The microbiome of the marine flatworm Macrostomum lignano provides fitness advantages and exhibits circadian rhythmicity

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Article

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The microbiome of the marine flatworm *Macrostomum lignano* provides fitness advantages and exhibits circadian rhythmicity

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Abstract

The close association between animals and their associated microbiota is usually beneficial for both partners. Here, we used a simple marine model invertebrate, the flatworm *Macrostomum lignano*, to characterize the host-microbiota interaction in detail. This analysis revealed that the different developmental stages each harbor a specific microbiota. Studies with gnotobiotic animals clarified the physiological significance of the microbiota. While no fitness benefits were mediated by the microbiota when food was freely available, animals with microbiota showed significantly increased fitness with reduced food supply. The microbiota of *M. lignano* shows circadian rhythmicity, affecting both the total bacterial load and the behavior of specific taxa. Moreover, the presence of the worm influences the composition of the bacterial consortia in the environment. In summary, the *Macrostomum*-microbiota system described here can serve as a general model for host-microbe interactions in marine invertebrates.

Key words *Macrostomum lignano*; microbiome; flatworms; microbial

Introduction

Almost all animals live together with associated bacteria. From the perspective of host-associated microorganisms, whose entirety forms the so-called microbiota, the animal host provides a suitable ecological niche (LEDERBERG AND MCCRAY 2001). However, this link between host and associated microbiota is crucial also for various life history traits of the host, with particular emphasis on behaviour, development and growth, as well as fecundity and lifespan (MCFALL-NGAI *et al.* 2013) (COLLINS *et al.* 2012; SOMMER AND BÄCKHED 2013). More precisely, the natural microbiota often supports the development and metabolism of the host and thus offers an important fitness advantage.

Very impressive examples of such symbiotic associations have been described in different animal groups. In particular, those associations were the focus in which the bacterial colonizers make it possible to use a certain food source in the first place. In this way, the ecological niche of the host is expanded, sometimes dramatically. There are intracellular as well as extracellular symbionts, which open specific food resources and are therefore often transmitted vertically. Examples for such symbioses are the Aphid/Buchnera association or a series of very specific associations in Heteroptera species (Douglas 1998, 2009, Wilson and Duncan 2015, Hansen and Moran 2014). The most impressive examples, however, are the wood-digesting insects, among which the termites stand out with their specific microbiota (Ohkuma and Brune 2010, Ohkmura et al. 2015. However, these very striking and impressive examples do not seem to represent the normal case of host-microbiota interaction, where this level of specificity is usually not reached. A typical, very well studied example is provided here by Drosophila, where the micobiota is taken up from the environment during development and therefore also exhibits considerable variability. Nonetheless, this microbiota imparts a significant fitness advantage in these systems as well (STORELLI *et al.* 2018). Despite the very successful efforts of the last years, the highly important group of marine invertebrates is strongly underrepresented in microbiota related research. Here, some ecologically very important sessile invertebrates such as corals and sponges are the exception (van Oppen and Blackall 2019, Hentschel et al. 20212) . These are the marine invertebrates, in particular the ecologically important non-sessile representatives of this group. Furthermore, very specialized systems of marine invertebrate hosts and their microbiota that allowed the hosts to populate otherwise extremely hostile niches such as hydrothermal vents, have been studied in detail (PETERSEN *et al.* 2011; RUBIN-BLUM *et al.* 2017). Besides these highly interesting and well characterized systems, in which the host almost completely relies on this highly sophisticated symbiotic interaction with specialized bacteria, most marine invertebrates harbor an indigenous microbiota that is more diverse and that has been recruited from environmental bacteria.

To understand the incorporation of the microbiota and the importance as well as the dynamics of microbiota colonization in these non-sessile marine invertebrates, we chose the marine flatworm *Macrostomum lignano* as a model. *M. lignano* has been developed into a versatile model organism because of its advantageous features such as small size, short generation time, ease of culture, transparent body, and available genomes (SCHÄRER *et al.* 2005; PFISTER *et al.* 2007; MOUTON *et al.* 2009; LENGERER *et al.* 2014; MOUTON *et al.* 2018). *M. lignano* inhabits coastal regions of temperate marine environments. As an estuarine and intertidal inhabitant, *M. lignano* is often exposed to a highly variable environment (MA *et al.* 2020). We characterized the microbiomes of different developmental stages, but also of the environment, especially of the food they graze on. With this, we could identify candidate resident members of the microbiota and showed daily cycling of the abundances of the microbiota. Moreover, using gnotobiotic animals we were able to show that the microbiota do not have influence on lifespan, but enhance the fitness in times of food restriction. Finally, we show that major parts of the *M. liganano* microbiota show a robust circadian cycling.

Results

Microbiome composition of *M. lignano*

Macrostomum lignano has a distinct microbiome

Flatworms (*Macrostomum lignano*) were reared in the lab in artificial seawater (Guillard's F/2 medium) and fed with the diatom *Nitszchia curvilineata*. We analyzed the microbial composition of the following sources: 1) The algae with the medium before contact with worms (denoted "Algae"), 2) the medium after 3 weeks of colonization with worms, but after removal of them (denoted "pre-conditioned medium", PCM), 3), eggs laid by the mature worms (denoted as "Eggs"), 4) worms collected 1-2 days after hatching (denoted "immature worms", IMWs), and 5) worms cultured on the algae for at least 3 weeks (denoted "mature worms", MWs). The bacterial taxa were defined for all samples via the V1-V2 region of the bacterial 16S rRNA gene.

We found complex microbiota associated with the different developmental stages of *M. lignano* kept under these conditions. In our studies, 4120 amplicon sequence variants (ASVs) were identified (Additional file 1) and the top 50 ASVs, which represented over 99% of the

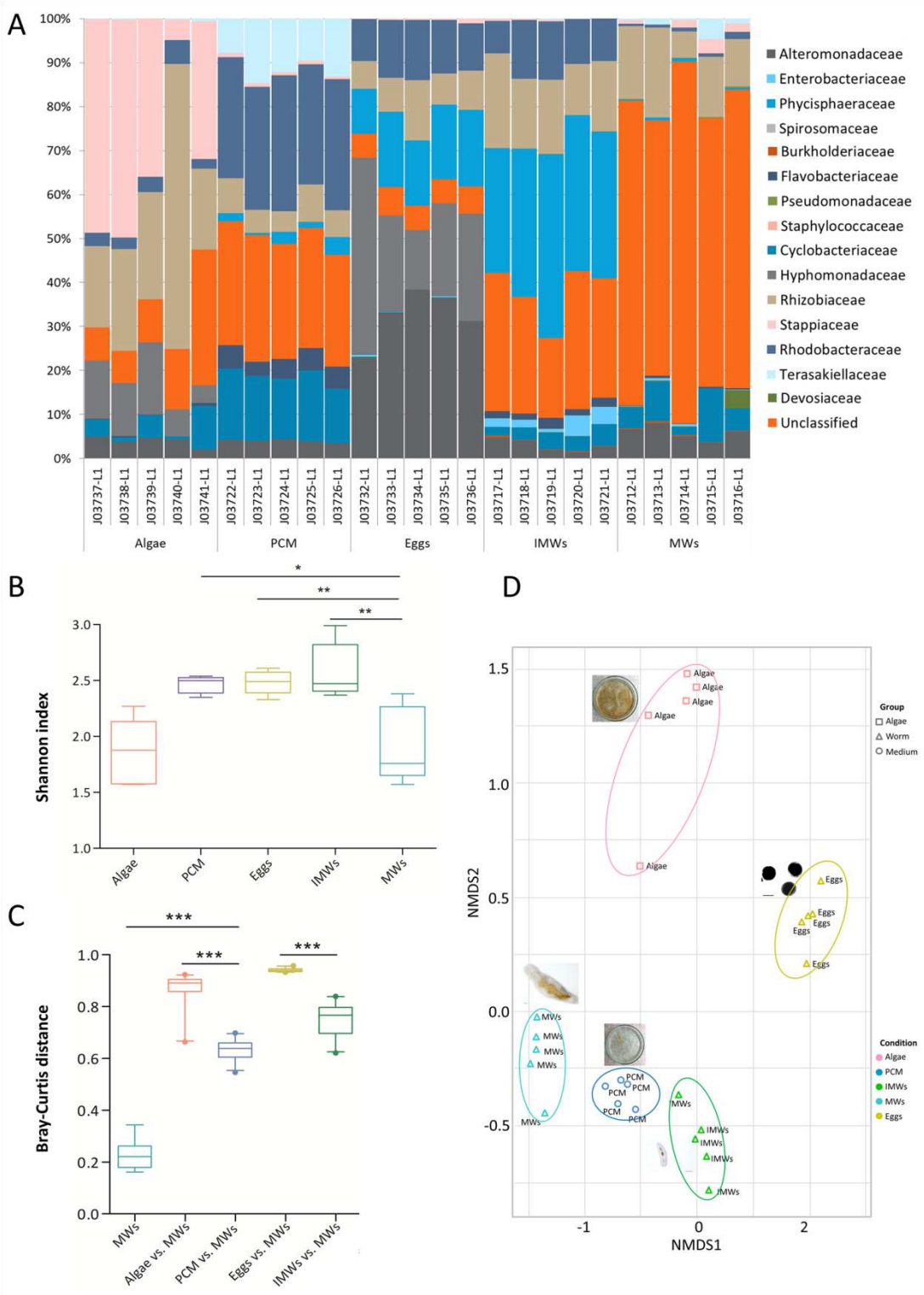

Figure 1: Bacterial composition in samples taken from algae, PCM and different developmental M. lignano stages. *In A, relative bacterial abundances of algae, pre-conditioned medium (PCM), eggs, immature and mature worms (IMWs and MWs). In B, Shannon index analysis shows the ⍰-diversity of bacterial samples from different sources. In C, Bray-Curtis distances of all samples in comparison with MWs are shown. D, shows the non-metric multidimensional scaling (NMDS) plot based on the Bray-Curtis distances analyses*

*(stress value = 0.069). The different microbial colonizer taxa are listed as bar charts with colour coded assignments. Each group employed 5 mature or 10 immature animals with five biological replicates. Asterisks denote significant differences between samples (one-way ANOVA with Turkey Post-hoc test), *, p<0.05, **, p<0.01,***, p<0.001.*

total bacterial abundance, were selected for analysis of composition at the family level (Fig. 1). Algae, without any contact with worms, showed a specific microbial community compared to all other samples that had contact with worms (Fig. 1A). Here, Stappiaceae, Rhizobiacea, and Hyphomonadaceae showed the highest relative abundances (Fig. 1, Algae vs. PCM, p<0.0001). However, the fact that worms lived for a certain time on this medium substantially and significantly changed the bacterial community, with Terasakiellaceae, Rhodobacteracea, Cyclobacteriaceae, and unclassified bacterial families found in higher concentrations in all tested PCM samples (Fig. 1A, Algae vs. PCM, p<0.0001). This difference is also evident in worm samples. The surface washed eggs contain 6 main bacteria groups, including Rhodobacteraceae, Rhizobiaceae, Phycisphaeraceae, Hyphomonadaceae, Alteromonadaceae, and unclassified bacterial groups. This bacterial composition was apparently different from that of the parental mature worms, where unclassified bacteria account for roughly 60% (Fig. 1A, Eggs vs. MWs, p<0.0001). While comparing IMWs with MWs, it became apparent that Rhodobacteriaceae, Phycisphaeraceae, and Enterobacteriaceae were significantly increased in all examined IMWs groups, while unclassified bacteria families were reduced to ~ 30% (Fig. 1A).

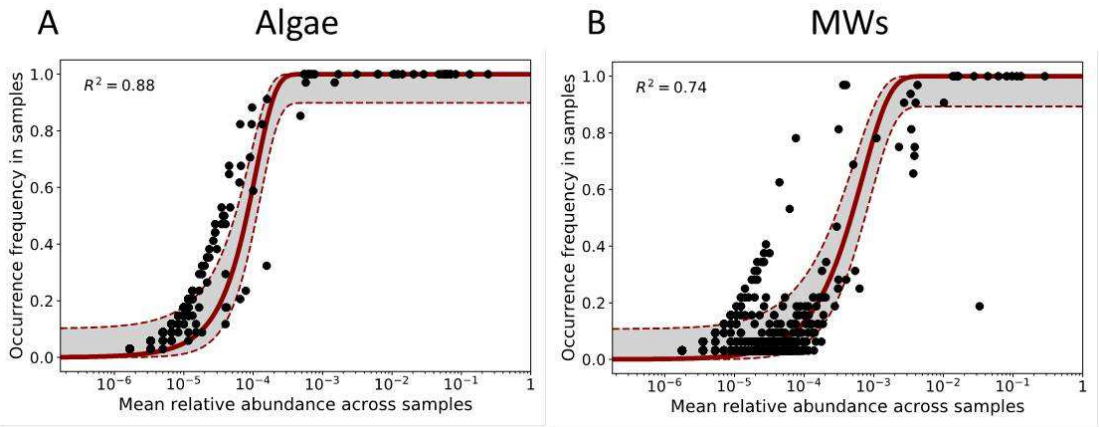

Figure 2: Relationship between mean bacterial taxa abundance across all samples and their frequency in algal and mature worm samples (MWs). *Individual bacterial taxa are represented by dots. The solid line represents the neutral community expectations with the 95% confidence interval (gray area bordered by the dotted lines). In A the comparison of the algal samples and in B the comparison of the mature worm derived samples is given.*

Estimating the α-diversities based on the Shannon index showed that the pre-colonization medium and early developmental stage animals (eggs and immature worms) have higher

bacterial diversities than adult animals and algae samples (Fig. 1B). SMWs retained the same bacterial diversity as mature worms despite an almost complete gut excretion (Fig. 3C, SMWs vs. MWs, $p>0.05$). β-diversity evaluation by Bray-Curtis dissimilarity analysis clearly showed higher diversity in all samples associated with the presence of worms (Fig. 1C). Displaying the dissimilarities as an NMDS plot with two dimensions revealed that all replicates of a peculiar sample type cluster together. All worm-associated samples show greatest distances to the algae samples (Fig. 1D). The first and second axis separate all worms from algae and pre-conditioned medium (PCM) samples, indicating that the microbial community of the worm is distinct from its environment (Fig. 1D, $p\leq0.0003$). Eggs, IMWs, and MWs all show separate clusters (Fig. 1D, $p<0.001$). These results were supported by a Bray-Curtis PCoA distance analysis (Additional figure 1).

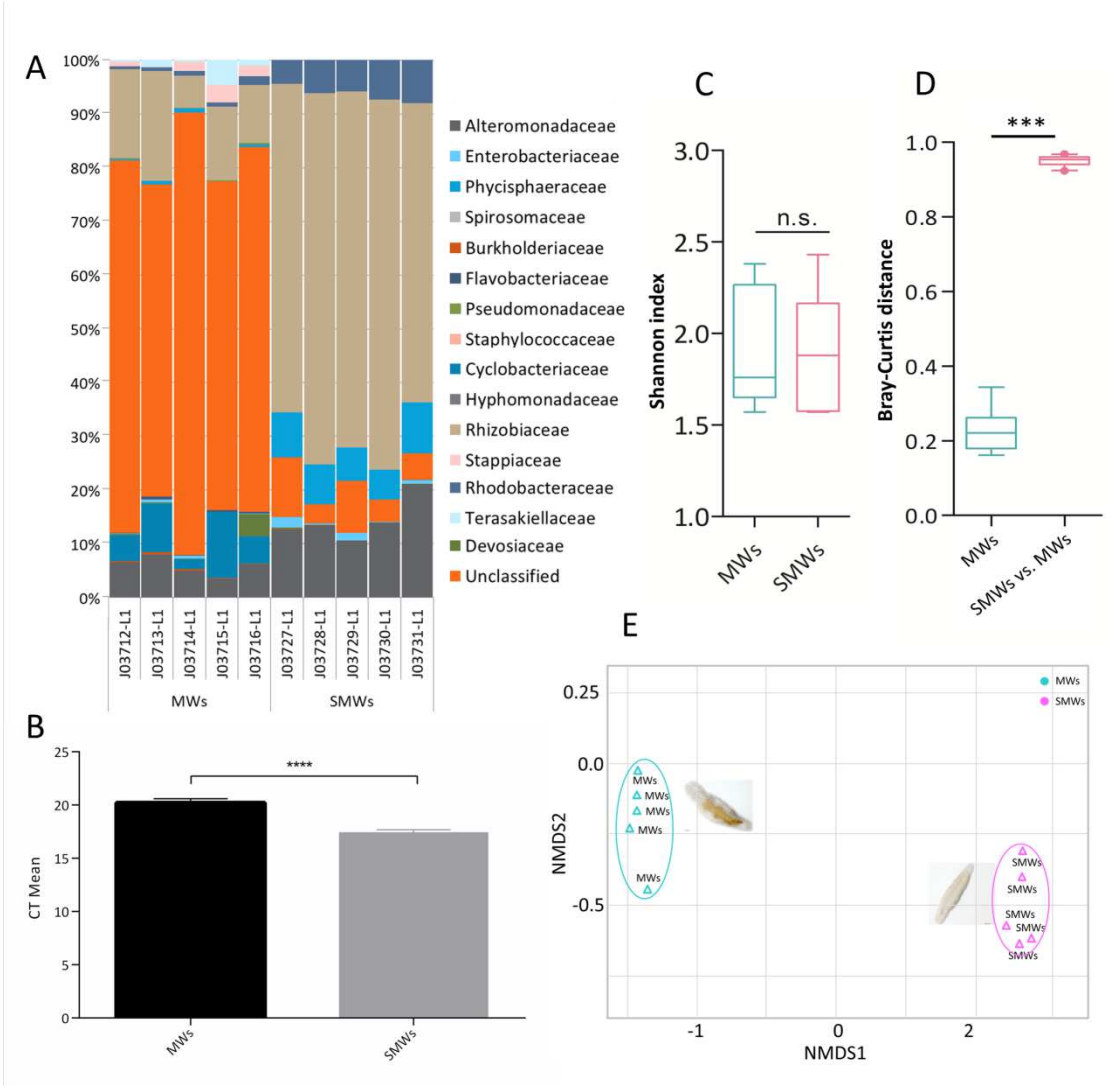

Figure 3 Diversity and bacterial load of mature worms (MWs) and starved mature worms (SMWs). *In A and B, the relative bacterial abundances and bacterial load of MWs and SMWs are shown. C and D, Shannon index and Bray-Curtis distances analysis shows the diversity of microbial community of bacterial samples from MWs and SMWs. E, shows the non-metric*

*multidimensional scaling (NMDS) plot based on the Bray-Curtis distances analyses. Each group employed 5 mature animals with five biological replicates. Asterisks denote significant differences between samples (t test), ***, p<0.001, ****, p<0.0001 and n.s. no significant difference.*

To analyze the composition of the microbiota and identify bacterial taxa that are specifically enriched or depleted, we tested the data against the null hypothesis of a neutral model (Sieber et al. 2019). The bacterial community isolated from the algal samples followed mostly the expectations of the neutral model (Fig. 2A). In contrast, the situation is more complex for the community derived from the MWs. Here, most bacteria were found according to these expectations indicating a stochastic type of colonization, but some are substantially enriched whereas others are substantially depleted (Fig. 2B). This implied that worm-related samples (IMWs, MWs, PCM) may also contain more of these outlier bacteria groups, indicative for specific host bacteria interactions. We therefore further tested the colonization pattern of immature worms (IMWs) and the pre-conditioned medium (PCM) samples. Including a few uncertain bacterial species, we found 21, 30 and 7 kinds bacterial taxa that were not compatible with the neutral model in IMWs, MWs and PCM samples, respectively. Except Phycisphaera and Cyclobacterium, the other outlier taxa exhibit obvious specificity. For example, Enterobacillus, Shewanella, Flavobacterium, Curvibacter, Hyphomonas, Pelomonas,

Taeseokella, Marinovum, Phyllobacterium, Caulobacter and Janibacter present in the IMWs;

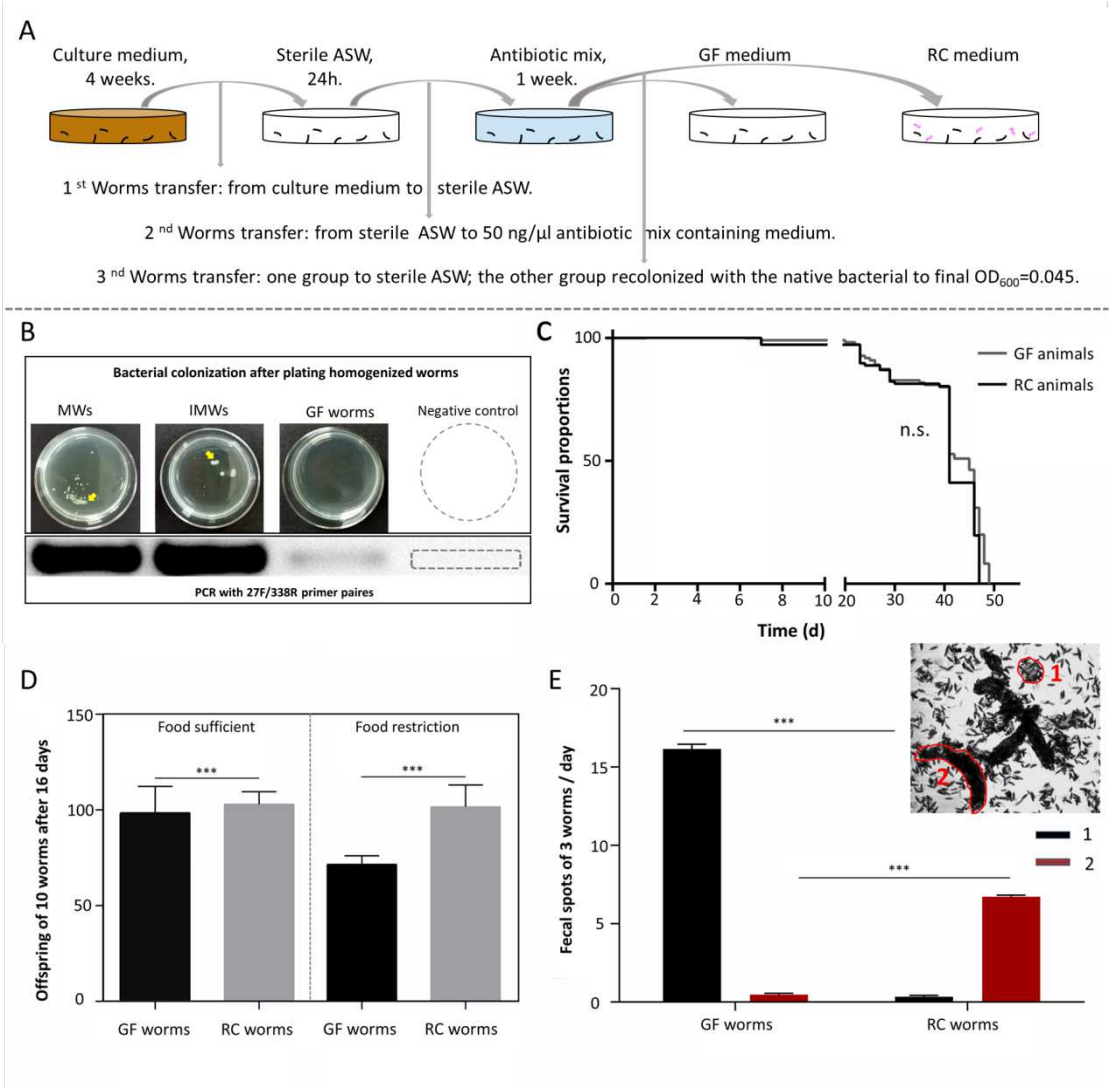

Figure 4 Survival, fitness and fecal output of germ free (GF) and recolonized (RC) worms. *A, shows the workflow to obtain germ free and recolonized animals. In B, the bacterial colonization and PCR results of GF and RC animals are shown. C, shows the survival curves of GF and RC animals. In D, the population size after 16 days was quantified, starting with 10 mature worms under standard laboratory conditions (20 ℃)). Numbers of offspring of GF and RC animals under good food conditions (left) and under reduced food availability (right) are shown. In E, fecal spots were quantified in RC animals and GF animals. Feces output was classified in two categories: (1) small, half-baked and transparent clumps; (2) integrated and brunet clumps. Unpaired two-tailed Student's t-test. ***, p<0.001; n.s. denotes no significant differences with Log-rank (Mantel-Cox) test., N = 15.*

Legionella, Qipengyuania, Massilia, Arenimonas, Halomonas, Limnobacter, Dermacoccus, Polynucleobacter, Salmonella, Constrictibacter, Corynebacterium, Paracoccus, Sulfuritalea, Paenirhodobacter, Streptococcus, Polaromonas and Humibacillus present in the MWs; while Ponticoccus, Alcanivorax and Devosia present in the PCM samples (Additional file 2-4).

To elucidate if the microbiota of *M. lignano* also contains residential bacteria, we tested the bacterial composition of worms that were starved for 5 days and thus unable to replenish the microbial community by feeding (named SMWs). In the SMWs the amount of unclassified bacterial families was reduced, while increased relative amounts of Rhodobacteraceae, Rhizobiaceae, Phycisphaeraceae, and Alteromonadaceae were seen compared to MWs (Fig. 3A, SWMs vs. MWs, $p<0.0001$). Interestingly, some bacterial families that are found in MWs were hardly found in SWMs, including Stappiaceae, Terasakiellaceae, and Rhodobacteraceae. As expected, starved animals showed a reduced total bacterial load if compared with non-starved ones (Fig. 3B, MW vs. SMWs, $p<0.0005$). Comparing the α-diversities based on the Shannon index showed no significant differences between MWs and SMWs (Fig. 3C). β-diversity based on Bray-Curtis dissimilarity showed higher bacterial diversity in SMWs compared to MWs (Fig. 3D). Displaying the dissimilarities as a two-dimensional NMDS plot showed that the respective replicates cluster together and that they can be easily separated by the first dimension (Fig. 3E).

Analyzing the relevance of the microbiota – comparing germ-free (GF) and re-colonization (RC) animals

To infer the relevance of the native microbiota of *M. lignano*, we generated germ-free (GF) animals through antibiotic treatment. To allow a direct comparison between germ-free (GF) and conventional worms, and to exclude the antibiotic treatment as a confounder, all animals were first made germ-free before one group was recolonized with the native microbiota and the other group remained sterile. The outline of the procedure is shown in Fig. 4A. For the worms of the GF group, no bacterial colonization was seen after plating homogenized worms on marine-agar plates and almost no bacteria were detected through amplification with the universal bacterial primers (Fig. 4B). Both germ-free and re-colonized animals can live about 50 days without food supply, which shows that the presence of a native microbiota had no effect on the lifespan under these conditions (Fig. 4C).

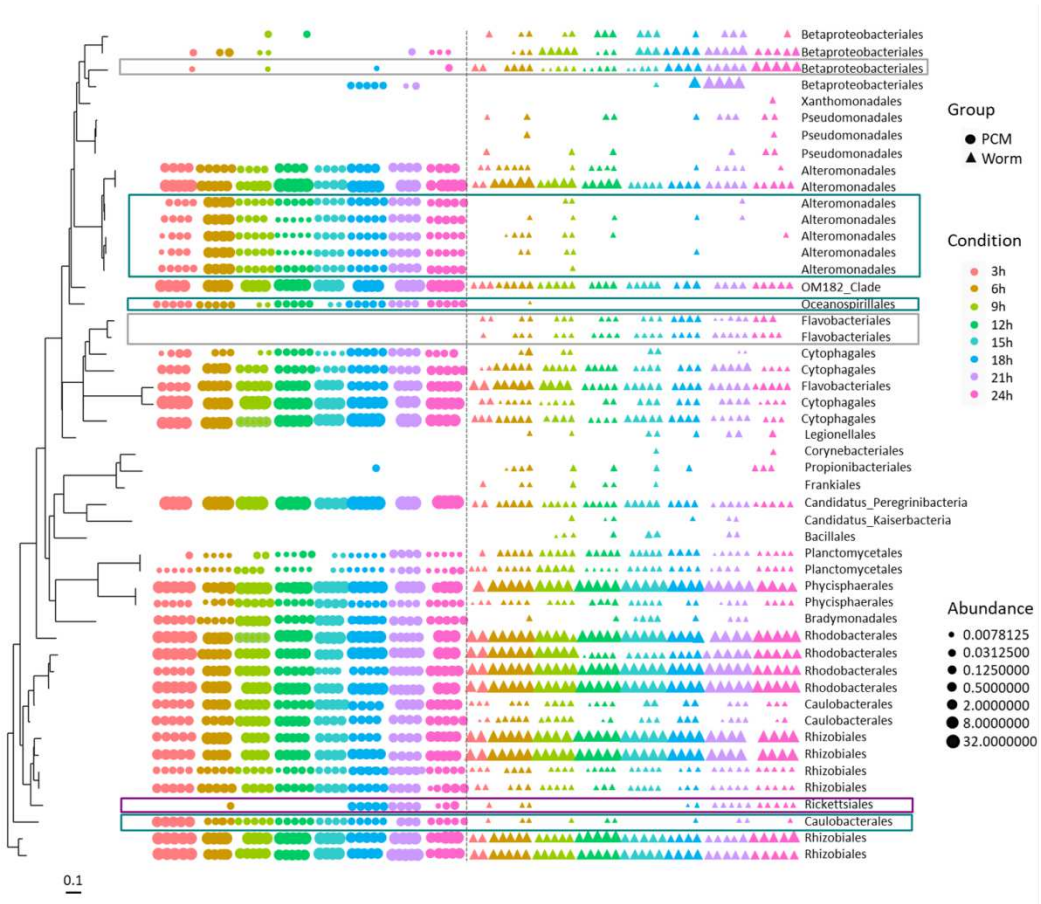

Figure 5 The phylogenetic relationships and bacterial abundances in both, worms and their living substrates (PCM) during a 24h period. *In A, the top 50 ASVs were used for phylogenetic reconstruction (SCHLIEP 2011). The round symbols show the relative bacterial abundances at the given time point in the PCM samples, the rectangle symbols show the relative bacterial abundances in the worm samples. The chosen time points during the 24 h observation time were highlighted by the colour code. N=3-5.*

We found that the presence of microbiota had no significant effect on the worm's development. All experimental juveniles grown either in sterile (SM) or re-colonization medium (RCM), developed into adults after about 6-7 days (data not shown). In order to quantify the effects on the fitness, as defined by the production of offspring, we measured the number of offspring produced by cohorts of 10 mature worms over a period of 16 days under standard laboratory conditions (20 °C; Fig. 4D). Constantly refreshing the medium ensures an optimal supply with nutrients. Under these conditions, almost identical numbers of offspring were generated in GF and RC worms (Fig. 4D, left). Reducing this constant replenishment led to slight starvation, and under these conditions, the GF worms showed a reduction in offspring production by about 35% (Fig. 4D, right). The recolonized worms showed an offspring production that was almost identical to that under ad libitum conditions. The difference between germ-free and recolonized worms was highly significant (Fig. 4B, right).

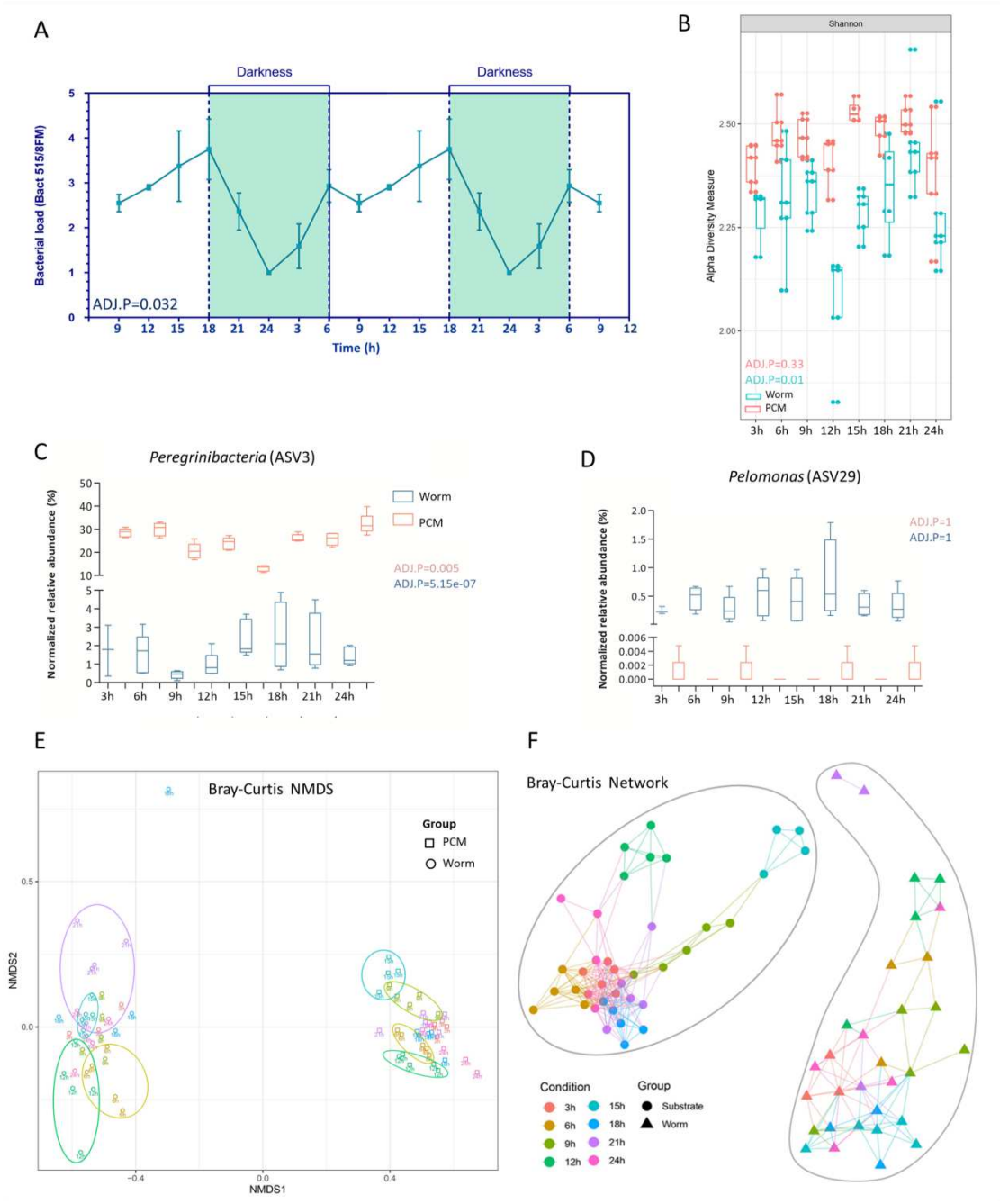

Figure 6 Microbial circadian rhythmicity of M. lignano, and composition characteristics of the microbiota of worms and PCM in the observation time points. *In A, the relative bacterial load is shown during the 24 hours observation period. In B, α-diversity measurement of these bacterial samples is based on Shannon index analysis. D, the relative abundances of the Peregrinibacteria and Pelomonas. E and F, show Bray-Curtis non-metric multidimensional scaling (NMDS) and network of worms and PCM in eight record time points. The Ellipsoids represent a confidence interval surrounding each group, stress value = 0.065. The colour code indicates the time points in both E and F. The cycling statistical JTK outputs permutation-based p-values (ADJ.P) were defined. N=5.*

We subsequently tested the feces output of the mature worms. They usually produce two different types of feces, rod-shaped dark fecal spots (2, Fig. 4E, inset) and smaller, round and oblique spots (1, Fig. 4E, inset). RC animals produced far more type 2 spots than type 1 spots (Fig. 4E). For GF animals the ratio was exactly the opposite, with more than 90% being type 1 spots. These differences between GF and RC worms were highly significant ($p<0.001$ each).

The microbiota of *M. lignano* shows a circadian rhythmicity

To evaluate whether the worm microbiota and/or its individual members show circadian rhythmicity, we kept the animals for two weeks in a climate chamber with a 12h/12h day/night rhythm, with the light phase ranging from 6 o'clock to 18 o'clock. Thereafter, worms were isolated and analyzed every 3 hours within 24 hours periods. These samples were used to evaluate the relative and total bacterial abundances of different bacterial orders in the MWs and the PCM samples, respectively. We performed a neighbor-joining (NJ) analysis with the top 50 ASVs, which covered over 99% of the detected bacteria -representing 21 orders (Fig. 5 and additional file 5). With this analysis, differences between the PCM (left-side) and the MW (right side) communities became apparent. Some bacteria were only apparent in the environment and never or rarely observed in the worm samples. This group comprises Alteromonadales, Oceanospirillales, and Caulobacterales, whereas other orders were specifically found in worm samples, e.g. some orders of the beta-Proteobacteriales and Flavobacteriales (Fig. 5). Moreover, the time-resolved analysis revealed substantial changes during the day both, in the PCM and the MW communities (Fig. 5). Here, the Rickettsiales showed the most interesting phenotype as they accumulated only at later time points of the day, which might indicate that they strictly depend on the circadian rhythms of the host (Fig. 5). Based on these results, we focused on circadian rhythmicity of the entire bacterial community as well as of selected bacterial groups (Fig. 6). The material from isolated worms was used to quantify the bacterial load via qPCR (Fig. 6A). According to these quantifications, the microbiota of *M. lignano* showed a clear circadian rhythm in the observation period (Fig. 6A). The lowest bacterial load was detected at midnight (24 h), where the values were approximately 2.2 fold lower than at the maximum at 18 h (Fig. 6A and additional Figure 2). The worms' microbiota is characterized by relatively low diversity of the bacterial community when compared to culture medium (PCM) at all time points analyzed (Fig. 6B). The lowest bacterial diversity of the worms was detected at 12 h based on the Shannon index (Fig. 6B). Peregrinibacteria (ASV3; Fig. 6C) and *Pelomonas* (ASV29; Fig. 6D) were selected as representatives to show this difference. Peregrinibacteria were mainly found in the PCM, while *Pelomonas* was at least 100 fold more abundant in the worm samples (Fig. 6C and D). Furthermore, we also analyzed if the abundances of these bacteria show circadian rhythmicity. Using the JTK program package, this cycling was obvious for the Peregrinibacteria found in the

worm, but also of those found in the medium. Interestingly, this cycling showed a nearly mirror-image distribution of minima and maxima (Fig. 6C). In contrast, no cycling was observed in the worm and environmental samples for *Pelomonas* (Fig. 6D).

Bray-Curtis and Weighted Unifrac distance analysis separated the microbial communities of worms from those of their living substrates at every time point (Fig. 6E, F and additional figure 3). The changes in the community observed in the PCM changed over time, where especially the samples 9 h and 15 h are completely apart from those taken at 6 h and 12 h. Very similar patterns were observed for the corresponding worm samples (Fig. 6E, F).

Discussion

Host-microbe interactions usually open up opportunities, i.e. ecological niches, for the host that would otherwise be closed to it. The form and narrowness of this association varies dramatically in marine invertebrates. Very intimate associations are found, for example in sponges (PITA *et al.* 2018; RUBIN-BLUM *et al.* 2019), bobtail squids (KREMER *et al.* 2013), corals or chemosymbiotic invertebrates (PETERSEN *et al.* 2016; JACKLE *et al.* 2019). The other side of this spectrum contains looser associations that arise through uptake or colonization by environmental bacteria, and not necessarily essential for both partners. In the work presented here, we found an example that falls between these two extremes and that we believe is representative of most invertebrate host-microbiota associations. The bacterial partners are taken up from the environment and confer the host a fitness advantage under particularly demanding conditions. A first and very important result is the realization that all host (*Macrostomum lignano*) associated samples show a profile that is uniquely distinguishable from the environmental samples.

One of the key questions we posed in this regard was whether or not the microbiota confers a fitness advantage for flatworms. The importance of a microbiota for its hosts can only be assessed by using gnotobiotic animals (MARQUES *et al.* 2006; DOUGLAS 2019). However, to free these experiments from additional influences of antibiotic treatment, it is recommended to compare only gnotobiotic with recolonized animals (MOOSER *et al.* 2018). Comparison of the recolonized and germ-free animals revealed several similarities, which included life span without food intake on the one hand, and offspring production when food was abundantly available on the other. Interestingly, we found a fitness advantage under restricted conditions. Here, the food intake was reduced, and the germ-free animals showed a significantly reduced fitness, whereas the recolonized animals showed a fitness exactly corresponding to that of animals kept under optimal feeding conditions. Such a situation, in which an improved response to food stress was observed, was demonstrated in other models such as *Drosophila*

(WALTERS *et al.* 2020). In general, it can be argued that this host-microbiota interaction can strongly influence the ecological niche of the host and in many cases leads to a widening of this niche, which opens up new colonization opportunities as periods of limited access to food tend to be the normal case for most organisms (LEMOINE *et al.* 2020).

A more detailed analysis of the microbiota of the different worm samples showed that the different developmental stages (eggs, immature worms and mature worms) had clearly distinguishable bacterial communities. All these communities differed significantly from those found in the environment, but also from those found in the conditioned medium. A picture like this, where the composition of the microbiota changes between developmental stages, appears to be seen for most organisms and may reflect the colonization history or the different physiological states of the corresponding host developmental stages (ALFANO *et al.* 2019; MOORE AND TOWNSEND 2019; WANG *et al.* 2019). To distinguish residents and transient inhabitants, we have adopted a simple experimental approach. We let the worms starve for a few days and then looked at the remaining microbiota. This revealed a considerable shift in the composition of the respective populations. On the one hand, there was a significant reduction of bacterial abundance, but at the same time there was also a very significant increase in certain bacterial taxa. These include the Rhodobacteraceae, Rhizobiaceae, Phycisphaeraceae, and Alteromonadaceae, which can therefore be considered as more resident parts of the microbiota. This composition of relatively few resident bacteria groups and a predominant number of transient ones could also be shown for other invertebrate host species (PAIS *et al.* 2018), while no residents were identified in other studies (HAMMER *et al.* 2017). The specificity of colonization, especially of the most important life stage of *M. lignano*, the adult worms, is also supported by modeling colonization using the neutral model approach. This showed that in contrast to the environment, there are specifically enriched and specifically depleted bacterial taxa.

Another finding of this work is also very interesting and very unexpected. Substantial differences were found between the bacterial consortia of the environmental samples and those of the conditioned medium. Although both have the same initial situation, the presence of the worms seems to lead to a substantial remodelling of the bacterial consortia in their environment. This means that not only the environment influences the microbiota of the flatworm, but also the holobiont consisting of *M. lignano* and its microbiota has a clear and long-lasting influence on the microbial composition of its environment. This aspect has hardly been considered so far, but could turn out to be a means to enable true co-evolution between host and microbiota (KLOSE *et al.* 2015). Similar types of influences have also been reported for fruit flies, where the presence of flies had a dramatic impact on the microbial consortia on the substrate (Wong et al. 2015).

Furthermore, we showed that the composition of the *M. lignano* microbiota is subject to a pronounced circadian rhythm. This applies to both the bacterial abundance and the relative composition of the microbiota. The differences between minima and maxima are more than threefold in terms of bacterial abundance, indicating that the influence of the microbiota on the host and vice versa change significantly during the day. Moreover, the composition of the microbiota was clearly distinct between different times of the day. These differences were not congruent with the differences in abundances that were maximal between the time points 18 h and 24 h, whereas maximal differences in terms of the microbial composition were seen between the time points 12 h and 21 h. The latter two timepoints had very similar abundances but were either on an ascending (12 h)- or a descending curve (21 h). Circadian fluctuations have already been identified for other, mostly vertebrate host microbiota systems (FRAZIER AND CHANG 2020). Circadian fluctuations of this kind show that host and microbiota influence each other, thus forming a complex interaction network. Interestingly, it has recently been shown that rhythms of host metabolism in the gut are programmed by the microbiota (KUANG *et al.* 2019), which open completely new fields of research in the complex interaction between host, microbiota, and the environment. Diurnal rhythms of the microbiota of few invertebrates have also been shown, e.g. in the sea anemone *Nematostella vectensis* (LEACH *et al.* 2019), but the underlying mechanisms as well as the physiological consequences remain to be elucidated.

Taken together, we characterized the microbiota of the marine flatworm *Macrostomum lignano*. Most importantly, we were able to demonstrate a fitness advantage of a microbiota under conditions of reduced nutrient availability. Moreover, the different developmental stages of the flatworm show different microbiota, each of them being clearly different from the microbial consortium of the environment. We also found that the host, together with its microbes, can shape the microbial community in its environment. A small group of bacteria was identified as candidate resident members of the microbiota. Finally, we could identify a robust circadian rhythm of the microbial abundance, but also of the composition of the microbial community of *M. lignano*, pointing to a complex interplay between host and its associated microbiota.

Methods

Marine-agar plates

To make the marine-agar plates, the following procedure was used: 10 g of tryptone, 1 g of KCl, 4 g of $MgCl_2 \cdot 6H_2O$, 10 g of NaCl, and 15 g of agar were filled up with 1 L of deionized water. The solution was stirred thoroughly and adjusted to pH 7.5. After autoclaving and cooling the liquid was poured into 60 mm sterile petri dishes under the clean bench. After they solidified at room temperature (RT), the plates were stored at 4 °C before use.

Animal culturing

Macrostomum lignano was originally collected from sediments of the Adriatic Sea and obtained from the Department of Zoology and Limnology, University of Innsbruck, Austria, and then reared in petri dishes, fed with *Nitszchia curvilineata* and cultured in Guillard's F/2 medium (Sigma G0154). Both animal and diatom cultures were incubated at RT (20 °C). 3-4 weeks old adults and the appropriate developmental stages were used for the current studies.

Antibiotic treatment for both algae and worms

Antibiotics (Anti) containing sterile Guillard's f/2 medium included 50 µg/ml ampicillin, 50 µg/ml streptomycin, 50 µg/ml neomycin, 50 µg/ml rifampicin and 50 µg/ml spectinomycin. The algae medium was treated with Anti Guillard's f/2 for 4 weeks and the medium was replaced by fresh medium at 2-day intervals. The newly hatched juveniles were washed in the sterile Guillard's f/2 medium for 2 days. Then the animals were transferred into the sterile algae medium for 4 weeks until animals were grown up. The medium was renewed every 2-3 days. The germ free (GF) animals were cultured in sterile Guillard's f/2 medium for 2 days to get rid of potential antibiotics effects before measuring the lifespan. We used the specific 16s rRNA gene primers (forward primer 27F (AGAGTTTGATCMTGGCTCAG) / reverse primer 338R (TGCTGCCTCCCGTAGGAGT)) for PCR and only a very weak band was detected in the PCR product of GF animals.

Recolonization

For recolonization, the 3-4 weeks worm cultured medium was filtered through a 0.8 µm filter and adjust with sterile Guillard's f/2 medium to the final OD_{600} of 0.045. It was the normal bacterial concentrations under lab conditions. For the GF group, a 0.2 µm filter was used and the final OD_{600} was 0. Worms were grown in the 24 - well plates that each well was filled with 1 ml of filtered homogenates. The GF worms recolonized with bacterium were named as "recolonization" (RC) worms.

Worm lifespan

In total Fifty worms (10 animals per replicate) were used in the experiment of the lifespan. To lower the risk of contamination and other interfering factors, e.g. evaporation, the medium was changed every day.

Feces output measurement

In order to slower feces decay during the observation time (24 h), we employed 3 animals per group (5 replicates). All animals were cultured in 24-well plates with a sterile medium for 10 days. The medium was replaced every day.

Gut pH staining

The pH measurements were performed by using the indicator dyes, m-cresol purple (657890, Merck, Sigma-Aldrich, Steinheim, Germany), and bromocresol purple (B5880, Merck, Sigma-Aldrich, Steinheim, Germany). Animals were stained with these 2 dyes using 0.1 % final concentration at a ratio of 1:1 for 4 hours. Before taking photographs, they were anesthetized with 7.14% $MgCl_2$ for 20 min.

***M. lignano* and substrate DNA extraction for 16S rRNA gene sequencing**

The worms were picked up from on the algae lawns. To remove adhesions on the surface, 5 mature or 10 immature animals taken from culture medium were carefully cleaned with sterile Guillard's F/2 medium before their DNA was extracted. The animals were starved for 5 days in the starvation group. These samples together with 4 weeks culturing algae and its living substrates were prepared for comparing the natural microbiomes of "mature worms". Total DNA extractions were followed by the instructions of the DNeasy ®Blood &Tissue kits (Qiagen, Hilden, Germany). DNA was eluted with 50 µl of elution buffer and storaged at -80 °C. The 16S rRNA gene primer pair 27F/338R was used to test the quality of DNA samples in a 20 µl duplex PCR reaction with Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, USA). The following PCR program was followed: a 3 min pre-denaturation step at 98 °C, followed by 35 cycles (98 °C for 10 s, 58 °C for 30 s and 72 °C for 1 min), and thereafter a final 5 min elongation step at 72 °C. The PCR products were checked by 2% agarose gel, the DNA samples having clear positive bands of correct size were chosen for sequencing of the bacterial 16S V1-V2 region on the MiSeq platform (Illumina) at the Institute for Clinical Molecular Biology, Kiel University. PCRs including both positive and negative controls were carried out under sterile conditions. For the negative control, sterile deionized water was used instead of template DNA.

The required materials and clean bench were all sterilized using 70% ethanol or followed by 20 min UV irradiation prior to sample isolation. Besides the centrifugation step, all remaining DNA isolation steps were carried out under a clean bench to avoid contamination in the laboratory environment.

Bacterial qPCR

The total bacterial DNA load of worms were measured using real-time quantitative PCR, which is based on the amplification of conserved segments of the 16S rRNA genes. The reaction component including 5 µl of 2x qPCR BIOSyGreen Mix Hi-ROX (London, UK), 0.5 µl of sense

primer (5 µM), 0.5 µl of anti-sense primer (5 µM), 1 µl of template DNA (2 ng/µl) and 3 µl of HPLC H_2O. The amplification program was as follows: 1 cycle (95 °C, 10 min), 40 cycles (95 °C, 15 sec; 60 °C, 20 sec; 17 °C, 35 sec). The whole reaction was performed using the StepOnePlus™. The same DNA samples were used for Miseq sequencing and qPCR amplification. The primer set for 16S were as follows: 8FM (5'-AGAGTTTGATCMTGGCTCAG-3'); Bact515R (5'-TTACCGCGGCKGCTGGCAC-3') (PAIS *et al.* 2018). In this case, ribosomal protein L12 (rpl12) of *M. lignano* served as a reference gene. The primer pair was: 5'-GACAAGGTTAACGACGGCTC-3'; 5'-TATAGCAGCCGGTGTGTCAA-3' (Rivera-Ingraham et al., 2016).

Circadian rhythmicity of the microbiota of *M.lignano*

Experiments were set up in a controlled incubator room at 20°C with 4 µmol·m^{-2} ·S^{-1} luminous intensity, 66% RLF humidity and 12 h/12 h day/night rhythms. To avoid the discrepancy generated from the samples, all animals were 4-week-old adults with 1-week constant adaption. The study rhythm was carried out in a 24 hrs scale, 10 animals per group with 5 replicates were used. They were raised under identical conditions and picked up every 3 hrs. The corresponding substrate samples were simultaneously picked up with the worms. To decrease the risk of contamination, animal culturing always was done using the sterilized petri dishes within germ-free plastic chambers. All handling procedures were performed under the clean bench.

Analysis of bacterial communities

Raw reads were first filtered by fastp using parameters -M 20 -q 20 to discard low-quality sequences (CHEN *et al.* 2018). To harness the full potential of deep-sequenced high quality reads and to gain insight into the strain-level host-associated microbiome diversity, amplicon sequence variants (ASVs) were resolved from error-corrected biological sequences down to the level of single-nucleotide differences, instead of clustering reads into operational taxonomic units (OTUs) on a 97% similarity (CALLAHAN *et al.* 2017). Error prediction, feature counting, and chimera detection and removal were performed using the Dada2 pipeline (CALLAHAN *et al.* 2016). Taxonomy was assigned to each ASV referring to a Dada2-formatted training dataset (CALLAHAN *et al.* 2016) derived from the Silva v132 release (QUAST *et al.* 2013). Phylogenetic reconstruction was performed using the phangorn package (SCHLIEP 2011). Further bacterial community analysis was conducted using R packages (RCORETEAM 2020) Phyloseq (MCMURDIE AND HOLMES 2013) and DEseq2 (LOVE *et al.* 2014) in RStudio (RSTUDIOTEAM 2020). To compensate for the uneven sequencing effort across different samples, ASV counts in individual samples were normalized to the median value of total counts in the analyzed groups and reported as either normalized counts or percentage proportions.

Statistical analysis

In all experiments, statistical analysis was performed with Prism version 7.0. The unpaired two-tailed Student's t-test was used for the analysis of differences between two groups. One-way ANOVA followed by a Turkey post-hoc test was used for the multiple comparisons. Two-way ANOVA performed to analyze 2 different categorical independent variables on one continuous dependent variable. All values of histograms were means ± SD. The survival curves of the lifespan were analyzed with the log-rank (Mantel-Cox) test. The levels of significant difference were defined as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. The cycling statistical p-value assignments were used JTK non parametric test (HUGHES *et al.* 2010).

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Author contribution

YM, IB, JFB, UB, and TR designed the study and planned the experimental design, YM, JH, MS, JvF performed the experiments. All authors contributed to writing or editing the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

The authors declare that all data supporting the findings of this study are available in the manuscript. Other relevant data are available from the corresponding author upon request.

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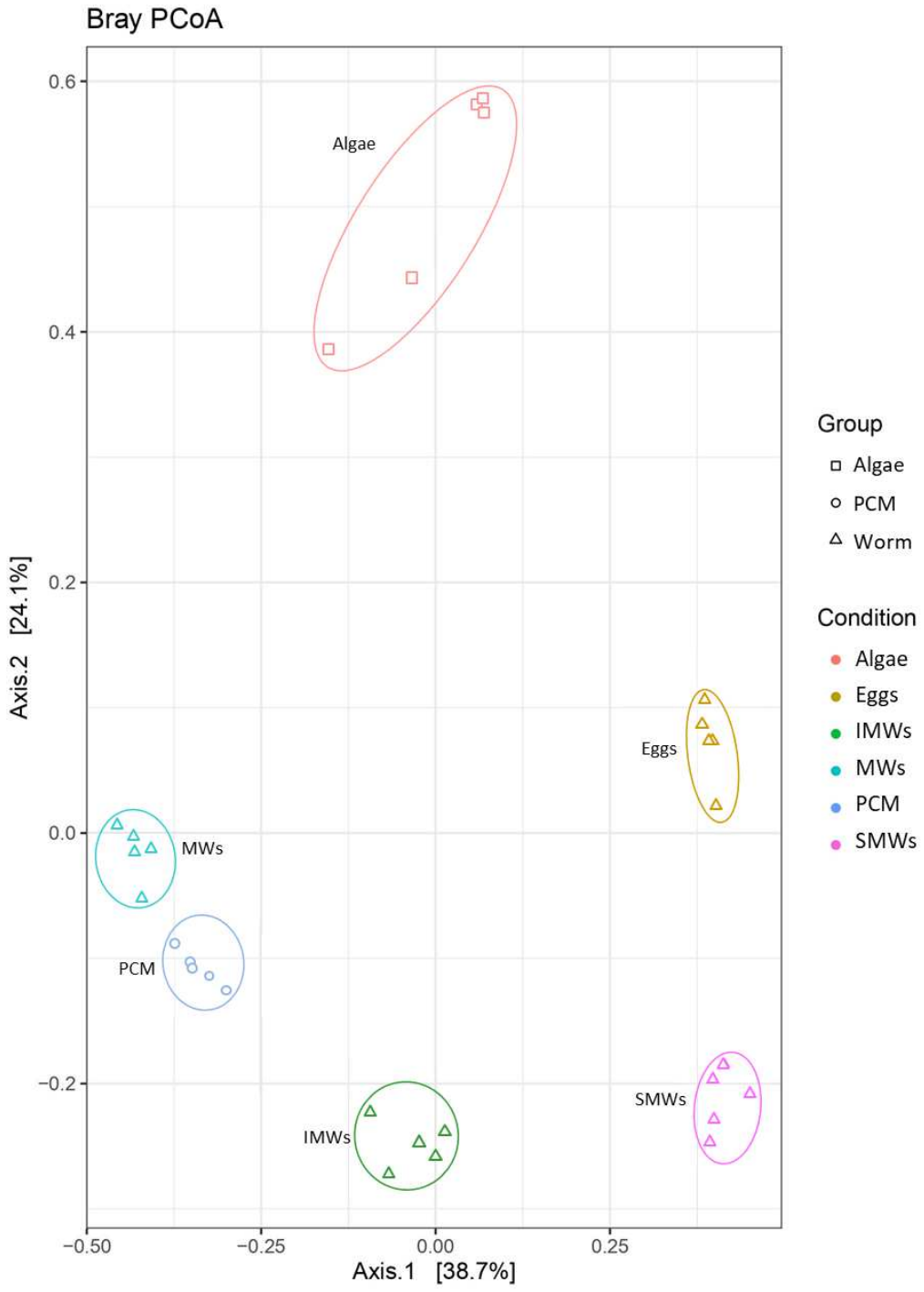
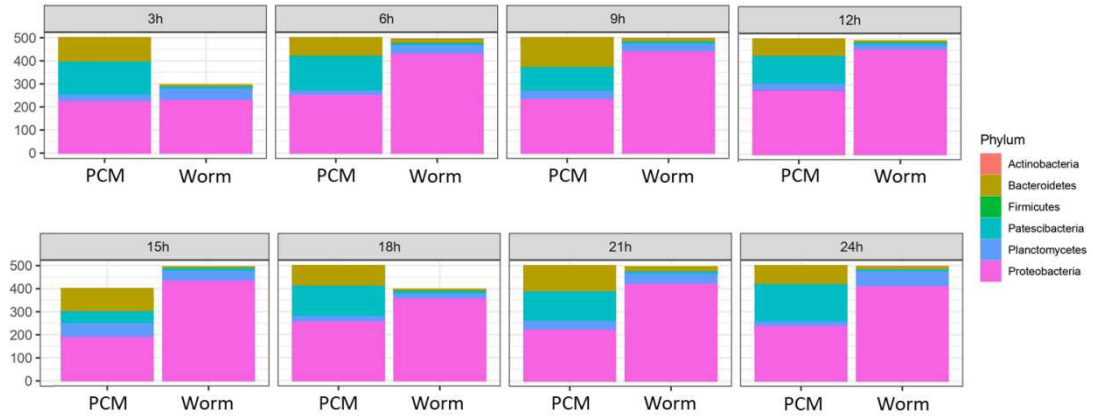
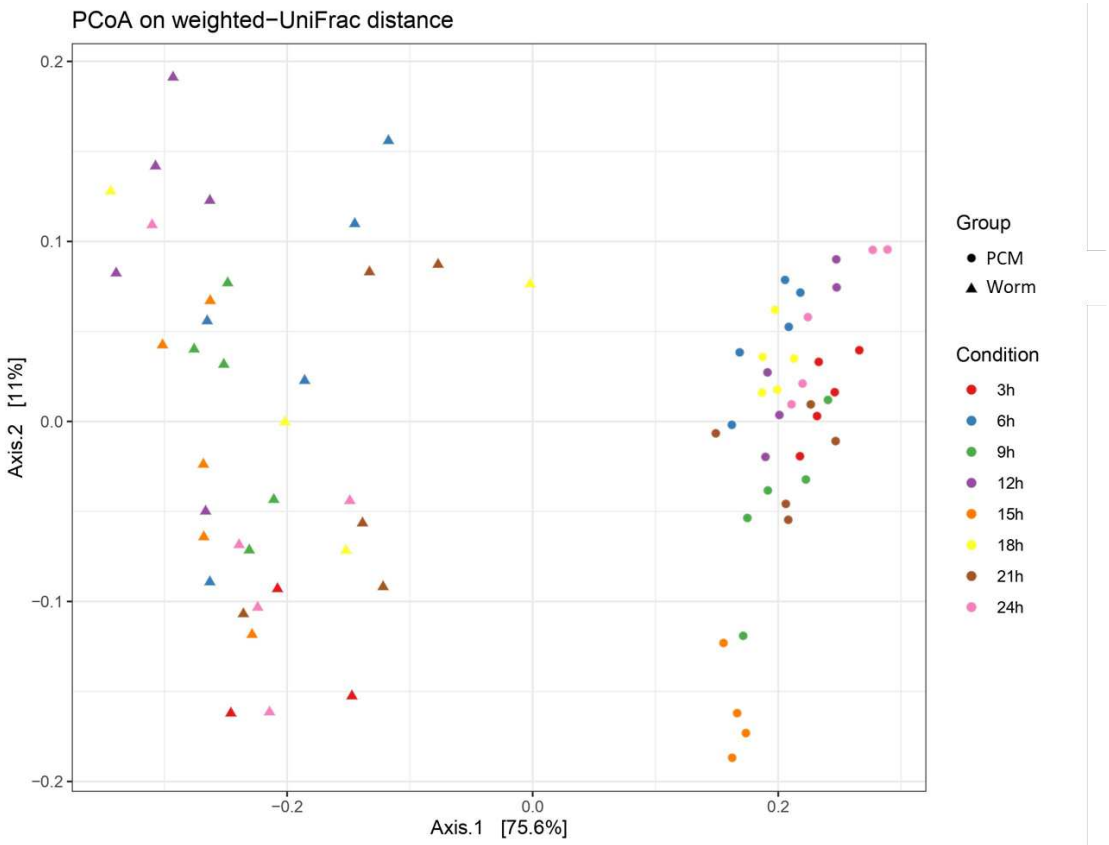
Supplemental Material



Additional figure 1 Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity. The different developmental worm samples have separated from each other, which all departure from the algae medium and their living law. MWs, mature worms; SMWs, starved mature worms; IMWs, immature worms; PCM, pre-conditioned medium. The animal samples employ 5 mature or 10 immature worms with 5 biological replicates.



Additional figure 2 The phylum bacterial abundance of worms and their living substrate (PCM) during eight observation time points.



Additional Figure 3 Principal coordinate analysis (PCoA) plot with weighted UniFrac distance analysis of worms and their living substrate (PCM) during eight observation time points.

Additional Figure 4 PH of *M.lignano* and medium samples. The pH measurements by using the indicator dyes, 0.1 % m-cresol purple (657890, Merck, Sigma-Aldrich) and 0.1 % bromocresol purple (B5880, Merck, Sigma-Aldrich) at a ratio of 1:1 for 4 hours. PH indicator present worms' gut yellow colour ($5.2 \leqslant PH < 6.8$) and the medium violet ($7.4 < PH \leqslant 9.0$). MW, mature worms; SMWs, starvation mature worms; IMW, immature worms.

Additional file 1 The total amplicon sequence variants (ASVs) identified in algae and worm associated samples.

Additional file 2 The nonneutral model annotation of immature wrom (IMWs).

Additional file 3 The nonneutral model annontation of mature worms (MWs).

Additional file 4 The nonneutral model annotation of pre-conditioned medium (PCM)

Additional file 5 The relative ASVs abundance of worms and their living medium during a day.