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Strain-dependent and host genotype–dependent priority effects in gut microbiome assembly affect host fitness in *Daphnia*

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Abstract

The arrival order of species can strongly influence the early assembly of ecological communities. Such events, known as priority effects, are documented in various ecological settings, but remain understudied within the context of the gut microbiome. Given the fundamental link between the gut microbiome and host health, exploring the potential role of priority effects in shaping the assembly and development of microbial communities within the gut becomes imperative. Using the freshwater planktonic crustacean *Daphnia magna* as a model system, we manipulated the immigration order of three bacterial strain pairs in two germ-free genotypes and quantified gut microbiome composition and host fitness at two time points, namely day 8 and day 12. Priority effects were observed; however, their presence, amplitude, and direction (suppressive or facilitative) were found to be contingent on the identity of bacterial strain and host genotype. These findings were accompanied by notable differences in *Daphnia* life history traits across inoculation order treatments, shedding light on the tangible consequences of priority effects triggered by the sequence of bacterial strain arrival in the gut environment, for host fitness. Our results thus highlight the complex nature of priority effects in gut community assembly, their strain/genotype specificity, and their potential impact on the host.

Gut microbiome composition has been shown to influence a broad array of host traits, including digestion, physiology, metabolism, immunity, and behavior (Koropatkin et al. 2012; Buffie and Pamer 2013; Lee and Hase 2014; Gensollen et al. 2016; Sharon et al. 2016). Alterations in gut community structure and composition have also been implicated in health in humans (Martinez et al. 2008; Sharma and Tripathi 2019), animals (Fouhse et al. 2016; Xu et al. 2021) and in responses to environmental stressors in free-living systems (Macke et al. 2017; Ziegler et al. 2017; Pita et al. 2018). It is therefore crucial to understand the determinants of gut microbiome assembly. Although host diet (Smith et al. 2015; Youngblut et al. 2019), genotype (Smith et al. 2015; Macke et al. 2017; Fan et al. 2020), lifestyle (Campbell et al. 2020) and antibiotic use (Pérez-Cobas et al. 2013) have been identified as drivers influencing gut community assembly, only a limited fraction of the observed variation in gut microbiome composition can often be explained, most often less than 30% (Falony et al. 2016; Martínez et al. 2018). While part of this unexplained variation is likely caused by stochastic processes, part is conceivably also caused by hitherto largely neglected ecological and eco-evolutionary interactions.

One such phenomenon is priority effects, where arrival order and timing of species determine the early assembly of ecological communities (Connell and Slatyer 1977; Alford and Wilbur 1985; De Meester et al. 2002, 2016; Fukami 2015). Priority effects occur when the first arriving colonists rapidly grow in numbers due to an abundance of space and resources and an absence of competitors. As the population reaches carrying capacity, the scope for population growth of competing species is reduced. This numerical advantage can result in strong inhibitory priority effects in which the first arriving

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species suppresses later arriving species. Such priority effects are expected to be strong when niche overlap is high and differences in fitness low (Fukami 2015; Fukami et al. 2016). In such cases, if the first arriving species changes the environment to its own benefit, these effects may persist and become permanent (De Meester et al. 2016). Conversely, niche modification by early colonists can also facilitate the establishment and growth of late-arriving immigrants, resulting in facilitative priority effects (Connell and Slatyer 1977; Monier and Lindow 2005; Poza-Carrion et al. 2013).

The importance and mechanisms of priority effects in gut microbiome community assembly are insufficiently studied. Yet, there are some clear indications that priority effects might be important. In one example, Martínez et al. (2018) observed the gut community to most closely resemble donor communities inoculated first in germ-free mice. Likewise, in humans, studies show that breastmilk enriches the gut microbiome early in life with taxa such as *Bifidobacterium* spp. (Sela et al. 2008; Solís et al. 2010), which effectively degrade the complex oligosaccharides present in breastmilk, likely depleting the intestinal lumen of these resources for later immigrants thus suppressing their successful establishment (Koenig et al. 2011; Marcobal et al. 2011). Conversely, facilitative priority effects have also been observed. For example, prior arrival of certain *Bacteroides* spp. release valuable mucosal carbohydrates, which are utilized by late arriving enteric pathogens, *Clostridium difficile* and *Salmonella enterica*, facilitating their successful colonization (Ng et al. 2013).

The role of priority effects in microbiome assembly has been shown through manipulative experiments in the phyllosphere of *Arabidopsis* (Carlström et al. 2019), in the microbiome of green macroalga *Ulva australis* (Nappi et al. 2022) and in nectar-inhabiting yeast communities of *Mimulus aurantiacus* (Peay et al. 2012; Dhami et al. 2016). However, there remains only limited experimental evidence for the occurrence of priority effects in gut microbiomes (Martínez et al. 2018; Sprockett et al. 2018). The influence of priority effects during gut microbiome assembly on host fitness is even less studied. Most studies so far have focused on well-known mutualistic or clearly antagonistic (i.e., host–parasite) interactions when examining the consequences of priority effects at the microbiome level. For instance, in the host plant *Medicago lupulina*, varying strain arrival order in the soil mediated strong priority effects for nitrogen-fixing bacteria that impacted the amount of nitrogen fixing nodules and above-ground biomass of the host (Boyle et al. 2021). Likewise, in the pacific chorus frog (*Pseudacris regilla*), sequential exposure to the trematode parasites *Ribeiroia ondatrae* and *Echinostoma trivolvis* resulted in strong inhibitory priority effects, resulting in varying disease outcomes in the host (Hoverman et al. 2013). Despite such exemplary studies with host-associated organisms, there is a dearth of research specifically investigating the impact of arrival order effects during gut microbiome assembly on host traits. Additionally, the degree to which priority effects and their impact on host fitness are strain or host genotype dependent is not known.

The water flea *Daphnia* is a widespread freshwater cladoceran genus that occupies a pivotal role in food webs as primary consumers of phytoplankton and preferred prey of fish (Miner et al. 2012). The species *D. magna* is an excellent model system for experimental manipulation of inoculation order in the gut. The species is known to harbor abundant bacteria, both on its surface and in its gut (Qi et al. 2009). Controlled inoculation of bacterial species into the gut at different times is possible because one can sterilize the eggs (Callens et al. 2016) to produce germ-free animals. *D. magna* are nonselective filter feeders that readily take up bacteria from their surrounding waters (Macke et al. 2017), thereby enabling the easy introduction of bacteria into the gut. Several studies have shown that the gut microbiome is important for the fitness of *D. magna*. For instance, studies with germ-free *D. magna* document increased mortality with a significant reduction in fitness of the surviving individuals (Sison-Mangus et al. 2015; Callens et al. 2016; Mushegian and Ebert 2017). This allows for the assessment of host fitness consequences of gut microbiome priority effects. Lastly, the cyclical parthenogenetic reproduction mode of *D. magna* allows the use of clonal lineages, where one can manipulate inoculation order against a common host genotypic background. This approach allows for a straightforward assessment of host genotype–dependent and bacterial strain–dependent responses. Additionally, prior research has established that the gut microbiome composition of *D. magna* is influenced by factors such as host genotype, host–microbe interactions, environment (diet, stressors) and host–parasite interactions (Callens et al. 2016; Macke et al. 2017; Akbar et al. 2020; Bulteel et al. 2021) thus laying the groundwork for further investigation.

In this study, we experimentally manipulated the arrival order of bacterial strains into the gut of germ-free *D. magna* to directly evaluate whether and to what extent priority effects influence gut community assembly and host fitness. While the use of diverse communities would replicate a setting closer to natural systems, we here chose to work with specific strain pairs as it allowed the design of strain-specific qPCR probes, thus enabling a more quantitative analysis. As we expected highly variable responses in the capacity of single strains to grow in the sterile *D. magna* gut and wanted to explore variation in priority effects between strain combinations, we selected three strain pairs for our analysis. To this end, three bacterial pairs originally isolated from *D. magna* genotype LRV4 were each inoculated into two *D. magna* host genotypes, namely LRV4 and O1-08 (Fig. 1a) either simultaneously, with a time-lag of 48 h, or in isolation (Fig. 1b). The gut microbiome composition and four host life history traits were determined after 8 and 12 d (Fig. 1b). With this design, we explored (1) whether inoculation order of individual gut bacterial strains impacted gut composition in adult *Daphnia*; (2) to what extent these effects were bacterial strain dependent, that is, depend on

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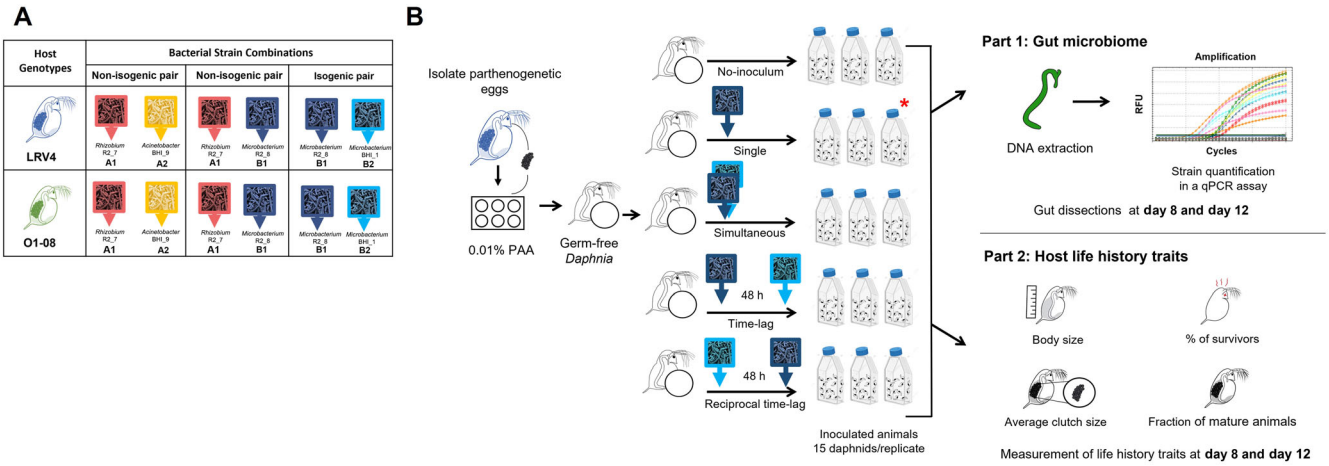

Fig. 1. (**a**) Summary of *Daphnia* genotypes and gut bacterial strain combinations used in the priority effects experiment. (**b**) Schematic representation of the experimental design. Germ-free host individuals were generated by treating parthenogenetic eggs with 0.01% peracetic acid (PAA). The axenic hatchlings (< 24 h old) were then subjected to the following treatments: no-inoculum control (no exposure to bacteria), single inoculations of bacterial strains, simultaneous, time-lag and reciprocal time-lag inoculations of two strains, for a total of three strain-pair combinations. Time-lags involved 48 h. This experiment was run full factorial for each bacterial strain pair combination in 2 host genotypes and 2 time points, with 3 replicates per treatment consisting each of 15 *Daphnia* individuals. Gut dissections were conducted at two time points (day 8 and day 12) to extract DNA. The obtained DNA was subsequently used for bacterial 16S rRNA gene quantification in a multiplex qPCR assay using target-specific probes. For the single inoculations (red asterisk), sampling was only done at day 12. Life history traits of the *Daphnia* host were measured on day 8 and day 12 (*see* the text).

the identity of the inoculated bacterial strains; (3) or host genotype dependent, that is, depend on the clonal identity of the host *Daphnia*; and (4) whether and to what extent differences in the order of exposure to different bacterial strains translated into host fitness differences, and whether these effects were strain and host genotype dependent.

Materials and methods

Experimental design

The experimental design is visualized in Fig. 1. We use *D. magna* as a model host system and use bacterial strains previously isolated from a host clone of the same species as inocula. We employ two host genotypes namely *D. magna* clone LRV4 and O1-08 (*Daphnia* genotype information in Supporting Information) to quantify host genotype–dependent effects, and three gut bacteria strain pair combinations for strain-dependent effects (Fig. 1a). One of the host genotypes (LRV4) is the genotype from which the bacterial strains were isolated. The experiment consisted of a full-factorial design combining five bacterial inoculation treatments (+ a no inoculation control) with three gut bacterial strain pair combinations in two *Daphnia* host genotypes, characterized at two time points, namely day 8 and day 12 of the experiment. The following treatments were set up: (1) single inoculations wherein each strain was introduced in isolation to determine their overall performance in *Daphnia* gut in the absence of competition; (2) simultaneous inoculation, where each strain of the pair was introduced simultaneously at equal inoculum densities; (3) time-lag inoculation, where one strain was added first followed by the second strain after a 48-h time-lag; and (4) reciprocal time-lag inoculation where the previous order was reversed. In addition, a no-inoculum treatment was maintained as a negative control to assess *Daphnia* traits in the absence of any added inoculum. For each of these treatments, independent experimental units were set up separately for analysis at two time points, namely day 8 and day 12. With three replicates per treatment, this resulted in 216 experimental units consisting each of 15 daphnids, totaling 3240 *Daphnia* individuals.

There are two overarching endpoints: gut microbiome strain densities and host life history traits (Fig. 1b).

Selection of bacterial strains

Selection of the specific strains was based on the following considerations. First, the bacterial strains used in the experiment were all isolated from the guts of *D. magna* genotype LRV4, allowing us to perform experiments with the host genotype from which the bacterial strains were isolated as well as with another host genotype. Second, both isogenic and non-isogenic strain pairs were established, since isogenic bacterial strains are expected to occupy a more similar niche in comparison to non-isogenic strains and possibly display stronger priority effects through niche pre-emption (Fukami 2015). Third, strain pairs were chosen such that their 16S rRNA gene sequences shared a sufficiently similar ± 250 bp region to allow the use of a single primer pair for polymerase amplification while also sufficient nucleotide differences to allow distinction. This allowed us to obtain a proxy for the abundance of the inoculated strains with target-specific probes in a multiplex qPCR assay. Lastly, the chosen bacterial pairs also differed

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in colony morphology, allowing for visual differentiation of the strains when plated.

Based on these criteria, the experiment was carried out with four bacterial strains that were among the most dominant groups isolated from the gut of host genotype LRV4: *Rhizobium* R2_7, *Acinetobacter* BHI_9, *Microbacterium* R2_8, and *Microbacterium* BHI_1. This enabled us to create two pairs that involved two different genera (*Rhizobium* R2_7–*Acinetobacter* BHI_9 and *Rhizobium* R2_7–*Microbacterium* R2_8) and a pair that involved two strains from the same genus (*Microbacterium* R2_8-*Microbacterium* BHI_1). For details on bacterial strain isolation, identification, and selection, see Supporting Information Section 2.

Experimental setup and sample analysis

The experimental workflow consisted of (1) isolation and sterilization of *Daphnia* eggs, (2) microbial inoculation and incubation, (3) gut dissection and DNA extraction, (4) qPCR assay, and (5) quantification of life history traits (Fig. 1). Detailed information on each of these steps is given in full in Supporting Information, Sections 4–8. In short, *Daphnia* eggs were isolated from non-sterile mothers and sterilized using 0.01% peracetic acid (Callens et al. 2020). The sterile hatchlings were then transferred into 50-mL falcon tubes (15 hatchlings per replicate unit) containing 30 mL sterile ADaM medium and 0.5×10^5 sterile algae cells mL^{-1}. The appropriate bacterial strain combination was then inoculated to each experimental unit at a standardized total concentration of 10^6 cells mL^{-1}. The second exposure in the (reciprocal) time-lag treatments was executed 48 h after the first inoculation, again at 0.5×10^6 cells mL^{-1}.

After 96 h of incubation, all the *Daphnia* individuals per falcon tube were transferred to culture flasks containing 450 mL sterile ADaM medium with 10^5 axenic algae cells mL^{-1}. All experimental units were incubated at 20°C with a 16:8 h L : D photoperiod, with their positions randomized. Gut dissections were performed on day 8 and day 12. Given that the average maturation time of healthy *D. magna* at 20°C is approximately 8 d when kept under favorable laboratory conditions and that egg development time in the brood pouch is approximately 3 d at 20°C (De Meester 1995; Van Doorslaer et al. 2009), we here assessed gut microbiome composition in animals that were close to maturation (day 8) or would have matured and likely have produced their first clutch (day 12) under normal conditions. In this way, it was ensured that the priority effects observed lasted long enough to have the capacity to affect important life history trajectories. A minimum of seven guts per experimental unit were collected for DNA extraction. DNA extraction was performed using the DNeasy® PowerSoil® Pro-Kit (Qiagen). Bacterial strain densities in the gut were quantified with strain-specific probes in a multiplex qPCR assay. For host fitness measurements, the following life history traits in *D. magna* were measured in all treatments and at both time points: percentage of survivors, body size, fraction of mature animals, and clutch size.

Primer and probe details

The primers used for amplification comprised the universal 16S forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) (Weisburg et al. 1991) and a custom reverse primer R206 (5′-CGGAAATGGGGTGGTTG-3′) specifically designed to maintain an amplicon size of 200 bp, ensuring high amplification efficiency. Detailed information on primer design and testing in Supporting Information Section 7.1.

For quantification of the bacterial strains in the gut extracts, four strain-specific hydrolysis probes (Table 1) targeting regions in the first 200 bp region of the bacterial 16S rRNA gene were designed to accurately detect the four strains used in our experiment: *Rhizobium* R2_7 (probe A1; FAM), *Acinetobacter* BHI_9 (probe A2; HEX), *Microbacterium* R2_8 (probe B1; HEX), and *Microbacterium* BHI_1 (probe B2; FAM). The selected probe sequences included an average length of 20–28 nucleotides, GC content of 35–65%, and melting temperatures in the range of 66–70°C (5–10°C higher than T_m of primer). Additionally, the formation of self-dimers, hairpin structures, and heterodimers were also screened. 5′-nuclease double quenched probes with an internal quencher molecule (ZEN) labeled with FAM or HEX dyes (PrimeTime qPCR probes) were purchased from Integrated DNA Technologies (IDT). For probe design and testing we refer to Supporting Information Section 7.2.

qPCR reaction and cycling conditions

Amplification and detection were carried out on the CFX96 Touch Real-Time PCR detection system (BioRad Laboratories NV) using the CFX Maestro™ software. An initial denaturation step at 95°C was maintained for 3 min followed by 40 cycles of denaturation and a combined annealing and

Table 1. Specifications of the designed probes based on a final reaction composition of 50 mM KCl, 3 mM $MgCl_2$, and 0.8 mM dNTPs.

No	Target species	Target sequence 5′–3′	Probe sequence 5′–3′	Length	GC%	T_m °C
A1	*Rhizobium* R2_7	GTGCCCTACGGAATAGCT	AGCTATTCCGTAGGGCAC	18	55.6	60.8
A2	*Acinetobacter* BHI_9	AATCTGCCTATTAGTGGGGGACAACA	TGTTGTCCCCCACTAATAGGCAGATT	26	46.2	67.3
B1	*Microbacterium* R2_8	TCTGGGATAAGCGCTGGA	CTGTTTCCAACTGTTATCCCAGAG	18	55.6	62.5
B2	*Microbacterium* BHI_1	CTCTGGGATAACAGTTGGAAACAG	TCCAGCGCTTATCCCAGA	24	45.8	62.9

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extension step at 95°C (15 s) and 61°C (30 s), respectively. For singleplex and multiplex reactions, a total of 20 μL reaction mixtures were prepared comprising 10 μL PrimeTime® Gene Expression master mix (Integrated DNA technologies, Leuven), 0.5 μL primer (20 μM F27/R206), 1 μL of each probe (3 μM; PrimeTime custom qPCR probes, IDT Leuven) and 2 μL of template. Ultra-pure distilled water (Qiagen) was added to the reaction components to make up the final volume to 20 μL. No-template controls (NTCs) were prepared with 2 μL of ultrapure distilled water as template. Concentrations were calculated from the obtained C_q values. Detailed information on development of qPCR assay in Supporting Information Section 7.

Due to the large sample size, several targets were incorporated in the same plate and all samples were spread across multiple runs using a "gene-maximization" set-up (qbase+). An inter-run calibration was performed to correct for among-run variation. A 100-fold diluted sample of gDNA of each of the four strains was used as an inter-run calibrator (IRC) in all runs. The differences in the C_q values of each IRC among runs was used as a measure of inter-run variation and used to correct all C_q values.

For quantification, a standard curve for each of the strains (serially diluted) was constructed with the logarithm of the starting quantity plotted on the x-axis and the C_q value obtained for each dilution on the y-axis. The equation of a linear regression line, $y = mx + b$, was then used to determine the concentrations (starting quantities) of the unknown targets:

$$N_n = 10^{(n-b/m)}, \text{where } n = C_q \text{ value}$$

$$\text{Quantity} = 10^{(C_q - b/m)}$$

The concentrations of all strains were determined using the C_q value obtained from the qPCR assay in the above equation. For all treatments, average C_q values of the three technical replicates were calculated and used to measure 16S rRNA concentrations as a proxy for bacterial cell abundances. In addition, because samples differed in the total amount of guts analyzed, the results for each of the samples were standardized to bacterial densities per gut by dividing the total DNA quantified by qPCR with the number of guts used in the sample.

Statistical analysis

For gut microbiome composition, the effect of inoculation order on bacterial abundances of each of the two strains per strain combination were analyzed. Analyses were preformed separately per individual strain in each strain combination. First, the main effects of inoculation order, host genotype, time point and their interactions on bacterial densities were tested. Due to strong host genotype and time point effects, a more targeted analysis within each host genotype and time point was performed (*see* Fig. S5). In both cases, a linear mixed model was constructed with the *lmer* function of the *lme4* package (Bates et al. 2015). To account for the staggered design of the experiment, in which egg collection and hence experimental treatments were performed on different days, the batch of eggs was added to the model as a random factor. Contrasts were set globally using the *set_sum_contrasts* function of the *afex* package (Singmann et al. 2015) and significance tested with type III sum of squares using the *Anova* function of the *car* package (Fox and Weisberg 2019). Tukey correction was performed for pairwise post hoc comparisons using the *emmeans* package in R for significant interactions (Lenth and Lenth 2018), thus allowing the assessment of differences between the simultaneous, time-lag and reciprocal time-lag inoculations. Cohen's d was calculated as a measure of effect size for the differences in bacterial densities and host traits between the inoculation treatments.

All four life history traits were analyzed separately following the same scheme as the bacterial densities (*see* Supporting Information Fig. S6). Normality of residuals and homogeneity of variance was checked and formally tested with the Shapiro–Wilk test and the Levene test, respectively (Fox and Weisberg 2019).

All statistical analyses were performed in R version 4.0.2.

Results

Effects of inoculation order on gut bacterial density

Overall, of the six combinations tested (three bacterial strain combinations × two host genotypes), there were four instances (66.7%) where differences in inoculation order led to significantly different final gut compositions in at least one of the two time points. The magnitude and direction of priority effects (suppressive or facilitative) varied widely, with the three bacterial pairwise combinations yielding different patterns.

For the non-isogenic strain pair *Rhizobium* R2_7 (A1) and *Acinetobacter* BHI_9 (A2), the single inoculations revealed that while *Rhizobium* R2_7 could grow in the gut of both *Daphnia* genotypes, no growth was observed for *Acinetobacter* BHI_9 (Fig. 2a). In line with these observations, growth in all pairwise inoculation treatments was observed only for *Rhizobium* R2_7, while *Acinetobacter* BHI_9 remained undetected. The mean abundances of *Rhizobium* R2_7 did not differ significantly among the pair-wise inoculation treatments, except for day 8 in *Daphnia* genotype O1-08 (Fig. 2a, *see* Supporting Information ANOVA Table S9). Here, *Rhizobium* R2_7 abundances were significantly higher in the time-lag (p-value = 0.0135; Cohen's $d = 1.39$) and reciprocal time-lag inoculation treatments (p-value = 0.022; Cohen's $d = 1.54$) compared to the simultaneous inoculation treatment (Fig. 2a). However, no significant differences in densities were exhibited between the time-lag and reciprocal time-lag inoculation for *Rhizobium* R2_7.

In the non-isogenic pair *Rhizobium* R2_7 (A1) and *Microbacterium* R2_8 (B1), a different pattern was detected (Fig. 2b). The single inoculation treatments show that both strains grow

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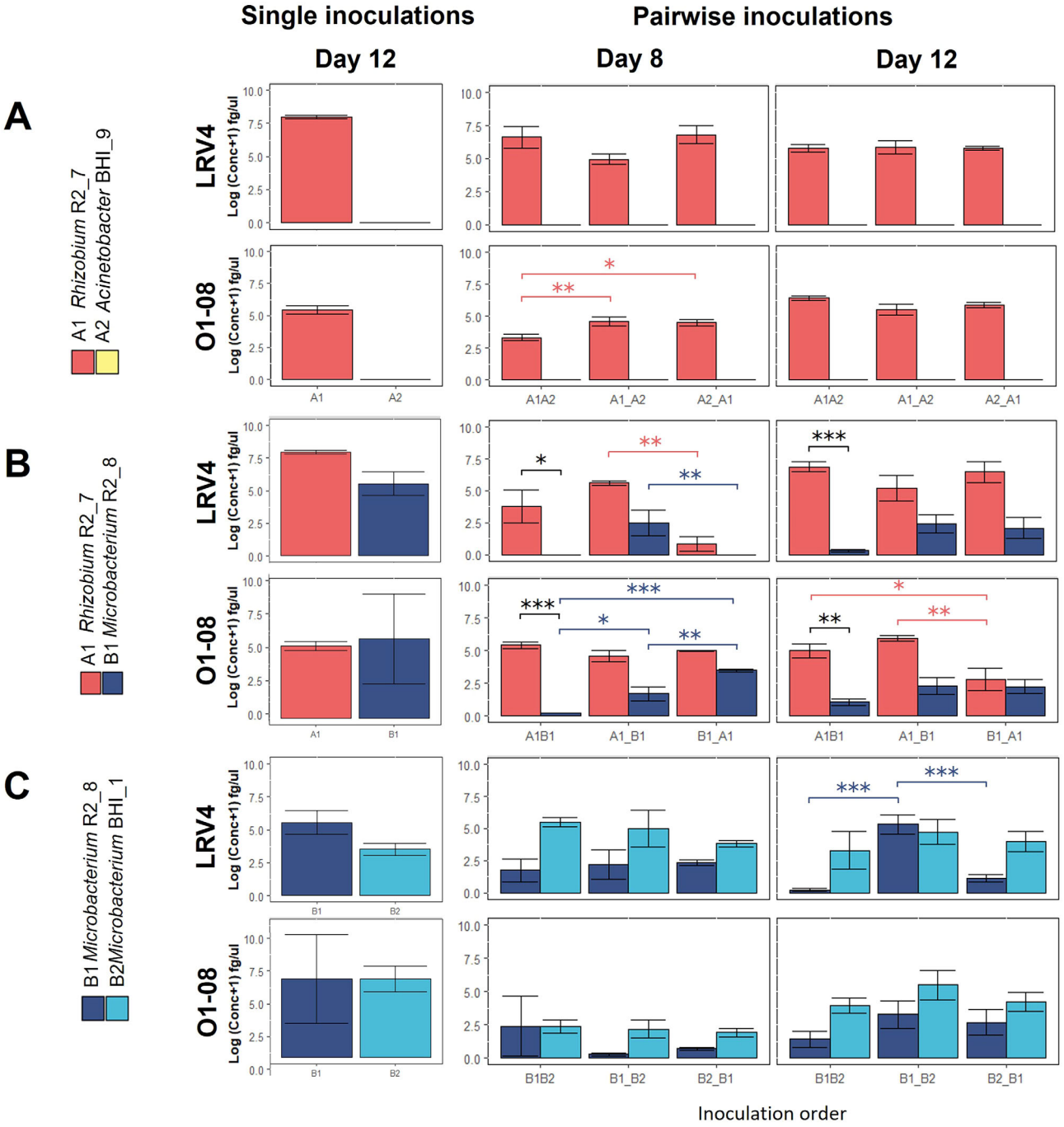

Fig. 2. Mean bacterial strain densities (per *Daphnia* gut) plotted against treatment for (**a**) non-isogenic strain combination *Rhizobium* R2_7 (A1) and *Acinetobacter* BHI_9 (A2), (**b**) non-isogenic strain combination *Rhizobium* R2_7 (A1) and *Microbacterium* R2_8 (B1) and C) isogenic strain combination *Microbacterium* R2_8 (B1) and *Microbacterium* BHI_1 (B2). Absolute concentrations of single inoculations (left); absolute concentrations of strains in pair-wise inoculations (middle: 8 d; right: 12 d). Bacterial strain abundances were measured for two *Daphnia* genotypes (LRV4 and O1-08) and for two time points (day 8 and day 12). Error bars represent the standard error from the mean. Single inoculations were only sampled at day 12. X1X2 = simultaneous inoculations: X1_X2 = X1 inoculated 48 h before X2 (time-lag inoculation); X2_X1 = X2 inoculated 48 h before X1 (reciprocal time-lag inoculation). $N = 3$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Significant values are marked with colored asterisks to match the color of the strain for easier comparison between treatments.

similarly well in the gut of both host genotypes. When inoculated simultaneously at equal densities, however, *Rhizobium* R2_7 displayed significantly higher abundances than *Microbacterium* R2_8 (Fig. 2b). In both host genotypes, we observed significant differences in bacterial density between time-lag and reciprocal time-lag inoculations, in addition to

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differences between simultaneous and time-lag inoculations. *Rhizobium* R2_7 densities were higher when inoculated first than when inoculated second for host genotype LRV4 on day 8 (p-value = 0.003; Cohen's $d = 3.072$) and for host genotype O1-08 on day 12 (p-value = 0.003; Cohen's $d = 1.64$) (Fig. 2b). Similarly, for *Microbacterium* R2_8, inhibitory priority effects were observed for host genotype O1-08 on day 8, with higher abundances when introduced first (p-value = 0.003; Cohen's $d = 1.74$) than when inoculated after *Rhizobium* R2_7. In host genotype LRV4, however, a facilitative priority effect was observed for *Microbacterium* R2_8 on day 8, with lower abundances when inoculated first and higher when inoculated after *Rhizobium* R2_7 (p-value = 0.006; Cohen's $d = 3.70$). Priority effects for this strain combination were thus observed to occur in an opposite direction depending on host genotype. At day 12, no significant differences in abundances of *Microbacterium* R2_8 were observed between the time-lag and reciprocal time-lag treatments in any of the two host genotypes.

In the isogenic pair *Microbacterium* R2_8 (B1) and *Microbacterium* BHI_1 (B2), single inoculations revealed that both strains performed similarly well in the gut when inoculated in isolation (Fig. 2c). Inhibitory priority effects were only recorded for *Microbacterium* R2_8 in *Daphnia* genotype LRV4 on day 12, where this strain displayed higher densities when introduced first rather than second (p-value < 0.0001; Cohen's $d = 2.40$).

Effects of gut microbiome inoculation order on *Daphnia* life history traits

Daphnia life history traits (body size, percentage of survivors, fraction of mature animals and average clutch size) were assessed at two time points, namely day 8 and day 12, for all experimental units. For day 8, measurements were recorded for body size and survival only as animals had not reached reproductive maturity. For day 12, all four traits were recorded. The responses observed were found to be specific to the bacterial strain pair combination and host genotype identity (Fig. 3; Supporting Information Table S10). Significant differences in body size between inoculation order treatments were recorded for genotype O1-08 at day 8 for the *Rhizobium* R2_7- *Microbacterium R2_8* strain pair (Fig. 3; Supporting Information ANOVA Table S10). For the isogenic strain pair *Microbacterium* R2_8–*Microbacterium* BHI_1, significant differences in reproductive traits, that is, fraction of mature animals and average clutch size were recorded at day 12 for genotype LRV4 (Fig. 3; Supporting Information ANOVA Table S10). For this same strain pair, significant differences in percentage of survivors were noted at day 8 for genotype O1-08 (not shown in figure, *see* Supporting Information ANOVA Table S10). For the non-isogenic species pair *Rhizobium* R2_7–*Microbacterium* R2_8, significant differences in body size at day 8 were observed for host genotype O1-08 (Fig. 3b) between the simultaneous and reciprocal time-lag inoculations (p-value = 0.035; Cohen's $d = 2.43$) and the time-lag and reciprocal time-lag inoculations (p-value = 0.0359; Cohen's $d = 2.67$). For the isogenic pair *Microbacterium* R2_8–*Microbacterium* BHI_1, there was a significant difference in the percentage of survivors for genotype O1-08 between the simultaneous and reciprocal time-lag inoculations at day 8 ($p = 0.07$; Supporting Information ANOVA Table S10). In addition, differences in inoculation order resulted in significant differences in average clutch size in host genotype LRV4 at day 12 (p-value = 0.049). For the same species pair, a significant difference in fraction of mature animals was also observed between the simultaneous and reciprocal time-lag inoculation (p-value = 0.003; Cohen's $d = 1.91$) and time-lag and reciprocal time-lag inoculations (p-value = 0.005; Cohen's $d = 1.97$) in host genotype LRV4 at day 12 (Fig. 3c). For a comparison of significant inoculation order effects on the gut microbiome and corresponding host-level life history trait changes for each bacterial strain pair and host genotype, refer to Table 2.

Discussion

Using a systematic design of manipulated exposure to microbial inocula, our study provides evidence, in some cases, for the role of inoculation order in gut community assembly of *Daphnia*, a key ecological interactor in freshwater lakes and ponds (Stollewerk 2010). Our approach involved two-strain gut community assembly experiments with three different pairs of bacterial strains and two different host genotypes to reveal to what extent community assembly in the gut microbiome is context dependent, changing with the order in which bacteria are taken up by the host. Our results revealed that the occurrence, amplitude and even direction (suppressive or facilitative) of priority effects is highly contingent on the identity of bacterial strains and *Daphnia* host genotype. At the host level, we observed that *Daphnia* life history traits can be significantly affected by the microbiome composition established as a consequence of differences in inoculation order of the two-strain gut communities. This was again highly dependent on the strain combination and the host genotype used.

Our results highlight the important role of arrival history in gut microbiome assembly. Priority effects in gut community assembly have previously only been demonstrated in the mouse gut, where Martinez et al. (2018) show how arrival order and timing of complex bacterial communities in environmentally and genetically controlled mice have a lasting impact on gut microbiome assembly and composition. Consistent with their results, our study with axenic *Daphnia* reveals how colonization history can be a key factor influencing early assembly in the gut. However, we note that while priority effects do occur in our study, our results suggest that their occurrence and outcome is contingent on host genotype and strain-specific interactions. These observations are in alignment with studies in free living systems (Fukami et al. 2007; van Gremberghe et al. 2009; Knope et al. 2011; Tucker and

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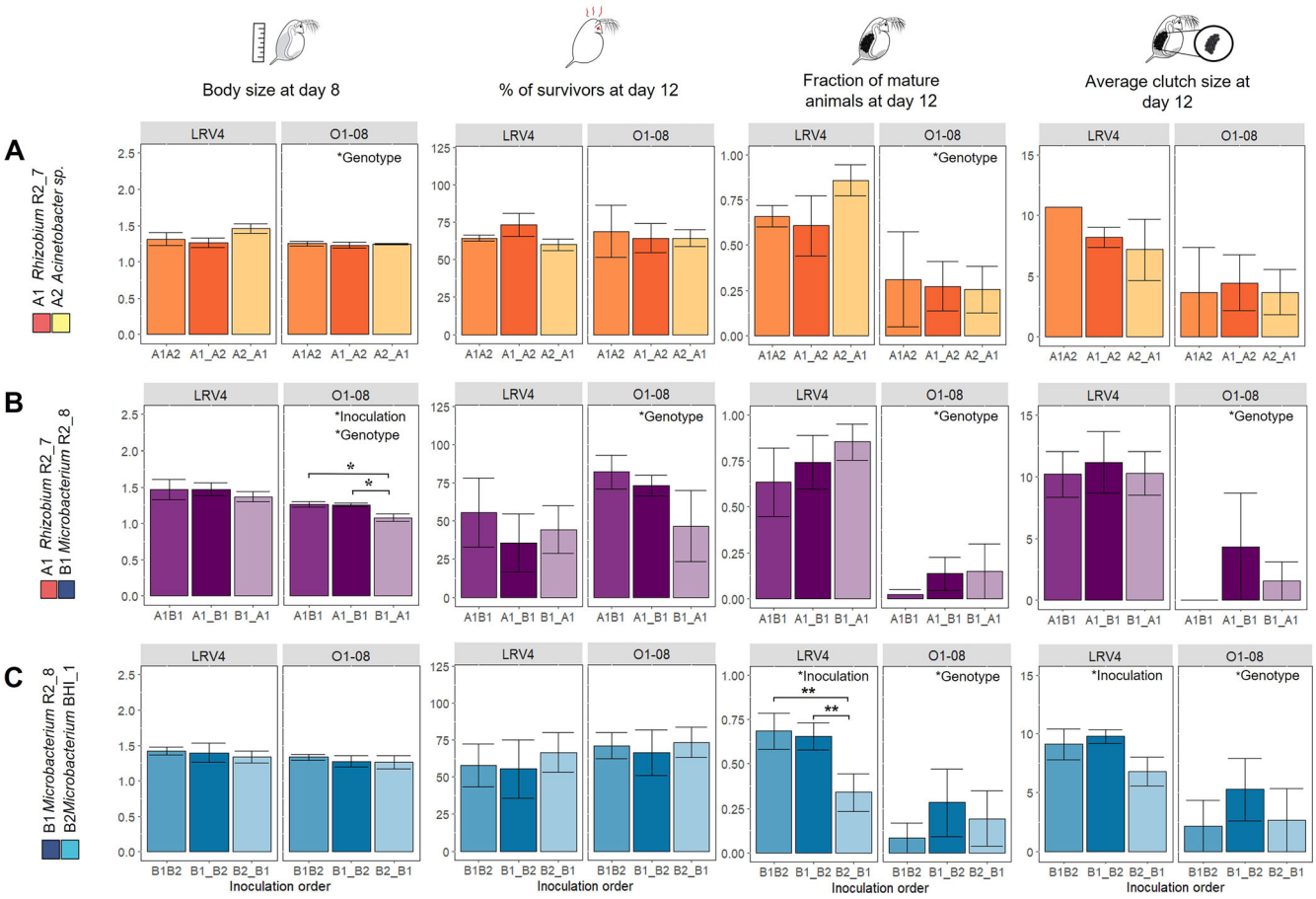

Fig. 3. Mean values ± one standard error of host body size at day 8 (mm), survival at day 12 (percentage of survivors at day 12), fraction of mature animals at day 12 (fraction of survivors that is mature) and clutch size (number of eggs per female at day 12), for two host (*Daphnia magna*) genotypes (LRV4 and O1-08) in the two-strain bacterial inoculations (simultaneous, time-lag, and reciprocal-time-lag) of (**a**) non-isogenic strain combination *Rhizobium* R2_7–*Acinetobacter* BHI_9; (**b**) non-isogenic strain combination *Rhizobium* R2_7–*Microbacterium* R2_8; and (**c**) isogenic strain combination *Microbacterium* R2_8–*Microbacterium* BHI_1. X1X2 = simultaneous inoculations: X1_X2 = X1 inoculated 48 h before X2 (time-lag inoculation); X2_X1 = X2 inoculated 48 h before X1 (reciprocal time-lag inoculation). $N = 3$. Genotype indicates significant genotype effects. Inoculation indicates significant inoculation effects. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For a full range of comparison of life history traits of *Daphnia* in the no-inoculum treatment, all pairwise treatments and daphnids with their natural microbiota refer to Supporting Information Fig. S7.

Fukami 2014; Nappi et al. 2022), where the strength of priority effects is subject to strain- or species-specific responses.

The presence of priority effects is often reflected in higher abundances of the species or strain that arrives first compared to later arriving taxa (so-called inhibitory priority effects; van Gremberghe et al. 2009; Devevey et al. 2015) because it is expected that the early colonist has an advantage in terms of space and resources and can monopolize resources by the time subsequent colonists arrive (Fukami 2015; De Meester et al. 2016). Most priority effects reported in literature are indeed inhibitory in nature (Peay et al. 2012; Devevey et al. 2015), with a few studies demonstrating facilitative effects (Alford and Wilbur 1985; Halliday et al. 2020). Our results show, however, that depending on the bacterial strains, priority effects can take very different forms. In the *Rhizobium* R2_7-*Microbacterium* R2_8 pair in host genotype LRV4, we observed standard inhibitory priority effects at day 8, wherein higher densities were recorded for *Rhizobium* R2_7 when introduced first into the gut in contrast to when this strain was inoculated after *Microbacterium* R2_8. This is in line with numerical advantages attributed to early colonization, potentially reinforced by strain-induced change of the environmental conditions in the gut (Fukami 2015) or evolutionary adaptation of the early colonist (De Meester et al. 2016; Scanlan 2019). For *Microbacterium* R2_8 in these same settings, however, a facilitative priority effect (Fukami 2015) was observed. *Microbacterium* R2_8 showed poor growth when introduced first, while growth was enhanced when this strain was inoculated following *Rhizobium* R2_7. This suggests that *Microbacterium* R2_8 potentially benefits from the prior establishment of *Rhizobium* R2_7. Both exemplary interactions underscore the degree to which gut microbiome assembly can

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Table 2. Summary of the statistical results obtained at the level of bacterial abundances and at the level of host traits, given per strain combination, *Daphnia* genotype and sampling time point (Time point). Treatment comparison indicates the combination of treatments for which a significant effect ($p < 0.05$) was obtained using a post hoc test in the targeted analysis (*see* Statistical analysis). Mean diff represents the mean measurement difference between the significant treatment comparison. Effect size is given as Cohen's *d*. NS indicates nonsignificant values for all possible comparisons. In case specific treatment combinations result in significant differences, these treatments are listed by the code of the strain for which the difference was observed followed by the two treatments for which the density of that strain differs (for codes of treatments, *see* Fig. 2). Shaded rows indicate cases in which at least one level (gut microbiome or host) was significantly affected by inoculation order. Bold indicates cases in which both the host and microbiome level were significantly affected by inoculation order.

				Bacterial abundance			Host trait			
	Strain combination	*Daphnia* genotype	Time point	Treatment comparison	Mean diff	Effect size	Treatment comparison	Trait	Mean diff	Effect size
A1A2	*Rhizobium*_R27–*Acinetobacter*_BHI9	LRV4	Day 8	NS	NS	NS	NS	NS	NS	NS
			Day 12	NS	NS	NS	NS	NS	NS	NS
		O1-08	Day 8	**A1: A1A2-A1_A2**	**1.26**	**1.39**	NS	NS	NS	NS
				A1: A1A2-A2_A1	**1.17**	**1.54**	NS	NS	NS	NS
			Day 12	NS	NS	NA	NS	NS	NS	NS
A1B1	*Rhizobium*_R27–*Microbacterium*_R28	LRV4	Day 8	**A1: A1_B1-B1-A1**	**−4.47**	**−3.07**	NS	NS	NS	NS
				B1: A1_B1-B1_A1	**−2.48**	**−3.70**	NS	NS	NS	NS
			Day 12	NS	NS	NS	NS	NS	NS	NS
		O1-08	Day 8	**B1: A1B1-A1_B1**	**1.47**	**1.38**	NS	NS	NS	NS
				B1: A1B1-B1_A1	**3.26**	**19.55**	**A1B1-B1_A1**	**Body size**	**−0.18**	**−2.43**
				B1: A1_B1-B1_A1	**1.79**	**1.74**	**A1_B1-B1_A1**	**Body size**	**−0.17**	**−2.67**
			Day 12	**A1: A1B1-B1_A1**	**−2.19**	**−1.00**	NS	NS	NS	NS
				A1: A1_B1-B1_A1	**−3.14**	**−1.64**	NS	NS	NS	NS
B1B2	*Microbacterium*_R28–*Microbacterium*_BHI1	LRV4	Day 8	NS	NS	NS	NS	NS	NS	NS
			Day 12	**B1: B1B2-B1_B2**	**0.90**	**1.31**	NS	NS	NS	NS
				NS	NS	NS	**B1B2-B2_B1**	**Fraction of mature animals**	**−0.34**	**−1.91**
				B1: B1_B2-B2_B1	**−4.20**	**−2.40**	**B1_B2-B2_B1**	**Fraction of mature animals**	**−0.31**	**−1.97**
		O1-08	Day 8	NS	NS	NS	NS	NS	NS	NS
			Day 12	NS	NS	NS	NS	NS	NS	NS

be contingent on the arrival order of the different bacterial strains in the gut, resulting in alternative community assembly trajectories. Our findings align with those of Martinez et al. (2018) where priority effects in the mouse gut were observed to occur in both directions. Although mostly inhibitory (90%) in nature, they also observed facilitative priority effects in the mouse gut, with bacterial groups increasing in abundance when introduced second.

The results from the bacterial strain combination *Rhizobium* R2_7–*Microbacterium* R2_8 also illustrate how priority effects are contingent on host genotype. While *Rhizobium* R2_7 showed inhibitory and *Microbacterium* R2_8 facilitative priority effects at day 8 in host genotype LRV4 and these priority effects proved to be transient and disappeared by day 12, the results for the same bacterial strain combination and treatments were very different for host genotype O1-08. Here, we observed inhibitory priority effects for both strains, but at different time points for the two strains. *Microbacterium* R2_8 showed inhibitory priority effects at day 8 that were transient and absent by day 12, *Rhizobium* R2_7 showed inhibitory priority effects only at day 12. The isogenic strain pair *Microbacterium* R2_8–*Microbacterium* BHI_1 showed more similar densities when inoculated simultaneously. While theory predicts that similarities in fitness might lead to stronger priority effects, only weak priority effects were detected in this strain pair and only in one of the host genotypes. Here, our results contrast with the observations of Martinez et al. (2018), where priority effects were demonstrated in the gut independent of host genotype. Our results align with the findings of Leopold and Busby (2020), who reported that the effects of species arrival order on the foliar microbiome assembly of the black cottonwood (*Populus trichocarpa*) varied with host genotype. Such host genotype effects have also been demonstrated in fungal parasite assemblages of the host *Plantago lanceolata*.

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Notably, the facilitative priority effects observed in the fungal assemblages were found to be dependent on the specific genotype of the host (Halliday et al. 2020). Given that *Daphnia* body sizes varied across treatments (*see* further), one may question to what extent our results might have been influenced by variation in gut size among *Daphnia* individuals. We believe that this interference was negligible. First, our interpretation of priority effects is also corroborated when we plot relative abundances (*see* Supporting Information Fig. S9 in Supporting Information). Second, we plotted total bacterial densities against body size of our experimental animals and did not observe a significant positive correlation (Supporting Information Fig. S8).

In the *Rhizobium* R2_7–*Acinetobacter* BHI_9 strain pair, no priority effect was observed. Rather, in this strain combination, only strain *Rhizobium* R2_7 was observed to colonize the gut, while no growth was observed for *Acinetobacter* BHI_9 in the gut of both single and pairwise inoculations. The lack of growth of *Acinetobacter* BHI_9, despite this strain being isolated from a *Daphnia* gut microbiome, might be explained by the fact that this strain may need specific conditions (e.g., an existing biofilm) or resources that are, in natural gut microbiomes, provided by other species in the community. Although a vast majority of *Acinetobacter* strains possess the ability to consume a wide range of organic compounds as their carbon source (Towner 2006), there remain certain species within this group that demonstrate strain-specific nutrient preferences (Glover et al. 2022). It is thus conceivable that *Acinetobacter* BHI_9 may thrive better in the presence of a more speciose microbiome, possibly relying on positive growth promoting interactions (Kehe et al. 2021), modified environmental conditions (e.g., pH) and by-products from other microbiome members for its survival and growth.

Several studies have observed that the presence of a microbiome and variation in gut microbiome composition has fitness consequences for hosts (Berendsen et al. 2012; Shreiner et al. 2015; Vandenkoornhuyse et al. 2015; Bahrndorff et al. 2016). Microbiome-mediated stress tolerance in *Daphnia* has been demonstrated for exposure to cyanobacterial toxins (Macke et al. 2017) and mercury (Fong et al. 2019). Likewise, previous studies have shown that germ-free *Daphnia* demonstrate reduced fitness and high mortality (Sison-Mangus et al. 2015; Callens et al. 2016), which can be restored in the presence of specific bacterial strains, such as *Limnohabitans* and *Aeromonas* sp. (Peerakietkhajorn et al. 2015, 2016; Sison-Mangus et al. 2015). In line with these studies, we observe notable mortality in the no-inoculum treatment, with one replicate unit of genotype LRV4 and two replicate units of genotype O1-08 dying off completely. In this study, we also observed differences in *Daphnia* life history traits among treatments in which the animals were exposed to the same bacterial strains in different chronological order (simultaneous and the time-lag treatments). Also, for life history impact, the differences were found to be strain and host genotype dependent. In the bacterial strain pair *Rhizobium* R2_7–*Microbacterium* R2_8, differences in order of exposure resulted in significant differences in body size in host genotype O1-08, while in the bacterial pair *Microbacterium* R2_8–*Microbacterium* BHI_1, this led to differences in clutch size and percentage of mature animals in host genotype LRV4. These strain-dependent and host genotype–dependent responses might reflect a combination of priority effects in bacterial abundances, context-dependent microbe–microbe interactions (Rao et al. 2021) and differences in the degree and way bacterial strains affect host traits, potentially through differences in functional pathways and metabolite production.

Overall, there is alignment between the impact of inoculation order on microbiome community composition and the observed differences in life history traits of the host. In the *Rhizobium* R2_7-*Acinetobacter* BHI_9 pair, no priority effects were detected in the gut and no differences in host traits were detected. For bacterial strain pair *Rhizobium* R2_7–*Microbacterium* R2_8, the strongest priority effects at the level of the microbiome were observed for host genotype O1-08 at day 8, and this was matched by differences in body size of the host individuals of that genotype (Table 2). For bacterial strain pair *Microbacterium* R2_8–*Microbacterium* BHI_1, priority effects at the level of the microbiome were observed for host genotype LRV4 at day 12, and this was matched by differences in percentage of mature animals in that host genotype (Table 2). Notably, a priority effect that is stronger at day 8 than at day 12 is more likely to affect growth, whereas a priority effect that accumulates with age might more likely affect reproduction. Our observations strongly suggests that the differences in host life history traits observed are indeed linked to priority effects at the level of the microbiome.

The overall match between priority effects for microbiome composition and differences in host traits does not imply, however, that all priority effects at the level of the microbiome translate into host life history trait differences. For instance, the microbiome priority effects observed for host genotype LRV4 for the bacterial strain pair *Rhizobium* R2_7–*Microbacterium* R2_8 did not translate into differences in host life histories (Table 2). Concerning differences between the simultaneous and (reciprocal) time-lag inoculations, the difference in fraction of mature animals observed for host genotype O1-08 for the bacterial strain combination *Microbacterium* R2_8–*Microbacterium* BHI_1 is not matched by a significant difference in bacterial densities (Table 2). Here, a higher maturity was recorded for the simultaneous treatment in comparison to the reciprocal time-lag inoculation. This might be explained by the fact that bacterial inoculum densities at the start of the experiment were twice as high for the simultaneous than for the reciprocal time-lag inoculation treatments. While bacterial densities at day 8 or day 12 were not different, they might have been higher during crucial early life stages. Cases of observed priority effects for gut microbiome assembly that are not translated into changes in life history traits in hosts might

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reflect that both bacterial strains have a similar functional effect on host life histories or that the observed variation in densities does not make a difference strong enough to affect host life history.

We observed a strong main host genotype effect for life history trait in all three bacterial strain combinations. Differences in reproductive traits were striking, suggesting a much higher fitness for host genotype LRV4 than for host genotype O1-08: clutch sizes were generally twice as high for genotype LRV4 than for genotype O1-08, and the percentage of mature animals at day 12 in the presence of the bacterial strains were generally low (around 25%) for host individuals of genotype O1-08, while much higher (50–75%) for animals of genotype LRV4. This is, however, still very low as compared to control treatments where animals were grown with their own source of natural microbiota (Supporting Information Fig. S7), suggesting that the inoculation with two strains imposed by this study resulted in suboptimal growth conditions in *Daphnia*. Interestingly, the two genotypes did not strongly differ in fitness when grown with their own microbiome (*see* Supporting Information Fig. S7). The differences in fitness we observe between the two host genotypes when inoculated with single or pairs of strains (e.g., a much lower percentage of animals that reach maturity on day 12) likely reflect the disruptive effect of having no or a very poorly functioning microbiome. Given that the observed differences in fitness between the two genotypes are likely associated to the microbiome, it is noteworthy that the bacterial strains employed in our experiment were isolated from host genotype LRV4, which is the genotype that shows the higher fitness when grown in the presence of these bacteria. This indicates that the fitness effect of the microbiome might in part depend on a match between the bacterial strains and the *Daphnia* genome, either mediated by the capacity of bacteria to grow in the gut or by their functional effects. Our data suggest the latter, as we did not observe strong differences in bacterial densities between the two host genotypes. Our results thus indicate that *Daphnia* genotypes might perform better with microbiome strains isolated from individuals of the same genotype, which is consistent with other studies (Houwenhuyse et al. 2021; Jackrel et al. 2021; O'Brien and Harrison 2021) that point to a potential coadaptation between host genotypes and their microbiomes. While the pattern is intriguing, the hypothesis of co-adaptation to explain our observations remains speculative and requires targeted experiments to be tested. One alternative explanation is that *Daphnia* genotype O1-08 suffers more from having a low gut microbiome diversity compared to genotype LRV4.

Using two-strain gut community assembly experiments, we show that gut microbiome assembly in *Daphnia* can be contingent on arrival order of bacterial strains, and that the effect of arrival order on community assembly trajectories is itself contingent on the identity of both the bacterial strains as well as host genotype. Arrival order had long-lasting effects affecting gut microbiome composition in *Daphnia* individuals and had significant effects on host fitness. The importance of priority effects for gut microbiome assembly and its impact on host fitness may have important implications for the ecology of a wide range of species and ecosystems, as the alternative community assembly trajectories in the gut microbiome might translate into altered interactions of host individuals with conspecifics, other species, and the environment (Decaestecker et al. 2024). Given the importance of gut microbiome composition on plant, animal, human functioning, and health (Berendsen et al. 2012; Lee and Hase 2014; Shreiner et al. 2015), a better understanding of priority effects also has important implications in an applied context, for instance in the context of microorganism-based interventions. Knowledge of the occurrence of priority effects can be used to restore and restructure dysbiotic gut communities to sustain desired microbiome species while resisting unsought pathogens (Weidlich et al. 2021) and thus foster better health for humans, animals, and plants. Furthermore, the success of microorganism-based interventions such as pre-biotics, probiotics, fecal transplants, and bioaugmentation tools in ecological restoration efforts, water quality management, and human health can depend on the removal of resident communities and the targeted introduction of microbial inoculants with well-designed sequences and timing of exposure. The complexity of processes affecting community assembly (Leibold et al. 2022) and the multifaceted relationship between gut microbiome composition and host functioning and fitness (Rosshart et al. 2017; Fong et al. 2019; Bulteel et al. 2021; Houwenhuyse et al. 2021) require a targeted research approach so as to be able to predict better the implications of priority effects and other assembly processes in microbiomes for individual health and functioning as well as for ecological and evolutionary trajectories (Decaestecker et al. 2024).

Our results provide strong proof-of-principle that priority effects can be important in gut microbiome assembly in *Daphnia*, that they can take different shapes depending on the strain combination and host genotype and affect *Daphnia* fitness. We acknowledge that in nature, priority effects may operate more at the community level rather than at the level of specific strains, because situations in which a *Daphnia* would early on in its life be exposed to only one bacterial strain might be rare. However, such situations can also occur, for instance, if neonates at birth are exposed to high number of one or a few bacterial strains that are released by the mother or from the ephippial case of dormant *Daphnia* eggs (Mushegian and Ebert 2017). Yet, future work where priority effects in gut microbiome assembly are assessed at the level of entire communities will be important to assess how, in nature, early exposure might determine the trajectory of gut microbiome community assembly and to what extent gut microbiome communities are resistant to change when exposed to changing microbial communities in their

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environment. We expect that in settings with diverse communities, inhibitory priority effects, where the resident community reduces establishment success of immigrant strains, may be common. In such diverse settings, however, initial assembly may also be complex, with simultaneous exposure to multiple bacterial strains, resulting in less chance effects in terms of which strain colonizes the gut first. Another aspect that needs to be studied is to what extent resistance to change in microbiome communities is reduced under changing environmental and abiotic conditions, such as warming or exposure to pollutants, or changes in the food spectrum such as a shift in phytoplankton community structure.

Data availability statement

The 16S rRNA sequences of the strains used in our study are available in GenBank under the accession numbers PP417717, PP417718, PP417719, PP417720. All experimental data are submitted together with supporting information.

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Conflict of Interest

None declared.

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