Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Kathrine Obrestad

The effects of gut microbiota on fitness in *Daphnia magna*

Master's thesis in Biotechnology (MBIOT5)

Supervisor: Olav Vadstein, Sigurd Einum and Ingrid Bakke

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Kathrine Obrestad

Abstract

The assembly of microbial communities in the gastrointestinal tract is shaped by host genetics, conspecifics within same habitat, and environmental factors. Alternation of the microbial community composition can influence the hosts' physiological and mental health. Variable richness and relative abundance of the operational taxonomic units (OTUs) in the microbial composition are a result of the ecological processes: dispersal, speciation, selection and drift. The water flea *Daphnia magna* has been used to investigate how single genera or lack of microbiota affects the fitness. How the composition of the microbiota affects fitness has not been studied so far, except compositional variation in connection with death of the host. The hypothesis of this master thesis was that the composition of gut microbiota affects the fitness of *D. magna*.

Fitness of *D. magna* was calculated for each culture as the total number of offspring divided by the cumulative number of mother days. Variation in the intestinal microbial community composition of individuals from different cultures was suggested to be explained by selection and drift, and not due to external perturbation. At the end of each phase, the intestines from the daphniids were dissected and the intestinal bacterial community composition was determined with Illumina sequencing of V3-V4 hypervariable regions of the 16S rRNA gene.

The registered fitness, compared with the intestinal microbial community composition, revealed differences in the relative abundance of the OTUs between daphniids with high and low fitness. The gut microbiota of daphnia was dominated by the phyla Proteobacteria and Bacteroidetes. The abundance of Bacteroidetes was particularly high in the cultures with high fitness compared with the average abundance of Bacteroidetes in the collected intestines. Bacteroidetes was, therefore, considered to play a beneficial role in microbiotas' effect on the fitness. The most abundant genus, *Limnohabitants*, has been reported to have beneficial effects on the fecundity of *D. magna*. However, this experiment revealed the possibility that *Limnohabitants* may has a different effect alone than in combination with other intestinal bacteria. *Pseudomonas* and Comanadaceae (much likely *Hydrogenophaga* according to Ribosomal Database Project (RDP)) were present in the cultures with low fitness. Both have been reported to be toxic to daphnia, and were therefore potential reasons for lower fitness in these cultures. An experimentally verification is needed for the main findings in the presented study.

Sammendrag

Samlingen av mikrobielle samfunn i magetarmkanalen er formet av vertens genetikk, individer innen samme art og habitat, og miljøfaktorer. Endringer i sammensetningen til det mikrobielle samfunnet kan påvirke vertens fysiske og psykiske helse. Varierende artsrikdom og relativ forekomst av operasjonelle taksonomiske enheter (OTU) i den mikrobielle sammensetningen er et resultat av de økologiske prosessene: spredning, artsdannelse, seleksjon og drift. Vannloppen *Daphnia magna* har tidligere blitt brukt til å undersøke hvordan enkelte slekter eller mangelen på mikrobiota påvirker fitness. Det er derimot ikke blitt undersøkt hvordan sammensetningen av mikrobiotaen påvirker fitness, unntatt variasjoner i sammensetningen knyttet til vertens død. Hypotesen i denne masteroppgaven var at sammensetningen av mikrobiotaen i tarmen påvirker fitnessen til *D. magna*.

Fitness hos *D. magna* ble kalkulert for hver kultur som antall avkom dele på det kumulative antallet mordager. Variasjon i sammensetningen til det mikrobielle samfunnet i tarmen hos individer fra ulike kulturer kan trolig forklares med seleksjon og drift, og ikke på grunn av ytre forstyrrelser. På slutten av hver fase ble tarmene fra dafniene dissekert og sammensetningen av det mikrobielle samfunnet i tarmen ble bestemt med Illumina sekvensering av V3-V4 hypervariable regioner i 16S rRNA genet.

Den registrerte fitnessen ble sammenlignet med den mikrobielle sammensetningen i tarmen, noe som avslørte forskjeller i relativ forekomst av OTUene mellom dafnier med høy og lav fitness. Tarmmikrobiotaen hos dafnier var dominert av rekkene Proteobacteria and Bacteroidetes. Forekomsten av Bacteroidetes var spesielt høy i kulturene med høy fitness sammenlignet med den gjennomsnittlige forekomsten av Bacteroidetes i de innsamlede tarmene. Bacteroidetes var derfor vurdert til å spille en fordelaktig rolle i mikrobiotaens effekt på fitness. Slekten det var mest av i dafnia-tarmene var *Limnohabitants*, som har blitt rapportert å ha en fordelaktig effekt på fekunditeten hos *D. magna* når den opptrer alene. I dette eksperimentet, derimot, ble muligheten for at *Limnohabitants* kan ha en annen effekt alene enn i kombinasjon med andre tarmbakterier vist. *Pseudomonas* and Comanadaceae (trolig *Hydrogenophaga* ifølge Ribosomal Database Project (RDP)) var til stede i kulturene med lav fitness. Begge har blitt rapportert som giftige for dafnier, og er derfor potensielle årsaker til lavere fitness i disse kulturene. Det er behov for en eksperimentell verifisering av hovedfunnene i denne studien.

List of abbreviations

AdAM	Aachener Daphnien Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
FSC	Forward scatter
GA	Glutaraldehyde
MAGs	Metagenome-assembled genomes
NAL	N-acetylneuraminate lyase
Neu5Ac	N-acetylneuraminic acid
NTC	Non-template control
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Non-parametric multivariate analysis of variance
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal database project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SIMPER	Similarity percentage
SSC	Side scatter
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TRAP	Tripartite ATP-independent periplasmic
V3	Variable region 3
V4	Variable region 4

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1. Introduction

All animals contain a complex ecological system that constitute of both host cells and microbial cells (1, 2). In the human body, the ratio between human cells and microbial cells is around 1:1 (3, 4). The microbial cells reside throughout the animal body, including both internal and external surfaces. The majority are located in the gastrointestinal tract and termed microbiota (1, 5, 6). The gut of vertebrates is inhabited by both bacteria, archaea, fungi and viruses (7). The commensalistic and mutualistic microbes in the gut contribute with necessary functions in the animal body, such as acquisition of energy and nutritional resources (8-10), protection against invading pathogens (10, 11) and assistance in development (12, 13). For instance, in humans and mice, plant polysaccharides are a nutritional resource that only can be digested and utilized by the degradative activities of microbes (8, 14). On the other hand, parasitic microbes are also present in the microbiota and may cause disturbances in symbiotic microbial communities, e.g. reduction in the host fitness as a consequence of competition for limited resources among the microbes (15, 16). In humans, such disturbances may be related to diseases ranging from metabolic diseases (e.g. obesity and diabetes) to gastrointestinal disorders (e.g. inflammatory bowel diseases) and colorectal cancer (17). Therefore, the field of microbiome research needs more attention, to find the possibilities to improve the human health and combat diseases.

1.1 The composition of the gut microbiota changes over time

During and after birth, the human infant gut is mainly colonized by bacteria through transmission from mother to offspring, such as vaginal delivery and breastfeeding (18). Through development the composition of microbiota can be regulated by factors like host genetics (19), social circle (20), and environmental factors like dietary pattern and lifestyle (21). Transmission of microbes among hosts, and between host and their environment, are predicted to be among the strongest factors causing variability in the microbiome. One example of this phenomenon was reported by Rothschild et al. who found significant similarity in the microbiome of genetically unrelated humans sharing household (21). The composition of microbes in the gut is highly plastic, and easily perturbed by external factors such as antibiotic treatment or changes in diet (22). All these environmental factors are strong contributors in the alternation of the microbial community and

may influence the hosts' physiological and mental health (21, 23). However, not only external factors influence the formation of the structure in the microbiota. Ecological interactions among the microbes and between the microbes and their host are also important factors. Research of the human gut has shown specifically that two phyla, Firmicutes and Bacteroidetes, account for 90% of the microbiota (24). That being said, everyone has a unique combination of bacteria on the strain and species level in their gut.

1.2 Diversity in the microbial communities

A microbial community is defined as an assembly of multiple microorganisms that share the same habitat and interact with each other. Exploration of this composition over time gives knowledge of the community's biological dynamics, the functional interaction within and among the species and how the community can change in space and time. The complexity in a microbial community and variation among communities can be explained by diversity. Taxonomic diversity is described by the number of species (richness) and the relative abundance (evenness) in a community (25, 26). Four fundamental ecological processes can be involved in the formation of diversity: dispersal, speciation, selection and drift (25).

Dispersal is movement of organisms from one site to another (27). The process can be divided into emigration (leaving the natal habitat), transfer (movement) and colonization (settlement in a novel habitat) (28). Successful establishment involves that an immigrant reproduces in the new habitat, and is not just the presence of its' taxon (27). The composition of the community left or colonized by dispersers will decide the effect of the dispersal on the community dynamics (29). For instance, the immigrants will produce higher increase in richness and diversity in species-poor than in species-rich communities (27, 29). The richness in a community will increase until no new species are provided by the dispersers (29). The community dynamics is also influenced by the dispersal rate, where high dispersal may lead to a reduction of individuals that is adapted for the habitat (30).

The second process is speciation, or diversification, which involves creation of new species. Microbes are often assembled in large populations with high potential growth rate, and are exposed to strong selection regimes (31). This facilitates rapid adaptation to the environment through

mutations or recombination, involving horizontal gene transfer and diversification driven by phages. Microbes residing in the same ecological niche (e.g. the gastrointestinal tract) are more available for recombination by horizontal gene transfer (31). In the long run, speciation may cause an increase in the richness within communities.

Change in relative abundance in a community occurs through selection (31). Selection causes ecological dynamics by promoting reproduction of species that are better adapted (highest fitness) to the environments. The probability that progeny from these species survive is higher due to inherited fitness-related traits (29, 32). In a population, the reproduction or replication rate will, for that reason, vary. For microbes, this rate is driven by environmental filtering and microbial interactions. Environmental filtering is when the available resources and the condition of the environment select for growth of the microbes that are best adapted to the habitat. In this case, the host forms the microbiota through selection of microbial traits that support reproduction and survival. An example is the body temperature during fever, where the elevated temperature exceeds the pathogen's maximum temperature toleration and provides a thermal protection against specific microbes (31, 33). For microbial interactions, microbes will communicate with each other and with the host, involving competition, predation, parasitism and mutualism (29, 31). An example of mutualism is in the immune system, where the homeostasis in the microbial community is maintained by immune cells, while the microbes preserve the hosts' immunity (34). Through selection, the abundance of specific species may increase, while the richness in a community is reduced. For example, genetic traits that are not beneficial for the organism are probably not selected for further generations (35).

The last process, ecological drift, is defined as the stochastic change in a community structure (36), i.e. random birth or death (27). As a result, the drift will lead to random fluctuation of the species' abundances. Ecological drift in a small community may lead to local extinction (reduce local diversity) due to the loss of low-abundance taxa (37, 38).

1.3 Methods to study the microbial diversity

The diversity of microbial communities is assessed either by culture-based or culture-independent methods (39). Culture-based methods include isolation and culturing of microorganisms on suitable substrates, while in culture-independent techniques the nucleic acids (DNA or RNA) are extracted directly from the sample (40). Since the culture-independent methods were introduced in the late 90s, the application has increased due to a high fraction of species that are not able to grow under specific environmental conditions, and because it is less time-consuming (40, 41). The microbial diversity and dynamics can be uncovered by investigating the taxonomic and phylogenetic classification of DNA sequences, using polymerase chain reaction (PCR) and sequencing of amplicons. The microbial species in the samples can be characterized by targeting and amplifying the hypervariable regions of small-subunit ribosomal RNA gene (16S rRNA) (42-44). This rRNA gene is frequently used because it is highly conserved and present in all species of bacteria (45). After 16S rRNA amplicon sequencing, the sequences are identified by assigning them into operational taxonomic units (OTU) based on sequence similarity (typically 97% similarity) (44, 46). However, there is a potential risk for misidentifying an OTU due to the presence of mosaicism and the availability of nearly identical 16S sequences in species with different physiology and taxonomy (45). There is also a chance of reduced presence of some genotypes due to bias in PCR amplification (e.g. generation of chimeras) and genotypes that may fall off in the collected DNA sample because they are less abundant or available (40).

1.4 *Daphnia magna* as model organism for investigations of fitness and microbiota

Animal model organisms are used to improve the understanding of how biological mechanisms of the hosts are affected by modulations in the host microbiota (23). These model organisms comprise invertebrates and lower vertebrates, and are selected because of their ease to handle, the low diversity microbiomes, the availability of sequenced genome and genomic tools, and the opportunity to conduct cost-efficient experiments in a short timescale (23, 47, 48). The water flea *Daphnia magna* is a renowned model organism in ecology and ecotoxicology (47). This crustacean has a length ranging from 1 to 5mm, and lives in freshwater habitats like ponds and lakes (49). *D. magna* primary consumes phytoplankton and bacteria in a size range of 1 to 50µm through their

filtration apparatus, and are prey for fish and other predators, such as invertebrates and vertebrates (50-52). Normally, *D. magna* is present as parthenogenetic females, where the egg cells can develop into embryos without fertilization by sperm (51, 53). However, abiotic factors like photoperiod and quantity of food can cause a switch from clonal to sexual reproduction. The clonal reproduction, in addition to their short generation time, make them suitable for investigations of functions and fitness properties within genotypes at multiple environmental conditions (47, 54).

D. magna is used as model organism in the investigations of how the microbiota structure is affected by genotypes and different environmental conditions (e.g. temperature) (55, 56). The relation between the microbiota and the host fitness was reported in a study by Sison-Mangus et al. where germ-free water fleas were both smaller, less fecund and had higher mortality than those with microbiota. The same study reported the potential the genus *Aeromonas* had to increase the body size of *D. magna* (57). Bacteria in the gut of *D. magna* mainly belong to Betaproteobacteria, Gammaproteobacteria and facultative anaerobic Bacteroidetes species. A study by Peerakietkhajorn et al., showed that *D. magna* re-infected with *Limnohabitans* strain DM1 and *L.planktonicus* II-D had a greater number of viable juveniles than bacteria-free water fleas (58). There was also observed high mortality among *D. magna* fed *Hydrogenophaga* sp. or *Pseudomonas* sp. (59). The resilience that the microbiota offer has shown to be crucial when the effect of acute and chronic exposure of antibiotics and other pharmaceuticals have been investigated. This was also connected to the water flea's ability to recover and re-establish the important interaction between host and microbiota (60-64).

1.5 Project aim

Earlier studies have shown how single genera and lack of microbiota affect the fitness of *D. magna*, but not how the composition of the microbiota affects fitness, except compositional variation in connection to host death (54, 57, 65). This forms the knowledge base of the current project.

The hypothesis was that the composition of gut microbiota affects the fitness of *D. magna*. The effect of variation in the composition of the gut microbiota on fitness was compared among

D. magna cultures. Daily data on number of offspring in cultures with known numbers of maternal individuals were used to calculate fitness. The composition of the gut microbiota was identified by 16S rDNA amplicon sequencing. To test the hypothesis of the project, the thesis was separated into five secondary goals:

1. To select daphniids suitable for the experiment
2. Allow for establishment of variation in the microbial community composition in independent cultures, without external perturbations
3. Evaluate the correlation between fitness and gut microbiota in cultures during a three-week experiment with daily registration of fitness
4. Establish a procedure for how to obtain bacteria-free neonates, which allowed for direct manipulation of the microbiota
5. Experimental test of the hypothesis by controlled colonization of bacteria-free neonates with microbiota from cultures with high or low fitness

Based on the secondary goals, the experiment was divided into four phases. In Phase 0, daphniids were obtained from two aquaria reared under the same conditions. Daphniids with good health according to movement and size were chosen. In Phase 1, the cultures were treated equally for five weeks with respect to food and medium replacement, with the assumption that this period was sufficient to obtain variation in the microbiota due to drift and selection. In Phase 2, the offspring production and maternal mortality were investigated daily for 21 days. The daphnia intestines were collected at the end of the phase for 16S rDNA amplicon sequencing. These data allowed me to evaluate if there was a correlation between fitness and composition of the gut microbiota. Phase 3 was an experimental test of the hypothesis of the project. The fitness-data were used to select replicate cultures with high and low fitness. To test the hypothesis, bacteria-free neonates were added in medium containing microbiota from cultures with either high or low fitness for potential re-colonization.

A separate aim was to investigate the effect of environmental microbes, including cultivation medium and feed, on the colonization of the gut and the ability of bacteria-free *D. magna* to reproduce the microbial community in the cultivation medium.

2. Materials and methods

2.1 Biological material and cultivation conditions

D. magna used in this experiment was of clone 47, a clonal descendant from a lineage collected in a pond at Værøy Island, northern Norway (67.687°N, 12.672°E) in 2014. The clone was hatched and kept in the laboratory for several years. The cultivation medium, Aachener Daphnien Medium (AdAM), contained necessary nutrients to obtain optimal growth of the individuals. The recipe is found in Appendix A. Throughout the experiment, the daphniids were fed by a diluted solution of the Shellfish Diet 1800® (Reed Mariculture Inc, USA) three times every week. The dilution and amount were decided according to volume of medium (Appendix B). Normally, the daphnia individuals resided in the jars were fed 1.25ml diluted Shellfish diet. For all the phases, the daphniids were kept in an incubator with standardized environmental conditions (20±1°C, continuous 10% light). The jars were repositioned every second day to achieve similar light-conditions. In the three phases (1, 2 and 3), half of the medium (i.e. 125ml) in each of the 250ml jars was removed once a week to maintain a good environment without destroying the microbiota in the medium. Three times per week, 42ml autoclaved medium was added to the jars. There was not removed any medium until 250ml was reached.

2.2 Experimental design

To investigate the relationship between the microbiota and host fitness, an experiment divided into four phases was performed (Figure 1). Phase 0 encompassed the two start-up cultures. Phase 1 started with four individuals distributed into each of 20 different jars and kept for five weeks to allow for divergence in the microbial communities. Phase 2 continued with starting up two new replicates from each of the 20 jars, receiving medium and four juveniles from the original jar (i.e. total of 40 jars). The offspring production and maternal mortality of *D. magna* in each jar were registered for 21 days. Phase 3 used these data to select medium from 12 cultures according to fitness. The medium was separated into two replicates (i.e. 24 cultures) and inoculated with two-three bacteria-free neonates. The offspring production and maternal mortality of these neonates were registered for 18 days to investigate how the fitness was influenced by direct manipulation of the microbiota.

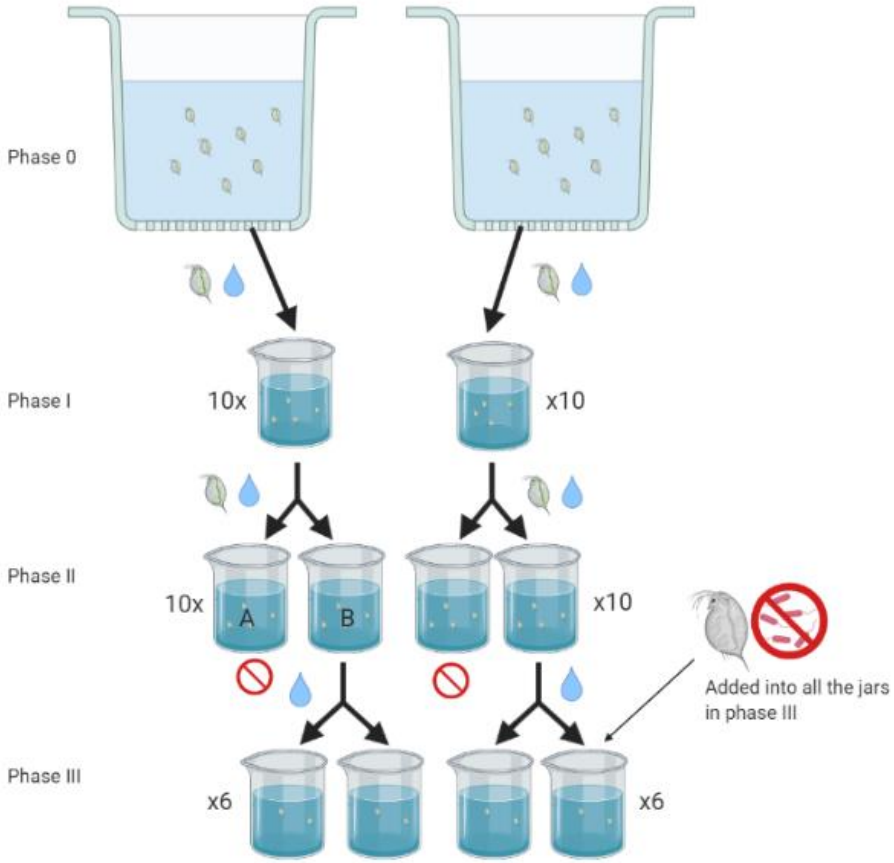

Figure 1: Daphniids were collected from two aquaria (Phase 0) and reared into AdAM medium in 20 jars (Phase 1), four individuals in each. Ten jars had individuals collected from the first aquarium and the other ten cultures were collected from the second aquarium. Both aquaria had the same conditions. After five weeks, medium and four juveniles from each of the 20 jars were separated into two replicates (Phase 2). The fitness was calculated for each culture as the total number of offspring divided by the cumulative number of mother days. These data were used to select six replicates with high fitness and six with low fitness. (A) The jars that were not selected were sampled and terminated. (B) Medium from each of the selected jars was distributed into two replicates (Phase 3). These jars were added two to three bacteria-free neonates, and the offspring production and maternal mortality were registered for 18 days.

2.2.1 Phase 1: Establishing variations in microbiota among cultures

The experiment started with two aquaria (Phase 0) that contained the crustacean *D. magna.* Both aquaria were treated in the same way prior to the experiment. From these aquaria four individuals (two juveniles and two adults) were transferred to each of the 20 jars. Ten cultures had individuals collected from the first aquarium and the other ten cultures were collected from the second

aquarium. Once a week, the newborns were counted. The four mother individuals added to the culture at the start were left in the jar to allow for continued reproduction and to maintain the existing microbiota, while the newborns were removed. If one of the mother individuals died, one neonate in the jar was allowed to grow up and be a fecund mother (i.e. the dead mother individual was replaced by a neonate to obtain a total number of four reproducing individuals). After five weeks, sampling of the cultivation medium in each jar for later quantification of bacterial densities was performed. The mother individuals left were stored at –20°C until characterization of the gut microbial composition.

2.2.2 Phase 2: Maintenance of the gut microbiota and measurement of the fitness of *D. magna* in the cultures

After five weeks, the content of each of the 20 jars was distributed into two autoclaved 250ml jars, establishing two replicates from each (i.e. 40 cultures in total). The biofilm formed by the microorganisms on the wall of the original jar was included by using a sterile Q-tip around the glass wall. The Q-tip was placed into the cultivation medium in the new culture to release the potential microbes. The same procedure was performed for the second replicate with a new sterile Q-tip. Four neonates (<24h old) from the original jar were transferred into each replicate culture to act as maternal individuals. Their mortality and number of offspring were registered daily for 21 days to obtain a measure of fitness. The newborns were removed upon counting. The cumulative number of “mother days” was calculated for each culture by summing up the number of live mothers per day for the experimental duration. From these data, fitness was calculated for each culture as the total number of offspring divided by the cumulative number of mother days. After 21 days, samples were collected for analysis. Bacterial densities were analyzed in the cultivation medium, and bacterial community composition was analyzed in both cultivation medium and intestines of daphnia.

2.2.3 Phase 3: Inoculation of bacteria-free neonates in cultures with high and low fitness

The collected fitness data from Phase 2 were used to select high and low fitness cultures. From the 40 cultures in Phase 2, six cultures were characterized as high fitness and six as low fitness. Each of these cultures was distributed in two autoclaved jars (i.e. 125ml in each), giving a total number of 24 jars. A sample of the biofilm on the wall was transferred in the same way as for Phase 2 (Section 2.2.2). Each of the jars received two to three bacteria-free neonates (<24h old, Section 2.3). Data were collected daily and fitness was calculated for each culture in the same way as in Phase 2 (Section 2.2.2). The newborns were removed upon counting. After 18 days, cultivation medium was sampled for later quantification of bacterial densities. Cultivation medium and daphnia intestines were collected for later characterization of bacterial composition by amplicon sequencing.

2.3 Method to obtain bacteria-free neonates

To achieve the bacteria-free neonates to be used in experiments, the eggs from *D. magna* had to be disinfected. Females without eggs were fed and isolated for 24 hours. Isolation was performed to only obtain eggs that were recently released from the ovary. These eggs are covered by an external membrane which has shown to improve the hatching success after disinfection (66). To obtain the most efficient disinfection of the eggs different glutaraldehyde (GA) concentrations, treatment lengths and presence/absence of external membrane were tested (Table 1). The disinfection was developed based on Peerakietkhajorn et al. (54).

Table 1: Different treatment methods to obtain efficient disinfection of eggs from *D. magna.* Eggs with/without an external membrane were treated with different concentrations of glutaraldehyde (GA, 50%) for different durations. The external membrane is present for eggs recently released from the ovary of *D. magna*.

Trial	**GA-concentration (%)**	**Treatment-duration (min)**	**External membrane**
1	0.1	45	Yes
2	0.1	30	No
3	0.1	30	Yes
4	0.025	30	Yes

The eggs produced over night were taken out from the mothers' brood chamber under microscope by using disinfected forceps. The eggs were stored in a petri dish with 25ml AdAM (approximately 20 eggs in each). 5-10 eggs were placed in their own petri dish and used as control. The rest were added 12.5µl GA (50%) per 25ml AdAM (0.025% final concentration). The eggs were treated for 30 minutes. The petri dishes were moved slightly during the treatment to make sure that all the eggs were disinfected. The eggs were collected and transferred to sterile petri dishes with sterile-filtrated AdAM-medium. This was repeated one more time before the eggs were stored in an incubator at 20°C until hatching. GA cross-links proteins, which leads to inhibition of membrane transport, enzyme activity and synthesis of RNA, DNA and proteins inside the bacterial cells (54, 67). After the eggs had hatched, the cultivation medium (80µl) from each of the petri dishes was plated on agar to verify the absence of bacteria. Glass beads were used to spread the cells evenly over the growth medium. The glass beads were removed, and the plates were incubated in room temperature 2-3 days.

2.4 Characterization of bacterial community composition by PCR-amplicon sequencing

2.4.1 Sampling of daphnia guts and cultivation medium from the cultures

At the end of each phase the mother individuals were collected in a tube, whereafter intestines were dissected out and frozen until further analysis. In Phase 2 and 3, bacteria in the cultivation medium from the jars were collected on Sterivex filter and stored at -20°C until DNA extraction. During the DNA extraction, the filters were opened and the paper on the inside were cut into small pieces by using sterile forceps and sterile scalpel.

Prior to the dissection, water fleas were washed twice with sterile-filtrated AdAM in a petri dish. The intestine was pulled out by using sterile forceps under microscope. Two forceps were used, one to hold the head of the water flea, and the other to pull off the body (Figure 2). The intestines from the individuals that belonged to the same jar (i.e. four intestines) were assembled in a tube with 500µl sterile-filtrated AdAM and stored at –20°C until DNA extraction.

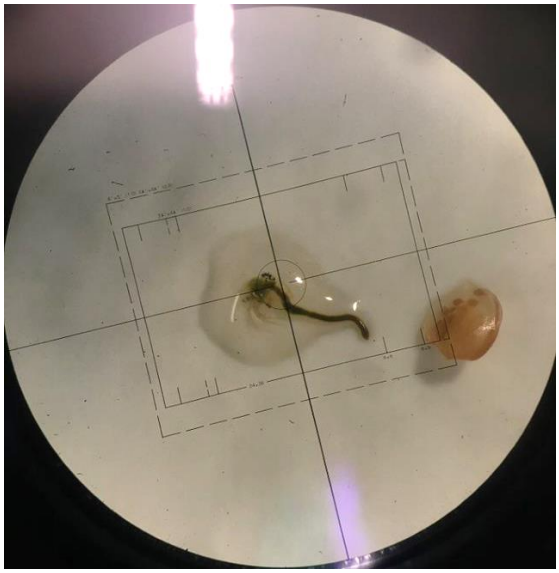

Figure 2: The intestine of *D. magna* after dissection under microscope. The remaining body is to the right.

2.4.2 DNA-extraction of daphnia guts and cultivation medium

The genomic DNA from the intestines and small filter-pieces were extracted by using Qiagen DNeasy® PowerSoil® Kit (100) according to the manufacturer's protocol. The PowerBeads from the kit were used to homogenize the samples. The positive control was added a known bacterial culture, and the negative control was only added DNA-extracting solutions. The positive and negative controls were included to compare and identify possible background contamination. The isolated DNA solutions were stored at –20°C until the concentration of the DNA was determined.

The quality and quantity of the DNA were measured by Thermo Scientific™ NanoDrop™ (One Microvolume UV-Vis Spectrophotometer). Before the samples were measured, 1µl HCl was added to remove potential proteins that had dried on the pedestals. The upper pedestal arm was lowered, and a dry lint-free lab wipe was used to remove liquid from both upper and lower pedestal. Before the dsDNA was measured, 1µl blank (PCR-free water) was added to the lower pedestal. After the water was wiped off, the samples with newly extracted DNA were measured. PCR-free water was used to wash the pedestals after use.

2.4.3 Amplification of 16S rDNA by PCR

The "universal" primers, ill338F and ill805R (Sigma-Aldrich; Table 2), with high coverage for bacteria, were used to amplify 16S rDNA by targeting the V3-V4 hypervariable regions. The mastermix was made in a batch according to Table 3. The total volume in each PCR-tube was 25µl, where 24µl was mastermix and 1µl was template. A positive control with DNA from a known bacterial culture and a negative control without any template (no template control = NTC) were also included to make sure that none of the reagents were contaminated. After vortexing and centrifuging, the tubes were situated into T100™ Thermal Cycler (Bio-Rad) and ran through the program explained in Table 4.

Table 2: Primer sequences (Sigma-Aldrich) used in the amplification of the bacterial 16S rRNA gene by targeting V3 and V4 regions. Sequences marked as bold represent the Illumina adapters. A= adenine, T= thymine, C= cytosine, G= guanine, N= A, T, C or G, W= A or T, K= G or T and V= G, C or A.

Primer name	**Nucleotide sequence**
Ill338F	5'-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN** CCT ACG GGW GGC AGC AG-3'
Ill805R	5'-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN** GAC TCA NVG GGT ATC TAA KCC-3'

Table 3: The reagents and their quantity in the mastermix used for amplification of the 16S rRNA gene. 25µl reaction volume per sample.

Components	**Supplier**	**Quantity x1**
PCR-grade water	-	16.4373µl
5x Phusion buffer HF (7.5mM MgCl2)	Phusion Kit Illumina	5.0µl
Ill338F (10um)	Sigma Aldrich	0.75µl
Ill805R (10um)	Sigma Aldrich	0.75µl
dNTP (10mM each)	VWR	0.625µl
MgCl2 (50mM)	Phusion Kit Illumina	0.25µl
Phusion Hot Start DNA polymerase	Phusion Kit Illumina	0.1875µl
Template	-	1.0µl

Table 4: The PCR- program (cycling conditions) used for amplification of bacterial 16S rDNA.

Temperature	Time	
98°C	1 min	
98°C	15 sec	x 35 cycles
53°C	20 sec	
72°C	20 sec	
72°C	5 min	
4°C	1 min	
10°C	∞	

2.4.4 Verification and quality control of PCR by agarose gel electrophoresis

The PCR products were run in an agarose gel electrophoresis to verify that the samples were amplified and had expected size according to a ladder. The 1% agarose gel was made by mixing and microwaving 2g agarose with 200ml 1xTAE (Tris-acetate-EDTA) buffer (Appendix C) until the agarose was dissolved. The dissolved agarose was added GelRed® (5µl GelRed per 100ml dissolved agarose, Biotium) for detection of the DNA. After approximately 3 minutes the gel was solidified and 1xTAE was filled to marked line in the electrophoresis chamber. The samples (4µl) were mixed with a 6x DNA loading dye (1µl, Thermo Fisher Scientific) before they were added into the wells. This allowed tracking of the DNA migration during the electrophoresis. GeneRuler™ 1kb Plus (5µL, Thermo Fisher Scientific) was used as a DNA size standard on the gel. The gel included positive and negative controls, both from the kit-extraction and the PCR amplification, to compare and investigate possible contamination. The gel electrophoresis was run at 110V for one hour. At the end, the gel was visualized with 200ms exposure in a UV-chamber by using the program GeneSnap (SynGene, Cambridge).

2.4.5 Illumina sequencing

2.4.5.1 Principle of Illumina sequencing

The genetic variation of the prokaryotic 16S rRNA gene is often analyzed by amplicon sequencing. The set-up for Illumina sequencing is illustrated in Figure 3. First, the sequencing library is prepared by fragmentation of the genomic DNA. Afterwards, a region of interest-specific primer

with overhang of sample-specific adapters is ligated to the 5' and 3' end of all the fragments through a limited-cycle PCR program. The addition of these adapters allows for high-throughput sequencing where hundreds of sequences can be analyzed in a single run (68). Secondly, a cluster is generated by bridge amplification. Afterwards, DNA polymerase will incorporate the fluorescently labeled terminators (ATP, TTP, GTP and CTP) with the complimentary base in the selected DNA strand. When the terminator binds to the proper base, the cluster excites, and a fluorescent signal is detected. The color will vary according to terminator and allow for identification of the base. This process is repeated until the whole base sequence is characterized. To identify the taxonomy of the newly sequenced reads, the result is aligned to a reference genome (68, 69).

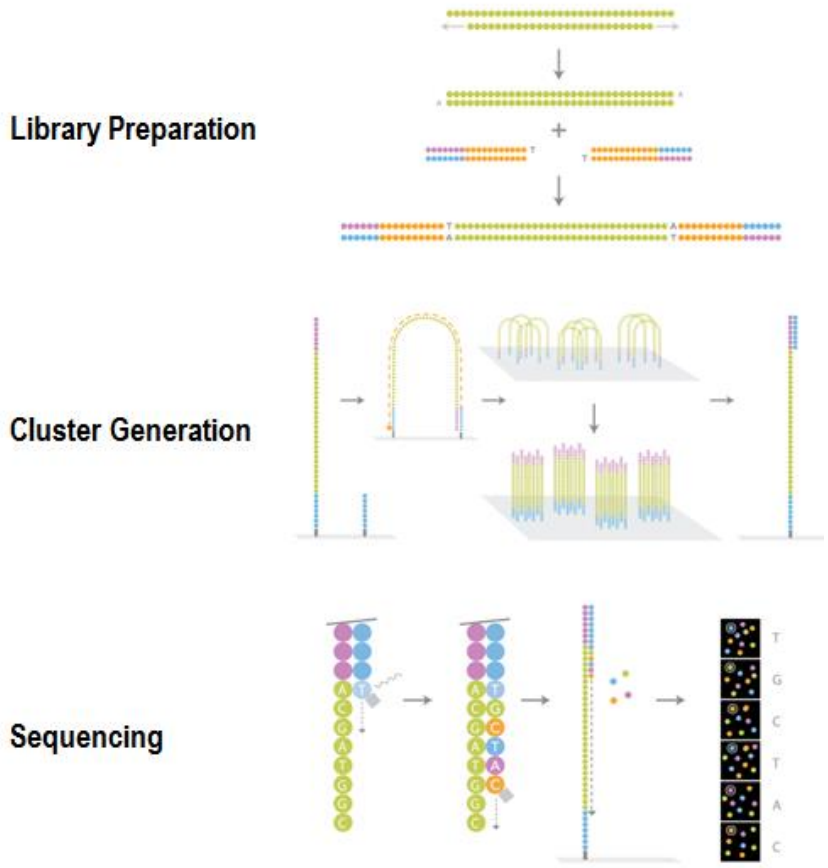

Figure 3: The workflow of Illumina sequencing. A sequencing library is prepared by fragmentation of the genomic DNA and addition of adapters. Further, a cluster is formed by bridge amplification. Afterwards, DNA polymerase incorporates the fluorescently labeled terminators (A=ATP, T=TTP, G=GTP and C=CTP) with the proper base. The cluster excites and a fluorescent signal is detected and used for identification of the base. The process continues until whole base sequence is identified. Adapted from Hagmann (70).

2.4.5.2 Preparation of amplicon library and performance of the Illumina sequencing

Prior to sequencing, the PCR-products were purified and normalized with The SequalPrep™ Normalization plate (96) kit (Invitrogen) according to the manufacturer's protocol. The purification removes impurities, such as primers and salts, from the samples. After normalization, a 96-well plate was added mastermix (17.5µl, Table 5), index i7 (2.5µl) and index i5 (2.5µl). Both indices were provided by Nextera® XT Index Kit (Illumina), and ensured that the amplicons could be backtracked to a particular sample. Also 2.5µl of the PCR-product (Section 2.4.5.1) was added, which gave a total reaction-volume of 25µl. The negative controls from the DNA extraction and the PCR non-template controls were also included to detect possible contamination. After vortexed and centrifugated, the tubes were situated in T100™ Thermal Cycler (Bio-Rad) and ran through the program explained in Table 4 with 8 cycles instead of 35 cycles. To verify a successful indexing, the products were run through an agarose gel electrophoresis. The PCR-products were purified and normalized one more time as previously described.

Table 5: Quantities and reagents in the mastermix used to index one sample.

Components	**Supplier**	**Quantity x1**
PCR-grade water	-	11.437µl
5x Phusion buffer HF (7.5mM $MgCl_2$)	Phusion Kit Illumina	5.0µl
dNTP (10mM each)	VWR	0.625µl
Phusion Hot Start DNA polymerase	Phusion Kit Illumina	0.188µl
$MgCl_2$ (50mM)	Phusion Kit Illumina	0.25µl
Index 1 (orange top, N-series)	Illumina	2.5µl
Index 2 (white top, S-series)	Illumina	2.5µl
Template (normalized)	-	2.5µl

After normalization, all the PCR-products were collected into one tube. The total product was concentrated by using Amicon® Ultra 0.5mL Centrifugal Filter Devices (30 K membrane, Merck Millipore) according to manufacturer's protocol. An additional washing step was included after step four, where TE-buffer (500µl, Appendix C) was added to the sample before it was centrifugated at 14000xg for 10 min. This step was repeated. The concentration and the purity were measured by Thermo Scientific™ NanoDrop™ (One Microvolume UV-Vis Spectrophotometer). The collected product was run on an agarose gel electrophoresis to determine the size of the product. The amplicon library was sent for MiSeq sequencing (Illumina) at the Norwegian Sequencing Centre (NSC).

2.4.5.3 Processing of Illumina sequencing data

The USEARCH pipeline (https://www.drive5.com/usearch/, version 10) was used to process the Illumina sequencing data. Consensus sequences and quality scores (Q-score) were achieved by merging paired reads through fastq_mergepairs. The command was also used to remove primer-binding sequences and eliminate reads shorter than 390 base pairs. Quality filtering and demultiplexing (i.e. using the barcode to retrace the right sample after sequencing) was performed by using the fastq_filter command with an expected error threshold of 1. Prior to dereplication, the reads were sorted by decreasing abundance through the command sortbysize. The command Cluster_otus removed chimera and clustered the remaining reads into OTUs with a similarity level of 97% into OTUs. The taxonomic assignment was generated by aligning the sequences (at a confidence value of 0.8) from the Sintax script (71) to the RDP reference dataset (version 16). The resulting table contained the number of reads per OTU for each sample, where each OTU had a taxonomic assignment.

The taxonomic table was processed in Microsoft Excel. The resulting OTUs were compared with the negative controls from the extraction kit and PCR non-template controls. OTU 3 and OTU 5, representing *Propionibacterium* and *Escherichia/shigella* respectively, were removed from the table due to high availability in the negative controls and low probability for them to inhabit the intestine of *D. magna*. The OTUs classified as Chloroplast or only phylum Cyanobacteria/Chloroplast were also deleted because the chloroplasts are most likely to have

originated from the algae used as feed for *D. magna*. To obtain equal sequencing depth of all samples they were normalized to 27 000 reads per sample in three steps. First, the number of reads for each OTU was divided by the total number of reads for each sample. Second, relative abundance was multiplied with the desired number of reads. Third, the number was rounded off to the closest integer. The normalized table was further organized according to taxonomy by using the command sintax_summary.

2.5 Quantification of bacterial densities by flow cytometry

2.5.1 Principle of flow cytometry

A flow cytometer is an instrument that use lasers to detect the size, granularity and fluorescent features of cells. The instrument contains lasers (light source in blue and red), fluidics that transport the particles through the laser, optics that gather the light, detectors that register the light signals and a computer that analyze signals and transform them into readable data files. The set-up is illustrated by Figure 4. Particles pass through the laser, resulting in scattered light and fluorescent emissions. The scattered light is measured in two directions: forward scatter (FSC) and side scatter (SSC). The forward scatter indicates the size of the cells, while the side scatter indicates the shape and internal complexity of the cells. The instrument also has multiple fluorescent detectors that can detect and quantity fluorescent light released after excitation of antibodies, staining or dyes (72, 73).

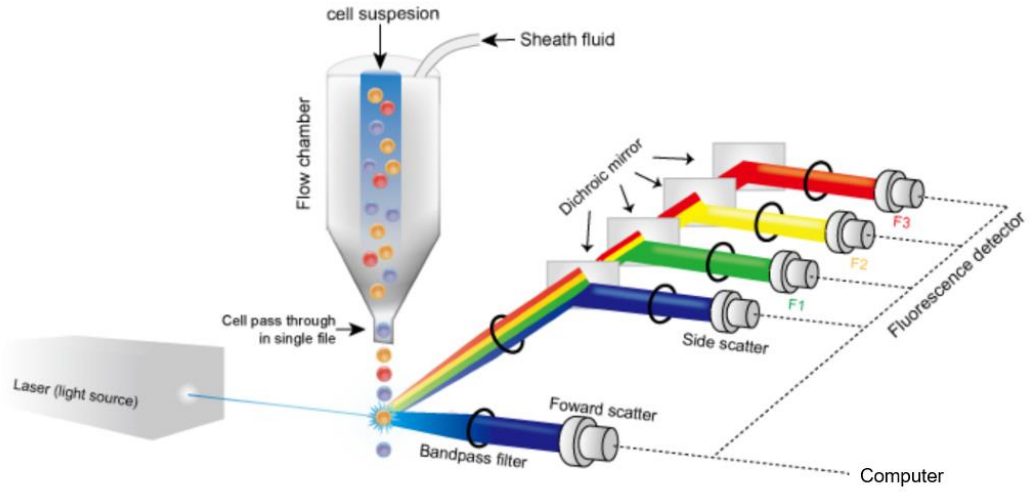

Figure 4: The set-up of a flow cytometer. Particles from the flow chamber (transported by fluid) will pass through a laser, resulting in scatter light and fluorescent emissions. The light and emissions are registered by detectors and analyzed by a computer. *Adapted from creative-diagnostics.com/flow-cytometry-guide.htm*

2.5.2 Sampling of cultivation medium and algal solution

Cultivation medium from the jars in Phase 2 and 3 were collected in cryo-tubes. To stop further metabolism of the bacteria, they were fixed with 1% GA (final concentration) and stored at room temperature for 30 min. The tubes were snap frozen in liquid nitrogen and stored at –20°C until further analysis. The food given to *D. magna* during the experiment was also sampled. The algae solution was diluted 1:41 and centrifuged at 3000xg for 1 minute to remove most of the algal cells. The supernatant was filtered through a polycarbonate membrane (OSMONICS) with pore size 1.0µm by using vacuum (Figure 5). This was performed to separate the algae, that contains chlorophyll, from the bacteria. The filtered solution was collected into two cryo-tubes and snap frozen in liquid nitrogen. The samples were stored at –20°C until analysis.

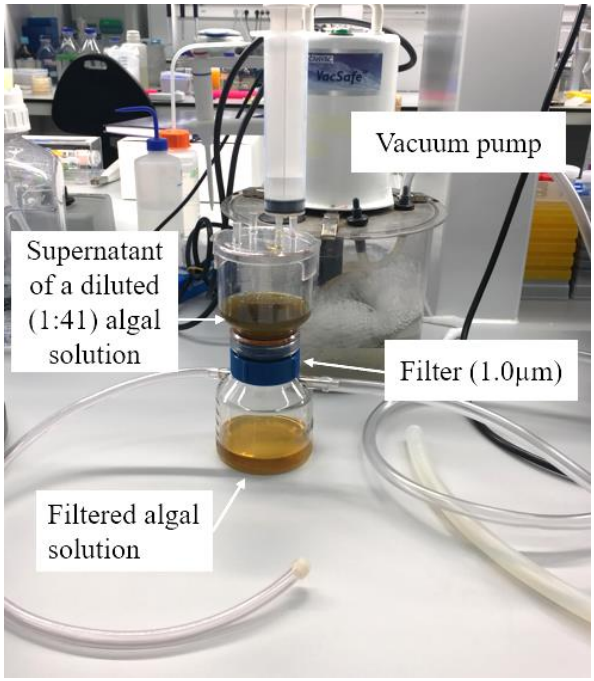

Figure 5: The set-up for the filtration of diluted algal solution by using a vacuum pump. The algae solution was used as feed for *D. magna*, and the filtration was performed to minimize the algal cells for further analysis.

2.5.3 Quantification of bacterial cells in the cultivation medium by flow cytometry

Before analyzing the samples, a daily calibration with Spherotech 8-peak and 6-peak validation beads (BD Accuri® Cytometers) was performed. The samples were diluted 1:4 with 1x Phosphate-buffered saline (PBS) filtered at 0.2µm. SYBR® Green I nucleic acid gel stain (10µl, Thermo Fisher Scientific) was also diluted with 490µl filtered 1xPBS and mixed. For detection of dsDNA, the samples were stained with SYBR® Green I (10µl stain per 1mL sample). A negative control with only filtered 1xPBS was also added SYBR® Green I. All samples added stain were mixed and incubated at 37°C for 20 minutes. In addition, one sample without stain was included to investigate the presence of natural fluorescent. dsDNA stained with SYBR® Green I absorbs the blue laser with a maximum wavelength of 497nm, the SYBR/DNA complex excites, and emits fluorescent light maximum on a wavelength of 520nm. FL1-H is the optical filter that is most suitable to register signals at this wavelength. FL3-H was also selected as an important optical filter for possible detection of chlorophyll at wavelength 662/669nm (73). The settings on the flow cytometer (BD Accuri™ C6 Plus, BD Bioscience) were decided to be medium fluidics with a time

of 2 minutes for each sample (35µl/min). The primary threshold was set to eliminate the events on FL1-H with intensity less than 1000.

The flow cytometry data from BD Accuri® C6 Software were processed using R (https://www.r-project.org/, version 3.6.2). The transform() function from the package flowCore ((74), version 1.11.20) was used to perform an arcsin transformation of the flow cytometry data. In addition, the flowCore package allowed for identification and counting of bacterial cell populations with the functions PolygonGate() and filter(). The signals from FL1-H and FL3-H were detected for each event per sample and plotted by using the function xyplot (FL3-H~FL1-H) from the flowViz package ((75), version 0.2.1). The bacterial cell-density was calculated by dividing number of cells on the total volume analyzed by the flow cytometer and multiplying this number with the dilution factor of the sample.

2.6 Statistical analysis

2.6.1 Statistical analysis of the fitness data

The data on fitness of daphnia collected during three phases (1, 2 and 3) were exported to the program R (https://www.r-project.org/, version 3.6.2). The resulting data were plotted against each other to analyze the reproducibility between the replicates in Phase 2 and between the replicates in Phase 3. Pearson's product-moment was used to calculate the linear correlation between the replicates.

2.6.2 Analysis of bacterial diversity

The normalized OTU table was used for further analysis. There are numerous indices available to quantify different aspects of diversity, such as richness, evenness, inequality and dominance. Diversity comprises richness and evenness in a community, and is measured on three levels: alpha, beta and gamma diversity (76). Alpha diversity represents the diversity within a habitat or sample (intra-community), while beta diversity is comparisons between habitats or samples (inter-community). Gamma diversity estimates the overall diversity in an ecosystem or a set of samples, and is a function of both alpha and beta diversity (77, 78).

The alpha (α)-diversity was quantified in Past (https://folk.uio.no/ohammer/past/, version 4.01). This table was exported to Microsoft Excel for analysis. The coverage of the sequencing was calculated by dividing the number of OTUs in a sample (richness) by the Chao-1 index. Chao-1 is a richness estimator that also takes unique OTUs with low abundance (singletons and doubletons) into account (79). One of the challenges for bacterial diversity is to estimate the proportion of rare OTUs. Hill numbers (^{q}D) is a family of indices that incorporate species richness and weight the taxa differently according to species frequency (80)

$$ {}^{q}D = \left(\sum_{i=1}^{S} p_i^q \right)^{\frac{1}{1-q}} \quad (1) $$

In this formula (1) p_i is defined as the proportion of individuals in OTU_*i* in a sample. *i* is a number from 1 to *S*, representing the *i*'th OTU. The exponent q is a diversity order (0, 1, …, ∞) that indicates the sensitivity to rare or dominated OTUs. The formula is insensitive for the OTU abundancy at q=0 and is then representing the number of taxa. When q=1 the OTUs are weighted according to OTUs' frequency.

$$ {}^{1}D = exp\left(-\sum_{i=1}^{S} p_i \, ln \, p_i \right) = \exp(H) \quad (2) $$

This formula (2) defines the exponential Shannon index (exp(H)) that comprises both unique OTUs and relative abundance (81). If the order (q) is larger than one, the formula will favor the more common OTUs (82). The term evenness is a synonym to equitability and quantifies how similar (ranging from 0 to 1) the OTUs in an environment/sample are distributed (83). To investigate significant difference in richness, abundancy of OTUs and evenness among the groups, a two-sample t-test was performed on the alpha-diversity data. F-test was used to confirm unequal or equal variance between the groups. The difference was significant when the p-value was lower than 0.05.

The beta (β)-diversity was calculated in the program Past. To estimate similarity between communities Dice-Sørensen and Bray-Curtis similarity were used. Dice is an index based on the presence/absence of species and is calculated by the number of shared species among the samples and the species that are unique for each sample. Bray-Curtis is a modified version of Dice and was developed by Bray and Curtis (1957) (79). Bray-Curtis use abundance of OTUs as an input, and weight abundant OTUs higher than rare OTUs (84). The output from the analysis was a similarity matrix for microbial community composition between all samples, where the similarity is a number between 0 (dissimilar) and 1 (identical). The resulting data were exported to Microsoft Excel. To evaluate the sample similarities within and between sample groups (phases, sample-types or fitness) the averages and standard deviations were calculated. The similarity between the samples was illustrated by a principle coordinates analysis (PCoA) for both Bray-Curtis and Dice similarity. PCoA represents the data graphically in two dimensions that explain most of the similarity between the samples. The plot illustrated that the samples were less equal if they were placed farther from each other (85). To find out whether the differences between the sample groups (phases, sample-types or fitness) were statistically significant, a permutational multivariate analysis of variance (PERMANOVA) was done based on Bray-Curtis similarities and Dice index. A significance level lower than 0.05 was considered as significant difference between the samples. By using both similarity indices, it was possible to distinguish between clustering caused by relative abundance or clustering caused by occurrence of OTUs.

SIMPER (Similarity Percentage), another multivariate analysis, was used to find the OTUs that contributed most to the differences between the microbial communities. The relative abundance of these OTUs were given for each group (phases, sample-types or fitness).

3. Results

The aim of this thesis was to investigate the relationship between host-microbiota and fitness in the water flea *D. magna*. Fitness was calculated for each culture as the total number of offspring divided by the cumulative number of mother days. The composition of the gut microbiota was characterized by 16S rDNA amplicon sequencing.

3.1 Evaluation and verification of a procedure to obtain successful disinfection of daphnia eggs

The most efficient treatment method to obtain bacteria-free neonates was found by investigating the hatching success of the eggs and verifying the absence of bacteria on the eggs. Trial 1 (0.1% GA, 45 min) gave eggs with low mortality (15 of 16 eggs hatched) after three days, but without the external membrane, all individuals died (Trial 2). Trial 3 (0.1% GA, 30 min) was performed in two rounds and had addition of feed after the eggs hatched. First round gave eggs with no mortality (4 of 4 eggs hatched) after two days, but there was only 1 viable neonate after 8 days. Second round gave 8 viable neonates out of 29 treated eggs. Trial 4 (0.025% GA, 30 min) resulted in 24 viable neonates out of 44 treated eggs. Trial 4 was suggested to be the most suitable procedure, and was therefore used for further experiment.

The absence of bacteria on the eggs was verified by adding medium from the petri dishes with treated eggs onto an agar plate. Three plates (parallel 1-3) were added medium from petri dishes with GA-treated eggs, and the fourth was incubated with medium from a petri dish containing eggs without treatment (Figure 6). A rough estimation of colony-forming unit (CFU) in the petri dishes with eggs/neonates indicated 6 600 CFU/ml in control, whereas the CFU was below the detection limit of 13 CFU/ml for the disinfected eggs. This confirms the absence of viable bacteria associated with the eggs treated with GA.

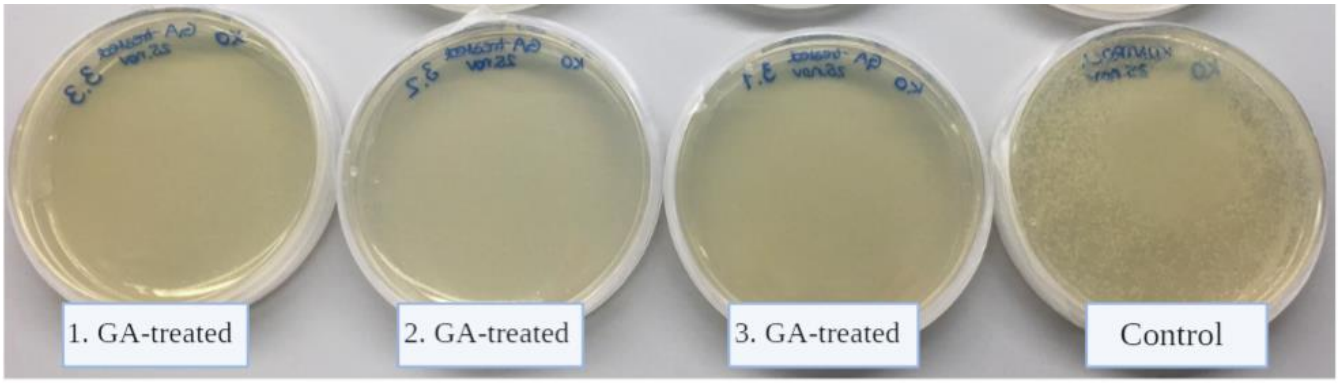

Figure 6: Effect of disinfection on the presence of culturable bacteria for three GA-treated and one control petri dish. The medium was plated the same day as the treatment. The agar plates were incubated for 2-3 days at room temperature.

The disinfection was repeated on new eggs, and three new water-samples were plated the day after treatment to get a stronger indication of any available bacteria. A rough estimation indicated 24 900 CFU/ml in the control, whereas the CFU was below the detection limit of 13 CFU/ml for the disinfected eggs (Figure 7).

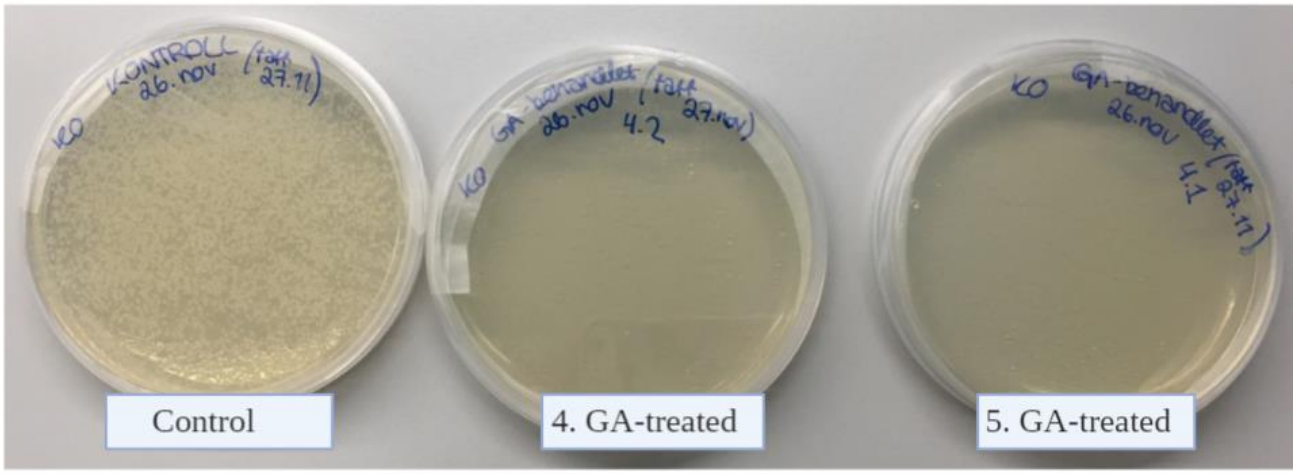

Figure 7: Effect of disinfection on the presence of culturable bacteria for one control and two GA-treated petri dishes. The medium was plated the same day as the treatment. The agar plates were incubated for 2-3 days at room temperature.

3.2 Phase 1: Variations in the gut microbiota and fitness among the cultures

In Phase 1, the number of offspring for 20 different jars (each initiated with four daphniids) was registered every 6th or 7th day for five weeks (Figure 8). The population growth increased the last three weeks of registration, e.g. from week 3 to week 4, 397 and 734 neonates were born, respectively. This corresponds to an increase of 46%.

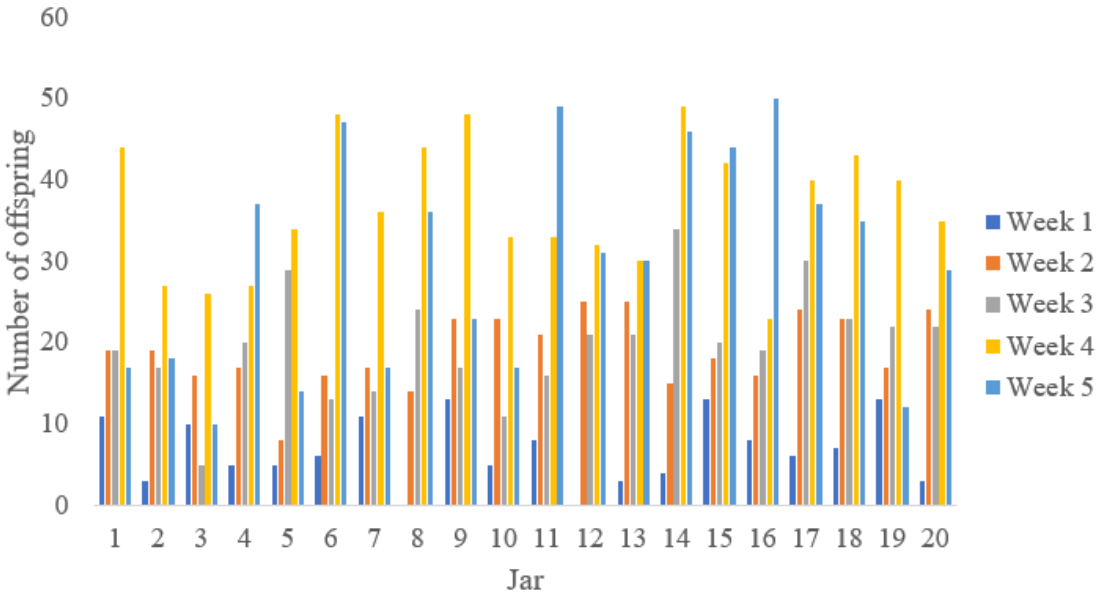

Figure 8: The weekly registration of number of offspring from the four daphniids in 20 different jars over five weeks (Phase 1).

After the five-week period, there were three jars with less than 90 offspring (jar 2, 3 and 10) and three with more than 136 offspring (jar 14, 15 and 17; Figure 9). Average was 112 (± 20 SD) offspring. The highest number of dead maternal individuals were found in jar 19 and 17, but also jar 9, 10, 14 and 16 (Figure 10). This indicates that there was no correlation between high number of deaths and low number of offspring.

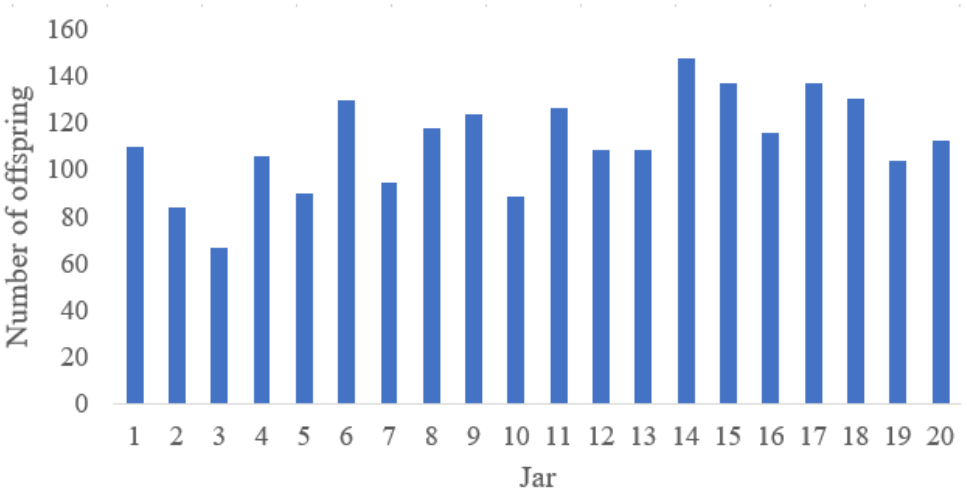

Figure 9: The total number of offspring from the four daphniids resided in each of the 20 jars during the time period of five weeks (Phase 1). The newborns were counted and removed once a week.

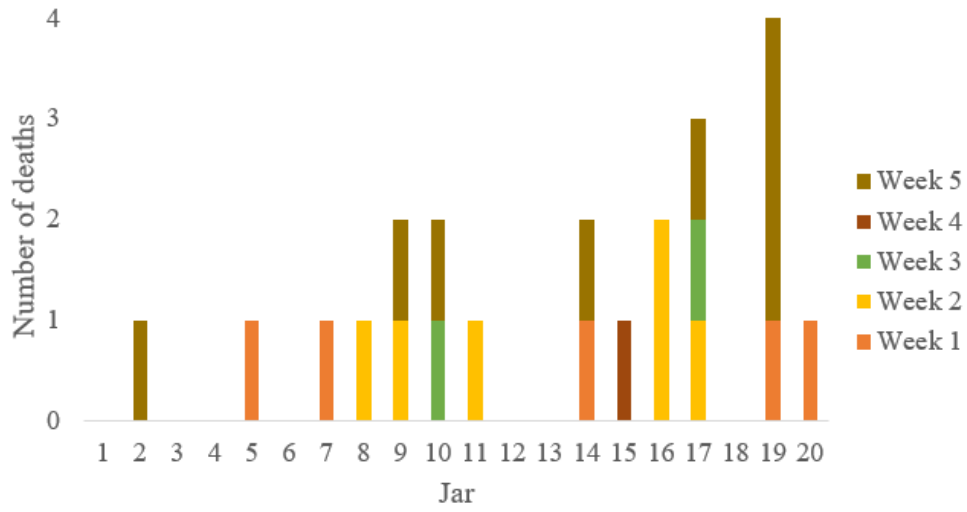

Figure 10: Number of dead daphnia mothers in each jar during the time period of five weeks (Phase 1). Each of the 20 jars had four mother individuals.

3.3 Phase 2: Evaluating the correlation between fitness and gut microbiota in the cultures

Medium from each of the 20 jars in Phase 1 was in Phase 2 transferred to two jars and inoculated with four juveniles from the original jar. Offspring production and maternal mortality were registered daily for 21 days. An illustration of the growth in two of the jars (7.2 and 8.1) during the time period of 21 days is given in Figure 11. These had the same conditions. The total growth was higher in jar 7.2 (267 offspring) than in 8.1 (131 offspring).

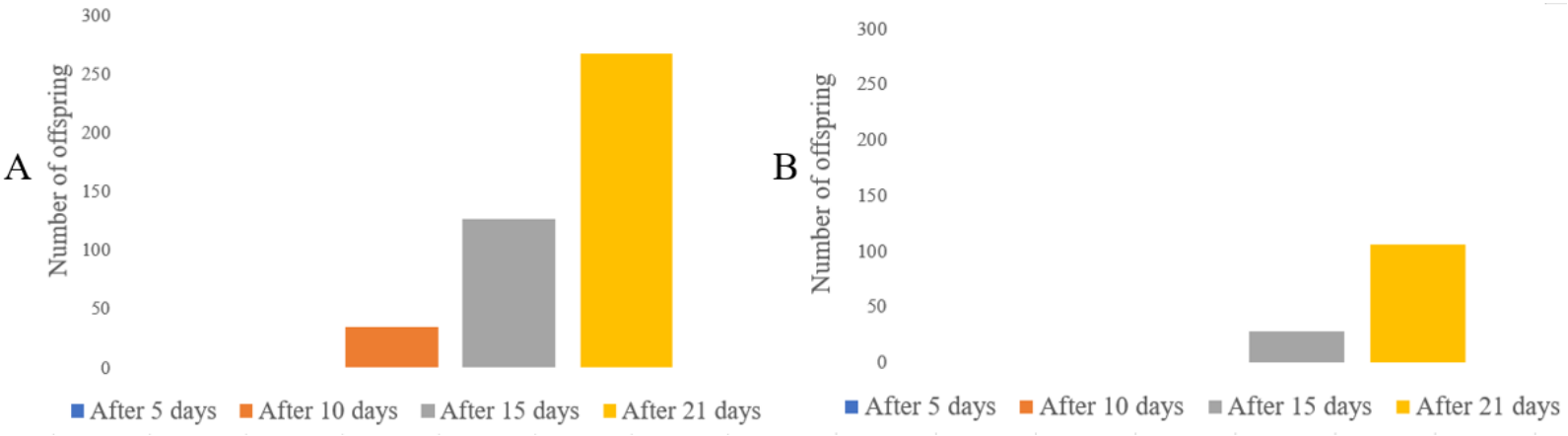

Figure 11: Total number of offspring from the four daphniids in jar 7.2 (A) and 8.1 (B) after 5, 10, 15 and 21 days (Phase 2).

The raw data (Appendix D, Table 17) were used to produce Figure 12, which shows my measure of fitness across cultures (number of offspring per mother days). There were some jars with high and low fitness that were used for further investigations (Section 3.4). Average number of offspring in Phase 2 was 164 (± 53 SD).

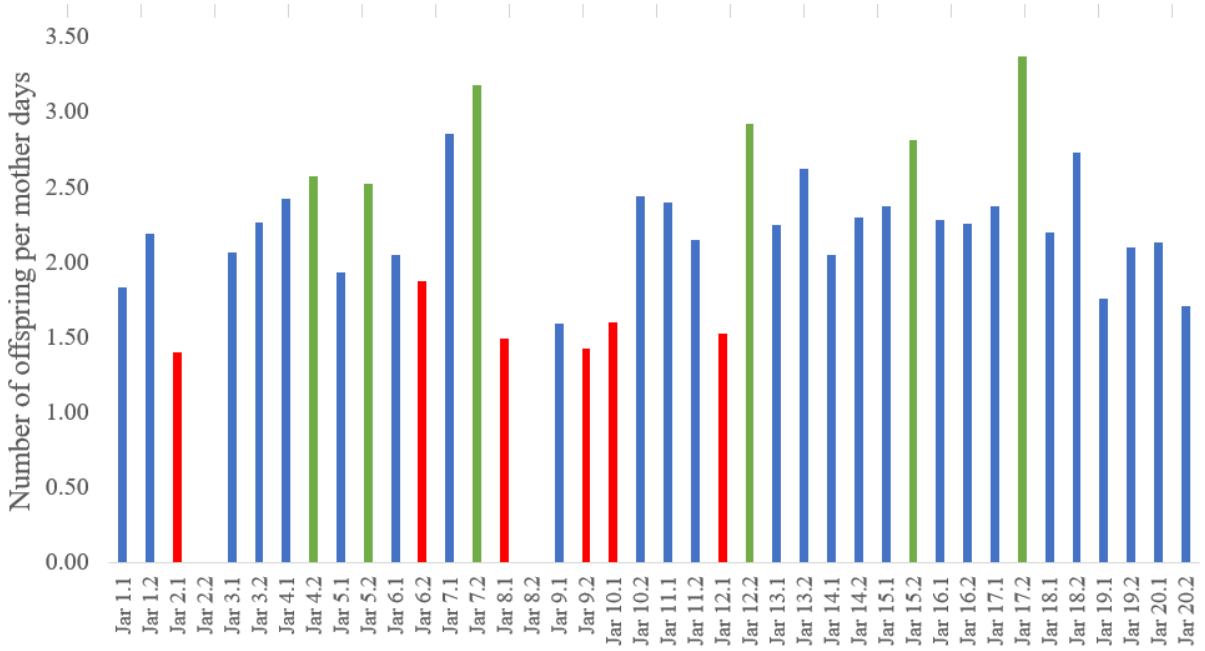

Figure 12: The number of offspring per cumulative number of mother days for all the jars included in Phase 2. The six jars with low fitness (■ , value less than 1.61) or high fitness (■ , value higher than 2.51) are indicated.

The raw data (Appendix D, Table 17) were also used in Figure 13 to illustrate the reproducibility between the replicates in Phase 2. The datapoints were number of offspring per mother days, where replicate 2 is a function of replicate 1. The samples with zero at the Y-axis, were jar 2.2 and 8.2. In these jars, all fecund mothers died at an early stage resulting in no offspring. The linear correlation between replicate 1 and replicate 2 was significant ($p = 0.0058$) based on Pearson's product-moment.

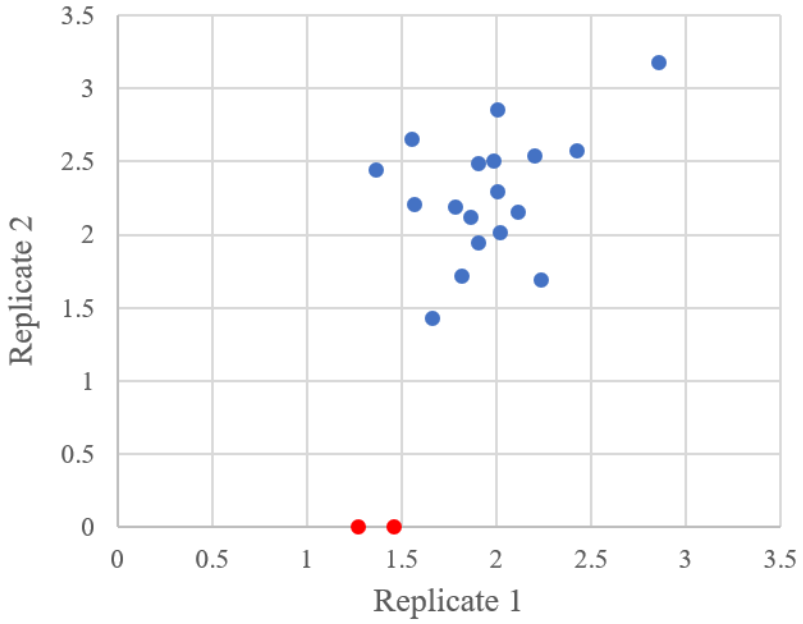

Figure 13: The reproducibility of fitness between replicate 1 and replicate 2 in Phase 2. The datapoints illustrate the number of offspring per mother days in each jar. Those with red color (●) are jar 2.2 and 8.2.

3.4 Phase 3: Experimental evaluation of bacteria-free neonates in cultures with high and low fitness

In Phase 3, number of offspring per mother days was used to select cultures from Phase 2 with high and low fitness. Those with a value ("number of offspring/mother days") lower than 1.61 were selected as replicates with low fitness (jar 2.1, 6.2, 8.1, 9.2, 10.1 and 12.1), and those with a value higher than 2.51 were selected as replicates with high fitness (jar 4.2, 5.2, 7.2, 12.2, 15.2 and 17.2) (Figure 12, Section 3.3). The medium from these cultures was distributed into two replicates and inoculated with bacteria-free neonates. The offspring production and maternal mortality were registered daily for 18 days. The raw data (Appendix D, Table 18) were used to make an illustration (Figure 14) of the reproducibility of the replicates according to the number of offspring per mother days. There was no significant correlation between the two replicates in Phase 3 ($p=0.8445$). In addition, the disinfected eggs from Phase 3 had high mortality, which made the performance of Phase 3 difficult due to few viable bacteria-free neonates. Throughout Phase 3, 6 out of 51 neonates died, resulting in a death rate of 12% for bacteria-free neonates. This was a higher mortality than for the symbiotic juveniles (Phase 2) with 7.5%. Phase 3 was therefore excluded from further analysis.

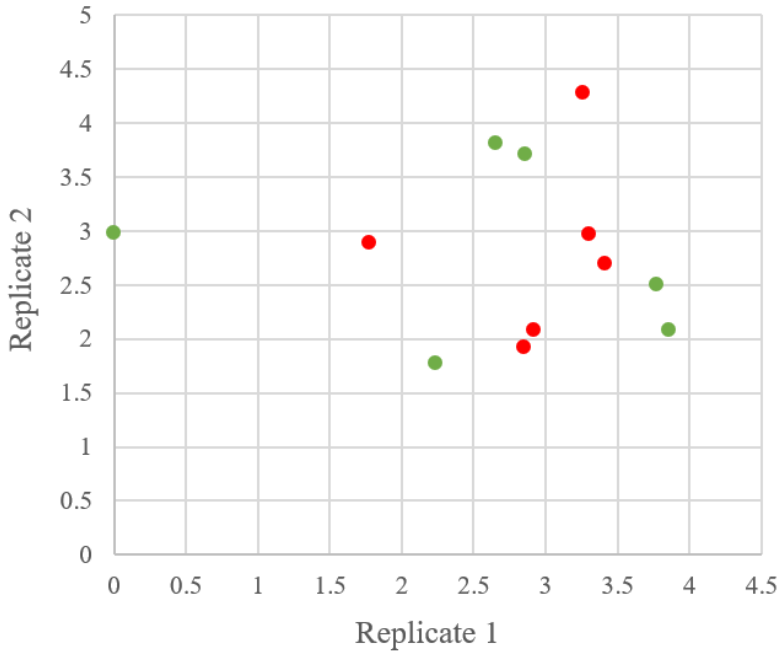

Figure 14: Reproducibility between replicate 1 and replicate 2 based on number of offspring per mother days for each jar (Phase 3). Datapoints originating from replicates with low ( ) and high ( ) fitness selected in the end of Phase 2.

3.5 The alpha diversity among sample-types, phases and fitness level

The 78 samples had a total number of 4 661 525 sequence reads in the raw data after quality filtering, chimera removal and removal of OTUs described as chloroplasts or DNA contamination (Section 2.4.5.3). 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, the total number of OTUs were 514.

The observed number of OTUs (richness) from all sample-types were compared with Chao-1 index (Figure 15). The sequencing covered between 38 to 100% of the actual OTUs available, with a mean of 81.4% (± 14% SD).

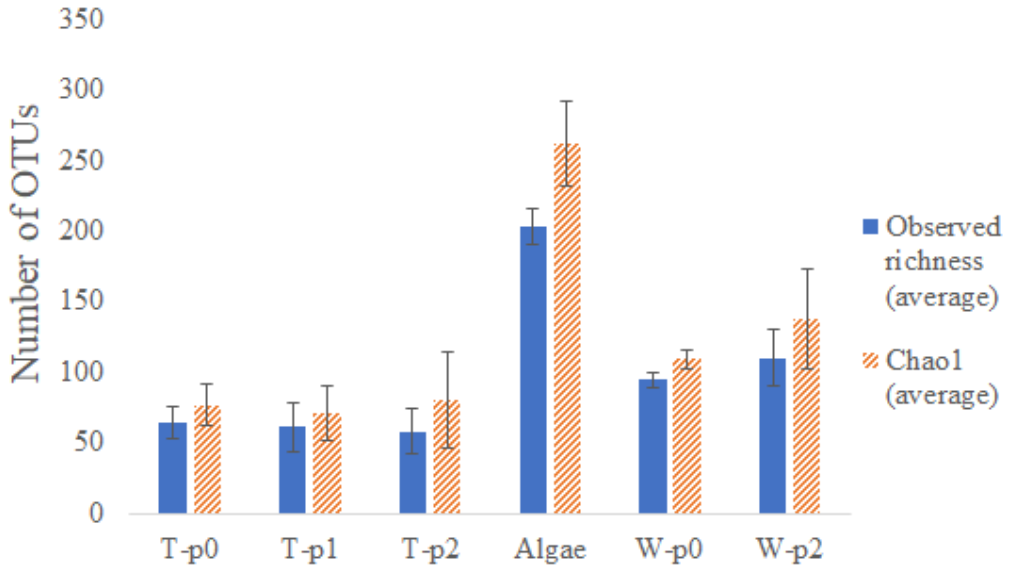

Figure 15: The number of OTUs (richness) and standard deviation (SD) for the sample-types and phases, compared with Chao-1 index. "T" indicates intestines from *D. magna*, and "W" indicates medium where the daphnia resided. The "p" means phase followed by the phase number (0, 1 or 2). "Algae" refers to the algal solution used as feed for *D. magna.*

The average richness (^{0}D) in the cultivation medium (108 OTUs) was around twice as large as the average richness in the intestines (59 OTUs). A two-sample paired t-test confirmed significant difference ($p<0.05$) between the two sample-types. The microbial communities (intestine and cultivation medium) had the same evenness (Figure 16B). Evenness (^{1}E) lower than 0.2 indicates an overall low evenness where few OTUs are abundant in the samples. ^{1}D indicates that cultivation medium has a higher diversity than intestine due to higher richness (Figure 16A). This difference was significant ($p<0.05$) according to two-sample paired t-test.

The alpha-diversity investigation was only performed on the intestines in Phase 1 and 2, due to low number of replicates for Phase 0. The average richness (^{0}D) was almost equal for both phases, with a mean of 61 and 58 OTUs, respectively. The evenness (^{1}E) was higher for Phase 1 than for Phase 2, which indicates that communities in Phase 1 were more evenly distributed than communities in Phase 2. There was no significant difference ($p>0.05$) in richness and evenness according to two-sample paired t-test on the two phases. However, ^{1}D indicated that diversity in Phase 1 was higher than in Phase 2 from an overall perspective of both richness and evenness. The two-sample paired t-test confirmed that the difference was significant ($p<0.05$).

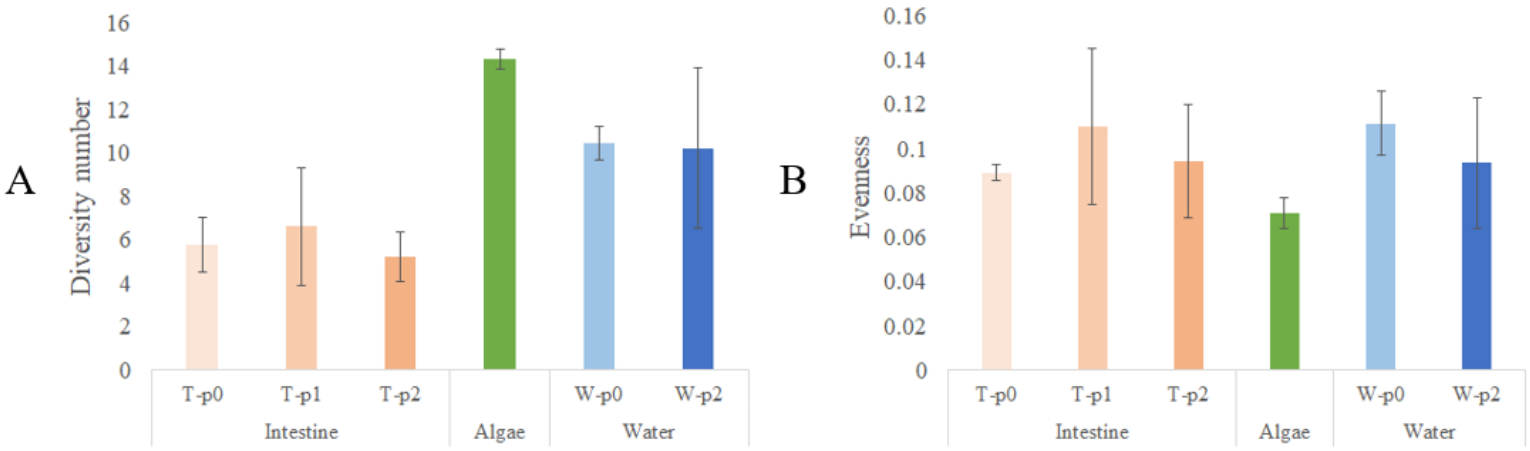

Figure 16: (A) The diversity number according to ^{1}D and the standard deviation (SD) for the different sample-types and phases. (B) The evenness (^{1}E) and standard deviation (SD) of the OTUs among sample-types and phases. "T" indicates intestines from *D. magna*, and "W" represents medium where the daphnia resided. "p" means phase followed by the phase number (0, 1 or 2). "Algae" refers to the algal solution that was used as feed for *D. magna*.

The richness in the groups of intestines with high and low fitness (Phase 2) had a mean of 62 and 53 OTUs per sample, respectively (Figure 17). The two-sample paired t-test showed no significant difference ($p>0.05$) in the richness between high and low fitness. The evenness of the OTUs was almost equal, but jars characterized as high fitness had a slightly higher evenness than those with low fitness (Figure 18B). ^{1}D indicates, therefore, that the group with high fitness has a higher diversity (according to both richness and evenness) than the group with low fitness (Figure 18A). The difference was not significant according to two-sample paired t-test ($p>0.05$).

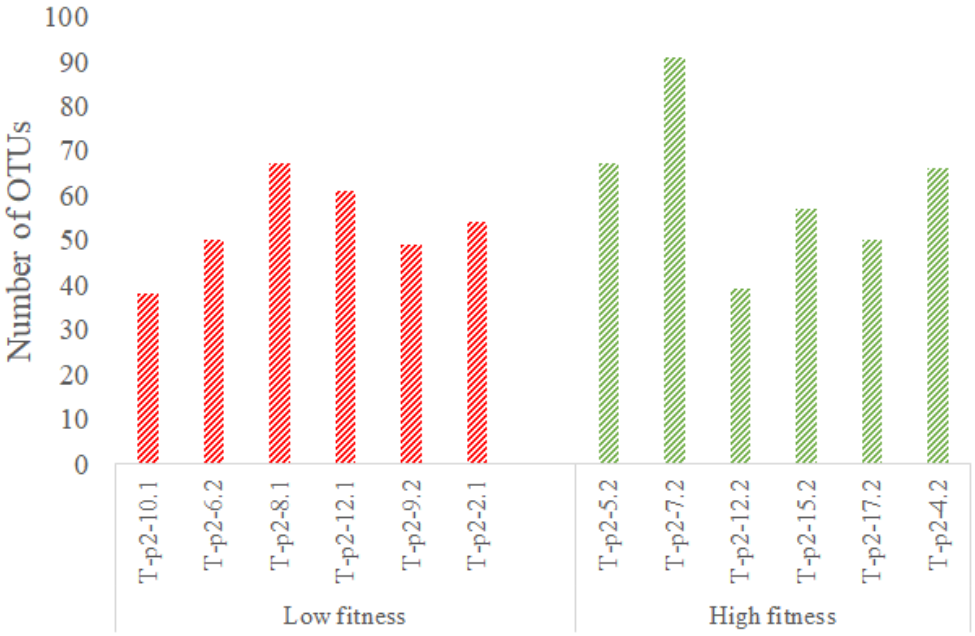

Figure 17: The number of OTUs (richness) among a group of intestines (Phase 2) with either high ( ) or low fitness ( ). "T-p2" represents intestine from Phase 2, followed by the jar-and replicate-numbers. Each sample comprise four intestines from individuals resided in same jar.

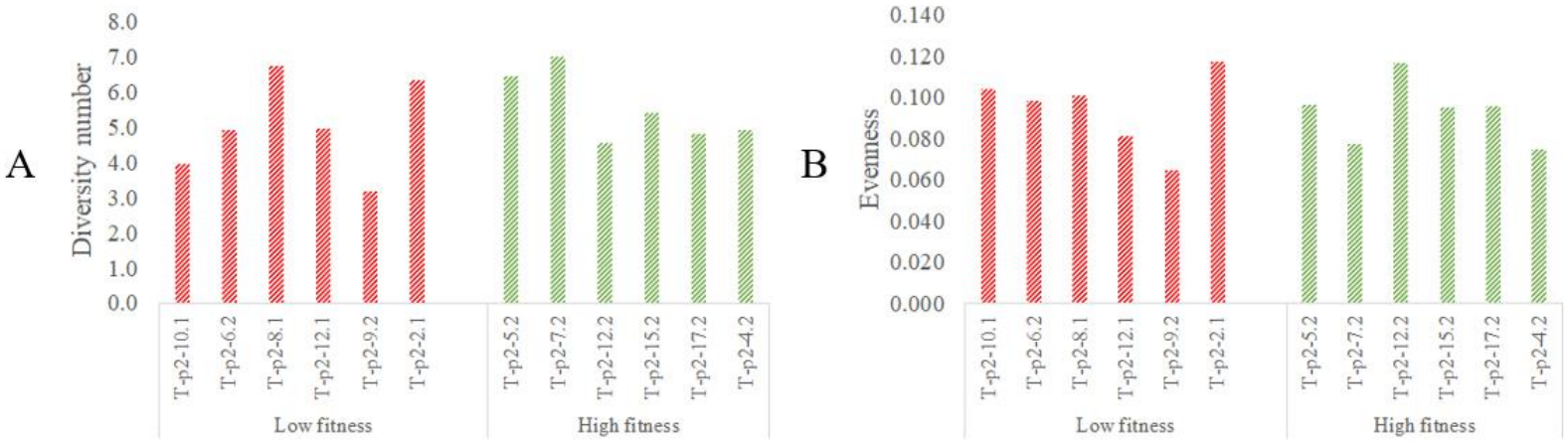

Figure 18: (A) The diversity number according to ^{1}D for two groups of intestines (Phase 2) with high ( ) and low ( ) fitness. (B) The evenness for the same two groups of intestines. "T-p2" represents intestines from Phase 2, followed by the jar-and replicate-numbers. Each sample comprise four intestines from individuals resided in same jar.

3.6 Comparison of bacterial community composition among intestines from different phases

The microbial communities in the intestines were identified by Illumina sequencing of the V3 and V4 regions of the bacterial amplicon (16S rDNA) to describe taxonomic variations among phases and fitness levels. The intestines from the different phases were investigated closer by PCoA based on Bray-Curtis similarity (Figure 19). There was no distinct clustering between the different phases, but there were some outliers in both Phase 1 and 2. These included three samples from Phase 1 (intestines from jar 3, 13 and 18) and two samples from Phase 2 (intestines from jar 1.1 and jar 3.1).

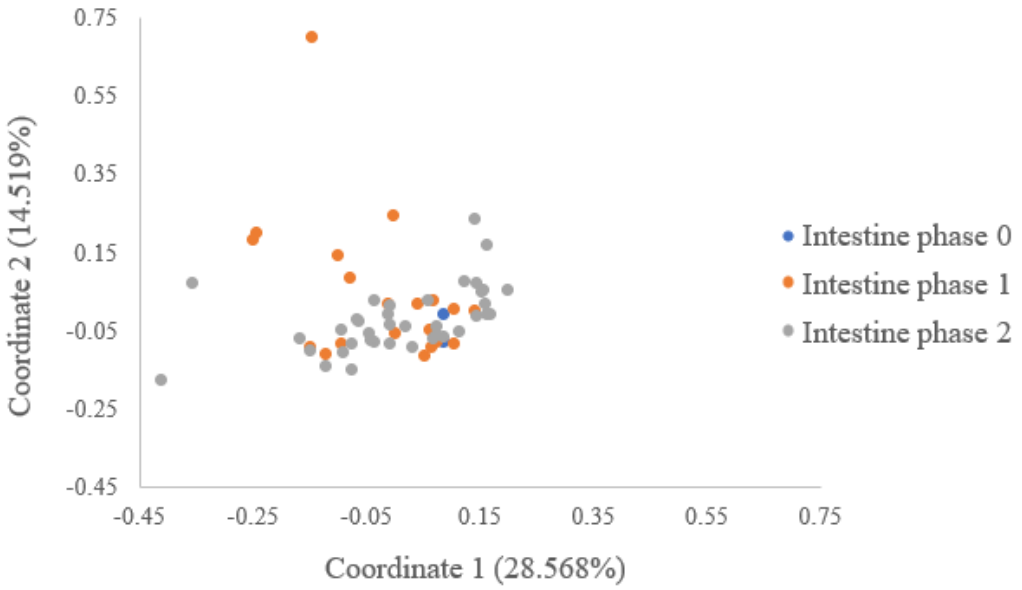

Figure 19: PCoA based on Bray-Curtis similarity of the OTUs in the intestines collected in Phase 0, 1 and 2. Intestines were collected from *D. magna*. The percentage after the coordinate number indicates how much of the variance in the data that is explained by that axis.

One-way PERMANOVA based on Bray-Curtis similarity confirmed a significant difference in the microbial community composition between intestines in Phase 1 and in Phase 2 (p=0.0448). There was no significant difference between Phase 0 and Phase 1 (p=0.3647) or between Phase 0 and Phase 2 (p=0.4462) according to Bray-Curtis similarity. One-way PERMANOVA based on Dice index performed on the same samples showed significant difference between Phase 0 and Phase 1

(p=0.0327) and between Phase 1 and Phase 2 (p=0.0001), but not between Phase 0 and Phase 2 (p=0.07). This indicates that clustering between Phase 0 and Phase 1 was only caused by the difference in occurrence of OTUs, while the clustering between Phase 1 and Phase 2 was caused by differences in both occurrence and relative abundance of the OTUs.

The similarity among the intestines was investigated by Pasts' similarity and distance indices for both Bray-Curtis (Figure 20) and Dice index (Figure 21). There was high similarity in the microbial community composition between the intestines in the start-up aquaria (Phase 0) based on Bray-Curtis similarity (82%) and Dice index (71%). This indicates that 71% of the OTUs were present in both aquaria and 82% of these OTUs had similar abundance. For intestines in Phase 1, 60% of the OTUs were present in all the samples and 63% of these OTUs had similar abundance in the samples. The intestines in Phase 2 also had 60% of the same OTUs in each sample, but the similarity in relative abundance of the OTUs was 70%. The phases were compared to each other, and the similarity based on Dice (Figure 21) indicated that 57% of the OTUs were similar between phases. However, Phase 0 and Phase 2 had a more similar abundance of these OTUs than Phase 1 and Phase 2 according to Bray-Curtis similarity.

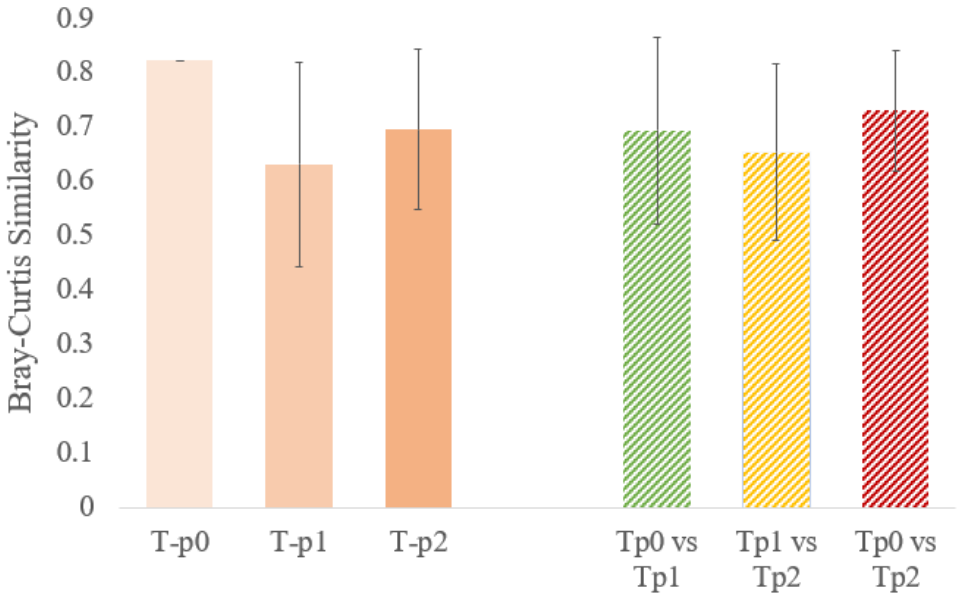

Figure 20: Bray-Curtis similarity and the standard deviation (SD) within each phase, and between the phases. The phases indicate different timepoints throughout the experiment where the intestines where dissected. "T" refers to intestines from *D. magna*, and "p" represents the phase followed by the phase-number.

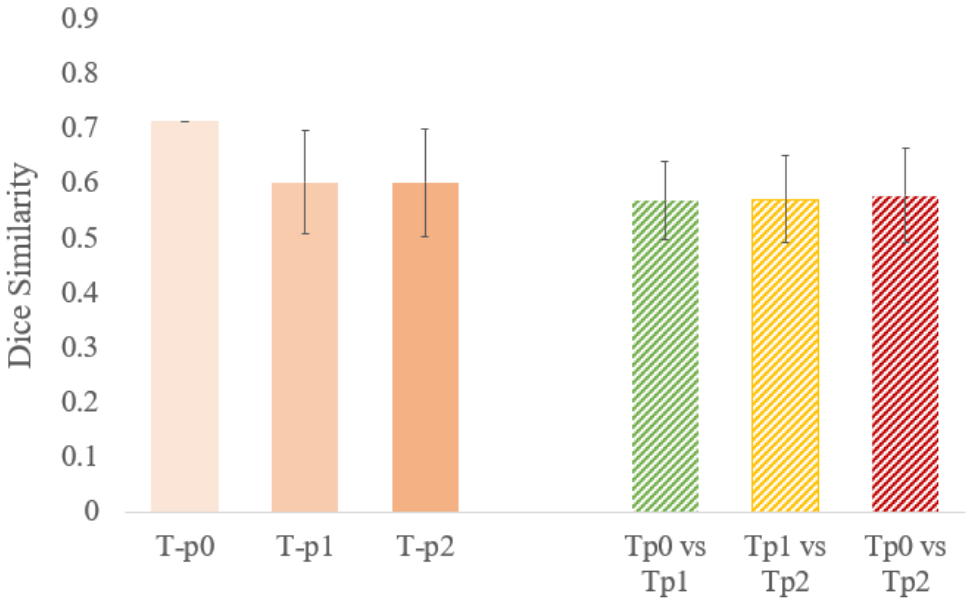

Figure 21: The similarity, based on Dice (Sørensen), and the standard deviation (SD) within each phase, and between the phases. The phases indicate different timepoints throughout the experiment where the intestines where dissected. "T" refers to intestines from *D. magna*, and "p" represents the phase followed by the phase-number.

In total the five most significant OTUs calculated by SIMPER explained 61% of the difference in the community composition between Phase 0 and Phase 1 (Table 6), and 69% between Phase 1 and Phase 2 (Table 7). Phase 0 has more reads of *Limnohabitans* and *Pedobacter* than Phase 1, but less of Bacteroidetes, *Pseudomonas* and Rhodobacteraceae. While, Phase 2 has more reads of Bacteroidetes, *Limnohabitans* and Comamonadaceae than Phase 1.

Table 6: The OTUs contributing the most to the taxonomic differences between intestines in Phase 0 and Phase 1, and their relative abundance given as fraction in each phase.

OTU_id	**Taxonomy**	**Contribution %**	**Avg. rel. abundance Phase 0**	**Avg. rel. abundance Phase 1**
OTU_1	*Limnohabitans* (Proteobacteria)	19.51	0.44	0.37
OTU_2	*Pedobacter* (Becteroidetes)	15.61	0.25	0.19
OTU_4	Bacteroidetes	13.90	0.06	0.14
OTU_9	*Pseudomonas* (Proteobacteria)	6.09	0.01	0.04
OTU_8	Rhodobacteraceae (Proteobacteria)	5.77	0.02	0.05

Table 7: The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic difference between intestines in Phase 1 and Phase 2, and their relative abundance given in fraction in each phase.

OTU_id	**Taxonomy**	**Contribution %**	**Avg. rel. abundance Phase 1**	**Avg. rel. abundance Phase 2**
OTU_1	*Limnohabitans* (Proteobacteria)	23.61	0.37	0.40
OTU_4	Bacteroidetes	15.87	0.14	0.15
OTU_2	*Pedobacter* (Bacteroidetes)	12.62	0.19	0.19
OTU_6	Comamonadaceae (Proteobacteria)	11.01	0.08	0.13
OTU_9	*Pseudomonas* (Proteobacteria)	6.00	0.04	0.01

3.7 Comparison of the bacterial community composition between cultures selected as high or low fitness

Taxonomic data from the jars with high and low fitness clustered separately in PCoA based on Bray-Curtis similarity (Figure 22), indicating differences in the microbial community composition. However, there were some exceptions, like jar 12.1, which was clustered with the cultures with high fitness, and jar 4.2, which was clustered with low fitness. Jar 4.2 had the lowest "number of offspring/mother days" among the jars selected as high fitness. There was also an outlier in the y-axis, jar 2.1, which was registered as the jar with the lowest fitness among those selected as low fitness. One-way PERMANOVA with Bray-Curtis similarity confirmed the significant difference in microbial community composition between high and low fitness (p=0.0234). PERMANOVA based on Dice index gave no significant difference (p=0.1517). This indicates that the clustering in Figure 22 was caused by the differences in the relative abundance of the OTUs and not by changes in OTU inventory.

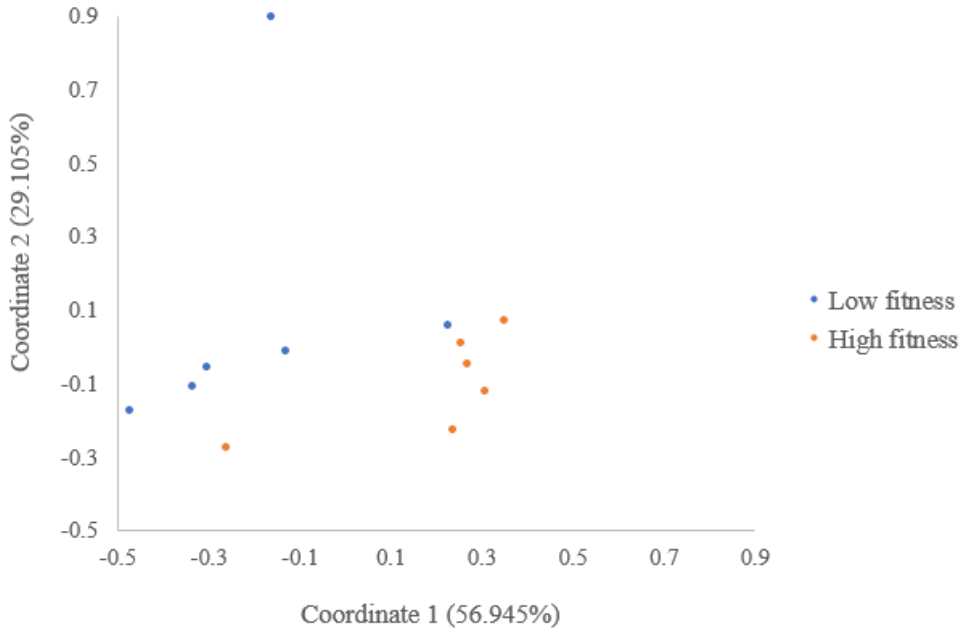

Figure 22: PCoA based on Bray-Curtis similarity of two groups of intestines (Phase 2) selected as high or low fitness according to population growth per mother days in each jar. Intestines were collected from *D. magna*. The percentage after the coordinate number indicates how much of the variance in the data that is explained by that axis.

Similarity among and between cultures described as high fitness and low fitness were investigated by Pasts' similarity and distance indices. The similarity analysis was based on Bray-Curtis similarity (Figure 23) and Dice index (Figure 24). Among the jars characterized as high fitness, 65% of the OTUs were present in all the jars and 78% of these OTUs had similar relative abundance within the jars. Among the jars characterized as low fitness, 57% of the OTUs were present in all the jars and 68% of these OTUs had similar abundance. For the similarity between high fitness and low fitness, 62% describes the relative occurrence of OTUs and 69% describes the relative abundancy of these OTUs.

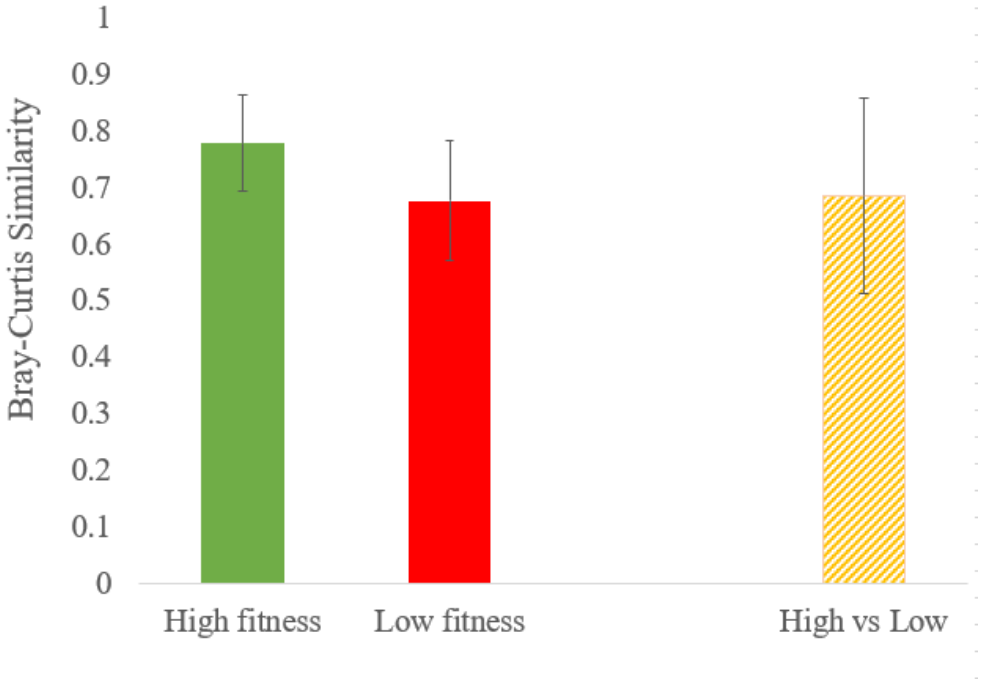

Figure 23: Bray-Curtis similarity and the standard deviation (SD) among and between intestines selected as high or low fitness according to population growth per mother days in each jar. Intestines were collected from four *D. magna*.

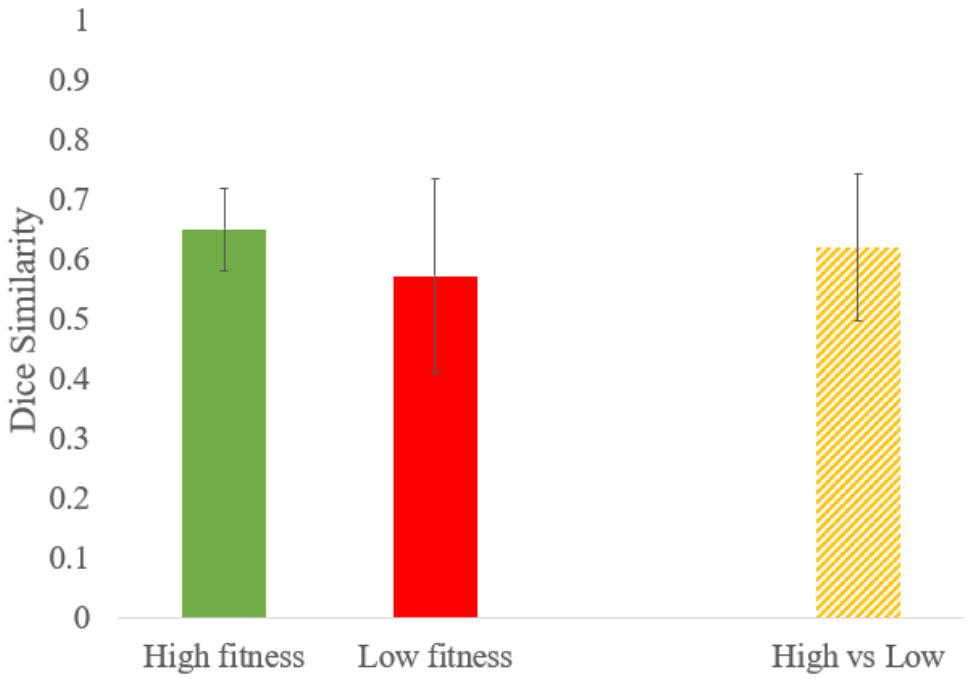

Figure 24: The similarity according to Dice (Sørensen) index and the standard deviation (SD) among and between intestines selected as high or low fitness according to population growth per mother days in each jar. Intestines were collected from four *D. magna*.

The taxonomic variations (at genus level) between samples selected as high and low fitness in Figure 25 confirmed the similarity in OTU inventory and difference in relative abundance. The classification "unassigned" involves OTU_4 (Bacteroidetes), OTU_6 (Comamonadaceae) and OTU_10 (Betaproteobacteria), among others. Among the jars with high fitness, jar 17.2 and 7.2 had the highest population growth and jar 5.2 and 4.2 had the lowest (Figure 12, Section 3.3). Among the jars with low fitness, jar 2.1 and 9.2 had the lowest population growth, and jar 6.2 and 10.1 had the highest (Figure 12, Section 3.3).

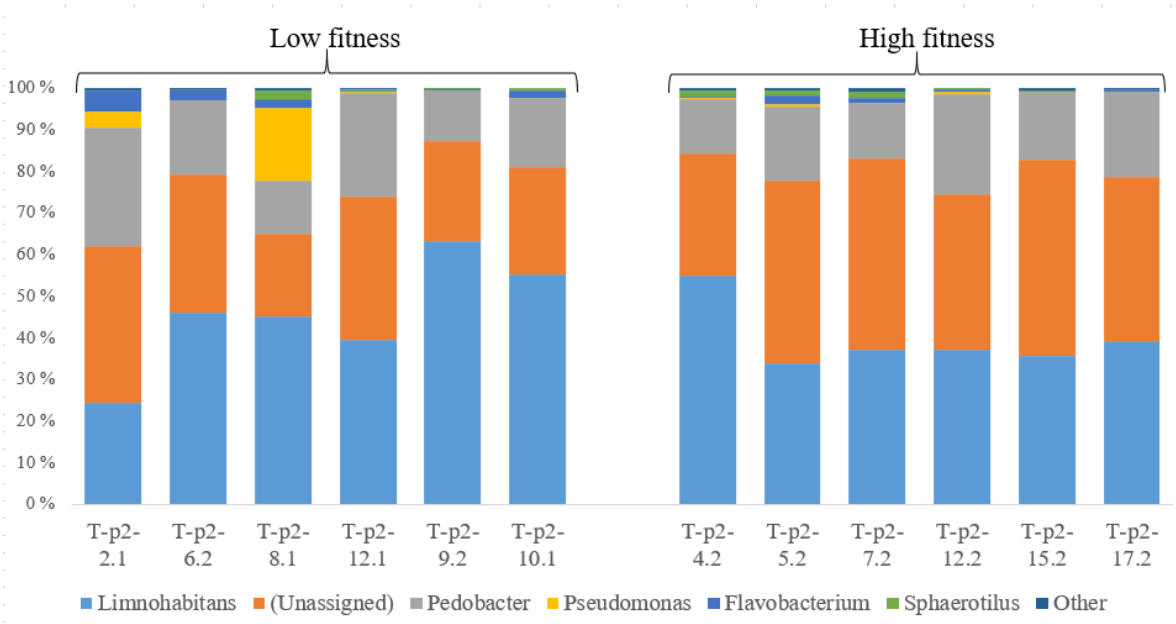

Figure 25: The taxonomic overview at genus level for intestines selected as low and high fitness decided according to population growth per mother days in each jar. "T-p2" refers to intestine in Phase 2, followed by the number of the jar.

In total, the six most significant OTUs, calculated by the statistical method SIMPER, explained 82% of the difference in the community composition between individuals with high and low fitness (Table 8). Those characterized as high fitness had a higher abundance of Bacteroidetes, while those with low fitness had a higher abundance of *Limnohabitans*, *Pedobacter*, Betaproteobacteria and Comamonadaceae. There were also some proportions of *Psudomonas* in some of the intestines with low fitness (i.e. especially jar 2.1 and 8.1). The OTU characterized as Bacteroidetes (OTU_4) was specified as either Flavobacteria or Sphingobacteria at class level, according to Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/). Betaproteobacteria (OTU_10) was classified as order Burkholderiales, and either Burkholderiaceae or Oxalbacteraceae at family level by RDP.

Table 8: The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic difference between intestines with high fitness and low fitness, and their relative abundance given in fraction for each group.

OTU_id	Taxonomy	Contribution %	Avg. rel. abundance High	Avg. rel. abundance Low
OTU_4	Bacteroidetes	28.38	0.230	0.074
OTU_1	*Limnohabitans* (Proteobacteria)	22.16	0.396	0.456
OTU_2	*Pedobacter* (Bacteroidetes)	10.03	0.175	0.188
OTU_6	Comamonadaceae (Proteobacteria)	9.97	0.087	0.137
OTU_10	Betaproteobacteria (Proteobacteria)	5.81	0.002	0.037
OTU_9	*Pseudomonas* (Proteobacteria)	5.66	0.002	0.033

3.8 Comparison of bacterial community composition between cultivation medium and intestines

To evaluate the differences in taxonomy between sample-types, PCoA based on Bray-Curtis similarity was performed. The plot indicates two distinct clusters between the microbial communities from cultivation medium (includes Phase 0 and Phase 2) and from the intestines (includes Phase 0, 1 and 2; Figure 26). There was high significant difference ($p=0.0001$) between OTUs present in the intestine and in the cultivation medium according to one-way PERMANOVA based on both Bray-Curtis similarity and Dice index. The high significance was due to differences in OTU inventory and the relative abundance of the OTUs in the different habitats. The samples from the cultivation medium in Phase 0 were clustered together with the samples from the cultivation medium in Phase 2. In addition to the outliers in Figure 19 (Section 3.6), Figure 26 shows two samples from the cultivation medium collected in Phase 2 (jar 2.1 and 8.2) that stand out from the rest.

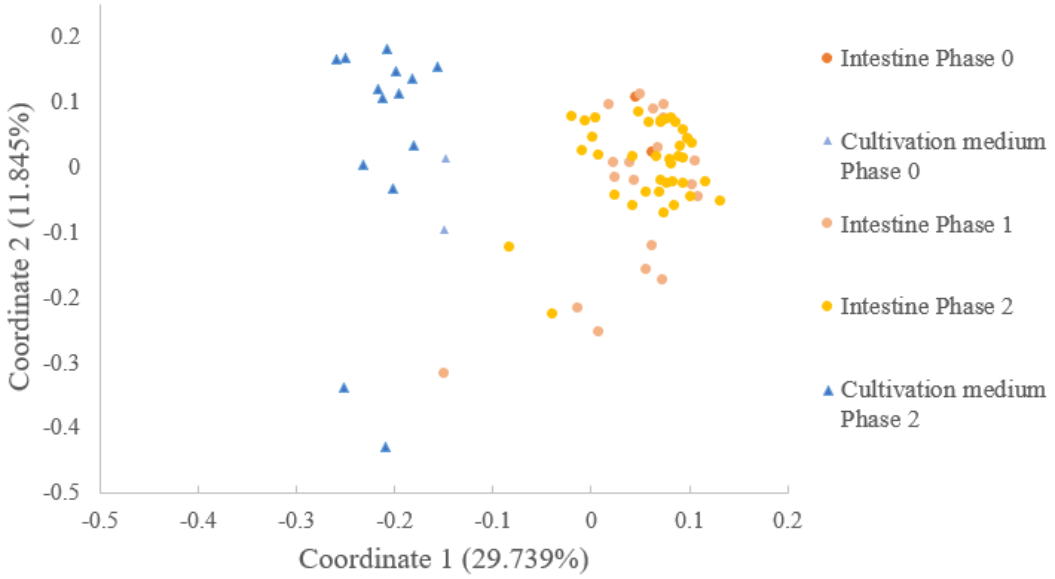

Figure 26: PCoA based on Bray-Curtis similarity of the OTUs in the intestines (Phase 0, 1 and 2) and cultivation medium (Phase 0 and 2). Intestines were collected from *D. magna*, and "Cultivation medium" is where the daphniids resided. The percentage after the coordinate number indicates how much of the variance in the data that is explained by that axis.

Similarity and distance indices were performed to investigate the similarity among a sample-type and between sample-types. The similarity was decided by Bray-Curtis similarity (Figure 27) and Dice index (Figure 28). Among the intestines, 68% of the similarity was explained by the relative abundance and 60% explained by the same OTU inventory. Among the samples collected from the cultivation medium, the main similarity (74%) was explained by the occurrence of the same OTUs. The relative abundance among the cultivation medium was 51%. This means that the cultivation medium has a more similar OTU inventory than the intestines, but the relative abundance of the OTUs were more similar for intestines than for the cultivation medium. The similarity between intestine and cultivation medium was mainly explained by the OTU inventory, where 50% of the OTUs were present in both sample-types. However, these were distributed differently according to Bray-Curtis similarity (34%).

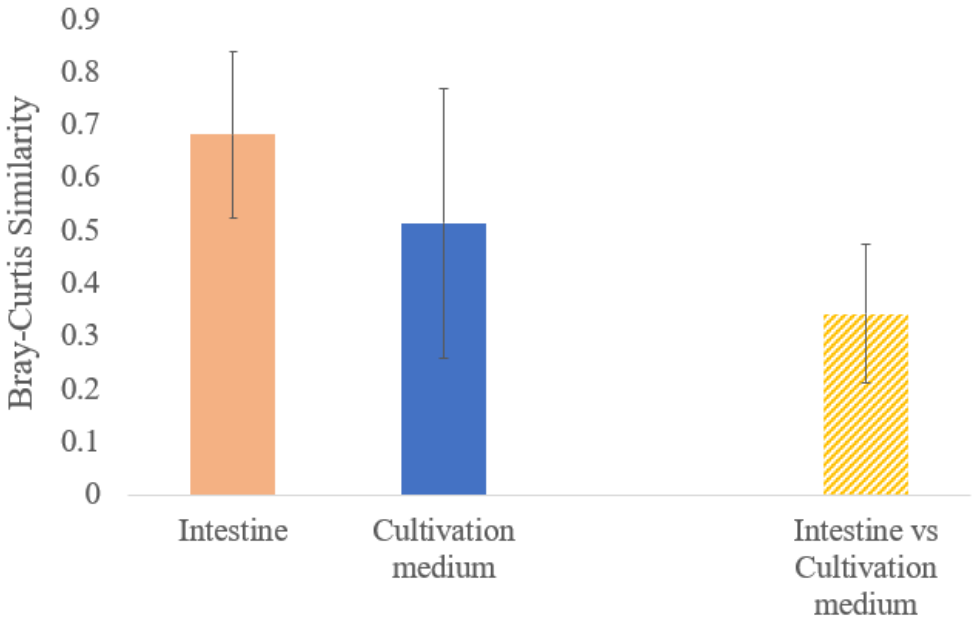

Figure 27: Bray-Curtis similarity and standard deviation (SD) within and between sample-types (intestine and cultivation medium). Intestines were collected from *D. magna* (Phase 0, 1 and 2) and cultivation medium is where daphniids resided (Phase 0 and 2).

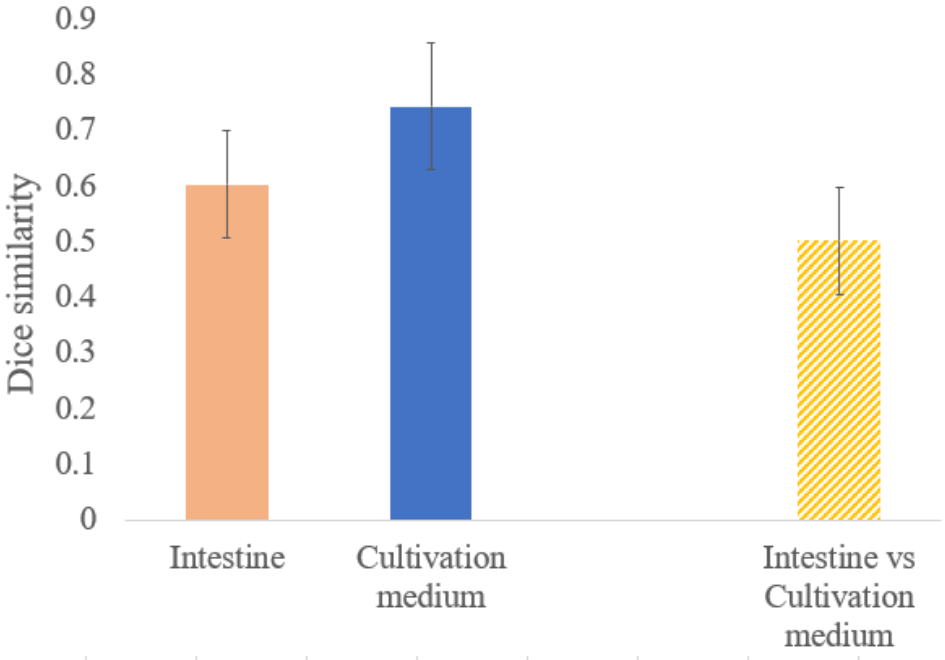

Figure 28: The similarity according to Dice (Sørensen) index and the standard deviation (SD) within sample-types and between sample-types (intestine and cultivation medium). Intestines were collected from *D. magna* (Phase 0, 1 and 2) and cultivation medium is where daphniids resided (Phase 0 and 2).

In total the five most significant OTUs explained 70% of the difference in the community composition between intestines and cultivation medium (Table 9). The phylum Bacteroidetes, the genus *Limnohabitans* and the family Comamonadaceae were most dominant in the intestines, while the genera *Flavobacterium* and *Pedobacter* dominated in the cultivation medium.

Table 9: The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic difference between intestines and cultivation medium, and their relative abundance given in fraction for each group.

OTU_id	**Taxonomy**	**Contribution %**	**Avg. rel. abundance Intestine**	**Avg. rel. abundance Cultivation medium**
OTU_1	*Limnohabitans* (Proteobacteria)	25.72	0.400	0.065
OTU_7	*Flavobacterium* (Bacteroidetes)	15.16	0.006	0.206
OTU_2	*Pedobacter* (Bacteroidetes)	11.79	0.191	0.251
OTU_4	Bacteroidetes	11.04	0.154	0.008
OTU_6	Comamonadaceae (Proteobacteria)	6.13	0.134	0.105

The cultivation medium from the cultures selected as high and low fitness was also compared with each other. The difference in the relative abundance of the OTUs was marginally significant according to one-way PERMANOVA based on Bray-Curtis similarity ($p=0.0521$). There was no significant difference according to PERMANOVA based on Dice index ($p=0.4201$). In total the five most significant OTUs explained 58% of the difference in the community composition between the cultivation medium from cultures with high and low fitness (Table 10). The genera *Pedobacter* and *Flavobacterium* dominated in the cultivation medium with high fitness, while the family Comamonadaceae, the class Cyanobacteria and genus *Limnohabitants* dominated in the cultivation medium with low fitness.

Table 10: The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic difference between cultivation medium with high or low fitness, and their relative abundance given in fraction for each group.

OTU_id	**Taxonomy**	**Contribution %**	**Avg. rel. abundance Cultivation medium (High fitness)**	**Avg. rel. abundance Cultivation medium (Low fitness)**
OTU_2	*Pedobacter* (Bacteroidetes)	26.29	0.374	0.197
OTU_7	*Flavobacterium* (Bacteroidetes)	12.99	0.230	0.193
OTU_6	Comamonadaceae (Proteobacteria)	6.94	0.093	0.107
OTU_15	Cyanobacteria (Cyanobacteria/ Chloroplast)	5.97	0.023	0.049
OTU_1	*Limnohabitans* (Proteobacteria)	5.64	0.065	0.070

Taxonomic variation (at class level) collected in the different sample-types and phases is given in Figure 29. Betaproteobacteria and Sphingobacteria were the most dominated classes in both intestine and cultivation medium. The intestines had highest level of Betaproteobacteria, but they were also present in the cultivation medium. Flavobacteria was at a low level in the intestines, but at high level in the cultivation medium. The Sphingobacteria were evenly distributed in all the sample-types.

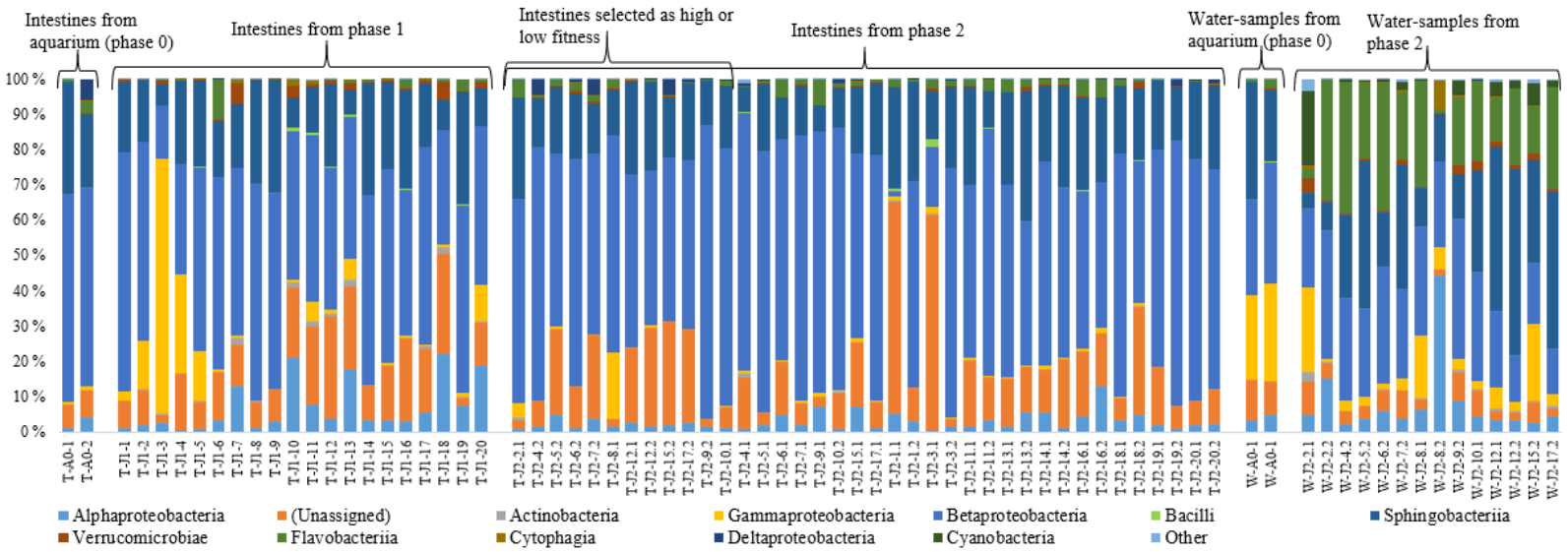

Figure 29: Taxonomic overview at class level for the different sample-types, phases and fitness. Twelve intestines (Phase 2) were selected as high or low fitness according to population growth per mother days. "T" represents intestines collected from four *D. magna*, while "W" is the medium where the daphniids resided. "A0" refers to the start-up aquaria (Phase 0), and "J" stands for the jars, followed by the phase-number (Phase 1 or 2). The next numbers represent the jar- and replicate number of the jar.

3.9 Variation in bacterial cell density among the cultivation medium in different cultures

To evaluate if number of bacteria cells had effect within or among the samples (sample-type, phases and fitness) the bacterial cell densities were determined by flow cytometry. Two samples of the cultivation media in Phase 0, and 14 samples of the cultivation media in Phase 2 were collected. The fluorescence detected per cell or particle was used to form PolygonGates, that were further used for identification and counting of bacterial cell populations (Figure 30). The areas with high intensity of the fluorescent SYBR® Green I (red color) in Figure 30 indicate potential bacterial cell populations, e.g. the aquarium in (A) may illustrates three different bacterial cell populations. Figure 30 also shows fluorescent signals beyond the normal FL1-H signal, which was, according to the sample without staining (Appendix E, Figure 36Ai), natural fluorescent. In addition, little to no contamination were present in the filtered 1xPBS that was used to dilute the samples (Appendix E, Figure 38Bii).

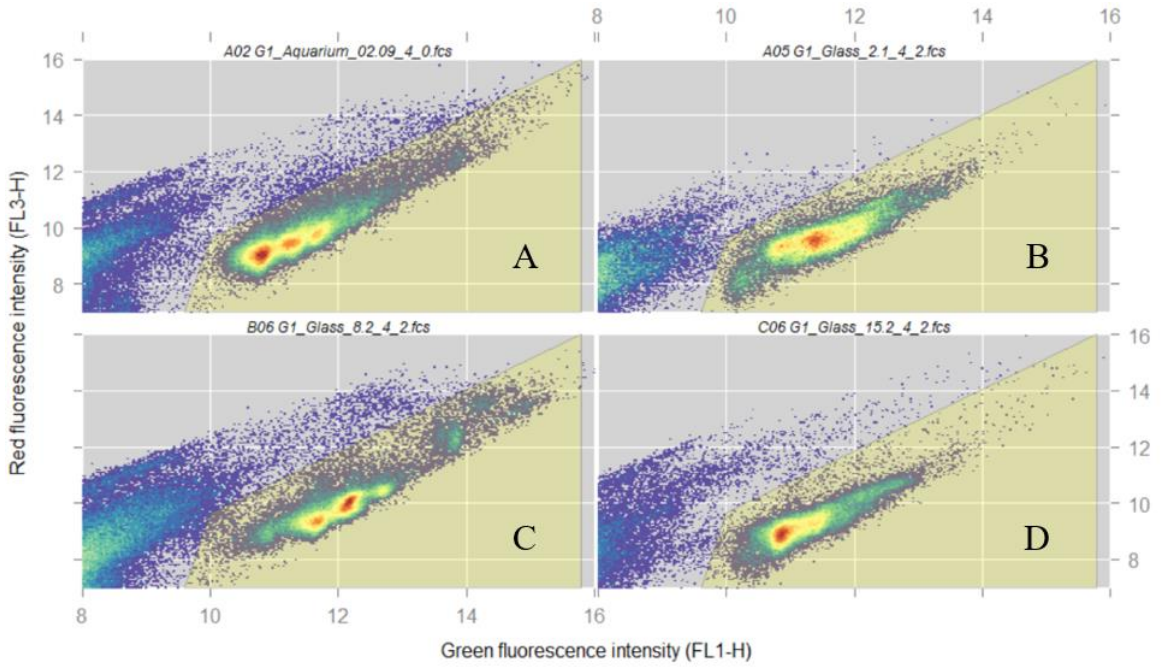

Figure 30: Examples of fluorescent emissions from samples of the cultivation medium (Phase 0 and Phase 2) stained with SYBR® Green I detected by flow cytometry. The four samples were collected from (A) an aquarium (B) jar 2.1, (C) jar 8.2, and (D) jar 15.2. The cultivation medium is where *D. magna* resided. Green fluorescence intensity (FL1-H) was plotted against the red fluorescence intensity (FL3-H). PolygonGates (yellow areas) were used to identify bacterial population cells. Different color shows the intensity of the fluorescent SYBR® Green I (red= high, yellow= middle and blue/green= low).

The cell-density (number of cells per unit volume) within the PolygonGates was calculated with respect to dilution factor for each sample (Figure 31). The aquaria had a higher cell-density (average 4 807 977± 186 337 SD) than the jars (average 3 189 442± 1 330 900 SD). Besides the aquaria, jar 2.2 and 4.2 represented the highest cell-density, and jar 9.2 and 12.2 represented those with lowest cell-density. Cultures selected as high fitness had a higher average cell-density (2 723 609± 966 817 SD) than those selected as low fitness (2 129 784± 697 334 SD). The difference was not significant according to two-sample paired t-test.

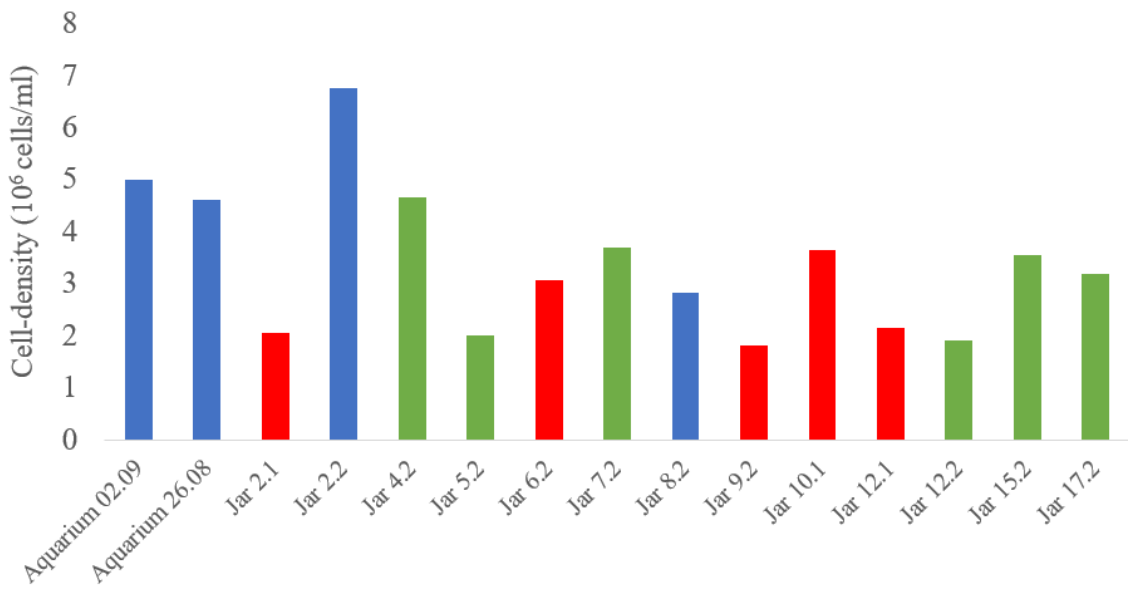

Figure 31: Cell-densities (10^6 cells per ml), with respect to the dilution factor, for cultivation media in Phase 0 (aquaria) and Phase 2 (jars). Medium was collected from the jars where daphniids resided, involving the jars selected as high (■) and low fitness (■). The data for sample 8.1 was not included.

3.10 Evaluation of how bacteria present in the feed influences the intestinal microbial composition

The daphniids were fed with Shellfish Fish 1800 (Reed Mariculture Inc, USA). The fluorescence detected within the PolygonGate (yellow area) gives an indication of high availability of bacteria in the algal solution (Figure 32). There is also a clear fluorescent signal at higher wavelength than SYBR® Green I, that may be caused by chlorophyll.

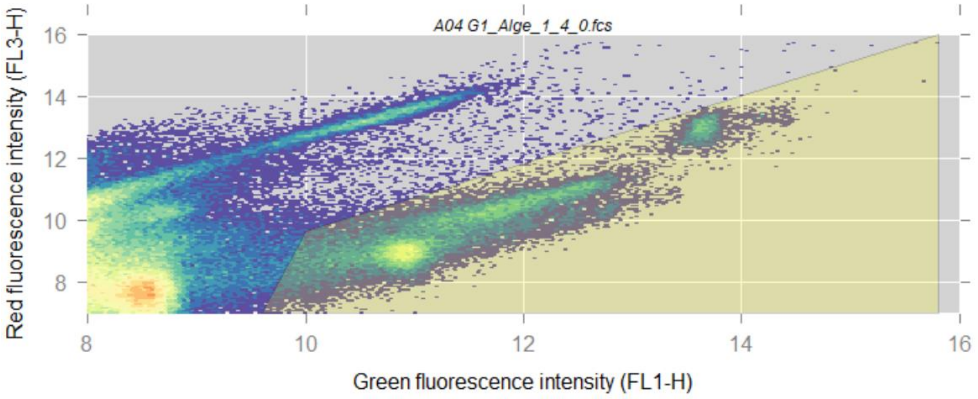

Figure 32: Fluorescent intensity of the algal solution stained with the fluorescent SYBR® Green I detected by flow cytometry. The green fluorescence intensity (FL1-H) was plotted against the red fluorescence intensity (FL3-H). The PolygonGate (yellow area) was used to identify potential bacterial population cells in the feed for *D. magna*. Different colors indicate quantity of detected fluorescent (red= high, yellow= middle and blue/green= low).

Prior to sequencing, the algal solution was distributed into two identical samples. The average of both samples is illustrated in taxonomic overview (Figure 33), indicating that the phylum Firmicutes were the most abundant in the feed.

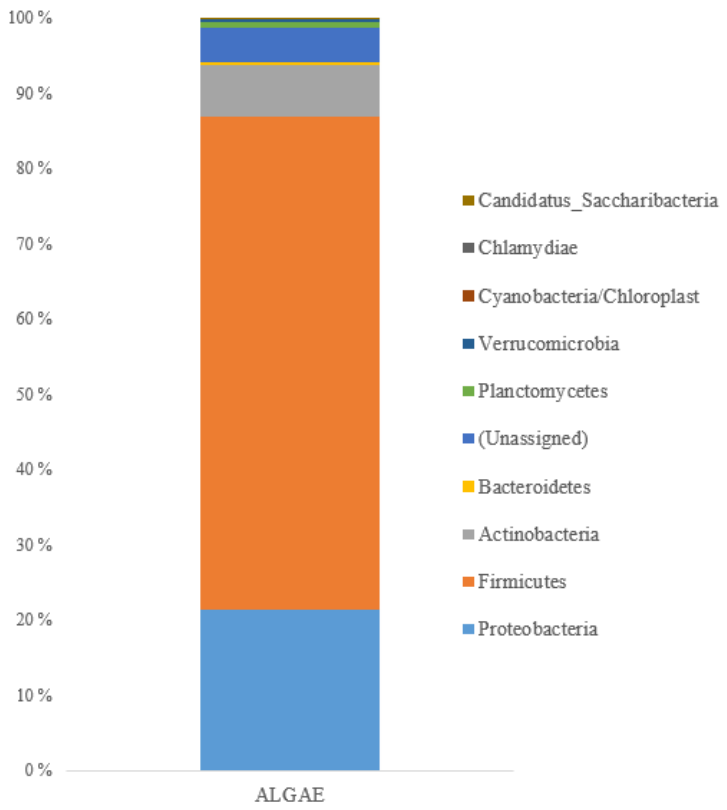

Figure 33: Taxonomic overview of the phyla resided in the algal solution used as feed for *D. magna*. The overview is an average of two identical algal samples.

To compare the difference between feed and cultivation medium (from Phase 0 and Phase 2), one-way PERMANOVA based on Bray-Curtis similarity and Dice index was performed. Both similarity indices were significantly different (p= 0.0082 and p= 0.0075, respectively), indicating difference in occurrence of OTUs and relative abundance of OTUs in the microbial composition of the samples. One-way PERMANOVA based on Bray-Curtis similarity and Dice index confirmed the significant difference between the microbial community composition in the feed and in the intestines from Phase 1 (p=0.0046 and p=0.0042, respectively) and from Phase 2 (p=0.0009 and p=0.0017, respectively), as well. This indicates that the difference was caused by both occurrence and relative abundance of OTUs.

The similarity and distance indices in Past were also performed based on Bray-Curtis similarity and Dice index. Similarities and standard deviations (SD) among sample-types and between sample-types according to Bray-Curtis similarity are illustrated in Figure 34. As expected, the

occurrence (78%) and relative abundance (88%) of OTUs among the algal samples were almost identical because both samples were taken from the same original solution. The similarity in relative abundance of OTUs between intestines and algal solution (1.7%) was lower than the similarity between the cultivation medium and algal solution (2.6%). However, neither the intestines nor cultivation media had any strong correlation to the algal solution according to Bray-Curtis similarity.

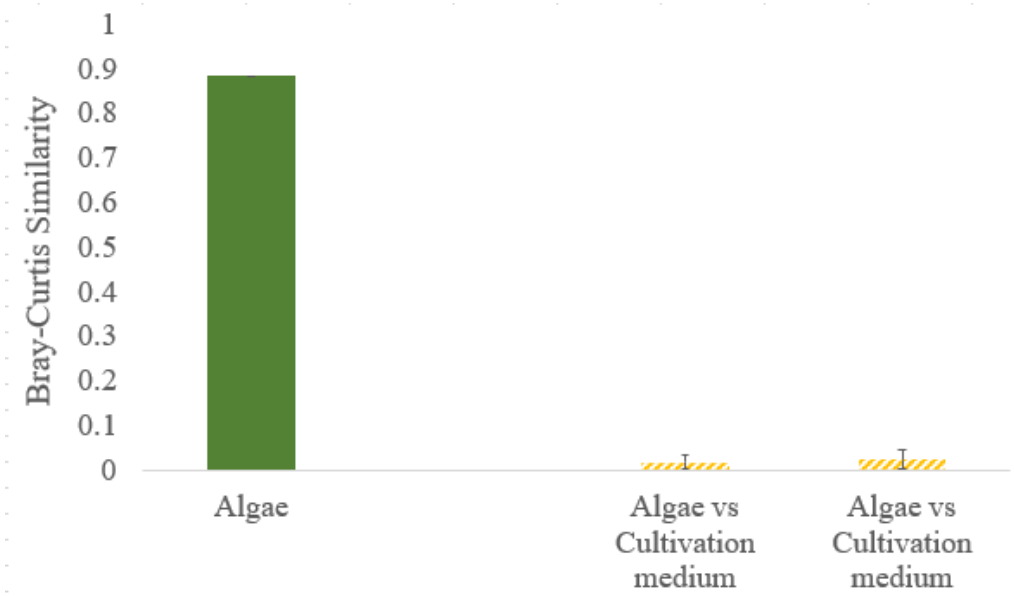

Figure 34: Bray-Curtis similarity and standard deviation (SD) among algal samples (collected from the same algal solution) and between the algal solution and intestines, and between the algal solution and the cultivation medium. Intestines were collected from *D. magna* (Phase 0, 1 and 2). Cultivation medium is where the daphniids resided (Phase 0 and Phase 2). "Algae" refers to the algal solution used as feed for the daphniids.

Similarity among sample-types and between sample-types based on Dice index are illustrated in Figure 35. Compared with Bray-Curtis, there was a higher similarity in the occurrence of OTUs between the algal samples and the intestines (13%), and between the algal samples and cultivation medium (20%). This indicates that the similarity was better explained by the occurrence of the same OTUs than the relative abundance.

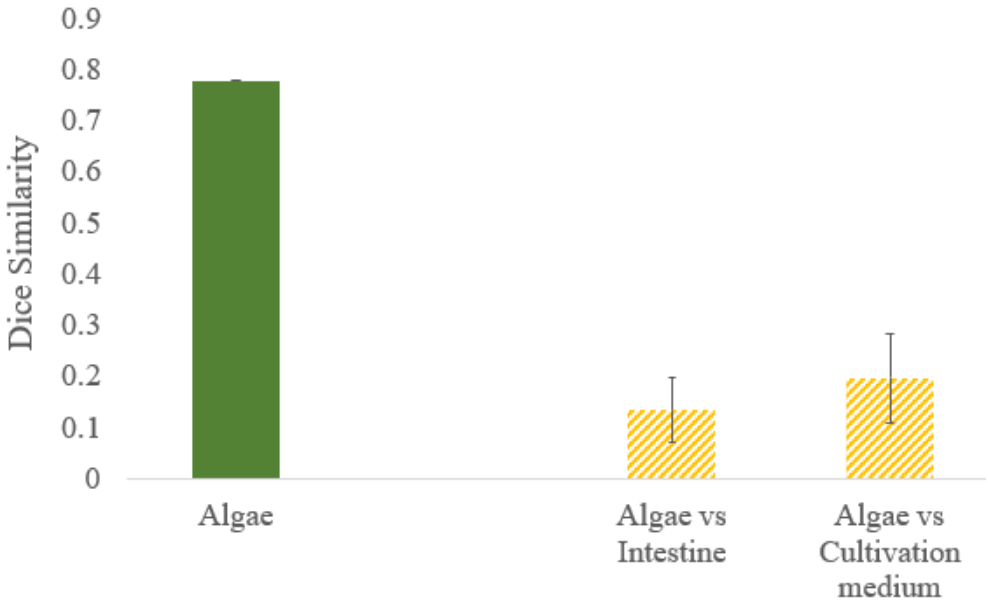

Figure 35: The similarity according to Dice (Sørensen) index and standard deviation (SD) among algal samples (collected from the same algae solution), between the algal samples and intestines, and between algal samples and the samples collected from the cultivation medium. Intestine were collected from *D. magna* (Phase 0, 1 and 2). Cultivation medium is where the daphniids resided (Phase 0 and 2). "Algae" refers to the algal solution used as feed for the daphniids.

In total, the five most significant OTUs contributing with 63% of the difference in the microbial community composition of the intestines and algal solution (Table 11). The main OTUs in the intestines were *Limnohabitans*, *Pedobacter* and Bacteroidetes, but not *Lactobacillus* and *Atopostipes* that dominated in the feed.

Table 11: The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic differences between intestines (Phase 0,1 and 2) and algal solution (feed) and their relative abundance given in fraction for each sample-types.

OTU_id	**Taxonomy**	**Contribution %**	**Avg. rel. abundance Intestine**	**Avg. rel. abundance Algae (feed)**
OTU_1	*Limnohabitants* (Proteobacteria)	19.71	0.393	0.003
OTU_23	*Lactobacillus (*Firmicutes)	13.36	0.001	0.263
OTU_26	*Atopostipes* (Firmicutes)	12.46	0.000	0.245
OTU_2	*Pedobacter* (Proteobacteria)	9.70	0.193	0.002
OTU_4	Bacteroidetes	7.44	0.146	0.000

In total the five most significant OTUs explained 54% of the difference in the microbial community composition between the algal solution and the cultivation medium (Table 12). *Lactobacillus, Atopostipes* and *Alkalibacterium* were found in the algal solution, while none of them were found in the cultivation medium. *Flavobacterium* and *Pedobacter*, that were abundant genera in the cultivation medium, were only present at minor levels in the algal solution.

Table 12: The results from SIMPER analysis showing the OTUs contributing the most to the taxonomic differences between cultivation medium (Phase 0 and 2) and algal solution (feed) and their relative abundance given in fraction for each sample-types.

OTU_id	**Taxonomy**	**Contribution %**	**Rel. Abundance Algae (feed)**	**Rel. Abundance Cultivation medium**
OTU_23	*Lactobacillus (*Firmicutes)	13.47	0.263	0.000
OTU_2	*Pedobacter* (Proteobacteria)	12.80	0.002	0.252
OTU_26	*Atopostipes* (Firmicutes)	12.53	0.245	0.000
OTU_7	*Flavobacterium* (Bacteroidetes)	9.20	0.000	0.180
OTU_39	*Alkalibacterium* (Firmicutes)	5.73	0.112	0.000

4. Discussion

4.1 The procedure to obtain bacteria-free neonates for direct manipulation of the microbiota

The procedure with lowest GA-concentration (0.025%) and shortest treatment-length (30 min; Trial 4) was the best conditions for obtaining viable bacteria-free neonates with low mortality. Peerakietkhajorn et al. concluded that 0.25% GA was the best alternative in their experiment, but reported also that embryos exposed to 0.025% GA had 100% hatching and no detectable bacterial 16S rRNA genes. The same study showed that GA-concentration of 0.25% reduced the hatching success with 19% (54). This may be a reason for the higher mortality among the eggs exposed to 0.1% GA compared with 0.025% GA in my experiment, even though the difference was small (i.e. 49 and 55% survival, respectively). However, the results were difficult to use in the determination of the best procedure, due to unstructured experimental setup. The experiment should have included the same number of treated eggs, more documentation of long-term effects and several replicates for each concentration of GA. However, the most important in this part was to obtain enough viable bacteria-free neonates to inoculate all 24 cultures in Phase 3. Therefore, Trial 4 was the best alternative based on Peerakietkhajorn et al. which reported that daphnia eggs exposed to low concentration of GA had a higher survival (54). Despite the fact that the trial with least exposure of GA was selected, only 50% of the treated daphniids were viable. For my experiment, the effect GA has on bacterial cells (i.e. cross-links proteins, resulting in inhibition of membrane transport, enzyme activity and synthesis of RNA, DNA and proteins) is considered to include for the cells in daphnia, as well (54, 67). This made the performance of Phase 3 problematic (discussed in Section 4.2).

4.2 Variation in intestinal microbial community composition in *D. magna* caused by ecological processes

Microbial ecological experiments are commonly performed by following the diversity in a community for several generations (86). The ecological processes are the most fundamental reasons for variation in structure and diversity within a microbial community (87). This experiment shows evidence of these ecological processes in microbial communities, such as selection for their

environments, drift within cultures and dispersal between sample-types. Speciation is an ecological process assumed to have impact only in the long run and was therefore not considered in this experiment.

Normally, an environment contains multiple niches that differ in conditions of growth. These different sets of growth conditions are favored by different types of organisms. If the growth conditions are uniform, then natural selection may eliminate all except the fittest individuals. The clones used for this experiment were descendants of daphniids collected 6 years ago. These were in the beginning introduced to a novel environment. Throughout years with laboratory conditions and homogenous diet, diversification and selection in the gut may have caused reduction in the diversity of the microbial community (86, 88-90). Therefore, the microbial composition in daphniids gut used in this experiment may not reflect the free-living *D. magna* completely.

The composition of the intestinal microbial community in Phase 0 was difficult to compare with the other phases due to only two samples. Phase 1 and Phase 2 with 20 and 40 replicates, respectively, were more reliable (91). Throughout these phases, it was assumed that each daphnia had established a microbiota that was adapted to the environment. A study by Callens et al. supported this by suggesting that OTUs in the gut of *D. magna* variated in abundance according to their environments (92). Variation of the OTU inventory and relative abundance in the microbial taxa within the intestine for the different phases were probably caused by the ecological processes' selection and drift. Prior to the experiment, individuals in Phase 0 had, for a long time, lived in the same environment. This suggests the possibility that the gut microbiota in the daphniids was adapted for the environment. The medium and daphniids from Phase 0 were transferred into an environment with less volume (jar). A significant change in presence of OTUs occurred throughout the five weeks in the new environment. For instance, the presence of the genera *Limnohabitants* and *Pedobacter* decreased from Phase 0 to Phase 1, but there was an increase in the Bacteroidetes, Rhodobacteraceae and *Pseudomonas* (Table 6). Lower density, more available feed (66) and less dispersal between intestine and cultivation medium (due to autoclaved medium) may also have contributed in changes in the microbial community composition between the two phases.

The microbiota adapts to the environment over time based on the change in OTU inventory and relative abundance of the intestinal microbial community composition in Phase 2. The presence of the genus *Limnohabitants* in the intestines from Phase 2 had increased to approximately the same as the content in Phase 0. Bacteroidetes and Comamonadaceae had also increased from Phase 1 to Phase 2, while the presence of *Pedobacter* was the same (Table 7). Number of OTUs were the same between Phase 1 and Phase 2, but the abundancy varied. In addition, Phase 1 had OTUs that were more evenly distributed than Phase 2 according to evenness. This reflected an assumption that Phase 2 had selected for the OTUs that were more required in its' environment (93). Statistical analysis indicated no significant difference in evenness or richness, but a significant difference in ^{1}D. This suggested that evenness and richness separately did not have any effect on the difference, but that they had an effect combined.

Another evidence of the microbiotas' adaption to its' environment over time was the similarity between the intestinal microbial community composition between Phase 0 and Phase 2. This can be supported by no significant difference in OTUs inventory (Dice-index) and relative abundance (Bray-Curtis similarity) between the two phases, but can also have been caused by drift. At the end of Phase 2, the microbial community composition in the intestines was more similar to the one observed in Phase 0, which may be due to the time they were allowed to reside in each environment. However, due to few replicates in Phase 0, it was difficult to know if the microbial community composition was representative for the composition in the aquaria.

In Phase 3, the aim was to test the hypothesis by controlled colonization of bacteria-free neonates with microbiota from cultures with high and low fitness. Analysis of this phase was excluded due to difficulties with disinfection of the daphnia eggs (discussed in Section 4.1) and low reproducibility of the microbiota in the jars. Due to high mortality among the bacteria-free eggs, several rounds with disinfection were needed to obtain the desired number of bacteria-free neonates. Some of the jars in Phase 3 were prepared for inoculation of these bacteria-free neonates before the disinfection was completed, and leaved jars without individuals of daphnia to maintain the microbiota. This resulted in loss of transmission of microbes from one host to another. In zebrafish, this interhost dispersal was shown to overwhelm effects of host factors (e.g. immune-

deficiency), and eliminate variation between the wild-type and the immune-deficient hosts (94). It was therefore assumed that the dispersal of microbes between bacteria-free neonates and symbiotic juveniles could contribute in the colonization of the bacteria-free neonates. However, low reproducibility could be due to divergence of the microbiota in the cultures, as a consequence of drift. This could, further, be the reason why the community composition did not correspond with the expected microbiota in the cultures selected as high or low fitness (Figure 14).

4.3 The microbiota in the feed and cultivation medium did not reflect the microbial composition in daphnia gut

There were two ways to obtain dispersal of microbes to daphnia guts in this experiment, either between medium and animals in each culture, or from the outside, such as feed. The medium added to the cultures was autoclaved and could therefore not bring any microbes. There were not any dispersal limitations for the daphniids in the cultures due to high production of offspring and efficient dispersion of the bacteria due to consumption by daphnia (95). Dispersal of microbes among hosts and between host and environment may cause microbiome variations (96). *D. magna* consumes bacteria, the microbes from the feed or cultivation medium will therefore end up in the intestine. In addition, excrements from *D. magna* will be released into the cultivation medium, suggesting a higher probability for finding some of the same microbes in the two habitats.

The bacterial taxonomic variation in the feed (algae solution) has earlier shown to be important for the microbial composition in the digestive tract of *D. magna* (59, 97). However, one-way PERMANOVA performed on the intestines and feed indicated a significant difference in both occurrence and relative abundance of OTUs in the microbial community composition between these two sample-types. According to SIMPER performed on intestine and feed (Table 11), none of the most significant OTUs were present in both sample types. In marine fish larvae, it was shown that the microbiota in the cultivation medium and live food can affect the microbiota in the larvae, even though the microbial compositions in the medium or feed were highly different from the microbiota in the larvae (98). This may also apply in this case. Analysis of the algal solution and cultivation medium showed a 20% similarity in OTU inventory (Dice index). The similarity can

be explained by the release of feed into the cultivation medium when *D. magna* was fed. Still, there was a significant difference according to both Bray-Curtis similarity and Dice index, and none of the most significant OTUs were present in both sample-types, according to SIMPER (Table 11). The consumption rate performed by the daphniids may be an explanation.

Alpha-, beta-, gamma-Proteobacteria and Cytophaga–Flavobacteria in the cultivation medium were detected in the gut homogenates of Copepods and Cladocera (*Daphnia pulex*) by Peter and Sommaruga (99). This correlation between the cultivation medium and intestinal microbial composition was absent in this study. The difference between the microbial composition in the intestine and cultivation medium was significant for both OTU inventory and relative abundance. Bray-Curtis (Figure 27) and Dice similarity (Figure 28) explained that 50% of the available OTUs were similar in both the intestines and cultivation medium, but the abundance varied in the two sample-types (34%). The cultivation medium had almost twice as many OTUs than intestines, indicating the difference in the taxonomic richness. SIMPER (Table 9) illustrated that *Limnohabitants, Flavobacterium, Pedobacter,* Bacteroidetes and Comamonadaceae were present both in the intestines and the cultivation medium, but *Pedobacter* and Comamonadaceae were the only genera that were almost equally distributed in both sample-types. The microbial composition in the intestines may varied according to host selection (100, 101), which indicates that the habitat has restrictions for which microbes that can colonize the gut according to both host-microbe interactions and microbe-microbe interactions (102). The cultivation medium, on the other hand, was assumed to have less of these restrictions. Colonization of the medium was not dependent on the host-microbe interaction and reflects a community with more diverse conditions, open for several taxa. The difference in selection regimes in the intestine and cultivation medium indicates that different habitats require different microbial traits (100).

4.4 The microbial abundance in the gut microbiota effects the fitness of *D. magna*

Microbial composition (described by 16S rDNA amplicon sequencing) was compared between cultures with high and low fitness. Significant difference in the relative abundance of the OTUs between the cultures was confirmed by PERMANOVA based on Bray-Curtis similarity, but there

was no significant difference in OTU inventory (Dice similarity). The significant reproducibility in fitness among the replicates in Phase 2 (Figure 13) supports the correlation between microbiota and fitness. The replicates received microbiota from the same culture, and therefore, their microbiota should be relatively similar. Hence, the microbiota should affect the fitness equally in the two replicates.

Cultures with high fitness (62 OTUs) represented the most diverse communities with nine OTUs more than cultures with low fitness (53 OTUs). In humans, a rich and diverse microbial community promotes a healthy gut microbiota in balance (103-105). On the other hand, studies performed on *D. magna* have reported that individuals exposed to sediments with low species-richness, or toxic Cyanobacteria, had a diverse gut microbiota (20, 93). However, exposure to toxic Cyanobacteria on susceptible genotypes led to decrease in fitness, indicating that the diverse gut microbiota was not promoting the tolerance (93). In my experiment, differences in richness and evenness between the cultures with high and low fitness were not statistically significant. The two communities contained 62% of the same OTUs, and had 69% similarity in the relative abundance. The relative abundance illustrated by SIMPER (Table 8) indicated that Bacteroidetes (OTU_4) dominated in those cultures with high fitness, while *Limnohabitants* (OTU_1), *Pedobacter* (OTU_2), Comamonadaceae (OTU_6), Betaproteobacteria (OTU_10) and *Pseudomonas* (OTU_9) were common in the cultures with low fitness. The effects four of these OTUs have on the daphnia fitness are discussed in the following sections.

The phylum Bacteroidetes constitutes major parts of the microbial community in the digestive tract of the animals. In humans, Bacteroidetes is mutualistic and contributes with the degradation of organic matter with high molecular weight, such as proteins and carbohydrates (106, 107). Distribution of Bacteroidetes (OTU_4) in the cultures with high and low fitness were 23 and 7%, respectively. The average relative abundance in all the intestines collected throughout this experiment was 15%, suggesting that Bacteroidetes played an important and beneficial role in microbiotas' effect on the fitness. In humans, butyrate is a product of the fiber fermentation, performed by gut Bacteroidetes and Firmicutes, and was associated with beneficial effects against e.g. colon cancer development, type 2 diabetes, obesity and cardiovascular disease (106, 108-110).

The polymer-degrading enzymes produced by Bacteroidetes is, on the other hand, revealed as pathogenic in animals (106). Therefore, more research is needed to support the positive effect Bacteroidetes may have on daphnia fitness.

At genus level, *Limnohabitants* (OTU_1) dominated the intestinal bacterial community in *D. magna*. The genus that belongs to the family Comamonadaceae is a stable member of the digestive tract in daphnia, also through starvation and alternative diets (58, 88, 111). *Limnohabitants* accounts for 40 and 46% of the relative abundance in the cultures with high and low fitness, respectively. *Limnohabitants* has been reported to increase the fecundity and population size of aposymbiotic *D. magna* after re-infection (54, 58). The average abundance of *Limnohabitants* collected for all the intestines (Phase 0, 1 and 2) was 40%, indicating that higher fitness was not induced by increased abundancy of *Limnohabitants*. The level of *Limnohabitants* between the two cultures was minimal, however, the difference might indicate that *Limnohabitants* has different effects alone than in combination with other intestinal bacteria. The intestinal bacteria may have interacted with *Limnohabitants*, and caused a reduction of the beneficial effect *Limnohabitants* has alone. A potential microbe-microbe interaction has been reported between *Pedobacter* and *Limnohabitants* (112). In my experiment, *Pedobacter* was the second most abundant genus in the intestinal bacterial community in *D. magna*, but the presence in cultures with high and low fitness was equal. *Pedobacter* encodes the enzyme N-acetylneuraminate lyase (NAL) that is responsible for the reversible cleavage and biosynthesis of the most abundant sialic acid, N-acetylneuraminic acid (Neu5Ac). Sialic acid can be utilized as a nutrient source by *Limnohabitants* due to presence of genes encoding sialic acid tripartite ATP-independent periplasmic (TRAP) transporter (112-114). *Pedobacter* and *Limnohabitants*, together with two other metagenome-assembled genomes (MAGs) collected from the core microbiota in *D. magna*, were reported to contribute with nutrient acquisition and metabolism pathways, suggesting their presence as beneficial (112).

Normally, several genera in the family Comanadaceae are present in the daphnia microbiota (e.g. *Limnohabitants*, *Hydrogenophaga* and *Pelomonas)* (111, 115). Abundance of Comanadaceae (OTU_6) in the cultures associated with high and low fitness were 8.7 and 13.7%, respectively. It was difficult to know which genus OTU_6 represented, but according to RDP, *Hydrogenophaga*

may be a good candidate. *Hydrogenophaga* was suggested to be present in daphniids with low somatic growth and low fecundity (111). This corresponded with the features of the daphniids with low fitness in my experiment, and can be one of the factors causing differences in the fitness. Martin-Creuzburg et al. indicated their toxicity by reporting that all *D. magna* fed *Hydrogenophaga* sp. died after two days (59). However, the abundancy between high and low fitness was minimal and the average abundance of Comanadaceae was 13.4%, which is around the same level as for low fitness. This indicates the minimal effect of this bacterium on the fitness.

Pseudomonas (OTU_9) was also inhabiting the daphnia microbiota but at a lower level. The opportunistic taxon is common in the intestinal bacterial community of fish, crabs, terrestrial crustacean and insects (116-119). Part of the *Pseudomonas* community in the gut has been reported as harmful for the fruit fly *Drosophila* (120, 121) and nematode *Caenorhabditis elegans* (121). In this experiment, there was 16x higher abundance of *Pseudomonas* in the cultures with low fitness (3.3%) than cultures with high fitness (0.2%), especially in jar 2.1 and 8.1. These jars were, together with jar 9.2, those with lowest number of offspring per mother days. 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. The abundance of *Pseudomonas* in cultures with high and low fitness were compared with the average abundance of *Pseudomonas* in all intestines (0.8%), indicating that *Pseudomonas* may influenced the daphnia fitness negatively. The virulence of *Pseudomonas* was documented by Le Coadic et al., where *D. magna* exposed to high bacterial concentrations of *Pseudomonas aeruginosa* or its' secreted compounds (rhamnolipids or elastase) died rapidly (122). According to RDP, OTU_9 was likely to represent the species *Pseudomonas peli* or *Pseudomonas anguilliseptica.* These species have not been documented as pathogens specifically, but *Pseudomonas* without species specification has been reported as toxic to *D. magna* (59). The abundance of *Pseudomonas* was suggested to increase during antibiotic treatment, starvation and before death (65, 88, 123). The presence of *Pseudomonas* in jar 2.1 (4%) and 8.1 (18%) may be explained by interhost dispersal. The microbial community composition of the daphnia resided in the replicates of jar 2.1 and 8.1 (i.e. jar 2.2 and 8.2) may contained high amounts of *Pseudomonas* that may have dispersed from host to host during Phase 1. Later this could have resulted in death among individuals in jar 2.1 and 8.1 as well.

4.5 Further prospects

In the present study, there were some challenges with the disinfection of the daphnia eggs due to high mortality and low hatching success compared with an other project with the same procedure (54). To obtain a protocol that is suitable for future studies, an organized experiment with more factors (e.g. different chemicals, concentrations, treatment-lengths and presence/absence of external membrane on daphnia eggs) must be performed on several replicates. The experiment should have registration of hatching success, survival rate and long-term effects on the bacteria-free neonates.

The bacteria-free neonates in this experiment were colonized with microbiota from cultures with high and low fitness. However, the procedure was not optimal to test the hypothesis and to evaluate the neonates' ability to reproduce the microbiota in the cultivation medium. This still needs to be investigated, to support the correlation showed in this study between fitness and microbiota in *D. magna*. Successful performance of Phase 3 can also give information about the efficiency of host-host dispersal and dispersal between host and cultivation medium.

How single genera effects fitness has been investigated for some bacteria. Bacteroidetes was shown to be one of the phyla providing beneficial effects to daphnia fitness in combination with other intestinal microbes. Still, the effect of Bacteroidetes alone on the daphnia fitness is unknown. Investigation of the effect of Bacteroidetes alone could reveal detailed information about how it promotes the fitness. The OTU that dominated Bacteroidetes was difficult to classify on lower taxonomic rank, but is required before the effect of Bacteroidetes alone can be investigated. The present study also suggested some microbe-microbe interactions. Further research could give more information about this, and maybe explain why *Limnohabitants* had a less beneficial effect in combination with other intestinal microbes than alone.

5. Conclusion

In the present study it was confirmed that there was a correlation between the intestinal microbial composition and the fitness of the water flea *D. magna*. The fitness was calculated for each culture as the total number of offspring divided by the cumulative number of mother days, and the intestinal microbial community composition was identified by Illumina sequencing of V3-V4 hypervariable regions in 16S rRNA-gene.

The study indicated that the ecological processes of drift and selection allowed for variation in the microbial community as a result of time and changes in the environment. The variation in fitness was used to characterize twelve daphnia cultures as high or low fitness. The microbiota of these cultures was compared and revealed a significant difference in the relative abundance of OTUs. Bacteroidetes was the OTU that had a particularly high abundance in the cultures with high fitness. Unfortunately, the OTU characterized as Bacteroidetes was problematic to determine at a lower taxonomic rank, which made it difficult to find the exact reasons for its' beneficial effect. In this study, *Limnohabitants*, which earlier has been showed to improve daphnia fecundity (58), was at a higher level in the cultures with low fitness than for those with high fitness. One possible reason is that *Limnohabitants* may have a different effect in combination with other intestinal bacteria (i.e. a microbe-microbe interactions) than alone. The reduction in fitness observed in the cultures with low fitness may be caused by *Pseudomonas* and Comanadaceae (much likely *Hydrogenophaga* according to RDP), that earlier has been reported to cause high mortality among *D. magna* (59).

The procedure with lowest GA-concentration (0.025%) and shortest treatment-length (30 min) was the best conditions for obtaining viable bacteria-free neonates with low mortality in the present study. However, due to unstructured experimental setup, more research is needed to obtain a universal protocol. The study also indicates low correlation between the intestinal microbial composition and the microbiota in the cultivation medium and feed. This may be a result of different selection regimes in the different habitats. The complexity of the host-microbe interactions and microbe-microbe interactions in the microbiota was revealed in this study. Experimental verification of the main findings of the present correlation based on in this study is needed.

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Appendix A Recipe cultivation medium

Aachener Daphnien Medium (ADaM) is a medium prepared for zooplankton species, such as cladocerans, rotifers and insect larvae (124). The medium was prepared by mixing deionized water with sea salt and three different stock solutions (Table 13), developed by Klüttgen et.al (124). The medium was stored at 20°C.

Table 13: Components and quantities of the cultivation medium, Aachener Daphnien Medium (ADaM), used for the water flea *D.magna*. The recipe is developed by Klüttgen et.al (124).

Component	**Stock solution (g/l)**	**Quantity (ml)**
Deionized water	-	1000
Sea salt	61.5	100
$CaCl_2$ x $2H_2O$	117.6	23
$NaHCO_3$	25.2	22
SeO_2	0.07	1

Appendix B Dilution of shellfish diet

The Shellfish Diet 1800® (Reed Mariculture Inc, USA) was used as feed for *D. magna*. The diet had a concentration of 2 x 10^9 cells/ml and was diluted according to volume of the medium (Table 14). The diet added into the aquaria, jars and tubes was diluted $\frac{1}{16}$, $\frac{1}{41}$ and $\frac{1}{201}$, respectively. The diet-mixture contained six marine microalgae; *Isochrysis galbana, Pavlova lutheri, Tetraselmis suecica, Chaetoceros calcitrans, Thalassiosira weissflogii* and *Thalassiosira pseudonana* (125, 126).

Table 14: The dilution of Shellfish Diet 1800® and the number of cells per ml according to chamber-size (volume of the medium) where *D. magna* reside during the experiment.

	Aquarium (2,5 L)	**Jars (250 ml)**	**50 ml tube**
Shellfish diet (ml)	10	2,5	0,5
MQ-water (ml)	150	100	100
Cells/ml	13 x 10^7	5 x 10^7	10^7

Appendix C Buffer solutions

Tris-acetate-EDTA (TAE) buffer was used to make 1% agarose gel, which further was used for agarose gel electrophoresis. Prior to the gel preparation, the buffer was diluted to 1X TAE buffer by mixing 40ml 50X TAE buffer (Table 15) and 1960ml MQ-water. Tris-EDTA (TE) buffer (Table 16) was used during the washing steps when the pooled sample was concentrated.

Table 15: Components and quantities used to make 50X Tris-acetate-EDTA buffer

Component	**Quantity**
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA (pH 0.8)	100 ml
dH_2O	Up to 1 L

Table 16: Components and quantities used to make 1x Tris-EDTA buffer.

Component	**Quantity**	**Final concentration**
2M Tris-HCl pH 7.5	2.5ml	10.0mM
0.5M EDTA pH 8.0	1.0ml	1.0mM
dH_2O	496.6ml	-

Appendix D Raw data for Phase 2 and Phase 3

Offspring production and maternal mortality were registered daily for 21 days in the 40 jars from Phase 2 (Table 17). These data were used to calculate the fitness for each culture by dividing the total number of offspring on the cumulative number of mother days of *D. magna*. The value of "born per mother days" was used to select 12 jars, six with high fitness and six with low fitness. The individuals were removed, and the medium was inoculated with three-four bacteria-free neonates (Phase 3). Offspring production and maternal mortality were registered daily for 18 days (Table 18), and used to measure fitness.

Table 17: Offspring production and maternal mortality for the different jars and replicates (Phase 2). There were a total number of four *D. magna* individuals in each jar from the beginning.

Jar	Replicate	Number of born	Number of deaths	Mother days	Born per mother days
1	1	169	0	84	2.01
1	2	193	0	84	2.3
2	1	123	0	84	1.46
2	2	0	4	21	0
3	1	150	0	84	1.79
3	2	118	2	54	2.19
4	1	204	0	84	2.43
4	2	216	0	84	2.57
5	1	160	0	84	1.9
5	2	163	0	84	1.94
6	1	153	0	84	1.82
6	2	144	0	84	1.71
7	1	240	0	84	2.86
7	2	267	0	84	3.18
8	1	106	1	83	1.28
8	2	0	4	56	0
9	1	140	1	84	1.67
9	2	120	0	84	1.43
10	1	115	0	84	1.37
10	2	205	0	84	2.44

11	1	170	0	84	2.02
11	2	169	0	84	2.01
12	1	131	0	84	1.56
12	2	223	0	84	2.65
13	1	160	0	84	1.9
13	2	209	0	84	2.49
14	1	157	0	84	1.87
14	2	178	0	84	2.12
15	1	185	0	84	2.2
15	2	213	0	84	2.54
16	1	178	0	84	2.12
16	2	181	0	84	2.15
17	1	169	0	84	2.01
17	2	240	0	84	2.86
18	1	167	0	84	1.99
18	2	210	0	84	2.5
19	1	132	0	84	1.57
19	2	185	0	84	2.2
20	1	188	0	84	2.24
20	2	142	0	84	1.69

Table 18: Offspring production and maternal mortality for the different jars and replicates in Phase 3. There were a total number of two-three *D. magna* individuals in each jar from the beginning.

Jar	Replicate	Mother days	Number of offspring	Born per mother days
2.1	1	54	176	3.26
2.1	2	22	94	4.27
4.2	1	36	139	3.86
4.2	2	25	52	2.08
5.2	1	34	76	2.24
5.2	2	36	64	1.78
6.2	1	34	97	2.85
6.2	2	36	69	1.92
7.2	1	35	132	3.77
7.2	2	36	90	2.5
8.1	1	35	102	2.91
8.1	2	52	108	2.08
9.2	1	36	119	3.31
9.2	2	36	107	2.97
10.1	1	36	123	3.42
10.1	2	36	97	2.69
12.1	1	36	64	1.78
12.1	2	26	75	2.88
12.2	1	7	0	0
12.2	2	54	161	2.98
15.2	1	36	103	2.86
15.2	2	31	115	3.71
17.2	1	38	101	2.66
17.2	2	36	137	3.81

Appendix E Identification of bacterial cell populations by flow cytometry

Fluorescent emissions from the samples collected from the cultivation medium (Phase 0 and Phase 2) stained with SYBR® Green I and detected by flow cytometry (Figure 36, 37, 38 and 39). The fluorescence detected per cell or particle formed PolygonGates that was used for identification and counting of bacterial cell populations.

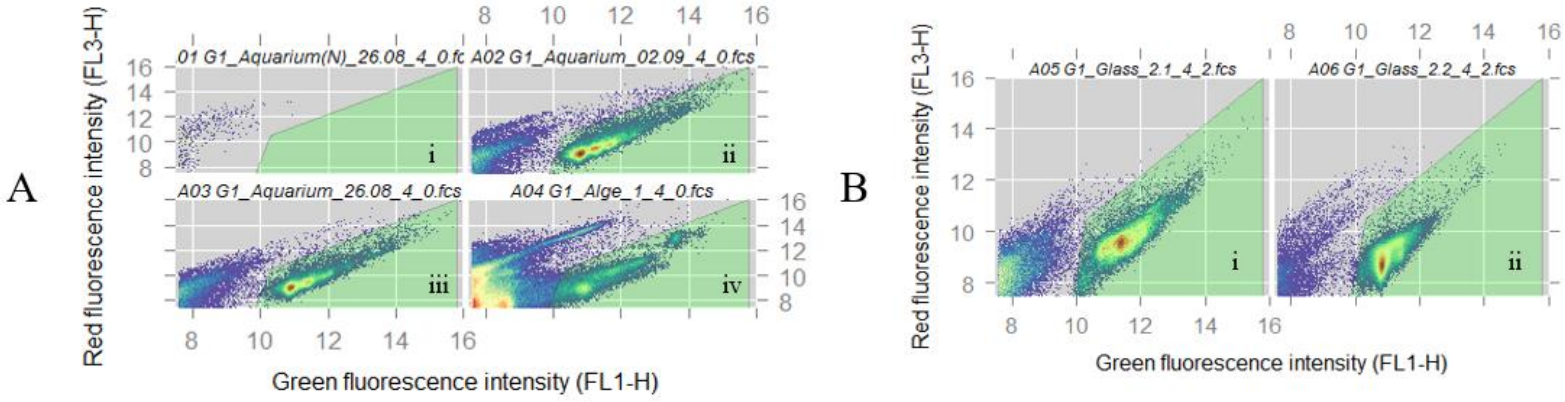

Figure 36: Fluorescent emissions from the cultivation media (Phase 0 and Phase 2) stained with SYBR® Green I, detected by flow cytometry. Cultivation medium is where *D. magna* resided. Green fluorescence intensity (FL1-H) was plotted against the red fluorescence intensity (FL3-H). PolygonGates (green areas) were used to identify bacterial population cells. Different color shows the intensity of the fluorescent SYBR® Green I (red= high, yellow= middle and blue/green= low). (A) Sample (i) is the first established aquarium (without stain), illustrating the presence of other fluorescents. Sample (ii) is the second established aquarium (stained). Sample (iii) is the first established aquarium (stained). Sample (iv) is the algae solution (stained). (B) Sample (i) is jar 2.1 (stained), and Sample (ii) is jar 2.2 (stained).

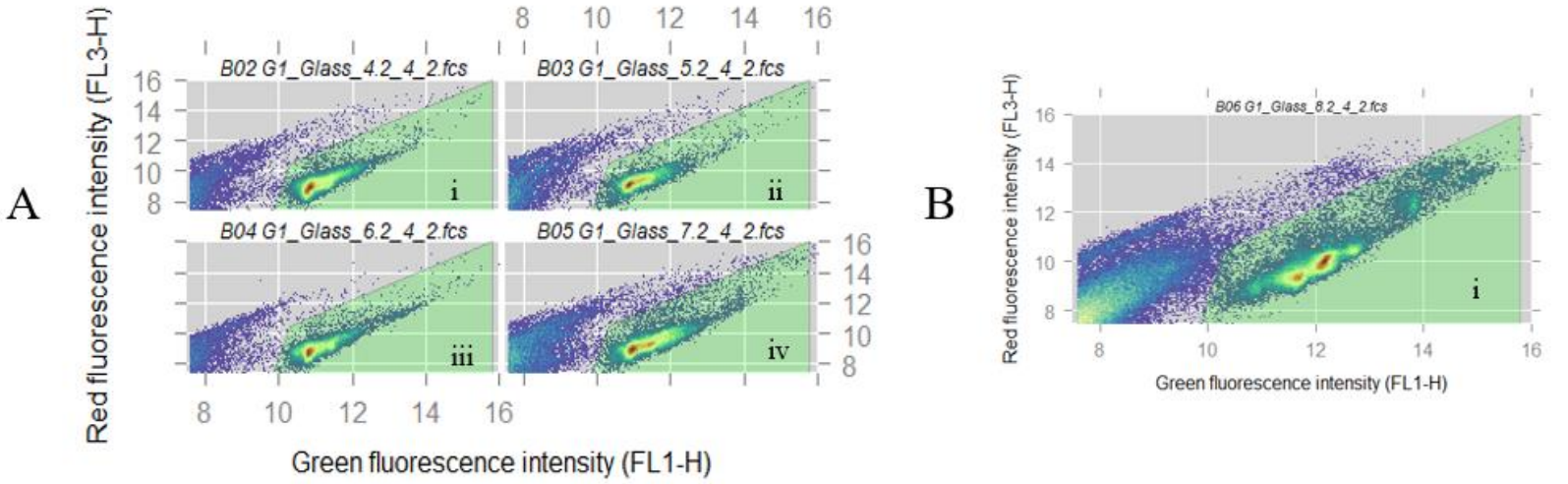

Figure 37: Fluorescent emissions from the cultivation media (Phase 0 and Phase 2) stained with SYBR® Green I, detected by flow cytometry. Cultivation medium is where *D. magna* resided. Green fluorescence intensity (FL1-H) was plotted against the red fluorescence intensity (FL3-H). PolygonGates (green areas) were used to identify bacterial population cells. Different color shows the intensity of the fluorescent SYBR® Green I (red= high, yellow= middle and blue/green= low). (A) Sample (i) is jar 4.2 (stained), Sample (ii) is jar 5.2 (stained), Sample (iii) is jar 6.2 (stained) and Sample (iv) is jar 7.2 (stained). (B) Sample (i) is jar 8.2 (stained).

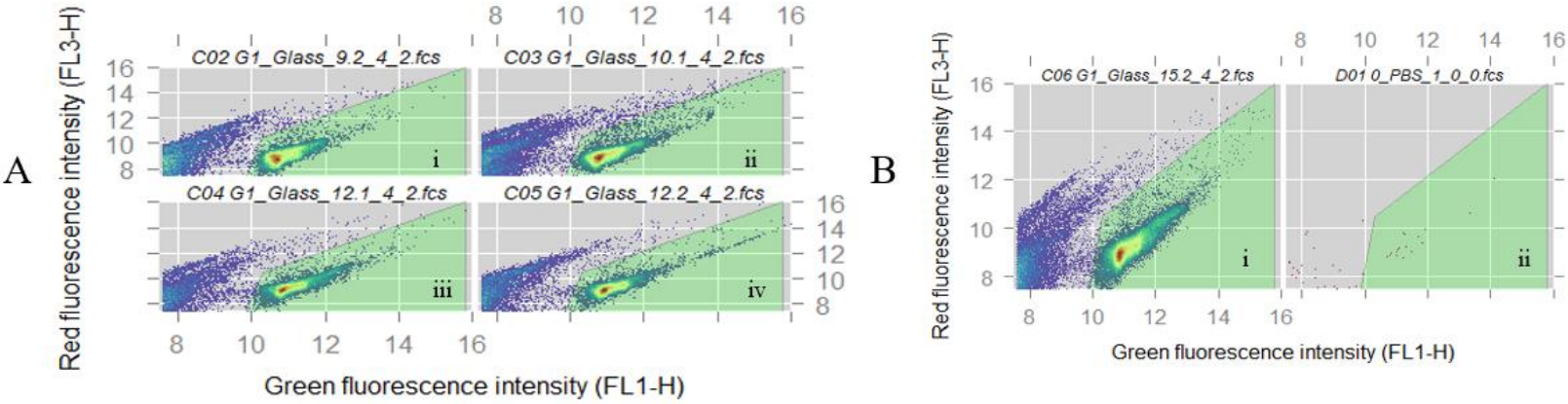

Figure 38: Fluorescent emissions from the cultivation media (Phase 0 and Phase 2) stained with SYBR® Green I, detected by flow cytometry. Cultivation medium is where *D. magna* resided. Green fluorescence intensity (FL1-H) was plotted against the red fluorescence intensity (FL3-H). PolygonGates (green areas) were used to identify bacterial population cells. Different color shows the intensity of the fluorescent SYBR® Green I (red= high, yellow= middle and blue/green= low). (A) Sample (i) is jar 9.2 (stained), Sample (ii) is jar 10.1 (stained), Sample (iii) is jar 12.1 (stained) and Sample (iv) is jar 12.2 (stained). (B) Sample (i) is jar 15.2 (stained) and Sample (ii) is the PBS that was used in the dilution of the samples.

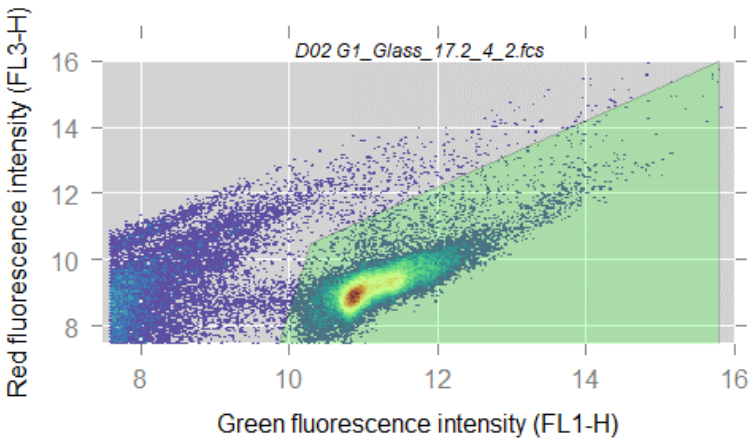

Figure 39: Fluorescent emissions from the cultivation medium (jar 17.2, Phase 2) stained with SYBR® Green I, detected by flow cytometry. Cultivation medium is where *D. magna* resided. Green fluorescence intensity (FL1-H) was plotted against the red fluorescence intensity (FL3-H). PolygonGates (green area) were used to identify bacterial population cells. Different color shows the intensity of the fluorescent SYBR® Green I (red= high, yellow= middle and blue/green= low).

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