Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean *Daphnia magna*

Heike M. Freese • Bernhard Schink

Abstract Small filter-feeding zooplankton organisms like the cladoceran *Daphnia* spp. are key members of freshwater food webs. Although several interactions between *Daphnia* and bacteria have been investigated, the importance of the microbial communities inside *Daphnia* guts has been studied only poorly so far. In the present study, we characterised the bacterial community composition inside the digestive tract of a laboratory-reared clonal culture of *Daphnia magna* using 16S rRNA gene libraries and terminal-restriction length polymorphism fingerprint analyses. In addition, the diversity and stability of the intestinal microbial community were investigated over time, with different food sources as well as under starvation stress and death, and were compared to the community in the cultivation water. The diversity of the *Daphnia* gut microbiota was low. The bacterial community consisted mainly of Betaproteobacteria (e.g. *Limnohabitans* sp.), few Gammaproteobacteria (e.g. *Pseudomonas* sp.) and Bacteroidetes that were related to facultatively anaerobic bacteria, but did not contain typical fermentative or obligately anaerobic gut bacteria. Rather, the microbiota was constantly dominated by *Limnohabitans* sp. which belongs to the Lhab-A1 tribe (previously called R-BT065 cluster) that is abundant in various freshwaters. Other bacterial groups varied distinctly even under constant cultivation conditions. Overall, the

intestinal microbial community did not reflect the community in the surrounding cultivation water and clustered separately when analysed via the Additive Main Effects and Multiplicative Interaction model. In addition, the microbiota proved to be stable also when *Daphnia* were exposed to bacteria associated with a different food alga. After starvation, the community in the digestive tract was reduced to stable members. After death of the host animals, the community composition in the gut changed distinctly, and formerly undetected bacteria were activated. Our results suggest that the *Daphnia* microbiota consists mainly of an aerobic resident bacterial community which is indigenous to this habitat.

Introduction

In aquatic ecosystems, bacteria play a key role in the degradation of organic matter which is partly mineralised to phytoplankton nutrients and partly incorporated into biomass up to higher trophic levels [2, 12]. Bacteria occur in pelagic waters, in sediments, on aggregates and also associated with higher organisms [2, 19, 21, 23, 56]. Bacteria associated with aquatic organisms can be substantially more abundant than those in ambient water [19, 23] and are also present in zooplankton guts [21, 48]. Intestinal microorganisms contribute to their host's digestion and nutrition by degradation and transformation of complex biopolymers. They may produce methane and nitrous oxide as side products [18, 47, 57], but may also improve the host's resistance to pathogens and infections [10, 18]. Nevertheless, the composition and stability of intestinal microbial communities in small aquatic filter feeders like cladocerans of the genus *Daphnia* and their interaction with their host is largely unknown.

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H. M. Freese (✉) • B. Schink
Department of Biology, Microbial Ecology,
University of Konstanz,
Universitätsstraße 10,
78464 Konstanz, Germany
e-mail: heike.freese@uni-konstanz.de

Daphnia spp. are often the dominant zooplankton constituent in standing freshwater systems where they act as a crucial link between primary and secondary production [49]. In addition, *Daphnia* spp., in particular *Daphnia magna*, ingest bacteria from ambient waters [28, 50] and can, overall, shape aquatic microbial communities [9, 37]. Carcasses of *Daphnia* were found to be rapidly colonised by an active bacterial community [60]. However, research on the importance of bacteria inside zooplankton guts, which Tang et al. [61] discussed in their recent review, was mainly done with copepods and intact organisms, including the carapaces with numerous epibiotic bacteria [16, 52]. Grossart et al. [17], on the other hand, demonstrated in transplantation experiments that bacterial communities associated with (intact) cladocerans (*Bosmina*) were much more stable than communities associated with copepods; the authors interpreted this result to be due to different gut-internal conditions. Although the cladocerans were investigated before and after gut clearance, the intestinal community itself was not directly investigated. Only one recent study [48] showed that bacteria occurred near the microvilli and gut wall of *Daphnia*, and it was suggested that the animals maintain a permanent gut microbiota. The major bacterial constituents of the microbiota, which were detected via CARD-FISH in a homogenate of *Daphnia pulex* guts, were predominantly similar to the bacterioplankton of the respective habitat [48].

Our present study aimed at analysing the microbial community inside the gut of *D. magna*. Since the *Daphnia* guts are small, we did not expect that the intestinal microbial community is dominated by anaerobic microorganisms which contribute to their host's digestion by fermentation as typical of larger animals. Rather, the intestinal community should be shaped by environmental factors such as the type of food, surrounding bacteria etc. To draw conclusion on the stability of the *Daphnia* microbiota, we first examined whether the microbiota of *D. magna* clonal cultures remains stable under constant cultivation conditions. In addition, the variability of the community due to starvation stress and death of the animals or to an alternative (xenic) food source was investigated. These experiments were performed with a *D. magna* clonal culture in order to exclude variability of the microbiota due to different host genotypes which can affect microbiota compositions (as known for other animals [e.g. 34]). As well, influences by residues of incorporated bacteria from outside and seasonal effects of the environment could be minimised this way. We used a culture-independent approach combining clonal analysis and terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting analysis of 16S rRNA genes to characterise the intestinal bacterial community composition of *D. magna* and to investigate the stability of the microbiota

over time and under starvation stress, in comparison to the cultivation water.

Materials and Methods

Cultivation of *Daphnia* and Experimental Setup

Stock cultures of a *D. magna* originating from Lake Binnensee (Northern Germany; isolated by [35]) were cultured in membrane-filtered (0.2 µm) and autoclaved water from Lake Constance (LCW) at 18°C with a saturating supply of the green alga *Scenedesmus obliquus* (SAG 276-3a) grown in 'Cyano' medium [29]. Before experiments were started, *Daphnia* were pooled for 5 h in LCW at 18°C. Afterwards, they were transferred to sterile 1-l glass beakers (ca. 20–25 animals per beaker) filled with 500 ml LCW supplemented with ca. 1 mg C l⁻¹ *S. obliquus* that had been centrifuged (3,400×g for 7 min), washed and resuspended with LCW. After 2 days, *Daphnia* were refed and, after another 2 days, were transferred to a new beaker with fresh LCW and fed again. Experiments were stopped after another 3 days of incubation (Dn—normal condition). Three experiments were conducted independently (E1–E3). Additionally, the effect of starvation was investigated. Previously normally fed *Daphnia* were transferred to fresh LCW and incubated without food. In E1, *Daphnia* were starved for 3 days whereupon most animals (nine of 15) were dead. In E2, starvation was stopped after 2 days when all individuals were still alive. In E3, starvation was not investigated but the microbial community after normal incubation was analysed in 15 and 30 guts, respectively. In parallel, *Daphnia* were fed with a xenic cryptophycean alga, *Cryptomonas* sp. SAG 26.80, as an alternative, high-quality food source. In another experiment, dissected guts of *Daphnia* which were starved for 1 day were incubated for 0, 24, 48, and 72 h to simulate dead animals but to avoid interference of otherwise associated bacteria. For each time point, 15 guts were placed directly into 15 ml LCW and, after the incubation, were carefully removed with forceps.

Daphnia Gut Preparation and Extraction of Microbial DNA

Before *D. magna* guts were dissected, organisms were washed twice with LCW, immobilised with 50 mg ml⁻¹ NaHCO₃ in LCW and washed again in LCW. The guts (including the hepatopancreas) were separated from body tissue using Dumont forceps (Fig. 1) and after a short washing step were transferred to PBS (phosphate-buffered saline; 8 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, 1.44 g l⁻¹ Na₂HPO₄·12H₂O, 0.24 g l⁻¹ KH₂PO₄, pH 7.6). Wash water as well as LCW did not yield any PCR or T-RFLP products (data not shown).

Figure 1 Dissection of a gut of *Daphnia magna*. Animal before preparation of the gut (a) and the dissected gut including the hepatopancreas (b) are shown. Bar equals 1 mm

Preliminary experiments revealed that 10–15 guts had to be pooled for successful molecular biological analysis. Dissected *Daphnia* guts in 1 ml PBS were concentrated by centrifugation (13,000 rpm, 5 min, microcentrifuge) and frozen at -20°C . Cultivation water (200 ml) was filtered (5 μm , polyamide gauze; Franz Eckert GmbH, Germany) to exclude molted carapaces. Afterwards, bacteria were concentrated (12,900 $\times g$, 10 min), washed with PBS and frozen at -20°C . Bacterial DNA was extracted from guts and water following a protocol modified after Moré et al. [43]. A small amount of zirconium beads (0.1 mm diameter, sterilised at 180°C for at least 5 h), 200 μl PBS and 65 μl SDS solution (10% sodium dodecyl sulphate, 0.5 M Tris/HCl pH 8.0; 0.1 M NaCl) were added to each pellet. Cells and guts were disrupted by bead-beating (6.5 m s^{-1} , 45 s, BIO101/Savant FastPrep® FP120) and cooled immediately on ice. Cell debris was centrifuged (15,300 $\times g$, 4 min) and the supernatant was collected. The bead-beating step was repeated with 200 μl PBS at 6.5 m s^{-1} for 30 s, and both supernatants were pooled. Proteins were precipitated with ammonium acetate (2 vol 7.5 M to 5 vol sample, 4°C), and DNA in the supernatant was precipitated with 0.7 vol of isopropanol. After centrifugation of precipitated DNA (20,800 $\times g$, 60 min, 4°C) isopropanol was removed and DNA pellets were washed with 70% ethanol at -20°C before DNA was dried and resuspended in 35 μl buffer EB (Qiagen Elution buffer).

Other DNA extractions (e.g. with the MP Biomedicals FastDNA® SPIN Kit for Soil or Gentra Puregene Tissue Core Kit B) as well as modifications of the abovementioned method (e.g. additional incubation with mutanolysin for 30 min at 37°C) were also tested, but less DNA was recovered (data not shown).

Cloning and Phylogenetic Analysis

Clone libraries of the intestinal bacterial community were obtained from experiment E3 (samples Dn15 and Dn30). The 16S rRNA gene PCR products from gut DNA extracts were obtained with the primer set 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') and for the treatment Dn30 additionally with 907r (5'-CCG TCA ATT CMT TTG AGT TT-3') [36]. The PCR products were ligated into pCR®2.1 vector according to the manufacturer's instructions (TA Cloning® kit, Invitrogen). Clones with inserts were amplified by PCR using the vector-specific primers M13f (5'-TGT AAA ACG ACG GCC AGT-3') and M13r (5'-CAG GAA ACA GCT ATG ACC-3'). Ninety five clones were randomly picked and 96-well sequencing was performed at GATC Biotech AG (Konstanz, Germany) and produced sequences of 700 to 800 bp. The quality of sequences was checked with Bioedit software version 7.0.5.3 [20], and a BLAST search was performed to obtain related sequences [1]. Reference sequences were from bacterial strains which had either been published or are included in SILVA's 'The All-Species Living Tree' [62], or which were isolated in our laboratory from *D. magna* guts. Additionally, 16S rDNA shotgun sequences from entire *Daphnia* which were assigned to bacteria [52] as well as some of their closest matches were included. Sequences were aligned with the SINA Webaligner. Phylogenetic trees were calculated with MEGA4's UPGMA method (Fig. 2) and checked with the neighbour-joining method which yielded the same overall topology (data not shown) [58]. The robustness of tree topology was also tested by bootstrap analysis (2,000 replicates) [15]. Evolutionary distances were computed with the Maximum Composite Likelihood method [59].

Nucleotide sequences of the clones obtained in this study have been deposited in GenBank (accession numbers HM561429–HM561504).

Plasmids of selected clones were extracted with the Qiaprep Spin Miniprep Kit (Qiagen) to be screened by T-RFLP. These clones were used as references for the T-RFLP analysis of the bacterial community in the *Daphnia* gut.

T-RFLP Analysis

For T-RFLP analysis, 16S rRNA genes were amplified using the primers 27f and 907r (see above). The forward primer 27f was 5'-end labelled with 6-carboxyfluorescein (Biomers). PCR was carried out with 50- μ l reaction mixtures containing 1–2 μ l gut DNA extract or 1 μ l of a 1:10 dilution of cloned plasmid (including 16S rRNA gene sequence inserts), each primer at 250 nM concentration, each deoxyribonucleoside triphosphate at 30–100 μ M concentration, 5 μ l 10 $\times$ ThermoPol buffer and 1 U *Taq* DNA Polymerase (New England BioLabs). Negative control reaction mixtures without DNA were used for each amplified set. Cycling conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 45°C for 45 s and 72°C for 1.5 min, a single final extension cycle at 72°C for 20 min and a final soak at 4°C. Aliquots (5 μ l) of 16S rRNA amplicons were analysed by gel electrophoresis on 1% agarose gels and visualised after staining with ethidium bromide. All PCR products and also the negative controls were purified with the MinElute PCR purification kit (Qiagen). Always two to three parallel PCR products of the same bacterial community were pooled.

Prior to digestion, amplicon concentrations were determined photometrically. DNA [200 ng for amplicons from the environmental DNA extract (with Dn E1 100 ng since concentration was too low) or 50 ng for clonal amplicons or 17 μ l for negative controls], 2 μ l incubation buffer and 0.5 U of restriction enzyme *MspI* (Fermentas) were combined in a total volume of 20 μ l and digested for 3 h at 37°C. The optimal digestion time was estimated with isolated bacterial strains as that time resulting in the highest fluorescence intensity of T-RF (terminal-restriction fragment) and in the lowest percentage of pseudo-peaks (data not shown). The restriction enzyme was inactivated by incubation at 80°C for 20 min. Fluorescently labelled T-RFs were size-separated in triplicate on an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems) using an internal size standard (0.5 μ l diluted in 10 μ l water; GeneScan 500 ROX; Applied Biosystems) and 1 μ l of digested DNA. Samples were denatured at 94°C for 5 min, immediately cooled on ice and spun down. T-RF sizes between 50 and 500 bp with peak heights of ≥ 25 fluorescence units were determined and analysed using GeneMapper software 4.0 (Applied Biosystems). T-RF

Figure 2 Phylogenetic tree of cloned and sequenced 16S rRNA gene fragments of *Daphnia magna* microbiota. Clones are prefixed with 'Dn', additionally labelled with their T-RF and highlighted. Bacterial strains which were isolated from the *Daphnia* gut were included, too (highlighted). Clones were compared mainly with cultured bacterial strains for which NCBI accession numbers are given behind each name. Clones were also compared with bacterial 16S rDNA shotgun sequences associated with entire *Daphnia* (*D. magna* (Dm), *D. pulex* (Dp1) and *D. pulicaria* (Dp2) [52]) which were kindly provided by D. Ebert. The tree was constructed in MEGA using the UPGMA method, while evolutionary distances were computed with the Maximum Composite Likelihood method. The bar indicates the number of nucleotide substitutions per nucleotide site. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches

peak heights of triplicates were normalised according to Dunbar et al. [13] to the smallest DNA quantity before a mean of reproducible T-RFs was calculated. Different samples were not normalised since it cannot be guaranteed that the entire DNA was completely distributed on detected peaks. In addition, T-RFLP profiles were analysed with the Additive Main Effects and Multiplicative Interaction model (AMMI) and Interaction Principal Component Analysis (IPCA) using the T-REX program (<http://trex.biohpc.org/>) [7]. Relative peak height was chosen as parameter and no noise filtering was used because the filtering algorithm is not suitable for samples with a low number of T-RFs.

Results

Phylogenetic Analysis of the *Daphnia* Gut Microbiota

Three separate clone libraries were generated from the 16S rRNA gene fragments amplified from gut DNA with *Bacteria*-specific primer pairs. The 77 randomly selected clones were assigned to three distinct phylogenetic groups, β -proteobacteria, γ -proteobacteria and Bacteroidetes (Fig. 2). The gut libraries were found to be dominated by *Limnohabitans*-related clones (73% of all clones; Table 1). These clones of the three Dn clusters (HM561429–HM561483) were related closest (99.9–98.6%, on average 99.2%) to *Limnohabitans planktonicus*, a recently described facultatively anaerobic Gram-negative bacterium, and slightly less related (98.7%) to *Limnohabitans parvus* [30]. These species are representatives of the recently defined Lhab-A1 tribe of the betI-A clade and betI lineage which, together with the Lhab-A2 tribe, were previously defined as the freshwater R-BT065 cluster [45]. Maximal sequence differences of *Daphnia* gut clones belonging to the *Limnohabitans* group were 5.3%. Most of the remaining microbiota clones were related to *Leptothrix*, *Ideonella* and *Pseudomonas* spp. Four *Daphnia* gut clones were closely related (99.2–96.1%) to an *Acinetobacter* strain

Table 1 Distribution and similarity of cloned 16S rRNA gene fragments from the guts of *Daphnia magna* with the most similar published bacterial strains in NCBI

Phylum	Class	Order	Family	Bacteria	Accession number	Clones	% Similarity		References
							Min	Max	
Proteobacteria	β-Proteobacteria	Burkholderiales	Comamonadaceae	<i>Limnohabitans planktonicus</i>	FM165535	56	94.7	100	Kasalicky et al. 2010 IJSEM
				II-D5T					
				<i>Ideonella dechloratans</i>	X72724	4	98.5	98.8	Malmqvist et al. 1994 Syst Appl Microbiol
				Anox B					
γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae		<i>Leptothrix</i> sp. S1.1	DQ241397	8	97.8	98.0	Battaglia-Brunet et al. 2006 Geomicrobiol
				<i>Pseudomonas gessardii</i>	NR_024928	2	100	100	Verhille et al. 1999 Int J Syst Bacteriol
				CJP 105469					
				<i>Pseudomonas breunneri</i>	NR_025103	2	99.6	99.7	Baida et al. 2001 Res Microbiol
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Moraxellaceae	CFML 97-391					
				<i>Acinetobacter johnsonii</i> S35	AB099655	4	98.9	99.5	Malik et al. 2003 AEM
				<i>Pedobacter composti</i>	AB267720	1	98.6	98.6	Lee et al. 2009 IJSEM

isolated from a rainbow trout intestine [26]. Some clones were also associated (>95%) to another intestinal strain, *Comamonas odontotermitis*, isolated from the gut of the termite *Odontotermes formosanus* [6]. Only one clone was assigned to the Bacteroidetes, i.e. to *Pedobacter composti* [38]. In addition, a bacterial strain closely related to *Pedobacter* sp. and a strain affiliated to the CFB phylum were isolated from the gut of *D. magna*, too (Fig. 2). The reference strain *P. composti* was the only strictly aerobic strain. All other reference strains assigned to the gut clones have been described as facultatively anaerobic, but it is not always clear if these bacteria are denitrifiers or fermenters.

T-RFLP Analysis of the Intestinal Bacterial Community

Structure and diversity of the microbial community in *D. magna* guts were assessed in parallel by T-RFLP. When *Daphnia* were cultivated under standard conditions, the T-RFLP profile of 16S rRNA gene fragments from *Daphnia* microbiota consisted of seven to overall 14 different reproducible T-RFs (Fig. 3), more than the number of phylogenetically different types of bacteria identified via the clone library. Double amounts of gut material used for DNA extraction did not increase the diversity and numbers of T-RFs. Three major T-RFs and two minor T-RFs could be assigned to phylogenetic groups from the gut clone library (Table 2). Overall, *in silico* determination of clone T-RFs revealed significant differences of 3 to 5 bp, compared to the directly assessed T-RFs. Comparison of unidentified T-RFs with the T-RFLP Phylogenetic Assignment Tool resulted in a broad range of probable species hits, which even belonged to different phyla, for single T-RFs. Thus, no possible identity could be assigned to unidentified T-RFs. However, few T-RFs were also originated from bacterial strains isolated from the gut of *D. magna* (Table 2).

Clones affiliated to closely related *Ideonella* sp. and *Leptothrix* sp. shared the same T-RF which was constantly detected in the *Daphnia* gut (Fig. 3). Clones of *Limnohabitans* sp. differed slightly in their sequences, resulting in two separate T-RFs distinguished by two base pairs (Table 2). In the digestive tract of living *Daphnia*, the first *Limnohabitans* T-RF which also had the highest clone frequencies always produced the highest T-RF signal. The second *Limnohabitans* T-RF, although it was repeatedly detected in the gut, was less pronounced but accounted for a higher percentage in the cultivation water. The related taxa *Pseudomonas* sp. and *Acinetobacter* sp. originated two separate T-RFs but were not constantly detected. Although the *Daphnia* microbiota always generated three stable T-RFs (*Limnohabitans*-1, *Ideonella/Leptothrix*, unidentified D145) when incubated under normal conditions, the other T-RFs of the bacterial gut community changed

Figure 3 Relative fluorescence of T-RFs from MspI digested bacterial 16S rRNA genes amplified from DNA extracts of *Daphnia magna* microbiota incubated with *Scenedesmus obliquus* in different experiments (E1–3; E3_30: double amount of dissected guts), with xenic *Cryptomonas* sp., and incubated without food in comparison to the respective cultivation water and the algal culture used for feeding. T-RFs identified via clones were named after these clones, if T-RFs were identified via bacterial isolates from the gut, the name was placed in brackets. Accurate relative fluorescence values of T-RFs can be found in Supplementary Table 1

between the experiments although the conditions remained equal (Fig. 3). Some peaks were repeatedly detected while others, e.g. T-RF 277 which was also originated from the Actinobacterium *Microbacterium kitamiense* strain DD4 (HQ113380), were found only occasionally. Few highly variable T-RFs (e.g. *Acinetobacter* spp.) contributed even more than 15% to the overall composition, but most variable T-RFs occurred just with low intensities.

Comparison of Intestinal and Exogenous Bacterial Community Composition

The *Daphnia* microbiota did not originate predominantly from bacteria associated with the food alga *S. obliquus*. Only one high T-RF peak of the concentrated algal food suspension was also detected in the microbial gut community (Fig. 3). On the other hand, the *in silico* restriction of the rRNA gene of a *S. obliquus* chloroplast [NCBI

Table 2 Lengths (numbers of base pairs, bp) of analysed terminal-restriction fragments (T-RF, mean, minimum and maximum) and T-RFs theoretically deduced from clone sequences and sequences from

phylogenetically related bacteria from NCBI (Theo T-RF) as well as the T-RF name in the community profile (Community T-RF) and the percentage of clones producing the T-RF

Clone or isolate identity	T-RF (bp)	Min T-RF (bp)	Max T-RF (bp)	Theo T-RF (bp)	Community T-RF	% of clones
<i>Ideonella</i>	136.1	135.8	136.2	139	D136	6
<i>Leptothrix</i>	136.0			139	D136	10
<i>Limnhabitans-1</i>	481.5	481.1	481.9	486	D481	59
<i>Limnhabitans-2</i>	482.9			488		12
<i>Pseudomonas</i>	485.8	485.4	486.4	490	D485	6
<i>Acinetobacter</i>	485.9	484.9	487.0	491 (490)	D485–486	6
	486.3	485.9	487.0	491	D486	
<i>Pedobacter</i>	–			540		1
<i>Flavobacterium</i> DD5b	82.2	82.1	82.4		D82	
<i>Sphingomonas</i> DD7	147.5	147.4	147.6		D147	
<i>Microbacterium</i> DD4	277.0	276.6	277.3		D277	

DQ396875, 8] resulted in a T-RF of 462 bp which would be in the same position as the big *Scenedesmus* T-RF after correction of the base pair shift. Interestingly, the bacterial community composition (T-RFs) differed distinctly between microbiota, cultivation water and algal food suspension. Although cultivation waters were supplemented with *S. obliquus*, algal T-RFs were not detected in these waters (Fig. 3). The bacterial diversity (number of T-RFs) was nearly the same in cultivation water and in *Daphnia* guts, whereas LCW before supplementation with algae or *Daphnia* did not yield any T-RFs (data not shown). Some T-RFs like D82 occurred constantly in the cultivation water, and the same was also affiliated with the CFB *Flavobacterium* sp. DD5b (HQ113381) while others varied highly. However, most (60%) T-RFs from the cultivation water did not occur in the *Daphnia* gut even if they appeared repeatedly at highest intensities.

If a xenic *Cryptomonas* culture was added as the sole food source, some other different T-RFs dominated the cultivation water, and many unique T-RFs were found to be associated with the algae (Fig. 3). Nevertheless, the intestinal bacterial community was distinctly dominated by *Limnohabitans* T-RFs which occurred neither in the cultivation water nor associated to *Cryptomonas* sp., and none of the unique or cultivation water-dominating T-RFs were detected in the gut. In addition, some T-RFs were represented exclusively in the *Daphnia* microbiota; even major gut T-RFs were detected in cultivation waters only at low intensity, if at all.

Effect of Starvation and Death of *Daphnia* on the Bacterial Community Composition

Starvation of *D. magna* reduced the diversity (i.e. T-RF number) of the microbial gut community in comparison to normal incubation (Fig. 3). The most stable and dominant 'normal' bacterial gut T-RFs which were assigned to *Limnohabitans* sp., *Ideonella* sp. or *Leptothrix* sp. remained in the living animals through starvation for 2 days. During longer starvation, most *Daphnia* died but few (six of 15) survived, and the T-RF pattern shifted distinctly. The intensity of the formerly dominant *Limnohabitans* T-RF was considerably reduced, and the microbiota was dominated by a previously insignificant T-RF which can be attributed to *Pseudomonas* sp., and one further T-RF was detected for the first time at all. In contrast to cultivation water of shorter starvation experiments, the microbial community diversity in the water of longer starved *Daphnia* increased distinctly with several novel T-RFs (cf. Fig. 3). The novel gut-dominating *Pseudomonas* T-RF was not detected in the water, in neither parallel nor below the threshold (data not shown). When incubating dissected *Daphnia* guts over time, also the contribution of *Limnohabitans*-1 T-RF to the intestinal

community was reduced, and the *Pseudomonas* T-RF was detected after 48 h (Fig. 4). In addition, several previously undetected T-RFs were found which dominated the bacterial community in the gut.

Similarities Between Bacterial Communities

AMMI analysis revealed that the average sample heterogeneity (i.e. beta diversity) across all T-RFLP datasets was high (10.9) and the bacterial communities differed distinctly. The differences between the sample origins accounted for 74% (1% noise) of the total variation among the T-RFLP patterns. Thus, bacterial communities grouped clearly according to their origin (Fig. 5). The intestinal microbiota of all living *Daphnia* (*Scenedesmus*-fed, *Cryptomonas*-fed and starved) clustered distinctly separate from the other bacterial communities. Within this cluster, the bacterial communities were relatively widespread indicating some variability of the microbiota. However, the microbiota of *Cryptomonas*-fed *Daphnia* was located centrally in the cluster and just 8.6% of the variability within the cluster was due to the sample origin. In contrast, bacterial communities in the cultivation water differed considerably more due to the different cultivation histories (interaction signal 75%).

The bacterial communities in the gut of dead *Daphnia* also clustered distinctly separate. Especially the gut microbiota of *Daphnia* starved to death clustered not only separately with the first and second IPC values which explained 50% of the variance but their third IPC values covering additional 13% of variation were also clearly different (-8.3) from all other samples (between -1.6 and 1.5, data not shown). Thus, the microbiota differed from the microbiota of living *Daphnia* as well as from the bacterial communities in dissected guts (i.e. simulated dead animals). The bacterial community in dissected guts changed distinctly within 24 h but remained relatively stable afterwards (sample heterogeneity 1.6% and 17% of variability explained by time). Obviously, death of the *Daphnia* highly affected the intestinal microbial community resulting in the development of previously negligible gut bacteria.

Discussion

Composition and Diversity of the *Daphnia* Microbiota

The present study analyses for the first time the identity and stability of the intestinal microbial community of *D. magna* via clone libraries and T-RFLP. The bacterial community in the digestive tract of laboratory-reared *D. magna* was distinctly different from the gut microbiota of most other organisms, no matter whether terrestrial or aquatic [e.g. 14 and papers cited therein, 27, 33, 40]. Most *Daphnia* gut

Figure 4 Relative fluorescence of T-RFs from MspI digested bacterial 16S rRNA genes amplified from DNA extracts of *Daphnia magna* microbiota and incubation water from dissected guts incubated

for 0, 24, 48, and 72 h. T-RFs identified via clones were named after these clones, if T-RFs were identified via bacterial isolates from the gut, the name was placed in *brackets*

clones were affiliated to β -proteobacteria, mainly to *Limnohabitans* spp. (Comamonadaceae) that are commonly found in diverse freshwater systems but so far not inside digestive tracts [30, 55]. Several 16S rDNA sequences obtained by shotgun sequencing of bacteria associated with intact *Daphnia* [52] were also affiliated to these bacteria. Only one intestinal strain, *C. odontotermitis*, isolated from the gut of the termite *O. formosanus* [6] was also related (>95%) to some of our clones. In contrast, the γ -proteobacterial clones related to *Acinetobacter* and *Pseudomonas* were commonly detected in gut systems of

other aquatic animals such as fish and crabs [26, 40] or terrestrial crustaceans and insects [3, 24, 33] but, as in the *Daphnia* gut, they were detected there only as a minor group. Clones of typical intestinal bacterial groups such as Lactobacilli, Clostridia or Enterobacteriaceae (literature see above) were not detected. One bacterial strain belonging to the Enterobacteriaceae, *Serratia* sp. strain DD3, was isolated from the *Daphnia* gut but its T-RF was never detected. In addition, only one clone represented the CFB phylum but was related to an aerobic bacterial strain [38].

Figure 5 AMMI analysis of bacterial T-RFLP profiles from the digestive tract of *Daphnia magna* (filled symbols) incubated with *Scenedesmus obliquus* in different experiments (E1–3; E3_30: double amount of dissected guts), with xenic *Cryptomonas* sp., incubated without food and from dissected guts incubated up to 72 h, in comparison to the respective cultivation water (empty symbols)

The diversity of the intestinal microbial community was less than in other terrestrial and aquatic organism (ref. see above) but appears also to be lower than that in gut homogenates of environmental *D. pulex* analysed by CARD-FISH or that of bacterial shotgun sequences from intact *Daphnia* [48, 52]. Although the microbiota diversities of some predatory or plant sap-sucking insects were also low, they were composed differently (mainly of Firmicutes and γ -proteobacteria) than the *Daphnia* microbiota [22, 39]. However, the T-RFLP profiles of the *Daphnia* microbiota indicated a higher diversity than the clone library analysis, and T-RFLP most likely even underestimated the species diversity of the microbial community [13, 41]. Moreover, some undetected phylogenetic groups, e.g. α -proteobacteria and CFB, were represented by bacterial strains which we isolated from *Daphnia* guts. Nevertheless, laboratory maintenance of *Daphnia* may have artificially changed the intestinal microbial community since long-term cultivation with a homogeneous diet can reduce bacterial diversity [e.g. 31]. However, laboratory rearing of insects characterised by a less diverse microbial community had little effect on the associated bacterial community [22, 39]. In addition, *Daphnia* cannot survive without bacteria [51]. Thus, at least important (symbiotic) bacteria had to be maintained, although some diversity of the intestinal bacterial community may be lost. Qi et al. [52], on the other hand, demonstrated that bacteria associated with cultured *Daphnia* spp. were not only diverse but also remarkably similar across different *Daphnia* species cultivated on different continents, indicating a long-lasting stability of these associations. Some of these common bacteria, e.g. Comamonadaceae, were detected also in the gut of *D. magna* (this study) and even associated to the environmental cladoceran *Bosmina* [17]. Therefore, the intestinal bacterial community of cultured *Daphnia* may not be exactly identical with that of free-living animals but may likely represent the most important bacteria.

Overall, no evidence of obligately anaerobic bacteria or typical inhabitants of anoxic guts were found; rather, the *Daphnia* gut was dominated by clones affiliated to aerobic or facultatively anaerobic bacteria. Thus, the microbiota of *Daphnia* differed considerably from those of larger animals where an anaerobic microbial community contributes to their host's digestion. The small gut diameter (<200 μ m) and a correspondingly high surface/volume ratio as well as a fast throughput of food particles (retention time ~30 min) [cf. 4, 44] indicates that oxygen is available in almost the entire *Daphnia* gut. This was also confirmed by microelectrode measurements of oxygen profiles through the gut (unpublished data). Thus, even if some of the gut bacteria are potentially capable of fermentation, we conclude that mainly aerobic, oxygen-consuming bacteria live in the intestinal tract of *Daphnia* although they may produce suboxic microzones where fermentation takes

place. However, this hypothesis has to be examined in more detail with physiological experiments.

Dominance of *Limnohabitans* sp. in the *Daphnia* Gut

Although β -proteobacteria are rather irrelevant in the digestive tracts of most animals, they appear to be typical intestinal bacteria in *Daphnia* and may even maintain a symbiotic relationship to their host. A CARD-FISH analysis of gut homogenates of environmental *D. pulex* also revealed β -proteobacteria as one major group [48]. These bacteria were also a dominant bacterial group associated with another cladocera (*Bosmina*) [17]. In addition, the majority of bacterial shotgun sequences from intact *Daphnia* were assigned to β -proteobacteria and, on average, 50% of all detected proteobacteria sequences also belonged to the family Comamonadaceae [52]. Notably, several of their few identified 16S rRNA gene sequences were affiliated to *Limnohabitans* sp. In the digestive tract of *D. magna*, these microorganisms constantly dominated the intestinal bacterial community as stable members. They dominated not only at constant conditions but also when *Daphnia* were starved or incubated with an alternative xenic food source in which *Limnohabitans* was not detected, neither associated with the algae nor in the cultivation water. *Limnohabitans* sp. are members of the R-BT065 cluster (tribes Lhab-A1 and -A2) typically comprising 5–30% (up to 50%) of the bacterioplankton of non-humic freshwater lakes [54, 55]. These bacteria have not been detected as intestinal bacteria before, but the gut-inhabiting microorganisms were closely related to the typical planktonic bacteria (>99%). They are known to utilise algal dissolved organic matter [46], thus the high density of algae may favour these bacteria in the *Daphnia* gut. Furthermore, they use acetate at a notably low rate [5], which is a typical fermentation product and one of the most important short-chain fatty acids absorbed by animal hosts [18, 47]. Members of the R-BT065 cluster are abundant and highly active (glucose or amino acid incorporation) also under anoxic conditions [5, 53] and could therefore be active in anoxic gut compartments. Although these bacteria are subject to predation by protists [54], it is not yet known if this is true also for *Daphnia* predation. Moreover, it was demonstrated that the proportion of metabolically active *Limnohabitans* cells even increased under heavy grazing pressure, and they responded rapidly to environmental changes [i.e. 25, 46, 54]. Consequently, the *Daphnia* gut appears to be a perfect habitat for *Limnohabitans* sp. of the Lhab-A1 and -A2 tribes (R-BT065 cluster). Due to the growth conditions in the gut environment, *Daphnia* may even serve as a reservoir where these microorganisms are enriched and constantly released into the pelagic environment. On the other hand, the stability and dominance of these

bacteria indicate that they might have an important function for *Daphnia*, a subject worth to be treated in a separate study.

Variability of the Intestinal Microbial Community of *Daphnia*

Even though the intestinal community in *Daphnia* was always dominated by *Limnohabitans* and few other stable members, less prevalent members varied over time even when constantly fed a pure culture of algae. Some community members occurred sporadically, although at times at high intensity, while others were detected repeatedly. Peter and Sommaruga [48] detected in gut homogenates most major bacterial groups of the surrounding water, except for Actinobacteria. Thus, these variable community members could reflect bacteria from the cultivation water which were accumulated in the gut through filtration. However, in our study, the surrounding community was distinctly different from the gut microbiota. Even when *Daphnia* were exposed to *Cryptomonas* and bacteria associated with it, only few novel bacteria were detected, and the intestinal community remained highly similar. In contrast, bacteria from the cultivation water or associated with the algae were not detected inside the gut at all. Nevertheless, a change of the microbiota may be caused by uptake and stimulation of inactive or underrepresented bacteria as this was described, e.g., for the guts of earthworms [11]. However, such bacteria would need to integrate quickly into established *Daphnia* microbiota to compensate for the short residence time of the gut content (~30 min; [44]). When dissected guts of *D. magna* were incubated in sterile water, the intestinal bacterial community also changed distinctly, and even novel members occurred which had not been detected previously in the guts. Thus, the variable members more likely represent less numerous bacteria that were not detected by T-RFLP [41] but were activated and thus increased their abundance over time as known, e.g., for pelagic bacteria or human gut microbiota [18, 32]. In lake water with its more diverse bacterial communities, exogenous bacteria may have a bigger impact on the intestinal microbial community. However, Grossart et al. [17] demonstrated that bacteria associated with the cladoceran *Bosmina* remained highly similar also when transplanted into another lake. Thus, it can be assumed that the intestinal microbial community in *Daphnia* is composed, at least to a major part, of resident bacteria and is less susceptible to reflect the surrounding bacterial community. However, the importance of the surrounding bacterial community for the first inoculation of *Daphnia* guts and the establishment of the gut microbiota remains to be investigated.

Independent of the variability of the total community, *Limnohabitans*-related bacteria constantly dominated the

gut, especially when *Daphnia* were starved. However, if *Daphnia* were starved to death or dissected guts were incubated (simulating dead *Daphnia*), *Limnohabitans* sp. decreased while novel bacteria appeared. One of the new dominating T-RFs probably represented bacteria related to *Pseudomonas* sp. Previous colonisation of the gut by these novel bacteria was possibly prevented by the established microbiota [10, 18], and the invading bacteria might also be digested by the host. Starvation may have reduced the fitness of the microbiota (or host) thus allowing higher densities of *Pseudomonas* sp. which are known to be lethal or pathogenic, e.g., for insects [3]. Feeding higher amounts of these bacteria to *Daphnia* resulted in a decrease of animal biomass [42]. Thus, *Limnohabitans* sp. and other stable members may cause a colonisation resistance of the digestive tract of *Daphnia* which may also explain the obvious differences between the internal and the ambient microbial communities. However, whether the present intestinal community in the gut of *Daphnia* confers resistance against colonisation by surrounding and possibly pathogenic bacteria remains to be studied.

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