

ORIGINAL ARTICLE

Food availability affects the strength of mutualistic host–microbiota interactions in *Daphnia magna*

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The symbiotic gut microbial community is generally known to have a strong impact on the fitness of its host. Nevertheless, it is less clear how the impact of symbiotic interactions on the hosts' fitness varies according to environmental circumstances such as changes in the diet. This study aims to get a better understanding of host–microbiota interactions under different levels of food availability. We conducted experiments with the invertebrate, experimental model organism *Daphnia magna* and compared growth, survival and reproduction of conventionalized symbiotic *Daphnia* with germ-free individuals given varying quantities of food. Our experiments revealed that the relative importance of the microbiota for the hosts' fitness varied according to dietary conditions. The presence of the microbiota had strong positive effects on *Daphnia* when food was sufficient or abundant, but had weaker effects under food limitation. Our results indicate that the microbiota can be a potentially important factor in determining host responses to changes in dietary conditions. Characterization of the host-associated microbiota further showed that *Aeromonas* sp. was the most prevalent taxon in the digestive tract of *Daphnia*.

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Introduction

The digestive tract of animals is host to a diverse community of symbiotic microorganisms, collectively called the gut microbiota. It is well known that the gut microbiota can have a positive impact on the fitness of its host through various mechanisms like producing essential nutrients (Tokuda *et al.*, 2013), enhancing resistance to disease (Belden & Harris, 2007) or breaking down indigestible substances (Ohkuma, 2008) and toxic compounds (Kohl & Dearing, 2012). In some cases, however, host fitness can also be reduced by the microbiota through competition for resources (Douglas, 2010) or via a dysfunctional symbiotic microbial community causing disease (Clemente *et al.*, 2012).

The fitness impact of services provided by symbiotic interactions on the host is often strongly dependent on the ecological context (Daskin & Alford, 2012). Several studies have shown that the hosts' diet, especially food quality, can be an important influencing factor on interactions between the host and its gut microbiota. For example, de

Vries *et al.* (2004) found that bacteria belonging to the genus *Erwinia*, present in the gut of western flower thrips, were parasitic under a high-quality diet but mutualistic when the thrips were given a nutrient-poor diet, indicating that these symbionts benefit their host only in nutritionally poor environments. In contrast, Ben-Yosef *et al.* (2008) found that the gut microbiota of Mediterranean fruit flies (*Ceratitis capitata*) can negatively affect longevity and egg deposition, but only when the hosts feed on a low-quality diet. These contrasting results imply that the strength and direction in which host–microbiota interactions change under different dietary conditions cannot be easily predicted.

Although most research has focused on food quality, several studies found that food quantity can affect the composition and functionality of the gut microbiota (Dillon *et al.*, 2010; Freese & Schink, 2011). In mice, for example, starvation is known to alter the gut microbiota in a way that it confers health benefits to its host (Zhang *et al.*, 2013). Furthermore, the gut microbiota is known to positively influence physiological responses of the host to starvation (Crawford *et al.*, 2009). As strong fluctuations in food availability commonly occur in natural populations (Müller-Navarra & Lampert, 1996), this factor might be an important driver of host–microbiota interactions and can thus be highly relevant from an ecological perspective. Here we

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address this issue using the water flea *Daphnia magna* and its symbiotic microbial community as a model system. *D. magna* is a keystone species in many freshwater habitats and has been widely used as an experimental system to study ecological and evolutionary interactions (for example, Decaestecker et al., 2007; Miner et al., 2012). *Daphnia* is known to harbor high numbers of bacteria on the surface of its body and in its gut (Grossart et al., 2010; Eckert & Pernthaler, 2014), and Qi et al. (2009) showed that several bacterial taxa are consistently found in affiliation with *Daphnia*, even in geographically separated populations, indicating a highly specific association between *Daphnia* and its microbiota. Notably, the β -proteobacterial genus *Limnohabitans* (Comamonadaceae) seems to be a major constituent of the *Daphnia* microbiota (Freese & Schink, 2011; Eckert & Pernthaler, 2014; Peerakietkhajorn et al., 2015). Sison-Mangus et al. (2014) showed, by comparing bacteria-free *Daphnia* with conventional ones, that the relationship between *D. magna* and its microbiota is mutualistic, as removing the microbiota results in decreased growth, survival and reproduction of the host. It is, however, not known how this interaction is affected by variation in environmental conditions, such as food availability.

Food availability is known to profoundly affect life-history characteristics and population dynamics in *D. magna* (Vanoverbeke, 2008). Food limitation has been shown to decrease growth and reproduction while increasing longevity (Pietrzak et al., 2010). Here, we investigate the effects of variation in food availability on host-microbiota interactions in *Daphnia* by comparing symbiotic with germ-free hosts raised in environments with different levels of food availability. Dependent on the specific mode-of-action of the microbiota and how this will interact with variation in food availability, different outcomes are possible. If, for example, the microbiota helps its host to acquire nutrients, increasing the amount of food will not correspond to an equal increase in nutrient uptake when the microbiota is removed. Under this hypothesis, we expect that germ-free *Daphnia* will show a lower response to an increase in food availability than symbiotic *Daphnia*. It is, however, also possible that the microbiota competes with its host for nutrients when food availability is limited. If this is the case, we expect symbiotic *Daphnia* to have a lower fitness than germ-free *Daphnia* under food limitation. To identify bacterial taxa possibly responsible for the observed effects on their host, we characterized the *Daphnia* microbiota by sequencing 16S rDNA.

Materials and methods

Cultivation of algae

An axenic batch culture of *Scenedesmus obliquus* was grown in Wright's cryptophyte medium (Guillard & Lorenzen, 1972) for 1 week, after which

the algae were harvested. The presence of bacteria in the cultures was checked by 4',6-diamidino-2-phenylindole-stained preparations under a fluorescence microscope at $\times 1000$ magnification and by plating 100 μ l on nutrient agar in triplicate and checking for bacterial growth after 5 days of incubation at 37 °C. Ash-free dry weight (expressed as mg C) of the culture was determined following Moheimani et al. (2013).

Daphnia cultivation and obtaining of germ-free animals

We used *D. magna* clone KNO15.04 in all experiments. This clone was isolated from a pond in Knokke, Belgium (51°20'05.62" N, 03°20'53.63" E) and has been maintained in the lab for multiple generations. Stock cultures were kept in 500 ml jars filled with Aachener Daphnien medium (ADaM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 273 mg l⁻¹; NaHCO_3 : 56 mg l⁻¹; sea-salt: 333 mg l⁻¹; Klüttgen et al., 1994) in a temperature-controlled room at 19 \pm 2 °C, under a light regime of 16:8 h light:dark, and on a diet of saturating amounts of *S. obliquus*. Before the start of each experiment, reproduction of the animals was synchronized by maintaining individuals of the same age for at least two generations under identical conditions.

To obtain germ-free *Daphnia* individuals, a protocol that we recently developed and which is published for the first time was applied. Females carrying parthenogenetic eggs were dissected under a stereo-microscope and the eggs were collected in a petri dish containing ADaM. Only recently deposited eggs, which are characterized by the presence of an external membrane, were isolated. Preliminary experiments showed that the timing of egg isolation is very important for hatching success: eggs isolated <12 h after deposition had a significant lower hatching rate than those who were isolated later (but still surrounded by an external membrane; personal observation). From here onwards, all experimental manipulations were performed under sterile conditions in a laminar flow hood. To remove all microbial organisms from the eggs, they were placed in a petri dish containing 10 ml of a 0.01% peracetic acid (PAA) solution. These were gently agitated for 10 min, after which the eggs were transferred to another petri dish containing sterile ADaM to remove any PAA residues. Afterwards, the disinfected eggs were transferred to six-well (cell culture) plates. Each well contained ~50 eggs in 8 ml of sterile ADaM. Well plates were incubated at 20 \pm 0.5 °C. Eggs were allowed to hatch during 48 h, and the resulting germ-free individuals were used in the experiment.

Verification of symbiont removal

Because we applied a newly developed method for obtaining germ-free *Daphnia*, removal of bacteria from the experimental animals needed to be verified after applying the disinfection protocol. To do this,

the presence of bacterial 16S rDNA on disinfected *Daphnia* was checked using PCR. This was tested on pooled samples of 20 neonate juveniles hatched from PAA-disinfected eggs. As a positive control, 20 neonate juveniles from a stock laboratory culture were used. Both the disinfection treatment and the positive control were tested in triplicate. A blank sample was also included in the analysis as negative control. Preliminary tests on samples containing a known number of *E. coli* cells showed that when applying the following protocol, the detection threshold is 10^4 bacterial cells.

To lyse the cells, *Daphnia* were first homogenized in 0.5 ml of double-distilled H₂O. Then, 30 µl of lysozyme (50 mg ml⁻¹) was added to each sample and these were incubated for 1 h at 37 °C. To each tube, a small amount of glass beads (0.1 mm diameter, acid washed) was added, and they were placed on a bead-beater (2 min, 30 m s⁻¹). Afterwards, 50 µl of proteinase K (1 mg ml⁻¹) was added and the samples were incubated for 30 min at 55 °C. Finally, 25 µl lysis buffer (10% sodium dodecyl sulfate; 0.5 M ethylenediaminetetraacetic acid) was added and samples were incubated for 1 h at 55 °C.

DNA was extracted from the lysate by adding 500 µl of phenol–chloroform–isoamylalcohol (25–24–1) to the samples, followed by centrifugation (13 200 rpm, 10 min) and transfer of the aqueous supernatant to a new micro-centrifuge tube. Phenol–chloroform extraction was repeated once more on the supernatant. DNA was precipitated by adding 0.1 vol of sodium acetate (3M) and an equal volume of isopropanol to the tube, followed by a centrifugation step (25 min; 14 000 rpm; 4 °C). The supernatant was discarded and the pellet was washed with 70% ice-cold ethanol, centrifuged (10 min; 14 000 rpm; 4 °C), dried and dissolved in 15 µl tris-EDTA buffer. Extracted DNA was stored at -20 °C until further processing.

PCRs were performed on 5 µl of extracted DNA using Platinum *Taq* DNA polymerase (Life Technologies, Waltham, MA, USA) according to the manufacturers' instructions. Universal bacterial primers 8F and 1492R (Weisburg *et al.*, 1991) were used to amplify a 1484 bp fragment of the 16S rDNA. PCR reactions were run for 30 cycles (94 °C 30 s, 50 °C 30 s and 72 °C 60 s). The presence of amplified 16S rDNA was checked under UV light on 10 µl of PCR product loaded on a 1% agarose gel stained with GelRed (Biotium, Hayward, CA, USA).

Experimental setup

Germ-free *Daphnia* were placed individually in a 50-ml falcon tube containing 5 ml of sterile ADaM. Each tube was randomly assigned to either the symbiotic ($n=240$) or germ-free ($n=240$) treatment. A microbiota inoculum was prepared by homogenizing one adult *Daphnia* per ml in sterile ADaM. In the symbiotic treatment, axenic juveniles were

re-colonized with *Daphnia* microbiota by adding 100 µl of this inoculum. Individuals in the germ-free treatment also received 100 µl of the inoculum, but which had been autoclaved prior to administration. All *Daphnia* were incubated for another 24 h, after which sterile ADaM was added to obtain a final volume of 40 ml medium in all tubes.

To test for the effects of different food quantities on host-microbiota interactions, both symbiotic and germ-free animals were administered daily one of four different food amounts: 2 mg C l⁻¹ (high), 0.5 mg C l⁻¹ (intermediate), 0.1 mg C l⁻¹ (low) or no food (starvation). This resulted in a total of eight treatments (presence or absence of symbionts × four different food amounts, with 30 individuals per treatment). All experimental units were kept in an incubator at 20 ± 0.5 °C and a 16:8 h light:dark regime. Every 24 h during 3 weeks, survival and reproduction (time of egg deposition and number of juveniles per brood) were registered and released juveniles were removed.

To measure the effect of the different treatments on *Daphnia* body size, a similar experiment was performed with circa 30 *Daphnia* per treatment. After 10 days, all surviving animals were fixated with 4% formaldehyde and mounted in glycerol on microscope slides. Carapace size (from the underside of the head to the base of the caudal spine) was measured under a stereo-microscope using the Infinity Analyze 6.3.0 software (Lumenera, Ottawa, ON, Canada).

Identification of symbiotic bacteria

To identify the bacteria present on *Daphnia* in our laboratory cultures, we characterized the composition of symbiotic bacterial communities of both entire *Daphnia* and their guts by sequencing 16S rDNA. For each sample, 20 neonate KNO15.04 *Daphnia* were kept in 2 l containers filled with ADaM medium and given a daily amount of 2 mg C l⁻¹ *S. obliquus*. After 10 days, adult *Daphnia* were collected from each culture and kept for 24 h in 50 ml of 0.22 µl filtered water originating from their respective container to remove any non-symbiotic bacteria. To characterize the entire community of host-associated microbiota, 15 *Daphnia* were pooled in a micro-centrifuge tube. To specifically characterize the gut microbiota, the digestive tracts of 15 *Daphnia* were pooled after dissecting them under a stereo-microscope using sterilized dissecting needles. Both communities were characterized in triplicate.

DNA was extracted using the protocol described above and 16 rDNA fragments were amplified using a nested primer design. PCR reactions were performed using high-fidelity Platinum Pfx DNA polymerase (Life technologies) according to the manufacturer's instructions. For the external amplification, a PCR reaction was run for 25 cycles (95 °C 30 s, 50 °C 30 s, 68 °C 60 s) using primers Eub8F and

984yR (Bakke *et al.*, 2011). The PCR products were subsequently purified and amplified in a second PCR reaction for 25 cycles (94 °C 30 s, 45 °C 30 s, 68 °C 60 s) using primers 343 F (Wilson *et al.*, 1990) and 798 R (Rochelle *et al.*, 1995) modified by adding ligation adaptors and sample identification sequences to the 5'-end. PCR products were purified and forward 16S rDNA amplicons were sequenced using 454 pyrosequencing.

Data analysis

Normal distribution and equal variance of data were tested using a Shapiro–Wilk and a Bartlett's test, respectively. Differences in size and production of juveniles between treatments were analyzed using a two-way analysis of variance with microbiota, food quantity and their interaction as fixed factors. A Tukey honest significant difference test was used to make *post hoc* pairwise comparisons. The proportions of reproducing individuals were tested using a χ^2 -test. All these analyses were performed with R 3.1.0.

The intrinsic rate of natural increase (r_m) was calculated using the equation $\sum e^{-rx} l_x m_x = 1$, with x = time in days, l_x = age-specific survival (number of living females on day x /number of females alive at the start) and m_x = age-specific reproduction (number of offspring released at day x /number of females alive at day x) (Birch, 1948).

Longevity and age at first reproduction were analyzed with a Cox proportional-hazards model regression using the SAS 9.4 software (PHREG procedure). Microbiota, food quantity and their interaction were declared as fixed factors. The survival times of individuals that were still alive at the end of the experiment and the time at first reproduction of individuals that died before reproducing were coded as censored. As ties in survival and reproduction times were numerous, we used the Efron approximate likelihood. Pairwise comparisons were performed using the CONTRAST statement, which provided both the hazard ratios (HR) between groups for the variable of interest, and the associated P -values.

Sequences of 16S rDNA were demultiplexed, quality-filtered and clustered into operational taxonomic units (OTUs) at 97% pairwise sequence similarity using the QIIME pipeline (Caporaso *et al.*, 2010). As some samples contained a low number of sequences, only operational taxonomic units with a mean relative abundance of >1% in either the entire or gut microbiome were used to assess community composition. Operational taxonomic units were taxonomically classified by searching for the sequence with the highest similarity using the SINA aligner (Pruesse *et al.*, 2012). Nucleotide sequences obtained in this study have been submitted to the European Nucleotide Archive (accession numbers LN869908–LN869916).

Results

Removal of symbionts from *Daphnia*

In all samples containing PAA-disinfected *Daphnia*, no fragments of amplified 16S rDNA were observed on an agarose gel after PCR. In all positive controls a band of the amplified 16S rDNA fragment was clearly visible (Supplementary Figure 1). This indicates that the PAA treatment was capable of removing all bacteria from *Daphnia*, or at least reducing them to an amount undetectable by PCR.

Effects of symbiotic bacteria and food quantity on *daphnia* survival

Both food quantity ($X^2_3 = 111.3$, $P < 0.001$) and the microbiota ($X^2_1 = 50.9$, $P < 0.001$) had a significant main effect on survival, and there was a significant interaction between these two factors ($X^2_3 = 9.9$, $P < 0.05$). Within each food treatment, symbiotic *Daphnia* always survived significantly longer than germ-free ones and the effect of the microbiota was the strongest under intermediate food quantities (starvation: HR = 18.8, $P < 0.001$; low: HR = 35.5, $P < 0.001$; intermediate: HR = 214.3, $P < 0.001$; high: HR = 19.1, $P < 0.001$) (Figure 1, Table 1). For symbiotic *Daphnia*, survival was significantly higher when they received low or intermediate food quantities compared with starved individuals or those receiving high food quantities (all pairwise comparisons, $P < 0.001$) and symbiotic *Daphnia* in the high food quantity treatment showed a higher survival than starved ones (HR = 0.03, $P < 0.001$). For germ-free *Daphnia*, survival in the low food quantity treatment was significantly higher compared with all other treatments (all pairwise comparisons, $P < 0.001$). Germ-free individuals receiving an intermediate or high food quantity showed no difference in survival ($P = 0.11$). *Daphnia* from the germ-free/starvation treatment showed the lowest survival of all (all pairwise comparisons, $P < 0.001$).

Effects of symbiotic bacteria and food quantity on body size

Both food quantity ($F_{2,169} = 180$; $P < 0.001$) and microbiota ($F_{1,170} = 587.13$; $P < 0.001$) had a significant main effect on growth, and there was a significant interaction between these two factors ($F_{2,169} = 88.28$; $P < 0.001$) (Figure 2). For symbiotic animals, body size increased significantly with increasing food quantity (Tukey *post hoc*, P -adj. < 0.05). For germ-free animals, growth was slightly (but significantly) higher when food quantity increased from low to intermediate (Tukey *post hoc*, P -adj. < 0.05). However, no difference in size was observed between animals fed with intermediate and high food quantities. Within a particular food treatment, symbiotic animals were always significantly larger than germ-free ones (Tukey *post hoc*, P -adj. < 0.05), but the difference between symbiotic

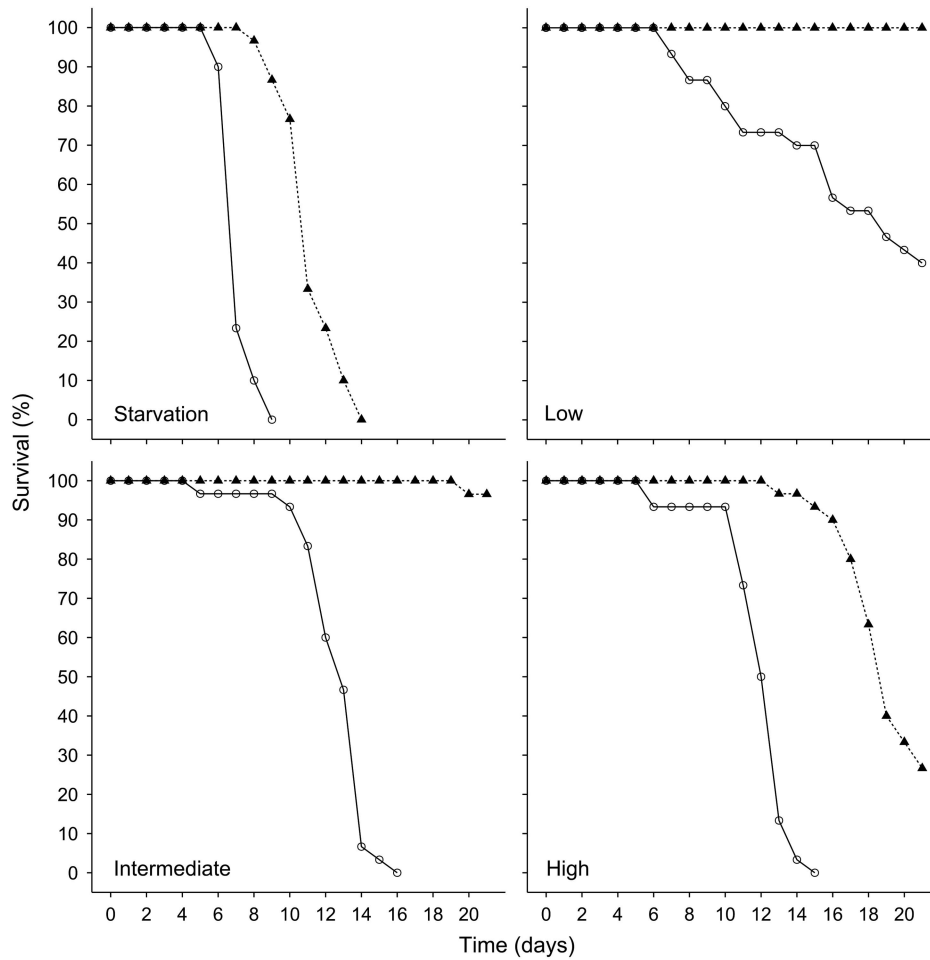


Figure 1 Percentage of surviving *Daphnia* exposed to different microbiota- (open circles = germ-free, black triangles = symbiotic) and food quantity (starvation = no food, low = 0.1 mg C l⁻¹ per day, intermediate = 0.5 mg C l⁻¹ per day, high = 2 mg C l⁻¹ per day) treatments.

Table 1 Summary of life-history parameters

Parameter	Food quantity	Symbiotic	Germ-free
Mean survival time (days)	High	18.9 d (s.d. = 2.0 d; n = 30)	12.1 d (s.d. = 1.9 d; n = 30)*
	Intermediate	20.9 d (s.d. = 0.4 d; n = 29)	12.8 d (s.d. = 2.0 d; n = 30)*
	Low	21.0 d (s.d. = 0.0 d; n = 30)	16.6 d (s.d. = 5.1 d; n = 30)*
	Starvation	11.3 d (s.d. = 1.5 d; n = 30)	7.2 d (s.d. = 0.8 d; n = 29)*
Mean body size (mm)	High	1.78 mm (s.d. = 0.11; n = 29)	1.13 mm (s.d. = 0.15; n = 29)*
	Intermediate	1.68 mm (s.d. = 0.10; n = 29)	1.18 mm (s.d. = 0.14; n = 29)*
	Low	1.13 mm (s.d. = 0.07; n = 29)	1.04 mm (s.d. = 0.09; n = 26)*
	Starvation	NA	NA
Observed egg deposition (%)	High	100% (n = 30)	6.70% (n = 30)*
	Intermediate	100% (n = 29)	3.30% (n = 30)*
	Low	0% (n = 30)	0% (n = 30)
	Starvation	0% (n = 30)	0% (n = 29)
Mean time to first clutch (days)	High	6.5 d (s.d. = 1.0 d; n = 30)	7.0 d (s.d. = 0.0 d; n = 2)*
	Intermediate	7.5 d (s.d. = 1.0 d; n = 29)	9.0 d (s.d. = 0.0 d; n = 1)*
	Low	NA	NA
	Starvation	NA	NA
Mean number of juveniles	High	4.6 (s.d. = 3.0; n = 30)	0.0 (s.d. = 0.0; n = 30)*
	Intermediate	7.3 (s.d. = 3.2; n = 28)	0.0 (s.d. = 0.0; n = 30)*
	Low	0.0 (s.d. = 0.0; n = 30)	0.0 (s.d. = 0.0; n = 30)
	Starvation	0.0 (s.d. = 0.0; n = 30)	0.0 (s.d. = 0.0; n = 30)

An asterisk indicates a significant difference between symbiotic and germ-free *Daphnia* within a food treatment.

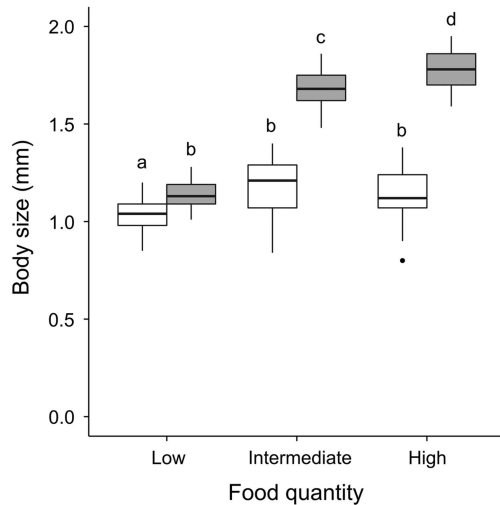


Figure 2 Boxplot of *Daphnia* body size exposed to different microbiota- (white = germ-free, gray = symbiotic) and food quantity (low = 0.1 mg C l⁻¹ per day, intermediate = 0.5 mg C l⁻¹ per day, high = 2 mg C l⁻¹ per day) treatments. Letters above samples represent differences as determined by a Tukey HSD *post hoc* test. Different letters are significant at 5%.

and germ-free animals was more pronounced under intermediate and high food levels than under low food levels.

Effects of symbiotic bacteria and food quantity on reproduction

The combined food- and microbiota treatments had a significant effect on egg production ($X^2 = 223.29$, $P < 0.001$). Both symbiotic and germ-free *Daphnia* receiving a low food quantity or starvation treatment never deposited eggs during the experiment (Table 1). In all treatments receiving a high- or intermediate food quantity, egg production was observed at least once. Within the high- and intermediate food quantity treatments, egg deposition was observed in 100% of symbiotic *Daphnia* during the experiment. However, only a small fraction of the germ-free individuals produced eggs (Table 1; high food quantity: 6.70%; intermediate food quantity: 3.30%).

There was a significant effect of both food availability ($X^2_1 = 11.7$, $P < 0.001$) and the microbiota ($X^2_1 = 18.3$, $P < 0.001$) on the age at first reproduction, the interaction between these two factors was not significant ($X^2_1 = 0.01$, $P = 0.91$). Symbiotic *Daphnia* receiving a high food quantity reproduced significantly earlier than those receiving an intermediate food quantity (HR = 2.5, $P < 0.001$). Furthermore, *Daphnia* receiving the same amount of food always reproduced significantly earlier in the presence of symbionts than when germ-free (high: HR = 0.01, $P < 0.001$; intermediate: HR = 0.01, $P < 0.001$) (Figure 3).

Only in symbiotic *Daphnia* did the eggs fully develop and were the juveniles released from the brood chamber (Table 1). Over the course of the

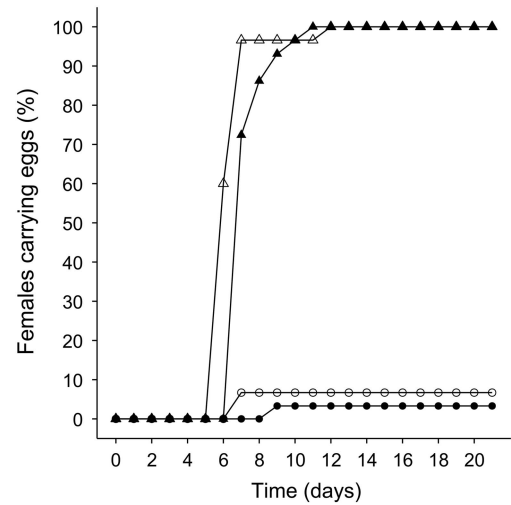


Figure 3 Percentage of *Daphnia* where production of first clutch eggs was observed in different microbiota (triangles = symbiotic; circles = germ-free) and food quantity (open = high (2 mg C l⁻¹ per day); filled = intermediate (0.5 mg C l⁻¹ per day)) treatments. Data from the starvation- and low food quantity treatments are omitted from the graphs because here no egg deposition was observed.

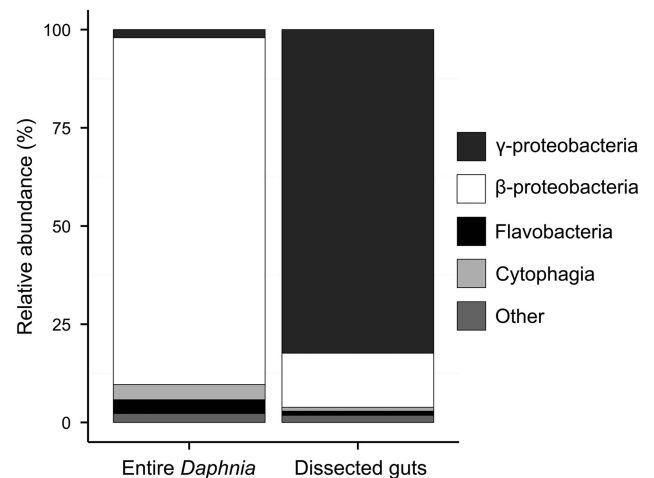


Figure 4 Class-level taxonomic composition of the bacterial communities found on entire *Daphnia* (left) and in dissected guts of *Daphnia* (right). The relative abundance of each class was calculated from mean values over three samples.

experiment, individuals receiving an intermediate food quantity produced a significantly higher number of juveniles than those receiving high amounts of food ($t_{28,30} = 3.27$, $P < 0.05$). However, the intrinsic rate of natural increase was very similar in the high food ($r_m = 0.16$) and intermediate food treatment ($r_m = 0.15$). This is attributable to an early peak in reproduction in the high food treatment compared with a later and more spread-out reproduction in the intermediate food treatment.

Identification of symbiotic bacteria

The entire bacterial community on *Daphnia* was found to be dominated by β -proteobacteria (mainly

Table 2 List of bacteria found in the microbiome of entire *Daphnia*

Phylum	Class	Order	Family	Bacterium	Accession number	Identity	Rel. ab. (%)	Reference
Proteobacteria	β-proteobacteria	Burkholderiales	Comamonadaceae	uncultured <i>Chlorochromatium</i> sp.	HM128814	95.27%	2.4 (0.7) %	Zhang et al., 2013
				uncultured <i>Hydrogenophaga</i> sp.	EU801511	95.80%	14.7 (2.7) %	Shaw et al., 2008
				uncultured <i>Limnohabitans</i> sp.	HM561445	96.56%	65.8 (0.6) %	Freese & Schink, 2011
Bacteroidetes	γ-proteobacteria	Aeromonadales	Aeromonadaceae	<i>Limnohabitans</i> sp. 2KL-7	HE600663	95.79%	1.1 (0.5) %	Kasalicky et al., 2013
				uncultured <i>Aeromonas</i> sp.	HM778882	94.15%	2.0 (1.9) %	Roeselers et al., 2011
				Bacteroidetes bacterium 3–6	AB578880	96.99%	2.6 (2.3) %	–
				Uncultured <i>Flavobacterium</i> sp.	DQ264455	97.54%	1.3 (0.4) %	DeSantis et al., 2007
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i> sp. HME6126	HM776984	100%	1.2 (1.0) %	–

Only OTUs with a mean relative abundance > 1% are included.

Table 3 List of bacteria found in the gut microbiome of *Daphnia*

Phylum	Class	Order	Family	Bacterium	Accession number	Identity	Rel. abundance (%)	Reference
Proteobacteria	β-proteobacteria	Burkholderiales	Comamonadaceae	uncultured <i>Acidovorax</i> sp.	JX120471	89.73%	1.4 (0.8) %	Handley et al., 2013
				uncultured <i>Limnohabitans</i> sp.	HM561445	96.56%	13.6 (12.3) %	Freese & Schink, 2011
Bacteroidetes	γ-proteobacteria	Aeromonadales	Aeromonadaceae	uncultured <i>Aeromonas</i> sp.	HM778882	94.15%	80.3 (15.2) %	Roeselers et al., 2011
				uncultured <i>Flavobacterium</i> sp.	DQ264455	97.54%	1.1 (0.9) %	DeSantis et al., 2007

Only OTUs with a mean relative abundance > 1% are included.

Limnohabitans sp. and *Hydrogenophaga* sp.), whereas γ -proteobacteria, flavobacteria and cytophagia occurred at lower relative abundances (Figure 4, Table 2). The gut microbiota, on the other hand, was found to be consistently dominated by γ -proteobacteria (mainly *Aeromonas* sp.), whereas the occurrence of other taxa was lower and more variable (Figure 4, Table 3).

Discussion

In this study we investigated the interaction between *Daphnia* and its microbiota under different levels of food availability. We observed a consistent mutualistic effect of the microbiota on its host. However, the relative impact of this mutualistic interaction on the hosts' fitness was dependent on food availability. This is reflected by the smaller difference in host growth and reproduction between symbiotic and germ-free *Daphnia* when food was limited compared with treatments where food availability was higher. The impact of the microbiota on host survival was also dependent on food availability and was the strongest under intermediate food quantities.

Our experiments clearly showed that removing the microbiota of *D. magna* had an overall negative effect on host growth, survival and reproduction. Although in this study we used a newly developed method to obtain germ-free *Daphnia* without the use of antibiotics, results from non-nutritionally stressed treatments are similar to what Sison-Mangus *et al.* (2014) found with respect to growth, survival and reproduction. This confirms the generality of the mutualistic relationship between *Daphnia* and its microbiota.

Characterization of the symbiotic bacterial community showed *Limnohabitans* sp. to be the dominant taxon, this is in accordance with earlier studies who also found this taxon to be a major constituent of the *Daphnia* microbiota (Freese & Schink, 2011; Eckert & Pernthaler, 2014; Peerakietkhajorn *et al.*, 2015; Gorokhova *et al.*, 2015). However, by specifically characterizing the gut microbiota, we found that in our cultures *Aeromonas* sp. is the dominant taxon in the digestive tract. Previous mono-association experiments with either *Limnohabitans* sp. or *Aeromonas* sp. showed that inoculating germ-free *Daphnia* with these bacteria had a positive effect on host fitness, although for *Limnohabitans* sp. this was found to be restricted to specific strains (Sison-Mangus *et al.*, 2014; Peerakietkhajorn *et al.*, 2015b). Interestingly, the dominant *Aeromonas* sp. sequence found in our *Daphnia* guts most closely matched a zebrafish gut symbiont (Roeselers *et al.*, 2011). In zebrafish, *Aeromonas* sp. is known to be a major constituent of the gut microbiota and mono-association experiments with this bacterium showed that it increases the expression of digestive enzymes and abundance of secretory cells in the gut epithelium. Its presence

furthermore enabled the uptake of protein macromolecules and influenced gut motility (Bates *et al.*, 2006). Interestingly, Gorokhova *et al.* (2015) found a strong decrease in feeding activity, digestion efficiency and carbon uptake in *Daphnia* of which the gut microbiota was affected by antibiotic exposure. Possibly, *Aeromonas* sp. or *Limnohabitans* sp. present in the *Daphnia* microbiota had similar effects on gut maturation as those found in zebrafish. These findings suggest that in our experiment the presence of the microbiota positively affected *Daphnia* through an enhanced nutrient acquisition, a hypothesis that is supported by the fact that the effect of the microbiota on host fitness was greater when food availability increased. Alternatively, the microbiota could also be important for neutralizing effects from harmful substances produced by the hosts' metabolism. Higher food intake can result in the release of higher concentrations of such harmful substances and reduce survival if these are not removed. For example, nitrogenous metabolic waste products are known to be efficiently degraded by symbiotic microbial communities in different organisms (Hoffmann *et al.*, 2009; Sabree *et al.*, 2009). This could also explain the stronger impact of the microbiota on the host when food availability increases.

In the starvation treatment, however, we observed a pronounced effect of the microbiota on host survival, suggesting that the microbiota have an additional role. Symbiotic *Daphnia* might make more efficient use of nutrients obtained via the egg than germ-free *Daphnia*, as they survived for a longer period in the absence of food. In mice, for example, the microbiota is known to shape metabolic and physiological adaptations of the host to periods of nutrient deprivation by enhancing utilization of stored fat reserves (Crawford *et al.*, 2009). It is also known that bacteria can act as a supplementary food source for *Daphnia*. These are, however, nutritionally inferior to algae (Martin-Creuzburg *et al.*, 2011; Taipale *et al.*, 2012). In our experiment, we controlled for the effect of feeding on bacteria by inoculating autoclaved microbiota to germ-free treatments. The starvation treatment is of particular interest here, as no food was added, which can support further bacterial growth. Nevertheless, we still found a pronounced effect on host survival, indicating that the presence of a live, metabolically active microbiota is essential.

Both symbiotic and germ-free *Daphnia* showed a clear response to food availability. However, an increase in food availability from intermediate to high was found to have less-pronounced effects on *Daphnia* growth and reproduction than when food availability increased from low to intermediate. In our experiment the saturating food concentration, at which an increase in food availability does not correspond to an increase in food intake, was probably situated somewhere between the intermediate and high concentration of algae, resulting

in a lower response. Nevertheless, an increase in caloric intake was associated with an overall increase in growth and reproduction. Survival, on the other hand, decreased with increasing food quantities. This discrepancy can be associated with a trade-off between growth and reproduction on one side, and survival on the other. Such a trade-off is commonly observed in *Daphnia* (Glazier & Calow, 1992; Dudycha, 2003). For example, Pietrzak *et al.* (2010) found that investments in early reproduction were costly in *Daphnia* and result in a shortened lifespan. In our experiment a similar pattern was observed. This was especially clear in the symbiotic treatments where under high food conditions *Daphnia* showed an early peak in reproduction and a reduced longevity compared with those receiving intermediate amounts of food. The intrinsic rate of natural increase remained, however, almost identical in both treatments.

In conclusion, we showed a consistent mutualistic effect of the *Daphnia* microbiota on its host, but food availability was found to affect the strength of these host-microbiota interactions. This shows that the effect of the microbiota on *Daphnia* can vary according to dietary conditions. The microbiota can thus be an important factor in determining responses of *Daphnia* to changing environmental circumstances. Further research is needed on both the response of host-microbiota interactions to other environmental factors and the precise nature of this interaction to be able to accurately quantify the functional importance of the microbiota for *Daphnia*.

Conflict of Interest

The authors declare no conflict of interest.

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