacterium* sp., *Parabacteroides* sp., *Sphingobacterium faecium*, *Clostridium perfringens*, *Pseudomonas* sp., *Bacteroides* sp. and *Pseudomonas* sp. commonly existed in samples from lake and paddy field but did not appear in pond. At 120d, the specific microbes were more in pond (74%) than paddy field (33.4%) for former gut and the common species accounted for 18.6%, the specific microbes were more in paddy field (44%) than pond (34.4%) for later gut and the common species accounted for 26.1% (**Fig.S2**).

The LEfSe analysis was also conducted to identify representative microbes among various groups. For initial groups, representative genera were Weissella, Cetobacterium, Chryseobacterium, Epulopiscium, Escherichia, Flavobacterium, Lactococcus, Leuconostoc, Plesiomonas, Romboutsia, Sarcina and Stenotrophomonas. For groups cultured in different habitats, it showed F1L contained more species differed from other groups including Cetobacterium, Lactobacillaceae, Bacteroides, Parabacteroides, Plesiomonas and several species belonging to phyla Firmicutes presented higher LDA score. For F1F, representative genera were Sutterella, Bacteroides and Clostridiales. For samples from lake, Xanthomonadaceae and Pseudomonadales were representative genera, especially at 60d. The representative microbes in ponds were numerous and belonging to various phyla, especially the phyla Proteobacteria, also there were some unassigned species were indicated in pond (**Fig.S3**).

Functional predictions

The nearest sequenced taxon index (NSTI) were developed to quantify the availability of nearby genome representatives for groups (**Table S2**). Totally 41 predicted functional categories which represented 7 pathway maps in KEGG level 2 were indicated by PICRUSt.

Cultural periods had significant effect on metabolism especially the amino acid and carbohydrate metabolism, membrane transport as well as replication and repair. At 60d, the functional microbiota related to amino acid and carbohydrate metabolism was distinct in lake compared to pond and paddy field (Fig.S4).

Discussion

Turtles had same general microbiota regardless of origin, body size and habitats, and also presented fast adaption after allocated to different habitats[29]. The differentiation on growth, behavior and physiology of the homologous turtles appeared under different living habitats in a short period. Environmental changes can substantially influence the gut microbiome[30, 31]. The differences might be attributed to living space[6], water quality, food composition and abundance[32, 33], prey and predation conditions for different habitats[34, 35]. Considering the similarity of natural conditions as geographical location, climate, rainfall and temperature for the mentioned three habitats, the food intake and relative living space might be the main factors determining the growth and physiology of turtles in this study[36, 37]. Wild turtles are predominantly carnivorous and prey on small fish, mollusks, crustaceans, insects or their larvae, occasionally some plant seeds, but the food abundance was affected by water environment, competitor or enemy [38, 39]. In present study, turtles in paddy fields and ponds were regularly fed with artificial feeds, but no feeds were provided for turtles in lake during the experiment. Besides, gastropods and insect larvae commonly existed as supplementary food in lake and paddy field, but rarely in ponds[40]. The stocking density in lake was undoubted lower than in paddy fields and ponds and the lake environment was relatively stable with capacious water and less disturbance. Otherwise, more competitors existed in the lake, predators and parasite, but negligible inter-specific competition[41]. Paddy field in this study was a complicated habitat of which environmental features were comprised of common field and pond. The paddy field provided spacious living space and rice plants served as shelter for turtles. The high growth rate of turtles might be attributed to relative low stocking density in paddy field and enough food. He *et al.* (2017) demonstrated the taste of turtles cultured in paddy field was better than turtles in ponds based on texture and chewiness of meat, which might also due to the broad space of paddy fields for turtle activities[18]. All of these indicated the extensive living space of paddy fields could promote the growth and quality with proper amount of food.

Food and feeding strategy obviously affected the morphology and function of digestive system[42], previous study demonstrated that the relative gut length was shorter in stress conditions such as food shortage[35]. Sufficient feeds might enhance digestive function and promote the development of gut at earlier feeding stage, but continuous regular feeding with sufficient food might decline the appetite and digestive activities, along with changes of gut morphology and structure[43, 44]. The gut presented obvious adaptation to habitat, the relative length of gut was significant lower in ponds compared to paddy fields and lake. It might be related to the complicate food composition in lake and paddy field which increased nutrient absorption and prolonged intestinal transit time[45, 46]. Although the turtles in ponds were fed apparently satiation during the experiment, the fixed and simple artificial feeding might not be accorded with ingestion habit for turtles and the food species or types also influenced the internal environment and gut microbial communities[47].

Gut microbiota was closely associated with host physiological metabolism, nutrients utilization, nutritional status, immunity, even the body health[48, 49]. The microbes originally derived from parents, then living environment played important roles in forming procession of gut microbial community and micro-ecological system [21, 50]. There were significant differences in gut microbial composition under different habitats[51, 52]. In general, the microbial population was less diverse in diseased organism compared to healthy ones. The gut microbial species were more in paddy field and pond than lake at 60d, while the species were fewer and no obvious differences were found among three habitats at 120d. It might due to obvious reduction of the feed intake at 120d. The composition and abundance of gut microbial communities varied under different habitats to adapt the heterogeneous habitats[53]. Food was deemed to a main factor which influenced the gut morphology, homeostasis and microbiota, which provided nutrients for body, also act as fermentation substrate for gut microbes[54, 55]. The microbial gut communities varied a lot when fed with diets in different compositions[56].

Ambient water conditions like temperature and diets change affected microbiome composition[57, 58], suitable diet was conducive to improve the intestinal environment and increasing the abundance of probiotics[49]. The PICRUSt functional predictions revealed both the cultural periods (different seasons) and habitats had significant effects on metabolism, especially the amino acid and carbohydrate metabolism, which also indicated the key role of food intake on gut microbial community[59]. Meanwhile, gut microbiota would further influence the metabolic activity of host[60].

Most of previous studies focused on factors that affected gut microbial community, such as genotype, rearing conditions and diets[61–63]. However, the causality between microbial community and specific diseases were ambiguous[64, 65]. Healthy individuals often had intricate and stable gut microbial community and the pathogenic bacteria might disturb the homeostasis and microbial balance which may present as reduction of gut microbial species and richness. Adversely, in several recent studies, more bacteria and higher alpha diversity were observed in diseased intestines compared to healthy ones and the richness of bacteria couldn't utterly indicate the health status[66]. The representative microbes which could reflect the balance of microbial communities and contribute to intestinal health should be concerned, and it might also vary in different species or life stage.

For turtles in this study, the dominant phyla were Proteobacteria, Bacteroidetes, Firmicutes and Fusobacteria in different habitats, which were similar with other freshwater fish as crucian carp (*Carassius auratus*), grass carp (*Ctenopharynodon idellus*), bighead carp (*Hypophthalmichthysnobilis*)[67] and marine turtles such as green turtles (*Chelonia mydas*)[29]. Previous studies indicated there was clear difference in composition between aquaculture reared and wild aquatic animals: in the wild species, Proteobacteria was always the most abundant phylum; whereas Firmicutes were the most abundant phylum in the aquaculture reared species [68, 69]. For turtles in this study, it was also found that Firmicutes was the most abundant phylum in turtle gut sampled from greenhouse at initial intensive aquaculture condition, whereas Proteobacteria was the most abundant phylum after cultivated in pond, lake and paddy field, especially in gut sampled at 120d. It also indicated that gut microbiota of turtles had both intrinsic and distinct environmental characteristic. Aeromonas, Chryseobacterium and Citrobacter commonly existed in European pond turtles kept in breeding centers and there were obvious differences in bacteria composition and abundance for turtles in different ages[70]. The composition and abundance of gut bacteria also varied in different physical status, the virulence and prevalence of pathogens were always suppressed in healthy individuals[71]. Cetobacterium, Cyanobacterium and Clostridiaceae were more abundant in healthy fish, whereas Aeromonas, Vibrio and Shewanella OTUs were more abundant in diseased individuals[72]. *Enterococcus* spp. and *Citrobacter* spp. were the dominant bacteria in healthy turtles, while *Citrobacter* spp., *Aeromonas spp.* and *Bacillus* spp. dominated in the diseased ones[73]. *Lactococcus garvieae*, *Citrobacter freundii and Edwardsiella tarda* were commonly existed pathogenic bacteria in water environment[74]. In this study, *Edwardsiella* spp. occasionally existed in samples from ponds, but rarely found in paddy fields and lake. *Aeromonas* spp. and *Citrobacter* spp. were almost absent in all samples. *Bacillus* spp. were more abundant in paddy fields than lake and ponds at 60d. The *Pseudomonas* spp. widely existed and were rich in most of samples except later gut from ponds at 60d. In addition, the nonpathogenic bacterium as *Enterococcus faecium*, *Enterococcus hirae*, *Haemophilus segnis*, *Ochrobactrum anthropi* and *Pseudomonas* spp. could also induce fester of carapace and plastron when the cultural environment became worse. It revealed the relationship between gut microbial communities and body health were not static and the formation of gut microbial community was mutual adaptation with internal and external environment. Therefore, the relationship among microbial communities in gut, cultural water and soil should also be detected to reveal the adaptation for turtles in different habitats.

It was necessary to optimize feeding regime and cultural conditions to improve the economic and environmental sustainability of aquaculture. Burgeoning modes as cultured in reconstructive outdoor ponds and paddy fields occurred to replace the hothouse cultivation, especially in later life stage before coming into the market. In this study, turtles cultured in paddy fields presented maximum growth rate. Meanwhile, it could keep the rice yield and increase the value of turtles with remarkable decrease of fertilizers and pesticides utilization. All of these indicated that the co-culture mode was economic and ecological. The co-culture mode could be optimized by reasonable soil, water and fertilizer, especially the nitrogen fertilizer and feeding regime of turtles on basis of digestibility that could minimize nutrient outputs and decline the environmental impacts in intensive culture[75, 76]. The rice-turtle coculture was an economic and ecological integrated culture mode which might play important roles in paddy fields environment protection and food security, due to the sharply declined utilization of chemical fertilizer and pesticide compared to traditional planting modes. The mutual promotion for field environment and turtle health were preliminarily detected in present study but the effectiveness and potentiality should be investigated more systematically in future work.

Conclusion

The juvenile Chinese soft-shelled turtles could adapt to different habitats including natural lake, artificial ponds and paddy fields. The divergence on growth, appearance, physiological characteristics and gut microbial communities could be observed within a relative short term. The species of microbes were significantly diverse in paddy field than in pond and lake. The diversity and abundance of gut microbes were also higher for turtles from paddy field than from lake and pond. Significant divergence was found in summer, whereas relatively harmonious was detected in late autumn. The abundance of dominant phyla and genera were obviously different in various habitats in specific sampling time. Sampling time and habitat had significant effects on turtle metabolism, especially the

amino acid and carbohydrate metabolism. The rice-turtle co-culture was a potential ecological and economic farming mode which would play important roles in wild turtle protection, food security and paddy field environment improvement.

Methods

Experimental habitats and turtles rearing

The turtles (*Pelodiscus sinensis*, Janpanese strain) were intensive breeding in a standardized farm Xijiang Aquaculture co. LTD, located in Anqing, China. The turtles were stocked at cement tanks in hothouse with relative stable conditions (temperature was 30.0±1.0°C and water depth was about 0.5 m) before being allocated to different experimental habitats. The turtles were fed apparently satiation once a day with commercial feed containing 46% crude protein (Haihuang, Hangzhou, China). Thereafter, thousands of juvenile turtles in similar size of approximately 340 g were purchased and randomly divided into three groups that allocated to different experimental culture habitats as follows. Natural Lake (L): Bohu Lake was located in Anqing, Anhui Province, China (E116°22', N30°13'), which belonged to the Yangtze River basin. It covered 217 km^2 and the average water depth was about 3.5 m during July to October. It was abundant in fish, shellfish and other aquatic species. Two thousand marked turtles were released to the lake and no artificial feeds were provided. The artificial releasing would be conducive to the recovery of wild turtle population. Artificial Pond (P): The quadrate artificial ponds equipped with feeding and basking facilities was located in a standard aquafarm (116°54'E, 30°28'N, Xijiang Aquaculture co. LTD, Anhui, China). The experimental ponds were about 2000 m^2 and 1.5 m deep. One thousand turtles were allocated to the pond. The turtles were fed with commercial feed which contained 43% crude protein (Haihuang, Hangzhou, China) twice daily at 09:00 AM and 16:00 PM, respectively. Paddy Field (F): The experimental paddy fields (E116°21', N30°18') were about 2000 m^2 and surrounded by facilities to prevent escape. It was modified for turtle cultivation with a 200 m^2 pond (1.5m deep), which was about 10% of the total field area. Two hundred turtles were allocated to each paddy field. The turtles were fed with commercial feed twice a day same as ponds.The rearing experiment was conducted for 120 days from July to November. Air temperature was monitored at 11:00 AM every day during experiment, which varied in range of 22.5°C~35.8°C. Water temperature, pH and dissolved oxygen were monitored daily with a multi-parameter water quality analyzer (YSI ProPlus, Yellow Springs, Oh, USA). Besides, ammonium nitrogen, nitrite and nitrate nitrogen were measured weekly. Water were partially changed when it became worse for ponds and small ponds in paddy fields. The change interval was about 20 days in summer and 30 days in autumn.

Measurement and sampling

Turtles were randomly collected at initial 0d and 60d, then totally collected as much as possible at 120d. The turtles collected was randomly numbered from different habitats, the investigator who selected individuals for analysis was unaware of the grouping details, and an other investigator (also unaware of grouping details) conducted the anaesthetic and anatomy procedure. Every three male individuals with no trauma, bruises or scars from each habitat and cultural periods were collected for sampling. The turtles were anesthetized after 48h fasting by intramuscular injection with Tiletamine and Zolazepam (1:1) at dosage of 30 mg/kg, the turtles were in deep anesthesia and unconscious within 15-20 min after injection from left foreleg. The somatotype index including body weight, carapace length, carapace width and calipash lateral width were measured. Then turtles were quickly decapitated in unconscious state and dissected by a sharp bone shears, the livers, clumpy fat, guts were carefully removed on ice and weighted under sterile condition. Gut length, i.e. the length from end of esophagus to end of rectum was also measured without external tension. The gastric area (expressed as former gut "F") and rectum (expressed as later gut "L") were separated, rapidly frozen in liquid nitrogen, and then stored at -80°C until DNA extraction for microbial analysis. The grouping details were listed in **Table 2**. The specific growth rate (SGR), hepato-smatic index (HSI), fat-smatic index (FSI), gut-smatic index on weight (DSI$_W$) and length (DSI$_L$) were calculated. All operations on turtles were conducted in accordance with the institutional animal care guidelines and supervision of committees of Anhui Academy of Agricultural Sciences.

Bacterial DNA extraction and 16S rRNA gene amplicon sequencing

The bacterial DNA extraction was conducted by TIANamp Stool DNA Kit (DP328, TIANGEN, Beijing, China) according to the instructions. The variable region V4~V5 of 16S rRNA gene was amplified by bacterial primers 515F (5'-GTGCCAGCMGC CGCGGTAA-3') and 907R (5'-CCGTCAATTCMTTTRAGT TT-3') with overhang adapters attached. The PCR reactions (25 µL) contained approximately 2.5 µL DNA templates (5 ng/µL), 5.0 µL reverse/forward primer (1 µM), 12.5 µL 2×KAPA HiFi HotStart Ready Mix. PCRs were performed on Step One Plus Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with following program: 95°C

for 3 minutes, followed by 25 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C with a post-amplification extension of 10 min at 72°C. The products were confirmed by agarose gel electrophoresis (Peiqing, Shanghai, China). AMPure XP beads (Beckman Coulter, Indianapolis,IN,USA) and fresh 80% EtOH were used to purify the 16S V4 and V5 amplicon away from free primers and primer dimer species for index PCR. Dual indices and Illumina sequencing adapters were attached by using the Nextera XT Index Kit (FC-131-1002, Illumina, San Diego,CA,USA). Perform PCR on a thermal cycler using the following program: 95°C for 3 minutes, followed by 8 cycles of 30 sec at 95°C, 30 seconds at 55°C, 30 sec at 72°C with a post-amplification extension of 5 min at 72°C. AMPure XP beads were used as cleaning up the final library before quantification, normalization and pooling. The purified bacterial DNA samples were sent to Sangon Biotech co., Ltd (Shanghai, China) for Illumina Miseq Sequencing.

16S Metagenomics sequencing analysis

Methods were mainly referenced to those mentioned by Campos *et al*. (2018) and Abdelrhman *et al*. (2016)[29,77]. The obtained DNA reads were compiled in FastQC version 0.11.5 for further processing. QIIME version 1.9.1 was used for performing microbiome analysis from raw DNA sequencing data, including demultiplexing and quality filtering, OTU picking, taxonomic assignment, and phylogenetic reconstruction, diversity analyses and visualizations. The barcode and primer sequences were cut off after the samples are loaded, read pairs were merged using PANDAseq assembler version 2.10 for raw tags, the pairs sequences would be filtered if there was no overlap between them. And then the chimeras and host sequences were further filtered for clean tags. Singletons were removed before operational taxonomical units (OTUs) clustering (with an identity threshold of 97%). The valid data were clustered into OTU using UPARSE. The rarefaction curves for each sample were produced and diversity values were estimated. The distances among samples were calculated according the abundance and the samples were clustered on OTUs to evaluate the similarity. The cluster dendrogram and a phylogenetic tree were also built. Specific differences in community composition were determined using PCA based on Bray-Curtis distance matrix. OTUs were taxonomically classified using USEARCH (a unique sequence analysis tool) version 5.2.236 against GreenGenes databases and compiled into each taxonomic level. Meanwhile, the composition, abundance and diversity analysis on OTUs were conducted for the species richness and evenness, mutual or proper traits on OTUs for various samples or groups. Test of the significance of difference on OTUs composition were conducted LEfSe analysis to find the various species. The prediction of microbial community function was conducted by using PICRUSt to evaluate the abundance of function genes for samples.

Statistical analysis

All differences among biometric measurements were determined by analysis of variance using SPSS20.0. The measured data were subjected to one-way analysis of variance (ANOVA). Differences among treatments were tested by Tukey's multiple range test and results of $p < 0.05$ were deemed statistically significant. Duncan's multiple comparison was carried out to determine the difference among repeated groups. All statistics on gut microbiota were conducted by using R (version 3.2.2).

Abbreviations

16S rRNA: 16S ribosomal RNA; OTU:Operational taxonomic unit; PCA:Principal component analysis; ANOVA:Analysis of variance; LEfSe:Linear discriminant analysis coupled with effect size; LDA:Liner discriminant analysis; PICRUSt:Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Basel Declaration and was approved by the Experimental Animal Welfare and Ethical of Anhui Academy of Agricultural Sciences (NO.AAAS 2020-11).

Consent for publication

Not applicable.

Availability of data and materials

Authors' contributions

JXH and BLW designed the work, BLW drafted the manuscript, LH collected samples and performed the study, JC and YZ acquired important physiological and environmental data, JW revised the manuscript and provided effective suggestions on data analysis. All authors read and approved the final manuscript.

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References

1. Nagai N, Kobayashi H, Katayama S, et al. Preparation and characterization of collagen from soft-shelled turtle (*Pelodiscus Sinensis*) skin for biomaterial applications. J Biomater Sci Polym Ed. 2009;20:567–76.

2. Lu JF, Wan Q, Yin ZM, et al. Extraction and characterization of collagen from calipash of Chinese soft-shelled turtle (*Pelodiscus sinensis*). Journal of Fisheries of China. 2010;34:801–8. (in Chinese with English Abstract).

3. Rhodin AGJ, Iverson R, Bour U, Fritz A, Georges HB, Shaffer PPV, Dijk JB. 2017. Turtles of the world annotated checklist and atlas of taxonomy, synonymy, distribution, and conservation status. 8th ed. Baltimore:Johns Hopkins University Press.

4. Zhang J, Wang F, Jiang YL, et al. Modern greenhouse culture of juvenile soft-shelled turtle, *Pelodiscus sinensis*. Aquacul Int. 2017;25:1607–24.

5. Weng LP, Chen FD, Wang HH, et al. Investigation on the volatile flavor compounds of *Pelodiscus sinensis* grown in greenhouse by SPME-GC-MS. Food Industry. 2014;35:266–9. (in Chinese with English Abstract).

6. Chen X, Niu C, Pu L. Effects of stocking density on growth and non-specific immune responses in juvenile soft-shelled turtle, *Pelodiscus sinensis*. Aquacul Res. 2007;38:1380–6.

7. Zhou XQ, Niu CJ, Sun RY. The effects of vitamin E on non-specific immune response of the juvenile soft-shelled turtle *Pelodiscus sinensis*. Fisheries Sci. 2010;71:612–7.

8. Zhou XQ, Niu CJ, Sun RY. The effect of vitamin C on stress withstanding capability in the juvenile soft-shelled turtle (*Pelodiscus sinensis*). Aquacul Nutr. 2015;11:169–74.

9. Montalban-Arques A, Schryver PD, Bossier P, et al. Selective manipulation of the gut microbiota improves immune status in vertebrates. Front Immuno. 2015;6:512.

10. Standen B, Rodiles A, Peggs D, et al. Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*, following the application of a multi-species probiotic. Appl Microbiol Biot. 2015;99:8403–17.

11. Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ microbiol. 2006;8:1137–44.

12. Brandt KK, Amézquita A, Backhaus T, et al. Ecotoxicological assessment of antibiotics: A call for improved consideration of microorganisms. Environ Int. 2015;85:189–205.

13. Patil HJ, Ayana BP, Alon N, et al. Evidence of increased antibiotic resistance in phylogenetically-diverse aeromonas isolates from semi-intensive fish ponds treated with antibiotics. Front Microbiol. 2016;7:1875.

14. Adel M, Yeganeh S, Dawood MAO, et al. Effects of *Pediococcus pentosaceus* supplementation on growth performance, intestinal microflora and disease resistance of white shrimp, *Litopenaeus vannamei*. Aquacult Nutr. 2017;23:1401–9.

15. Chua MC, Benamor K, Lay C, et al. Effect of synbiotic on the gut microbiota of caesarean delivered infants: a randomized, double-blind, multicenter study. J Pediatr Gastr Nutr. 2017;65:102–6.

16. De Bruijn I, Liu Y, Wiegertjes GF, et al. Exploring fish microbial communities to mitigate emerging diseases in aquaculture. FEMS Microbiol Ecol. 2018;94:fix161.

17. Guerreiro I, Serra CR, Pousão-Ferreira P, et al. Prebiotics effect on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes of white sea bream (*Diplodus sargus*). Aquacul Nutr. 2018;24:153–63.

18. He JX, Chen J, Song GT, et al. Comparison of nutritional composition and quality between the paddy-cultured and pond-cultured *Pelodiscus sinensis* Japanese strain. Journal of Anhui Agricultural University. 2017;44:1005–9. (in Chinese with English Abstract).

19. Marques TM, Wall R, Ross RP, et al. Programming infant gut microbiota: influence of dietary and environmental factors. Curr Opin in Biotech. 2010;21:149–56.

20. Vrieze A, Holleman F, Zoetendal EG, et al. The environment within: how gut microbiota may influence metabolism and body composition. Diabetologia. 2010;53:606–13.

21. Snijders AM, Langley SA, Kim YM, et al. Influence of early life exposure, host genetics and diet on the mouse gut microbiome and metabolome. Nat Microbiol. 2016;2:16221.

22. Xiong JB, Nie L, Chen J. Current understanding on the roles of gut microbiota in fish disease and immunity. Zool Res. 2019;40:70–6.

23. Frei M, Becker K. Integrated rice-fish culture: Coupled production saves resources. Nat Resour Forum. 2005;29:135–43.

24. Tsuruta T, Yamaguchi M, Abe SI, et al. Effect of fish in rice-fish culture on the rice yield. Fisheries Sci. 2011;77:95–106.

25. Hu L, Zhang J, Tang JJ, et al. The productivity of traditional rice-fish co-culture can be increased without increasing nitrogen loss to the environment. Agr Ecosys Enviro. 2013;177:28–34.

26. Ghanbari M, Kneifel W, Domig KJ. A new view of the fish gut microbiome: advances from next-generation sequencing. Aquaculture. 2015;448:464–75.

27. Sullam KE, Essinger SD, Lozupone CA, et al. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol Ecol. 2012;21:3363–78.

28. De Bruijn I, Liu Y, Wiegertjes GF, et al. Exploring fish microbial communities to mitigate emerging diseases in aquaculture. FEMS Microbiol Ecol. 2017;94:fix161.

29. Campos P, Guivernau M, Prenafeta-Boldú FX, et al. Fast acquisition of a polysaccharide fermenting gut microbiome by juvenile green turtles Chelonia mydas after settlement in coastal habitats. Microbiome. 2018;6:69.

30. Stecher B, Maier L, Hardt W-D. 'Blooming' in the gut: how dysbiosis might contribute to pathogen evolution. Nat Rev Microbiol. 2013;11:277–84.

31. Yan Q, Li J, Yu Y, et al. Environmental filtering decreases with fish development for the assembly of gut microbiota. Environ Microbiol. 2016;18:4739–54.

32. Fazeli M, Hassanzadeh P, Alaei S. Cadmium chloride exhibits a profound toxic effect on bacterial microflora of the mice gastrointestinal tract. Hum Exp Toxicol. 2011;30:152–9.

33. Clarke SF, Murphy EF, O'Sullivan O, et al. Exercise and associated dietary extremes impact on gut microbial diversity. Gut. 2014;63:1913–20.

34. Lee SM, Wong WP, Hiong KC, et al. Nitrogen metabolism and excretion in the aquatic Chinese soft-shelled turtle, *Pelodiscus sinensis*, exposed to a progressive increase in ambient salinity. J Exp Zool Part A. 2010;305A:995–1009.

35. Zha Y, Eiler A, Johansson F, et al. Effects of predation stress and food ration on perch gut microbiota. Microbiome. 2018;6:28.

36. Kim SG, Han XY, Wei D, et al. Influence of Cu^{2+}-loaded silicate on the growth performance and microflora of crucian carp *Carassius auratus*. Dis Aquat Organ. 2009;85:239–43.

37. Baldo L, Joan LR, Toomingklunderud A, et al. Gut microbiota dynamics during dietary shift in eastern African cichlid fishes. PLoS One. 2015;10:e0127462.

38. Cremona F, Järvalt A, Bhele U, et al. Relationships between fisheries, foodweb structure, and detrital pathway in a large shallow lake. Hydrobiologia. 2018;820:145–63.

39. Koomen R, Herrmann E. The effects of social context and food abundance on chimpanzee feeding competition. AM J of Primatol. 2018;80:e22734.

40. Wang CF, Wang JP, Cao CG. Effect of rice-duck farming ecosystem on zoobenthos biodiversity in paddy field. Chinese Journal of Eco-Agriculture. 2008;16:933–7. (in Chinese with English Abstract).

41. Bartrons M, Arranz I, Cañedo-Argüelles M, et al. Fish shift the feeding behaviour and trophic niche diversification of their prey in subarctic Lake Mývatn. Iceland *Hydrobiologia*. 2018;816:896–906.

42. Lei SJ. Effects of ration level and feeding frequency on digestibility in juvenile soft-shelled turtle, *Pelodiscus sinensis*. J Zhejiang Univ B. 2006;7:580–5. (in Chinese with English Abstract).

43. Wu BL, Luo S, Wang JW. Effects of temperature and feeding frequency on ingestion and growth for rare minnow. Physiol Behav. 2015;140:197–202.

44. Serra CR, Júnior FM, Couto A, et al. Gut microbiota and gut morphology of gilthead sea bream (*Sparus aurata*) juveniles are not affected by chromic oxide as digestibility marker. Aquacul Res. 2018;49:1347–56.

45. Lin WY, Huang CH. Fatty acid composition and lipid peroxidation of soft-shelled turtle, *Pelodiscus sinensis*, fed different dietary lipid source. Comp Biochem Phys C. 2007;144:327–33.

46. Li XH, Zhou L, Yu YH, et al. Composition of gut microbiota in the gibel carp (*Carassius auratus gibelio*) varies with host development. Microbl Ecol. 2017;74:1–11.

47. Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334:105–8.

48. Merrifield DL, Shaw BJ, Harper GM, et al. Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (*Danio rerio*). Environ Pollut. 2013;174:157–63.

49. Zhou Z, Ringø E, Olsen RE, et al. Dietary effects of soybean products on gut microbiota and immunity of aquatic animals: A review. Aquacul Nutr. 2018;24:644–65.

50. Goodrich JK, Davenport ER, Beaumont M, et al. Genetic determinants of the gut microbiome in UK twins. Cell Host Microbe. 2016;19:731–43.

51. Rawls JF, Mahowald MA, Ley RE, et al. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell. 2006;127:423–33.

52. Nelson TM, Rogers TL, Carlini AR, et al. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environ Microbiol. 2013;15:1132–45.

53. Smith CC, Snowberg LK, Gregory CJ, et al. Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. ISME J. 2015;9:2515–26.

54. Stagaman K, Burns AR, Guillemin K, et al. The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. ISME J. 2017;11:1360–9.

55. Masuoka H, Shimada K, Kiyosueyasuda T, et al. Transition of the intestinal microbiota of dogs with age. Bioscience of Microbiota Food Health. 2017;36:27–31.

56. Schmidt V, Amaral-Zettler L, Davidson J, et al. Influence of fishmeal-free diets on microbial communities in Atlantic salmon (*Salmo salar*) recirculation aquaculture systems. Appl Environ Microb. 2016;82:4470–81.

57. Hatje E, Neuman C, Stevenson H, et al. Population dynamics of Vibrio and Pseudomonas species isolated from farmed tasmanian Atlantic salmon (Salmo salar L.): a seasonal study. Microb Ecol. 2014;68:679–87.

58. Zarkasi KZ, Taylor RS, Abell GC, et al. Atlantic salmon (Salmo salar L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet. Microb Ecol. 2016;71:589–603.

59. Maurice CF, Cl Knowles S, Ladau J, et al. Marked seasonal variation in the wild mouse gut microbiota. ISME J. 2015;9:2423–34.

60. Patrick S, Willemsen D, Popkes M, et al. Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. eLife. 2017;6:e27014.

61. Zhang C. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J. 2010;4:232–41.

62. Kuz'mina VV, Zolotareva GV, Sheptitskii VA. Effect of habitat conditions on proteinase and glycosidase activities in the gut microbiota of crucian carp in a wide range of pH. Russ J Ecol. 2014;45:303–9.

63. Wang S, Chen L, He M, et al. Different rearing conditions alter gut microbiota composition and host physiology in Shaoxing ducks. Sci Rep-UK. 2018;8:7387.

64. Turnbaugh PJ, Ley RE, Mahowald MA, et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444: 1027–31.

65. Zhao LP. The gut microbiota and obesity: from correlation to causality. Nat Rev Microbio. 2013;11:639–47.

66. Tran NT, Zhang J, Xiong F, et al. Altered gut microbiota associated with intestinal disease in grass carp (*Ctenopharyngodon idellus*). World J Microbiol Biot. 2018;34:71.

67. Li XM, Yan QY, Xie SQ, et al. Gut microbiota contributes to the growth of fast-growing transgenic common carp (*Cyprinus carpio L.*). PLoS One. 2013;8:e64577.

68. Ramírez C, Romero J. Fine flounder (*Paralichthys adspersus*) microbiome showed important differences between wild and reared specimens. Front Microbiol. 2017;8:271.

69. Wang AR, Ran C, Ringø E, et al. Progress in fish gastrointestinal microbiota research. Rev Aquac. 2017;10:626–40.

70. Nowakiewicz A, Ziółkowska G, Zięba P, et al. Aerobic bacterial microbiota isolated from thecloaca of the European pond turtle (*Emys orbicularis*) in Poland. J Wildlife Dis. 2015;51:255–9.

71. Ahasan MS, Waltzek TB, Huerlimann R, et al. Comparative analysis of gut bacterial communities of green turtles (*Chelonia mydas*) pre-hospitalization and post-rehabilitation by high-throughput sequencing of bacterial 16S rRNA gene. Microbiol Res. 2018;207:91–9.

72. Li T, Li H, Gatesoupe FJ, et al. Bacterial signatures of "Red Operculum" disease in the gut of crucian carp (*Carassius auratus*). Microb Ecol. 2017;74:510–21.

73. Zhu NY, Bei YJ, Zheng TL, et al. Comparison of bacteria floras in intestines of healthy and diseased Chinese soft-shelled turtles (*Pelodiscus sinensis*) and in aquatic water. Acta Agriculturae Zhejiangensis. 2014;26:1176–9. (in Chinese with English Abstract).

74. Barelli C, Albanese D, Donati C, et al. Habitat fragmentation is associated to gut microbiota diversity of an endangered primate: implications for conservation. Sci Rep-UK. 2015;5:14862.

75. Lee SM, Wong WP, Loong AM, et al. Postprandial increases in nitrogenous excretion and urea synthesis in the Chinese soft-shelled turtle, *Pelodiscus sinensis*. J Comp Physiol B. 2007;177:19–29.

76. Wang J, Qi Z, Yang Z. Evaluation of the protein requirement of juvenile Chinese soft-shelled turtle (*Pelodiscus sinensis*, Wiegmann) fed with practical diets. Aquaculture. 2014;433:252–5.

77. Abdelrhman KFA, Giovanni B, Cecilia M, et al. A first insight into the gut microbiota of the sea turtle *Caretta caretta*. *Front Microbiol* 2016;7.

Tables

Table1 The anatomical indices of turtles from different habitats and cultured days

Indices	0d			60d			120d	
	Field	Pond	Lake	Field	Pond	Lake	Field	Pond
BW	335.8±22.2	341.3±32.6	344.9±26.8	529.7±35.5^b	512.2±39.3^b	438.8±27.4^a	796.6±58.2^b	717.2±64.6^a
CL	12.89±0.09	12.91±0.06	12.92±0.07	15.96±0.26^b	15.87±0.30^b	15.30±0.28 ^{a}	17.71±0.35^b	17.13±0.38^a
CW	11.72±0.07	11.80±0.03	11.81±0.03	12.26±0.09	12.15±0.10	11.92±0.08	13.84±0.12	13.46±0.22
CLW	1.93±0.08	1.92±0.03	1.93±0.04	2.62±0.08	2.82±0.11	2.66±0.18	3.59±0.15	3.71±0.20
SGR	-	-	-	0.76	0.68	0.40	0.72	0.62
HSI	2.9±0.2	2.9±0.2	2.9±0.2	3.0±0.3^b	3.1±0.2^b	2.8±0.3^a	2.6±0.2^a	2.7±0.2^b
FSI	3.8±0.2	3.8±0.2	3.8±0.2	3.6±0.2^b	4.2±0.2^c	2.9±0.2^a	3.6±0.1^a	3.9±0.2^b
GSI$_W$	2.6±0.1	2.6±0.1	2.6±0.1	2.1±0.1^a	2.3±0.1^b	2.1±0.1^a	2.1±0.0^a	2.4±0.1^b
GSI$_L$	4.0±0.2	4.0±0.2	4.0±0.2	4.0±0.1	3.9±0.1	4.0±0.1	3.7±0.2^b	3.5±0.2^a

BW (g): body weight

CL (cm): carapace length

CW (cm): carapace width

CLW (cm): calipash lateral width

SGR (Specific Growth Rate,%/d):100×(Ln(BWT)-Ln(BW0))/T

HSI (Hepatosmatic Index,%)=100×liver weight / BW

FSI (Clumpy Fat Index,%)=100×clumpy fat weight/ BW

GSI$_W$ (Gut-smatic Index on Weight,%)=100×gut weight/BW

GSI$_L$ (Gut-smatic Index on Length) = gut length/ Carapace length

Table 2 Grouping details for samples from different habitats, cultured days and intestinal segment

Groups	Body weight (g)	Living habitats	Cultured days	Sampled gut segment
IF	340.5±6.7	Greenhouse	0d	Gastric area
IL		Greenhouse	0d	Rectum
F1F	530.3±5.6	Paddy Field	60d	Gastric area
F1L		Paddy Field	60d	Rectum
F2F	806.6±10.2	Paddy Field	120d	Gastric area
F2L		Paddy Field	120d	Rectum
P1F	515.0±7.3	Artificial Pond	60d	Gastric area
P1L		Artificial Pond	60d	Rectum
P2F	720.4±3.3	Artificial Pond	120d	Gastric area
P2L		Artificial Pond	120d	Rectum
L1F	350.3±5.1	Natural Lake	60d	Gastric area
L1L		Natural Lake	60d	Rectum

Figures
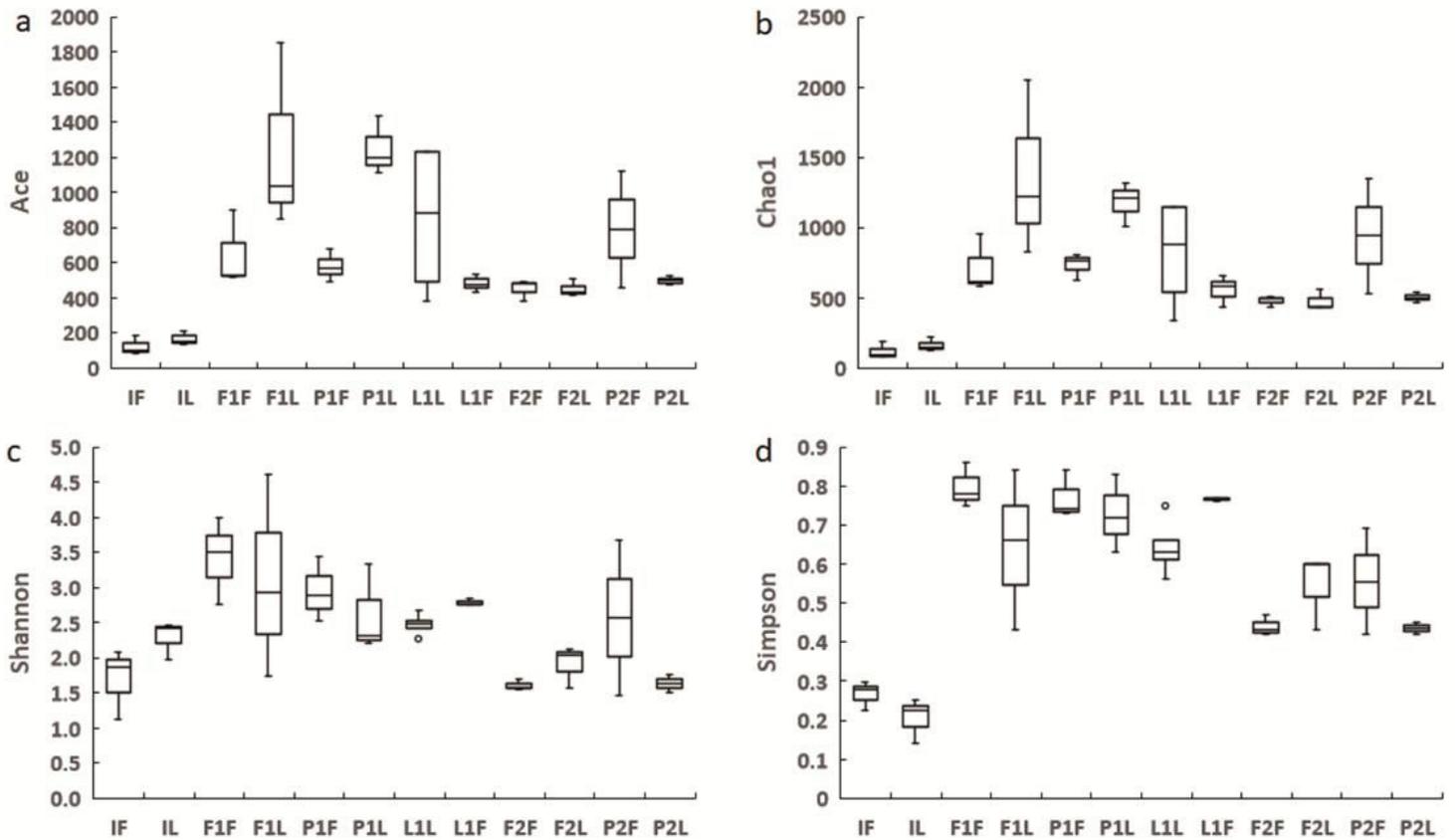
Figure 1

Figure1
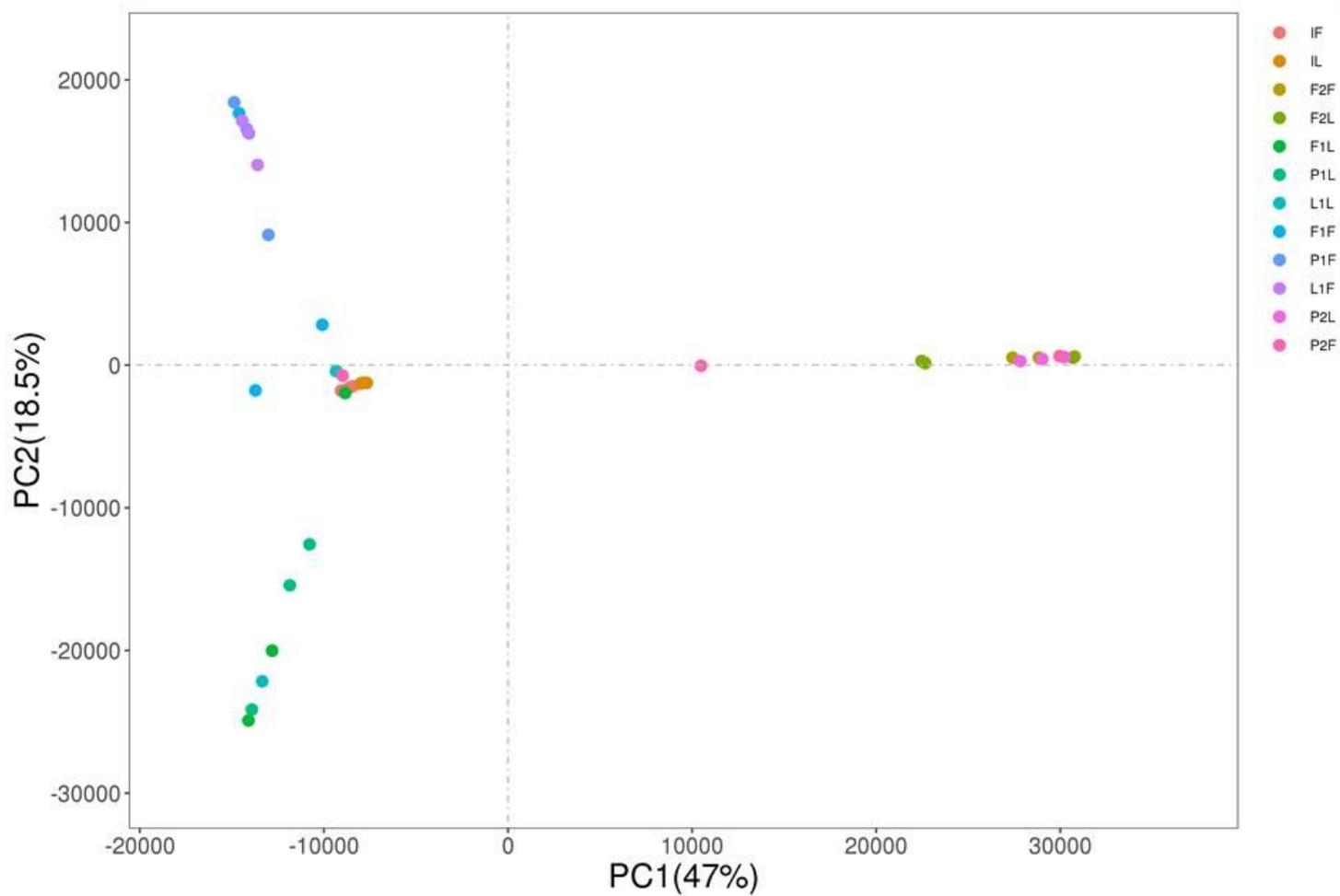
Figure 2

Figure2
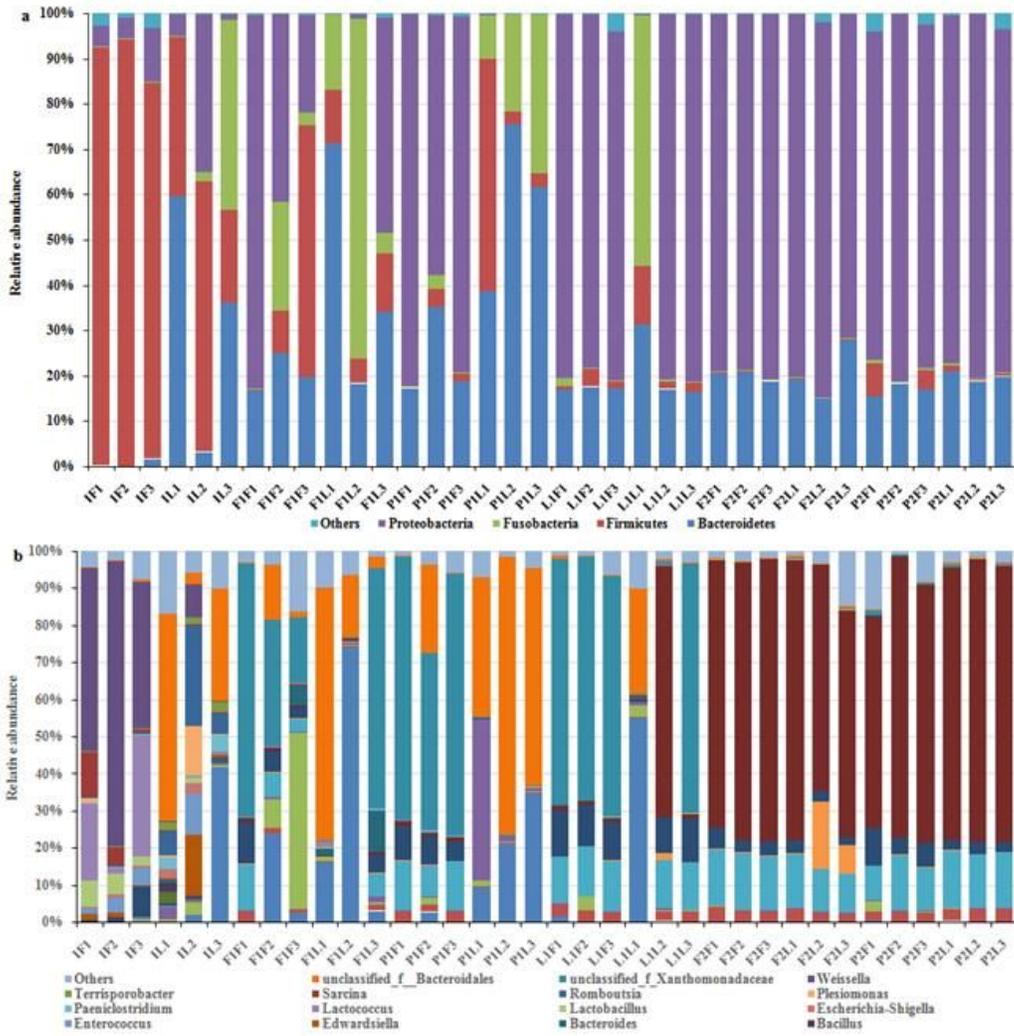
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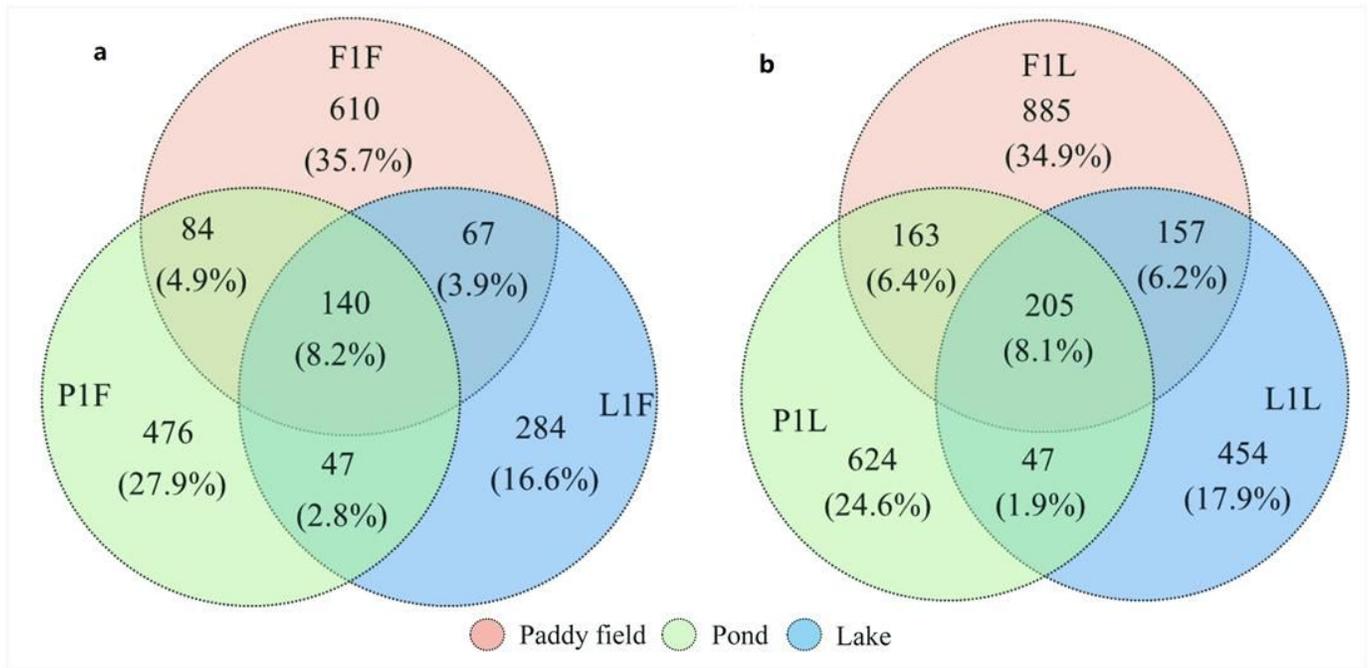
Figure 4

Figure4

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