

Title	Role of symbiotic bacteria on life history traits of freshwater crustacean, <i>Daphnia magna</i>
Author(s)	Peerakietkhajorn, Saranya
Citation	大阪大学, 2015, 博士論文
Version Type	VoR
URL	https://doi.org/10.18910/54011
rights	
Note	

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Osaka University

Doctoral Dissertation

**Role of symbiotic bacteria on life history traits of
freshwater crustacean, *Daphnia magna***

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June 2015

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Chapter 1

General introduction

In aquatic ecosystems, populations within a community are bound by a network of interactions. The most important interactions are of a trophic nature – “eat” or “be eaten” and the simplest connection is the food chain, which is started from producers that are photoautotrophs consumed by primary consumers such as herbivorous animals, zooplanktons, mollusks and nematodes. These animals are in turn eaten by secondary consumers including carnivorous crustaceans and planktonivorous fish. Bacteria are placed in this concept as decomposers, that is, they are responsible for the remineralization (“destruction”) of dead organic matters. Organisms within a food chain that can be assigned to the same position are collectively referred as a trophic level. The primary consumer is an important trophic level to change plant biomass to animal biomass and transfer energy from producer to the other higher trophic level (Figure 1). Therefore, changes of life history traits of primary consumers such as population size and longevity give great impact on the ecosystem, which have prompted researchers to study their ecologically important life history traits.

Daphnia is the best-studied freshwater zooplankton because it is an important keystone species in freshwater ecosystem and it is globally found in freshwater habitats. The ecologically important life history traits of *Daphnia* including fecundity, longevity and growth are known to be affected by abiotic factors such as pH, photoperiod, quality and quantity of food, metal ions and salinity (Stross and Hill, 1968; Vijverberg, 1976; Sterner, 1993; Caffrey and Keating, 1997; Heugens et al., 2006; Ghazy et al., 2009, 2011).

The biotic factors also involve in the changes of *Daphnia*'s life history traits. Previously, the studies of predation between fish and *Daphnia* revealed that fish kairomone increase population size and body length of *Daphnia* via GABA inhibition (Weiss et al., 2012). Competition, one of important biotic factors, controls the population size and biomass of *Daphnia* (Loureiro et al., 2013). Another ecologically important biotic factor is symbiosis, which concerns in interaction between *Daphnia* and their symbionts. Nevertheless, the study of symbiotic relationship is limited.

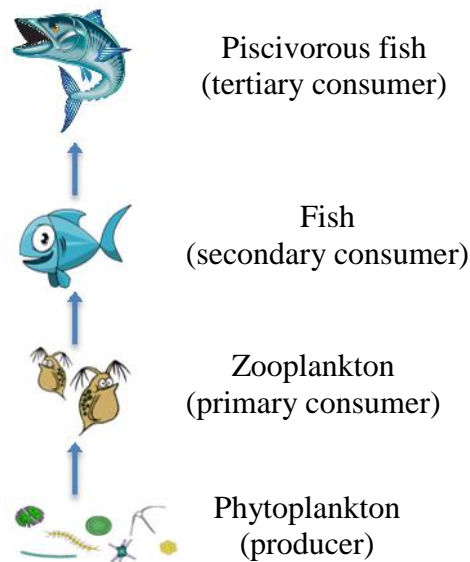


Figure 1 Trophic cascade. Cascade occurs when reciprocal effects of predator-prey alter the abundance, biomass or productivity of a population community. The trophic cascades in aquatic ecosystem are started from producers, which are photoautotrophs consumed by primary consumers, such as zooplanktons, mollusks and nematodes, then energy is transferred to the higher trophic levels via food consumption.

1.1 Biology of *Daphnia*

Daphnia is an important keystone species, which is found in many freshwater habitats, such as lakes and ponds. *Daphnia* have appendages, which are antennules,

antennae, maxillae, mandibles and 5 pairs of limbs on the trunk (Figure 2). The limbs are the filter apparatus for feeding and respiration. The abdominal claws are at the end of abdomen. Body of *Daphnia* is enclosed by a noncalcified shell called “carapace” which is mainly made of chitin. It has a double wall and hemolymph flows in between the walls. Apical spine is dominantly existent at the posterior of carapace for defences. Size of adult *Daphnia* varies from 1 mm to more than 5 mm upon the species and strains. Male *Daphnia* can be distinguished from females by their smaller size, larger antennules, modified post-abdomen, and first legs, which are armed with a hook used in clasping (Ebert, 2005).

Daphnia is a filter feeder, which gathers the small and suspended particles in the water with their filtering apparatus, consisting of the flattened phylopods. The movements of phylopods make the currents flows from anterior to posterior and *Daphnia* collect particles and transfer into the food grove using special setae. The food filtered by feeding apparatus is usually planktonic algae, but bacteria sometimes can be collected as well (Hebert, 1978). In most laboratories, green algae are normally fed to *Daphnia*, because of rich nutrients and easy culture in monoclonal chemostats (Ebert, 2005).

Gut of *Daphnia* is tubular lining with epithelium with microvilli along the gut. The gut comprises of three parts: esophagus, midgut and hindgut. And, there are two small ceca (diverticula) that are found at the beginning of midgut (Figure 2). The food passes through the gut by peristalsis of gut wall where epithelial cells are capable to absorb nutrient molecules (Ebert, 2005).

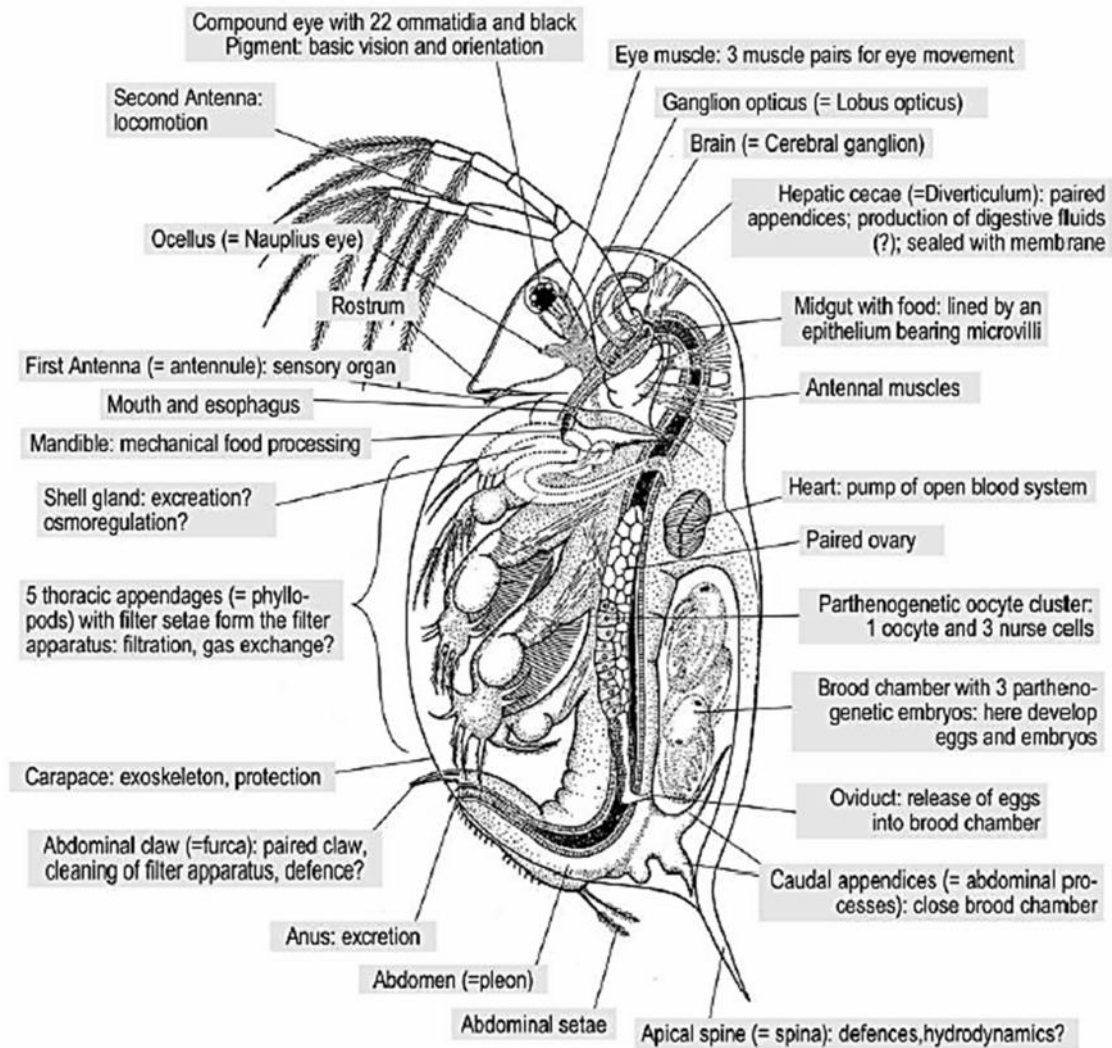


Figure 2 Anatomy of *Daphnia* (Ebert, 2005).

The most important life history trait of *Daphnia* is high fecundity by parthenogenesis (Hebert, 1978), leading to large population size that is fundamental to its role as a primary consumer. Under healthy conditions, a female produces a clutch of parthenogenetic eggs every adult molt. The eggs are placed in the brood chamber that is located at dorsoposterior under the carapace. The embryos hatch from the eggs after 1 day and remain in the brood chamber. After 3 days in the brood chamber, newborn *Daphnia* are released from brood chamber. The juvenile *Daphnia* looks like the adult *Daphnia*,

except that the brood chamber is not developed yet. The juveniles develop to be the adults within 5-10 days under normal conditions and lay first clutch eggs in their brood chambers. An adult female produce a clutch of eggs every 3-4 days in her entire life that may live around 2 months (Ebert, 2005). By the environmental changes such as temperature and light cycle *Daphnia* switch reproductive system from parthenogenetic reproduction to sexual reproduction (Figure 3).

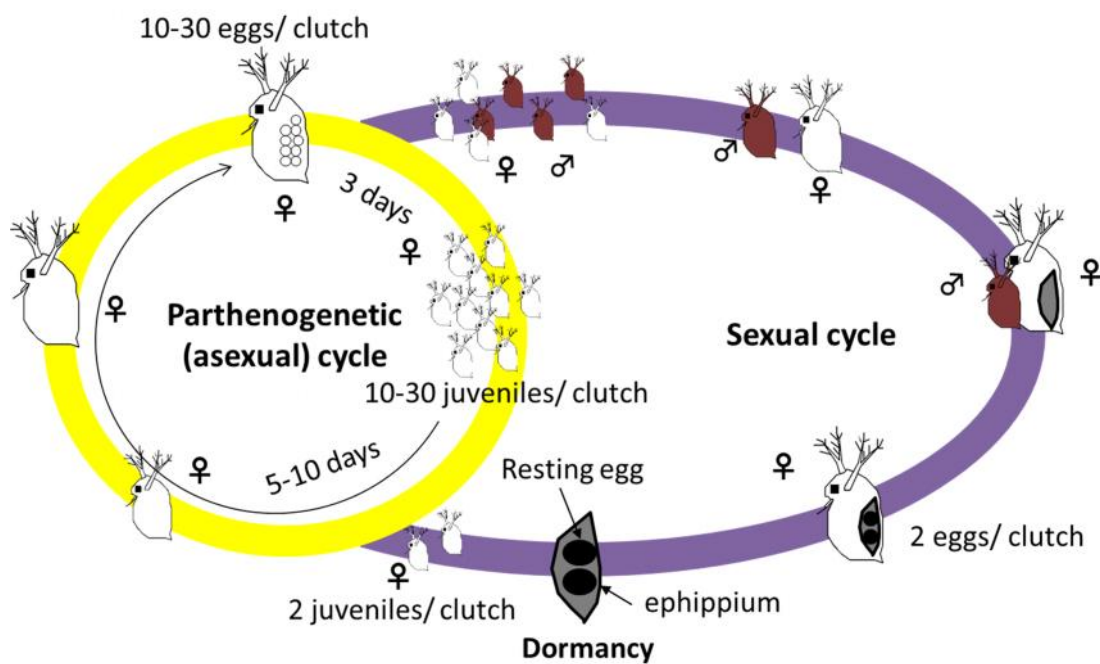


Figure 3 Life cycle of *Daphnia*. This diagram exhibits the parthenogenetic (asexual) and sexual life cycle of *Daphnia*. Under normal conditions, female *Daphnia* produce diploid eggs that develop to be female juveniles in parthenogenetic cycle. When *Daphnia* is stimulated by environmental stresses, such as photoperiod, temperature and salinity, female *Daphnia* produces diploid asexual eggs that develop to be male *Daphnia*. Furthermore, the stressed female adult produces haploid eggs that require fertilization by male. These eggs (resting eggs) are enclosed in protective hard shell called ephippium. The ephippia are released from females and sink to the bottom or float in the freshwater habitats. The resting eggs may endure during unfavorable conditions and hatching is induced by external stimuli, such as an appropriate photoperiod, rising of temperature, light or presence of water in dry pond. Only

females are hatched from resting eggs and develop to be the adults, which are able to produce parthenogenetic eggs under normal conditions.

1.2 *Daphnia* in bioenvironmental sciences

Daphnia is globally found in freshwater habitats and has been used as a model organism in many studies of environmental sciences: ecology, evolution, toxicology, since it has short life cycle, small size, and the culturing is simple and easy to handle in the laboratories (Little and Ebert, 2000; Ebert, 2008; Heinlaan et al., 2008; Weiss et al., 2012). In addition, *Daphnia* shows high chemical sensitivity and phenotypic plasticity that responds to environmental changes (Lüning, 1992; Tatarazako et al., 2003; Ebert, 2011). Therefore, *Daphnia* is one of model organisms for estimation of contaminated chemicals in freshwater.

Some guidelines of Organisation for Economic Co-operation and Development (OECD) specify to use *Daphnia* for evaluation of toxicity of insecticides, metal ions and the toxic chemicals released to aquatic ecosystems. Physiological effects, such as immobilization and reproduction of *Daphnia*, are evaluated to estimate the effective concentration (OECD 202 and 211). Furthermore, *Daphnia* is a subject to study in a model system of host-microbe coevolution to understand the parasitic relationship in ecosystems (Little and Ebert, 2000; Ebert, 2008; Stjernman and Little, 2011). The investigations of coevolution between *Daphnia* and parasites reveal the dynamic of population and specificity between hosts and parasites (Decaestecker et al., 2005; Duncan and Little, 2007; Ebert, 2008; Hall et al., 2011).

1.3 Molecular genetics of *Daphnia*

Whole genome sequence of *Daphnia* is available in database, which allows us to

study in molecular genetics of *Daphnia*. Since microinjection technique was established in *Daphnia*, which enabled us to analyse gene function by introduction of foreign DNAs and RNAs. The RNA interference and targeted mutagenesis by CRIPR-Cas system and TALEN are developed and used to knockdown or knockout, leading us to perform the loss-of-function analyses in *Daphnia* (Kato, Shiga, et al., 2011; Nakanishi et al., 2014; Naitou et al., 2015). Another way to clarify the function of target gene is to perform the gain-of-function analyses. The overexpression of targeted genes and transgenesis technique have been developed in *Daphnia* to study in vivo expression of target gene (Kato, Kobayashi, et al., 2011; Kato et al., 2012; Törner et al., 2014). These techniques allow us to study the function of interesting genes and the physiological mechanisms in *Daphnia*.

These genetic tools have been applied to *Daphnia* studies, which allow us to approach to molecular biology of *Daphnia*. The mechanisms of environmental sex determination have been studied using microinjection of mRNAs and dsRNAs. This study revealed *dsx* gene involving in environmental sex determination in *D. magna* (Kato, Kobayashi, et al., 2011). In addition, ecdysteroid activity was investigated and visualized in *D. magna* by injecting the plasmid DNA that codes for the ecdysone response element driving a reporter gene (Asada et al., 2014). By using microinjection of plasmid, transgenic *Daphnia* that harbors the green fluorescent protein (*GFP*) gene fused with the *D. magna* histone *H2B* gene, has been established. This transgenic line named HG line is useful to study the embryogenesis and oogenesis of *Daphnia* (Kato et al., 2012).

1.4 Symbiosis

The term of “symbiosis” is an interaction between the organisms, which is categorized to 3 groups: parasitism, commensalism and mutualism (Wells and Varel,

2011; Martin and Schwab, 2013). Parasitism is a relationship, in which one costs to another and one get benefits from host. This interaction has been studied widely in human, animals and plants to investigate the preventions of widespread diseases (Wells and Varel, 2011). The term of “commensal” comes from the mediaval Latin “commensalis”, which means “at table together” and commensalism generally refers to two organisms living together without cost but without obvious benefit (Hooper and Gordon, 2001). Mutualism is a beneficial interaction that two organisms interact with benefit to each other (Wells and Varel, 2011).

The studies concerning symbiotic relationship between terrestrial invertebrates and their symbionts have been widely studied, and revealed the symbionts play in mutual interaction to improve longevity, growth rate, body size and reproduction of insects. Symbiotic bacteria is required for larval growth and adult fecundity of pea aphids (*Acyrtosiphon pisum*) (Douglas, 1992). *Wolbachia*, a member of *Alphaproteobacteria*, regulate maturation of oocytes in parasitic wasp (*Asobara tabida*). Moreover, symbiotic bacteria regulate longevity of the Mediterranean fruit fly (*Ceratitidis capitata*) (Behar et al., 2008; Ben-Yosef et al., 2008). Disruption of gut microbiota of termites (*Zootermopsis angusticollis* and *Reticulitermes flavipes*) causes decrease of queen’s fecundity and severely affects to fitness and colony growth (Rosengaus et al., 2011). *Burkholderia*, a member of *Betaproteobacteria*, is capable to increase fecundity and body length of bean bug (*Riptortus pedestris*) (Kikuchi and Fukatsu, 2014). Furthermore, intestinal bacteria stimulate the larval development and survival in common fly (*Calliphora vomitoria*) (Wollman, 1911; Erkosar et al., 2013). *Lactobacillus plantarum* is directly responsible for microbiota-mediated mating preference of *Drosophila melanogaster* (Sharon et al., 2010). Symbiotic bacteria also show ability to resist the parasite colonization (Pan et al., 2012). Recently, an ecotoxicological study shows symbiont could degrade insecticide and

infected bug has higher insecticide resistance (Kikuchi et al., 2012).

In vertebrates, gut microflora provide beneficial nutrients for human health and function in immunity (Hooper and Gordon, 2001; Binn, 2013). Probiotics in gut are responsible for benefits to reproduction of osteichthyes. *Lactobacillus rhamnosus* functions as a probiotic to play with nutrition in zebrafish (*Danio rerio*) leading to inhibit follicular apoptosis, improve follicular survival, enhance germinal vesicle breakdown rate and increase ovulated eggs (Gioacchini et al., 2010, 2013). *L. rhamonosus* involve in endocrine system leading to induce oocyte maturation in female zebrafish (Gioacchini et al., 2010). Probiotics are able to modulate bacterial community in the gut of zebrafish and shift to increase the presence of lactic acid bacteria, *Streptococcus thermophiles* (Gioacchini et al., 2013). In marine teleosts, probiotics also regulate the reproduction of killifish (*Fundulus heterociltus*) resulting in enhancing of reproductive performance of killifish, such as, gonadal growth, fecundity and embryo survival. Moreover, probiotics are capable affecting larval development and growth of killifish larvae (Lombardo et al., 2011).

In *Daphnia*, several studies revealed the parasitic interactions between *Daphnia* and parasite controls *Daphnia*'s life history traits and function in coevolution of host-parasite (Mangin et al., 1995; Ebert et al., 2000; Little and Ebert, 2000; Decaestecker et al., 2005; Ebert, 2008). Recently, metagenomics of symbionts in *D. pulex*, *D. pulicaria* and *D. magna* have been investigated by using shotgun sequencing. This study revealed that the bacterial community compositions are stable among these 3 species and the majority of microbial community is Proteobacteria. Most sequences belong to Betaproteobacteria, family Comamonadaceae (Qi et al., 2009). However, the role of the microbiota to function on *Daphnia*'s life history traits still remained unknown.

1.5 Objective of this study

I aimed to clarify the role of symbiotic bacteria on *Daphnia magna*'s life history traits. First, I established a novel method to prepare aposymbiotic *Daphnia*, which allowed me to study the role of symbiotic bacteria on important life history traits of *Daphnia*. Second, I developed a method to reinfect aposymbiotic *Daphnia* with the dominant symbiont *Limnohabitans* and found the function of *Limnohabitans* on *Daphnia*'s life history traits.

In Chapter 1 of this dissertation, structure of trophic cascade in freshwater ecosystem, definition of symbiosis and recent studies concerning symbiotic relationship, biology of *Daphnia* were described. In Chapter 2, a method to prepare aposymbiotic *Daphnia* by glutaraldehyde exposure to embryos and phenotypes of aposymbiotic *Daphnia* were described, in addition to the role of symbionts on ecologically important life history traits of *Daphnia*: population size and longevity. Composition of the symbiotic bacteria was also explained in this chapter. In Chapter 3, results of study on the role of dominant bacteria on *Daphnia*'s important life history traits, fecundity and growth, were shown. In Chapter 4, I will discuss the roles of symbiotic bacteria and dominant bacterium on *Daphnia*'s life history traits that lead to drive trophic cascade. Finally, I will provide new insights into role of bacteria in freshwater ecosystems and show usefulness of an aposymbiotic *Daphnia* for symbiotic relationship between animals and bacteria.

Chapter 2

Role of symbiotic bacteria on life history traits of *D. magna* and bacterial community composition

2.1 Introduction

The microcrustacean *Daphnia* is a freshwater zooplankton that commonly inhabits ponds and lakes throughout the world. Under normal conditions populations consist entirely of parthenogenetic females with a high ability to propagate (Hebert, 1978), leading to large population sizes that are fundamental to *Daphnia*'s key role as a primary consumer in freshwater ecosystems. Life-history traits of female *Daphnia* such as size, weight, longevity and fecundity are affected by abiotic factors such as temperature, pH, photoperiod, quantity and quality of food, metal ions and salinity (Stross and Hill, 1968; Vijverberg, 1976; Sterner, 1993; Caffrey and Keating, 1997; Heugens et al., 2006; Ghazy et al., 2009, 2011). Factors such as photoperiod and quantity of food can even cause *Daphnia* to switch from parthenogenetic to sexual reproduction (Hebert, 1978).

Symbiosis, a biotic factor showing the interaction between different biological species, affects the life-history traits of many animal species (Gilbert, 2010). In insects, the closest relatives of crustaceans, some species of symbiotic bacteria have developed the ability to increase the longevity and fecundity of their hosts (Douglas, 1992; Dedeine et al., 2001; Behar et al., 2008; Ben-Yosef et al., 2008; Rosengaus et al., 2011). In *Daphnia*, most of the symbiosis-related research involves the relationship between *Daphnia* and parasites (Metchnikoff, 1884; Ebert et al., 2000; Little and Ebert, 2000; Decaestecker et al., 2005; Ebert, 2008) while a few studies have investigated the bacterial

community composition in *Daphnia* (Qi et al., 2009; Freese and Schink, 2011) by using metagenomic sequencing. In one of the latter studies the majority of bacteria sequenced from 3 *Daphnia* species were classified under the *Betaproteobacteria* genera of the family Comamonadaceae (Qi et al., 2009). The other study suggested that the diversity of the *Daphnia* gut microflora was relatively low and that the dominant bacterial species found was of the *Limnohabitans* species of the *Betaproteobacteria* genera (Freese and Schink, 2011). Both studies revealed that symbiotic associations are stable over long periods, suggesting that stable bacterial communities in *Daphnia* may play essential roles in their hosts' life history.

Glutaraldehyde (GA) is known to have a broad spectrum of activity, rapid antimicrobial action and is highly active in the presence of organic matter (Gorman et al., 1980; Salvesen et al., 1997). The mechanism of action of GA involves a cross-linking of protein at outer layers and inside the bacterial cell that leads to inhibit transport, enzyme activity and synthesis of RNA, DNA and protein (Munton and Russell, 1971, 1973; McGucken and Woodside, 1973; McDonnell and Russell, 1999).

This study aimed to develop a method to prepare aposymbiotic *D. magna* by sterilizing embryos using GA and investigated roles of symbiotic bacteria for two life history traits, population size and longevity.

2.2 Materials and Methods

2.2.1 *Daphnia* strain and culture condition

D. magna (Belgium strain) were obtained from National Institute for Environmental Studies (NIES, Tsukuba, Japan) (Oda et al., 2006). Eighty *Daphnia* were incubated in 5 L of M4 media (Elendt and Bias, 1990) at 23±1°C under 16 h light/ 8 h dark photoperiod. 1×10⁹ cells of *Chlorella* sp. were added daily on first week, then

2×10^9 cells daily.

2.2.2 Axenic *Chlorella*

Chlorella vulgaris Beijerinck were obtained from National Institute for Environmental Studies (NIES, Tsukuba, Japan), inoculated in MAM medium (0.0025% CaCl_2 , 0.0075% MgSO_4 , 0.0025% NaCl , 0.01% KNO_3 , 0.025% NH_4NO_3 , 0.2% casamino acids, 0.05% yeast extract and 0.05% malt extract) and incubated with shaking at 23°C under 16 h light/ 8 h dark photoperiod for 5 days. Then, the cells were collected, re-suspended in filtered M4 media prepared by filtration with 0.2 μm filter (Corning-500 mL filter system, Corning, NY, USA) and stored at 4°C. The 10^8 cells of axenic *Chlorella* were used for bacterial screening.

2.2.3 Preparation of aposymbiotic juvenile *Daphnia*

Adult *Daphnia* were transferred to the filtered M4 culture media and dissected to separate the early stage of *Daphnia* embryos (less than 24 hours) from brood chambers. The early stage of embryo is still covered with chorion, which protects *Daphnia* embryo from bacteria in environment and harm of GA. The collected embryos were separated into 3 treatments. The 20 – 35 embryos were placed in each well of a 6-well plate, washed twice with 1 ml of filtered M4 media, exposed to 1 ml of 0, 0.025% and 0.25% GA (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes to find the optimized concentration of GA for *Daphnia* sterilization, washed with the 1 ml of filtered M4 media twice, and then incubated in the 4 ml of filtered M4 media at 23°C under 16 h light/ 8 h dark photoperiod until they grow to be swimming juveniles (about 48 hours) and then used for experiments. The total number of *Daphnia* used in each experiment would be written in the later section.

2.2.4 Bacteria-free culture of aposymbiotic *Daphnia*

Except the longevity experiment, four aposymbiotic juvenile *Daphnia* were aseptically transferred into 100 ml of filtered M4 solution, aseptically cultured and fed 2×10^7 cells of axenic *Chlorella* daily on first week, then 4×10^7 cells daily, respectively. As a control, four symbiotic juveniles were cultured by the same culturing method.

2.2.5 Determination of longevity of *Daphnia*

Ten aposymbiotic or 10 symbiotic juvenile *Daphnia* were transferred into 100 ml of filtered M4 solution, aseptically cultured and fed 2×10^7 cells of axenic *Chlorella* daily on first week, then 4×10^7 cells daily, respectively. Newborn juveniles were removed every second day. The proportion of survival was observed every day. The experiments with symbiotic *Daphnia* and aposymbiotic *Daphnia* were performed twice and three times, respectively. The mean longevity was calculated as previously reported (Fletcher et al., 1990).

2.2.6 Re-infection by co-culture with symbiotic *Daphnia*

Two aposymbiotic juvenile *Daphnia* were aseptically transferred into 100 ml of M4 solution and then two symbiotic *Daphnia* were added. Culture condition of the co-cultured *Daphnia* was same as that of aposymbiotic *Daphnia*.

2.2.7 Re-infection by dipping in *Daphnia* extracts

Ten adult *Daphnia* were grinded roughly in 500 μ l of M4 media. The homogenate was incubated at room temperature for 5 minutes, 200 μ l of the supernatant were transferred to a new tube after standing, diluted 10 times and used for the re-infection experiment. To prepare filtered *Daphnia* extracts, the crude extracts were

filtered using 0.2 µm filter (Millex – LG 0.2 µm, Millipore, Ireland), then diluted 10 times. Then, the 0.25% glutaraldehyde-treated embryos were incubated in the *Daphnia* extract and filtrate of the *Daphnia* extract. After hatching, 4 aposymbiotic *Daphnia* dipped in crude extract and filtrate of crude extract were transferred into 100 ml of filtered M4 solution and cultured by the same way as aposymbiotic *Daphnia* culture.

2.2.8 DNA extraction

Genomic DNA extraction was performed using a standard protocol for *Drosophila* (Huang et al., 2000). First, four *Daphnia* and *Chlorella* were homogenized in a solution of 200 µl Buffer A (100 mM tris-HCl, 100 mM ethylenediaminetetraacetic acid [EDTA], 100 mM NaCl and 0.5% sodium dodecyl sulfate [SDS], pH 7.5) in a 1.5-mL tube and incubated at 65°C for 30 min. Second, the homogenate was mixed with 400 µl of LiCl/KAc solution (5 M potassium acetate: 6 M lithium chloride = 1:2.5), incubated on ice for 10 min and centrifuged for 15 min at 15000 rpm. Third, 500 µl of the supernatant was transferred to a new tube, mixed with 300 µl isopropanol and centrifuged for 15 min at 15,000 rpm. Finally, the supernatant was removed and the precipitate was washed with 70% ethanol, dried and resuspended in 50 µl of MilliQ, after which estimation of copy number of 16s rRNA genes and sequencing were conducted.

2.2.9 Quantitative polymerase chain reaction (qPCR)

PCRs were performed in MX3005P (Stratagene) using SYBR GreenER qPCR SuperMix Universal (Invitrogen), in the presence of a primer set: forward primer 5'-AGACACGGTCCAGACTCCTAC-3' and reverse primer 5'-TTTACGGCGTGGA-CTACCAG-3'. PCR amplifications were performed under the following conditions: 2 minutes at 95°C and followed by 40 two-temperature cycles (15 seconds at 95°C and 1

minute at 60°C). Average and standard error were calculated. The detection limit was 50 copies of 16S rRNA genes.

2.2.10 Sequencing

DNA extracts from *Daphnia* were used to amplify 16S rRNA genes using the primer set, which was used for the qPCR reaction. After PCR amplification, the PCR products (199-224 bp) were purified, cloned into a pCR4-TOPO vector (Invitrogen), sequenced and identified via a BLAST search of the NCBI database and SILVA rRNA database supported by Takara Bio Dragon Genomics Center (Yokkaichi, Mie, Japan).

2.2.11 Statistical analyses

All data were shown as mean \pm SE. The population size and longevity were tested using the Welch's *t*-test. The significance level for all of statistical analyses was $p = 0.01$.

2.3 Results

2.3.1 Generation of aposymbiotic *Daphnia*

To investigate the effect of symbiotic bacteria on life-history traits in *Daphnia magna*, we developed a method to generate aposymbiotic *Daphnia* by using the Belgian strain of *D. magna*, which was obtained from the National Institute for Environmental Studies (NIES, Tsukuba, Japan) (Oda et al., 2006). GA was used to disinfect *Daphnia* as an alternative to the antibiotics that have previously been used to disinfect *Daphnia* (D'Agostino and Provasoli, 1970). We tried to eliminate symbiotic bacteria by treatment of GA from early stage of *Daphnia* embryos (less than 24 h old) that were covered by chorion because this thick membrane could protect embryos from bacteria in environment

and harm of GA. The embryos were exposed to 0%, 0.025%, and 0.25% GA for 30 min and then incubated in filtered M4 solution (Elendt and Bias, 1990) for 48 h during which the chorion was shed, the embryo developed into the first instar juvenile. At this stage, more than 80% of treated *Daphnia* survived even when we used 0.25% GA.

To confirm removal of bacteria from *Daphnia*, DNA was extracted from the juveniles by using a standard protocol for *Drosophila* (Huang et al., 2000) and quantitative PCR of bacterial 16S rRNA genes were performed. We found that treatment of the *Daphnia* embryos with GA for 30 min was sufficient to remove bacteria from *Daphnia* (Table 1). The estimated copy numbers of bacterial 16S rRNA genes isolated from the GA-treated *Daphnia* were lower than the detection limit (<50 copies of 16S rRNA genes/ *Daphnia*), whereas non-treated *Daphnia* were found to have $2.43 \pm 0.90 \times 10^4$ copies per individual (Table 1). In addition, the copy numbers of bacterial 16S rDNA in GA-treated *Daphnia* remained under the detection limit after a 3-week culture, indicating that the bacteria in the GA-treated *Daphnia* had been removed. Even in 0.25% GA, over 80% of the embryos matured to swimming juvenile *Daphnia* (Table 1). To ensure the removal of the bacteria, 0.25% GA was used for the sterilization of embryos, and the resulting aposymbiotic juveniles were used in the subsequent experiments.

Table 1 Hatching rate of embryos exposed to varied concentrations of glutaraldehyde (GA) and copy numbers of 16S rRNA genes/juvenile *Daphnia*.

Concentration of GA	Hatching rate	Copy numbers of bacterial 16S rRNA genes / <i>Daphnia</i>
0%	98% (112/114)	$2.43 \pm 0.90 \times 10^4$
0.025%	100% (33/33)	ND
0.25%	81% (112/139)	ND

ND: not detectable.

2.3.2 Longevity of aposymbiotic *Daphnia*

I first investigated whether symbiotic bacteria have an effect on *Daphnia* longevity, because this is known to be the case in other animal species (Fukatsu et al., 2001; Behar et al., 2008; Ben-Yosef et al., 2008; Vorburger and Gousskov, 2011). The maximum life span of aposymbiotic *Daphnia* was 9 d shorter than that of symbiotic *Daphnia* (Figure 4). However, the mean longevity of aposymbiotic *Daphnia* (28.63 ± 2.2) was not significantly different to that of symbiotic *Daphnia* (p -value = 0.16; 33.50 ± 4.21). This may have been related to the mortality rate after the initial treatment with GA. Several individuals died within 3 days after the treatment of GA. Although further detailed analysis is needed, it is possible that aposymbiotic *Daphnia* have shorter longevity than symbiotic *Daphnia*.

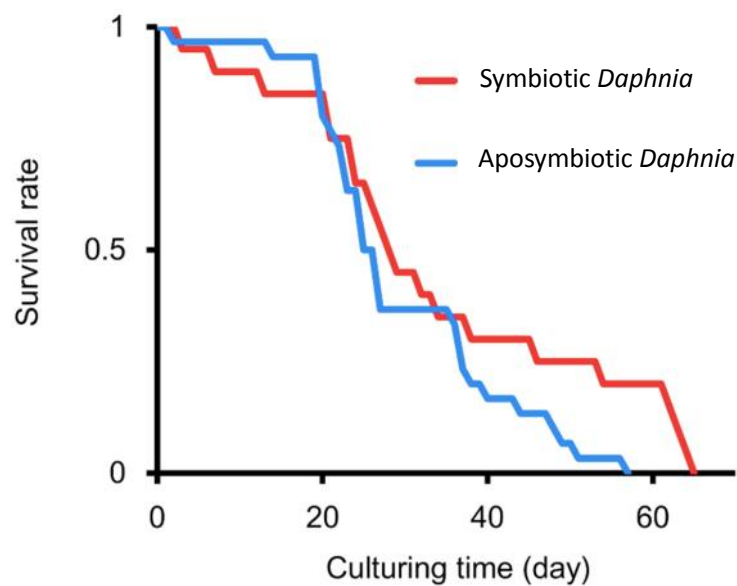


Figure 4 Survival curves of control and aposymbiotic *Daphnia*. The longevities of aposymbiotic and symbiotic *Daphnia* were determined in thrice- and twice-repeated experiments, respectively. Data are shown as survival rate versus age (day). Blue line indicated aposymbiotic *Daphnia*, and red line indicated symbiotic *Daphnia*.

2.3.3 Population dynamics of aposymbiotic *Daphnia*

Since population dynamics is an important factor in the role of *Daphnia* as a freshwater keystone species, I aseptically cultured aposymbiotic *Daphnia* for 21 d and investigated their population size. In contrast to the symbiotic *Daphnia*, the population size of aposymbiotic *Daphnia* did not increase during the culture (Figures 5A and 6A). The absence of bacteria in the aposymbiotic *Daphnia* was confirmed by qPCR at the beginning and end of the experiment (Figures 5B and 6B), suggesting that symbiotic bacteria play an important role in *Daphnia* population size.

2.3.4 Recovery of fecundity of aposymbiotic *Daphnia* by re-infection

To elucidate whether the smaller population size of aposymbiotic *Daphnia* was due to the loss of symbiotic bacteria, or due to reproductive system dysfunction because of the GA treatment, the aposymbiotic *Daphnia* were re-infected in 2 ways: (1) co-cultured with symbiotic *Daphnia* or (2) dipped in *Daphnia* extracts containing symbiotic bacteria.

I re-infected aposymbiotic *Daphnia* via co-culturing with symbiotic *Daphnia* by culturing 2 aposymbiotic *Daphnia* with 2 symbiotic *Daphnia* under bacteria-free conditions. After 21 d, the population size of the co-cultured *Daphnia* was approximately 30-fold higher than that of the aposymbiotic *Daphnia*, and 3-fold higher than that of the symbiotic *Daphnia* ($p < 0.01$; Figure 5A). To confirm that the re-infection was successful, qPCR analyses to detect bacterial 16S rRNA genes were performed at the beginning and end of the experiment. Aposymbiotic *Daphnia* co-cultured with symbiotic *Daphnia* contained a similar amount of bacteria as that of the symbiotic *Daphnia* (Figure 5B), suggesting that bacteria from the symbiotic *Daphnia* had transferred to and multiplied on the aposymbiotic *Daphnia*. Together, these results suggest that the transmission of

bacteria from the symbiotic *Daphnia* to the aposymbiotic *Daphnia* lead to an increase in the population size of the aposymbiotic *Daphnia*.

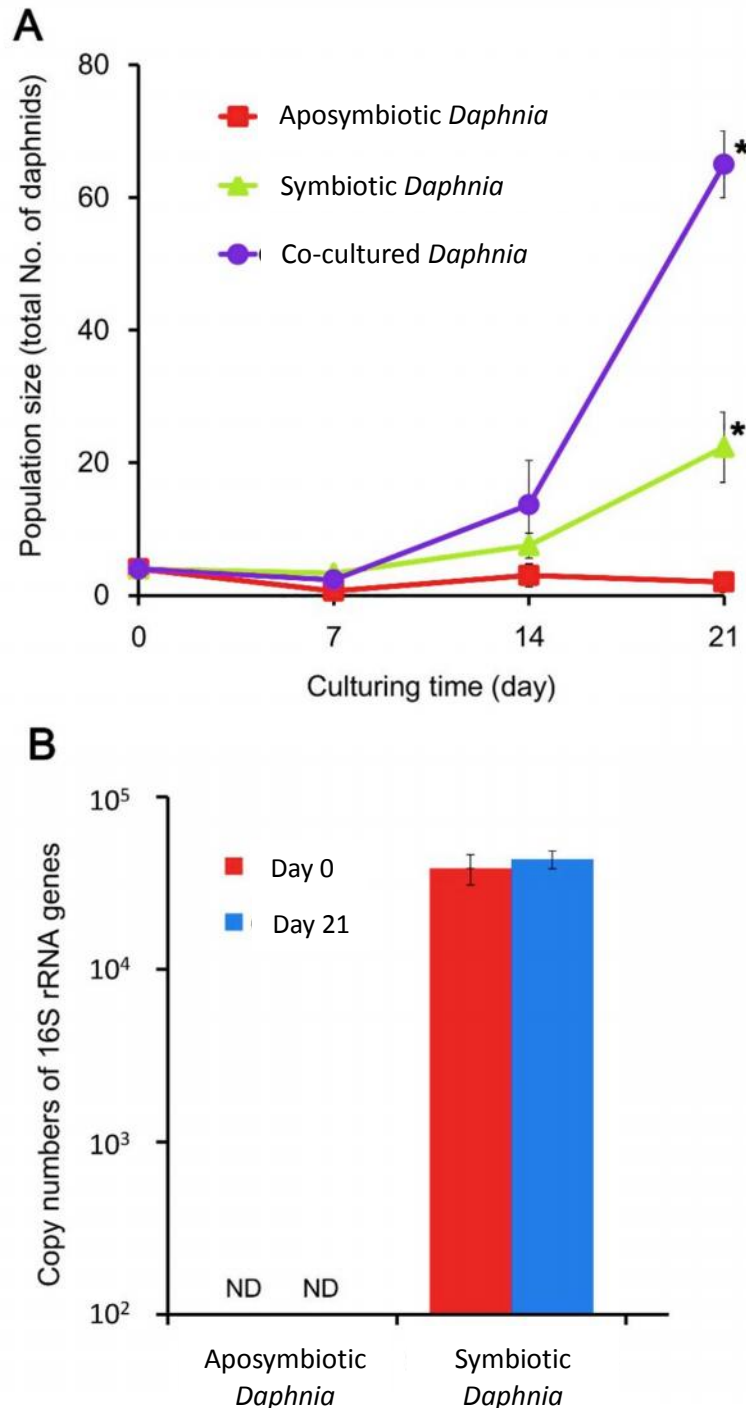


Figure 5 Re-infection experiment: co-culture with symbiotic *Daphnia*. (A) Temporal change in the population size. Square: aposymbiotic *Daphnia*; triangle: symbiotic *Daphnia*; circle: co-cultured *Daphnia*. An asterisk signifies that the population sizes were significantly different (Welch's *t*-test, $p <$

0.01) at 21 d. Data are presented as means \pm SE (aposymbiotic *Daphnia*: n = 3; symbiotic *Daphnia*: n = 6; co-cultured *Daphnia*: n = 3). (B) Copy numbers of 16S rRNA genes. Data are presented as means \pm SE (n = 3). ND signifies not detectable.

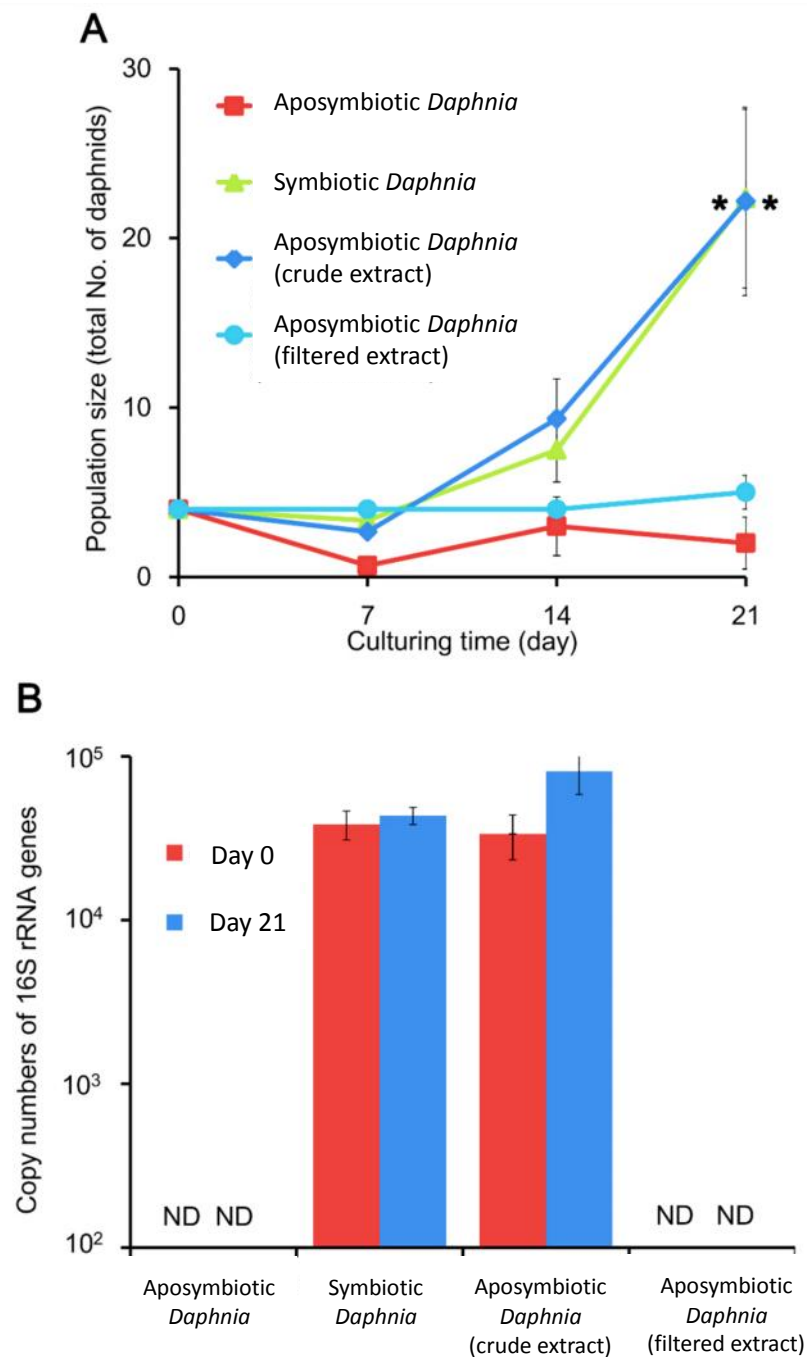


Figure 6 Re-infection experiment: dipping in *Daphnia* extracts. (A) Temporal change in the population size. Square: aposymbiotic *Daphnia*; triangle: symbiotic *Daphnia*; rhombus: aposymbiotic

Daphnia dipped into the crude extract; circle: aposymbiotic *Daphnia* dipped into the filtered extract. An asterisk signifies that the population sizes were significantly different when compared with aposymbiotic *Daphnia* (Welch's *t*-test, $p < 0.01$). Data are presented as means \pm SE (aposymbiotic *Daphnia*: $n = 3$; symbiotic *Daphnia*: $n = 6$; aposymbiotic *Daphnia* dipped in crude extracts: $n = 6$; aposymbiotic *Daphnia* dipped in filtered extracts: $n = 2$). (B) Copy numbers of 16S rRNA genes. Data are presented as means \pm standard error (SE; aposymbiotic *Daphnia*, symbiotic *Daphnia*, aposymbiotic *Daphnia* dipped in crude extracts: all $n = 3$, aposymbiotic *Daphnia* dipped in filtered extracts: $n = 2$). ND signifies not detectable.

I also re-infected aposymbiotic *Daphnia* with symbiotic bacteria by dipping embryos in crude extracts of untreated adult *Daphnia* which contained the bacterial symbionts. These re-infected *Daphnia* produced juveniles after 1 week, displayed a rapid population increase after 2 weeks (Figure 6A) in a manner similar to that of the symbiotic *Daphnia*, and possessed an increased number of bacteria according to the qPCR results.

To determine the possibility of small molecules such as nutrients in the *Daphnia* extracts affecting the results of population size, I dipped aposymbiotic *Daphnia* embryos into filtered *Daphnia* extracts that did not contain any bacteria. However, the filtered extracts did not affect the population size of aposymbiotic *Daphnia* and bacteria could not be detected 21 d in this sample. Together these results suggest that symbiotic bacteria play a critical role in the population growth of this species.

2.3.5 Sequencing of symbiotic bacteria

The results from the above experiments suggest that symbiotic bacteria can increase the population size in *Daphnia*. To identify the bacteria involved in this process, the bacterial community composition of the control and re-infected *Daphnia* was investigated by sequencing the bacterial 16S rRNA genes. I found that the majority of

bacteria in both the symbiotic *Daphnia* and re-infected *Daphnia* was *Betaproteobacteria* (Figure 7). The dominant bacterium was *Limnohabitans* sp., a *Betaproteobacteria* (Table 2 and 3). *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* were also identified but in very small numbers.

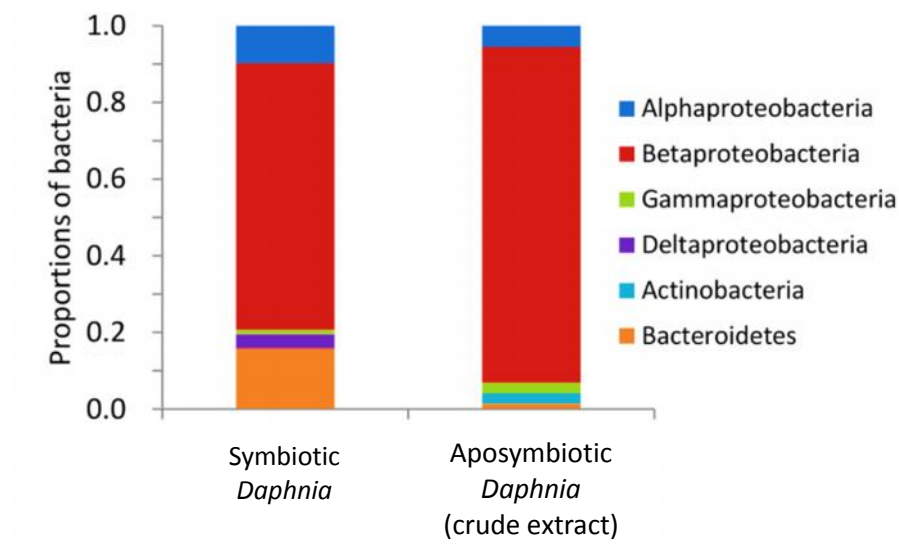


Figure 7 Taxonomic composition of symbiotic bacteria in symbiotic *Daphnia* and aposymbiotic *Daphnia* dipped in *Daphnia* extracts.

Table 2 Lists of bacteria found in aposymbiotic *Daphnia* dipped in *Daphnia* extracts.

Phylum	Class	Order	Family	Bacteria	Accession number	Clones	%Similarity	Accession number registered in this study	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Brevundimonas</i> sp. Eza32	JQ977273	1	100	-	
				<i>Brevundimonas diminuta</i>	KM009127	1	100	-	
				TR6					
			Rhizobiales	Rhizobiaceae	<i>Rhizobium grahamii</i> NH18	KJ921045	1	100	-
			Rhodobacterales	Rhodobacteraceae	<i>Rhodobacter</i> sp. A191	GQ484552	1	98	LC006866
		Betaproteobacteria	Burkholderiales	Comamonadaceae	Betaproteobacterium O-5-10	KF827201	2	100	-
	Burkholderiales bacterium				DQ922760	19	100	-	
	RCPCd10								
	<i>Limnohabitans</i> sp. 2KL-7				HE600663	40	100	-	
	<i>Limnohabitans</i> sp. Dn48				HM561454	1	98	LC006867	
	Bacterium B-17				HQ860533	1	99	LC006868	
	Gammaproteobacteria	Chromatiales	Chromatiaceae	Bacterium MayA002	JQ327531	1	98	LC006869	
Xanthomonadales				Xanthomonadaceae	Bacterium clone	JN641541	1	99	LC006870
			RRD14.0May-94						
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobineae	Bacterium CW2P2_12B	KC110409	1	100	-	
	Actinobacteridae	Actinomycetales	Microbacteriaceae	<i>Agrococcus</i> sp. DoB22	JQ359093	1	100	-	
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Chryseobacterium greenlandense</i> Hb1	JF899297	1	98	LC006871	

Table 3 Lists of bacteria found in symbiotic *Daphnia*.

Phylum	Class	Order	Family	Bacteria	Accession number	Clones	%Similarity	Accession number registered in this study	
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Alpha proteobacterium 4520-27F	FR648027	1	100	-	
				Bacterium CypR_69	JQ766928	2	100	-	
				Bacterium CLC8	HQ271262	1	100	-	
				<i>Rhizobium grahamii</i> NH18	KJ921045	2	100	-	
		Sphingomonadales	Sphingomonadaceae	Bacterium F1Q32TO06GY	GU508284	1	97	LC006872	
				Bacterium OTU-263	KF411929	1	97	LC006873	
		Betaproteobacteria	Burkholderiales	Comamonadaceae	Bacterium BB66	KF756603	1	100	-
					Bacterium 9B-72	JX298776	1	99	LC006878
	Beta proteobacterium 105T36				DQ110055	1	100	-	
	Bacterium C1Q				DQ856516	6	97	LC006874	
						1	95	LC006875	
	Burkholderiales bacterium RCPCd10				DQ922760	2	100	-	
	<i>Aquabacterium</i> sp. PM5_-0.3-19				JQ177862	1	97	LC006877	
	<i>Limnohabitans</i> sp. 2KL-7				HE600663	38	100	-	
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Oxalobacteraceae	<i>Duganella</i> sp. HME5	HQ829837	5	99	LC006876
				Rhodocyclales	Rhodocyclaceae	Bacterium WW1_LAB_F5	JQ413524	1	100
Bacterium Cyp1_93				JQ766608	1	100	-		
Bacterium DGGE gel band T 21				EU684012	3	100	-		
Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	Bacterium PL1	AF298766	1	100	-	
				Flavobacteriaceae	Bacterium 3C003625	EU802198	9	100	-
					Bacteroidetes bacterium CrystalBog2KG7	AY792294	1	100	-
	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	Bacterium B-17	HQ860533	2	100	-	

2.4 Discussion

In this study, I generated aposymbiotic *Daphnia* to investigate the effect of symbiotic bacteria on life-history traits in *Daphnia magna*. GA was used to disinfect *Daphnia* as an alternative to the antibiotics that have previously been used to disinfect *Daphnia*, despite their high toxicity (D'Agostino and Provasoli, 1970). GA is known to have a broad spectrum of activity, rapid antimicrobial action and is highly active in the presence of organic matter (Gorman et al. 1980; Salvesen et al. 1997). The mechanism of action of GA involves a strong binding with outer layers of bacterial cells, specifically with unprotonated amines of proteins on the cell surface (Munton and Russell, 1973; McDonnell and Russell, 1999). Furthermore, GA causes cross-linking of protein inside the bacterial cell and leads to inhibit transport in bacteria (McDonnell and Russell, 1999), to inhibit dehydrogenase activity (Munton and Russell, 1973) and periplasmic enzymes (McDonnell and Russell, 1999), and to inhibit RNA, DNA, and protein synthesis (McGucken and Woodside, 1973). Treatment of the *Daphnia* embryos with GA for 30 min was sufficient to remove bacteria from *D. magna*. In addition, the persistence of bacteria-free conditions was confirmed during the culture by real-time 16S rRNA gene PCR, suggesting that disinfection and axenic culture methods allowed me to accurately evaluate the effects of symbiotic bacteria on *Daphnia* in this study.

The results suggest that the population dynamics of aposymbiotic and symbiotic *Daphnia* differ. As *Daphnia* mortality during the experiments was limited, the difference between the population sizes was mainly due to differences in fecundity. The smaller population size of aposymbiotic *Daphnia* suggests that symbiotic bacteria play an essential role in *Daphnia* fecundity and population growth. Our results support most recent study with aposymbiotic *Daphnia* prepared by treatment of antibiotics mixtures to embryos for 2 days, reporting that bacteria-free *Daphnia* were smaller and less fecund

than *Daphnia* with microbiota (Sison-Mangus et al., 2014). In addition, a similar phenomenon has also been observed in aposymbiotic insects, such as the wasp *Asobara tabida*, the termite *Zootermopsis angusticollis*, and the aphid *Acyrtosiphon pisum* (Douglas, 1992; Dedeine et al., 2001; Rosengaus et al., 2011).

Symbiotic bacteria in *Daphnia* may play a role in *Daphnia* nutrition by producing enzymes and/or providing nutrients. Prokaryotic symbionts in the guts of termites are considered to play important roles in host nutrition (Ohkuma, 2008), and previous studies have shown that certain nutrients are important in *Daphnia* reproduction and growth. In *D. pulex*, 2 essential amino acids (arginine and histidine) are necessary for inhibiting the production of resting eggs, and increasing the production of asexual eggs (Fink et al., 2011; Koch et al., 2011). Growth and reproduction in *D. galeata* is limited by sterols and polyunsaturated fatty acids (DeMott and Müller-Navarra, 1997; Wacker and von Elert 2001; von Elert et al. 2003). In addition, vitamin B12 is required for normal reproduction in *D. pulex* (Keating 1985). As a result it is probable that symbiotic bacteria play nutritional roles in *Daphnia* by producing certain enzymes and/or by supplying nutrients, such as essential amino acids or lipids, to their hosts (Guarner and Malagelada, 2003; Dillon and Dillon, 2004). Therefore, the results of this study may be useful for further investigation of the symbiosis of *Daphnia* and bacteria.

No significant difference in longevity was observed between aposymbiotic and symbiotic *Daphnia*. This may have been related to the mortality rate after the initial treatment with GA. Several individuals died within 3 days after the treatment of GA, and the *p*-value was 0.16. Although further detailed analysis is needed, it is possible that aposymbiotic *Daphnia* have shorter longevity and lower fecundity than symbiotic *Daphnia*.

I further investigated the symbiotic relationship between *D. magna* and bacteria

by characterizing the bacterial community composition. The sequencing analysis of the PCR products revealed that the majority of the bacteria present in *Daphnia* were *Limnohabitans* sp. of the *Betaproteobacteria* class, which are usually found in freshwater habitats (Kasalický et al., 2010; Šimek, Kasalický, Hornák, et al., 2010; Šimek et al., 2011). This suggests that *Limnohabitans* sp. may be responsible for increasing fecundity in *D. magna*. These results are consistent with those from previous studies (Qi et al., 2009; Freese and Schink, 2011) suggesting that the symbiotic relationship between *Daphnia* and bacteria is stable and widely conserved, irrespective of spatiotemporal differences. In other words, our finding suggested the importance of widely distributed bacteria for the maintenance of the ecosystems by supporting the population size of zooplankton.

In order to investigate differences in life-history traits between aposymbiotic and symbiotic *Daphnia* under the same culturing conditions, symbiotic *Daphnia* were also cultured and maintained under aseptic conditions. However, this approach did not allow any bacteria to further infect the host during the culture, consequently leading to a lower abundance of symbiotic bacteria compared to normally cultured *Daphnia*. Therefore, it is possible that the results may underestimate the effects of bacterial symbionts, and could explain why the longevity of aposymbiotic *Daphnia* was not significantly different from that of symbiotic *Daphnia*.

2.5 Summary

In this chapter, I characterized the role of symbiotic bacteria on ecologically important life-history traits, such as population dynamics and longevity, in *D. magna*. By disinfection of the *Daphnia* embryos with glutaraldehyde, aposymbiotic *Daphnia* were prepared and cultured under bacteria-free conditions. Removal of bacteria from the

Daphnia was monitored by qPCR for bacterial 16S rRNA genes. The population of aposymbiotic *Daphnia* was reduced 10-folds compared to that of the symbiotic *Daphnia*. Importantly, re-infection with symbiotic bacteria caused *Daphnia* to regain bacteria and increase their fecundity to the level of the symbiotic *Daphnia*, suggesting that symbiotic bacteria regulate *Daphnia* fecundity. To identify the species of symbiotic bacteria, 16S rRNA genes of bacteria in *Daphnia* were sequenced. This revealed that 50% of sequences belonged to the *Limnohabitans* sp. of the *Betaproteobacteria* class and that the diversity of bacterial taxa was relatively low. I suggest that symbiotic bacteria have a beneficial effect on *D. magna*, and that aposymbiotic *Daphnia* are useful tools in understanding the role of symbiotic bacteria in the environmental responses and evolution of their hosts.

Chapter 3

Role of *Limnohabitans*, a dominant bacterium on *D. magna*'s life history traits

3.1 Introduction

In aquatic ecosystems, bacteria and zooplankton have been treated as separate functional units in aquatic food webs (Tang et al., 2010). Aquatic bacteria have individually been treated as free-living bacteria in studies observing their functions. Studies of zooplanktons have tended to focus on morphology, physiology, and interaction between them and their prey or predators. Since the finding of the “microbial loop” (Figure 8), bacteria and zooplankton have been regarded to be indirectly connected via nutrient cycling and trophic cascades (Azam and Malfatti, 2007). Aquatic bacteria utilize dissolved organic matter that is released from phytoplankton and are grazed by zooplankton, which means some energy from the microbial loop are returned to the conventional planktonic food chain (phytoplankton – zooplankton – fish links) (Azam et al., 1983). Nevertheless, the direct functional connection between zooplankton and aquatic bacteria remains largely unknown.

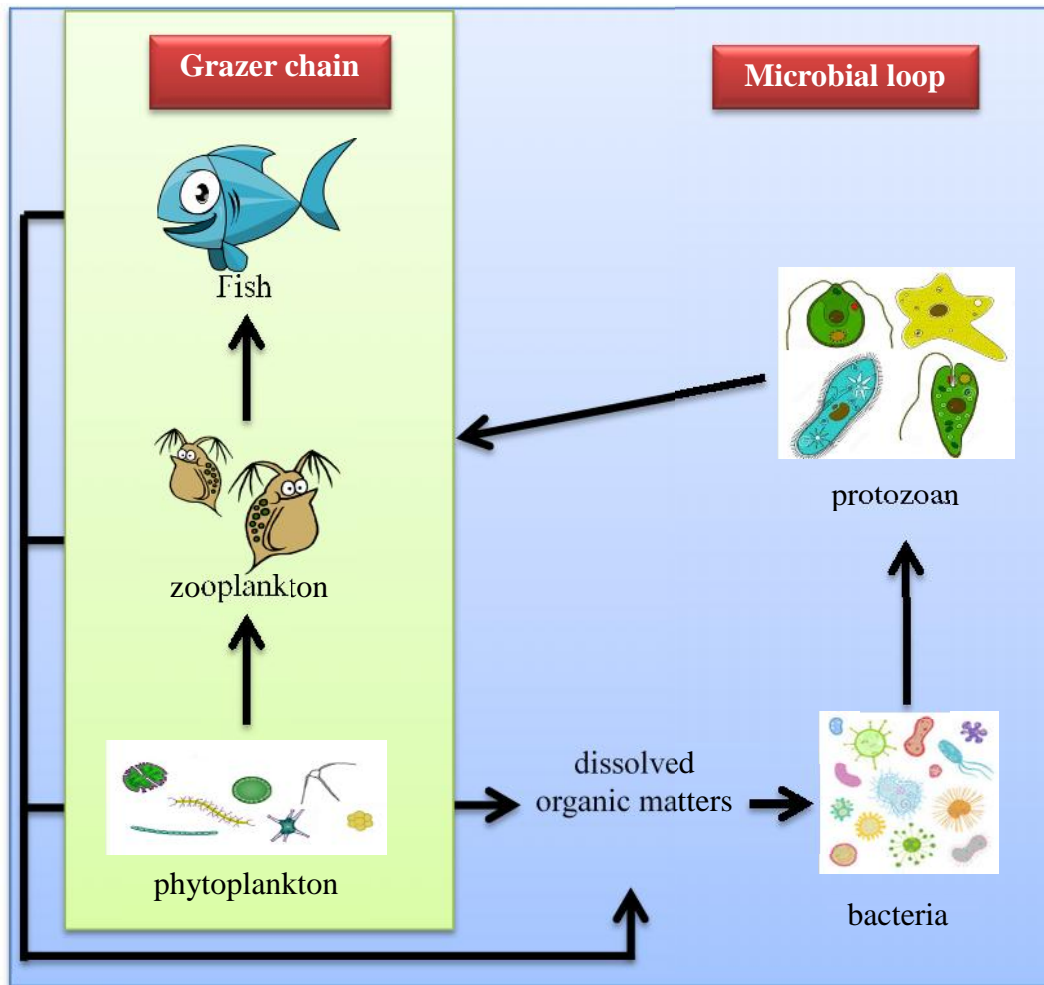


Figure 8 Microbial loop. The diagram depicts of the microbial loop. Organisms involving in the microbial loop including dissolved organic matters (DOMs) are bacteria, phytoplankton, protozoa, and zooplankton. The pathway is started from phytoplanktons fix carbon by photosynthesis and release DOMs (e.g. proteins, carbohydrates lipids and nucleic acids) to the aquatic habitats, then DOMs are utilized as a food source for bacteria. Bacteria are grazed by protozoan and transfer energy to zooplankton and the higher trophic level by consumptions. The DOMs are recycled back into the system as various organisms of all trophic levels die off and they are decomposed by bacteria or the other decomposers.

Recently, some quantitative studies revealed the bacterial abundance per unit of zooplankton body volume is between 10^7 and 10^{11} cells/ ml, which is higher than in the surrounding water (Hansen and Bech, 1996; Olsen et al., 2000; Heidelberg et al., 2002; Tang, 2005; Tang et al., 2010) , because the exoskeleton and gut lining of zooplankton provide suitable surfaces for bacterial attachment and enrich organic matters that support bacterial growth (Carman and Dobbs, 1997). In addition, intestinal bacteria of zooplankton, such as, *Homarus vulgaris*, *Hirondellea* sp., *Caprella kroyeri* and *Eucalanus bungii*, have been observed by SEM (Egidius, 1972; Schwarz et al., 1976; Nagasawa and Nemoto, 1988).

The crustacean zooplankton *Daphnia* is a keystone species in freshwater throughout the world. Sequencing of *Daphnia*'s symbiotic bacteria in Chapter 2 revealed that *Betaproteobacteria* is a major group of the microbial communities with the dominant bacterium being *Limnohabitans*, which is consistent with previous studies (Qi et al., 2009; Freese and Schink, 2011). Interestingly, *Limnohabitans* is an abundant and important member of freshwater bacterioplankton, inhabiting a broad range of freshwater habitats worldwide (Hahn et al., 2010; Kasalický et al., 2010, 2013) and maintaining growth and species diversity of bacterial communities (Šimek *et al.*, 2011; Horák and Corno, 2012). *Limnohabitans planktonicus* located on the filter combs of *Daphnia* as epibionts play important roles for the transfer of dissolved organic carbon to higher trophic levels in freshwater food webs (Eckert and Pernthaler, 2014).

To study symbiotic relationships between microbiota and *Daphnia*, I developed a method to prepare aposymbiotic *Daphnia* by treatment with sanitized reagents and found that microbial communities increase population size of aposymbiotic *D. magna* (see Chapter 2). In this chapter, I aimed to study the role of a major symbiotic bacterium, *Limnohabitans*, on *D. magna* life history traits by reinfection of aposymbiotic *Daphnia*.

3.2 Materials and Methods

3.2.1 *Daphnia* strain and culture condition

Daphnia magna strain NIES was obtained from the National Institute for Environmental Studies (NIES, Tsukuba, Japan). A single genetic clone of *D. magna* was isolated and has been subcultured parthenogenetically for more than 5 years. Eighty *Daphnia* were reared in 5 L of artificial *Daphnia* medium (ADaM) (Kluttgen et al., 1994) at $23 \pm 1^\circ\text{C}$ under a 16-h light/8-h dark photoperiod. In the first week, 5×10^8 cells of *Chlorella* sp. were added daily, and thereafter, 1×10^9 cells were added daily.

3.2.2 Strains of bacteria and preparation of bacterial suspension

Four *Limnohabitans* strains, 2KL-7, 2KL-3 (Kasalický et al., 2013), DM1 (V. Kasalický, unpublished), and *L. planktonicus* II-D5 (Kasalický et al., 2010) were used in this study. Each *Limnohabitans* was cultured in 50 ml of NSY medium (Hahn et al., 2004) for 2 days until logarithmic phase ($\text{OD}_{590} = 0.40$). *Escherichia coli* strain XL-10 Gold (Agilent technologies, USA) was grown until the logarithmic phase ($\text{OD}_{590} = 1.00$) in NSY. Each bacterium was collected by centrifugation at 6000 rpm for 5 min, and washed twice with filtered M4 media (Elendt and Bias, 1990) that were sterilized by filtration with a 0.2- μm filter, which were named “filtered M4 media” in this study (Corning-500 mL filter system, Corning, NY, USA). Each pellet was resuspended in filtered M4 media to adjust OD_{590} of each cell suspension to 1.72-1.82 for single reinfection experiments and to 1.56-1.64 for multiple reinfection experiments with calorimeter (WPA CO7500 colorimeter, WPA, Cambridge, UK). In order to prepare crude extract of *Daphnia*, 10 *Daphnia* were homogenized in 500 μl of filtered M4 media and then incubated for 5 min at room temperature.

3.2.3 Axenic *Chlorella*

Chlorella vulgaris Beijerinck was obtained from the National Institute for Environmental Studies (NIES, Tsukuba, Japan), inoculated in sterilized MAM medium (0.0025% CaCl₂, 0.0075% MgSO₄, 0.0025% NaCl, 0.01% KNO₃, 0.025% NH₄NO₃, 0.2% casamino acids, 0.05% yeast extract, and 0.05% malt extract), and incubated while being shaken at 23°C and under a 16-h light/8-h dark photoperiod for 5 d. The cells were then collected, washed twice, and resuspended in filtered M4 media and stored at 4°C. The 10⁸ cells of axenic *Chlorella* were used for bacterial screening.

3.2.4 Preparation of aposymbiotic *Daphnia*

Adult *Daphnia* were dissected to separate embryos from brood chambers. Collected embryos were divided into two groups for preparation of aposymbiotic and symbiotic *Daphnia*. For preparing aposymbiotic *Daphnia*, the embryos were exposed to 0.25% glutaraldehyde (GA) for 30 min to eliminate coexistent bacteria on the chorion and washed twice with filtered M4 media as described previously in Chapter 2. These GA-treated embryos were incubated in 2 ml of filtered M4 media in a 6-well plate at 23°C and under a 16-h light/8-h dark photoperiod for 48 h.

3.2.5 Single exposure of aposymbiotic *Daphnia* to a single strain bacterium

Four aposymbiotic juvenile *Daphnia* were transferred into each well of a 24-well plate containing 2 ml of filtered M4 media. For inoculation of *Limnohabitans* strains 2KL-7, 2KL-3, DM1 and *L. planktonicus* II-D5, 200 µl of each bacterial suspension was added in aposymbiotic *Daphnia* culture on the first day (day 1). Twenty microliters of crude extract containing *Daphnia* microbiota and 200 µl of *E. coli* were inoculated as infectant positive and negative controls, respectively. All groups were reared aseptically at

23 ± 1°C under a 16-h light/8-h dark photoperiod, fed every second day with 3 × 10⁷ cells of axenic *Chlorella* for 1 week, and thereafter with 6 × 10⁷ cells. Four GA-untreated symbiotic *Daphnia* were cultured under the same condition. At least one *Daphnia* in each well was used for bacterial screening.

3.2.6 Multiple exposures of aposymbiotic *Daphnia* to a single strain bacterium

Four aposymbiotic juvenile *Daphnia* were aseptically transferred and fed into each well of a 24-well plate in the same manner as single reinfection. For inoculation of *Limnohabitans* strains 2KL-7, 2KL-3, 200 µl of each bacterial suspension was added in culture of aposymbiotic *Daphnia* every second day. Twenty microliters of crude extract containing *Daphnia* microbiota and 200 µl of *E. coli* were inoculated as infectant positive and negative controls, respectively. Four GA-untreated symbiotic *Daphnia* were cultured under the same condition. One *Daphnia* in each well was used for bacterial screening.

3.2.7 Phenotypic analyses of reinfected *Daphnia*

For counting the number of juveniles per individual, the ovulated *Daphnia* were separated individually into a well of a new 24-well plate and cultured until the juveniles swam out from the brood chambers. For counting the number of eggs per individual, *Daphnia* were dissected after they laid parthenogenetic eggs in the brood chambers. For measurement of growth rate, the body length of *Daphnia* was measured day 1 and day 9 (Anderson, 1932).

3.2.8 Screening of bacterial 16S rDNA in *Daphnia* and *Chlorella* by quantitative polymerase chain reaction (qPCR) and sequencing.

Daphnia and *Chlorella* DNAs were extracted using a standard protocol for

Drosophila (Huang et al., 2000). First, *Daphnia* and *Chlorella* were homogenized in a solution of 200 µl Buffer A (100 mM Tris-HCl, 100 mM ethylenediaminetetraacetic acid [EDTA], 100 mM NaCl and 0.5% sodium dodecyl sulfate [SDS], pH 7.5) in a 1.5-ml tube and incubated at 65°C for 30 min. Second, the homogenate was mixed with 400 µl of LiCl/KAc solution (5 M potassium acetate: 6 M lithium chloride = 1:2.5), incubated on ice for 10 min and centrifuged for 15 min at 15,000 rpm. Third, 500 µl of the supernatant was transferred to a new tube, mixed with 300 µl isopropanol and centrifuged for 15 min at 15,000 rpm. Finally, the supernatant was removed and the precipitate was washed with 70% ethanol, dried, and resuspended in 50 µl of MilliQ, after which estimation of the copy number of 16S rRNA genes and sequencing were conducted.

qPCR of bacterial 16S rRNA genes was performed using a qPCR machine (MX3005P, Stratagene, CA, USA) and SYBR GreenER qPCR SuperMix Universal (Invitrogen). PCR amplifications were performed using primer set (forward primer 5'-AGACACGGTCCAGACTCCTAC-3' and reverse primer 5'-TTTACGGCGTGGACTACCAG-3') under the following conditions: 2 min at 95°C followed by 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). The detection limit was 50 copies of 16S rRNA genes.

To confirm the presence of *Limnohabitans* in reinfected *Daphnia*, intergenic spacer regions between 16S rRNA and 23S rRNA genes (IGS) of *Limnohabitans* strain DM1 and *L. planktonicus* II-D5 were amplified using primer set (1406F (5'-TGYACACACACCGCCCGT-3') and 23Sr (5'-GGGTTBCCCCATTTCRG-3') (Kasalický et al., 2013)) and using 1F (5'-CACATGCAAGTCGAACGGTAG-3') and 586R (5'-TGCAGTCACAAAGGCAGTTC-3') to distinguish between *Limnohabitans* 2KL-7 and 2KL-3. After PCR amplification, the PCR products were purified, cloned into a pCR4-TOPO vector (Invitrogen), sequenced, and aligned with the DNA sequence from

each *Limnohabitans*.

3.2.9 Statistical analyses

All data are presented as means \pm standard error (SE). Data of time of reproductive maturation and number of first clutch juveniles per individual were tested with Kruskal–Wallis followed by a Mann-Whitney U test for comparison of means. Data of growth rate and number of eggs per individual were tested with one-way ANOVA followed by Tukey’s honestly significant difference test at $\alpha = 0.05$ using the R statistical program R 3.0.1 (The R Core Team, 2013).

3.3 Results

3.3.1 Development of methods for reinfection of single *Limnohabitans* species to aposymbiotic *Daphnia*

To examine the role of *Limnohabitans* sp. in *Daphnia*, I developed methods for reinfection of single strain of *Limnohabitans* to aposymbiotic *Daphnia*, which were prepared by treatment of glutaraldehyde (GA) to embryos as described previously in Chapter 2. As infectants, I chose four *Limnohabitans* strains DM1, 2KL-3, 2KL-7 and *L. planktonicus*, all of which were found in the digestive tract of *Daphnia*, *D. magna* culture, or both (Qi et al., 2009; Freese and Schink, 2011, V. Kasalický, unpublished). *E. coli* and crude extracts of adult *Daphnia* containing the symbiotic bacteria were used as infectant negative and positive controls.

At first instar juvenile stage, aposymbiotic *Daphnia* were exposed to each single bacterial strain or crude extract. The exposed *Daphnia* were cultured without further addition of the bacterium to the culturing medium. In parallel with culture of the exposed

Daphnia, GA-treated aposymbiotic and GA-untreated symbiotic *Daphnia* were cultured. Until producing first clutch juveniles, all of the *Daphnia* were aseptically cultured to prevent bacterial contamination from the surrounding environment.

To investigate whether inoculated bacterial strain coexisted with *Daphnia* or not, I extracted DNA from the exposed *Daphnia* at the end of all experiments, and performed qPCR of bacterial 16S rRNA genes. The estimated copy numbers of bacterial 16S rRNA genes were lower than the detection limit (<50 copies of 16S rRNA genes/daphniid), whereas the symbiotic *Daphnia* were found to have $3.53 \pm 1.05 \times 10^4$ copies per individual. *Daphnia* exposed to any of *Limnohabitans* DM1, *L. planktonicus* II-D5, *E. coli* and crude extracts had similar copy numbers of bacterial 16S rRNA genes as the symbiotic *Daphnia*. In contrast, *Daphnia* exposed to either of *Limnohabitans* strains 2KL-7 and 2KL-3 were not detected 16S rRNA genes (Table 1).

Since single exposure was not sufficient for reinfection by *Limnohabitans* strains 2KL-7 and 2KL-3, I tried to increase the number of times of exposure. In addition to the exposure of aposymbiotic *Daphnia* at first juvenile instar stage, each bacterium was added to the culturing medium every second day until they produced first clutch juveniles. This multiple exposure enabled us to detect bacterial 16S rDNA in both of the exposed *Daphnia* (Table 2).

Finally, by sequencing of the intergenic spacer region of rDNA, I confirmed that each exposed *Daphnia* had inoculated *Limnohabitans* strain (data not shown), which meant that methods of reinfection by each *Limnohabitans* species were successfully developed. These methods were applied to phenotypic analyses of *Daphnia* for each *Limnohabitans* species.

Table 1 Copy number of 16S rDNA in *Daphnia* reinfected with bacteria by single exposure

Treatments	Copy number of 16S rDNA/ <i>Daphnia</i>
AD* (n=31)	ND**
AD+crude extract (n=16)	3.59±2.37 x 10 ⁴
AD+Limnohabitans DM1 (n=11)	1.47±0.53 x 10 ⁴
AD+ <i>L. planktonicus</i> II-D5 (n=11)	9.19±1.77 x 10 ³
AD+Limnohabitans 2KL-7 (n=3)	ND
AD+Limnohabitans 2KL-3 (n=3)	ND
AD+ <i>E. coli</i> XL 10-Gold (n=12)	3.16±1.08 x 10 ⁵
GA-untreated symbiotic <i>Daphnia</i> (n=23)	3.53±1.05 x 10 ⁴

*AD: Aposymbiotic *Daphnia*.

**ND: Copy number of 16S rDNA was under detection limit (<50 copies).

Table 2 Copy number of 16S rDNA in *Daphnia* reinfected with bacteria by multiple exposures

Treatments	Copy number of 16S rDNA/ <i>Daphnia</i>
AD* (n=3)	ND**
AD+crude extract (n=3)	1.98±0.61 x 10 ⁴
AD+Limnohabitans 2KL-7 (n=3)	3.00±0.39 x 10 ³
AD+Limnohabitans 2KL-3 (n=3)	5.57±1.94 x 10 ⁴
AD+ <i>E. coli</i> XL 10-Gold (n=3)	2.47±0.03 x 10 ⁵
GA-untreated symbiotic <i>Daphnia</i> (n=3)	1.12±0.32 x 10 ⁴

*AD: Aposymbiotic *Daphnia*.

**ND: Copy number of 16S rDNA was under detection limit (<50 copies).

3.3.2 *Limnohabitans* species can recover fecundity of *Daphnia*

The study in Chapter 2 found that symbiotic bacteria contribute to increased population size of *Daphnia*. I first characterized the role of *Limnohabitans* sp. on *Daphnia* fecundity by comparing the number of juveniles produced by the reinfected *Daphnia* with that produced by aposymbiotic *Daphnia* in their first clutches. Consistent with finding in Chapter 2, the number of juveniles produced by aposymbiotic *Daphnia* decreased 2-fold compared to wild-type *Daphnia* (Figure 9). Importantly I also found that aposymbiotic *Daphnia* produced abnormal juveniles unable to swim normally and that died during first instar juvenile stage, which were defined as “nonviable juvenile” in this study.

Daphnia with *Limnohabitans* strain DM1 or *L. planktonicus* II-D5 produced similar numbers of juveniles as GA-untreated symbiotic *Daphnia* (Figure 9). In contrast, *Daphnia* reinfected with any of *Limnohabitans* strains 2KL-7, 2KL-3 and *E. coli* produced nonviable juveniles as well as aposymbiotic *Daphnia*, which led to a reduction in number of juveniles produced in the first clutch (Figure 10). To test the possibility that the multiple exposures conferred an adverse effect on fecundity of *Daphnia*, I performed multiple exposures of GA-untreated symbiotic *Daphnia* to each bacterium and counted the number of juveniles. However, I found no decrease of fecundity in the *Daphnia* exposed to each bacterium (Figure 11). These results suggested that fecundity of aposymbiotic *Daphnia* could be recovered by reinfection of both *Limnohabitans* DM1 and *L. planktonicus* II-D5.

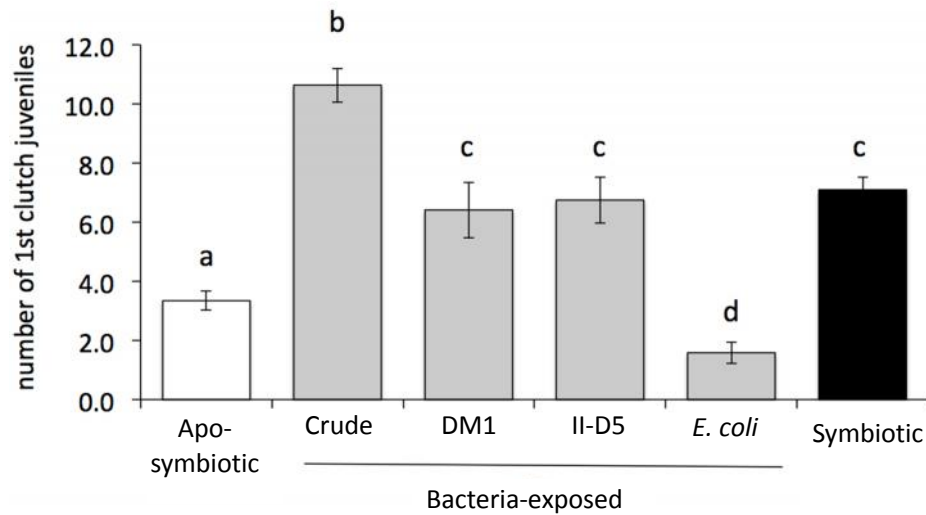


Figure 9 Fecundity of *Daphnia* reinfected with *Limnohabitans* strain DM1 and *L. planktonicus* by single exposure. Each bacterium or crude extract of *Daphnia* were inoculated at first instar juvenile stage. The number of juveniles in *Daphnia* was counted in their first clutch. Aposymbiotic *Daphnia* (aposymbiotic, n = 4 individuals per treatment, 24 replicates); aposymbiotic *Daphnia* reinfected with crude extract (bacteria exposed + crude, n = 4 individuals per treatment, 9 replicates), *Limnohabitans* strain DM1 (bacteria exposed + DM1, n = 4 individuals per treatment, 6 replicates), *L. planktonicus* strain II-D5 (bacteria exposed + II-D5, n = 4 individuals per treatment, 6 replicates) and *E. coli* (bacteria exposed + *E. coli*, n = 4 individuals per treatment, 6 replicates); and GA-untreated symbiotic *Daphnia* (symbiotic, n = 4 individuals per treatment, 18 replicates). Different letters above the bars indicate significant differences between treatments (Mann-Whitney U test, $p < 0.05$)

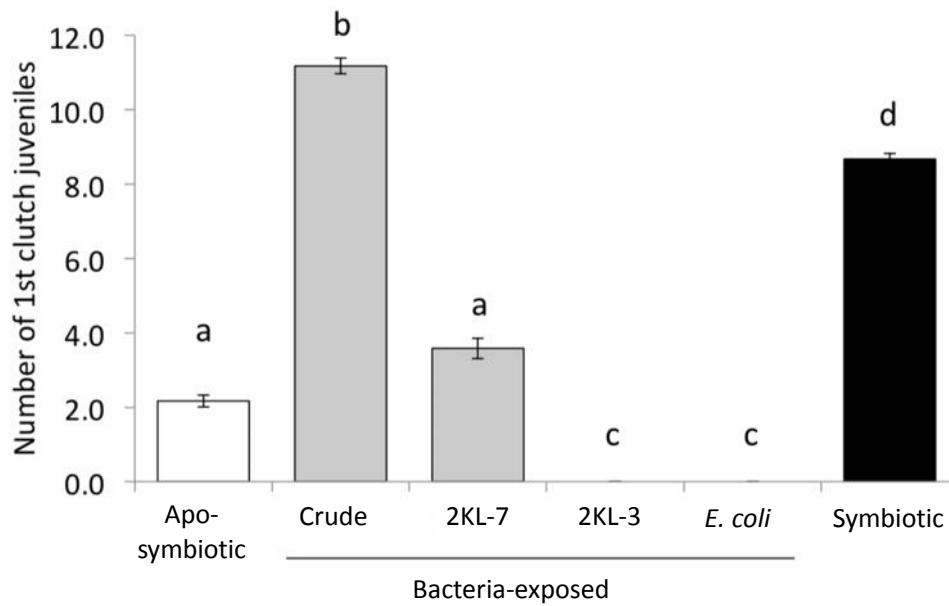


Figure 10 Fecundity of *Daphnia* reinfected with *Limnohabitans* strains 2KL-7 and 2KL-3 by

multiple exposures. Each bacterium or crude extract of *Daphnia* were inoculated every second day after first instar juvenile stage. The number of juveniles in *Daphnia* was counted in their first clutch. Aposymbiotic *Daphnia* (aposymbiotic, n = 4 individuals per treatment, 3 replicates); aposymbiotic *Daphnia* reinfected with crude extract bacteria-exposed, crude, n = 4 individuals per treatment, 3 replicates), *Limnohabitans* strain 2KL-7 (bacteria-exposed, 2KL-7, n = 4 individuals per treatment, 3 replicates), *Limnohabitans* strain 2KL-3 (bacteria-exposed, 2KL-3, n = 4 individuals per treatment, 3 replicates) and *E. coli* (bacteria-exposed, *E. coli*, n = 4 individuals per treatment, 3 replicates); GA-untreated symbiotic *Daphnia* (symbiotic, n = 4 individuals per treatment, 3 replicates). Different letters above the bars indicate significant differences between treatments (Mann-Whitney U test, $p < 0.005$).

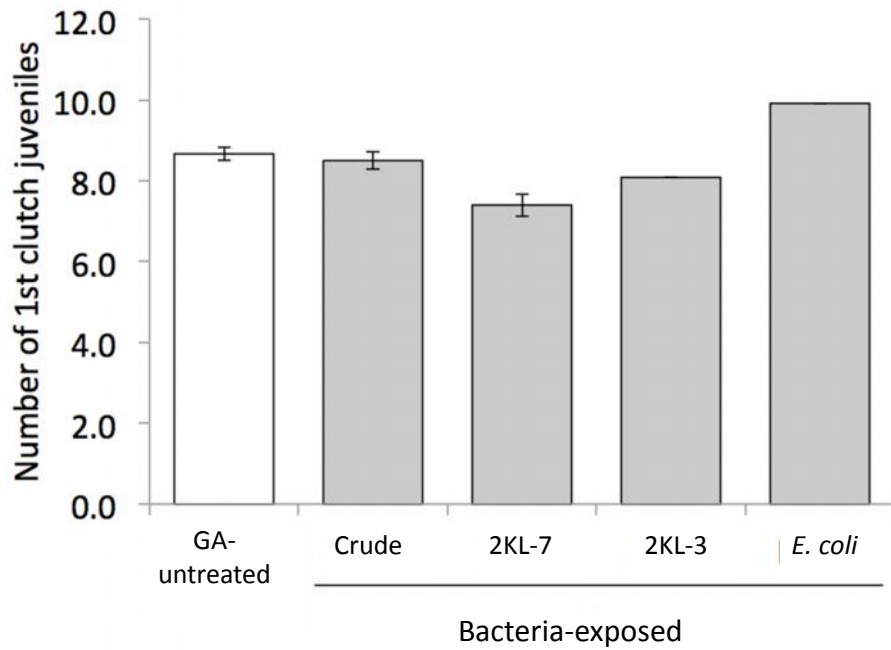


Figure 11 Effect of multiple exposures on fecundity of symbiotic (GA-untreated) *Daphnia*. Each bacterium or crude extract of *Daphnia* were inoculated at first instar juvenile stage. Each bacterium or crude extract of *Daphnia* were inoculated at first instar juvenile stage. The number of juveniles in *Daphnia* was counted in their first clutch. GA-untreated symbiotic *Daphnia* (GA-untreated, n = 4 individuals per treatment, 3 replicates); symbiotic *Daphnia* exposed to crude extract (bacteria-exposed, crude, n = 4 individuals per treatment, 3 replicates), *Limnohabitans* strain 2KL-7 (bacteria-exposed, 2KL-7, n = 4 individuals per treatment, 3 replicates), *Limnohabitans* strain 2KL-3 (bacteria-exposed, 2KL-3, n = 4 individuals per treatment, 3 replicates) and *E. coli* (bacteria-exposed, *E. coli*, n = 4 individuals per treatment, 3 replicates). There was no significant difference between groups (Kruskal-Wallis test, $p > 0.05$).

3.3.3 *Limnohabitans* sp. prevents the production of nonviable juvenile in *Daphnia*

Lethality of offspring produced by aposymbiotic *Daphnia* provided us a hypothesis that *Limnohabitans* sp. increases fecundity of *Daphnia* by preventing the production of nonviable juveniles rather than increasing egg production. To confirm this hypothesis, I first counted the number of first clutch eggs of GA-treated aposymbiotic and GA-untreated symbiotic *Daphnia* and found no difference in number of eggs between

aprosymbiotic and symbiotic *Daphnia* (Figure 12). I also confirmed that *Daphnia* reinfected with *Limnohabitans* sp. had similar numbers of first clutch eggs as the symbiotic *Daphnia*. Only through exposure to crude extracts did the reinfected *Daphnia* increase egg production. These data supported the hypothesis of a role of *Limnohabitans* strains on fecundity of *Daphnia*.

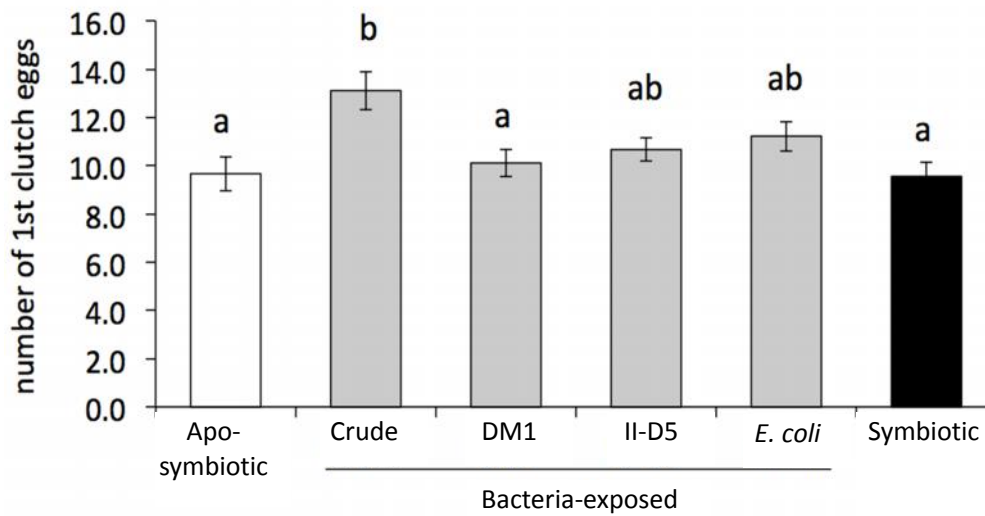


Figure 12 Egg production of *Daphnia* reinfected with *Limnohabitans* strain DM1 and *L. planktonicus* by single exposure. Each bacterium or crude extract of *Daphnia* were inoculated at first instar juvenile stage. The number of eggs in *Daphnia* was counted in their first clutch. Aposymbiotic *Daphnia* (aprosymbiotic, n = 3 individuals per treatment, 3 replicates); aposymbiotic *Daphnia* reinfected with crude extract (bacteria-exposed, crude, n = 3 individuals per treatment, 3 replicates), *Limnohabitans* strain DM1 (bacteria-exposed, DM1, n = 3 individuals per treatment, 3 replicates), *L. planktonicus* strain II-D5 (bacteria-exposed, II-D5, n = 3 individuals per treatment, 3 replicates) and *E. coli* (bacteria-exposed, *E. coli*, n = 3 individuals per treatment, 3 replicates); and GA-untreated symbiotic *Daphnia* (symbiotic, n = 3 individuals per treatment, 3 replicates). Different letters above the bars indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$).

3.3.4 *Limnohabitans* does not affect to growth rate of *Daphnia*

Because change of growth rate can also affect population size of *Daphnia*, I investigated the growth rates of GA-treated aposymbiotic *Daphnia*, GA-untreated symbiotic *Daphnia* and *Daphnia* reinfected with one of *Limnohabitans* DM1, *L. planktonicus* II-D5, or crude extract. Growth rates of *Daphnia* in all reinfections were not significantly different when compared with wild type *Daphnia* (Figure 13), suggesting the possibility that symbiotic *Daphnia* including *Limnohabitans* DM1 and *L. planktonicus* II-D5 did not concern the growth of *Daphnia*.

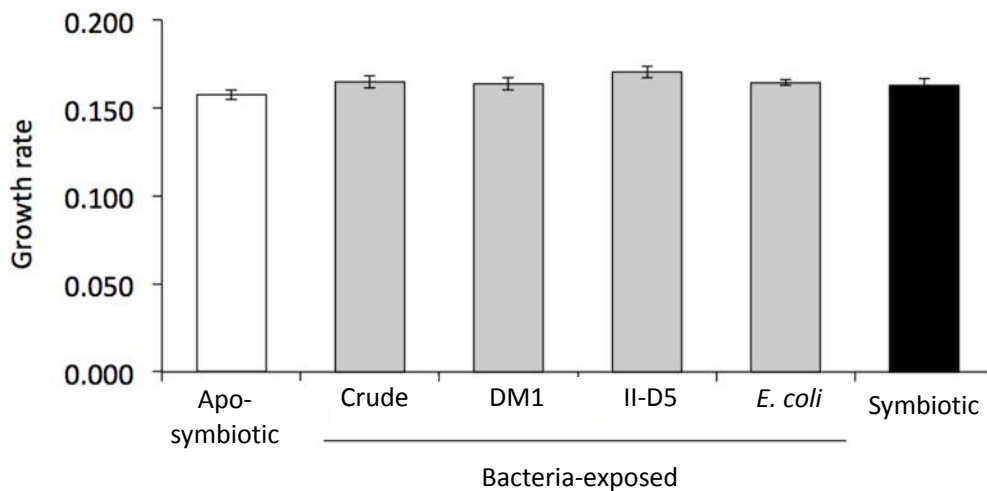


Figure 13 Growth rate of *Daphnia* reinfected with *Limnohabitans* strain DM1 and *L.*

***planktonicus* by single exposure.** Each bacterium or crude extract of *Daphnia* were inoculated at first instar juvenile stage. The number of eggs in *Daphnia* was counted in their first clutch. Aposymbiotic *Daphnia* (aposymbiotic, n = 3 individuals per treatment, 4 replicates); aposymbiotic *Daphnia* reinfected with crude extract (bacteria-exposed, crude, n = 3 individuals per treatment, 4 replicates), *Limnohabitans* strain DM1 (bacteria-exposed, DM1, n = 3 individuals per treatment, 2 replicates), *L. planktonicus* strain II-D5 (AD + II-D5, n = 3 individuals per treatment, 2 replicates) and *E. coli* (bacteria-exposed, *E. coli*, n = 3 individuals per treatment, 3 replicates); and GA-untreated symbiotic

Daphnia (symbiotic, n = 3 individuals per treatment, 2 replicates). There was no significant difference between groups (one-way ANOVA, $p > 0.05$).

3.4 Discussion

Here I first report the symbiotic relationship between *D. magna* and their major symbiont, *Limnohabitans* sp. Four strains of *Limnohabitans* sp. were used for reinfection by a single bacterial strain in order to investigate the role of *Limnohabitans* on *Daphnia* fecundity and growth. Recently, methods to prepare aposymbiotic *Daphnia* using the crosslinking reagent glutaraldehyde have been established and effects of the microbiota on *D. magna*'s life history traits were observed in Chapter 2. The results in this study suggested that *Limnohabitans* strain DM1 and *L. planktonicus* II D-5 are symbionts necessary for increasing fecundity of their host *D. magna*.

In this study, I found that *Daphnia* reinfected with *Limnohabitans* strain DM1 and *L. planktonicus* II-D5 produced more numbers of viable juveniles than that by aposymbiotic *Daphnia*. These strains may produce useful enzymes for digestion in *Daphnia*'s gut, increasing production of nutrients incorporated into parthenogenetic eggs during development of oocytes and leading to increased numbers of viable *Daphnia* juveniles. Previous studies revealed that certain nutrients are important in *Daphnia* reproduction. Arginine and histidine, essential amino acids, are necessary for production of asexual eggs and inhibition of resting eggs production (Fink et al., 2011; Koch et al., 2011). Sterols and polyunsaturated fatty acids are also considered essential nutrients for reproduction in *D. galeata* and *D. magna* (Demott and Muller-Navarra, 1997; Wacker and von Elert, 2001; von Elert et al., 2003; Martin-Creuzburg et al., 2006; Freese and Martin-Creuzburg, 2013). *D. magna* can assimilate fatty acids from some methanotrophic bacteria, *Methylomonas methanica* and *Methylosinus trichosporium* (Taipale et al., 2012).

Vitamin B12 is also required for normal reproduction in *D. pulex* (Keating, 1985). Symbiotic bacteria might mediate syntheses of these nutrients necessary for *Daphnia* reproduction.

Limnohabitans strains 2KL-7 and 2KL-3 could not recover *Daphnia* fecundity although *Limnohabitans* strain 2KL-7 was a major symbiont of *D. magna* NIES strain used in this study. *Limnohabitans* strains 2KL-7 and 2KL-3 are known to belong to the same sublineage of *Limnohabitans* sp. named LimC6 (Kasalický *et al.*, 2013). Aposymbiotic *Daphnia* exposed to crude extracts of *D. magna* NIES strain containing *Limnohabitans* strain 2KL-7 recovered their fecundity. Therefore, LimC6 might function in collaboration with other bacteria or associate with *Daphnia* without providing any benefit to the host, in contrast to *Limnohabitans* strain DM1 and *L. planktonicus* II-D5, both belonging to a different sublineage (Kasalický *et al.*, 2013).

Symbiotic bacteria did not seem to affect the growth rate of *Daphnia*, in contrast to a previous study (Sison-Mangus *et al.*, 2014). This contradiction might be due to difference of algae between the two studies. It is possible that some nutrients in *Scenedesmus* might be degraded by autoclave in the previous study and the nutrients of *Chlorella* we used might be sufficient for the growth of *Daphnia*, preventing us from observing positive effects of symbiotic bacteria on their growth.

3.5 Summary

In this chapter, I investigated symbiosis between a crustacean zooplankton *Daphnia magna* and its dominant bacterial symbiont *Limnohabitans*, an abundant and globally distributed freshwater Betaproteobacteria. Aposymbiotic juvenile *Daphnia* were prepared and exposed to any of four *Limnohabitans* sp.—*Limnohabitans* strains DM1, 2KL-3, 2KL-7, and *Limnohabitans planktonicus* strain II-D5, all previously found in *D.*

magna digestive tract or culture. Reinfected *Daphnia* were cultured until they produced the first clutch of juveniles. *Limnohabitans* strain DM1 and *L. planktonicus* strain II-D5 successfully reinfected *Daphnia* through single exposure at the first instar juvenile stage. In contrast to aposymbiotic *Daphnia* that produced non-viable juveniles, reinfected *Daphnia* produced viable juveniles and increased fecundity to levels of that of symbiotic *Daphnia*. Reinfected *Daphnia* did not increase their number of eggs nor growth rates. *Limnohabitans* strains 2KL-7 and 2KL-3 could not recover fecundity even in multiple exposures during culture. This study shows the functional evidence demonstrating that a single bacterium *Limnohabitans* regulates fecundity of the consumer *Daphnia* through symbiosis. The results indicated that symbiotic relationship between major bacterioplankton and zooplankton is important for maintaining the population of zooplankton and driving the trophic cascades in freshwater ecosystems.

Chapter 4

General discussion and conclusion

This study aimed to study the symbiotic relationship between primary consumer *Daphnia* and bacteria. First, I established a novel method for preparation of aposymbiotic *Daphnia* by exposure of GA, which is capable to eliminate bacteria on the surface of early-stage *Daphnia* embryos. The aposymbiotic *Daphnia* is a useful tool to allow me to clarify the role of symbiotic bacteria on *Daphnia*'s life history traits; longevity and population size. The results suggest that symbiotic bacteria are important to regulate population size of *Daphnia*. However, the difference of mean longevity between aposymbiotic and symbiotic were not statistically observed. Subsequently, I investigated microbial community composition in *Daphnia* body and found that the majority of microbiota was *Betaproteobacteria* and a dominant genus of *Betaproteobacteria* is *Limnohabitans*. In order to characterize the role of the dominant bacterium on ecologically important life history traits of *Daphnia*; fecundity and growth rate, the reinfection of aposymbiotic *Daphnia* with *Limnohabitans* strain was performed. The results suggest that fecundity of *Daphnia magna* is enhanced by *Limnohabitans* strain DM1 or *Limnohabitans planktonicus* II-D5. These microbial symbionts may play a nutritional role in their host by producing some necessary nutrients or enzyme to digest algae, which allows *Daphnia* to utilize the nutrients for their fecundity (Guarner and Malagelada, 2003; Dillon and Dillon, 2004; Ohkuma, 2008).

The effect of symbiotic bacteria on reproduction is not limited in *Daphnia*. Previous studies revealed the function of symbiotic bacteria is enhancing fecundity of

their host insects (Douglas, 1992; Dedeine et al., 2001; Kikuchi et al., 2007; Ben-Yosef et al., 2008; Rosengaus et al., 2011; Kikuchi and Fukatsu, 2014). Intestinal bacteria stimulate the larval development and survival in common fly (*Calliphora vomitoria*) (Wollman, 1911; Erkosar et al., 2013). In addition, *Lactobacillus plantarum* is directly responsible for microbiota-mediated mating phenotype of *Drosophila melanogaster* (Sharon et al., 2010). The human gut microflora provide benefit to human health involving nutritional and immune functions (Hooper and Gordon, 2001; Binn, 2013). The probiotic activity results in the production of the short chain fatty acids (SCFA) acetic, propionic acid, butyric acid and lactic acid. SCFA are absorbed to enhance the uptake of water and salts, and used as a source of energy by the host. Butyric acid is also the major source of energy of the epithelial cells lining the colon and can impact cell growth and differentiation (Binn, 2013). These suggest that function of symbiotic bacteria as maintenance of host population by modulating the life history traits of their hosts is conserved among animal species.

Interestingly, the results in Chapter 3 showed that *Limnohabitans* strain DM1 and *L. planktonicus* strain II-D5 could regulate fecundity of *Daphnia* and induce *Daphnia* to produce more viable juveniles, while *Limnohabitans* strain 2KL-7 and 2KL-3 could not recover *Daphnia* fecundity although *Limnohabitans* strains 2KL-7 was a major symbiont of *D. magna* NIES strain used in this study. *Limnohabitans* strains 2KL-7 and 2KL-3 are belonging to the same sublineage namely LimC6 (Figure 14). Aposymbiotic *Daphnia* exposed to *Daphnia* crude extracts containing *Limnohabitans* strain 2KL-7 recovered their fecundity. Therefore, LimC6 might function in collaboration with the other symbiotic bacteria or associate with *Daphnia* as a commensal without providing any benefit to the host. In contrast, *Limnohabitans* strain DM1 and *L. planktonicus* II-D5 are belonging to a different sublineage (Kasalický et al., 2013).

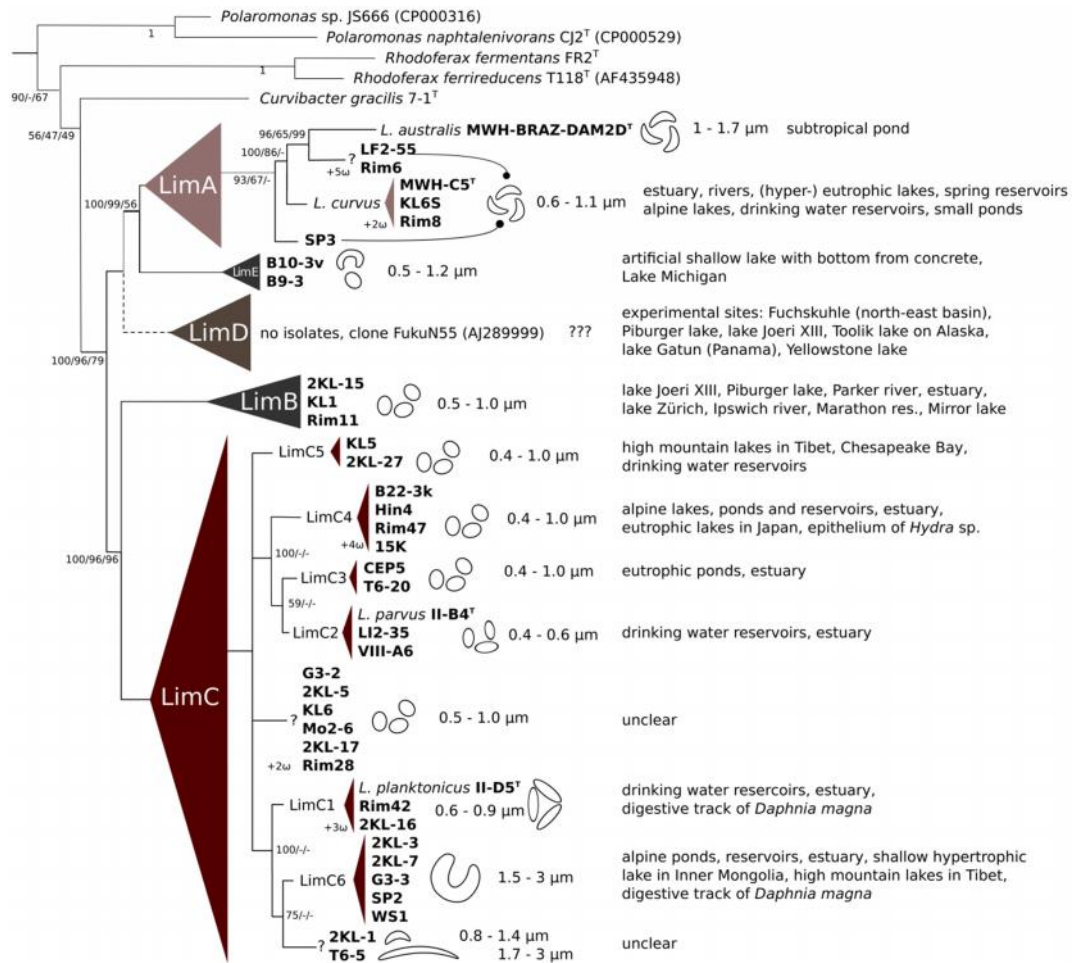


Figure 14 Phylogenetic tree of *Limnohabitans* genus based on 40 isolated strains (Kasalický et al., 2013). The simplified phylogeny schema was built on analyses of 16S rRNA gene and intergenic spacer regions between 16S rRNA and 23S rRNA genes (IGS) sequences. The phylogeny depicts *L. planktonicus* strain II-D5 belongs to LimC1 sublineage, while *Limnohabitans* strain 2KL-7 and 2KL-3 belong to sublineage LimC6.

One of the reasons for the different roles between *Limnohabitans* species in increasing fecundity of *Daphnia* may be that the different sublineages of *Limnohabitans* may have different metabolisms resulting in different function and relationship with *Daphnia*. To prove this hypothesis, the analysis of metabolites in *Limnohabitans* is necessary to predict nutrients affecting the fecundity of *Daphnia*. The candidate of

essential nutrients that *Limnohabitans* produces should be subjected to aposymbiotic *Daphnia* to confirm the effects of essential nutrients on *Daphnia*'s fecundity. Furthermore, investigation of metabolic pathways of each strain of *Limnohabitans* should be performed by genome sequencing to predict metabolic capabilities and mechanisms of *Limnohabitans* by following to a method previously established in the other bacterial species (Kwong et al., 2014), which uncover the factors that may describe the enhancement of *Daphnia*'s fecundity. Another reason may be that the other bacteria collaborate with *Limnohabitans* to enhance reproduction of *Daphnia*, hence, the collaboration between *Limnohabitans* and other symbiotic bacteria in *Daphnia* should be clarified.

Moreover, this study revealed the novel role of bacteria to regulate fecundity of freshwater zooplankton in aquatic ecosystem. Previously, *Limnohabitans* sp. as members of *Betaproteobacteria* are globally distributed and abundant in neutral and alkaline lakes (Šimek, Kasalický, Jezbera, et al., 2010) and are known to contribute to carbon flow within the grazer food chain (Šimek et al., 2014). This study indicates that *Limnohabitans* could also function as a mediator of carbon transfer leading to increase fecundity of *Daphnia*. This novel role also would contribute to carbon transfer to higher trophic levels to maintain the food chain core due to *Daphnia* plays a central role in freshwater ecosystems as a primary consumer (Figure 15). Eckert and Pernthaler (2014) found another pathway of carbon transfer through symbiosis between *Limnohabitans* and *Daphnia* where *L. planktonicus* function as epibionts attached on filter apparatus on the freshwater zooplankton *D. magna* (Eckert and Pernthaler, 2014). A recent study also showed that a single bacterial strain of *Aeromonas* sp. potentially increases body size of aposymbiotic *Daphnia* (Sison-Mangus et al., 2014). These suggest that complex and diverse symbiotic relationships between bacteria and *Daphnia* are required for ecosystem

maintenance. These findings may become a clue to solve a fundamental problem in ecology “How symbioses between bacteria and aquatic animals influence food webs in freshwater ecosystems is a fundamental question in ecology”.

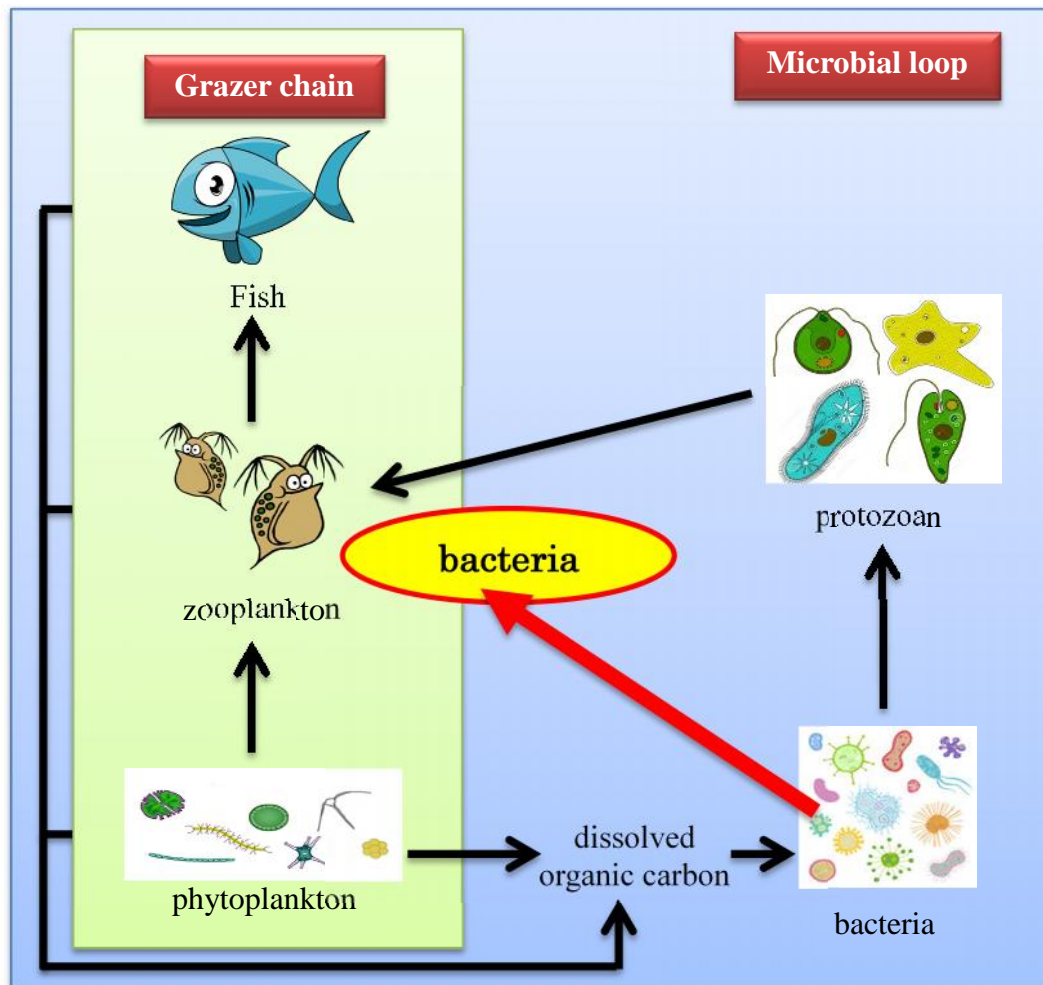


Figure 15 Carbon transfer pathway in freshwater ecosystem. Conventional concept of food chain considers zooplankton to function as a primary consumer, which transfers carbon from producer to the higher trophic level. In microbial loop, bacteria are decomposers and utilizers of dissolved organic matters, which transfer carbon to zooplankton through protozoan. This study revealed the hidden role of bacteria to regulate fecundity of zooplankton through symbiosis, in which zooplankton and bacteria are considered in one unit and function to enhance fecundity of zooplankton leading to maintain the food chain in freshwater ecosystem.

The further analyses are necessary to clarify the mechanisms of symbiosis in *Daphnia*. First, localization of the interesting symbiotic bacteria in *Daphnia* by using fluorescence in situ hybridization is necessary to clarify where bacteria function in *Daphnia* body for regulating fecundity of *Daphnia*. Second, bacterial genomic analyses or metabolic profiling in important symbiotic bacteria are important for prediction of essential nutrients produced by bacterial symbionts. Third, single strain of symbiotic bacteria should be isolated from *Daphnia* and the effects of minor symbiotic bacteria on *Daphnia*'s fecundity also should be investigated because some minor bacteria may collaborate with *Limnohabitans* and play an important role on *Daphnia*'s fecundity. Fourth, since many approaches of molecular genetics in *Daphnia* are available, the transcriptomics and proteomics involving reproduction of aposymbiotic *Daphnia* should be compared with those of symbiotic *Daphnia* to understand how symbiotic bacteria regulate *Daphnia* fecundity. Fifth, the candidate bacterial gene responsible for *Daphnia* fecundity can be introduced into *Daphnia* to analyze its function by using recently established genetic engineering tools. These analyses are needed to provide more evidences to answer how symbiotic bacteria regulate fecundity of *Daphnia*.

Daphnia can be used as a model for study of symbiosis in aquatic invertebrate, because diversity of symbionts in *Daphnia* is stable among species of *Daphnia* (Qi et al., 2009; Freese and Schink, 2011). Because host-microbe interaction is known to be one of factors driving host specificity and coevolution between bees and their gut symbionts (Kwong et al., 2014), the stability of bacterial community in *Daphnia* might become a model to study the host-microbe interaction and coevolution between hosts and symbionts.

Aposymbiotic *Daphnia* allows us to clarify the functions of single bacterial species on morphological, physiological, and behavioral effects of *Daphnia*. Furthermore,

we can obtain the accurate functions of symbiotic bacteria in *Daphnia*, to estimate the actual chemical resistance of the host (Kikuchi et al., 2012; P. Manakul, unpublished) and to investigate immunity responses to parasite (Pan et al., 2012). Therefore, aposymbiotic *Daphnia* developed in this study can be a useful tool to study in toxicology and immunity in *Daphnia*.

Conclusions

In this study, I firstly established a method to prepare aposymbiotic *Daphnia* by using glutaraldehyde. This method was simple, less time consuming and achieved high survival rate of treated *Daphnia*. Aposymbiotic *Daphnia* is a useful tool to study the role of symbiosis between *Daphnia* and their symbionts which is useful for further understanding the host-microbe interaction in freshwater ecosystem. Furthermore, I developed a method to reinfect *D. magna* with single strain of *Limnohabitans* and found a function of symbiotic relationship between *Daphnia* and *Limnohabitans* for increasing *Daphnia* fecundity and population size. These findings indicate a previously unidentified symbiotic relationship between major bacterioplankton and zooplankton for driving trophic cascade in freshwater ecosystems. Further analyses of the relationship between *D. magna* and symbiotic bacteria will be necessary for understanding the structure of freshwater ecosystem.

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Wollman, E. (1911) Sur l'élevage des mouches stériles. Contribution à la connaissance du rôle des microbes dans les voies digestives. *Ann. Inst. Pasteur* **25**: 79-88.

List of publications

1. **Peerakietkhajorn, S.**, Tsukada, K., Kato, Y., Matsuura, T. and Watanabe, H. (2015) Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, *Daphnia magna*. *Environmental Microbiology Reports*, 7: 364–372.
DOI: 10.1111/1758-2229.12260
2. **Peerakietkhajorn, S.**, Kato, Y., Kasalický, V., Matsuura, T. and Watanabe, H. (2015) Betaproteobacteria *Limnohabitans* strains increase fecundity in the crustacean *Daphnia magna*: Symbiotic relationship between major bacterioplankton and zooplankton in freshwater ecosystem. *Environmental Microbiology*, *in press*.
DOI: 10.1111/1462-2920.12919

Acknowledgements

I would like to express my sincere gratitude to Professor Dr. Hajime Watanabe, Department of Biotechnology, Graduate School of Engineering, Osaka University, for his kindness in giving me the great opportunity to research in Bioenvironmental Science Laboratory and his helpful comments to give the experiments a meaningful direction. Furthermore, I am equally thankful to Associate Professor Dr. Tomoaki Matsuura and Assistant Professor Dr. Yasuhiko Kato for their kind suggestions and guidance. I also would like to thank to Dr. Koji Tsukada for his helpful technical guidance.

I also would like to sincerely acknowledge Dr. Vojtěch Kasalický, Biology Centre of the ASCR, v.v.i., Institute of Hydrobiology, České Budějovice, Czech Republic, for the strains of *Limnohabitans* and his valuable suggestions.

I am deeply grateful to Professor Dr. Kiichi Fukui and Professor Dr. Toshiya Muranaka, Department of Biotechnology, Graduate School of Engineering, Osaka University, for their valuable comments and suggestions.

I would like to acknowledge the Department of Biotechnology, Osaka University and Monbukagakusho Scholarship for financially supporting.

Finally, I am also thankful to my family and friends for their understanding, encouragement and for having faith in me.