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Research Article

Stochastic variation in gut bacterial community affects reproductive rates in the water flea *Daphnia magna*

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Abstract

It is well-documented that perturbation of the gut bacterial community can influence the reproductive rates of the host. Less is known about how natural ecological processes can change the bacterial composition in the gut and how such changes influence the reproductive rate of the host. Here, we provide novel experimental insights into such processes using the clonally reproducing water flea, *Daphnia magna*. A total of 20 replicate cultures were reared for 5 weeks (Phase 1) to allow for divergence of bacterial communities through stochastic processes (i.e. drift, founder effects, and/or colonization). Duplicate cultures created from each of these were reared for 21 days (Phase 2) while recording reproductive rates. There was a significant repeatability in reproductive rates between these duplicates, suggesting that divergence of the bacterial communities during Phase 1 translated into reproductive rate effects during Phase 2. This was further supported by significant differences in the relative abundance of gut bacteria (investigated by amplicon sequencing of a part of the 16S rRNA gene) between cultures with high and low reproductive rate in Phase 2. These results are consistent with the hypothesis that stochastic processes can cause natural variation in the bacterial composition in the gut, which in turn affect host reproductive rates.

Keywords: *ecological processes*, gut bacterial community, *Daphnia magna*, reproductive rates, water flea

Introduction

All animals are complex ecological systems that constitute both host cells and microbial cells (Bromberg et al. 2015, Eisthen and Theis 2016). In humans, the ratio between human cells and microbial cells is around 1:1 (Sender et al. 2016, Rosner 2014). During the last two decades, we have gained a lot of new knowledge on host–microbe interactions. Intestinal bacteria have important positive functions that improve the reproductive rates of the hosts, including acquisition of energy and nutritional resources (Sonnenburg et al. 2005, Yatsunenko et al. 2012, Kamada et al. 2013a), protection against invading pathogens (Candela et al. 2008, Kamada et al. 2013a) and assistance in development (Bates et al. 2006, Haque and Haque 2017). On the other hand, parasitic microbes may negatively influence host reproductive rates, both directly and indirectly by outcompeting mutualistic microbes (Clemente et al. 2012, Kamada et al. 2013b).

The host's bacterial composition is regulated by genetics (Goodrich et al. 2014), social circle (Mushegian et al. 2019), and environmental factors like diet and lifestyle (Rothschild et al. 2018). Transmission of microbes among hosts, and between host and their environment, are assumed to be the factors causing most of the variability in the gut bacterial community. One example of this is significant similarity in the gut bacterial community of genetically unrelated humans sharing a household (Rothschild et al. 2018). This illustrates that also ecological interactions among the microbes can be important, and not only host–microbe interactions. A total of four fundamental ecological processes are involved in community assembly and have implications on both diversity and functionality; dispersal, speciation, selection, and drift (Vellend 2010). These processes result in both deterministic and stochastic changes in the microbiota.

Animal model organisms are used to improve our understanding of how the gut bacterial community is assembled and how it affects the host (Douglas 2019). These model organisms include both invertebrates and vertebrates, including the crustacean freshwater water flea *Daphnia magna* (Orsini et al. 2016). A comparison of conventional and germ-free animals documented the effect of the intestinal bacterial community on the fitness of *D. magna*, and showed that germ-free water fleas were smaller, less fecund, and had higher mortality than those with a gut bacterial community (Sison-Mangus et al. 2015). These authors suggested that species within the genus *Aeromonas* could contribute to an increase in the body size of *D. magna*. Peerakietkhajorn et al. (2016) showed that germ-free *D. magna* reinfected with *Limnohabitans* strain DM1 and *L. planktonicus* II-D had a higher number of viable juveniles than bacteria-free water fleas. In addition, high mortality is reported for *D. magna* fed *Hydrogenophaga* sp. or *Pseudomonas* sp. (Martin-Creuzburg et al. 2011). So far, all studies on the gut bacterial community of *D. magna* have involved strong perturbations of the gut bacterial community. Little knowledge exists on how natural ecological processes such as dispersal, drift, and selection can result in changes in the bacterial composition in the gut of hosts, and how these changes affect the reproductive rates of the host.

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The hypothesis of this study was that isolated populations of the same clone of *D. magna* could over time develop distinct bacterial communities due to stochastic processes, and that these differences in the composition of the gut bacterial community affect the fitness of *D. magna*. To allow for independent development of the gut bacterial community 20 independent cultures of *D. magna* were treated equally for 5 weeks (Phase 1), with the assumption that this period was sufficient to obtain variation in the gut bacterial community due to stochastic processes (Phase 1). Thereafter, two replicates from each of the 20 independent cultures were made, and the production of offspring and maternal mortality of these cultures were monitored daily for 21 days (Phase 2). The bacterial composition of the gut (Phases 1 and 2) and water (Phase 2) was determined by amplicon sequencing of a part of the 16S rRNA gene, and the daily average number of offspring per mother was used as an estimate of reproductive rates. We compared the gut bacterial community between cultures with high and low reproductive rates from Phase 2, and evaluated the possible significance of specific bacterial taxa. We also investigated to what extent the bacterial composition in the water from Phase 2 affected the gut bacterial community of *D. magna* in the same phase.

Materials and methods

Cultivation conditions and experimental design

The clone of *D. magna* used in this experiment (lab id EF 47) originated from an ephippium collected in a pond at Værøy Island, northern Norway (67.687° N, 12.672° E), and was hatched in December 2014. During subsequent rearing, and for the whole experimental period, live animals were kept in thermal cabinets at standardized environmental conditions (20°C, 24 h light) in a modified ADaM medium (Klüttgen et al. 1994), SeO_2 concentration reduced by 50% and sea-salt was increased to 1.23 g l^{-1}). The animals were fed Shellfish Diet 1800® (Reed Mariculture Inc, USA) three times per week. The jars used for cultivation were repositioned every second day throughout the experiment to achieve similar light-conditions.

The experiment consisted of two phases (hereafter, Phases 1 and 2, Fig. 1). In Phase 1, we allowed for stochastic ecological processes (e.g. drift) in 5 weeks to create variation in the bacterial community among a set of 20 cultures. The duration of 5 weeks approximates five generations of *D.magna* at the used temperature, and a minimum of 70 generations of a typical bacterial taxon due to short gut passage time and high mortality from predation. In Phase 2, duplicate cultures were inoculated with medium and animals from each of the 20 cultures in Phase 1. For the Phase 2 cultures, we tested if variation in reproductive rates was repeatable across replicates (i.e. whether duplicates were more similar to each other than to replicates originating from other Phase 1 cultures), and if cultures with high vs. low reproductive rates differed in their intestinal bacterial composition.

Phase 1 started with transfer of four individuals (two juveniles and two adults) from stock aquaria into 20 replicate glass jars that contained 125 ml of autoclaved medium. A total of three times per week, 42 ml autoclaved medium was added to the jars, and then once a week the volume was reduced back to 125 ml. This procedure ensured appropriate medium quality, while maintaining the bacterial community in the jars. During volume reductions we also counted and removed individuals that had been born during the preceding week, whereas the original individuals were left in the jars (distinguished by size). If any of the original mother individuals died, these were replaced by allowing newborn individuals to remain in the jar. Phase 1 was terminated after 5 weeks, and Phase 2 was initiated (see below). All adult individuals were sampled from the 20 jars and stored at −20°C until characterization of the bacterial composition in the gut.

Phase 2 was initiated by transferring 125 ml of medium, samples of biofilm, and four neonates (< 24 h old) from each of the 20 jars from Phase 1 into two autoclaved 250 ml jars, providing two replicates from each original jar (i.e. 40 cultures in total). The biofilm was transferred by rubbing a sterile Q-tip against the glass wall and transferring it into the culture water in the new culture. Mortality among the transferred individuals and number of offspring produced were registered daily for 21 days. Offspring were removed during counting. Medium replacement followed the same procedure as for Phase 1. The cumulative number of mother days was calculated for each culture by summing up the number of surviving individuals per day for the duration of the experiment. Thus, as an example, for jars where all daphniids survived during the experiment the cumulative number of mother days was 84 (four individuals multiplied with 21 days). From these data, reproductive rates were calculated for each culture as the total number of offspring produced during the 21 days divided by the cumulative number of mother days. The reproductive rate is thus the daily average of offspring per mother present in the jar. We then identified the six cultures with highest and the six with lowest reproductive rates, but excluded replicate cultures (i.e. if both replicates from a single source culture in Phase 1 were among the six lowest or highest, only one of them was chosen). These 12 cultures were sampled for culture water (1.7 ml in Eppendorf tube and 60 ml on Sterivex filter). All surviving adult individuals from all 40 cultures were sampled. The culture water samples collected in Eppendorf tubes were fixed with 1% glutaraldehyde (final concentration), stored at room temperature for 30 min and snap frozen in liquid nitrogen. These fixed samples, as well as Sterivex filters and surviving adult individuals were stored at −20°C until further analysis.

Characterization of bacterial community composition

Bacterial community composition was determined by high throughput sequencing of PCR amplicons of a ≈ 450 base pair long stretch of the 16S rRNA gene. Mother individuals that had been sampled at the end of Phases 1 and 2 had their intestines dissected out under microscope using sterile forceps. Up to four intestines (depending on survival in Phase 2) from the same jar were transferred to a tube with 500 µl sterile-filtrated ADaM and stored at −20°C until DNA extraction. During the DNA extraction from free-living bacteria collected on Sterivex filters, the filter capsules were opened and the filter inside were cut into small pieces using sterile tools. The genomic DNA from intestines and filters were extracted using Qiagen DNeasy® PowerSoil® Kit (100) according to the manufacturer's protocol and stored at −20°C until quality and quantity of the DNA extract were measured with a Thermo Scientific™ NanoDrop™.

PCR and Illumina sequencing were done according to Fossmark et al. (2020). The exception was that the V3 and V4 regions of the 16S rRNA gene was targeted for sequencing, by using forward primer ill338F (5′-tcg tcg gca gcg tca gat gtg tat aag aga cag nnnn CCT ACG GGW GGC AGC AG-3′) and reverse primer ill805R (5′-gtc tcg tgg gct cgg aga tgt gta taa gag aca g nnnn GAC TAC NVG GGT ATC TAA KCC-3′). Illumina adapter sequences are in

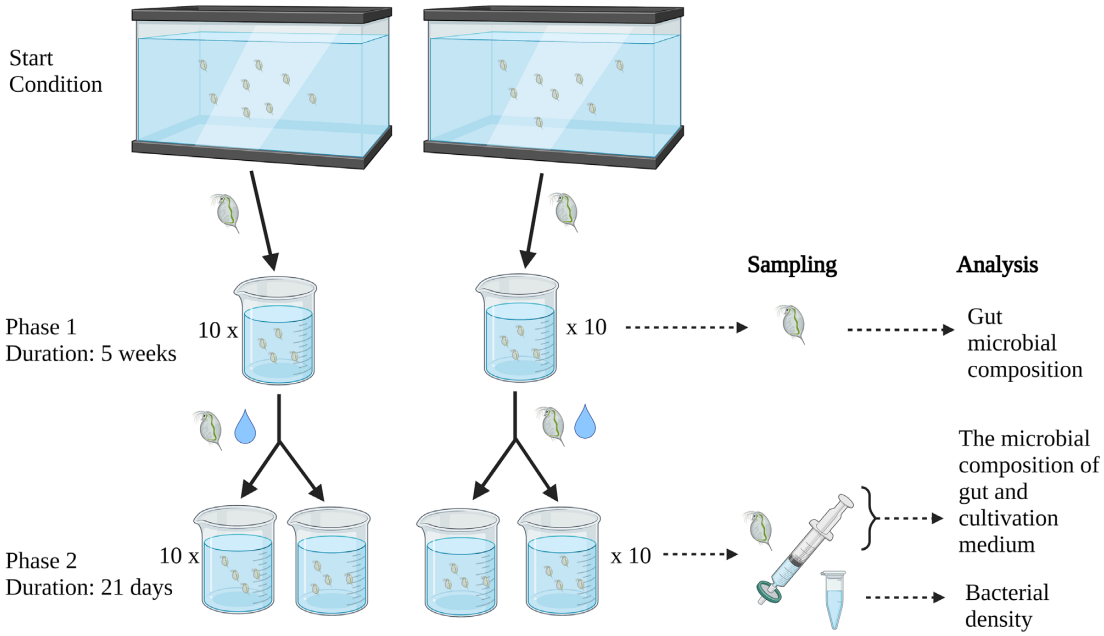

Figure 1. Experimental design. A single clone of *D. magna* that had been sourced from a common stock culture was allowed to propagate in two identical aquaria (start condition). From these, four individuals (two juveniles and two adults) were transferred into each of 20 jars (Phase 1). After 5 weeks of cultivation, medium and four neonates (< 24 h old) from each of the 20 jars were separated into two replicates (Phase 2), and live adults were sampled for analyses of gutbacterial community. After 21 days Phase 2 was terminated, and live adults and culture water were sampled for microbial analysis. The bacterial density was quantified for the culture water.

lower case letters. The PCR reaction was run for 35 cycles (98°C—15 s; 53°C—20 s; and 72°C—20 s) with 0.75 μl of each primer, 10 mM of each dNTP (VWR), 0.2 μl Phusion Hot Start DNA polymerase (Thermo Scientific), 50 mM $MgCl_2$ (Thermo Scientific), and reaction buffer (Thermo Scientic) in a total volume of 25 μl, including 1.0 μl DNA extract as template. Successful amplification of the PCR products was verified by gel electrophoresis. The amplicon library was sent for MiSeq sequencing (Illumina) at the Norwegian Sequencing Centre (NSC). The resulting Illumina sequencing data was deposited at the European Nucleotide Archive (accession numbers ERS10770888–ERS10770978—ongoing). The USEARCH pipeline was used to analyze the MiSeq data as previously described. The procedure was done according to Fossmark et al. (2020), with the exception that reads shorter than 400 base pair were excluded. The Ribosomal Database Project (RDP version 16; Cole et al. 2014) was used as reference to assign taxonomy to the OTUs at 97% sequence similarity.

The OTU table was compared with the negative controls from the extraction kit and nontemplate controls. A total of two OTUs, representing *Propionibacterium* and *Escherichia/Shigella*, were removed due to presence in the negative controls and low probability for them to inhabit the intestine of *D. magna*. Also, OTUs classified as chloroplast or phylum Cyanobacteria were removed, as the study had focus on heterotrophic bacteria. To obtain equal sequencing depth for all samples they were normalized to 27 000 reads, the lowest number of reads observed in one sample. The normalized table was summarized at various taxonomic levels with the command sintax_summary in USEARCH.

Quantification of bacterial densities

We used flow cytometry for quantification of bacterial densities. The 12 samples of culture water from jars with high and low reproductive rates were diluted 1:4 with 0.2 μm filtered phosphate-buffered saline (PBS), stained with SYBR® Green I (Thermo Fisher Scientific) diluted 5000x and incubated in the dark for 20 min at 37°C. Analysis was done with BD Accuri™ C6 flow cytometer (BD Bioscience), with a flow rate of 35 μl/min for 2 min and with a threshold of 1000 for the FL1 channel. FL1 (533/30 nm) is most suitable for detection of DNA/SYBR fluorescence, and FL3 (> 670 nm) was used for detection of cells with chlorophyll (McKinnon 2018). The flow cytometry data were imported into R (V 3.6.2; R Core Team 2020) using the flowCore package (V 1.11.20; Hahne et al. 2009). The data were arcsine hyperbolic transformed with the trandorm() function, and bacteria and algae were separated from noise by manual gating in xy-plots of FL1 vs. FL3. The bacterial cell-density was calculated by correcting for analyzed volume and dilution.

Data analysis and statistics

Repeatability of the reproductive rates-estimates between replicates (Phase 2) that originated from the same jar from Phase 1 were estimated in R (V.4.0.0; R Core Team 2020). We used the package ICC (Wolak et al. 2012) to estimate the intraclass correlation coefficient.

Alpha (α)- and beta (β)-diversity was quantified using Past (Hammer et al. 2001; version 4.01). The coverage of the sequencing was calculated by dividing the number of OTUs in a sample (richness) by the Chao-1 index. To quantify α-diversity we used Hill numbers (^{q}D) of order q and Evenness (^{1}E) based on Hill numbers, as they avoid the inherent problems with entropy-based indices (Jost 2006). To emphasize the impact of both richness and relative abundance we used Hill numbers of order 0 (i.e. richness) and 1 (i.e. Exp Shannon index), respectively. Evenness was calculated as $^{1}D/^{0}D$. To evaluate difference in α-diversity between groups of samples, a two-sample *t*-test was performed. F-test was used to evaluate equal variance between groups.

For analysis of beta (β)-diversity we used Dice-Sørensen and Bray–Curtis similarity. In this way, we could evaluate the signifi-

cance of presence/absence and relative abundance, respectively. The similarity between the samples was visualized by a principal coordinates analysis (PCoA) using both similarity indices. To evaluate the similarities within and between groups (phases, sample-types or reproductive rates) the averages and standard deviations were calculated. To test for statistical differences between groups of samples (phases, sample-types, or reproductive rates) a permutational multivariate analysis of variance (PERMANOVA) was done based on both Bray–Curtis and Dice-Sørensen similarities (Anderson 2001). SIMPER (Similarity Percentage) was used to identify which OTUs that contributed most to the differences between groups of communities (Clarke 1993).

Results

Reproductive rates of *D. magna*

The reproductive rates during Phase 2 varied considerably among jars (Table S1, Supporting Information), ranging from 0 (the populations in two jars went extinct prior to any reproduction) to 2.86 offspring per mother day, and with a mean of 1.98 (SD 0.62, Fig. 2). There was significant repeatability in this variation between jars that originated from the same source population in Phase 1, with an intraclass correlation coefficient of 0.45, and with a 95% CI (for alpha = 0.05) that did not overlap with zero (0.10–0.81).

The alpha and beta diversity and taxonomic composition of bacterial communities among sample types and phases

The 78 samples had a total number of 4661 525 sequence reads after quality filtering, chimera removal and removal of OTUs identified as chloroplasts/cyanobacteria or contamination. The number of reads for each sample ranged from 15 901 to 121 872 reads, with an average of 59 763 reads (± 18 252 SD). After the sequence reads were clustered into OTUs with a similarity level of 97% and normalized to 27 000 reads per sample to avoid bias due to sequencing depth, the total number of OTUs were 514. On average, the actual sequencing depth covered 81.4% (± 14% SD) of the estimated total OTUs (Chao-1), range 38%–100%.

The average richness (^{0}D) in the culture water (108 OTUs) was about two times higher than the average richness in the intestines for combined samples from Phase 1 and 2 (59 OTUs). A two-sample *t*-test confirmed significant difference ($P < .0001$) between the two sample-types. The microbial communities in intestines and culture water had similar evenness (^{1}E), with averages of 0.094 and 0.093, respectively, and were not significantly different ($P = .500$). An evenness lower than 0.2 indicates that a few OTUs were dominant in the samples. ^{1}D indicates that the culture water had a significantly higher diversity than intestine due to higher richness ($P < .0007$; two-sample *t*-test).

There was no significant difference in richness (^{0}D, $P = .539$) or evenness ($P = .058$) between the intestines in Phases 1 and 2. However, ^{1}D, including both richness and evenness, revealed that the diversity in Phase 1 (6.59 ± 2.71 SD) was higher than in Phase 2 (5.20 ± 1.12 SD; $P = .043$).

A comparison of the bacterial community composition in the intestines from the two phases revealed that the Bray–Curtis similarity within the groups was 63% and 70% for Phases 1 and 2, respectively, and 65% between the two phases. Similar data for Dice-Sørensen similarity was 60%, 60%, and 57%. A one-way PERMANOVA based on Bray–Curtis similarity revealed a significant difference in the microbial community composition of intestine samples between Phases 1 and 2 ($P = .0443$). PERMANOVA based on Dice-Sørensen similarity on the same samples provided stronger support for significant difference between Phases 1 and 2 ($P = .0001$). This indicates that the changes in community composition from Phase 1 to Phase 2 was caused mainly by differences in the OTU inventory, and due to loss of rare OTUs. This is consistent with the observed differences in alpha-diversity. The five OTUs contributing the most to the significant difference in the community composition between Phases 1 and 2 and that explained 69% of the difference, were identified with lowest classification to *Limnohabitans,* Bacteroidetes, *Pedobacter*, Comamonadaceae, and *Pseudomonas.*

Comparison of the bacterial community composition between *Daphnia* cultures with high or low reproductive rates

The richness of the intestine samples in the groups with high and low reproductive rates (Phase 2) had a mean of 60 (range 39–91) and 57 (range 38–70) OTUs per sample, respectively. The difference was not significant ($P = .726$). The evenness of the two groups was low and very similar (0.096 and 0.090 respectively; $P = .651$). Similarly, ^{1}D between the group with high and low reproductive rates was not significant different (5.51 and 5.04, respectively; $P = .511$).

Bacterial community composition of intestines from *D.* magna from the jars with high and low reproductive rates clustered separately in ordination with PCoA based on Bray–Curtis similarity (Fig. 3), indicating differences in the bacterial community composition between individuals with high and low reproductive rates. A total of 75.2% of the variance in the data was explained by the first two coordinates. PERMANOVA with Bray–Curtis similarity confirmed that the difference in bacterial community composition between high and low reproductive rates were statistically significant ($P = .033$). PERMANOVA based on Dice-Sørensen index gave no significant difference ($P = .584$). This indicates that the differences in community composition for individuals with high and low reproductive rates was caused by the differences in the relative abundance of the OTUs, and not by differences in the OTU inventory.

A comparison of the bacterial communities in the intestines from the two reproductive rate levels revealed that the Bray–Curtis similarity within the groups was 80% and 69% for high and low reproductive rates, respectively, and 70% between the two reproductive rate levels. Similar data for Dice-Sørensen was 65%, 60%, and 63%. The taxonomic variation at the genus level for samples with high and low reproductive rates confirmed the similarity in OTU inventory and difference in relative abundance (Fig. 4). The classification "unassigned" is mainly OTU_4 (Bacteroidetes), OTU_6 (Comamonadaceae), and OTU_10 (Betaproteobacteria).

A total of six OTUs explained 82.5% of the difference in the community composition between individuals with high and low reproductive rates (Table 1). Individuals with high reproductive rates had a higher abundance of Bacteroidetes, and lower abundance of *Limnohabitans*, *Pedobacter*, Betaproteobacteria, and Comamonadaceae than those with low reproductive rates. The OTU characterized as Bacteroidetes (OTU_4) was classified as either Flavobacteria or Sphingobacteria at the class level, according to Ribosomal Database Project (RDP; Cole et al. 2014). Betaproteobacteria (OTU_10) was classified as order Burkholderiales, and either Burkholderiaceae or Oxalbacteraceae at family level.

In addition to the gut bacterial community of the founder individuals of the cultures for Phase 2, the bacterial composition in the culture water and in the algal suspension are the

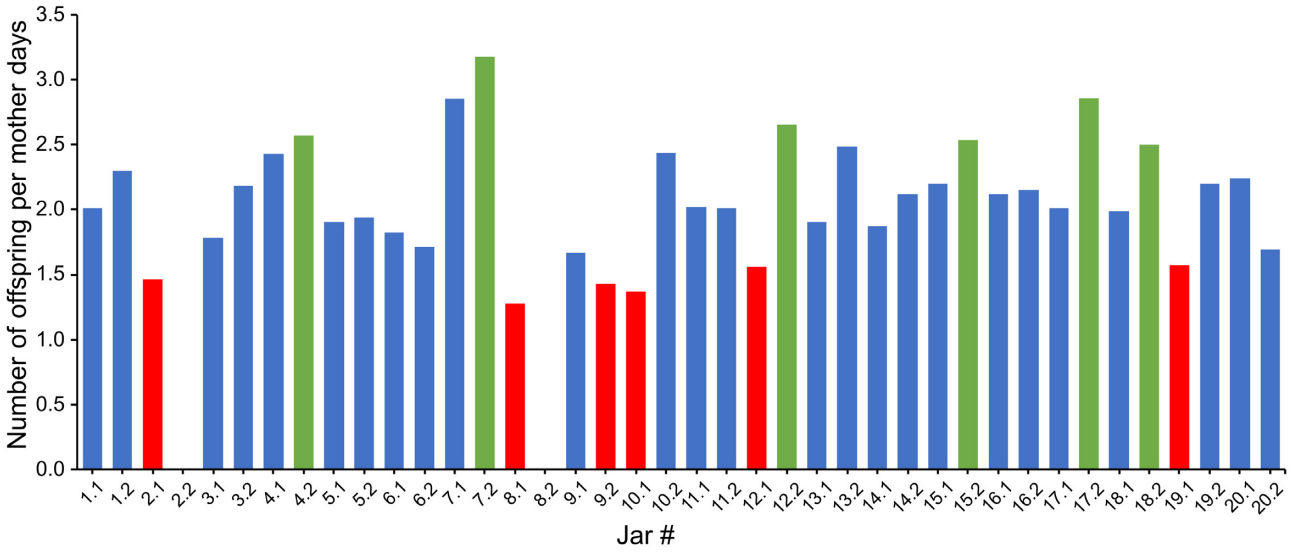

Figure 2. Reproductive rates (average number of offspring per mother and day) for jars in Phase 2. Jars are numbered such that the first number refers to the jar of origin in Phase 1. The six jars with low reproductive rates (■, value < 1.57) and the six with high reproductive rates (■, value > 2.50) that were selected for analyses of microbial community composition are indicated.

Table 1. The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic difference in intestine microbiota of *D. magna* sampled from jars with high and low fitness, and their relative abundance given in fraction for each group. *Lowest classification: g = genus, f = family, and c = class

OTU_id	Taxonomy	Contribution	Average relative abundance	
	Lowest classification* (Phylum)	%	High reproductive rates ± SD	Low reproductive rates ± SD
OTU_4	Unclassified (Bacteroidetes)	28.66	0.240 ± 0.086	0.082 ± 0.084
OTU_1	*Limnohabitans* (g) (Proteobacteria)	23.11	0.378 ± 0.102	0.449 ± 0.135
OTU_2	*Pedobacter* (g) (Bacteroidetes)	10.04	0.179 ± 0.044	0.189 ± 0.066
OTU_6	Comamonadaceae (f) (Proteobacteria)	9.09	0.094 ± 0.032	0.134 ± 0.046
OTU_10	Betaproteobacteria (c) (Proteobacteria)	6.09	0.004 ± 0.006	0.037 ± 0.080
OTU_9	*Pseudomonas* (g) (Proteobacteria)	5.56	0.002 ± 0.003	0.033 ± 0.062

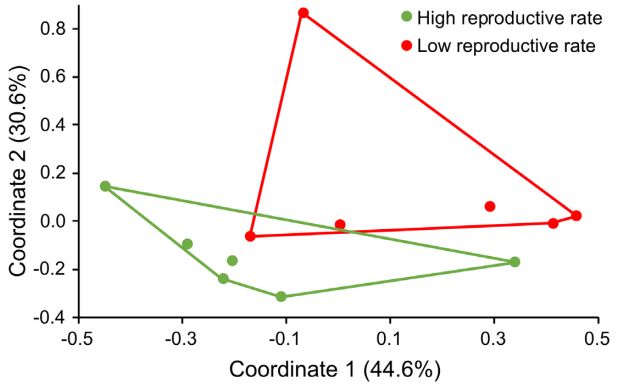

Figure 3. PCoA based on Bray–Curtis similarity of two groups of intestines from *D. magna* (Phase 2) classified as high or low reproductive rates. The percentage after the coordinate number indicates how much of the variance in the data that is explained by that axis.

main sources of the intestinal bacterial composition for *D. magna*. Ordination by PCoA indicates distinct differences between the bacterial communities from cultivation water and intestines at the end of Phase 2 (Fig. 5). PERMANOVA confirmed that the difference in microbial community composition was highly significant (P = .0001) between samples from intestine and culture water, based on both Bray–Curtis and Dice-Sørensen similarities. The Bray–Curtis similarity between intestine and culture water was 34% and the Dice-Sørensen similarity was 50%. Therefore, the differences in community composition were due to differences in both OTU inventory and the relative abundance of the OTUs in the different cultures. A total of five OTUs explained 68% of the difference in the community composition between intestines and culture water (Table 2). The phylum Bacteroidetes, the genus *Limnohabitans* and the family Comamonadaceae were most dominant in the intestines, whereas the genera *Flavobacterium* and *Pedobacter* dominated in the culture water.

The culture water from the cultures with high and low reproductive rates was also compared with each other. The difference in the relative abundance of the OTUs was marginally significant based on Bray–Curtis similarity (P = .052). There was no significant difference based on Dice-Sørensen similarity (P = .4201). The culture water from jars with high and low reproductive rates revealed that the Bray–Curtis similarity within the group was 51% and the Dice-Sørensen similarity was 74%.

The possible effect of bacteria cell density on reproductive rates was investigated using 12 samples of culture water that included jars with high and low reproductive rates. There was no statisti-

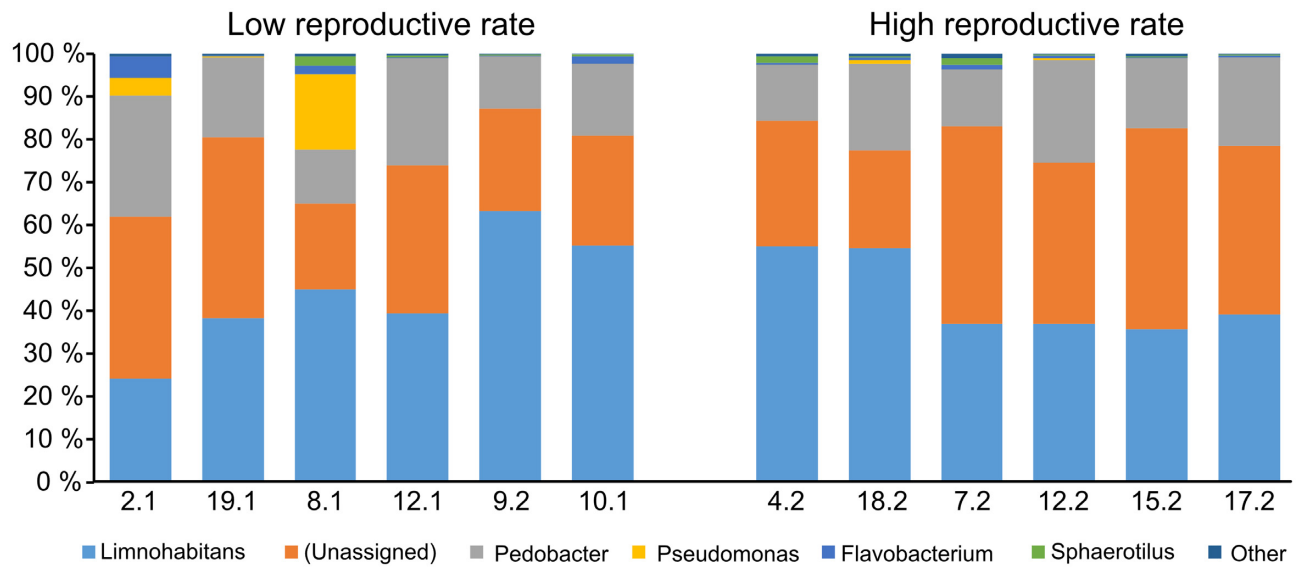

Figure 4. The taxonomic composition at the genus level of the bacterial community sampled from *D. magna* intestines of jars with low and high reproductive rates. Jars are numbered such that the first number refers to the jar of origin in Phase 1, and the second is the replicate number.

Table 2. The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic difference between intestines and culture water, and their relative abundance given in fraction for each group. *Lowest classification: g = genus, f = family

OTU_id	Taxonomy	Contribution	Avg. rel. abundance	
	Lowest classification* (Phylum)	%	Intestine	Culture water
OTU_1	*Limnohabitans* (g) (Proteobacteria)	24.53	0.389	0.065
OTU_7	*Flavobacterium* (g) (Bacteroidetes)	15.15	0.004	0.206
OTU_2	*Pedobacter* (g) (Bacteroidetes)	11.82	0.191	0.251
OTU_4	Unclassified (Bacteroidetes)	10.60	0.149	0.008
OTU_6	Comamonadaceae (f) (Proteobacteria)	5.64	0.116	0.105

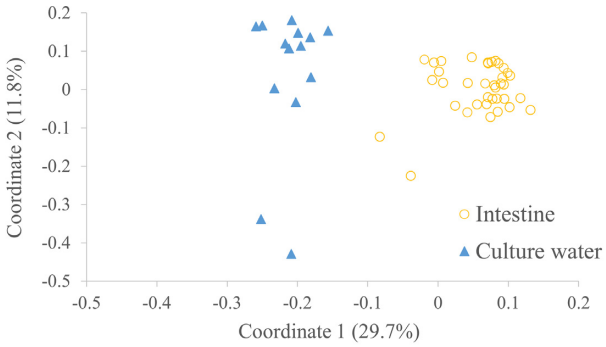

Figure 5. PCoA based on Bray–Curtis similarity of the OTUs in the intestine (circles) and culture water (triangles) from *D. magna* cultures. Data from Phase 2 include samples of both sample types from 14 jars, the intestines are from all jars in Phase 2. The percentage after the coordinate number indicates how much of the variance in the data, i.e. explained by that coordinate.

cal difference in cell-density between cultures with high and low reproductive rates (high: $2.72{\cdot}10^6 \pm 0.97{\cdot}10^6$ SD; low: $2.13{\cdot}10^6 \pm 0.70{\cdot}10^6$ SD, *t*-test: $P = .306$).

Discussion

The results of the present study are consistent with the hypothesis that variation in the bacterial composition in the gut that occur through stochastic processes (i.e. drift, founder effects, and/or colonization) can affect reproductive rates of *D. magna*. To test this hypothesis, we first reared populations for 5 weeks to allow for divergence in their bacterial community (Phase 1), and then studied the repeatability in reproductive rates between replicate cultures that originated from these founder populations (Phase 2). We also tested for an association between the reproductive rate in Phase 2 and the bacterial composition in the gut at the end of this phase. We found significant repeatability of reproductive rates between replicates (i.e. two replicates originating from the same founder population were more similar with respect to reproductive rate than two replicates originating from different founder populations), and a significant difference in the relative abundance of OTUs in *Daphnia* guts between cultures with high and low reproductive rates.

Our data suggest that stochastic ecological processes and selection are important for the bacterial composition in the gut in *Daphnia*. First, alpha-diversity which consider relative abundance (^{1}D) was significantly higher during Phase 1 than during Phase 2. This indicates that selection for OTUs that were more adapted to the conditions and gut ecosystem and/or drift was important for bacterial community composition. Second, a significant difference in the bacterial community composition between the gut and the culture water in Phase 2 suggest a different selection pressures in these two environments. The differences are strong, even though *Daphnia* release bacteria to the water during fecation, and take up bacteria from the environment during filter feeding. Our

finding is consistent with previous studies showing that although the bacterial community in the rearing water serves as a species pool from which aquatic organisms acquire gut bacterial communities (Vadstein et al. 2018a), the gut environment imposes a selection regime for the microbial community, which is different from the selection in the environment (Wong and Rawls 2012, Bakke et al. 2015, Smith et al. 2015, Foster et al. 2017). Finally, the difference in the bacterial composition in the gut between cultures with high and low reproductive rates in Phase 2 suggest that also stochastic processes during Phase 1 structured the bacterial community, because the selection pressures should be identical among replicates throughout the experiment (a single clone of *Daphnia* and identical rearing conditions).

Comparison of the bacterial community composition within the intestines between cultures with high and low reproductive rates revealed significant difference in the relative abundance of the OTUs between the two groups (Bray–Curtis similarity), but no significant difference in the OTU inventory (Dice-Sørensen similarity). Cultures with high reproductive rates were dominated by Bacteroidetes, whereas *Limnohabitans*, *Pedobacter,* Comamonadaceae, Betaproteobacteria, and *Pseudomonas* were more abundant in the cultures with low reproductive rates. Existing knowledge about these groups of bacteria provides insight into possible mechanisms for the observed differences in reproductive rates among independently cultivated groups of *D. magna*. The phylum Bacteroidetes constitutes a major part of the microbial community in the digestive tract of a large number of animals, including *Daphnia* (e.g. Freese and Schink 2011). In humans, Bacteroidetes is mutualistic and contributes with the degradation of organic matter with high molecular weight, including proteins and carbohydrates (Bäckhed et al. 2005, Thomas et al. 2011). Relative abundance of Bacteroidetes (OTU_4) was 3x higher in the cultures with high relative to low reproductive rates (24% and 8%, respectively), suggesting that Bacteroidetes have a beneficial role for reproductive rates. In humans, butyrate is a product of the fibre fermentation performed by gut Bacteroidetes and Firmicutes, and is associated with good health and normal development of gut epithelia (Ley et al. 2006). In addition, butyrate is known for beneficial effects against e.g. colon cancer development, type 2 diabetes, obesity and cardiovascular disease (Lupton 2004, Kim and Milner 2007, Thomas et al. 2011, Vital et al. 2017). On the other hand Bacteroidetes may also be pathogenic due to the production of polymer-degrading enzymes, which damage cellular components in the host (Thomas et al. 2011). More research is needed to verify a possible positive effect and mechanisms of Bacteroidetes on reproductive rates of *Daphnia*.

At the genus level, *Limnohabitans* (OTU_1) dominated the intestinal bacterial community in *D. magna* for individuals with both high and low reproductive rates, and accounted for 38% and 45% of the relative abundance in the cultures with high and low reproductive rates, respectively. The genus is a stable member of the digestive tract in *Daphnia*, independent of starvation and diets (Freese and Schink 2011, Peerakietkhajorn et al. 2016, Motiei et al. 2020). *Limnohabitans* is reported to increase the fecundity and population size of aposymbiotic *D. magna* after reinfection (Peerakietkhajorn et al. 2015, Peerakietkhajorn et al. 2016). The average abundance of *Limnohabitans* collected for all the intestines (Phases 1 and 2) was 40% and the difference in abundance was low between the two groups with high and low reproductive rates. This indicate that higher reproductive rates were not induced by increased abundance of *Limnohabitans*. Our data indicate that *Limnohabitans* has different effects alone than when present in combination with other intestinal bacteria. Several mechanisms may give such an effect, including functional redundancy and inhibition of activity. For example, a potential microbe–microbe interaction has been reported between *Pedobacter* and *Limnohabitans* (Cooper and Cressler 2020).

Normally, OTUs within several genera in the family Comanadaceae are present in the gut bacterial community of *Daphnia* (e.g. *Limnohabitans*, *Hydrogenophaga*, and *Pelomonas*; Gorokhova et al. 2015, Motiei et al. 2020). Abundance of Comanadaceae (OTU_6) in the cultures associated with high and low reproductive rates was 9.4% and 13.4%, respectively. It was difficult to identify which genus OTU_6 belongs to, but according to RDP *Hydrogenophaga* is a good candidate. It has been shown that *Hydrogenophaga* was present in daphniids with low somatic growth and low fecundity (Motiei et al. 2020). This is in accordance with the results from our experiment, and it can be one of the factors causing differences in the reproductive rates. Martin-Creuzburg et al., (2011) reported that all *D. magna* fed *Hydrogenophaga* sp. died after 2 days.

Pseudomonas (OTU_9) was also a part of the bacterial community in *Daphnia* gut. *Pseudomonas* in the gut community has been reported as harmful for the fruit fly *Drosophila* (Vodovar et al. 2005, Hilbi et al. 2007) and the nematode *Caenorhabditis elegans* (Hilbi et al. 2007). In the present experiment, there was a 16x higher abundance of *Pseudomonas* in the cultures with low reproductive rates (3.3%) than cultures with high reproductive rates (0.2%). The three jars with the lowest number of offspring per mother days had the overall highest relative abundance of *Pseudomonas* (jar 2.1, 8.1, and 9.2). In addition, replicates of culture 2.1 and 8.1 (i.e. jar 2.2 and 8.2) had a high mortality in the beginning of Phase 2. This indicates that *Pseudomonas* influenced the reproductive rates of *Daphnia* negatively. *Pseudomonas* without species identification has been reported to be toxic to *D. magna* (Martin-Creuzburg et al. 2011). It has been shown that the abundance of *Pseudomonas* increased during antibiotic treatment, starvation and before death (Freese and Schink 2011, Preiswerk et al. 2018, Akbar et al. 2020).

In conclusion, our data and current knowledge on microbe–host interactions support that the observed differences in the gut bacterial community contributed to the observed differences in reproductive rates between independently grown cultures of *D. magna*. The differences in microbial composition for individuals with high and low reproductive rates, was due to differences in the relative abundance and not the inventory of OTUs. Stochastic ecological processes (e.g. drift) likely cause these differences in the gut bacterial community at a time scale of weeks. This is the first study to demonstrate that stochastic processes can affect the bacterial composition in the gut in *Daphnia* under conditions with normal cultivation, and that this change significantly affect reproductive rates.

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Supplementary data

Supplementary data are available at FEMSEC online.

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