Anti-predation Defense Traits of Daphnia are Associated With the Gut Microbiota Composition Shaped by Fish Kairomone

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Research

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Anti-predation defense traits of *Daphnia* are associated with the gut microbiota composition shaped by fish kairomone

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Abstract

Background: Gut microbiota plays an important role in host physiology and fitness. The gut microbiota can promote host health by influencing life history traits, especially in arthropods. However, it is not clear whether the performance of host defense traits in response to predator pressure in natural food webs is related to their gut microbiota composition. In this study, we used *Daphnia magna* as a model organism to investigate the relationship of *D. magna* life history traits and gut microbiota alterations under predator kairomone based on *16S* rRNA amplicon sequencing.

Result: We showed that the microbiota composition of *D. magna* was significantly affected by their predator risk and development stage. The relative abundance of *Comamonadaceae* (mainly *Limnohabitans* sp.) significantly decreased in the presence of predator kairomone. Furthermore, the presence of predator kairomone significantly reduced the α diversity of gut microbiota in *D. magna* with the increase of instar. Among them, the OTUs belonged to *Epsilonbacteraeota* and *Firmicutes* in the presence of predator kairomone were significantly higher than those in the control group. The results of functional predictions showed that predation pressure promote the metabolic function of gut microbiota, such as metabolism of energy, cofactors, and vitamins. By analyzing the correlation between the induced defense traits of *D. magna* and the relative abundance of bacteria, we found that the increased abundance of *Comamonadaceae*, *Moraxellaceae*, and *Flavobacteriaceae* were linearly correlated with the partial defense traits of *D. magna*. Specifically speaking, body size was

positively correlated with an increased abundance of *Comamonadaceae*, whereas spine length was negatively correlated with an increased abundance of *Comamonadaceae* but was positively correlated with increased *Flavobacteriaceae* abundance.

Conclusions: Our results suggested that predation risk can affect the composition of the gut microbiota in *D. magna*, which may indirectly induce the production of defensive traits in *D. magna*. The results of this study revealed an important role of gut microbiota in the development of defensive traits of *Daphnia* in response to fish predators. The correlation between microbial abundance and defense traits is of great significance for further understanding the effect of host-microbiota interaction on individual anti-predation defense.

Keywords: *Daphnia magna*, Gut microbiota, Host-microbiota interaction, Predation risk, Inducible defense, Life-history traits

Background

The digestive tract of animals is host to a diverse community of symbiotic microorganisms, collectively called the gut microbiota. Evidence has accumulated that the gut microbiota is not just a random set of microorganisms, but rather a complex community that plays a critical role in host physiology and behavior [1-3]. Some studies have shown that gut microbiota may also contribute to host health by influencing life history traits of the host, in particular in arthropods, which reveal that gut bacteria have an over-whelming influence on growth, development, reproduction and survival. In the water flea *Daphnia magna* [4, 5] and in the fruit fly *Drosophila melanogaster* [6], germ-free individuals develop more slowly and are smaller than conventional animals, while in mosquitoes, axenic larvae fail to develop beyond the first instar [7]. In all these species, inoculating axenic larvae with gut bacteria can restore a normal developmental rate [5-7]. As a result, the gut microbiota is increasingly seen as a key driver of health.

Microbiome is highly plastic, and can respond rapidly to changes in host diet or environmental conditions, through changes in community composition, mutations, exchange of genetic material with bacteria from the environment, or changes in gene expression [8-10]. In natural animal populations, there is a relationship between gut microbial composition and their host nutritional status in the food web [11]. One important aspect of nutritional status affecting gut microbiota is related to shifts in diet quality and quantity [5, 12]. Akbar *et al.* [13] discovered that gut microbiota composition of *D. magna* is significantly different under different food conditions,

and the abundance of *Pseudomonadaceae* in gut microbiota of *D. magna* decreases significantly under lower food quality. Callens *et al.* [5] found that when food is sufficient or abundant, the microbiota has a strong positive effect on the growth and reproduction of cladocerans, while in the case of limited food, the microbiota has a weak effect on the growth and reproduction of *Daphnia*. In nature, however, in addition to changes in food quality and quantity, predation is also an important factor in the food chain. Increasing or decreasing predator density will change prey densities and indirectly affect the food resources availability for the prey [14-16]. On the other hand, chemical cues from predators have been shown to reduce activities in prey and subsequently induce prey morphological changes that could decrease the risk of being predated [17-19]. Moreover, predation stress also influences the physiological status of prey, for example, hormones released from stress could mediate immunological and behavioral responses in vertebrates [20]. It has been shown that nerve and immune system can play important roles in regulating gut microbiota communities [21, 22]. Gut microbiota provide their host with metabolic capabilities not directly encoded in the host genome, such as digestion of plant polysaccharides [23] or detoxification of food borne toxins [24, 25], and contribute to the normal development of the host, e.g. by fostering the maturation of the immune system [26]. Thus, changes the composition of host gut microbiota may not only depend on shifts in nutritional status, but also on the risk of predation.

In addition, gut microbiota is also affected by developmental stages [27, 28]. For example, Moll *et al.* [29] showed that newly emerged mosquito adults contain few or

93 no bacteria in their guts. Shifts in the microbial community in *Bombyx mori* are
94 apparent between early- and late-instar larvae, in concert with host developmental
95 changes [30]. The diversity and composition of amphibian gut microbial communities
96 differ between tadpoles and adults [31]. Further, studies have shown that the gut
97 microbiota communities are initially assembled by neutral or stochastic processes, and
98 become more fixed and stable later in host development, indicating an increased
99 importance of selective forces shaping the gut microbiome as animal development
100 proceeds [32-34]. Microbes will be selectively recruited through interactions with the
101 host and the already established microbiota [35], and then gradually create a stable
102 microbiome that is adapted to the environment.

103 Predators play an important ecological role in ecosystem, and the relationship
104 between predators and prey has been extensively studied [36, 37]. However, under
105 predation risk, does the gut microbiota of prey change with developmental stage
106 during the defense process? No study has reported on this issues. The freshwater
107 crustacean *D. magna* provide opportunity for such studies. *D. magna* has high
108 experimental traceability, short life cycle, strong clone reproduction, and rapid
109 response ability to environmental stress. Previous studies have shown that *D. magna*
110 can respond to chemicals released by their predators and exhibit different types of
111 defense, e.g., alterations in morphologies [38, 39], adjustment in life-history strategy
112 [40]. In this study, we used *D. magna* as the model organism and investigated the
113 effects of the presence of fish predation risk on the gut microbiota composition of *D.*
114 *magna* at different instars. We hypothesized that: (1) Kairomone released by fish

predators alters the relative abundance of the *D. magna* gut microbiota; (2) The development stage of *D. magna* can interact with fish kairomone to affect the diversity of the gut microbiota community; (3) Changes in *D. magna* defense traits are related to the composition of *D. magna* gut microbiota community under the influence of fish kairomone. To test these hypotheses, we placed *D. magna* in the medium with and without fish kairomone, and recorded key life history traits as well as the composition of the gut microbiota community of *D. magna* at different instars.

Methods

Experimental organisms and cultivation

Laboratory-cultured clone of *D. magna* that have been maintaining in laboratory under standard conditions for more than ten years were used in our experiments. Stock *Daphnia* clonal lineage was cultured in COMBO medium (refreshed four times a week) at a temperature of 25 ℃ under fluorescent light at 50 μmol photons m^{-2} s^{-1} with a 14 : 10 h light : dark cycle. They were fed daily with high-quality food green alga *Scenedesmus obliquus* (1.5 mg C L^{-1}), which was harvested from that cultured in 1 L of BG-11 medium in Erlenmeyer flasks under the same conditions.

Kairomone production

For harvesting fish kairomones, 14 small-sized fish (*Rhodeus ocellatus*, 3-5 cm in body length) were cultured in COMBO medium and fed with about 5000 *D. magna*. After 6 h, the fishes were transferred into 7 L medium to excrete kairomone for 18 h without any other food supply [41]. In order to prevent bacterial degradation,

the kairomone medium was filtered through a 0.22 μm glass fiber filter (Millipore, USA), and then stored at -20 °C until further usage.

Experimental design

Before the experiment, reproduction of the animals was synchronized by maintaining individuals of the same age for at least two generations under identical conditions. We used the third brood neonates born within 24 h for experiments to ensure that the test animals would be treated for the majority of their juvenile period, exclude the maternal effects [42], and minimize differences between experimental animals [43].

We set up the experiments with absence (control) and presence (treatment) of fish kairomone. The treatment with fish kairomone were produced by diluting the stock fish kairomone 20 times (i.e., on average one fish per 10 L) in the COMBO medium. Each treatment or control contained 250 replicates, and each replicate containing one individual was cultured in 50 mL of the COMBO medium with the green alga *S. obliquus* as food (1.5 mg C L^{-1}). The experiment was run for 21 days, and the instar of *D.magna* was recorded for 21 days. The neonates released by different ages of *D.magna* were removed after counted, and only the mothers were retained for later microbial analyze.

Some of the first instar *D. magna* (the newborn ones) and the fourth instar *D. magna* were respectively divided into three samples (each with 15 individuals) for the analysis of microbiota in the whole *Daphnia*, while some of the sixth, ninth and twelfth instar *D. magna* were respectively divided into three samples each containing

5 individuals for this analysis. After removed the *Daphnia* individuals, the remaining culture medium was filtered by 0.22 μm glass fiber filter (Millipore, USA) for the analysis of microbiota in the culture medium [44]. The microbiota was also analyzed in triplicate. Furthermore, at the sixth, ninth and twelfth instar, another 20-25 individuals of *D. magna* were collected and dissected guts for gut microbial analysis. To isolate the gut, the *D. magna* were put on sterile glass slide using flame sterilized tweezer, and guts were isolated under stereomicroscope using sterilized tweezer and syringe. Twenty to twenty-five guts were isolated for each sample and were immediately transferred to sterile Eppendorf tubes containing 100 μL sterile purified water and kept at -20 ℃ till further analysis. It is worth noting that the gut microbiota was analyzed in duplicate, as it was quite difficult to dissect the gut of *D. magna*, and thus there was not enough guts for microbiota analysis if we analyzed them in triplicate. In addition, ten *D. magna* were used for recording the life history traits (i.e., body size, spine length, time to maturation, number of broods, and total offspring number per female) every instar in absence and presence of fish kairomone, and the released neonates were removed after counted.

DNA extraction and PCR amplification

Microbial DNA was extracted using the HiPure Soil DNA Kits (Magen, Guangzhou, China) according to manufacturer's protocols. The full length 16S rDNA were amplified by PCR (95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72°C for 90s, with a final extension 72°C for 10 min) using primers 27F: AGAGTTTGATCCTGGCTCAG, 1492R: GNTACCTTGTTACGACTT.

The PCR reaction was carried out in a 50 μL reaction volume with TransGen High-Fidelity PCR SuperMix (TransGen Biotech, Beijing, China), 0.2 μM forward and reverse primers, and 5 ng template DNA.

Amplicons were evaluted with 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Sequencing libraries were generated using SMRTbell TM Template Prep Kit (PacBio, Menlo Park, CA, USA) following manufacturer's recommendation. The library quality was assessed with Qubit 3.0 Fluorometer (ThermoFischer Scientific,USA) and FEMTO Pulse system (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced on the PacBio Sequel platform. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP******).

Statistical analysis

The parameters of the response-functions were used to assess the impact of different treatments (presence or absence of fish kairomones) on *D. magna* body size and spine length / body size change with days. Specifically, we used two predictive functions to fit the data. An exponential rise to an asymptote function, $y = J_{max}\ (1\text{-}e^{-pt})$, was used to fit the relationships between *D. magna* body size and growth days at presence or absence of fish kairomones, where y is body size of *D. magna* at time t, J_{max} is the predicted asymptotes of the exponential rise functions (maximum value) when t approaches infinity, and p describes the initial rate of change in the response [45]. An exponential decline to an asymptote function, $y = L_{min} + \alpha e^{-rt}$, was used to fit

the relationships between spine length / body size of *D. magna* and growth days at presence or absence of fish kairomones, where *y* is spine length / body size of *D. magna* at time *t*, L_{min} is the predicted asymptotes of the exponential decline functions (minimum value) when *t* approaches infinity, and *r* describes the initial rate of change in the response [46]. The spine length of *D. magna* showed a "peak curve" trend, i.e., increasing first and then declining, thus a three-parameter Gaussian model: $y = K \times e^{[-0.5\left(\frac{t-t_k}{q}\right)^2]}$ was used to fit the spine length of *D. magna*, where *y* represents the spine length of *D. magna* at time *t*, *K* represents the theoretical maximum spine length, t_k represents the time needed to reach the theoretical maximum spine length, and *q* reflects the shape of the curve [47, 48].

The interactive effect of growth days and presence or absence of fish kairomones on the body size, spine length, and spine length / body size of *D. magna* were assessed using two-way ANOVA ($\alpha = 0.05$). The effect of presence or absence of fish kairomones on time to maturation, body size at maturation, total offspring number, brood number, and average offspring per brood of *D. magna* were assessed using one-way ANOVA ($\alpha = 0.05$). Post hoc analysis between various treatments and multiple comparisons were determined by Tukey's HSD ($\alpha = 0.05$). All data were expressed as the mean values ± SE, and all statistical analyses were performed using Sigmaplot 14.0.

Differences in the abundances of dominant bacterial genera between different growth days with presence or absence of fish kairomones were assessed by analysis of variance (ANOVA) with post-hoc Tukey's test ($\alpha = 0.05$). To observe the overall

diversity of microbiota, nonmetric multidimensional scaling (NMDS) with Bray-Curtis (Vegan 2.3-5) and weighted and unweighted UniFrac distances were calculated using Phyloseq. Spearman's correlation analysis between two variables (bacterial abundance in treatment and life history traits) was performed by bivariate analysis in Sigmaplot 14.0, where a slope that was significantly different from zero ($\alpha = 0.05$) indicated that the bacterial abundance had a significant effect on a given life-history trait. Other bioinformatic analysis was performed using Omicsmart, a real-time interactive online platform for data analysis (http://www.omicsmart.com).

Results

Body size, spine length, and reproduction traits

The time to maturation, body size at maturation, total offspring number, and brood number of *D. magna* were significantly affected by fish kairomone (Table 1). The body size, spine length, and spine length / body size of *D. magna* were also significantly affected by fish kairomone, and there was significant interaction between kairomone and growth days on these morphological parameters (Table 2). Compared with those not exposed to fish kairomone, the maturation time of *D. magna* exposed to kairomone was significantly earlier, and the body size at maturity was significantly reduced (Fig. 1, Table 1). Total offspring number and brood number of *D. magna* exposed to fish kairomone were significantly increased, however, there was no significant difference in the average offspring per brood in *D. magna* with or without fish kairomone (Fig. 1, Table 1). *D. magna* exposed to fish kairomone significantly

247 decreased body size and increased spine length and spine length / body size compared
248 to those not exposed to fish kairomone (Fig. 2, Table 2). Among them, both the body
249 size and the spine length of *D. magna* were affected by fish kairomone after three
250 days (3rd instar, Table S1, Fig. 2). With the extension of growth days, the body size of
251 *D. magna* exposed to fish kairomone was significantly smaller than those not exposed
252 to fish kairomone after five days (5th instar, Table S1, Fig. 2a). The body sizes of *D.*
253 *magna* exposed or not exposed fish kairomone were close to the maximum after 12
254 days (9th instar, Table S1, Fig. 2a). Furthermore, With the extension of growth days,
255 the spine length of *D. magna* exposed to fish kairomone was significantly increased
256 than those not exposed to kairomone (Fig. 2d). After 11 days (9th instar, Table S1),
257 the spine length of *D. magna* reached the maximum value regardless of exposure to
258 kairomone, and then began to decrease (Fig. 2d). The spine length / body size of *D.*
259 *magna* exposed to fish kairomone was significantly higher than that not exposed to
260 kairomone (Fig. 2g). With the extension of growth days, the spine length / body size
261 of *D. magna* in exposed or not exposed kairomone treatment was close to the
262 minimum after 12 days (9th instar, Table S1, Fig. 2g).

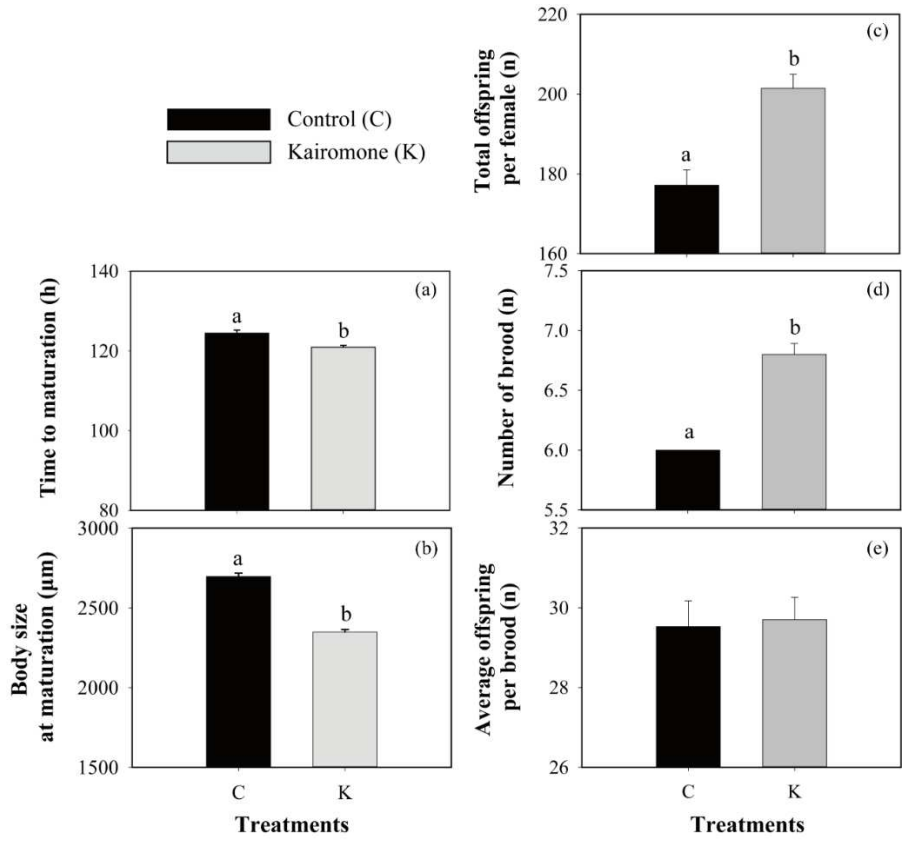


Fig. 1 Reproductive traits of *D. magna* in absence and presence of fish kairomone. The significant differences among different treatments are indicated by the different lowercase letters.

Table 1 The results of one-way ANOVA on reproductive traits of *D. magna*.

Life-history traits	DF	SS	MS	*F*	*P*
Time to maturation (Fig. 1a)	1	129.600	129.600	16.482	<0.001
Body size at maturation (Fig. 1b)	1	1167800.59	1167800.59	160.895	<0.001
Total offspring per female (Fig. 1c)	1	5773.448	5773.448	21.879	<0.001
Number of brood (Fig. 1d)	1	6.236	6.236	72.103	<0.001
Average offspring per brood (Fig. 1e)	1	0.306	0.306	0.0431	0.837

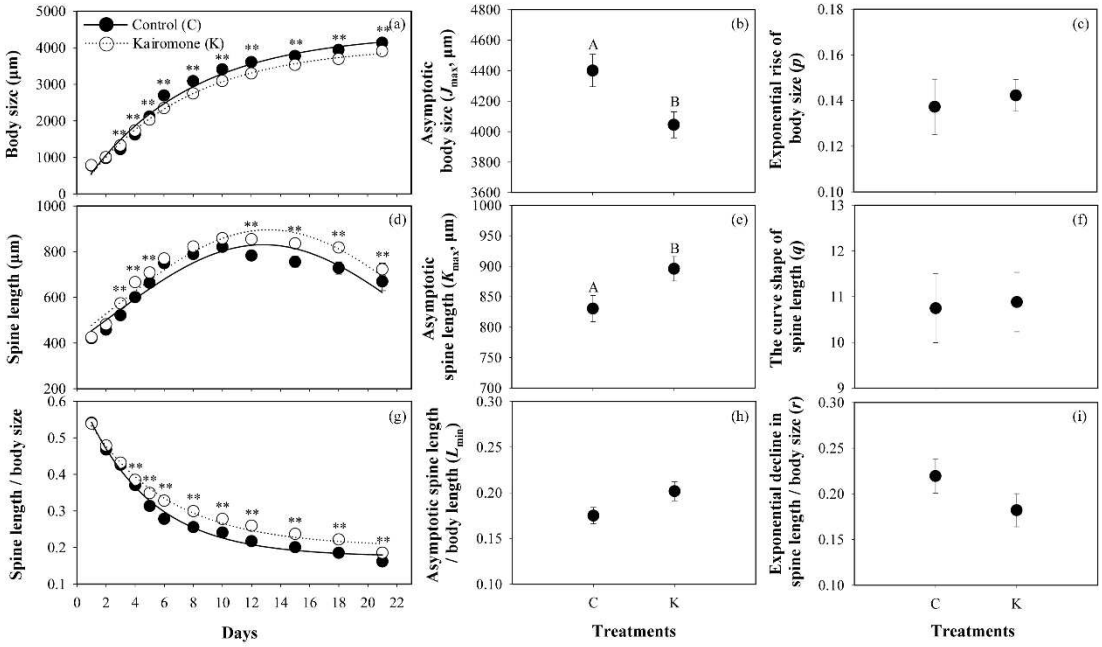


Fig. 2 Morphological traits of *D. magna* in absence and presence of fish kairomone change with days. The asterisks indicate significant differences between the control and fish kairomone treatments under different days (*$p < 0.05$, **$p < 0.01$). Some error bars are not visible in the figure as they are too short and are covered by the data symbol. The derived parameters (asymptote, the exponential rise rate, and the curve shape) of body size, spine length, and spine length / body size in the control (C) and fish kairomone (K) treatments were compared. The significant differences are indicated by using different uppercase letters.

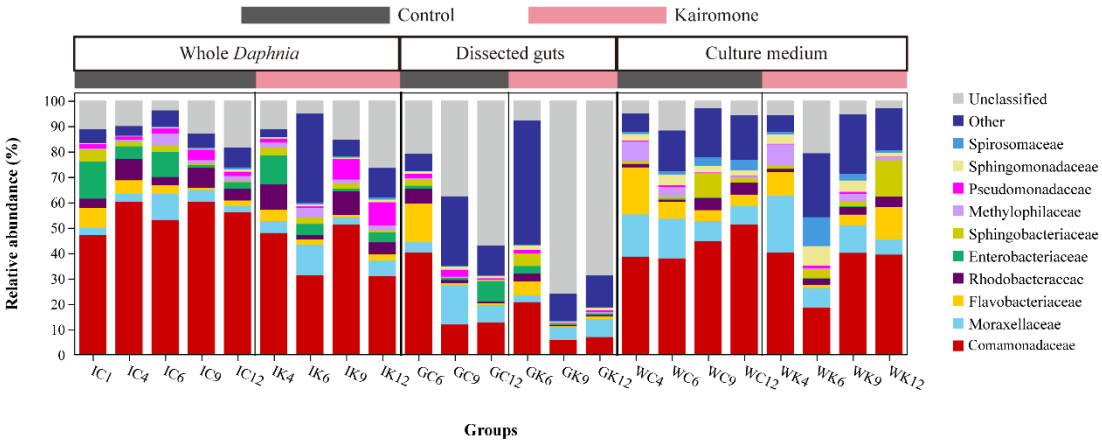
Table 2 The results of two-way ANOVA on morphological traits of *D. magna*.

Life-history traits	DF	SS	MS	*F*	*P*
Body size (Fig. 2a)					
Kairomone	1	2833534.400	2833534.400	690.170	<0.001
Days	11	568279909.279	51661809.934	12583.377	<0.001
Kairomone × days	11	3384809.082	307709.917	74.950	<0.001
Spine length (Fig. 2d)					
Kairomone	1	263622.719	263622.719	57.967	<0.001

Days	11	8418843.496	765349.409	168.290	<0.001
Kairomone × days	11	69952.937	6359.358	1.398	0.170
Spine length / body size (Fig. 2g)					
Kairomone	1	0.0883	0.0883	179.807	<0.001
Days	11	5.548	0.504	1027.106	<0.001
Kairomone × days	11	0.0303	0.00275	5.603	<0.001

Composition of the microbial communities in Daphnia individuals

The family *Comamonadaceae* (mainly *Limnohabitans* sp.) showed the highest relative abundance followed by *Moraxellaceae* (mainly *Acinetobacter* sp.) and *Flavobacteriaceae* (mainly *Flavobacterium* sp.) among all bacterial family (Fig. 3 and S1, Table S2). In the whole *Daphnia*, there was a significant interaction between kairomone and instar on the relative abundance of *Moraxellaceae* and *Flavobacteriaceae* (Table 3), and the relative abundance of *Comamonadaceae* were significantly decreased by kairomone (Fig. 3, Table 3). In the guts of *Daphnia*, there was no significant interaction between kairomone and *Daphnia* instar on the three bacterial family (Table 3). However, as the *Daphnia* instar increases, the relative abundance of *Comamonadaceae* and *Flavobacteriaceae* in the guts were significantly decreased (Fig. 3, Table 3). In the culture medium, there was a significant interaction between kairomone and instar on the relative abundance of the three bacterial family (Table 3); furthermore, the relative abundance of *Comamonadaceae* was significantly reduced under kairomone influence (Fig. 3, Table 3).



298

299 **Fig. 3** Relative abundance of bacterial at the family level. The left, middle, and right

300 sections represent whole *Daphnia* (left), dissected guts (middle), and culture medium

301 (right) of *D. magna* with different instars in absence and presence of fish kairomone,

302 respectively. The numbers "1", "4", "6", "9" and "12" represent different instars.

303

304 **Table 3** The results of two-way ANOVA on the relative abundance of bacterial.

Site of microflora detection	Bacterial specie (family level)	*F*	*P*
Whole *Daphnia*	*Comamonadaceae*		
	Kairomone	9.276	0.006
	instar	0.227	0.920
	Kairomone × instar	1.667	0.197
	Moraxellaceae		
	Kairomone	1.907	0.182
	instar	1.249	0.323
	Kairomone × instar	4.818	0.007
	Flavobacteriaceae		
	Kairomone	0.842	0.370
	instar	11.689	<0.001
	Kairomone × instar	16.893	<0.001
Dissected guts	*Comamonadaceae*		
	Kairomone	2.630	0.156
	Days	15.852	0.004
	Kairomone × days	0.0811	0.923
	Moraxellaceae		
	Kairomone	1.911	0.216
	Days	2.035	0.211

	Kairomone × days	1.412	0.314
	Flavobacteriaceae		
	Kairomone	3.274	0.120
	Days	13.543	0.006
	Kairomone × days	3.618	0.093
Culture medium	*Comamonadaceae*		
	Kairomone	19.169	<0.001
	Days	14.565	<0.001
	Kairomone × days	5.320	0.010
	Moraxellaceae		
	Kairomone	0.00421	0.949
	Days	14.690	<0.001
	Kairomone × days	4.190	0.023
	Flavobacteriaceae		
	Kairomone	2.501	0.133
	Days	22.109	<0.001
	Kairomone × days	14.185	<0.001

Diversity analysis in the microbiome

Alpha diversity, such as Shannon (Fig. 4a), of microbiota in dissected guts and whole *Daphnia* did not change significantly with the increase of *Daphnia* instar. However, in the presence of fish kairomone, the interaction with *Daphnia* instar (P = 0.008) significantly reduced the Shannon diversity of microbiota in dissected guts (Fig. 4a). The interaction between kairomone and *Daphnia* instar was not observed in Shannon diversity of whole *Daphnia* microbiota. We also detected the Shannon diversity of microbiota in the culture medium, and found that the increase of *Daphnia* instar did not change the Shannon diversity of microbiota in the culture medium, but the interaction between kairomone and *Daphnia* instar had a significantly effect on the Shannon diversity of microbiota in the culture medium (P = 0.005, Fig. 4a).

Daphnia gut microbiota communities were distinctively separated from the microbiota in whole *Daphnia* and culture medium in terms of NMDS using Bray-

Curtis distance matrix (Fig. 4b). Unweighted pair group method with arithmetic mean (UPGMA) tree of bacterial communities based on Bray-Curtis distance matrix showed that the samples in clade3 without kairomone and those treated with kairomone were in different branches (Fig. 4c), which indicated that predation risk was the main factor to determine the microbial composition variations.

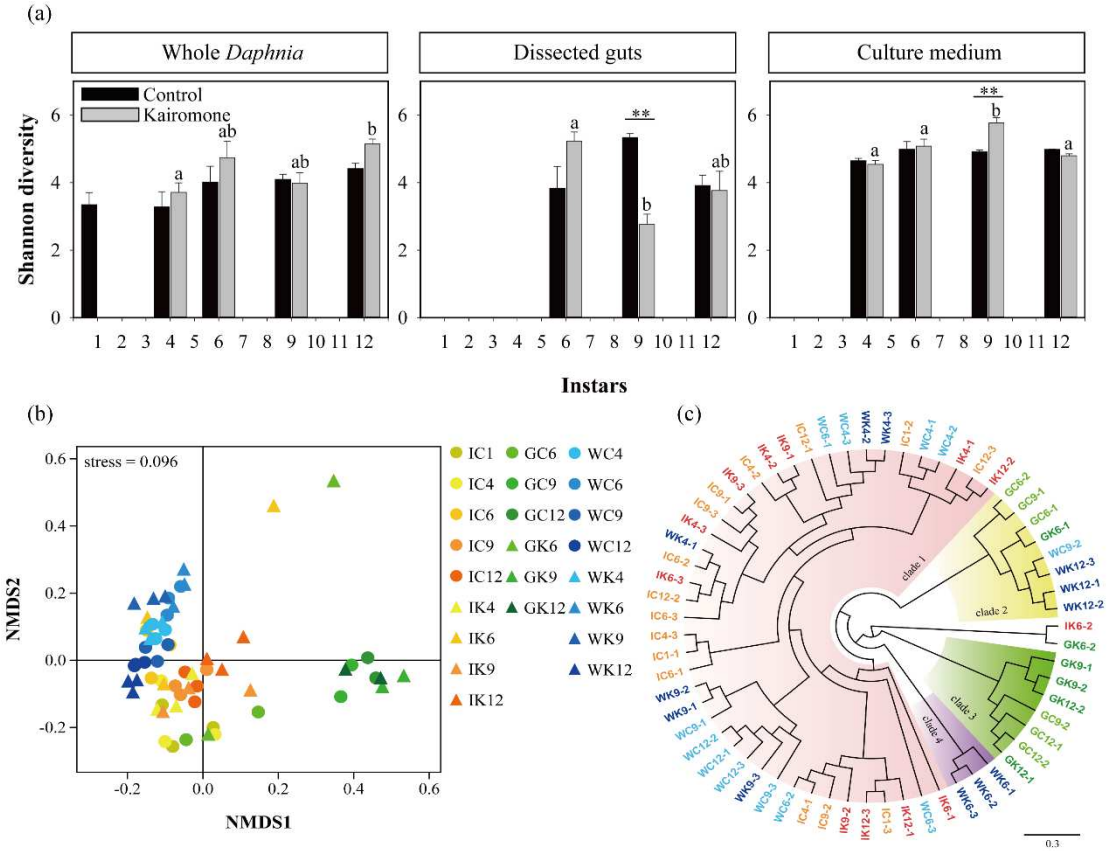


Fig. 4 Changes of alpha and beta diversity in *D. magna* bacterial communities. **(a)** Shannon diversity in whole *Daphnia* (left), dissected guts (middle), and culture medium (right) of *D. magna* in absence and presence of fish kairomone with different instars. Bars indicate mean values, and error bars indicate the standard error. The dark bars with asterisks indicate significant differences between the control and fish kairomone treatments under different instars (*p < 0.05, **p < 0.01). The significant

differences among different instars are indicated by the different uppercase (for the control) or lowercase (for the kairomone treatment) letters. **(b)** Two-dimensional non-metric multidimensional scaling (NMDS) plot of bacterial communities. Bray-Curtis distance matrix was used to generate the NMDS plots. "I" means "whole *Daphnia*" (red gradient), "G" means "dissected guts" (green gradient), and "W" means "culture medium" (blue gradient). The "C" (circle) and "K" (triangle) represent the control and fish kairomone treatments, respectively. **(c)** Unweighted pair group method with arithmetic mean (UPGMA) tree of bacterial communities. Bray-Curtis distance matrix was used to generate the UPGMA tree. "I" means "whole *Daphnia*" (red gradient), "G" means "dissected guts" (green gradient), and "W" means "culture medium" (blue gradient). The letters "C" and "K" represent the control and fish kairomone treatments, respectively.

Representative OTUs

There were small overlaps in OTUs of microbiota within whole *Daphnia*, dissected guts, and culture medium, while most OTUs in each part were unique (Fig. 5a). In the gut microbiota, OTUs between absence and presence of fish kairomone also overlapped small, and the number of unique OTUs was greater among the control and fish kairomone treatments (Fig. 5b). By detailed profiling of the OTU dynamics in the control and fish kairomone treatments, we found 9 OTUs belonged to, for example, *Arcobacter* and *Helicobacter* in *Epsilonbacteraeota*, that were found only in the gut microbiota exposed to fish kairomone. In addition, 295 OTUs belonging to

phylum *Firmicutes* were found in the gut microbiota exposed to fish kairomone. However, there were only 217 OTUs belonging to phylum *Firmicutes* found in the gut microbiota not exposed to fish kairomone. It is worth noting that the sum of these OTUs only accounted for a small proportion of the total relative abundance of all phyla (Additional file 2).

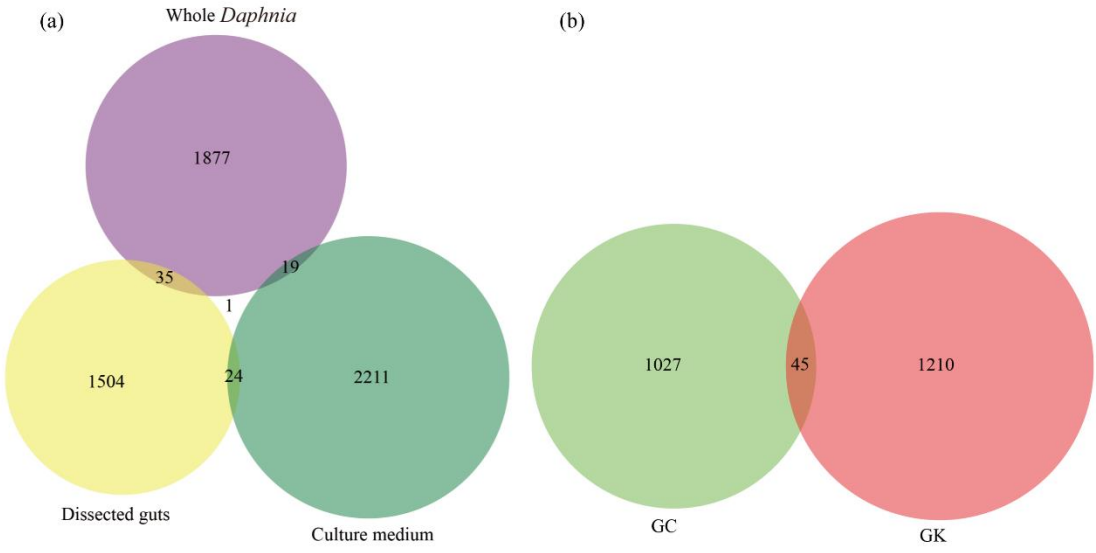


Fig. 5 Profiling of OTUs in *D. magna* microbial communities. Venn diagrams showing **(a)** number of OTUs in different parts, **(b)** number of OTUs in control (C) (green) and fish kairomone (K) (red) treatments in dissected guts.

Correlation between life history traits and bacterial abundance

The body size and the spine length of *D. magna* were significantly correlated with the relative abundance of *Comamonadaceae* in both whole *Daphnia* and culture medium microbiota (Fig. 6, Table S3). The body size and the spine length of *D. magna* increased and decreased by the high relative abundance of *Comamonadaceae*,

respectively (Fig. 6, Table S3). In the whole *Daphnia*, the relative abundance of other bacteria (*Moraxellaceae* and *Flavobacteriaceae*) were not correlated with both body size and spine length of *D. magna* (Fig. 6, Table S3). In the culture medium, the spines length of *D. magna* was also reduced by the high relative abundance of *Flavobacteriaceae* (Fig. 6, Table S3). In dissected guts of *Daphnia*, except that the spines length of *D. magna* was reduced by the high relative abundance of *Flavobacteriaceae* (Fig. 6, Table S3), the relative abundance of the three bacterium showed no correlation with the body size and spine length *D. magna* (Fig. 6, Table S3).

D. magna key life-history traits, such as the total offspring per female and the number of brood, were directly linked with the relative abundance of *Comamonadaceae* and *Moraxellaceae* in the whole *Daphnia* (Fig. 6, Table S3). The two traits (total offspring per female and number of brood) of *D. magna* were decreased by the high abundance of *Comamonadaceae* and increased by the high abundance of *Moraxellaceae* (Fig. 6, Table S3). In the culture medium, only the total offspring per female of *D. magna* was reduced by the high abundance of *Comamonadaceae* (Fig. 6, Table S3). In dissected guts of *Daphnia*, the abundance of the three bacterium showed no correlation with the two parameters (total offspring per female and number of brood) of *D. magna* (Fig. 6, Table S3).

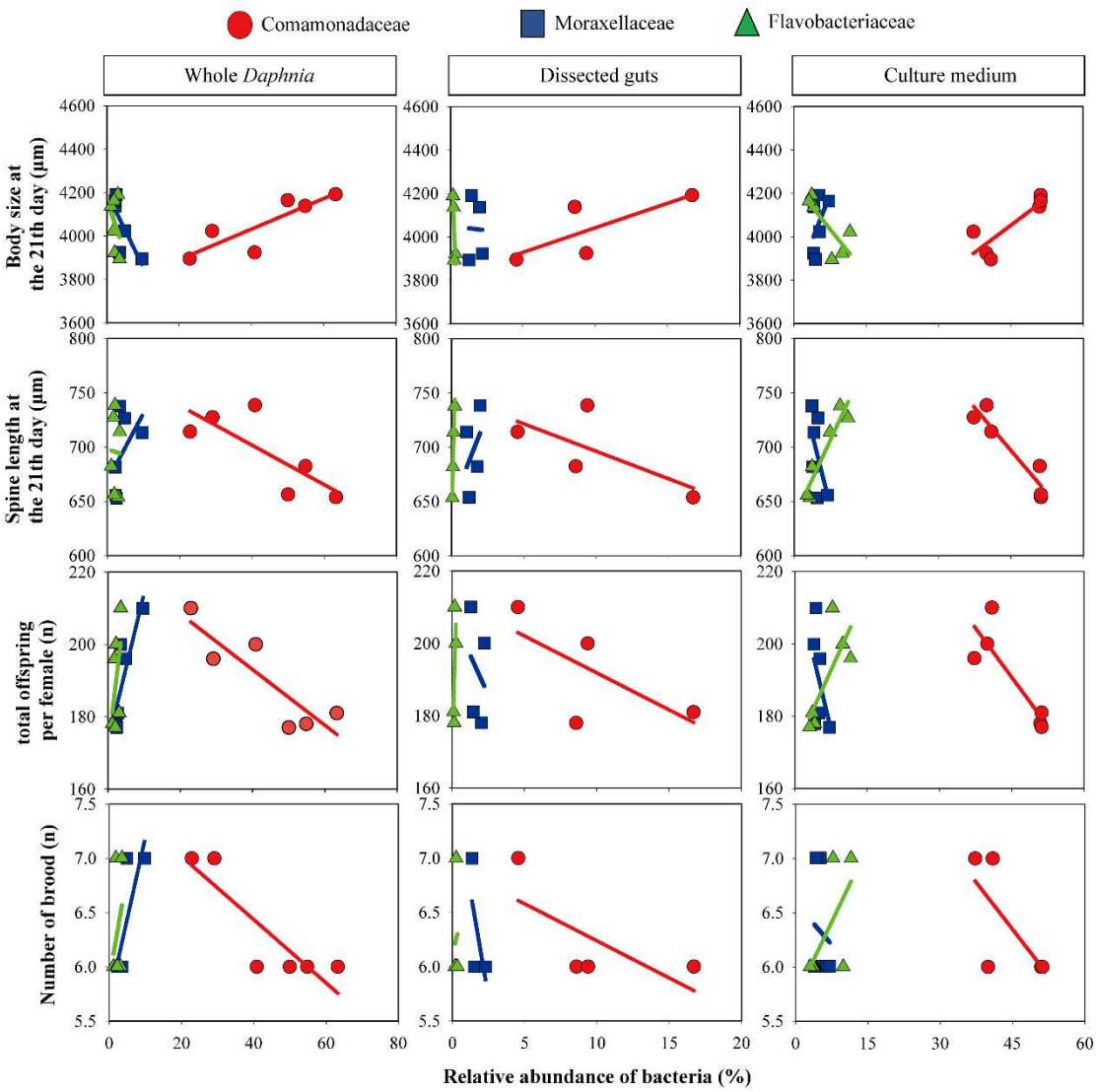


Fig. 6 Scatterplot of correlation between key life-history traits and family level abundances of bacteria. The data were fitted by linear regression model. The left, middle, and right sections represent whole *Daphnia* (left), dissected guts (middle), and culture medium (right) of *D. magna*, respectively. When the slope was significantly different from zero ($\alpha = 0.05$), this indicated a significant correlation. Values of R^2 and P are shown in Table S3.

Correlation between life history traits and microbial diversity in different microflora detection sites

For the body size and the spine length of *D. magna*, only the spine length showed significantly negative correlation with the microbial diversity detected in culture medium (Fig. 7, Table S4). For the total offspring per female and the brood number of *D. magna*, only the brood number was found positive correlation with the microbial diversity detected in the whole *Daphnia* (Fig. 7, Table S4). The body size and the total offspring number of *D. magna* had no significant correlation with microbial diversity detected in the three microflora detection sites (Fig. 7, Table S4).

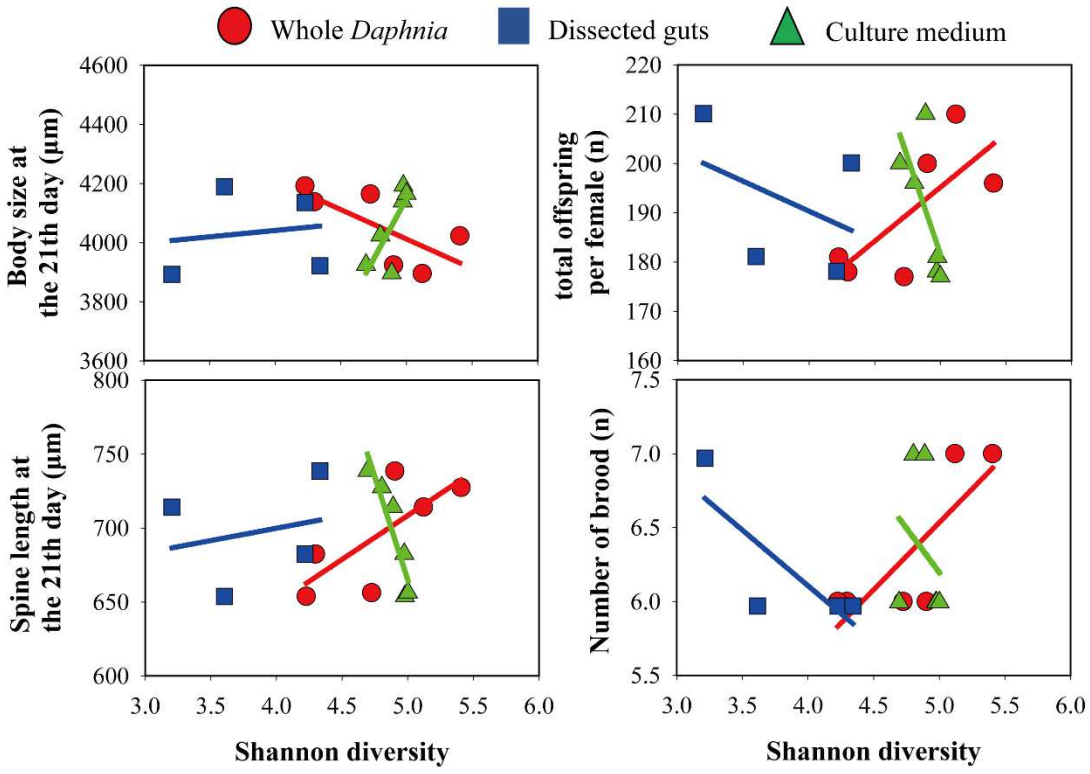


Fig. 7 Scatterplot of correlation between key life-history traits and microbial diversity in different microflora detection sites. The data were fitted by linear regression model. When the slope was significantly different from zero ($\alpha = 0.05$), this indicated a significant correlation. Values of R^2 and P are shown in Table S4.

Functional predictions

PICRUSt2 gave 36 predicted functional categories that represented 7 pathway maps in the KEGG level 2 functional modules. Multiple KEGG function abundance cluster analysis was conducted to check the changes of functions under the absence and presence of fish kairomone and different microflora detection sites (Fig. 8). When analyzing the effects of fish kairomone on microbial functional categories in whole *Daphnia* and culture medium, we found that there was little difference between different functional categories in whole *Daphnia* and culture medium in the presence or absence of fish pheromone (Fig. 8). However, when analyzing the effects of fish kairomone on microbial functional categories in dissected guts, we found that there was a significant effect on metabolic pathways, involved in enhancing the metabolism of cofactors, vitamins, and energy (Fig. 8). Furthermore, the presence of fish kairomone enhanced the functional categories folding, sorting, and degradation (Fig. 8).

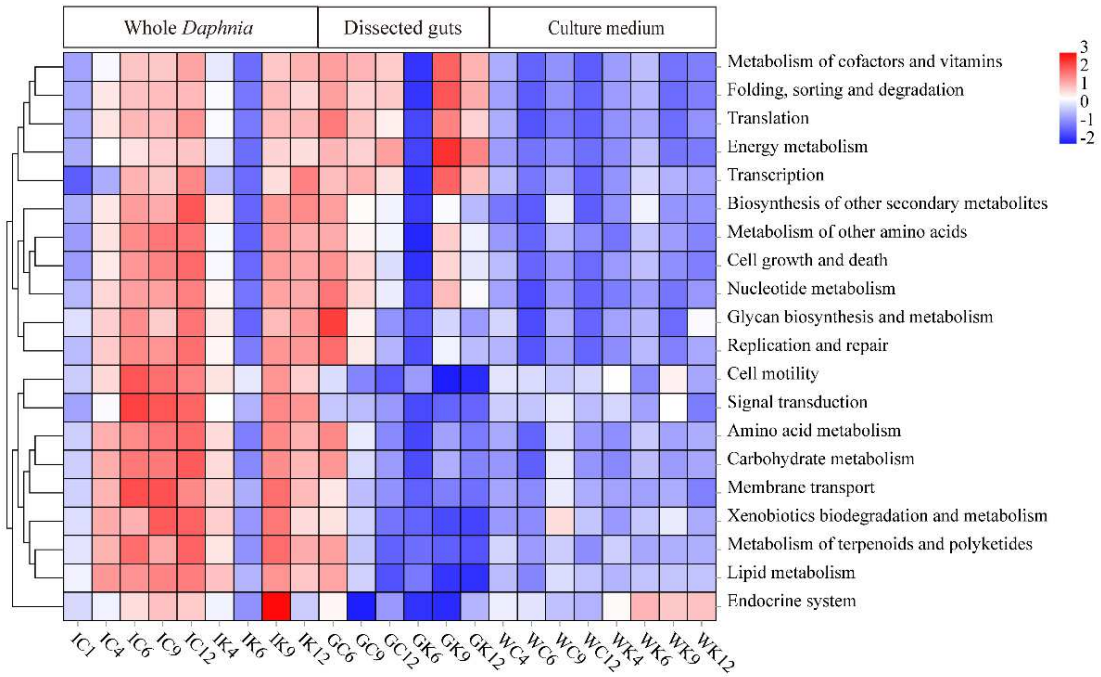


Fig. 8 Relative abundance of each predicted functional categories given in KEGG pathways (level 2). The left, middle, and right sections represent whole *Daphnia* (left), dissected guts (middle), and culture medium (right) of *D. magna* in absence and presence of fish kairomone, respectively. The numbers "1", "4", "6", "9" and "12" represent different instars.

Discussion

Over the past few years, evidences are accumulating that the gut microbiota can be a crucial mediator of life history variation, as well as of acclimatization and adaptation to changing environmental conditions [8, 9]. Predicting how, and to what extent, the gut microbiota may impact fitness requires to identify the links between variation in gut microbiota and host phenotype, and to understand how the microbiome communities are assembled [8]. In this study, we analyzed the composition of gut microbiota and the relative abundance of dominant bacteria related

to *D. magna* under the action of fish kairomone. As we hypothesized, with the increase of instars, fish kairomone changes the gut microbiota composition and decreases the α diversity of *D. magna* gut microbiota. The relative abundance of some bacteria decreases due to the presence of kairomone, among which, *Comamonadaceae* (mainly *Limnohabitans* sp.) is mainly reduced. Correlation analysis results showed that there was a linear correlation between the high relative abundance of *Comamonadaceae*, *Moraxellaceae*, and *Flavobacteriaceae* and the life history traits of *D. magna*. Among them, the body size of *D. magna* was positively correlated with the increased abundance of *Comamonadaceae*, while spine length of *D. magna* was negatively correlated with the increased abundance of *Comamonadaceae*, and positively correlated with the increased abundance of *Flavobacteriaceae*. Predation risk can affect the composition of gut microbiota during the growth *D. magna*, which may indirectly affect the defensive traits of *D. magna*. This result is of great significance for understanding the influence of host-microbial interaction on individual anti-predation defense.

D. magna gut microbiota

Peter and Sommaruga [49] detected most major bacterial groups of the surrounding water in gut homogenates of copepods and cladocerans, except for Actinobacteria. Thus, these different community members which were accumulated in the gut through filtration could reflect bacteria from the cultivation water. However, in our study, *D. magna* has a unique gut microbiota, and there were strong differences between microbial communities in the *Daphnia* gut and in the surrounding water.

Grossart *et al.* [50] demonstrated that bacteria associated with the cladoceran *Bosmina* still remained highly similar when transplanted to another lake. Thus, it can be assumed that the gut microbiota community in *Daphnia* is composed, at least to a major part, of resident bacteria and is less susceptible to reflect the surrounding bacterial community. In agreement with previous reports [5, 13, 51, 52], *Proteobacteria* and *Bacteroidetes* were the dominant bacterial phyla in *D. magna* guts in our study. Among them, *Proteobacteria* is dominant, and *Comamonadaceae* (mainly *Limnohabitans* sp.) is the main component of *Proteobacteria* phyla. *Comamonadaceae* family induced positive fitness effects in *Daphnia*, and *Limnohabitans* was the most abundant *Comamonadaceae*, as was described in previous studies [27, 53-56]. In this study, with the increase of *Daphnia* instar, the abundance of *Comamonadaceae* (mainly *Limnohabitans* sp.) in *Daphnia* guts was significantly reduced (Table 3). It was observed that the microbiota community composition did not remain stable over time. However, fish kairomone had no significant effect on the relative abundance of *Comamonadaceae* (mainly *Limnohabitans* sp.) in *Daphnia* guts, nor did the interaction with instars, although fish kairomones significantly increased the fecundity of *D. magna* (Fig. 1 and 2, Table 3). This seems to contradict the results of Peerakietkhajorn *et al.* [56, 57], which found bacteria in the genus *Limnohabitans* have been linked to increased fecundity and population size in *D. magna*. Possibly, this shift in the community composition is caused by two reasons. For one thing, *Daphnia* might spend costs on growth in the early stage, and turn to spend more energy on resisting predators in the later

487 developmental stage as time goes on. For another, *Daphnia* might uptake and
488 stimulate the inactive or underrepresented bacteria, as described in the guts of
489 earthworms [58]. These bacteria were not detected, but were activated and thus
490 increased in abundance over time, overriding the relative abundance of
491 *Limnohabitans*. In addition to *Limnohabitans*, other bacteria detected in *D. magna*
492 intestine include *Acinetobacter* and *Flavobacterium*. Furthermore, when comparing
493 the relative abundance of bacterial phyla among treatments, we found that
494 *Epsilonbacteraeota* only existed in the gut microbiota of *D. magna* exposed to fish
495 kairomone. This suggests that *Epsilonbacteraeota*, especially *Arcobacter* and
496 *Helicobacter* could be used as indicators for predation risk. In addition,
497 *Epsilonbacteraeota* have also been suggested to be associated with hypertension [59],
498 and the increased risk for hypertension is often associated with host psychological
499 stress [60, 61].

500 ***Effects of predation risk and Daphnia instar on D. magna gut microbiota***

501 In previous studies on humans and some vertebrates, researchers have shown that
502 stress can affect and alter the gut microbiota. For example, the fecal lactic acid
503 bacterial levels decreased significantly when students were facing academic stress
504 [62]; social disruption stressor could impact the gut microbiota community in mice
505 [63]; in aquatic organisms, the species richness and microbial diversity of overall gut
506 microbiota in perch significantly decreased with predator presence [12]. In our
507 experiment, the presence of predation risk interfered with the diversity of *D. magna*
508 gut microbiota, and the diversity of gut microbiota also significantly decreased with

509 the increase of *Daphnia* instar (Fig. 4a). As respiration rates of bacterial communities
510 are influenced by species richness and composition [64], more diverse communities
511 possibly contain a wider array of metabolic capabilities. However, coping with stress
512 is a process of expending energy for animals and may have an impact on metabolism.
513 For example, the haemoglobin concentration in the tissues of *Daphnia pulex*
514 decreased in the presence of kairomones [65]. A lower concentration of haemoglobin
515 could be responsible for a decreased efficiency of oxygen transport to body tissues,
516 which in turn leads to a decrease in energy for growth and reproduction [65]. In this
517 study, the presence of fish kairomone also affected the metabolic pathway of *D.*
518 *magna* (Fig. 8). Thus, animals might need to re-allocate metabolic substrates to other
519 tissues to cope with the increasing energy needs when facing stress, such as to
520 stimulate oxygen in gills [66], instead of spending them on a high-energy intestine
521 [67]. Alternately, there are differences in the composition of the gut microbiota
522 community at different *Daphnia* instars, and these differences may be due to the
523 physiological changes that occur during development of *Daphnia*. For example, in
524 contrast to the small body size of *Daphnia* juvenile, the increased body size after
525 juvenile growth could result in longer gut passage time and improved assimilation
526 efficiency [68]. Furthermore, the characteristics of transportation time and
527 morphological structure in the digestive system will affect the community
528 composition of gut microbiota. Longer gut passage time may make the gut microbiota
529 community have a longer time to use substrates [69, 70]. This may be the main reason
530 that *Daphnia* instar interacts with fish kairomone and significantly reduces the gut

531 microbiota diversity of *D. magna*.

532 ***Relationship between gut bacteria and D. magna life history traits***

533 In the study on the comparing germ-free and conventionally reared individuals of
534 *D. magna*, Sison *et al.* [4] first reported that the gut microbiota is an important factor
535 that affects life-history traits contributing to host fitness. Compared with
536 conventionally reared individuals, germ-free *D. magna* have smaller body size, less
537 fecundity, and higher mortality [4]. In the food chain, the body size and reproduction
538 quantity of prey are important traits to cope with predation pressure [41, 71, 72]. For
539 *Daphnia*, the significant reduction in body size and increase in fecundity can allow
540 them to increase the probability of reproduction before being eaten by visual, size-
541 selective predators such as fish [71-73]. Current study also shows that the presence of
542 fish kairomone significantly decreased the body size and increased the spine length,
543 the total offspring number of *D. magna* (Fig. 1 and 2, Table 1 and 2). We chose these
544 traits as representative parameters to establish the correlation between the abundance
545 and composition of gut microbiota and *D. magna* fitness, which were consist with
546 previous studies that used several key features such as fecundity, host survival time,
547 and body size to correlate host fitness with the microbiome [13, 51, 74, 75]. In our
548 study, the reproductive traits (the total offspring number and the brood number) of *D.*
549 *magna* were directly linked with the abundance of either *Comamonadaceae* or
550 *Moraxellaceae*; the growth traits (the body size and the spine length) of *D. magna*
551 were directly linked with *Comamonadaceae* abundance (Fig. 6, Table S3). Previous
552 studies have shown that some strains of bacteria can provide the essential elements for

host reproduction and growth to the benefit of the host [57, 75]. For example, the key components of *Daphnia* gut microbiota, *Limnohabitans*, *Aeromonas*, and *Acidovorax* [56, 76], have been linked to *Daphnia* obtaining essential amino acids [77, 78], polyunsaturated fatty acids, and sterols [79] that positively affect *Daphnia* growth and reproduction [78]. More specifically, these bacteria in *Daphnia*'s gut can produce some useful enzymes for digestion [57], and then increase the production of nutrients incorporated into the female and parthenogenetic eggs during development of oocytes, which may promote *Daphnia* growth and increase numbers of viable *Daphnia* juveniles. In our results, the presence of fish kairomone significantly decreased the relative abundance of *Comamonadaceae* (mainly *Limnohabitans* sp.), which could be the main reason for the body size reduction of *D. magna*. However, the reproduction quantity of *D. magna* increased significantly in the presence of fish kairomone, which may depend on the abundance and presence of *Moraxellaceae* (*Gammaproteobacteria*, *Pseudomonadales*). Although the knowledge of *Moraxellaceae* function in freshwater invertebrates is rarely, but some genus in *Pseudomonadales*, e.g., *Pseudomonas* sp., can widely colonize in *Daphnia* and have a positive effect on *Daphnia* growth and survival [80]. We speculated that *Moraxellaceae* plays the same role as *Comamonadaceae* in improving the fecundity of *Daphnia*, and *Moraxellaceae* is more competitive than *Comamonadaceae* in the presence of fish kairomone. Moreover, the host growth and reproduction may also acquire essential nutrients from more available pathways.

It should be noted that the correlation in our results was more pronounced in

whole *Daphnia* and culture medium microorganisms, but not in *Daphnia* gut, which may be because *Daphnia* feeding behavior plays an important role in structuring *Daphnia*-associated microbial communities [55, 81]. For shaping the gut environment, host metabolism and immunity might further extend to influence the external environment of *Daphnia*, for instance through the shedding of immune effectors [82], thus affecting the bacterioplankton community structure. This is in accordance with the results of Mack *et al.* [83], who found that diet or microbial inoculation has less influence on the gut microbiota, while has greater influence on the surrounding environmental microbiota.

Conclusions

In this study, we found that *D. magna* not only developed defense traits, but also changed gut microbiota in response to predation risk. The change in the abundance of microbiota was mainly manifested in the abundance of *Comamonadaceae* which was significantly reduced under the influence of fish kairomone. As for defense traits, the body size of *D. magna* was positively correlated with the increased abundance of *Comamonadaceae*. The spine, total offspring number, and brood number of *D. magna* were negatively correlated with the increased abundance of *Comamonadaceae*. In addition, the co-action of *Daphnia* instar and fish kairomone significantly reduced the diversity of gut microbiota, which may be related to the high diversity of gut microbiota that may consume more *Daphnia* resources which are necessary for its growth. This study suggests that gut microbiota play an important role in the

development of defensive traits in *Daphnia* in response to fish predators. Linking defense characteristics to the change of microbiota may help us to better predict host-microbiota interactions under multiple stressors, and further analysis is needed to better understand these relationships.

Abbreviations

ANOVA: Analysis of variance; NMDS: Non-metric multidimensional scaling; UPGMA: Unweighted pair group method with arithmetic; C: Absence of fish kairomone; K: Presence of fish kairomone; Site of microflora detection-I: Whole *Daphnia*; Site of microflora detection-G: Dissected guts; Site of microflora detection-W: Culture medium.

Supplementary Information

Additional file 1: Table S1. Measurement time and corresponding instar of *D. magna*. **Table S2.** List of bacteria genus found in the microbiome of *D. magna*. **Table S3.** Correlation between different traits and different dominant bacterial groups. **Table S4.** Correlation between different traits and microbial diversity in different microflora detection sites. **Fig. S1.** Relative abundance of bacteria at the different level of *D. magna*. The left, middle, and right sections represent whole *Daphnia* (left), dissected guts (middle), and culture medium (right) of *D. magna* with different instars in absence and presence of fish kairomone, respectively. The numbers “1”, “4”, “6”, “9” and “12” represent different instars.

Additional file 2: The metadata of OTUs.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data of raw sequences for microbial sequence data were deposited in…

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

All authors read and approved the final manuscript for submission. QL and ZY designed the experiments. QL, SA, QMZ, and ZHD conducted the experiments and collected the data. QL, YFS, and ZHD analyzed the data. QL, LG, and SA wrote the initial draft of the manuscript, while ZY provided substantial feedback.

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