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Eco-evolutionary dynamics and priority effects in the community assembly of *Daphnia* gut microbiome



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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Science (PhD): Biology

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5 years: what a ride! Looking back at my PhD, what a beautiful journey it was! I still remember very vividly my father's words as I left for Belgium, "*It feels like shooting arrows into the dark*". And honestly, he wasn't wrong, new country, new people, new work and a pandemic thrown into the mix, I had no idea how this would all unfold and could only hope for the best. But with a lot of courage (and maybe a bit of stubbornness), I took the leap. And well, looking back today, I can proudly say that it was the right decision, for this PhD has been nothing short of a rewarding and fulfilling journey. And yet, this journey could not have been complete without the support and guidance of so many wonderful people I've met along the way. Here, I want to celebrate and thank each one of them for the role they played in shaping both this work and the person I've grown to become today.

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Summary

Traditionally, ecology and evolutionary biology have been studied as two separate disciplines, considering that each process occurs independently at distinct time-scales; ecological processes occurring much rapidly in contrast to slow evolutionary change. However, with time, there has been a shift away from this perspective to a realization that ecological and evolutionary processes can occur at the same time scales and therefore influence each other. The field of eco-evolutionary dynamics focuses on these interactions and has in the past years gained strong momentum integrating theoretical models, conceptual frameworks, proof-of-principle experiments and genomics. In this thesis, I focus on integrating the microbiome into the framework of eco-evolutionary dynamics to study the role of such feedbacks on the gut microbiome and the resultant impact on individual host fitness scaling up to host populations.

All plants and animals host a diverse array of micro-organisms that have been closely linked to host health and fitness. These microorganisms occupy different areas of the host, their function differing based on their site of origin within the host. At the same time they also differ in their contribution to the host, ranging from host symbionts, pathogens, parasites to simply neutral associations. In humans and most organisms, the most diverse of these communities reside in the gut and have major documented implications for the host. For instance, the gut microbiome has been shown to influence a broad array of host traits, including digestion, physiology, metabolism, immunity, brain development, behavior and even adaptation to environmental change. Given the intimate link between host health and gut microbiome composition, it is crucial to understand how these communities assemble in the gut and stabilize in composition. In this thesis, I focus on a specific eco-evolutionary process, namely priority effects in the community assembly of the gut microbiome using *Daphnia* as a model organism. Given the use of this model for my study, it is crucial that I first understand what key members comprise the gut microbiome of *Daphnia* and thereby contribute to its fitness.

Thus, in **Chapter 1**, I start by investigating the presence of a core microbiome in *Daphnia magna*. I employ a meta-analysis approach to identify core taxa using an integrated dataset compiled from 12 published studies, comprising gut and whole animal samples of both laboratory and field animals across three continents. My analysis demonstrates that the *D. magna* microbiome is defined by its high variability with only a few conserved associations, namely *Limnohabitans planktonicus* emerging as the most stable taxon in laboratory settings. However, this pattern is tempered by the observation that field-grown animals exhibit a more diverse microbiome with a weaker presence of this taxon, challenging its status as a core member.

Given the variable nature of the *Daphnia* gut microbiome, in **Chapter 2**, I sought to isolate naturally occurring strains directly from the gut of native *Daphnia* clones for use in our study. For this, a gravid female was dissected to extract the gut, which was further homogenized and plated onto 5 media sources (ranging from simple to complex nutrient compositions) to isolate a wide range of bacterial strains native to the *Daphnia* gut. From a total of 76 strains isolated, 4 strains were selected for my study to investigate the role of priority effects or species arrival order in gut community assembly. A bottom-up approach was employed for this community assembly study where I started with an empty habitat patch, i.e., sterile *Daphnia* gut, and introduced bacterial strains either simultaneously at equal inoculum densities, or sequentially with a 48h time-lag. Strong priority effects were observed wherein there occurred a significant effect of strain arrival order on the gut community composition, however, the presence, amplitude and direction (inhibitory or facilitative) of these effects were found to strain-specific and host-genotype dependent. Life history traits were also monitored for these inoculation order treatments to understand the potential consequences of priority effects occurring in the gut on host fitness. Significant differences in host survival and reproductive output was documented across inoculation order treatments, shedding light on the tangible consequences of priority effects for host fitness.

Due to strong priority effects observed during gut microbiome assembly in Chapter 2, in **Chapter 3,** I sought to understand the role of evolution in the contribution to these processes. More specifically, I aimed to understand if prior adaptation to the *Daphnia* gut environment would improve bacterial strain fitness that would strengthen the outcome of priority effects. To this end, two bacterial strains originally isolated from *Daphnia* genotype LRV4 (in chapter 1) were experimentally evolved for 12 days to the gut of a new *Daphnia* genotype (O1-08) to obtain a pre-exposed population. Next, the order of introduction of these pre-exposed (evolved) population was alternated with the ancestral strains in the gut of germ-free animals of the new host genotype to test for evolution-mediated priority effects. My results showed that this brief evolutionary period did not produce significant fitness improvements in the evolved population of both strains in their new host environment. For *Microbacterium* R2_8 however, a marked reduction in fitness was witnessed

when re-introduced into the original host genotype following evolution, indicating a rapid loss of ancestral compatibility. Furthermore, manipulating inoculation order did not reveal clear priority effects. Instead, *Rhizobium* R2_7 strains were observed to dominate while suppressing the growth of *Microbacterium* R2_8, regardless of adaptation status or arrival order.

In the last chapter, **Chapter 4,** I investigate priority effects in a broader context and test their occurrence in zooplankton community assembly. In this study, I focus on the two-way interaction between hosts and their microbial communities in determining priority effects, using an experimental zooplankton community system. Specifically, I test whether the host-conditioned medium mediates priority effects in host community assembly and, conversely, whether host priority effects influence the assembly and composition of the surrounding bacterioplankton community. Priority effects were imposed by sequentially manipulating the arrival order of two species of zooplankton, namely, *D. magna* and *D. pulicaria*. These arrival order treatments were crossed with host-conditioned medium treatments following which the species and bacterioplankton community composition was quantified. Strong priority effects were observed between *D. magna* and *D. pulicaria* with the first-arriving species dominating the community. Interestingly, the type of host-conditioned medium applied did not alter the outcome of species arrival suggesting that host arrival order exerted a more prominent effect in contrast to the biochemical influence of the host-conditioned medium. Conversely, for the reciprocal interaction at the bacterioplankton level, the host arrival order imposed a significant divergence pattern in the bacterioplankton community composition, indicating the importance of host arrival order in host as well as bacterioplankton community dynamics.

Lastly, I integrate the main findings of my thesis within a broader community assembly framework highlighting their potential applications in microbiome-based interventions. I additionally propose a conceptual framework for incorporating priority effects into both experimental design and the development of such therapeutics. I conclude by identifying uncharted territories where such processes may be overlooked and outline promising directions for future research.

Samenvatting

Traditioneel werden ecologie en evolutionaire biologie als twee afzonderlijke disciplines bestudeerd, omdat men ervan uitging dat elk proces onafhankelijk van elkaar op verschillende tijdschalen plaatsvindt; ecologische processen werden gedacht veel sneller te verlopen in vergelijking met de langzame evolutionaire veranderingen. In de loop der tijd is dit perspectief echter verschoven naar het besef dat ecologische en evolutionaire processen op dezelfde tijdschaal kunnen plaatsvinden en elkaar daardoor kunnen beïnvloeden (Hairston Jr. et al., 2005; Hendry, 2016a, 2016b; Schoener, 2011; Urban et al., 2008). Eco-evolutionaire dynamieken is een vakgebied dat zich richt op deze interacties en dat de afgelopen jaren sterk aan momentum heeft gewonnen door de integratie van theoretische modellen, conceptuele raamwerken (De Meester et al., 2016; Matthews et al., 2014), proof-of-principle experimenten (Bassar et al., 2010; Koskella & Brockhurst, 2014; Pantel et al., 2015) en genomica (Becks et al., 2012; Comeault et al., 2015; Hand et al., 2015). In dit proefschrift richt ik me op de integratie van het microbioom in het raamwerk van eco-evolutionaire dynamieken om de rol van dergelijke feedbacken op het darmmicrobioom en de resulterende impact op de individuele fitheid van de gastheer te bestuderen, opschalend naar gastheerpopulaties in (semi-)natuurlijke omgevingen.

Alle organismen herbergen een diverse reeks micro-organismen die nauw verbonden zijn met de gezondheid en conditie van de gastheer. Deze micro-organismen bezetten verschillende delen van de gastheer, waarbij hun functie verschilt afhankelijk van hun oorsprong binnen de gastheer. Tegelijkertijd verschillen ze ook in hun bijdrage aan de gastheer, variërend van gastheersymbionten, pathogenen en parasieten tot simpelweg neutrale associaties. Bij mensen en de meeste organismen bevindt de meest diverse van deze gemeenschappen zich in de darmen en heeft deze belangrijke gedocumenteerde implicaties voor de gastheer. Zo is aangetoond dat het darmmicrobioom een breed scala aan gastheereigenschappen beïnvloedt, waaronder spijsvertering, fysiologie, metabolisme, immuniteit, hersenontwikkeling, gedrag en zelfs aanpassing aan veranderingen in de omgeving. Gezien de nauwe band tussen de gezondheid van de gastheer en de samenstelling van het darmmicrobioom, is het cruciaal om te begrijpen hoe deze gemeenschappen zich in de darmen vormen en hun samenstelling stabiliseren. In dit proefschrift richt ik mij op een specifiek eco-evolutionair proces, namelijk prioriteitseffecten in de gemeenschapsvorming van het darmmicrobioom met behulp van *Daphnia* als modelorganisme. Omdat ik dit model voor mijn onderzoek gebruik, is het van cruciaal belang om eerst te begrijpen welke genera het hoofdbestanddeel van het darmmicrobioom van *Daphnia* vormen en welke daarmee bijdragen aan de gastheerconditie.

Daarom begin ik in **hoofdstuk 1** met het onderzoeken van de aanwezigheid van een kernmicrobioom in *Daphnia magna*. Ik gebruik een meta-analyse om kerntaxa te identificeren met behulp van een geïntegreerde dataset samengesteld uit 12 gepubliceerde studies, bestaande uit darmmonsters en monsters van hele dieren van zowel laboratorium- als velddieren van drie continenten. Mijn analyse toont aan dat het *D. magna*-microbioom wordt gedefinieerd door zijn hoge variabiliteit met slechts enkele behouden associaties, waarbij *Limnohabitans planktonicus* naar voren komt als het meest stabiele taxon in laboratoriumomgevingen. Dit patroon wordt echter getemperd door de observatie dat in het veld gekweekte dieren een diverser microbioom vertonen met een zwakkere aanwezigheid van dit taxon, waardoor de status ervan als kernlid in twijfel wordt getrokken.

In **hoofdstuk 2** gebruikte ik 4 stammen geïsoleerd uit het darmmicrobioom van *Daphnia magna* genotype LRV4 in een experimentele studie naar de rol van prioriteitseffecten of de volgorde van aankomst van soorten in de samenstelling van de darmgemeenschap. Voor deze studie naar gemeenschapssamenstelling werd een bottom-upbenadering gebruikt, waarbij ik begon met een leeg habitatgebied, d.w.z. een steriele darm van *Daphnia*, en bacteriestammen introduceerde, hetzij gelijktijdig met gelijke inoculumdichtheden, hetzij sequentieel met een vertraging van 48 uur. Er werden sterke prioriteitseffecten waargenomen, waarbij de volgorde van aankomst van de stammen een significant effect had op de samenstelling van de darmgemeenschap. De aanwezigheid, amplitude en richting (remmend of faciliterend) van deze effecten bleken echter stamspecifiek en afhankelijk van het gastheergenotype. Levensgeschiedeniskenmerken werden ook gemonitord voor deze inoculatievolgordebehandelingen om de potentiële gevolgen van prioriteitseffecten in de darm op de gastheerfitness te begrijpen. Er werden significante verschillen in gastheeroverleving en reproductieve output gedocumenteerd tussen inoculatievolgordebehandelingen, wat licht werpt op de tastbare gevolgen van prioriteitseffecten voor de gastheerfitness.

Vanwege de sterke prioriteitseffecten die werden waargenomen tijdens de opbouw van het darmmicrobioom in hoofdstuk 2, probeerde ik in **hoofdstuk 3** de rol van evolutie in de bijdrage aan deze processen te begrijpen. Meer

specifiek wilde ik begrijpen of eerdere adaptatie aan de darmomgeving van *Daphnia* de fitness van de bacteriestam zou verbeteren, wat de uitkomst van prioriteitseffecten zou versterken. Hiertoe werden twee bacteriestammen, oorspronkelijk geïsoleerd uit *Daphnia*-genotype LRV4 (in hoofdstuk 1), gedurende 12 dagen experimenteel geëvolueerd naar de darm van een nieuw *Daphnia*-genotype (O1-08) om een vooraf blootgestelde populatie te verkrijgen. Vervolgens werd de volgorde van introductie van deze vooraf blootgestelde (geëvolueerde) populatie afgewisseld met de voorouderlijke stammen in de darm van kiemvrije dieren om te testen op evolutie-gemedieerde prioriteitseffecten. Mijn resultaten toonden aan dat deze korte evolutionaire periode geen significante fitnessverbeteringen opleverde in de geëvolueerde populatie van beide stammen in hun nieuwe gastheeromgeving. Voor *Microbacterium* R2_8 werd echter een duidelijke afname in fitness waargenomen bij herintroductie in het oorspronkelijke gastheergenotype na evolutie, wat wijst op een snel verlies van voorouderlijke compatibiliteit. Bovendien leverde het manipuleren van de inoculatievolgorde geen duidelijke prioriteitseffecten op. In plaats daarvan werd waargenomen dat *Rhizobium* R2_7-stammen domineerden en de groei van *Microbacterium* R2_8 onderdrukten, ongeacht de adaptatiestatus of de aankomstvolgorde.

In het laatste hoofdstuk, **hoofdstuk 4**, onderzoek ik prioriteitseffecten in een bredere context en test ik hun aanwezigheid in de vorming van zoöplanktongemeenschappen. In deze studie concentreer ik me op de tweerichtingsinteractie tussen gastheren en hun microbiële gemeenschappen bij het bepalen van prioriteitseffecten, met behulp van een experimenteel zoöplanktongemeenschapssysteem. Specifiek test ik of het door de gastheer geconditioneerde medium prioriteitseffecten bemiddelt in de vorming van de gastheergemeenschap en, omgekeerd, of prioriteitseffecten van de gastheer de vorming en samenstelling van de omringende bacterioplanktongemeenschap beïnvloeden. Prioriteitseffecten werden opgelegd door de aankomstvolgorde van twee soorten zoöplankton, namelijk *D. magna* en *D. pulicaria*, sequentieel te manipuleren. Deze aankomstvolgordebehandelingen werden gekruist met door de gastheer geconditioneerde mediumbehandelingen, waarna de soort en de samenstelling van de bacterioplanktongemeenschap werden gekwantificeerd. Er werden sterke prioriteitseffecten waargenomen tussen *D. magna* en *D. pulicaria*, waarbij de eerst arriverende soort de gemeenschap domineerde. Interessant genoeg had het type gastheer-geconditioneerd medium dat werd toegepast geen invloed op de uitkomst van de aankomst van soorten, wat suggereert dat de volgorde van aankomst van de gastheer een prominenter effect had dan de biochemische invloed van het gastheer-geconditioneerde medium. Omgekeerd, voor de wederzijdse interactie op bacterioplanktonniveau, legde de volgorde van aankomst van de gastheer een significant divergentiepatroon op in de samenstelling van de bacterioplanktongemeenschap, wat wijst op het belang van de volgorde van aankomst van de gastheer voor zowel de dynamiek van de gastheer als die van de bacterioplanktongemeenschap.

Tot slot integreer ik de belangrijkste bevindingen van mijn proefschrift in een breder kader van community assembly, waarbij ik hun potentiële toepassingen in microbioom-gebaseerde interventies benadruk. Daarnaast stel ik een conceptueel kader voor om prioriteitseffecten te integreren in zowel experimenteel ontwerp als de ontwikkeling van dergelijke therapieën. Ik sluit af met het identificeren van onontgonnen terrein waar dergelijke processen mogelijk over het hoofd worden gezien en schets veelbelovende richtingen voor toekomstig onderzoek.

List of abbreviations

IEC	Intestinal Epithelial Cells
AMPs	Antimicrobial Peptides
IgA	Immunoglobulin A
SCFA	Short Chain Fatty Acids
16S rRNA	16S Ribosomal Ribonucleic Acid
HMO	Human Milk Oligosaccharides
SNV	Single Nucleotide Variations
FMT	Fecal Microbiota Transplants
ADaM	Aachener Daphnien Medium
AGE	Agarose Gel Electrophoresis
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment search Tool
C_q	Quantification cycle
DNA	Deoxyribonucleic Acid
ENA	European Nucleotide Archive
FAM	Fluorescein
GC	Guanine-Cytosine content
gDNA	Genomic Deoxyribonucleic Acid
HEX	Hexachlorofluorescein
IRC	Inter-run calibration
ITS	Internal Transcribed Spacer Region
L:D	Light: Dark photoperiod
LRV	Langerodevijver pond
MC	Mac Conckey Agar
MDS	Multidimensional Scaling
NA	Nutrient Agar
NCBI	National Centre for Biotechnology Information
NTC	No template control
OD	Optical Density
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
qPCR	Quantitative Polymerase Chain Reaction
R2A	Reasoners 2A Agar
RNA	Ribonucleic Acid
SI	Supporting Information
T_m	Melting temperature
TSA	Tryptic Soy Agar
V4	Fourth hypervariable region of the 16S rRNA gene
VSC	Vlaams Supercompter Centrum

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GENERAL INTRODUCTION

General Introduction

Background

The gut microbiome has emerged as a critical area of research due to its close link to host health and phenotype. This complex and dynamic ecosystem harbors a vast array of microorganisms, often accounting for the highest density and diversity of species in contrast to other parts of the body (1,2). The gut microbiome plays essential roles in numerous host physiological processes including digestion, metabolism (3–5), shaping of the immune system, resistance against pathogens (6–8) and even brain development and behavior (9–11). Extensive research has demonstrated the capacity of the gut microbiome in the digestion and metabolism of complex carbohydrates and dietary elements into bioactive components that otherwise remain inaccessible due to a lack of available host enzymes. For instance, host-associated microbiota possess the ability to break down indigestible carbohydrates such as cellulose, hemicellulose, pectin, lignin and resistant starch into short chain fatty acids (SCFAs) such as acetic, propionic and butyric acids (12,13). Additionally, these microbial communities contribute to host nutrition through the biosynthesis of essential amino acids and vitamins such as biotin, thiamine, cobalamin, riboflavin, nicotine, vitamins B and K (14,15), thereby supplementing dietary intake and supporting metabolic biosynthesis. The role of the microbiome in extending the host adaptive potential is also being widely explored with studies demonstrating how host-associated microbiomes can enhance host resilience to environmental stressors including elevated temperatures, dietary variations and osmotic challenges (16). Conversely, disruptions in gut microbiome composition, termed "*dysbiosis*", has been implicated in chronic diseased states and metabolic disorders such as Crohn's disease, atherosclerosis, type II diabetes and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (17–20). Understanding the factors that bring about this shift from a stable to dysbiotic state thus becomes imperative.

While researchers have identified numerous determinants that influence gut microbiome composition, including host genetics, immunological status, delivery mode, diet, age, geographical location, use of antibiotics and lifestyle (21–28), these factors collectively account for less than 30% of the variation observed in the gut community composition, leaving the remaining 70% of the variation largely unexplained (29). This substantial unexplained variance represents one of the most pressing challenges in microbiome research that limits our understanding of the mechanisms that determine whether an individual's microbiome will promote health or contribute to disease susceptibility. A significant challenge to uncovering the community assembly of such host-associated communities is the lack of focus on foundational ecological and evolutionary principles and the feedbacks that occur between them, that can markedly influence and shape gut community assembly.

Microbiomes in the context of eco-evolutionary dynamics

Traditionally, ecology and evolutionary biology have been studied as separate disciplines, considering that each process occurs independently at distinct time-scales with ecological processes occurring more rapidly in contrast to slow evolutionary change. However, with time there has been a shift away from this perspective to a realization that ecological and evolutionary processes can occur at the same time scales and therefore influence each other (30–34). The field of eco-evolutionary dynamics focuses on these interactions and has in the past years gained strong momentum integrating theoretical models, conceptual frameworks (35,36), proof-of-principle experiments (37–39) and genomics (40–42).

The phenotype is often core to understanding eco-evolutionary dynamics (43) as it is the phenotype that undergoes evolutionary change (via genetic changes) to impact ecological interactions between species and their environment. These changes can influence how individuals respond to environmental conditions, how they interact with conspecifics and competing species and even how they impact the ecosystem itself. With increasing evidence for microbiomes strongly impacting host phenotypes (44), the integration of the microbiome into the framework of eco-evolutionary dynamics becomes essential. In the context of the gut microbiome, the host-associated microbes are likely to evolve rapidly in the host environment due to their short generation times, large population sizes, rapid mutation rates and capacity for horizontal gene transfer. In line with this expectation, rapid evolutionary changes have been recently reported demonstrating how residents can evolve over time in the host environment (45–48). For instance, host genetic factors such as the immune system (production of antimicrobial peptides, secretory antibodies) and gut physiological environment (strong acidity, production of bile acids) have often been shown to impose selection pressures on the gut microbiota resulting in adaptive evolution of the gut commensals (43). Likewise, the host diet has also been implicated in driving evolution in gut bacteria (23,49). For instance, the gut microbiota of Japanese residents have been shown to acquire through horizontal gene transfer, porphyrin degrading enzymes from marine bacterial species, *Phocaeicola*

plebeius, that allow for the degradation of seaweed, an important component of their daily diet (50). Evolutionary responses have also been reported in relation to microbial interspecific interactions in the gut. Interspecific competition over limited resources has been observed to drive the production of toxins by the abundant gut species *Bacteroides fragilis* to inhibit the growth of potential competitors of the order Bacteroidales, thereby creating a protective niche for themselves in the human colon (51).

Given that evolution can significantly shape community assembly (52), it is possible that evolutionary changes in microbial communities within the host can impact microbiome assembly. Imposed changes in the gut microbiome assembly may result in compositional shifts that can confer fitness advantages to the host. This can occur through microbial aid in digestion and nutrition acquisition (53,54), pathogen and disease tolerance (55,56), and stress resilience (57,58), which can collectively influence the host adaptive potential to the external environment. Host genotypes carrying these beneficial microbes can gain fitness benefits and become more successful over time, which can further shape how the host evolves (59–61). As a result, there may be natural selection for host genotypes that are linked to specific microbial genomes resulting in the evolution of host genes involved in the acquisition and control of beneficial symbiotic microbiota (59). As such, these eco-evolutionary dynamics impacting gut community assembly may alter host phenotypes, impacting how hosts interact with their environment and other species, ultimately influencing host population, community dynamics and ecosystem-level processes (43).

Community assembly of the gut microbiome

A fundamental goal of community ecology is to understand the processes that contribute to community composition and structure. Community assembly in general is a complex process encompassing a multitude of mechanisms that are deterministic, stochastic or a combination of both. In the past century community ecologists have proposed multiple concepts to explain observed patterns of species distribution and abundance. Vellend (2010) (62) integrated these concepts to categorize these mechanisms into four major processes, namely dispersal, selection, drift and diversification (Figure 1) which includes both niche-based processes driven by fitness differences between organisms and neutral processes driven conversely by stochastic or chance events. In gut microbiome assembly, previous work has mainly aimed to elucidate deterministic processes to explain diversity patterns while often overlooking stochastic ecological elements. However, recent advances in microbiome ecology increasingly acknowledge that both deterministic and stochastic forces operate in tandem to shape microbial community assembly dynamics in the gut (63).

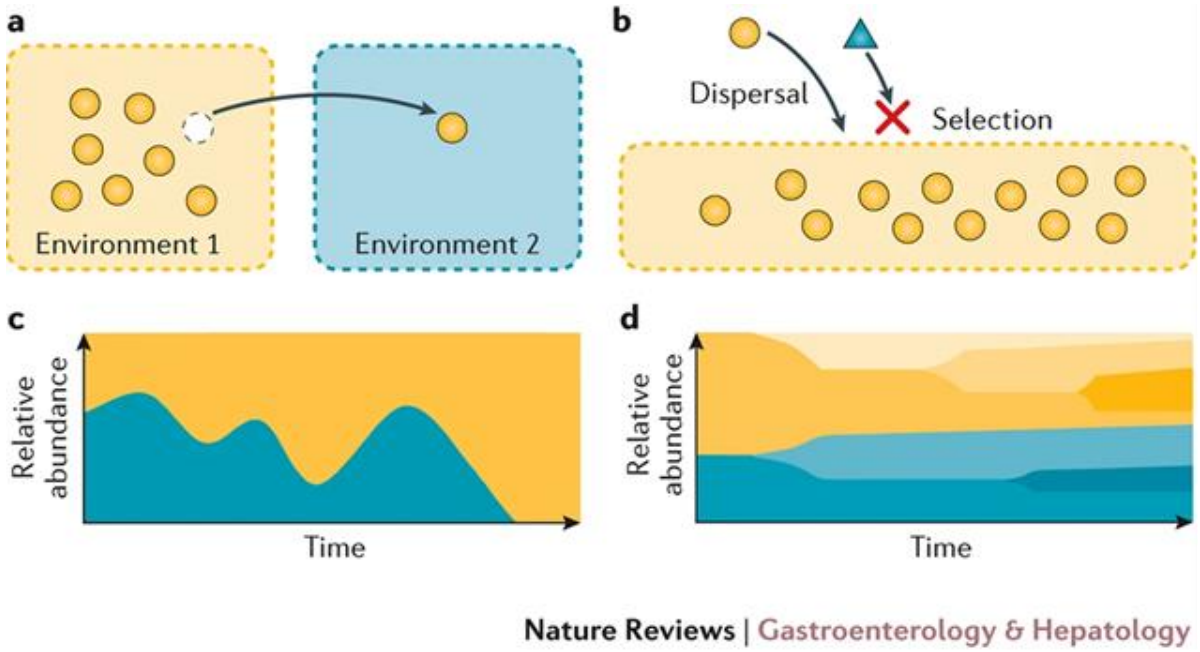

Figure 1: Schematic overview of the four processes categorized by Vellend (2010) in the contribution of community assembly (a) dispersal of an organism from one environment to another (b)selection pressures in the environment driving the selection of one species over the other (c) stochastic events driving changes in the distribution of species across time and (d) mutation and recombination over time within a population resulting in the appearance of new strains. Retrieved from Sprokett et al. 2018.

Acquisition of the gut microbiome via dispersal

It is largely assumed that the infant gut is sterile *in utero*, thereby marking birth the start point for microbial acquisition and microbiome assembly (64). In early life, the gastrointestinal tract of newborns represent empty habitat patches rich in space and resources, open for rapid colonization (65). Initial colonization of microbes into the host gut is known to occur primarily during birth, when infants pass through the birth canal of the mother. During this process, the infant is exposed to various maternal fluids, including amniotic fluid, vaginal secretions and maternal fecal bacteria which then serve as the primary inoculum for gut colonization (66–68). Infants delivered vaginally through the birth canal tend to acquire microbial species largely of maternal origin such as *Lactobacillus*, *Prevotella*, *Bifidobacterium* or *Atopobium* spp. which are dominant members of the vaginal microbiome (69). In contrast, studies show that human babies delivered by cesarian section often display microbes more prevalent on the skin and hospital environment including *Staphylococcus* spp., *Propionibacterium* spp. and *Corynebacterium* spp. (69,70). Beyond birth mode, skin contact, breast feeding, use of formula, antibiotics and environmental interactions contribute to seeding of the human infant gut (71). Additionally, indigenous cultural practices associated with raising infants, collectively referred to as infant-care-associated behaviors (ICABs) (63) which include customs such as administration of pre-lacteal foods (honey, ghee, jaggery) (72,73), anointing newborns with oils (74), premasticating solid foods before feeding (75) have been indicated in microbial acquisition during early life.

While early life events play a critical role in establishing the foundational gut microbiome, microbial acquisition continues throughout adulthood, in response to environmental exposures, lifestyle factors and dietary choices. During their life span, organisms are constantly exposed to new microbial taxa through diverse sources such as food, water, pets, social circle, occupational settings and interpersonal contact (76). Among these, diet plays a particularly significant role (77) in shaping the gut microbiome, with studies providing evidence for significant gut compositional shifts within 24h of initiation of a new diet (23). In addition, the use of antibiotics, onset of diseased states and infections (26,78) can often disrupt microbial homeostasis, creating opportunities for new microbial members to establish or for pathogenic organisms to dominate. Thus variations in these factors often result in variation in dispersal which can in turn bring about differences in gut microbiome composition.

Ecological selection during gut microbiome assembly

Once the microbes have been introduced, the question arises: how do they survive host habitat filters such as stomach acidity and immune defenses to successfully establish in the gut. Given that properties of bacteria can have negative consequences on host health, the immune system is designed to select against these elements. The host innate immune response is thereby developed to act as the first line of defense against incoming microbes (79). Genome-wide association studies have reported significant correlations between gut microbiome composition and host immune responses (80–82), indicating the active role of host genetic factors in the contribution to gut community composition. Additionally, the specificity and memory of adaptive immunity, further refines these responses to maintain a balance between tolerance and defense (83,84). Direct manipulations of the adaptive immune system have been linked to changes in gut microbiome composition (85–87) shedding light on the influence of the host in the selection of gut microbiota.

Ingested microbes enter through the oral cavity and reach the stomach via the esophagus, where they are subjected to a highly acidic stomach environment designed to limit microbial entry into the lower intestinal tracts. Much of what is currently known about gut microbial colonization is based on studies in humans, which serve as a valuable reference system for understanding general mechanisms of microbial community assembly and host interactions. Unlike adults which exhibit extremely acidic pH (88), the gastric pH of newborns at birth is typically neutral to alkaline (89) which allows for microbes to survive the passage through the stomach. Moreover, the transit time along the stomach is relatively short which may potentially reduce the exposure time to acid, giving the microbes an increased chance for survival. From the stomach, the microbes then reach the intestines which goes on to become the permanent residence for most of these microbes. In contrast to the stomach, the intestines harbor an increased diversity of microbes, with the bacterial diversity and density gradually increasing from the small to the large intestines (90). However, it is not until the ileum, where the peristaltic activity and acidity is reduced, that the microbial abundance and diversity surges significantly (91). In the colon, the slow transit time and availability of nutrients that bypass enzymatic digestion in the small intestine, support the establishment of the most abundant microbial communities (92) that eventually become permanent members of the gut community. The intestinal epithelial cells (IEC) produce antimicrobial peptides (AMPs) which include defensins, cathelicidins, ribonucleases, bacteriocins (93) and large-scale secretory immunoglobulin A

(IgA) which then serve to either limit or encourage the growth of microbial species in the gut (94). In the large intestine, the soluble mucins on the outer layer of mucus lining the IECs play a key role in shaping gut microbiota by providing a nutrient source and binding site for colonizing species. Gut bacterial species capable of expressing enzymes such as glycoside and polysaccharide hydrolases necessary for the utilization of mucins are thus able to reside in the gut (95). For a newborn, however, the immune system is still undergoing development, which releases the constraints of the host habitat filters during colonization. As the infant matures, the adaptive immune system of the host shows improved activity, exerting increasing selection pressures on the microbial communities, causing previous homogenous communities to diverge into site-specific microbiota (65,96).

In addition, dietary inputs providing nutritional support can act as a key maternal habitat filter (97). Nutrients derived from breast milk and colostrum provide an important source of pre-biotics which fuel the survival of specific bacterial immigrants in the gut. Human milk oligosaccharides (HMOs) in breast milk, for instance, which comprises a diverse mix of unconjugated glycans, are not absorbed by infants and instead are digested by a subset of microbiota in the colon (98). These complex sugars in breast milk thus select for HMO degrading specialists thereby driving the dominance of primary fermenters belonging to the family *Bifidobacteriaceae* in breastfed infants (99). The occurrence of antimicrobial factors such as lysozymes, lactoferrins and secretory immunoglobulins (IgA) in breastmilk (100,101) further impose additional selection on the gut microbiome composition. Conversely, infants receiving formula milk which lack these bioactive components have been shown to exhibit an altered gut composition favoring pro-inflammatory taxa, increased gut permeability and reduced concentrations of fecal short chain fatty acids (102–104). As infants transition from breast milk to solid food rich in proteins and dietary fiber, there occur further changes in the microbiota, marked by an increased microbial richness and diversity that were positively associated with dietary variety (105,106). Additionally, studies have reported an increase in the relative abundance of *Bacteroidetes* (107,108) and a decline in *Bifidobacterium* species (109) during this period. These observations thus underscore strong diet-driven selection pressures that shape gut microbiome community structure from early life.

Such diet-induced alterations are not limited to infancy and childhood but are also known to occur in response to dietary patterns in adulthood. The type of food consumed including protein, fats, dietary fibers, probiotics and fermented foods has been shown to influence gut microbiome composition, demonstrating how microbes respond to nutrient-specific conditions. Across the dietary spectrum, the nature of the diet ranging from Western-style diet (typically rich in saturated fats, refined sugar and low in dietary fiber), Mediterranean diet (abundant in polyphenols, antioxidants and plant-derived fibers) or vegan and vegetarian diets enriched in fermented plant-based components all provide distinct nutrient substrates that selectively enrich for different microbial communities (77).

Microbial diversification and ecological drift

Diversification refers to the generation of novel genetic and phenotypic variation within microbial communities through mechanisms such as mutation and horizontal gene transfer (HGT). In the gut environment, diversification enables microbes to adapt to host-imposed selection pressures and exploit new ecological niches (46,48,110,111). Single nucleotide variations (SNVs) and horizontal gene transfers are particularly prominent in the gut microbiome, facilitating the spread of traits such as antibiotic resistance, carbohydrate utilization and immune evasion (112–115). Such diversification can occur in response to interactions with the host, microbe-microbe interactions or changes in the gut environmental conditions which can further impact microbiome composition and community assembly (43). These processes can lead to the emergence of functionally distinct strains from the common ancestor, thereby enhancing intra-species diversity and contributing to the overall functional plasticity of the gut ecosystem.

Lastly, ecological drift is an important stochastic factor for community assembly, which causes the relative abundances of species to fluctuate at random, resulting in changes in population size that occur regardless of species identity as predicted by the neutral theory of Hubbell (2001)(116). This process is distinct from genetic drift which refers to random changes in allele frequencies within populations over generations (117). Ecological drift thus represents key stochastic processes in gut community assembly, particularly during early colonization or following disturbances (63). Unlike selection, which is governed by deterministic fitness differences, ecological drift operates independently of adaptive traits and can strongly influence community structure when population sizes are small and competitive differences weak (62). In the gut, ecological drift is especially more pronounced during infancy, after antibiotic treatment and in germ-free animals which are all characterized by low microbial diversity and minimal competitive contexts (118). In such settings, small initial differences in the abundance of colonists or random survival events can lead to markedly different community outcomes, even under similar environmental conditions.

Priority effects in gut community assembly

While the Vellend model (62) provides a theoretical foundation for understanding community assembly, one particularly significant process operating within this framework is the phenomenon of *priority effects*, where the order and timing of species arrival into a habitat can significantly alter community trajectories and assembly (119). Such events, where the differential entry of species into the habitat influences succession trajectories in a community, have been studied in the past (120–122). As such, it has become increasingly clear that such historical contingencies can modulate species interactions thereby impacting the establishment success of late colonizers in the community (123). Furthermore, these effects have been shown to not only influence community structure such as species richness and composition, but also key ecosystem functions such as productivity, decomposition and nutrient and energy flow (124–126).

With increasing evidence for priority effects in community assembly, the role of species arrival order and timing becomes a crucial area of exploration for gut community assembly dynamics. In recent years, studies have provided clear evidence for priority effects in complex microbial communities and environments such as that of the gut microbiome, where such effects have been shown to influence microbiome assembly (118,127). For instance, the inoculation of recovery associated bacterial species (RABs) such as *Bacteroides thetaiotaomicron* and *Bifidobacterium adolescentis* into the gut of murine models post antibiotic treatment have been reported to synergistically provide a > 100-fold boost in microbial abundance and diversity (128). The inoculation of these colonizers into a disturbed habitat (post antibiotic treatment) can be viewed as a priority effect wherein their seeding in the gut was essential to trigger a chain of food-web interactions that eventually accelerated density recovery and eventually led to the recovery of the overall system (128). Likewise, Martinez et al. (2021) manipulated the inoculation order of microbial seed communities in the gut of germ-free mice to understand how colonization history and priority effects led to individuality in gut microbiome composition. Their study showed strong evidence for the occurrence of priority effects in the mouse gut which in turn produced a measurable alteration in the long-term community trajectory of the gut microbiome. Despite growing interest, the role of priority effects in gut microbiome assembly remains understudied, with empirical evidence remaining limited and scarce (63).

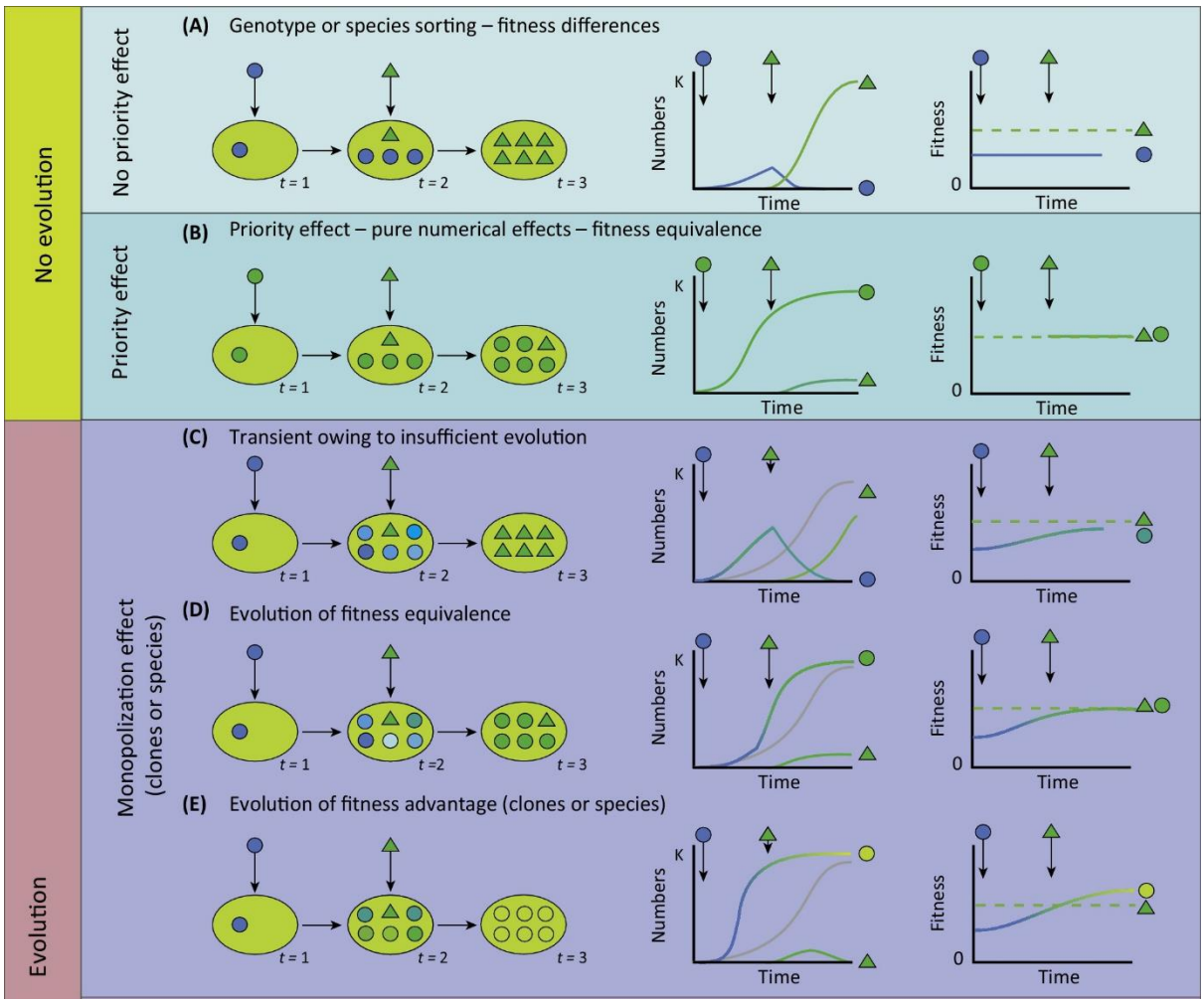

Figure 2: Fitness Differences, Adaptation, and the Time Interval between Arrivals of Genotypes or Species Determine the Opportunity for Monopolization Effects. Different genotypes or species are indicated as circles and triangles in the left column. The match between the colors of the colonists and the habitat indicates their relative fitness (i.e., green has higher fitness in the green habitat than blue). We show a single patch at three points in time. In the middle column, we show numbers of descendants of colonists and immigrants (genotypes or species) through time, with colonization and immigration events indicated by arrows. Line color indicates phenotype. The gray line indicates the baseline growth rate of the maladapted blue colonist for comparison. In the right column, we display the fitness of colonists (solid line) relative to the green pre-adapted immigrant (hatched line) for scenarios (A–E). Note that, for the case of sexual or hybridizing species, we reserve the right column to depict the frequencies of neutral loci. We assume no evolution in (A) and (B) and evolution in the rest of the scenarios. In (A), the green triangle increases and outcompetes the maladapted blue genotype or species despite arriving later (clonal or species sorting). In (B), the green circles establish before the green triangle arrives, and this numerical dominance lasts for potentially long periods via neutral genetic or community dynamics. In (C), the blue colonist adapts to the green habitat, which accelerates its rate of increase relative to a nonevolving colonist (gray line). It reduces the growth rate of the pre-adapted green immigrant and, thus, imposes a monopolization effect, but this effect is transient because, ultimately, the higher fitness of the later immigrant will allow it to dominate. Integrated from De Meester et al 2016.

Mechanisms of priority effects

Priority effects occur when microorganisms pre-empt or modify a given ecological niche and thereby alter the colonization ability of subsequent microbial immigrants (123). These mechanisms are termed niche pre-emption and niche modification which have been categorized as the two main processes driving priority effects in community assembly. Additionally, priority effects can be either inhibitory or facilitative in nature, either suppressing the growth and establishment success of late colonizers or promoting their colonization.

Niche pre-emption

Niche pre-emption occurs when the early arriving species gain priority to the untapped space and resources in the habitat, thereby exhibiting rapid population growth in an environment devoid of competitors. As the species grows and multiplies, this numerical advantage poses a challenge for late immigrants, as the carrying capacity of that habitat is saturated, limiting available space and resources necessary for late colonizers to survive. These priority effects are often inhibitory in nature and are hypothesized to be strong when niche-overlap is high between interacting species (123). As such, species have to be competitively similar for niche pre-emption to result in priority effects. In the murine gut, *Bacteroides* species arriving early are able to penetrate and saturate deep colonic crypts and sustain their survival in the gut. These protected niches being already saturated can no longer hold subsequently arriving species, that in turn are cleared by the murine immune response (127).

Local adaptation can additionally contribute to the stabilization of pre-emption driven priority effects as emphasized in the *monopolization* hypothesis (129). Local evolutionary adaptation of the early colonizers to the habitat can augment transient numerical priority effects by increasing or equalizing their fitness relative to the late immigrants. Due to this fitness advantage, the primary colonists are able to accelerate their initial rate of increase to better monopolize resources in the habitat, thereby promoting their long term dominance (129). The degree of this *monopolization* mediated by genetic adaptation is often dependent on a race between the local adaptation of early colonists and the immigration of more fit genotypes(129) which further emphasizes the importance of arrival timing in community assembly.

Niche modification

Niche modification occurs when primary colonists modify the environment in ways that either inhibits or benefits late arriving species. Thus through niche modification, early arriving species are able to alter available ecological niches within the habitat to influence which species can colonize the community later (123). Depending on their effects, the niche modification process can either be facilitative or inhibitory in nature. Facilitation, also known as *niche facilitative modification* (130) is often observed in host associated microbiomes through cross-feeding mechanisms, whereby strains in close physical proximity benefit from each other's metabolic by-products (131). In the context of priority effects, early arriving microbes that can degrade complex organic molecules into simpler components that others can use, can facilitate other species, thereby promoting their growth in the community (128,132). Such metabolic cooperation between members of the gut microbiome can thus lead to improved functional productivity and influence community assembly trajectories over time (133).

In contrast, inhibition or *niche inhibitory modification* (130) occurs when early arrivers modify the environment in ways that slows or prevents the establishment success of newcomers (120). In the gut, such effects are often mediated by the host immune response. For instance, gut bacterial phyla such as *Bacteroidetes* and *Firmicutes* produce short chain fatty acids that stimulate mucus and intestinal epithelial cells (IECs) to produce antimicrobial peptides (AMPs), limiting the successful colonization of invading pathogens (134). Conversely, pathogens like *Salmonella enterica* subsp. *enterica* serovar *typhimurium* has been reported to induce host immune responses to restructure the chemical and microbial environment of the gut over a 16-day period thereby suppressing the growth of beneficial gut symbionts (135).

Daphnia *as a model system*

The freshwater cladoceran genus *Daphnia* (Phylum Arthropoda; Subphylum Crustacea), commonly known as water fleas, is a well established model system extensively employed in ecology, evolution, ecotoxicology and reproductive biology (136–140). These planktonic crustaceans are ubiquitous in nature, found worldwide in freshwater ecosystems and brackish water bodies, including temporary pools, small ponds and large lakes (139,141). In such ecosystems, *Daphnia* is known to be of pivotal ecological importance, occupying a central position in aquatic food webs as highly efficient grazer of phytoplankton and preferred prey for planktivorous fish and invertebrate predators (138).

A hallmark feature of *Daphnia* is its phenotypic plasticity, often expressed in response to external environmental cues (142–144). For instance, some species alter diurnal migration behavior and develop extravagant morphological defense phenotypes in response to predators (145,146). Likewise, some species exhibit dramatic physiological changes such as the rapid upregulation of hemoglobin levels in response to environmental hypoxia (147). The most significant phenotypic plastic trait in *Daphnia* is their switch between asexual and sexual reproduction. This unique feature comprises a cyclical parthenogenetic reproductive cycle, which allows for both asexual and sexual reproduction in this organism often in response to environmental stimuli (148). A key advantage of this reproduction cycle is the production

of asexual diploid parthenogenetic eggs during favorable environmental conditions which result in the development of identical female clones (139). As a result, studies can employ isoclonal lines for the study of phenotypes while excluding genetic variation, thereby allowing the separation of genetic from non-genetic effects. Conversely, their ability to switch to sexual reproduction to produce dormant resting eggs during unfavorable conditions allows for studies in resurrection ecology, providing insights into the evolutionary history of the populations (149). These studies have revealed the potential of different *Daphnia* species to undergo rapid adaptive evolution to diverse environmental selection pressures such as predation pressure, toxic cyanobacteria, parasite infection and heavy metal pollution (60,150–152). Lastly, their short generation time, large brood sizes, ease of culture and low maintenance cost in the laboratory make them a widely used model organism across a broad range of fields (138,139).

Compared to other crustacean species, relatively large genomic resources are available for *Daphnia*, with the first publication of the genome sequence of *Daphnia pulex* in 2011 (153), followed by several genomes of other *Daphnia* species being released in previous years (154,155). With enhanced genetic knowledge and information, their utility as model systems in the fields of ecological, evolutionary and environmental genomics has risen significantly. The total number of annotated genes is 15,721 for *D. magna* (154) and approximately 30,907 for *D. pulex* (153). The high number of functional genes in *Daphnia*, which is estimated to be 30% higher than in humans and 3 times higher than in other invertebrates is assumed to contribute to *Daphnia's* ecological and evolutionary success by providing the phenotypic flexibility needed to rapidly respond and adapt to environmental change (139,153).

The gut system of Daphnia

The alimentary canal of *Daphnia* comprises a simplified system which consists of a straight tube with a single pair of blind-end caeca (156). The gut constitutes mainly three parts: a foregut or esophagus, a midgut section or a mesenteron carrying a pair of short anterior diverticula and a short hindgut or rectum. The foregut or the esophagus originates from the embryonic ectoderm and comprises of low cuboidal epithelial cells, the apical surface of which is covered by a 1-2μm thick flexible chitinous intima (cuticle lining) which separates the gut lumen from the epithelial cells (156). The esophagus is surrounded by circular muscle with striated myofibrils typical of body wall musculature, which controls the foregut movement together with numerous dorso-ventral dilator muscles that insert at right angles to the long axis of the tube (156). The midgut or the mesentron, the primary site of digestion and nutrient absorption, has a different developmental origin arising from endodermal cells. This region consists of simple columnar to cuboidal epithelial cells, their apical cell surface being modified as a regular border of microvilli throughout the entire length of the midgut. This region is not cuticle lined. Instead, the gut epithelium is enclosed by a felt-like peritrophic membrane which is present surrounding the portion of the midgut posterior to the digestive caeca (156). This membrane is a consistent feature of crustacean digestive systems, documented in 14 different orders (157), being structurally formed of chitinous microfibrils embedded in a protein-mucopolysaccharide matrix (157). A similar structure is also observed in many insects, where midgut epithelial cells secrete a semi-permeable envelope otherwise known as peritrophic membrane/matrix which is typically continuously renewed as it is shed (158). The peritrophic matrix partitions the midgut into the endo-and ectoperitrophic spaces, confining microorganisms to the former, preventing their direct contact with the midgut epithelial cells (158). Functionally, this layer is key in separating food and microbes in the lumen from the apical surface of the epithelial cells. The paired digestive anterior diverticula in the midgut are simple evaginations of the midgut with the wall of each caecum consisting of a simple epithelial layer, a thick beaded basement lamina and a peripheral muscle network. The hindgut located at the distal end of the post abdomen is short, lined by a chitinous intima and formed of simple cuboidal epithelium similar in structure to the foregut (156). The hindgut is surrounded by *constrictores recti* muscles (159) that extend toward the posterior mid-gut region. The anus is at the terminal end and is controlled by dilator muscles (159).

As such, in terms of microbial colonization, micro-organisms in the gut of *Daphnia* are most prominently located in the lumen (Figure 3A) and found attached to the microvilli of the midgut section (Figure 3B,C) (160). In the midgut, more specifically, they are likely to be detected within the endoperitrophic space, closely associated with food particles. Additionally, the physicochemical conditions can also vary along the gut, creating distinct microenvironments; for instance, in *Daphnia*, the pH ranges from 6 to 6.8 in the anterior midgut and 6.6 to 7.2 in the posterior midgut (161) which may harbor differential microbial communities in different gut regions.

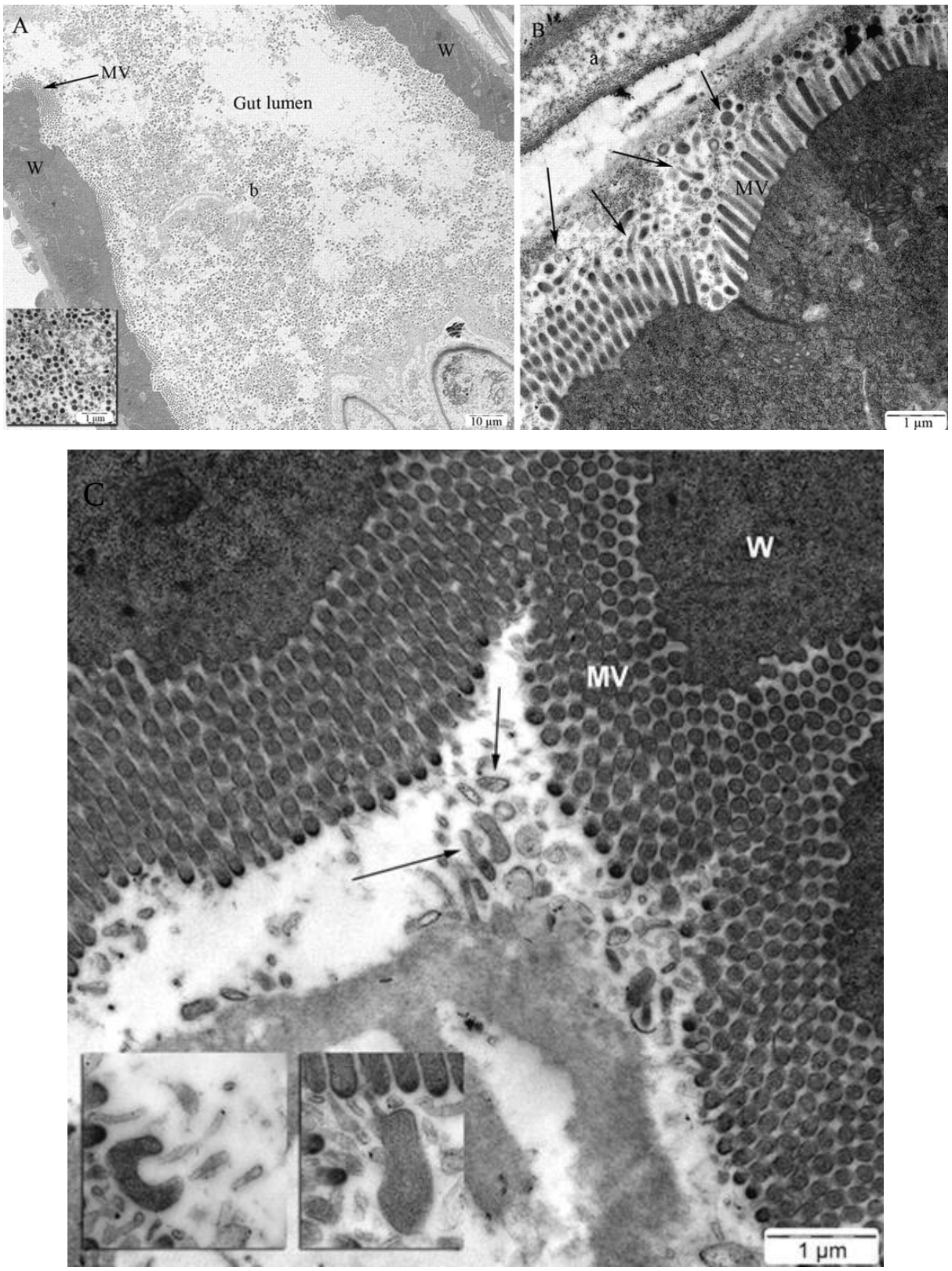

Figure 3: TEM micrographs of a cross-section of the gut of *D. pulex* showing colonizing bacteria (arrows) at (A) 4000X (B) 20,000X and (C) 20,000X magnification. MV: Microvilli; W: Gut wall; b: bacteria (160).

Unlike higher order organisms carrying anaerobic gut environments, the anatomical characteristics of the *Daphnia* gut, namely in terms of its short length, narrow diameter (< 200µm), high surface-to-volume ratio and rapid transit time for food particles (approximately 30 minutes of retention time) indicate that oxygen is likely present along the entire length of the gut. This assumption is further supported by microelectrode-based measurements of oxygen profiles across the gut (162) which suggest that the *Daphnia* gut microbiome predominantly comprise aerobic and facultatively anaerobic

microbes which is in contrast to the fermentative and obligately anaerobic microbes typical of anoxic guts in higher organisms.

While the *Daphnia* gut system does not fully replicate complex host-microbiome systems reflective of higher order organisms, it represents a unique model system that nonetheless offers tractability in design and execution for gut microbiome studies. Unlike vertebrate systems which rely on the use of fecal samples or gut regurgitates, the gut of *Daphnia* can be entirely dissected (Figure 4) for a comprehensive overview of its contents. As a result, these systems offer critical analysis of in-situ components such as spatial structure, host-specific factors and dynamic host-microbiota interactions (163) that are generally inaccessible in models reliant on fecal sampling. Fecal-based samples, while common, tend to provide only partial insights into the interactions occurring in the lumen of the gut, with significant interactions in the cryptic niches, mucus-associated interactions and proximal regions of the gut often being overlooked (17,63).

In addition, the generation of germ-free animals is often necessary for gut microbiome studies which enables the experimental manipulation of microbial communities in the gut to study their effects on gut microbiome assembly. In this context, *Daphnia* serves as a particularly suitable model, with well established and standardized protocols available for generating germ-free animals (53,164). Following germ-free rearing, the controlled introduction of microbial species into the gut becomes the next critical step. Given the generalist non-selective filter-feeding nature of *Daphnia* (165,166), this process is greatly simplified in *Daphnia,* allowing for the rapid uptake of free-living microbes from their surrounding waters. As *Daphnia* feed, microbes from the ambient water are ingested via the filtering apparatus (165). The microbes then colonize the gut and typically appear throughout the entire length of the gut and more concentrated around the microvilli (160). The transparent carapace of *Daphnia* allows for the real-time visualization of their feeding and facilitates the tracking of fluorescently-labeled bacteria in the gut, offering a powerful platform for investigating gut colonization. Additionally, their parthenogenetic reproduction cycle allows for the production of genetically identical clones that is crucial in microbiome studies to dissect interactions between genotype × environment interactions. These features thus make *Daphnia* a suitable model system for investigating host-microbe interactions, offering an ideal system for experimental research in gut microbiome ecology and evolution.

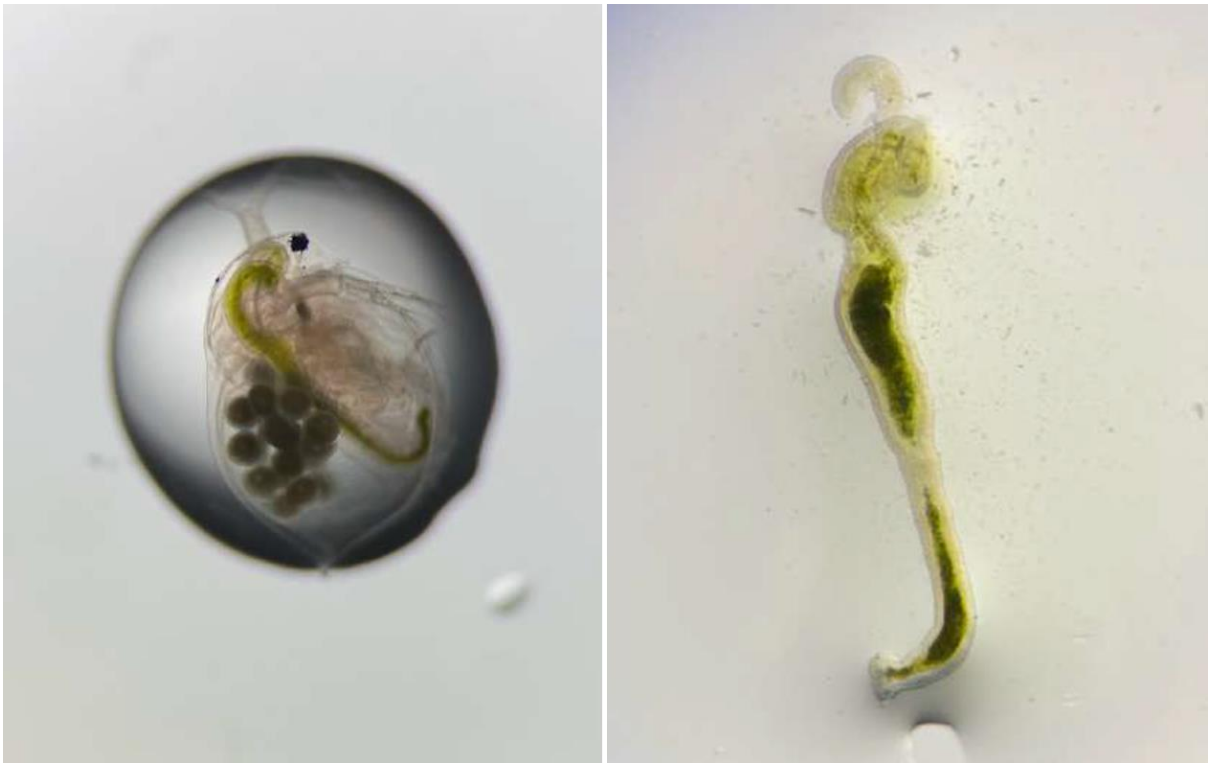

Figure 4: Extraction of the gut from *D. magna*. *Daphnia* before gut extraction (left) the extracted gut following dissection (right). Photo credit: Cato Curias.

Thesis outline

Despite growing interest in priority effects in community assembly, direct empirical evidence on the occurrence of these processes in complex and dynamic systems such as that of the gut microbiome remains limited and largely circumstantial. While a few studies have highlighted the potential of priority effects in gut microbiome assembly, comprehensive experimental investigations still remain scarce. In my thesis, I thus aimed to address this gap by investigating the role of priority effects in community assembly of the gut microbiome and the implications of such priority effects on both host fitness and on an extended phenotype, being community composition of free-living microbial communities in the medium using *Daphnia* as a model system. Although vertebrate models have traditionally dominated microbiome research, there has been a rising interest in the use of alternative models such as the invertebrate system *Daphnia* owing to their suitability in the design of large-scale, cost-effective experiments, along with the unique advantage of working with clones, which enables experimental designs that standardize or manipulate genetic identity and diversity. To further our understanding on the importance of priority effects for gut microbiome assembly, five research questions were addressed in this thesis:

(1) Does *Daphnia* have a core microbiome?

(2) How do priority effects shape gut microbiome assembly?

(3) Do compositional changes in the microbiome driven by species arrival order in the host gut translate into fitness differences in the host?

(4) Are priority effects in the gut microbiome (partially) evolution-driven?

(5) Do priority effects in the host community cascade to second-order effects at the level of the community assembly of free-living microbial communities in the medium?

These five questions translate to the following four research chapters (Figure 5):

With the emerging use of *Daphnia* as a model system for studying host-microbiota interactions, gaining a clear view on the taxon composition of its gut microbiome, particularly the core taxa that consistently associate with the host, is essential. In my first chapter, I thus explore the concept of the *core microbiome* in *D. magna*. More specifically, I test whether *D. magna* is characterized by a microbiome comprising species shared by most individuals or whether the microbiome is highly variable among individuals without a real core of recurring microbial taxa. To test for the existence of a core microbiome, I systematically analyzed an integrated dataset compiled from 12 published studies comprising sequences of the V4 hypervariable region of the 16S rRNA gene of both *D. magna* gut and whole microbiome samples of both laboratory and field origin across five countries.

In my second chapter, I experimentally manipulated the arrival order of bacterial strains into germ-free *D. magna* to 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, I 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 I 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, I selected three strain pairs for the experiment. 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, either simultaneously, with a (reciprocal) time-lag of 48h, or in isolation. The gut microbiome composition and four host life history traits were determined after 8 and 12 days. With this design, I explored (1) whether inoculation order of individual gut bacterial strains influences gut composition in adult *Daphnia*; (2) to what extent these effects were bacterial strain-dependent, i.e., depend on the identity of the inoculated bacterial strains, (3) or host genotype-dependent, i.e., 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.

In chapter 3, I investigate the role of bacterial evolution within the host and the consequent effect of strain evolution in mediating priority effects during gut community assembly. I first conducted an experimental evolution trial. Two bacterial strains originally isolated from the gut of one *Daphnia* genotype (LRV4) were allowed to grow and adapt for 12 days in the gut environment of a second *Daphnia* host genotype (O1-08) to allow within-host adaptation. These pre-exposed strains were subsequently re-isolated and used in a priority effects experiment, in which their inoculation order

into the host gut was manipulated to assess to what extent species arrival order influences gut microbiome assembly. I varied inoculation order treatments across four bacterial strain pair combinations comprising of both ancestral strains, both evolved strains and two mixed pairs comprising one ancestral and one evolved strain. With this design, I thus sought to determine whether within-host adaptation of bacterial strains strengthens priority effects in the gut thereby contributing to gut community assembly.

In my fourth chapter, I test for both the degree to which microbiome-mediated conditioning of the medium can impact priority effects at the host community level and to what extent priority effects at the host community level can mediate community assembly of free living microbial communities in the medium. This experiment was inspired by an earlier study, in which clear-cut priority effects among large-bodied zooplankton species were observed (Louette et al 2006). We here tested one of the possible mechanisms that can potentially explain this strong priority effect, which is a fitness advantage of the first arriving species through beneficial conditioning of the medium with bacterial strains that confer a fitness advantage to the species. In this experiment, I employed an experimental community assembly approach using two freshwater zooplankton species, namely, *D. magna* and *D. pulicaria* to specifically assess (i) if arrival order of the zooplankton species imposes priority effects between the two species, (ii) whether altering the identity of the host-conditioned medium influences the strength and direction of the priority effects, and if so, (iii) which fraction of the conditioned medium (the microbial component, the chemical/metabolic fraction, or a combination of both) has the largest effect, and iv) whether priority effects at the host level or medium conditioning by the host influence microbial community assembly at the bacterioplankton level, testing for a second-order effect of host priority effect on an extended phenotype, being the community composition of another taxon in the environment.

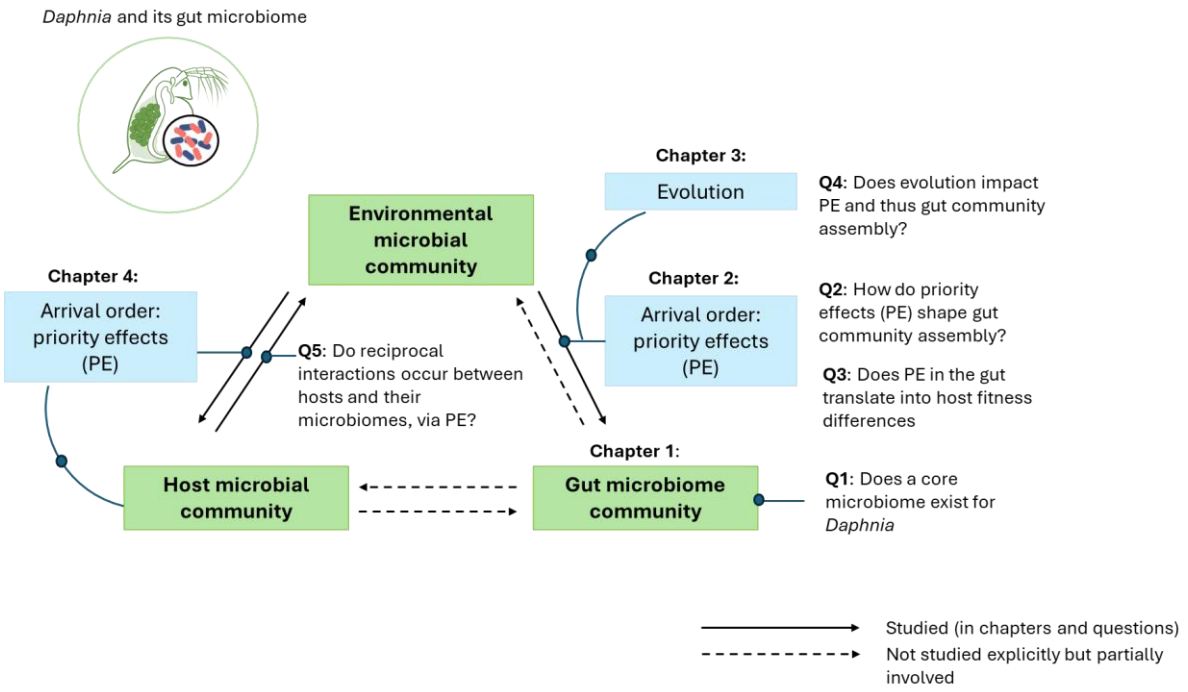

Figure 5: Schematic overview of thesis chapters illustrating conceptual links.

References

1. Quigley EMM. Gut Bacteria in Health and Disease. Gastroenterol Hepatol (N Y). 2013 Sep;9(9):560–9.

2. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLOS Biology. 2016 Aug 19;14(8):e1002533.

3. Bull MJ, Plummer NT. Part 1: The Human Gut Microbiome in Health and Disease. Integr Med (Encinitas). 2014 Dec;13(6):17–22.

4. de Vos WM, Tilg H, Van Hul M, Cani PD. Gut microbiome and health: mechanistic insights. Gut. 2022 May;71(5):1020–32.

5. Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. BMJ. 2018 Jun 13;361:k2179.

6. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol. 2013 Jul;14(7):685–90.

7. van Schaik W. The human gut resistome. Philosophical Transactions of the Royal Society B: Biological Sciences. 2015 Jun 5;370(1670):20140087.

8. Khan I, Bai Y, Zha L, Ullah N, Ullah H, Shah SRH, et al. Mechanism of the Gut Microbiota Colonization Resistance and Enteric Pathogen Infection. Front Cell Infect Microbiol 2021 Dec 23;11:7162999.

9. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The Central Nervous System and the Gut Microbiome. Cell. 2016 Nov 3;167(4):915–32.

10. Sampson TR, Mazmanian SK. Control of Brain Development, Function, and Behavior by the Microbiome. Cell Host & Microbe. 2015 May 13;17(5):565–76.

11. Kim GH, Shim JO. Gut microbiota affects brain development and behavior. Clin Exp Pediatr. 2022 Nov 8;66(7):274–80.

12. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome. 2019 Dec;7(1):1–15.

13. Gomaa EZ. Human gut microbiota/microbiome in health and diseases: a review. Antonie van Leeuwenhoek. 2020 Dec 1;113(12):2019–40.

14. LeBlanc JG, Milani C, de Giori GS, Semsa F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol. 2013 Apr;24(2):160-8.

15. LeBlanc, J.G., Chain, F., Martín, R. *et al.* Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microb Cell Fact 16, 79 (2017)

16. Suzuki TA, Ley RE. The role of the microbiota in human genetic adaptation. Science. 2020 Dec 4;370(6521):eaaz6827.

17. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The Treatment-Naive Microbiome in New-Onset Crohn's Disease. Cell Host Microbe. 2014 Mar 12;15(3):382–92.

18. Jie Z, Xia H, Zhong SL, Feng Q, Li S, Liang S, et al. The gut microbiome in atherosclerotic cardiovascular disease. Nat Commun. 2017 Oct 10;8(1):845.

19. Sharma S, Tripathi P. Gut microbiome and type 2 diabetes: where we are and where to go? J Nutr Biochem. 2019 Jan 1;63:101–8.

20. Zhu X, Li B, Lou P, Dai T, Chen Y, Zhuge A, et al. The Relationship Between the Gut Microbiome and Neurodegenerative Diseases. Neurosci Bull. 2021 Oct 1;37(10):1510–22.

21. Fan P, Bian B, Teng L, Nelson CD, Driver J, Elzo MA, et al. Host genetic effects upon the early gut microbiota in a bovine model with graduated spectrum of genetic variation. ISME J. 2020 Jan;14(1):302–17.

22. Macke E, Callens M, Massol F, Vanoverberghe I, De Meester L, Decaestecker E. Diet and Genotype of an Aquatic Invertebrate Affect the Composition of Free-Living Microbial Communities. Front Microbiol 2020 Mar 17; 11:380.

23. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan;505(7484):559–63.

24. Smith CC, Snowberg LK, Gregory Caporaso J, Knight R, Bolnick DI. Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. ISME J. 2015 Nov;9(11):2515–26.

25. Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C, Stalder G, et al. Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. Nat Commun. 2019 May 16;10(1):2200.

26. Hasan N, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ. 2019 Aug 16;7:e7502.

27. Yuan X, Chen R, Zhang Y, Lin X, Yang X. Gut microbiota: effect of pubertal status. BMC Microbiology. 2020 Nov 3;20(1):334.

28. Pérez-Cobas AE, Gosalbes MJ, Friedrichs A, Knecht H, Artacho A, Eismann K, et al. Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut. 2013 Nov 1;62(11):1591–601.

29. Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, et al. Population-level analysis of gut microbiome variation. Science. 2016 Apr 29;352(6285):560–4.

30. Hairston Jr NG, Ellner SP, Geber MA, Yoshida T, Fox JA. Rapid evolution and the convergence of ecological and evolutionary time. Ecology letters. 2005;8(10):1114–27.

31. Urban MC, Leibold MA, Amarasekare P, De Meester L, Gomulkiewicz R, Hochberg ME, et al. The evolutionary ecology of metacommunities. Trends in ecology & evolution. 2008;23(6):311–7.

32. Schoener TW. The newest synthesis: understanding the interplay of evolutionary and ecological dynamics. science. 2011;331(6016):426–9.

33. Hendry AP. Eco-evolutionary dynamics. Princeton university press; 2016.

34. Hendry AP. Introduction and conceptual framework. In: Eco-Evolutionary Dynamics. Princeton University Press; 2016. p. 1–25.

35. Matthews B, De Meester L, Jones CG, Ibelings BW, Bouma TJ, Nuutinen V, et al. Under niche construction: an operational bridge between ecology, evolution, and ecosystem science. Ecological Monographs. 2014;84(2):245–63.

36. De Meester L, Vanoverbeke J, Kilsdonk LJ, Urban MC. Evolving perspectives on monopolization and priority effects. Trends in Ecology & Evolution. 2016;31(2):136–46.

37. Bassar RD, Marshall MC, López-Sepulcre A, Zandonà E, Auer SK, Travis J, et al. Local adaptation in Trinidadian guppies alters ecosystem processes. Proceedings of the National Academy of Sciences. 2010;107(8):3616–21.

38. Koskella B, Brockhurst MA. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS microbiology reviews. 2014;38(5):916–31.

39. Pantel JH, Duvivier C, Meester L De. Rapid local adaptation mediates zooplankton community assembly in experimental mesocosms. Ecology letters. 2015;18(10):992–1000.

40. Becks L, Ellner SP, Jones LE, Hairston Jr NG. The functional genomics of an eco-evolutionary feedback loop: linking gene expression, trait evolution, and community dynamics. Ecology letters. 2012;15(5):492–501.

41. Comeault AA, Flaxman SM, Riesch R, Curran E, Soria-Carrasco V, Gompert Z, et al. Selection on a genetic polymorphism counteracts ecological speciation in a stick insect. Current Biology. 2015;25(15):1975–81.

42. Hand BK, Lowe WH, Kovach RP, Muhlfeld CC, Luikart G. Landscape community genomics: understanding eco-evolutionary processes in complex environments. Trends in Ecology & Evolution. 2015;30(3):161–8.

43. Decaestecker E, Van de Moortel B, Mukherjee S, Gurung A, Stoks R, De Meester L. Hierarchical Eco-Evo Dynamics Mediated by the Gut Microbiome. Trends Ecol Evol. 39.2 (2024): 165-174.

44. Lynch JB, Hsiao EY. Microbiomes as sources of emergent host phenotypes. Science 2019 Sep 27 ;365(6460):1405-1409. doi: 10.1126/science.aay0240. PMID: 31604267.

45. Groussin M, Poyet M, Sistiaga A, Kearney SM, Moniz K, Noel M, et al. Elevated rates of horizontal gene transfer in the industrialized human microbiome. Cell. 2021 Apr 15;184(8):2053-2067.e18.

46. Matamouros S, Hayden HS, Hager KR, Brittnacher MJ, Lachance K, Weiss EJ, et al. Adaptation of commensal proliferating Escherichia coli to the intestinal tract of young children with cystic fibrosis. Proceedings of the National Academy of Sciences. 2018 Feb 13;115(7):1605–10.

47. Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev. 2013 Apr;26(2):185–230.

48. Ghalayini M, Launay A, Bridier-Nahmias A, Clermont O, Denamur E, Lescat M, et al. Evolution of a Dominant Natural Isolate of Escherichia coli in the Human Gut over the Course of a Year Suggests a Neutral Evolution with Reduced Effective Population Size. Appl Environ Microbiol. 2018 Mar 1;84(6):e02377-17.

49. Dapa T, Ramiro RS, Pedro MF, Gordo I, Xavier KB. Diet leaves a genetic signature in a keystone member of the gut microbiota. Cell Host & Microbe. 2022 Feb 9;30(2):183-199.e10.

50. Hehemann JH, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature. 2010 Apr;464(7290):908–12.

51. Chatzidaki-Livanis M, Geva-Zatorsky N, Comstock LE. Bacteroides fragilis type VI secretion systems use novel effector and immunity proteins to antagonize human gut Bacteroidales species. Proceedings of the National Academy of Sciences. 2016 Mar 29;113(13):3627–32.

52. Leibold MA, Govaert L, Loeuille N, De Meester L, Urban MC. Evolution and Community Assembly Across Spatial Scales. Annu Rev Ecol Evol Sys. 2022;53(1):299–326.

53. Callens M, Watanabe H, Kato Y, Miura J, Decaestecker E. Microbiota inoculum composition affects holobiont assembly and host growth in Daphnia. Microbiome. 2018 Mar 22;6(1):56.

54. Schwarzer M, Gautam UK, Makki K, Lambert A, Brabec T, Joly A, et al. Microbe-mediated intestinal NOD2 stimulation improves linear growth of undernourished infant mice. Science. 2023 Feb 24;379(6634):826–33.

55. King KC, Brockhurst MA, Vasieva O, Paterson S, Betts A, Ford SA, et al. Rapid evolution of microbe-mediated protection against pathogens in a worm host. The ISME Journal. 2016 Aug 1;10(8):1915–24.

56. Ford SA, King KC. In Vivo Microbial Coevolution Favors Host Protection and Plastic Downregulation of Immunity. Molecular Biology and Evolution. 2021 Apr 1;38(4):1330–8.

57. Lemoine MM, Engl T, Kaltenpoth M. Microbial symbionts expanding or constraining abiotic niche space in insects. Current Opinion in Insect Science. 2020 Jun 1;39:14–20.

58. Janssens L, Van de Maele M, Delnat V, Theys C, Mukherjee S, De Meester L, et al. Evolution of pesticide tolerance and associated changes in the microbiome in the water flea *Daphnia magna*. Ecotoxicology and Environmental Safety. 2022 Jul 15;240:113697.

59. Koch H, Schmid-Hempel P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proceedings of the National Academy of Sciences. 2011 Nov 29;108(48):19288–92.

60. Decaestecker E, Gaba S, Raeymaekers JAM, Stoks R, Van Kerckhoven L, Ebert D, et al. Host–parasite 'Red Queen' dynamics archived in pond sediment. Nature. 2007 Dec;450(7171):870–3.

61. Houwenhuyse S, Stoks R, Mukherjee S, Decaestecker E. Locally adapted gut microbiomes mediate host stress tolerance. ISME J. 2021 Aug;15(8):2401–14.

62. Vellend M. Conceptual Synthesis in Community Ecology. The Quarterly Review of Biology. 2010 Jun;85(2):183–206.

63. Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat Rev Gastroenterol Hepatol. 2018 Apr;15(4):197–205.

64. Chavez-Arroyo A, Radlinski LC, Bäumler AJ. Principles of gut microbiota assembly. Trends in Microbiology. 2025 Jul 1;33(7):718–26.

65. Costello EK, Carlisle EM, Bik EM, Morowitz MJ, Relman DA. Microbiome Assembly across Multiple Body Sites in Low-Birthweight Infants. mBio. 2013 Oct 29;4(6):10.1128/mbio.00782-13.

66. Neu J, Rushing J. Cesarean versus Vaginal Delivery: Long term infant outcomes and the Hygiene Hypothesis. Clin Perinatol. 2011 Jun;38(2):321–31.

67. Wassenaar TM, Panigrahi P. Is a foetus developing in a sterile environment? Letters in Applied Microbiology. 2014 Dec 1;59(6):572–9.

68. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep. 2016 Mar 22;6(1):23129.

69. Palmer C, Bik, E. M., DiGiulio, D. B., Relman, D. A., & Brown, P. O. Development of the human infant intestinal microbiota. *PLoS biology*, 2007, 5.7: e177.

70. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences. 2010 Jun 29;107(26):11971–5.

71. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. Science Translational Medicine. 2016 Jun 15;8(343):343ra82-343ra82.

72. Choudhry UK. Traditional Practices of Women From India: Pregnancy, Childbirth, and Newborn Care. Journal of Obstetric, Gynecologic, & Neonatal Nursing. 1997;26(5):533–9.

73. McKenna KM, Shankar RT. The Practice of Prelacteal Feeding to Newborns Among Hindu and Muslim Families. Journal of Midwifery & Women's Health. 2009;54(1):78–81.

74. Darmstadt GL, Saha SK, Ahmed ASMNU, Choi Y, Chowdhury MAKA, Islam M, et al. Effect of Topical Emollient Treatment of Preterm Neonates in Bangladesh on Invasion of Pathogens Into the Bloodstream. Pediatr Res. 2007 May;61(5):588–93.

75. Han CS, Martin MA, Dichosa AEK, Daughton AR, Frietze S, Kaplan H, Gurven MD, Alcock J. Salivary microbiomes of indigenous Tsimane mothers and infants are distinct despite frequent premastication. PeerJ. 2016 Nov 3;4:e2660. doi: 10.7717/peerj.2660. PMID: 27833819; PMCID: PMC5101600.

76. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature. 2018 Mar;555(7695):210–5.

77. Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med. 2017 Dec;15(1):1–17.

78. Iizumi T, Battaglia T, Ruiz V, Perez Perez GI. Gut Microbiome and Antibiotics. Archives of Medical Research. 2017 Nov 1;48(8):727–34.

79. Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. Nature. 2016 Jul;535(7610):65–74.

80. Kostic AD, Howitt MR, Garrett WS. Exploring host-microbiota interactions in animal models and humans. Genes Dev. 2013 Apr 1;27(7):701-18. doi: 10.1101/gad.212522.112. PMID: 23592793; PMCID: PMC3639412.

81. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic Determinants of the Gut Microbiome in UK Twins. Cell Host & Microbe. 2016 May 11;19(5):731–43.

82. Leamy LJ, Kelly SA, Nietfeldt J, Legge RM, Ma F, Hua K, et al. Host genetics and diet, but not immunoglobulin A expression, converge to shape compositional features of the gut microbiome in an advanced intercross population of mice. Genome Biol. 2014 Dec;15(12):1–20.

83. Stagaman K, Burns AR, Guillemin K, Bohannan BJM. The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. The ISME Journal. 2017 Jul 1;11(7):1630–9.

84. O'Riordan KJ, Moloney GM, Keane L, Clarke G, Cryan JF. The gut microbiota-immune-brain axis: Therapeutic implications. Cell Rep Med. 2025 Mar 6;6(3):101982.

85. Dimitriu PA, Boyce G, Samarakoon A, Hartmann M, Johnson P, Mohn WW. Temporal stability of the mouse gut microbiota in relation to innate and adaptive immunity. Environmental Microbiology Reports. 2013 Apr;5(2):200–10.

86. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3+ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis. Immunity. 2014 Jul 17;41(1):152–65.

87. Zhang H, Sparks JB, Karyala SV, Settlage R, Luo XM. Host adaptive immunity alters gut microbiota. The ISME Journal. 2015 Mar 1;9(3):770–81.

88. Martinsen TC, Bergh K, Waldum HL. Gastric Juice: A Barrier Against Infectious Diseases. Basic & Clinical Pharmacology & Toxicology. 2005;96(2):94–102.

89. Omari TI, Davidson GP. Multipoint measurement of intragastric pH in healthy preterm infants. Archives of Disease in Childhood - Fetal and Neonatal Edition. 2003 Nov 1;88(6):F517–20.

90. Mentella MC, Scaldaferri F, Pizzoferrato M, Gasbarrini A, Miggiano GAD. Nutrition, IBD and Gut Microbiota: A Review. Nutrients. 2020 Apr;12(4):944.

91. Wilson M. Microbial Inhabitants of Humans: Their Ecology and Role in Health and Disease. Cambridge University Press; 2005. 480 p.

92. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. J Med Microbiol. 2005 Nov;54(Pt 11):1093–101.

93. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic Bacteria Direct Expression of an Intestinal Bactericidal Lectin. Science. 2006 Aug 25;313(5790):1126–30.

94. Hasan N, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ. 2019;7:e7502.

95. Tailford LE, Crost EH, Kavanaugh D, Juge N. Mucin glycan foraging in the human gut microbiome. Front Genet. 2015;6:81.

96. Olm MR, Brown CT, Brooks B, Firek B, Baker R, Burstein D, et al. Identical bacterial populations colonize premature infant gut, skin, and oral microbiomes and exhibit different in situ growth rates. Genome Res. 2017 Apr;27(4):601–12.

97. Garrido D, Dallas DC, Mills DA. Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications. Microbiology. 2013;159(Pt_4):649–64.

98. Bode L. Human milk oligosaccharides: Every baby needs a sugar mama. Glycobiology. 2012 Sep 1;22(9):1147–62.

99. Solís G, de los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe. 2010 Jun 1;16(3):307–10.

100. Rogier EW, Frantz AL, Bruno ME, Wedlund L, Cohen DA, Stromberg AJ, Kaetzel CS. Lessons from mother: Long-term impact of antibodies in breast milk on the gut microbiota and intestinal immune system of breastfed

offspring. Gut Microbes. 2014;5(5):663-8. doi: 10.4161/19490976.2014.969984. PMID: 25483336; PMCID: PMC4615880.

101. Planer JD, Peng Y, Kau AL, Blanton LV, Ndao IM, Tarr PI, et al. Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. Nature. 2016 Jun;534(7606):263–6.

102. Huërou-Luron IL, Blat S, Boudry G. Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. Nutrition Research Reviews. 2010 Jun;23(1):23–36.

103. O'Sullivan A, Farver M, Smilowitz JT. Article Commentary: The Influence of Early Infant-Feeding Practices on the Intestinal Microbiome and Body Composition in Infants. Nutr Metab Insights. 2015 Jan 1;8s1:NMI.S29530.

104. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host & Microbe. 2015 May 13;17(5):690–703.

105. Homann CM, Rossel CAJ, Dizzell S, Bervoets L, Simioni J, Li J, et al. Infants' First Solid Foods: Impact on Gut Microbiota Development in Two Intercontinental Cohorts. Nutrients. 2021 Aug;13(8):2639.

106. Laursen MF, Sakanaka M, von Burg N, Mörbe U, Andersen D, Moll JM, et al. Bifidobacterium species associated with breastfeeding produce aromatic lactic acids in the infant gut. Nat Microbiol. 2021 Nov;6(11):1367–82.

107. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci USA. 2011 Mar 15;108(supplement_1):4578–85.

108. Vallès Y, Artacho A, Pascual-García A, Ferrús ML, Gosalbes MJ, Abellán JJ, et al. Microbial Succession in the Gut: Directional Trends of Taxonomic and Functional Change in a Birth Cohort of Spanish Infants. PLOS Genetics. 2014 Jun 5;10(6):e1004406.

109. Thompson AL, Monteagudo-Mera A, Cadenas MB, Lampl ML, Azcarate-Peril MA. Milk- and solid-feeding practices and daycare attendance are associated with differences in bacterial diversity, predominant communities, and metabolic and immune function of the infant gut microbiome. Front Cell Infect Microbiol. 2015 Feb 5;5:3. doi: 10.3389/fcimb.2015.00003. PMID: 25705611; PMCID: PMC4318912.

110. Lourenço M, Ramiro RS, Güleresi D, Barroso-Batista J, Xavier KB, Gordo I, et al. A Mutational Hotspot and Strong Selection Contribute to the Order of Mutations Selected for during Escherichia coli Adaptation to the Gut. PLOS Genetics. 2016 Nov 3;12(11):e1006420.

111. Zhao S, Lieberman TD, Poyet M, Kauffman KM, Gibbons SM, Groussin M, et al. Adaptive Evolution within Gut Microbiomes of Healthy People. Cell Host Microbe. 2019 May 8;25(5):656-667.e8.

112. Barroso-Batista J, Sousa A, Lourenço M, Bergman ML, Sobral D, Demengeot J, et al. The first steps of adaptation of Escherichia coli to the gut are dominated by soft sweeps. PLoS Genet. 2014 Mar;10(3):e1004182.

113. Yaffe E, Relman DA. Tracking microbial evolution in the human gut using Hi-C reveals extensive horizontal gene transfer, persistence and adaptation. Nat Microbiol. 2020 Feb;5(2):343–53.

114. Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ. Ecology drives a global network of gene exchange connecting the human microbiome. Nature. 2011 Dec;480(7376):241–4.

115. Shterzer N, Mizrahi I. The animal gut as a melting pot for horizontal gene transfer. Can J Microbiol. 2015 Sep;61(9):603–5.

116. Hubbell SP. Neutral Theory in Community Ecology and the Hypothesis of Functional Equivalence. Functional Ecology. 2005;19(1):166–72.

117. Crow JF. Sewall Wright (1889-1988). Genetics. 1988 May 1;119(1):1–4.

118. Martínez I, Maldonado-Gomez MX, Gomes-Neto JC, Kittana H, Ding H, Schmaltz R, et al. Experimental evaluation of the importance of colonization history in early-life gut microbiota assembly. eLife. 2018;7:e36521.

119. Chase JM. Community assembly: when should history matter? Oecologia. 2003 Aug 1;136(4):489–98.

120. Connell JH, Slatyer RO. Mechanisms of Succession in Natural Communities and Their Role in Community Stability and Organization. Am Nat. 1977 Nov;111(982):1119–44.

121. Alford RA, Wilbur HM. Priority Effects in Experimental Pond Communities: Competition between Bufo and Rana. Ecology. 1985;66(4):1097–105.

122. Grman E, Suding KN. Within-Year Soil Legacies Contribute to Strong Priority Effects of Exotics on Native California Grassland Communities. Restoration Ecology. 2010;18(5):664–70.

123. Fukami T, Mordecai EA, Ostling A. A framework for priority effects. J Veg Sci. 2016;27(4):655–7.

124. Dickie IA, Fukami T, Wilkie JP, Allen RB, Buchanan PK. Do assembly history effects attenuate from species to ecosystem properties? A field test with wood-inhabiting fungi. Ecology Letters. 2012;15(2):133–41.

125. Körner C, Stöcklin J, Reuther-Thiébaud L, Pelaez-Riedl S. Small differences in arrival time influence composition and productivity of plant communities. New Phytologist. 2008;177(3):698–705.

126. Tan J, Pu Z, Ryberg WA, Jiang L. Species phylogenetic relatedness, priority effects, and ecosystem functioning. Ecology. 2012;93(5):1164–72.

127. Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K, Mazmanian SK. Bacterial colonization factors control specificity and stability of the gut microbiota. Nature. 2013 Sep 19;501(7467):426–9.

128. Chng KR, Ghosh TS, Tan YH, Nandi T, Lee IR, Ng AHQ, et al. Metagenome-wide association analysis identifies microbial determinants of post-antibiotic ecological recovery in the gut. Nat Ecol Evol. 2020 Sep;4(9):1256–67.

129. De Meester L, Vanoverbeke J, Kilsdonk LJ, Urban MC. Evolving Perspectives on Monopolization and Priority Effects. Trends in Ecology & Evolution. 2016 Feb;31(2):136–46.

130. Debray R, Herbert RA, Jaffe AL, Crits-Christoph A, Power ME, Koskella B. Priority effects in microbiome assembly. Nat Rev Microbiol. 2022 Feb;20(2):109–21.

131. Henriques SF, Dhakan DB, Serra L, Francisco AP, Carvalho-Santos Z, Baltazar C, et al. Metabolic cross-feeding in imbalanced diets allows gut microbes to improve reproduction and alter host behaviour. Nat Commun. 2020 Aug 25;11(1):4236.

132. Enke TN, Datta MS, Schwartzman J, Cermak N, Schmitz D, Barrere J, et al. Modular Assembly of Polysaccharide-Degrading Marine Microbial Communities. Current Biology. 2019 May 6;29(9):1528-1535.e6.

133. Rakoff-Nahoum S, Foster KR, Comstock LE. The evolution of cooperation within the gut microbiota. Nature. 2016 May;533(7602):255–9.

134. Chang PV. Chemical Mechanisms of Colonization Resistance by the Gut Microbial Metabolome. ACS Chem Biol. 2020 May 15;15(5):1119–26.

135. Borton MA, Sabag-Daigle A, Wu J, Solden LM, O'Banion BS, Daly RA, et al. Chemical and pathogen-induced inflammation disrupt the murine intestinal microbiome. Microbiome. 2017 Apr 27;5(1):47.

136. Altshuler I, Demiri B, Xu S, Constantin A, Yan ND, Cristescu ME. An Integrated Multi-Disciplinary Approach for Studying Multiple Stressors in Freshwater Ecosystems: Daphnia as a Model Organism. Integrative and Comparative Biology. 2011 Oct 1;51(4):623–33.

137. Stollewerk A. The water flea Daphnia - a "new" model system for ecology and evolution? J Biol. 2010;9(2):21.

138. Miner BE, De Meester L, Pfrender ME, Lampert W, Hairston NG. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc R Soc B: Biol Sci. 2012 May 22;279(1735):1873–82.

139. Ebert D. Daphnia as a versatile model system in ecology and evolution. Evo Devo. 2022 Aug 8;13(1):16.

140. Reilly K, Ellis LJA, Davoudi HH, Supian S, Maia MT, Silva GH, et al. Daphnia as a model organism to probe biological responses to nanomaterials—from individual to population effects via adverse outcome pathways. Front Toxicol. 2023 Apr 14;5:1178482.

141. Benzie JA. Guides to the Identification of the Microinvertebrates of the Continental Waters of the World: Cladocera: the Genus Daphnia (including Daphniopsis). SPB Acad. Publ.; 2005.

142. Becker D, Barnard-Kubow K, Porter R, Edwards A, Voss E, Beckerman AP, et al. Adaptive phenotypic plasticity is under stabilizing selection in Daphnia. Nat Ecol Evol. 2022 Oct;6(10):1449–57.

143. Ghadouani A, Pinel-Alloul B. Phenotypic plasticity in Daphnia pulicaria as an adaptation to high biomass of colonial and filamentous cyanobacteria: experimental evidence. Journal of Plankton Research. 2002 Oct 1;24(10):1047–56.

144. Scoville AG, Pfrender ME. Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. Proceedings of the National Academy of Sciences. 2010 Mar 2;107(9):4260–3.

145. Laforsch C, Tollrian R. Inducible Defenses in Multipredator Environments: Cyclomorphosis in Daphnia Cucullata. Ecology. 2004;85(8):2302–11.

146. Tollrian R, Heibl C. Phenotypic plasticity in pigmentation in *Daphnia* induced by UV radiation and fish kairomones. Functional Ecology. 2004 Aug;18(4):497–502.

147. Lee TM, Westbury KM, Martyniuk CJ, Nelson WA, Moyes CD. Metabolic Phenotype of Daphnia Under Hypoxia: Macroevolution, Microevolution, and Phenotypic Plasticity. Front Ecol Evol. 2022 Feb 4;10: 822935.

148. Decaestecker E, De Meester L, Mergeay J. Cyclical parthenogenesis in Daphnia: sexual versus asexual reproduction. In: Lost sex: The evolutionary biology of parthenogenesis. Dordrecht: Springer Netherlands, 2009. p. 295-316.

149. Cuenca Cambronero M, Orsini L. Resurrection of Dormant Daphnia magna: Protocol and Applications. J Vis Exp. 2018 Jan 19;(131):56637.

150. Rogalski MA. Tainted resurrection: metal pollution is linked with reduced hatching and high juvenile mortality in Daphnia egg banks. Ecology. 2015;96(5):1166–73.

151. Cousyn C, De Meester L, Colbourne JK, Brendonck L, Verschuren D, Volckaert F. Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. Proceedings of the National Academy of Sciences. 2001 May 22;98(11):6256–60.

152. Zhang H, Jiang X. Resurrection of dormant zooplankton grazers reveals multiple evolutionary responses to toxic cyanobacteria. Limnology and Oceanography. 2022;67(9):2000–11.

153. Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, et al. The Ecoresponsive Genome of Daphnia pulex. Science. 2011 Feb 4;331(6017):555–61.

154. Lee BY, Choi BS, Kim MS, Park JC, Jeong CB, Han J, et al. The genome of the freshwater water flea Daphnia magna: A potential use for freshwater molecular ecotoxicology. Aquat Toxicol. 2019 May;210:69–84.

155. Cornetti L, Fields PD, Ebert D. Genomic characterization of selfing in the cyclic parthenogen Daphnia magna. Journal of Evolutionary Biology. 2021 May 1;34(5):792–802.

156. Schultz TW, Kennedy JR. The fine structure of the digestive system of *Daphnia pulex* (Crustacea: Cladocera). Tissue and Cell. 1976 Jan 1;8(3):479–90.

157. Georgi R. Feinstruktur peritrophischer membranen. Z Morph Tiere. 1969 Sep 1;65(3):225–73.

158. Engel P, Moran NA. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev. 2013 Sep 1;37(5):699–735.

159. Binder G. Das Muskelsystem von Daphnia. Internationale Revue der gesamten Hydrobiologie und Hydrographie. 1931;26(1–2):54–98.

160. Peter H, Sommaruga R. An evaluation of methods to study the gut bacterial community composition of freshwater zooplankton. J Plankton Res. 2008 Sep 1;30(9):997–1006.

161. Ebert D. Ecology, Epidemiology, and Evolution of Parasitism in Daphnia [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2005. Chapter 2, Introduction to Daphnia Biology. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2042/

162. Freese HM, Schink B. Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean Daphnia magna. Microb Ecol. 2011 Nov 1;62(4):882–94.

163. Carlström CI, Field CM, Bortfeld-Miller M, Müller B, Sunagawa S, Vorholt JA. Synthetic microbiota reveal priority effects and keystone strains in the Arabidopsis phyllosphere. Nat Ecol Evol. 2019 Oct;3(10):1445–54.

164. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, et al. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. ISME J. 2016 Apr;10(4):911–20.

165. Peterson BJ, Hobbie JE, Haney JF. Daphnia grazing on natural bacteria. Limnology and Oceanography. 1978;23(5):1039–44.

166. Geller W, Müller H. The filtration apparatus of Cladocera: Filter mesh-sizes and their implications on

CHAPTER 1:

Investigating the core microbiome concept: *Daphnia* as a case study

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Under revision at Environmental Microbiome

Abstract

Host-associated microbiomes play an important role in the ecology and fitness of organisms. Given their significance, it is much debated to what extent these associations are widespread and even obligatory. Such frequent associations are captured by the concept of the core microbiome. The cladoceran *Daphnia* is a pivotal genus in freshwater ecosystems occupying a central position in the food webs of standing waters. With its unique standing in pelagic waters, *Daphnia* serves as a key grazer, regulating algal populations and nutrient cycling, making its microbiome essential to understanding ecosystem function and stability. In recent years, *Daphnia* has become an increasingly popular study system for exploring host–microbiota interactions. There is, however, limited knowledge on the baseline taxa that consistently inhabit this host and potentially contribute to its fitness. Identifying whether such a host-associated "core microbiome" exists for *Daphnia* and, if so, which microbial taxa it comprises is important both for enhancing our ecological understanding of this genus and its ecosystem function and for interpreting future experiments. We compiled a dataset on *Daphnia magna* microbiome based on 12 published studies, comprising gut and whole microbiome samples of both laboratory-cultured and field-grown animals across five countries spanning three continents. To identify core taxa, we employ quantification metrics based on prevalence and a combination of prevalence and relative abundance. Our analysis demonstrates that the *D. magna* microbiome is highly variable, yet, a consistent association with specific taxa, notably *Limnohabitans planktonicus*, is observed especially under laboratory conditions. However, this pattern is tempered by the observation that field-grown animals exhibit a more diverse microbiome with a weaker presence of *L. planktonicus*, challenging its status as a core member. Our analysis suggests that the *D. magna* microbiome is defined by its high variability and few conserved associations, with *L. planktonicus* being the most stable taxon in laboratory settings but not necessarily a core member in natural environments. These findings underscore the need for caution when using laboratory results to interpret natural microbiome compositions and emphasize the need for further research on field-grown animals to better understand the structuring of microbial communities under natural settings.

Keywords: core microbiome, *Daphnia*, *Limnohabitans planktonicus*

Introduction

All organisms host complex microbial communities, forming symbiotic relationships that often contribute to shaping host health and fitness (1). These microbiomes have been shown to extend beneficial functions to the host, including nutrient uptake and digestion, production of essential amino acids, host immune system development and direct protection against pathogens, toxins and other environmental stressors (2). To gain a deeper understanding of these interactions, the concept of the core microbiome was introduced. This concept aims to identify key microbial taxa (and their associated genes) that are characteristic of a specific environment or body site of a host and consistently inhabit this site across all or the vast majority of host individuals (3). This is in contrast with transient or satellite species, whose frequency in the microbiome is more variable, resulting in a fraction of the microbiome that occurs depending on factors such as diet, host genetic background, physiological status and external environmental conditions (3,4). Although the original definition of the core microbiome focused on microbial members that were consistently shared among the vast majority of populations (3), the definition has evolved over the years to encompass different aspects, often identifying key microbes in terms of their spatial distribution, temporal constancy, ecological impact, and overall functional contribution to host well-being (see overview in Table S1, Supporting Information [SI]). These different definitions emphasize two key criteria, namely widespread occurrence and importance for host fitness, which has to some extent complicated the debate on the existence of core microbiomes. This also parallels the dual definition of "symbiosis" defined either by "persistent interactions in close physical association" (original definition) or by "mutualistic interactions in close physical association" (popular definition) which echoes the ongoing debate in microbiome research with regard to how core membership is defined (5). Specifically, the core microbes defined can vary depending on whether the emphasis is placed on consistency and widespread occurrence across hosts (analogous to persistence in symbiosis) or on their functional contributions to host fitness (analogous to mutualistic interactions in symbiosis). Here, we use the original definition of the core microbiome defined by its consistent presence, rather than by its impact on host fitness. The core microbiome thus identified may contribute to host fitness, but here, we do not use it as a necessary criterion to identify a microbial taxon as a core microbiome member. A better understanding of the core microbiome of a host holds promise for understanding how the microbiome contributes to host biological functioning, helps dissect patterns of microbial colonization, factors shaping host–microbe interactions, and offers critical insights for experimental design allowing the selection of key strains that can serve as reliable indicators of host function and their response to environmental change.

The water flea *Daphnia* is a ubiquitous planktonic crustacean genus found in standing open waters worldwide, with a cosmopolitan distribution spanning all continents (6). These generalist filter feeders occupy a central position in the food web of freshwater systems as effective grazers of phytoplankton and fish prey (7). Owing to their convenient features for experimentation and their high responsiveness to both anthropogenic and abiotic (pollution, urbanization, climate warming, salinization (8–11)) as well as natural and biotic stressors (competitors, parasites, predators;(12–14)), *Daphnia* species, especially *D. magna* and *D. pulex*, are common model systems in the fields of ecotoxicology, ecology, evolution, and eco-evolutionary dynamics (7,15). In recent years, *Daphnia* has also become popular as a model system to study host–microbiome interactions owing to its short life cycle, clonal reproduction, and ability to create germ-free animals that allow manipulation of the (gut) microbiome composition, thus providing a unique opportunity to study the interplay between host genotypes and their microbiomes. *Daphnia* is known to harbor microbial communities both on the surface of its body and in its gut (16), and this microbiome has been reported to affect *Daphnia* reproduction and survival (17,18). For example, the microbiome of *Daphnia* has been shown to contribute to the digestion of phytoplankton (19), tolerance against toxic cyanobacteria and heavy metals (1,20,21) and to provide the host with essential vitamins and amino acids (22). However, very little is known about the identity of the microbial taxa that consistently inhabit the host. Some bacterial phyla that have been suggested to be consistently associated with *Daphnia* include Proteobacteria, Actinobacteria and Bacteroidetes (23). Specific bacterial groups such as *Limnohabitans*, *Aeromonas*, *Ideonella*, *Pseudomonas*, *Pedobacter*, *Flavobacterium*, *Polaromonas*, Comamonadaceae and unclassified Burkholderiaceae have been regularly reported in association with *Daphnia* (24–26). The degree to which these taxa consistently occur across individuals isolated from ecologically different habitats and form a stable association with the *Daphnia* microbiome is unknown. Although some earlier work suggested the presence of a core community in *Daphnia* microbiomes (24,27,23), a recent study has, however, suggested that *Daphnia* microbiomes may be more dynamic in nature, varying with the conditions of their external environment (28).

In this study, we explore the concept of the core microbiome and explicitly test whether a core microbiome occurs in the model organism *D. magna*. More specifically, we test whether *D. magna* is characterized by a microbiome

comprising species shared by most individuals or whether the microbiome is completely variable among individuals. To test for the existence of a core microbiome, we systematically analyzed an integrated dataset compiled from 12 published studies comprising sequences of the V4 hypervariable region of the 16S rRNA gene of both *D. magna* gut and whole microbiome samples of both laboratory and field origin across five countries.

Methods

Search strategy

A total of 145 hits spanning a period of 33 years (1989-2022) were obtained in a literature search (performed in June 2022) in the NCBI PubMed and Google Scholar databases via the search terms "*Daphnia* microbiome" (57 hits), "*Daphnia* gut microbiome" (18 hits), "*Daphnia* microbiota" (51 hits), and "*Daphnia* gut microbiota" (19 hits). After removing duplicates and reviews and retaining only those papers with relevant 16S rRNA sequence-based data on *Daphnia* microbiomes (both whole-animal and gut samples), either from animals collected in nature or screened as part of experiments, 34 studies were retained (Figure S1, SI). From these studies, pertinent information such as *Daphnia* species and genotype, geographical origin of the *Daphnia* clone, and the sequenced variable region were compiled in a table (Table S2, SI). A second round of pruning (Figure S1, SI) was performed to retain only studies that used the 16S rRNA V4 hypervariable region for metabarcoding, as this region was the most frequently targeted across the dataset and allowed for data standardization via a single pipeline for analysis. Focusing on a single hypervariable region was carried out to allow data standardization and reduce biases introduced by non-overlapping regions. As a result, from the 34 studies previously selected, 12 studies that reported on microbial community composition using the V4 region were retained. These 12 studies included a total of 656 samples originating from three continents (five countries; see Table S2, SI), involving the gut as well as whole-body microbiomes of host animals directly collected from their source habitat or grown in the laboratory. Of the 12 datasets, 11 examined the microbiome of laboratory-cultured animals, while one study included both laboratory and field-grown individuals. All field-grown animals in this study were from the same lake, i.e., Lake Bysjön, Southern Sweden (55°40′28.5″N, 13°32′45.4″E). All studies focused on the microbiome of *D. magna.* These studies were used in our analysis to identify the extent to which there is a core microbiome in this species. An overview of the analytical steps based on these 12 studies is visualized in Figure 1.

Data standardization

To account for the variability in the different studies in terms of sequencing methods and taxonomic classification of the microbial taxa, data standardization was conducted. For all the 12 selected studies, raw sequences were obtained in fastq format from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) using their specific BioProject/Accession numbers. These sequences were analyzed in mothur (v.1.48.0) using the MiSeq standard operating procedure (29) to maintain a consistent classification scheme. All sequences were first extracted from the fastq files. Using the *make.contigs* function, reverse reads were converted into their reverse complements and aligned to the forward reads to form contigs. A *screen.seqs* was run to maintain a maximum length of 250 bp (V4 region). Duplicate sequences were merged using the *unique.seqs* command. A customized reference alignment was created to maintain only the V4 hypervariable region. This was accomplished by trimming the SILVA reference database at a starting point of 11895 bp and an end point of 25318 bp (determined using the primers 515F and 806R) using the *pcr.seqs* command. Next, with the *align.seqs* command, the sequences were aligned to the customized reference alignment, and a *summary.seqs* was run to determine whether all the sequences were aligned securely. The *screen.seqs* data were rerun with a start at 1968 bp and an end of 11550 bp to ensure that all the sequences overlapped in the same V4 variable region. All overhangs at both ends following alignment were removed with the *filter.seqs* command. A second *unique.seqs* was run to identify duplicates and merge them together. All the sequences were subsequently split by group, allowing differences of up to 2 nucleotides, using the *pre.cluster* command and sorted by abundance. Chimera removal was carried out using the *vsearch* executable (*chimera.vsearch*). The remaining sequences were classified with the Bayesian classifier using the *classify.seqs* commands. Sequences from undesired 16S rRNA gene fragments arising from chloroplasts and mitochondria, Archaea or other unknown groups were removed with *remove.lineage*. The sequences were then clustered into operational taxonomic units (OTUs) using the *cluster.split* command at a cutoff value of 0.03, thereby allowing sequence clustering on the basis of a 97% similarity threshold (29). This threshold was set to facilitate comparison across studies; we opted for this approach instead of using amplicon sequence variants (ASVs), which resolve sequences to single nucleotide differences and may overestimate diversity and underestimate shared taxa. As a last step, the *make.shared* and *classify.otu* commands were run to make an OTU count table with corresponding taxonomic information. Additionally, to identify the most important OTUs, a species-level identification

was carried out. FASTA sequences of these OTUs were extracted using the *otu.rep* function in mothur, specifying the method abundance. The identity of the sequences was then determined with a BLAST search in the rRNA/ITS databases for 16S rRNA gene sequences (Bacteria and Archaea) of NCBI, optimized for highly similar sequences (megablast). Identification was based on the highest maximum score, > 98% identity and the lowest E value.

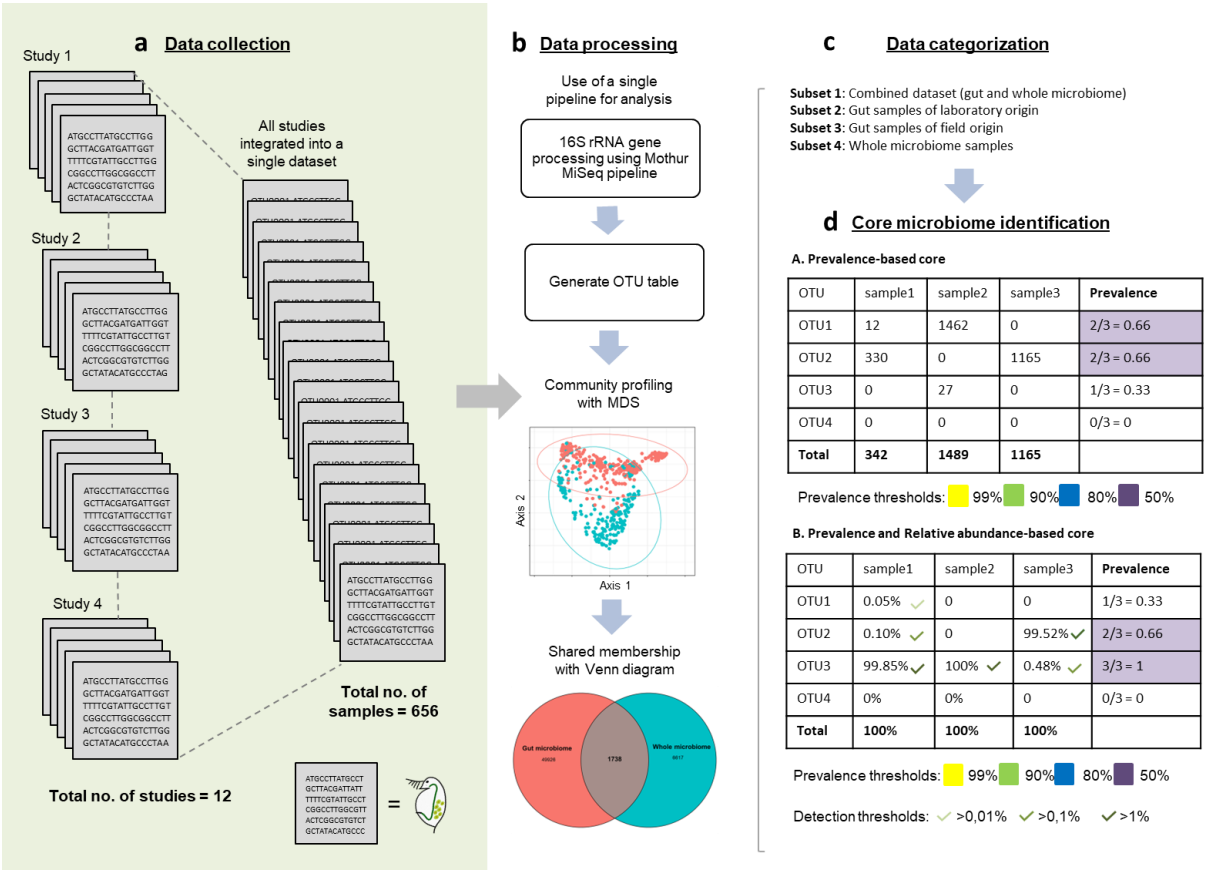

Figure 1: Schematic overview of the analysis starting from the 12 selected studies. a) Data collection: Raw fastq files from 12 published studies were collected and integrated to form a single dataset comprising a total of 656 *Daphnia* microbiome samples. b) Data processing: This dataset was processed using the mothur MiSeq SOP pipeline to generate an OTU table which was subsequently used for community profiling and data categorization. c) Data categorization: Based on the preliminary analysis, the dataset was categorized into the four subsets described. d) Core microbiome identification: Core species in the *D. magna* microbiome were determined for both combined and separate subsets of gut and whole-animal microbiomes of laboratory and field origin. Metrics used to identify core taxa were based on prevalence only or a combination of prevalence and relative abundance (See Methods for details).

Data exploration and grouping of samples for analysis

Because our dataset consisted of samples from different countries, sample types (gut and whole animal samples) and origins (laboratory vs field), a multidimensional scaling (MDS) based on Bray–Curtis distances was performed using the *ordinate* function from the *phyloseq* package in R (30) to explore and visualize whether these different groups differed in community composition (Figure 2). Significant differences between the communities were determined with a permutational multivariate analysis of variance (PERMANOVA), using the *adonis2* function of the *vegan* package in R (31,32) specifying *by = "margin"* to calculate type III sum of squares. Given the results of this exploratory analysis, in addition to an analysis of the complete dataset, core microbiome species were also identified for different subsets of the data, which resulted in the following hierarchical categorization. Next to the (1) *combined dataset,* comprising samples sourced from both guts and whole-animals of both laboratory and field origin across all five countries, we also analyzed the subset of (2) *gut samples,* comprising all samples sourced from host guts. The latter dataset was further divided into (2a) *gut samples of laboratory origin only* and (2b) *gut samples* of *field origin only*. In the latter dataset, all host individuals were retrieved from the same population. Finally, we separately analyzed (3) *whole-animal samples,* comprising all samples of the full microbiome. This subset consisted of only laboratory-based samples, as the dataset

did not contain whole-animal microbiome samples from field animals. To visualize the degree to which taxa were shared among countries, between field and laboratory origins or between whole-body and gut samples, Venn diagrams were generated using the *eulerr* package (33) in R (version 4.0.5, R Core team 2021-03-31).

Statistical analyses

All statistical analyses were carried out using the *microbiome* (34), *vegan* (31) and *phyloseq* package (30) in R (version 4.0.5, R Core team 2021-03-31). Core/unique members were detected using two quantification metrics, namely a (i) prevalence-based metric and a (ii) metric combining prevalence and relative abundance (as explained below; also Figure 1). Given that fine-scale data such as operational taxonomic units (OTUs) can often incorporate potential 'noise' in metabarcoding data, the core identification metrics were also analyzed at higher taxonomic ranks (phylum and order levels) via the agglomeration function of the *phyloseq* package *tax_glom*. In this study, we present results from the perspective of a community composition-based analysis and define core members in *Daphnia* from a "common core" standpoint, i.e. shared and recurrent co-occurrences of host and microbiota (Table S1, SI).

Core quantification metrics

Prevalence-defined core taxa

A prevalence-based metric was used to identify microbial taxonomic groups that were widespread and consistently present in all samples or across a preset percentage of *D. magna* samples, irrespective of their relative abundance in the communities. For this metric, a prevalence threshold was set to define the percentage of samples (i.e., host individuals) in which the taxa had to be found in order to be considered a member of the core microbiome. We used 50%, 80%, 90% and 99% as prevalence thresholds; 99% was used to allow for a by-chance absence of a taxon that is otherwise present in all samples. The *core* function of the *microbiome* package (34) was used for the analysis.

Abundance and prevalence-based core taxa

Prevalence is a good metric to assess whether a given taxon is widespread among hosts, but it poorly reflects its relative dominance within the microbiome of its host. To gauge this, the relative abundances of taxa comprising the host microbiome can be used. However, in our study, relative abundance was not used as a sole criterion for core identification, given that this metric only states whether a given taxon dominates the microbiome when it is present, but not whether it occurs in all or many host individuals. While an approach based on relative abundance could highlight abundant taxa, it would represent the selection of core taxa from a single or few samples that rather reflect condition-specific enrichment and not necessarily consistent dominance across samples. Instead, a combined abundance-prevalence-based metric with a double criterion was used, namely, through a minimum relative abundance threshold and a minimum prevalence threshold. The prevalence threshold was used to include only taxa present in a defined proportion of the samples, whereas the relative abundance detection threshold was used as a criterion to only pick those that met a minimum relative abundance threshold in the host individuals in which this taxon was present. Taxa that were sufficiently widespread with a sufficiently high relative abundance were identified as core members of the microbiome. We used all combinations of the above prevalence thresholds (50%, 80%, 90% and 99%) and three thresholds for relative abundance (10^{-4} or 0.01%; 10^{-3} or 0.1%; 10^{-2} or 1%) as criteria to identify core members of the microbiome. Higher thresholds for relative abundances did not result in the identification of any core member of the microbiome at the selected prevalence threshold range.

Results

Available data

Considering all the samples derived from both the gut and whole animals, irrespective of country of origin and whether they were laboratory-cultured or field-sampled, a total of 656 samples were obtained, comprising 423 gut samples and 233 whole animal samples. A total of 25,166,733 sample read counts were available for analysis, which represented 142,150 bacterial operational taxonomic units (OTUs) among 1,061 genera belonging to 32 phyla. After removing singletons, i.e., OTUs that had only one read count across all samples combined, and excluding samples with fewer than 10 total reads, 25,083,005 sample read counts remained representing 58,441 OTUs across 654 samples (Table 2).

Hierarchical categorization of datasets

MDS plots revealed distinct clustering patterns in the microbiome community composition across the total dataset (Figure 2). Samples from the five countries formed five overlapping clusters with notable separation of United Kingdom and Sweden compared to the rest (Figure 2A). A relatively clear distinction was observed between gut and whole animal samples (Figure 2B), and a complete separation between samples from laboratory-cultured and field-grown animals (Figure 2C). PERMANOVA results revealed that country had a significant effect on microbial composition (pseudo-F = 33.398, $p < 0.001$) explaining 15.97% of the observed variance in the microbiome composition across the combined dataset (Table 1). Likewise, sample type i.e., gut versus whole body and sample origin i.e., laboratory versus field also significantly influenced microbial community composition accounting for 0.87% (pseudo-F = 7.326, $p < 0.001$) and 5.79% (pseudo-F = 48.493, $p < 0.001$) of the observed variation in the total dataset, respectively (Table 1). Interaction terms produced undefined results (SumofSq = 0, $R^2 = 0$, pseudo-F = -Inf), indicating insufficient variation or data sparsity in these combinations. As a result, interaction terms were not interpretable and thus the model focused on main effects to explain the variation in the dataset.

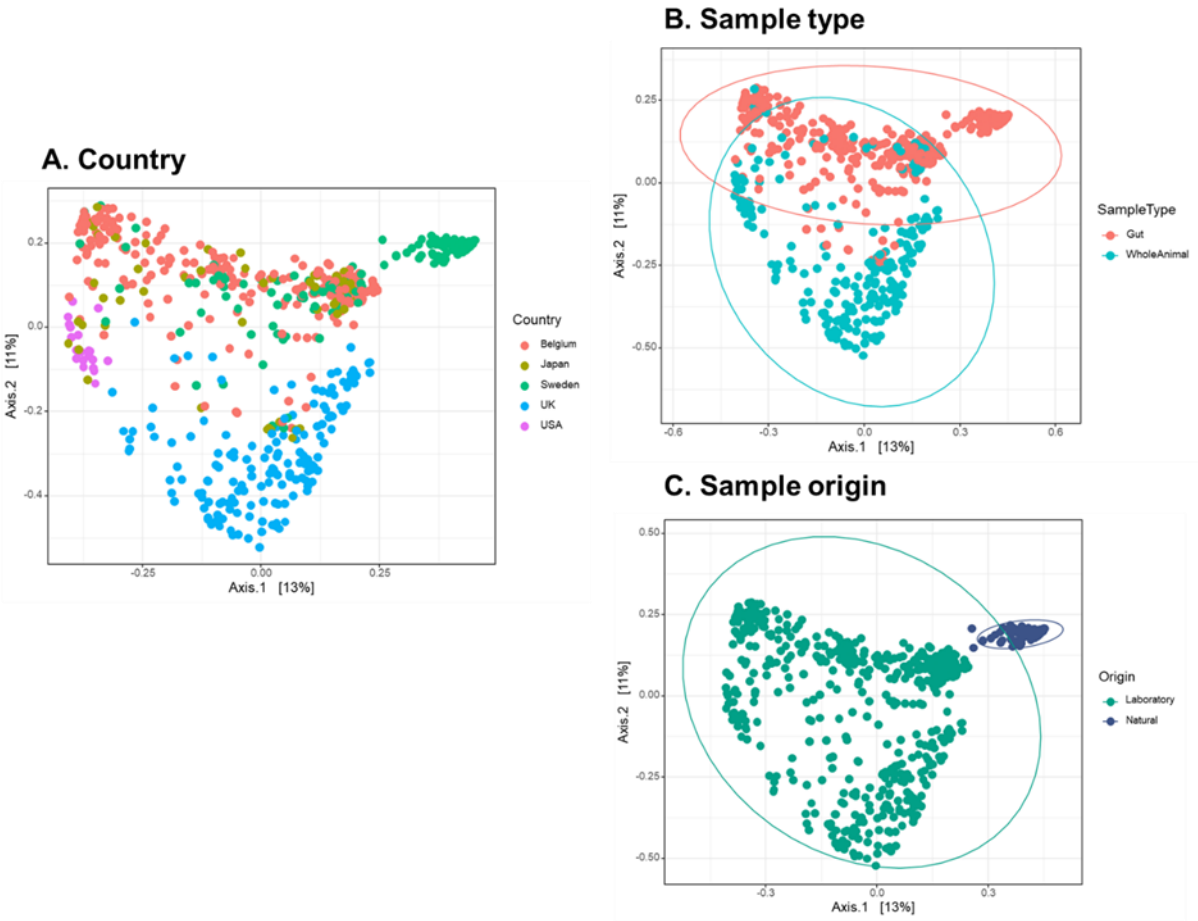

Figure 2: Multidimensional scaling (MDS) of the microbiome data of all samples based on Bray Curtis dissimilarity index illustrating differences in microbial communities between A) Countries, B) Sample type: whole-body versus gut and C) Sample origin: laboratory-cultured versus hosts grown in the field. Distances between points represent dissimilarities in microbial community composition, with closer points indicating more similar microbiomes as a function of the first two axes in multidimensional space that capture most (in total 24%) of the variation in the dataset. All field-grown animals were retrieved from the same lake.

Table 1: Results of PERMANOVA, based on Bray Curtis Beta diversity metric assessing the amount of variation in microbiome community composition that can be explained by the categorization variables- country, sample type (gut vs. whole microbiome) and sample origin (laboratory vs. field samples). Df: degrees of freedom associated with the effect; SumofSq: inertia of the dissimilarity matrix explained by the effect; R^2: squared coefficient of determination (obtained from the inertia explained by the effect and the total inertia of the dissimilarity matrix); F: F-statistic associated with the effect; Pr (>F): p-value associated with the sequential permutational F-test, based on permutation of the raw data.

Categorization variable	Df	SumofSq	R^2	F	Pr (>F)
Country	4	40.822	0.15972	33.3982	**0.001** ***
Sample type	1	2.239	0.00876	7.3266	**0.001** ***
Sample origin	1	14.818	0.05798	48.4937	**0.001** ***
Residual	647	197.703	0.77354		

Shared taxa among categories

To explore the unique and shared fractions of taxa among the different categories in the combined dataset, a Venn diagram was constructed to visualize shared membership between samples of the different countries, sample types (gut versus whole-body) and sample origin (laboratory-cultured versus field-grown). Overall, a low number of taxa were observed to be shared between categories (Figure 3A). From a total of 58,441 OTUs present in the total dataset across five countries (Belgium, Sweden, Japan, UK and USA), only 106 OTUs (0.18%) were common to all countries. Similarly, there occurred only 1,738 OTUs (2.97%) shared between gut and whole-body microbiome samples (Figure 3B). A comparison of *D. magna* microbiomes of laboratory and field origin revealed that there was an overlap of 2,383 OTUs (4.07%) (Figure 3C). This reflects a high variability in microbiome composition among hosts derived from different settings, and suggests that a more detailed analysis focusing on specific categories is warranted.

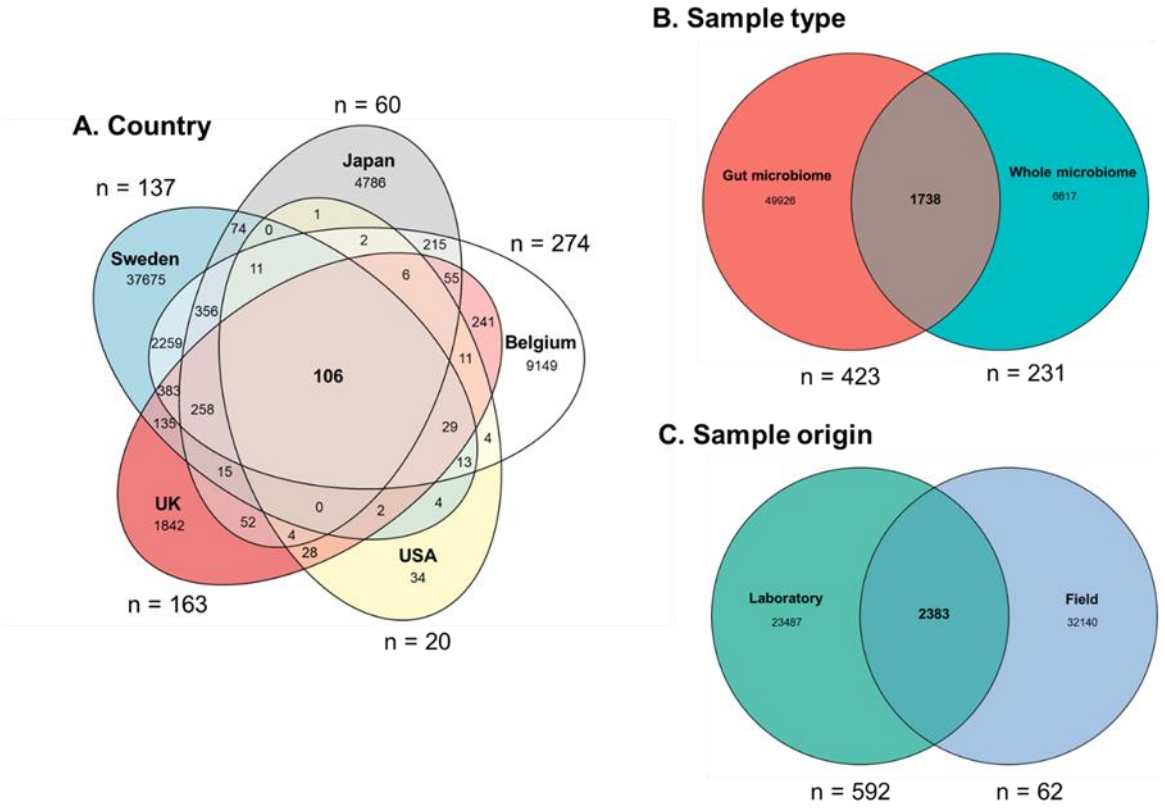

Figure 3: Venn diagrams representing shared membership between A) countries, B) sample type (gut and whole-animal samples), and C) sample origin (laboratory and field origins).

Core microbiome taxa in the combined dataset

No phylum or order met the criterium of occurring in 99% of the samples in this dataset. The phyla Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes occurred in 90% of the samples. At the order level, these were members of Burkholderiales, Chitinophagales, Flavobacteriales and Rhizobiales that occurred in 90% of the samples. The same phyla and orders as mentioned for the prevalence criterium also met the combined criteria of 10^{-4} relative abundance and 90% prevalence. Using the more stringent threshold of 10^{-2} relative abundance at 90% prevalence, only a single phylum, Proteobacteria, and a single order, Burkholderiales, were retained. Table S3 in SI lists all taxa for the various combinations of relative abundance and prevalence thresholds used.

At the OTU level, no taxon was detected at a strict prevalence threshold of 99%. At the 90% prevalence threshold, one particular OTU (OTU1), labeled as *unclassified Comamonadaceae* belonging to the class Betaproteobacteria and family Comamonadaceae, was detected. This OTU was identified as *Limnohabitans planktonicus* in a BLAST search using the rRNA/ITS databases for 16S rRNA sequences in NCBI. At the 80% prevalence threshold, 6 OTUs were identified, including two additional members of the family Comamonadaceae identified as *Variovorax* sp. and *Ideonella paludis, Escherichia/Shigella* sp., *Staphylococcus* sp. and unclassified Chitinophagaceae. Further lowering the prevalence threshold to 50% resulted in the detection of 27 taxa representing eight members of unclassified *Comamonadaceae,* two *Flavobacterium* sp., *Formosimonas* sp., *Acinetobacter* sp., unclassified *Chitinophagaceae, Pseudomonas* sp., unclassified *Rhodobacteriaceae, Escherichia/Shigella* sp., *Shinella* sp., *Polynucleobacter* sp., *Emticicia* sp., *Streptococcus* sp., unclassified *Microbacteriaceae, Staphylococcus* sp., *Bosea* sp., *Hydrogenophaga* sp., *Brevundimonas* sp. *Sphingomonas* sp. and *Bradyrhizobium* sp.. Combining the relative abundance and prevalence thresholds at the level of OTUs, it was observed that only *Limnohabitans planktonicus* (OTU1) met the combined threshold of 10^{-4} relative abundance and 90% prevalence. Other relatively abundant taxa (threshold 10^{-4}) that met the 50% prevalence threshold were unclassified members of the families *Comamonadaceae, Chitinophagaceae, and Rhodobacteraceae* and the genera *Formosimonas, Pseudomonas, Escherichia/Shigella, Shinella, Emticicia, Streptococcus* and *Staphylococcus.* At a relative abundance threshold of 10^{-3} and a prevalence threshold of 90% and 80%, only *Limnohabitans planktonicus* was detected. At the highest relative abundance threshold of 10^{-2}, *Limnohabitans planktonicus* was detected in only 50% of the samples.

Core microbiome taxa in the gut microbiome

The results of the core microbiome analysis (prevalence-based and combined criteria of relative abundance and prevalence) on all gut microbiome samples were nearly identical to those of the combined dataset detailed above, and are given in SI section 1. Below, we discuss the separate core microbiome analysis of gut microbiome samples of laboratory-cultured and field-grown animals.

Core microbiome in the gut communities of laboratory origin

This subset consisted of 361 *D. magna* gut samples of laboratory origin, representing 19,030 OTUs belonging to 763 genera and 26 phyla (Table 2). Similar to previous results on the combined dataset, higher-order analysis at the "phylum" and "order" levels revealed that the most prevalent phyla across 90% of the samples were Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes and the most prevalent orders- Burkholderiales, Chitinophagales, Flavobacteriales, Pseudomonadales, Enterobacterales, Rhizobiales and Micrococcales. The same phyla were also selected based on the combined criteria of prevalence and relative abundance at a detection threshold of 10^{-4} across 90% of the samples. The phyla Betaproteobacteria and Bacteroidetes met the more stringent threshold of 90% prevalence and 10^{-3} relative abundance, and only Betaproteobacteria occurred at 90% prevalence and 10^{-2} relative abundance.

At the OTU level, *Limnohabitans planktonicus* (OTU1) exhibited widespread occurrence being identified across almost all samples at 99% prevalence. Other prevalent bacterial taxa identified included *Escherichia/Shigella sp*. (OTU13) and *Ideonella paludis* (OTU8), which were found across 90% of the samples. Taxa identified at 80% prevalence included *Staphylococcus* sp., *Variovorax* sp., *Ideonella paludis*, *Pseudomonas* sp., *Streptococcus* sp., *Polynucleobacter* sp., and unclassified *Microbacteriaceae* and *Chitinophagaceae*. Based on the combined prevalence and relative abundance metrics, *Limnohabitans planktonicus* was identified at 99% prevalence and an abundance threshold of 10^{-4} . For the same detection threshold, *Escherichia/Shigella* sp. was identified at 90% prevalence, whereas *Ideonella paludis* and *Staphylococcus* sp. were detected when the prevalence threshold was lowered to 80%. Using a relative abundance

threshold of 10^{-3}, *Limnohabitans planktonicus* was the only taxon identified at both 90% and 80% prevalence. At the same detection threshold, taxa identified at 50% prevalence included *Formosimonas* sp., *Variovorax* sp., *Ideonella paludis*, *Pseudomonas* sp., *Polynucleobacter* sp., *Staphylococcus* sp. and unclassified *Microbacteriaceae*, *Chitinophagaceae*, *Rhodobacteraceae* and *Micrococcales*. At 10^{-2} *Limnohabitans planktonicus* was identified as a core member, and at a 80% prevalence threshold only.

Core microbiome in the gut communities of field origin

This subset was constituted from 62 gut samples originating from animals directly sampled from the same lake and comprised a total of 342,523 OTUs belonging to 493 genera and 22 phyla (Table 2). The most prevalent phyla, present across 99% of the samples in this dataset, included Proteobacteria, Plantomycetes, Verrucomicrobia, Bacteroidetes, Actinobacteria, Firmicutes and Bacteria_unclassified. The same high level of prevalence was observed for the orders Burkholderiales, Pirellulales, Rhodobacterales, Verrucomicrobia_unclassified, Solirubrobacterales, Rhizobiales, Xanthomonadales, Sphingomonadales, Planctomycetales, Mycobacteriales, Bacteria_unclassified, Acidimicrobiales, Actinobacteria_unclassified, Verrucomicrobiales, Bacillales, Rhodospirillales, Micrococcales, Propionibacteriales, Alphaproteobacteria_unclassified, Planctomycetacia_unclassified, Clostridiales, Gammaproteobacteria_unclassified, Legionellales, and Proteobacteria_unclassified. With the double criterion combining prevalence and relative abundance, the phyla detected at a threshold of 10^{-4} in 99% of the samples included Planctomycetes, Proteobacteria and Actinobacteria, which belong to the orders Pirellulales, Rhodobacterales, Solirubrobacterales, Mycobacteriales, Rhizobiales, Acidimicrobiales and Actinobacteria_unclassified. At a detection threshold of 10^{-3} and 99% prevalence, these included the orders Pirellulales, Rhodobacterales, and Solirubrobacterales. At the most stringent threshold of 10^{-2} at 99% prevalence, only the phylum Planctomycetes, comprising the order Planctomycetacia, was detected.

At the OTU level, the taxa that were widespread were quite diverse, with 27 different taxa detected at 99% prevalence and 153 and 527 taxa detected at prevalence thresholds of 80% and 50%, respectively (Table S3, in the SI). *Limnohabitans planktonicus* was only detected at a prevalence threshold of 80%. The combined relative abundance and prevalence metric revealed 15 dominant taxa at relative abundance thresholds of 10^{-4} and 99% prevalence. These were unclassified *Pirellulales* (OTU 7, OTU 43, 87, 336), unclassified *Rhodobacteraceae* (OTU 11; OTU 71, OTU 75), unclassified *Solirubrobacterales* (OTU 21), *Mycobacterium sp*. (OTU 74), unclassified *Rhizobiales* (OTU 98), unclassified *Pirellulaceae* (OTU 127), *Ilumatobacter* (OTU 138), unclassified *Actinobacter* (OTU 147, OTU 197), and unclassified *Acidimicrobiales* (OTU 182). Likewise, 50 and 143 taxa were detected for the same relative abundance threshold at 80% and 50% prevalence, respectively (see Table S3 in the SI). At a relative abundance threshold of 10^{-3}, 4 taxa were detected at 99% prevalence, namely, unclassified *Pirellulales* (OTU 7, OTU 43), unclassified *Rhodobacteraceae* (OTU 11) and unclassified *Solirubrobacterales* (OTU 21). At a detection threshold of 10^{-2}, only unclassified *Pirellulales* (OTU 7) remained at 99% prevalence. Unclassified *Solirubrobacterales* (OTU 21) and unclassified *Rhodobacteraceae* (OTU 11) were detected at only 90% prevalence.

Core microbiome in whole-animal samples of laboratory origin

For the whole-animal samples, only samples of laboratory origin were available. This subset comprised a total comprised 8,355 OTUs belonging to 620 genera and 26 phyla from 231 whole animal samples (Table 2). When evaluating prevalence at the phylum and order level, the phyla Proteobacteria and Bacteroidetes occurred at 90% prevalence, as did the orders Burkholderiales, Chitinophagales, Flavobacteriales and Cytophagales. Using the double criterion combining prevalence and relative abundance, the phyla Proteobacteria and Bacteroidetes and their respective orders Betaproteobacteria and Chitinophagales met the relative abundance criterium of 10^{-4} and the prevalence criterium of 90%. Only Proteobacteria met the relative abundance threshold of 10^{-3} and 90% prevalence, whereas at the 10^{-2} relative abundance threshold, this phylum was detected only at 80% prevalence.

The OTUs *Formosimonas*, *Limnohabitans planktonicus*, unclassified *Chitinophagaceae*, *Flavobacterium* and *Emticicia* sp. met the 90% prevalence criterion, while *Variovorax* sp., *Brevundimonas* sp., *Bosea*, *Shinella*, *Escherichia/Shigella* sp., *Sphingorhabdus* sp. and unclassified *Comamonadaceae* and *Rhodobacteraceae* met the 80% prevalence criterion. Using the 50% prevalence threshold, 57 taxa were identified (see SI, Table S3). *Limnohabitans planktonicus*, *Formosimonas* sp. and unclassified *Chitinophagaceae* were the three OTUs that met both the 10^{-4} relative abundance and 90% prevalence criteria, while *Flavobacterium* sp. *and Emticicia* sp. met the 10^{-4} relative abundance and 80%

prevalence criteria. When the relative abundance threshold was further increased to 10^{-3} and 10^{-2}, *Limnohabitans planktonicus* was detected only at 90% and 80% prevalence threshold, respectively.

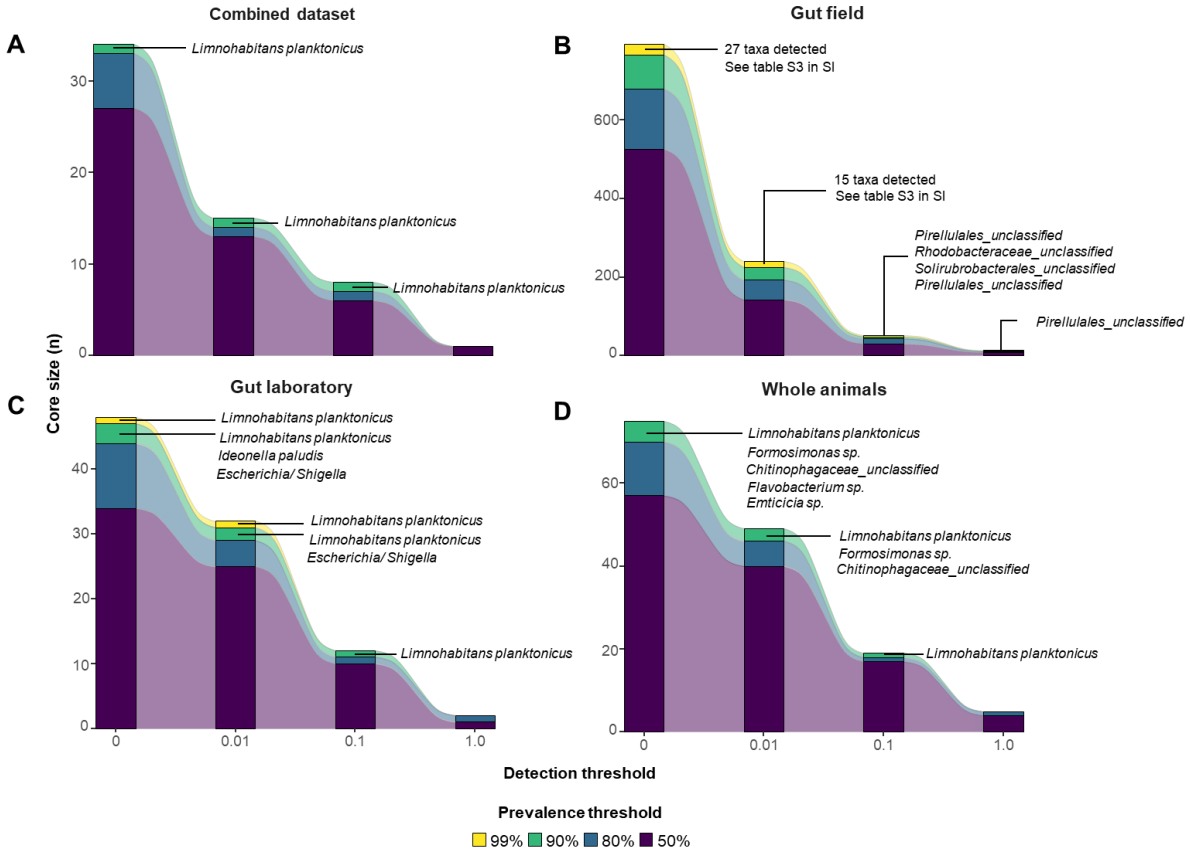

Figure 4: Core size (no. of OTUs) assigned based on prevalence thresholds (99%, 90%, 80%, 50%) and a combined prevalence and relative abundance detection threshold (>0.01%, 0.1%, 1%) in the A) combined dataset, B) gut samples of field origin, C) gut samples of laboratory origin and D) whole animal samples. For the core taxa identified based on prevalence alone, the detection threshold was set to 0. This means that any taxon with a non-zero relative abundance value in the defined proportion of the samples (e.g. 99% prevalence) was included. The core species identified in 99 and 90% of the samples have been labeled (see Table S3 in the SI for details). The alluvial segments or the streamlines between the bars do not represent interpolation but are solely for illustrative purposes to enhance readability and visualization.

Table 2: Summary information of the sample subsets used in the study and the number of core taxa assigned to each subset based on the core quantification metric employed.

Characteristics	**Dataset**				
	Combined dataset	**Gut samples**	**Gut samples (Lab origin)**	**Gut samples (Field origin)**	**Whole animal samples (lab origin)**
Total no. of samples	656	423	361	62	233
Total no. of reads	25,166,733	14,743,383	11,148,436	3,594,947	10,423,350
Total no. of singletons	83,709 (58.88%)	64,836 (55.65%)	15,352 (44.65%)	51,753 (59.98%)	20,013 (70.54%)
Total no. of OTUs detected	142,150	116,500	34,382	86,276	28,508
Total phyla	32	29	28	22	26
Total genera	1061	957	820	566	653
After removing singletons and low read samples					
Total no. of samples	654	423	361	62	231
Total no. of reads	25,083,005	14,678,547	11,133,084	3,543,194	10,403,318
Total no. of OTUs	58,441	51,664	19,030	34,523	8,355
Total phyla	32	29	26	22	26
Total genera	962	865	763	493	620
Core Metric	**Core size : No. of taxa assigned to core**				
Prevalence-based					
99%	0	0	1	27	0
90%	1	1	3	86	5
80%	6	6	10	153	13
50%	27	31	34	527	57
Prevalence-relative abundance					
0.0001 (0.01%)					
99%	0	0	1	15	0
90%	1	1	2	32	3
80%	1	2	4	50	6
50%	13	18	25	143	40
0.001 (0.1%)					
99%	0	0	0	4	0
90%	1	0	1	5	1
80%	1	1	1	13	1
50%	6	6	10	30	17
0.01 (1%)					
99%	0	0	0	1	0
90%	0	0	0	2	0
80%	0	0	1	3	1
50%	1	1	1	8	4

Discussion

In this study, we aimed to assess the extent to which the microbiome of the water flea *Daphnia*, and more specifically *D. magna*, harbors a set of highly prevalent and abundant taxa that can be considered a core microbiome, and identify the taxa that constitute that core. To that end, we compiled data on *D. magna* microbiomes across 656 individuals from 12 published datasets (Table S2, SI) targeting the V4 hypervariable region of the 16S rRNA gene. Core taxa were quantified based on prevalence thresholds and a combination of prevalence and relative abundance thresholds. Overall, our findings indicate that a large proportion of the microbiome in *D. magna* displays very variable prevalences and relative abundances, with only a low number of taxa (1-5) that are widespread (90% prevalence) and moderately abundant, as also observed in other organism groups (35). Of these, *Limnohabitans planktonicus* of the order Burkholderiales (phylum Proteobacteria) stands out as the most commonly observed taxon especially in laboratory-cultured samples. A key observation is that the gut microbiome of field-grown animals consistently differs from laboratory-cultured animals and does not feature *L. planktonicus* as the most prevalent and abundant taxon, even though it is still quite widespread. We discuss these results in the following paragraphs and highlight implications of the notable differences between microbiome composition of field-grown and laboratory-cultured animals.

The most prevalent microbiome phyla in *D. magna*, occurring in at least 90% of the gut as well as whole-animal samples, included Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. These phyla were mainly represented at high prevalence by the orders Burkholderiales, Chitinophagales, Flavobacteriales and Rhizobiales. These phyla were shared among the microbiomes of laboratory-cultured *D. magna*, irrespective of their geographical origin or sample type (i.e., gut or whole animal samples) (Figure S2, SI). The gut microbiome samples of field origin were, however, more diverse than the gut microbiomes of laboratory-cultured animals, and displayed high prevalence of the phyla Planctomycetes, Verrucomicrobia and Bacteria_unclassified, with the key orders being Pirellulales, Rhodobacterales, Solirubrobacterales, Rhizobiales, Sphingomonadales, Planctomycetales, Mycobacteriales and Acidimicrobiales. Houwenhuyse et al. (36) similarly reported that, upon bringing field-collected *Daphnia* to the laboratory, the diversity of the microbiome decreases strongly. This reduction in diversity may likely be a consequence of the relatively constant and homogenous culture conditions maintained in the laboratory, with a stable temperature, light:dark regime, pH of the culture medium and a simplified and standardized diet. This phenomenon has also been observed in laboratory-cultured tardigrades, whose microbial communities are predominantly composed of bacterial genotypes originating from food, media, or laboratory reagents (37). This is in contrast to field environments, where *Daphnia* indiscriminately filter feed on a diverse and assorted range of bacterioplankton communities further broadening the range of microbial taxa available for ingestion and subsequent gut colonization.

Overall, the phylum Proteobacteria constituted the majority (67.85%) of the bacterial sequences in the combined dataset containing both gut and whole animal samples. On average, 38.99% of all detected Proteobacteria sequences in the combined dataset belonged to the family Comamonadaceae. Likewise, this family constituted, on average, 40.52% of the bacterial sequences in the *D. magna* gut samples and 37.15% of those in the whole microbiome samples. This is in line with other studies that sequenced other parts of 16S rRNA gene hypervariable regions and are therefore not included in the current integrated analysis (16,24,23,38,39)); in these studies members of the Comamonadaceae were observed to be dominant taxa in the microbiome of *Daphnia*. At the OTU level, one single taxon, namely *Limnohabitans planktonicus* (OTU1) belonging to the family Comamonadaceae stands out in terms of prevalence and relative abundance. This pattern in particularly evident in laboratory-cultured animals where this taxon is consistently identified as the most common and abundant taxon in all data-sets of laboratory origin, even though the host individuals were cultured in five independent laboratories in five different countries. In invertebrates, microbiomes are often dominated by only a few species (40–43). Even species originating from microbially diverse environments, such as soil and marine waters, have been shown to selectively filter environmental microbes and exhibit simple microbiomes that are dominated by one-species associations (44,45). A number of vertebrates also have rather species-poor microbiomes (46–48). For instance, many avian and mammalian microbiomes are dominated by Firmicutes, particularly by the genera *Staphylococcus* and *Streptococcus*, and Proteobacteria (46,49,50). Likewise, gut microbiomes of terrestrial mammals also display a high proportion of *Streptococcus* and Proteobacteria (51,47).

The genus *Limnohabitans* may be common in the *Daphnia* gut microbiome either due to its potential contribution to host fitness or its dominance in freshwater systems, where it typically comprises 5–30% (up to 50%) of the bacterioplankton communities in non-humic freshwaters (52). *Limnohabitans* belongs to the R-BT lineage of the class Betaproteobacteria. This genus has been repeatedly reported on body parts such as the external surface, digestive tract,

and filtering apparatus of *Daphnia* (24,53,27,22,38). Since the natural substrates for the R-BT lineage are autochthonous algae, as witnessed by their enrichment during heavy phytoplankton blooms (52,54) and on algal extracellular exudates (55), the *Daphnia* gut emerges as an ideal habitat for *Limnohabitans*. The observation that the genus was especially widespread in all laboratory-cultured animals suggests strong selection pressure favoring this taxon. Given that laboratory-cultured *Daphnia* are generally fed with unicellular algae cultured at high densities, it is possible that these algae provide an ideal substrate for *Limnohabitans* to reach high densities in the culture medium. Laboratory culturing, often a preparatory stage to microbiome sampling, has often been shown to change the microbial community structure (56,57,36) and increase the abundance of parasitic and commensal microbes (58). In our study, we observe the high prevalence and dominance of *Escherichia/Shigella* sp., *Ideonella paludis, Staphylococcus* sp., *and Pseudomonas* sp. in the *D. magna* gut of laboratory origin. *Pseudomonas* sp. are often opportunistic bacteria (59) that have been reported in the microbiome of *Daphnia* immediately before death, in what was termed a "sit and wait (SAW) strategy", where these species wait and proliferate during vulnerable stages of the host (60). An increase in the abundance of *Pseudomonas* sp. has also been reported during antibiotic treatment (26), at which occasion they benefit from lower microbial diversity and a lack of competition. The strong association of *Escherichia sp*. with laboratory-reared *D. magna* reported is in contrast with the findings of Di Cesare et al.,(61), who found *E. coli* only to occur temporarily in the *Daphnia* gut, where the species potentially seek refuge during periods of unfavorable environments (61).

Laboratory studies have shown that the presence of *Limnohabitans* can contribute to host fitness. For instance, inoculation of germ-free animals with *Limnohabitans planktonicus,* strain II D5 and strain DM1 has been shown to restore fecundity in individuals that would otherwise fail to mature or produce short-lived and nonviable juveniles (25). In another study, an increase in host population size was linked to the dominance of *Limnohabitans sp*. in the *Daphnia* microbiome (17). *Limnohabitans planktonicus* has also been reported to be an epibiont inhabiting the filter combs of *Daphnia,* where it may play an important role in the transfer of dissolved organic carbon to higher trophic levels in freshwater systems (53). The functions underlying the beneficial *Limnohabitans–Daphnia* association remain largely elusive, yet recent studies suggest plausible mechanisms. For instance, the metabolic versatility encoded within the *Limnohabitans* genome (22) may facilitate the utilization of a broad spectrum of organic molecules, both within the digestive tract and on the carapace of the host. This may be especially important for non-selective generalist filter feeders such as *Daphnia*, which ingest a diverse array of particulate matter with varying organic matter composition. Moreover, it has been shown that the *Limnohabitans* genome harbors genes for arginine and threonine export, key amino acids crucial for *Daphnia* fitness (62,63). Finally, an analysis of annotated genes and functional pathways in *Limnohabitans* also revealed the presence of antibiotic resistance and detoxification genes, secretory and regulatory pumps and osmotic stress response systems, which may support for environmental stress tolerance mechanisms in the host (22). While these studies suggest multiple pathways through which *Limnohabitans* can contribute to host fitness, our observation that it is much less prevalent in field-grown samples casts doubts on this taxon playing an essential role. Alternatively, such dominance may not always reflect functional symbiosis but rather niche specialization driven by strong environmental filters typical of laboratory environments, such as stable abiotic parameters and a standardized homogenous diet. In particular, *Limnohabitans* are opportunistic fast-growing generalists (64,65) that may take advantage of the high algal densities fed to the daphnids maintained in culture, further favoring disproportionate growth of this taxon under laboratory settings. Notably, *Limnohabitans* strains such as 2KL-3 and 2KL-7 have been shown to differ in their effects as compared to the beneficial effect of *Limnohabitans* strain II-D2 and DM1 on *Daphnia* fecundity (25) highlighting the strain-specific nature of this genus (55). This suggests that *Limnohabitans* exhibits distinct ecological roles with their interactions with the host differing based on substrate utilization patterns and environmental preferences (25,54,66). Additionally, founder effects (67) and priority effects (68,69) during early establishment of laboratory cultures may shape microbiome assembly in ways that do not necessarily reflect ecological selection or functional relevance. Thus, the observed dominance of *Limnohabitans* may stem from reduced microbial competition in low-diversity laboratory microbiomes, passive introduction with food or water or altered host-microbe dynamics in artificial settings, rather than from an ecologically significant symbiosis.

For the whole-animal microbiome samples of laboratory-cultured animals, core taxa included *Limnohabitans planktonicus, Formosimonas* sp., *Flavobacterium* sp., unclassified *Chitinophagaceae* and *Emticicia* sp.. *Limnohabitans, Flavobacterium* and unclassified *Chitinophagaceae* have been shown to be not only dominant members of the *D. magna* microbiome (70) but also to be overrepresented on the external surfaces of ephippial cases in dormant resting eggs (71). The presence of unclassified *Chitinophagaceae* in whole-animal samples likely reflects their capacity to

degrade chitin, a principal constituent of the *Daphnia* carapace. The dominance of unclassified *Chitinophagaceae* in the microbiota has also been observed in antibiotic-treated daphnids (27,72). In such cases, these microorganisms may exert detrimental effects on host fitness by opportunistically degrading chitin in their host's carapace. Likewise, *Flavobacterium* sp., have also been detected in whole-community analyses of *Daphnia* (24,17,72). The dominance of this group has been observed in *Microcystis aeruginosa*-tolerant genotypes of *Daphnia* (1), suggesting their contribution to the degradation of cyanobacterial toxins. *Flavobacterium* sp. are frequently associated with the lysis and degradation of cyanobacteria in freshwater systems, with their relative abundances and metabolic activity increasing in magnitude during cyanobacterial blooms (73). In addition, the ability of *Flavobacterium* to digest polysaccharides (74), such as cellulose, hemicellulose and pectins, which are major constituents of the phytoplankton cell wall (16), might contribute to host digestion and nutrient acquisition. The presence of *Emticicia* may be relevant for the fitness of *Daphnia*, as a metagenomics study of key microbiota of *Daphnia* revealed that the genome of *Emticicia* sp. encodes for amino acid biosynthesis and transport, vitamin and mineral transport and carbohydrate breakdown, all of which are essential functions for the optimal survival and growth of *Daphnia.* (22). *Formosimonas* sp. are a group of free-living bacteria often recovered at high numbers from freshwater systems (75). Although not detected in other studies, the prevalence of this species in whole animal samples as host symbionts, parasites or neutral associations is unclear.

One interesting observation of the current study is that the microbiome of field-grown *D. magna* differed strongly from that of laboratory-cultured animals. A key difference is that *L. planktonicus* does not stand out as the most abundant and widespread taxon in the *D. magna* gut microbiome of field-grown hosts. We also observed that the diversity of OTUs that are shared among individuals in the field is much higher than that of laboratory-cultured animals (Figure 4). However, this latter observation needs to be interpreted with caution, given that all microbiomes of field-grown animals in our dataset were sampled from one single lake (Lake Bysön, Sweden), i.e. were exposed to the same environmental microbial community. This likely also explains why the among-individual differences in microbiome composition seems to be lower for field-grown animals than for laboratory-grown animals. If we only consider laboratory-cultured animals of genotypes isolated from the same lake as the field-grown animals, then the among-host individual variation in microbiome composition is actually larger than the variation among laboratory-cultured hosts (see Figure S3, SI). The core taxa identified at the highest prevalence (99%) in the gut samples of these field animals comprised various OTUs including unclassified *Pirellulales*, *Rhodobacteraceae*, *Solirubrobacterales*, *Hyphomicrobiaceae*, *Rhizobiales*, *Actinobacteria*, *Acidimicrobiales*, *Isophaeraceae* and the genera *Methylocystis*, *Roseimaritima*, *Mycobacterium, Ilumatobacter, Nocardioides* and *Aquihabitans*. While *L. planktonicus* was also detected in these samples, it occurred at a lower prevalence threshold (80%). We hypothesize that given the high diversity of the natural bacterioplankton, the indiscriminate filter feeding mechanism of *Daphnia* results in the uptake of a vast variety of bacterial taxa. Given that microbiomes may strongly affect the fitness and traits of their hosts, the distinct differences in microbiome composition of field and laboratory-cultured animals suggest that some caution may be needed when interpreting results of laboratory experiments to infer how *Daphnia* respond in the field (76). While standardization by growing animals in common garden experiments is often an essential element ensuring experimental control and straightforward interpretation of experimental results, it seems difficult to avoid that it is accompanied by a strong shift in microbiome composition. Our results clearly point towards the need to engage in more studies focusing on the microbiome composition of field-collected animals.

In summary, our findings indicate that the microbiome of *D. magna* is highly variable and can be easily influenced by the external environment. This is in contrast to studies on mice, zebrafish, bees, corals, and other organisms (77–80), where certain bacterial taxa have been detected as constant and stable members of the microbiome, but is in line with recent studies suggesting that core microbiomes may not be omnipresent (35). Our study shows that in *D. magna,* the proportion of core microbial taxa is quite low. In laboratory-cultured animals, it seems to be limited to one (*L. planktonicus*) or a few taxa that are widespread, although not always in high densities. Our results on field-grown hosts also temper the strong association between *Daphnia* and *L. planktonicus*, since for this dataset we have several other bacterial taxa that seem to be more prevalent than *L. planktonicus.* While *L. planktonicus* is still widespread (prevalence >80%), its prevalence and relative abundance in the field is lower than in laboratory-cultured animals. The emergent pattern is that the *D. magna* microbiome is highly dynamic and variable with *D. magna* possibly not being universally dependent on specialized microbes and its microbiome likely being strongly determined by what is taken up from the environment.

Our study suffers from some limitations. Our analysis was limited by the availability of only 12 datasets, comprising a total of 675 samples, which may not be sufficient to fully capture the vast diversity of the *Daphnia* microbiome. While

we did initially consider integrating datasets sequencing multiple hypervariable regions to increase the breadth of our analysis, specifically by incorporating V3-V4 and V3-V5 reads and truncating them to extract only the homologous V4 region, this approach proved technically challenging inflating operational taxonomic unit (OTU) counts and generating extremely large distance matrices that further increased computational burden. In addition, we also considered analyzing each hypervariable region separately and merging reads from these different regions into downstream data analysis. However, multiple studies have shown that different targeted hypervariable regions differ in their taxonomic assignment due to primer bias, difference in hypervariable region sequence length and sequence uniqueness across bacterial taxa (81–84). As a result, taxonomic assignments can vary between regions of the same species, especially at the lower taxonomic levels. This would mean that merging the individual analyses for the different regions would introduce inconsistencies due to the potential differences in taxonomic assignments for the same species (more prominent at the lower taxonomic levels).

We thus focused on the V4 hypervariable region of the 16S rRNA gene, as it was the most commonly targeted region across studies, while also enabling standardized processing for greater consistency and robust cross-study comparisons. This is in line with literature where most studies investigating the core microbiome focus on analyzing a single hypervariable region of the 16S rRNA gene (85–90) to allow data standardization and reduce biases introduced by non-overlapping regions. However, studies that do combine multiple hypervariable regions, although the input is processed as OTUs or ASVs for analysis, the results are typically collapsed to the genus or sometimes a higher taxonomic level to achieve cross study comparisons (91–95). While this approach reduces region-specific biases, it compromises ecological resolution given that distinct sequence variants are merged under the same genus. As such, the core microbiome is defined at the genus or family level which may not be accurate as there can arise multiple species within a single genus/family with distinct host functions, metabolic capabilities and ecological roles. In contrast, our synthesis focuses on a single hypervariable region (namely V4 region) of the 16S rRNA gene, thereby allowing OTU-level resolution to be retained for core microbiome analysis. We consider this more biologically relevant as we assume that the ecology of all members within an OTU will be highly similar and that members of an OTU are expected to share most of the adaptations required to persist in association with a particular host. As such, an OTU-based perspective provides a finer scale definition of a core microbiome which is in contrast to that achieved from collapsing OTUs into higher taxonomic levels. However, this choice also represents a limitation potentially underestimating overall microbial diversity that other hypervariable regions or full-length 16S sequencing could better resolve.

Variation in experimental design, sample collection methods, DNA extraction, amplification primers, library preparation protocols, and sequencing platforms across different laboratories and studies could potentially have introduced some heterogeneity and potential biases into the analysis. Given that our study is grounded on 16S rRNA sequencing data, we present results from a community composition-based analysis (96) and focus on identifying a "*common core*" based on consistent taxonomic presence across hosts. This approach follows the original definition of the core microbiome (3) and was selected to maintain clarity and comparability in a rapidly evolving and conceptually fragmented field. This is in contrast to a "*functional core*" which would require complementary data (e.g. metagenomics, transcriptomics, metabolomics) to assess fitness consequences of individual microbial taxa on the host. Future studies integrating multi-omic approaches hold promise for uncovering deeper insights into the functional components of the *Daphnia* microbiome. One further limitation is that all data considered here are snapshots, while the relative abundance of taxa can vary significantly over time (97,98). This does not interfere with our effort to identify core taxa given that dynamics in time would average out for the criteria of occurrences and relative abundances. It may rather explain that we observe no taxa that have a very high relative abundance (e.g., >10%) across many samples. Quite a number of taxa that were observed in our dataset may be transient and not permanent members of the microbiome. Additionally, a crucial factor affecting species richness in microbiome studies is the total number of sequences obtained from each sample. Multiple deep sequencing efforts have shown that rare taxa constitute a considerable portion of several microbial communities and are often overlooked by shallow sequencing efforts (99,100). To address variation in sequencing depths between samples, some studies suggest rarefaction (101), whereas others argue that such rarefaction result in important biases (102) and favor approaches based on alpha and beta diversities (103,104). In our study, sequence depth per sample varied widely. We did not normalize the sequencing depth for the samples, as we wanted to use all information and either would have to drop several studies or would have to resort to a low sequencing depth for all studies. While these limitations are real, we argue that the main conclusion of our analysis, being that the microbiome of *D. magna* is highly variable, is not affected by them. The key limitation revealed by our study is the surprisingly low number of studies on field-grown animals (only a single field study

identified in our analysis), which limits our capacity to draw strong conclusions for field animals and does not allow us to assess the influence of, for instance, geographic origin or the ecology of habitats on microbiome composition. Moving forward, future studies should address this shortcoming and focus more on field-sampled animals. One avenue to increase standardization and thus tractability of such studies is the use of germ-free animals that are exposed to medium directly harvested from natural systems. In addition, metagenomic studies that allow functional inference may shed more light on how widespread microbiome taxa can contribute to fitness of their host.

Conclusions

We conclude from our study that the *D. magna* microbiome is highly variable across host individuals, with most taxa being unique to a small subset of the analyzed host individuals. Yet, amid this large inter-individual variation, a relatively consistent association with a single taxon, namely, *Limnohabitans planktonicus*, which belongs to the family Comamonadaceae, is observed in both the *D. magna* gut and whole animal samples of laboratory origin across samples obtained from five independent laboratories and three continents. This robust association may point towards a symbiotic relationship, with *L. planktonicus* potentially exhibiting features that may contribute to host fitness. In contrast, this association could also be attributed to the fast-growing and generalist nature of this taxa, simply suggesting a neutral or opportunistic association in artificial environments with reduced microbial diversity and competition. Additionally, this putative strong association should be put into perspective due to the observation that the microbiomes of field-grown animals exhibit a relatively weaker presence of *L. planktonicus*, casting doubt on the extent to which one should consider it as a core species of the *D. magna* microbiome. Field-grown animals exhibit a more diverse microbiome in contrast to laboratory-cultured animals which display a reduced diversity when reared under constant and standardized long-term conditions. This suggests that transitioning from natural environments to controlled laboratory settings significantly diminish gut microbiome diversity, resulting in the loss of multiple microbial groups and a reduced core membership, which in turn might pave the path for the dominance and spread of *L. planktonicus*. Ultimately, our research highlights the striking scarcity of studies focused on natural microbiomes in their native environment (with only one field study found in our analysis). This glaring gap emphasizes the urgent need for further investigation into the microbiomes of field animals to truly understand the structuring of microbial communities under natural settings.

Availability of data and material

All raw sequences used in this study are previously published datasets available in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home). Please refer to the original publications or Table S2 in SI for additional information on their specific BioProject/Accession numbers. The dataset(s) supporting the conclusions of this article is(are) included within the article (and its additional file(s)).

Competing interests

The authors declare no competing interests.

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Author contributions

Conceptualization: AG, LDM, ED, RS, MC, FM, SH; Methodology: AG, MC, FM, LDM, ED; Bioinformatic analysis: AG, MC; Writing-original draft: AG, LDM; Writing-review and editing: all authors.

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References

1. Macke E, Callens M, De Meester L, Decaestecker E. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat Commun. 2017 Nov 20;8(1):1608.

2. Stock W, Callens M, Houwenhuyse S, Schols R, Goel N, Coone M, et al. Human impact on symbioses between aquatic organisms and microbes. Aquat Microb Ecol. 2021 Aug 26;87:113–38.

3. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project. Nature. 2007 Oct 1;449(7164):804–10.

4. Parfrey LW, Moreau CS, Russell JA. Introduction: The host-associated microbiome: Pattern, process and function. Mol Ecol. 2018 Apr;27(8):1749–65.

5. Wilkinson DM. At cross purposes. Nature. 2001 Aug;412(6846):485–485.

6. Benzie JA. Guides to the Identification of the Microinvertebrates of the Continental Waters of the World: Cladocera: the Genus Daphnia (including Daphniopsis). SPB Acad. Publ.; 2005.

7. Miner BE, De Meester L, Pfrender ME, Lampert W, Hairston NG. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc R Soc B: Biol Sci. 2012 May 22;279(1735):1873–82.

8. Almeida RA, Lemmens P, De Meester L, Brans KI. Differential local genetic adaptation to pesticide use in organic and conventional agriculture in an aquatic non-target species. Proc R Soc B: Biol Sci. 2021 Nov 17;288(1963):20211903.

9. Brans KI, Jansen M, Vanoverbeke J, Tüzün N, Stoks R, De Meester L. The heat is on: Genetic adaptation to urbanization mediated by thermal tolerance and body size. Glob Change Biol. 2017;23(12):5218–27.

10. Geerts AN, Vanoverbeke J, Vanschoenwinkel B, Van Doorslaer W, Feuchtmayr H, Atkinson D, et al. Correction: Corrigendum: Rapid evolution of thermal tolerance in the water flea Daphnia. Nature Clim Change. 2015 Oct;5(10):956–956.

11. Wersebe MJ, Weider LJ. Resurrection genomics provides molecular and phenotypic evidence of rapid adaptation to salinization in a keystone aquatic species. PNAS. 2023 Feb 7;120(6):e2217276120.

12. Steiner CF, Nowicki CJ. Eco-Evolutionary Dynamics in the Wild: Clonal Turnover and Stability in Daphnia Populations. Am Nat. 2019 Jul;194(1):117–23.

13. Decaestecker E, Gaba S, Raeymaekers JAM, Stoks R, Van Kerckhoven L, Ebert D, et al. Host–parasite 'Red Queen' dynamics archived in pond sediment. Nature. 2007 Dec;450(7171):870–3.

14. Zhang C, De Meester L, Stoks R. Rapid evolution of consumptive and non-consumptive predator effects on prey population densities, bioenergetics and stoichiometry. J Anim Ecol. 2024;93(7):906–17.

15. Ebert D. Daphnia as a versatile model system in ecology and evolution. Evo Devo. 2022 Aug 8;13(1):16.

16. Qi W, Nong G, Preston JF, Ben-Ami F, Ebert D. Comparative metagenomics of Daphnia symbionts. BMC Genomics. 2009 Apr 21;10(1):172.

17. Peerakietkhajorn S, Tsukada K, Kato Y, Matsuura T, Watanabe H. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, Daphnia magna. Environ Microbiol Rep. 2015 Apr;7(2):364–72.

18. Sison-Mangus MP, Mushegian AA, Ebert D. Water fleas require microbiota for survival, growth and reproduction. ISME J. 2015 Jan;9(1):59–67.

19. Mushegian AA, Ebert D. Presence of microbiota reverses the relative performance of Daphnia on two experimental diets. Zoology. 2017 Dec 1;125:29–31.

20. Sison-Mangus MP, Metzger CMJA, Ebert D. Host genotype-specific microbiota do not influence the susceptibility of D. magna to a bacterial pathogen. Sci Rep. 2018 Jun 20;8(1):9407.

21. Fong JC, De Guzman BE, Lamborg CH, Sison-Mangus MP. The Mercury-Tolerant Microbiota of the Zooplankton Daphnia Aids in Host Survival and Maintains Fecundity under Mercury Stress. Environ Sci Technol. 2019 Dec 17;53(24):14688–99.

22. Cooper RO, Cressler CE. Characterization of key bacterial species in the Daphnia magna microbiota using shotgun metagenomics. Sci Rep. 2020 Jan 20;10(1):652.

23. Motiei A, Brindefalk B, Ogonowski M, El-Shehawy R, Pastuszek P, Ek K, et al. Disparate effects of antibiotic-induced microbiome change and enhanced fitness in Daphnia magna. PLoS One. 2020;15(1):e0214833.

24. Freese HM, Schink B. Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean Daphnia magna. Microb Ecol. 2011 Nov 1;62(4):882–94.

25. Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. Betaproteobacteria Limnohabitans strains increase fecundity in the crustacean Daphnia magna: symbiotic relationship between major bacterioplankton and zooplankton in freshwater ecosystem. Environ Microbiol. 2016 Sep;18(8):2366–74.

26. Akbar S, Gu L, Sun Y, Zhou Q, Zhang L, Lyu K, et al. Changes in the life history traits of *Daphnia magna* are associated with the gut microbiota composition shaped by diet and antibiotics. Sci Tot Environ. 2020 Feb 25;705:135827.

27. Callens M, Watanabe H, Kato Y, Miura J, Decaestecker E. Microbiota inoculum composition affects holobiont assembly and host growth in Daphnia. Microbiome. 2018 Mar 22;6(1):56.

28. Eckert EM, Anicic N, Fontaneto D. Freshwater zooplankton microbiome composition is highly flexible and strongly influenced by the environment. Mol Ecol. 2021;30(6):1545–58.

29. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009 Dec;75(23):7537–41.

30. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE. 2013 Apr 22;8(4):e61217.

31. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al. vegan: Community Ecology Package [Internet]. 2024 [cited 2025 Jan 6]. Available from: https://cran.r-project.org/web/packages/vegan/index.html

32. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecology. 2001;26(1):32–46.

33. Larsson J, Godfrey AJR, Gustafsson P, algorithms) DHE (geometric, code) EH (root solver, Privé F. eulerr: Area-Proportional Euler and Venn Diagrams with Ellipses [Internet]. 2024 [cited 2024 Nov 21]. Available from: https://cran.r-project.org/web/packages/eulerr/index.html

34. Lahti L, Shetty S. Introduction to the microbiome R package. Preprint at https://microbiome github io/tutorials. 2018;

35. Hammer TJ, Sanders JG, Fierer N. Not all animals need a microbiome. FEMS Microbiol Lett. 2019 May 1;366(10):fnz117.

36. Houwenhuyse S, Callens M, Bulteel L, Decaestecker E. Comparison between the gut bacterial community of laboratory cultured and wild Daphnia. FEMS Microb Ecol. 2023; 11: fiad116; doi: 10.1093/femsec/fiad116.

37. Surmacz B, Stec D, Prus-Frankowska M, Buczek M, Michalczyk Ł, Łukasik P. Pinpointing the microbiota of tardigrades: What is really there? Environ Microbiol. 2024;26(6):e16659.

38. Akbar S, Li X, Ding Z, Liu Q, Huang J, Zhou Q, et al. Disentangling Diet- and Medium-Associated Microbes in Shaping Daphnia Gut Microbiome. Microb Ecol. 2022 Oct 1;84(3):911–21.

39. Gorokhova E, Motiei A, El-Shehawy R. Understanding Biofilm Formation in Ecotoxicological Assays With Natural and Anthropogenic Particulates. Front Microbiol. 2021;12: 632947; doi: 10.3389/fmicb.2021.632947.

40. Haynes S, Darby AC, Daniell TJ, Webster G, van Veen FJF, Godfray HCJ, et al. Diversity of Bacteria Associated with Natural Aphid Populations. Appl Environ Microbiol. 2003 Dec;69(12):7216–23.

41. Salem H, Bauer E, Kirsch R, Berasategui A, Cripps M, Weiss B, et al. Drastic Genome Reduction in an Herbivore's Pectinolytic Symbiont. Cell. 2017 Dec 14;171(7):1520-1531.e13.

42. Matsuura Y, Moriyama M, Łukasik P, Vanderpool D, Tanahashi M, Meng XY, et al. Recurrent symbiont recruitment from fungal parasites in cicadas. PNAS. 2018 Jun 26;115(26):E5970–9.

43. Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP, Fierer N. Caterpillars lack a resident gut microbiome. PNAS. 2017 Sep 5;114(36):9641–6.

44. Nyholm SV, McFall-Ngai M. The winnowing: establishing the squid–vibrio symbiosis. Nat Rev Microbiol. 2004 Aug;2(8):632–42.

45. Ohbayashi T, Takeshita K, Kitagawa W, Nikoh N, Koga R, Meng XY, et al. Insect's intestinal organ for symbiont sorting. PNAS. 2015 Sep 15;112(37):E5179–88.

46. Carrillo-Araujo M, Taş N, Alcántara-Hernández RJ, Gaona O, Schondube JE, Medellín RA, et al. Phyllostomid bat microbiome composition is associated to host phylogeny and feeding strategies. Front Microbiol. 2015;6: 447; doi: 10.3389/fmicb.2015.00447.

47. Xue Z, Zhang W, Wang L, Hou R, Zhang M, Fei L, et al. The Bamboo-Eating Giant Panda Harbors a Carnivore-Like Gut Microbiota, with Excessive Seasonal Variations. mBio. 2015 May 19;6(3):10.1128/mbio.00022-15.

48. McKenney EA, Maslanka M, Rodrigo A, Yoder AD. Bamboo Specialists from Two Mammalian Orders (Primates, Carnivora) Share a High Number of Low-Abundance Gut Microbes. Microb Ecol. 2018 Jul 1;76(1):272–84.

49. Waite DW, Deines P, Taylor MW. Gut Microbiome of the Critically Endangered New Zealand Parrot, the Kakapo (Strigops habroptilus). PLOS ONE. 2012 Apr 18;7(4):e35803.

50. Hird SM, Sánchez C, Carstens BC, Brumfield RT. Comparative Gut Microbiota of 59 Neotropical Bird Species. Front Microbiol. 2015;6. doi: Hird SM, Sánchez C, Carstens BC, Brumfield RT. Comparative Gut Microbiota of 59 Neotropical Bird Species. Front Microbiol. 2015;6; 1403. doi: 10.3389/fmicb.2015.01403.

51. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of Mammals and Their Gut Microbes. Science. 2008 Jun 20;320(5883):1647–51.

52. Šimek K, Kasalický V, Jezbera J, Jezberová J, Hejzlar J, Hahn MW. Broad Habitat Range of the Phylogenetically Narrow R-BT065 Cluster, Representing a Core Group of the Betaproteobacterial Genus Limnohabitans. Appl Environl Microbiol. 2010 Feb;76(3):631–9.

53. Eckert EM, Pernthaler J. Bacterial epibionts of Daphnia: a potential route for the transfer of dissolved organic carbon in freshwater food webs. ISME J. 2014 Sep 1;8(9):1808–19.

54. Šimek K, Kasalický V, Zapomělová E, Horňák K. Alga-Derived Substrates Select for Distinct Betaproteobacterial Lineages and Contribute to Niche Separation in Limnohabitans Strains. Appl Environ Microbiol. 2011 Oct 15;77(20):7307–15.

55. Horňák K, Kasalický V, Šimek K, Grossart HP. Strain-specific consumption and transformation of alga-derived dissolved organic matter by members of the Limnohabitans-C and Polynucleobacter-B clusters of Betaproteobacteria. Environ Microbiol. 2017;19(11):4519–35.

56. Hammer TJ, McMillan WO, Fierer N. Metamorphosis of a Butterfly-Associated Bacterial Community. PLOS ONE. 2014 Jan 23;9(1):e86995.

57. Staudacher H, Kaltenpoth M, Breeuwer JAJ, Menken SBJ, Heckel DG, Groot AT. Variability of Bacterial Communities in the Moth Heliothis virescens Indicates Transient Association with the Host. PLOS ONE. 2016 May 3;11(5):e0154514.

58. Lighthart B. Some Changes in Gut Bacterial Flora of Field-Grown Peridroma saucia (Lepidoptera: Noctuidae) When Brought into the Laboratory. Appl Environ Microbiol. 1988 Jul;54(7):1896–8.

59. Preiswerk D, Walser J-C, Ebert D. Temporal dynamics of microbiota before and after host death. ISME J. 2018; 12: 2076-2085 doi: 10.1038/s41396-018-0157-2.

60. Lennon JT, Jones SE. Microbial seed banks: the ecological and evolutionary implications of dormancy. Nat Rev Microbiol. 2011 Feb;9(2):119–30.

61. Di Cesare A, Riva F, Colinas N, Borgomaneiro G, Borin S, Cabello-Yeves PJ, et al. Zooplankton as a Transitional Host for Escherichia coli in Freshwater. Appl Environ Microbiol. 2022 Apr 13;88(9):e02522-21.

62. Hansen A, Moran N. Aphid genome expression reveals host–symbiont cooperation in the production of amino acids | PNAS [Internet]. [cited 2024 Oct 22]. Available from: https://www-pnas-org.kuleuven.e-bronnen.be/doi/abs/10.1073/pnas.1013465108

63. Macdonald SJ, Thomas GH, Douglas AE. Genetic and metabolic determinants of nutritional phenotype in an insect–bacterial symbiosis. Mol Ecol. 2011;20(10):2073–84.

64. Horňák K, Zeder M, Blom JF, Posch T, Pernthaler J. Suboptimal light conditions negatively affect the heterotrophy of Planktothrix rubescens but are beneficial for accompanying Limnohabitans spp. Environ Microbiol. 2012;14(3):765–78.

65. Jezberová J, Jezbera J, Znachor P, Nedoma J, Kasalický V, Šimek K. The Limnohabitans Genus Harbors Generalistic and Opportunistic Subtypes: Evidence from Spatiotemporal Succession in a Canyon-Shaped Reservoir. Appl Environ Microbiol. 2017 Oct 17;83(21):e01530-17.

66. Kasalický V, Jezbera J, Hahn MW, Šimek K. The Diversity of the Limnohabitans Genus, an Important Group of Freshwater Bacterioplankton, by Characterization of 35 Isolated Strains. PLOS ONE. 2013 Mar 7;8(3):e58209.

67. Santos J, Pascual M, Simões P, Fragata I, Lima M, Kellen B, et al. From nature to the laboratory: the impact of founder effects on adaptation. J Evol Biol. 2012 Dec 1;25(12):2607–22.

68. McCafferty J, Mühlbauer M, Gharaibeh RZ, Arthur JC, Perez-Chanona E, Sha W, et al. Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. ISME J. 2013 Nov;7(11):2116–25.

69. Martínez I, Maldonado-Gomez MX, Gomes-Neto JC, Kittana H, Ding H, Schmaltz R, et al. Experimental evaluation of the importance of colonization history in early-life gut microbiota assembly. eLife. 2018;7:e36521.

70. Pfenning-Butterworth A, Cooper RO, Cressler CE. Daily feeding rhythm linked to microbiome composition in two zooplankton species. PLOS ONE. 2022 Feb 3;17(2):e0263538.

71. Mushegian AA, Walser JC, Sullam KE, Ebert D. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. J Anim Ecol. 2018;87(2):400–13.

72. Cooper RO, Tjards S, Rischling J, Nguyen DT, Cressler CE. Multiple generations of antibiotic exposure and isolation influence host fitness and the microbiome in a model zooplankton species. FEMS Microbiol Ecol. 2017; 87: 400-413; doi: 10.1111/1365-2656.12709.

73. Williams TJ, Wilkins D, Long E, Evans F, DeMaere MZ, Raftery MJ, et al. The role of planktonic lavobacteria in processing algal organic matter in coastal East Antarctica revealed using metagenomics and metaproteomics. Environ Microbiol. 2013;15(5):1302–17.

74. Gavriilidou A, Gutleben J, Versluis D, Forgiarini F, van Passel MWJ, Ingham CJ, et al. Comparative genomic analysis of Flavobacteriaceae: insights into carbohydrate metabolism, gliding motility and secondary metabolite biosynthesis. BMC Genomics. 2020 Aug 20;21(1):569.

75. Chen WM, Xie PB, Young CC, Sheu SY. Formosimonas limnophila gen. nov., sp. nov., a new member of the family Burkholderiaceae isolated from a freshwater lake. Int J Syst Evol Microb. 2017; 67: 17-24; doi: 10.1099/ijsem.0.00156.

76. Houwenhuyse S, Bulteel L, Vanoverberghe I, Krzynowek A, Goel N, Coone M, et al. Microbiome mediated tolerance to biotic stressors: a case study of the interaction between a toxic cyanobacterium and an oomycete-like infection in Daphnia magna. OSF; https://osf.io/9n4mg (2023); doi: 10.31219/osf.io/ws7m6.

77. Wang J, Lang T, Shen J, Dai J, Tian L, Wang X. Core Gut Bacteria Analysis of Healthy Mice. Front Microbiol. 2019;10: 887; doi: 10.3389/fmicb.2019.00887.

78. Sharpton TJ, Stagaman K, Sieler MJ, Arnold HK, Davis EW. Phylogenetic Integration Reveals the Zebrafish Core Microbiome and Its Sensitivity to Environmental Exposures. Toxics. 2021;9:10; doi: 10.3390/toxics9010010.

79. Dew RM, McFrederick QS, Rehan SM. Diverse Diets with Consistent Core Microbiome in Wild Bee Pollen Provisions. Insects. 2020 Aug;11(8):499.

80. Hernandez-Agreda A, Gates RD, Ainsworth TD. Defining the Core Microbiome in Corals' Microbial Soup. Trends Microbiol. 2017 Feb 1;25(2):125–40.

81. Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, et al. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res. 2010 Dec;38(22):e200.

82. Pinto AJ, Raskin L. PCR Biases Distort Bacterial and Archaeal Community Structure in Pyrosequencing Datasets. PLOS ONE. 2012 Aug 15;7(8):e43093.

83. Tremblay J, Singh K, Fern A, Kirton ES, He S, Woyke T, Lee J, Chen F, Dangl JL, Tringe SG. Primer and platform effects on 16S rRNA tag sequencing. Frontiers microbiol. 2015 Aug 4;6:771.

84. Barb JJ, Oler AJ, Kim HS, Chalmers N, Wallen GR, Cashion A, et al. Development of an Analysis Pipeline Characterizing Multiple Hypervariable Regions of 16S rRNA Using Mock Samples. PLOS ONE. 2016 Feb 1;11(2):e0148047.

85. de Souza YPA, Schloter M, Weisser W, Huang Y, Schulz S. The seeds of Plantago lanceolata comprise a stable core microbiome along a plant richness gradient. Environ Microbiome. 2024 Feb 2;19(1):11.

86. Yu J, Jiang C, Yamano R, Koike S, Sakai Y, Mino S, et al. Unveiling the early life core microbiome of the sea cucumber Apostichopus japonicus and the unexpected abundance of the growth-promoting Sulfitobacter. anim microbiome. 2023 Oct 24;5(1):54.

87. Wang L, Zhang L, George TS, Feng G. A core microbiome in the hyphosphere of arbuscular mycorrhizal fungi has functional significance in organic phosphorus mineralization. New Phytologist. 2023;238(2):859–73.

88. Simonin M, Dasilva C, Terzi V, Ngonkeu ELM, Diouf D, Kane A, et al. Influence of plant genotype and soil on the wheat rhizosphere microbiome: evidences for a core microbiome across eight African and European soils. FEMS Microbiol Ecol. 2020 Jun 1;96(6):fiaa067.

89. Kellogg CA. Microbiomes of stony and soft deep-sea corals share rare core bacteria. Microbiome. 2019 Jun 10;7(1):90.

90. Xia F, Zhou X, Liu Y, Li Y, Bai X, Zhou X. Composition and predictive functional analysis of bacterial communities inhabiting Chinese Cordyceps insight into conserved core microbiome. BMC Microbiol. 2019 May 23;19(1):105.

91. Hoffbeck C, Middleton DMRL, Nelson NJ, Taylor MW. 16S rRNA gene-based meta-analysis of the reptile gut microbiota reveals environmental effects, host influences and a limited core microbiota. Mol Ecol. 2023;32(22):6044–58.

92. Mills S, Dessì P, Pant D, Farràs P, Sloan WT, Collins G, et al. A meta-analysis of acetogenic and methanogenic microbiomes in microbial electrosynthesis. npj Biofilms Microbiomes. 2022 Sep 23;8(1):73.

93. Ghosh TS, Shanahan F, O'Toole PW. Toward an improved definition of a healthy microbiome for healthy aging. Nat Aging. 2022 Nov;2(11):1054–69.

94. Huang C, Gin C, Fettweis J, Foxman B, Gelaye B, MacIntyre DA, et al. Meta-analysis reveals the vaginal microbiome is a better predictor of earlier than later preterm birth. BMC Biol. 2023 Sep 25;21(1):199.

95. Akinsuyi OS, Roesch LFW. Meta-Analysis Reveals Compositional and Functional Microbial Changes Associated with Osteoporosis. Microbiol Spectrum. 2023 Apr 12;11(3):e00322-23.

96. Sharon I, Quijada NM, Pasolli E, Fabbrini M, Vitali F, Agamennone V, et al. The Core Human Microbiome: Does It Exist and How Can We Find It? A Critical Review of the Concept. Nutrients. 2022 Jan;14(14):2872.

97. Neu AT, Allen EE, Roy K. Defining and quantifying the core microbiome: Challenges and prospects. PNAS. 2021 Dec 21;118(51):e2104429118.

98. Hegg A, Radersma R, Uller T. A field experiment reveals seasonal variation in the Daphnia gut microbiome. Oikos. 2021;130(12):2191–201.

99. Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, et al. Microbial diversity in the deep sea and the underexplored "rare biosphere." PNAS. 2006 Aug 8;103(32):12115–20.

100. Gibbons SM, Caporaso JG, Pirrung M, Field D, Knight R, Gilbert JA. Evidence for a persistent microbial seed bank throughout the global ocean. PNAS. 2013 Mar 19;110(12):4651–5.

101. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome. 2017 Mar 3;5(1):27.

102. McMurdie PJ, Holmes S. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. PLOS Computational Biology. 2014 Apr 3;10(4):e1003531.

103. Chiu CH, Chao A. Estimating and comparing microbial diversity in the presence of sequencing errors. PeerJ. 2016 Feb 1;4:e1634.

104. Martino C, Morton JT, Marotz CA, Thompson LR, Tripathi A, Knight R, et al. A Novel Sparse Compositional Technique Reveals Microbial Perturbations. mSystems. 2019 Feb 12;4(1):10.1128/msystems.00016-19.

Supporting Information

Table S1: Summary overview of definitions of the core microbiome in the literature.

No.	Term	Definition	Author/Reference
1.	**Core microbiome**	"The core human microbiome is the set of genes present in a given habitat in all or the vast majority of humans. Habitat can be defined over a range of scales, from the entire body to a specific surface area, such as the gut or a region within the gut"	[1]
2.	**Substantial core model**	As per this definition, the majority of individuals in a population have shared taxa that constitute a substantial portion of their microbiome	[2]
3.	**Minimal core model**	"All individuals share a few components, and any individual shares many components with a few other individuals, but very little is shared across all individuals"	[2]
4.	**No core model**	"Nothing is shared by all individuals, and most diversity is unique to a given individual"	[2]
5.	**Gradient model**	"Individuals next to each other on a gradient, for example, age or obesity, share many components, but individuals at opposite ends share little or nothing"	[2]
6.	**Subpopulation model**	"Different subpopulations, for example, those defined by geography or disease, have different cores, but nothing is shared across subpopulations"	[2]
7.	**Common core**	"The component of the microbiome that is found across a considerable proportion of hosts within a defined host population or species"	[3]
8.	**Temporal core**	"A temporally stable or predictable component of the microbiota"	[3]
9.	**Ecological core**	"The component of the microbiome that is disproportionally important for shaping the organization and diversity of the ecological community"	[3]
10.	**Functional core**	"The component of the microbiome that performs essential biological functions to the host, usually in respect to their biochemical, physiological or ecological services to the host"	[3]
11.	**Host-adapted core**	"A set of microbes that has coevolved with the host species or subpopulation and whose presence increases host fitness in at least some ecological contexts"	[3]

Table S2: List of studies collected for the meta-analysis. The green color indicates studies that were selected for the meta-analysis (i.e., studies with V4 region).

No.	*Daphnia* species	Clones	Treatment	Sample type	Country	Origin	Primers	Variable region	DNA extraction	Sequencing platform	Sequencing depth	Acc. no	Reference	Selection
1	*D. magna*	OM2NF2, T9, B7, KNO1504 10 animals pooled per replicate per clone	Treatment with toxic cyanobacteria	Gut	OHZ, Knokke, Heverlee, Belgium	Laboratory	515F-806R	V4	Power soil DNA isolation kit, MOBIO Laboratories	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads	Average of 51,176 reads/sample	PRJNA398629	Macke *et al.*, 2017[4]	selected
2	*D. magna*	BSW7, OM2F8 10 animals pooled per replicate per clone	Effect of microbial inoculum	Gut	Bysjön Lake,Sweden Heverlee Belgium	Laboratory	515F-806R	V4	Power soil DNA isolation kit, MOBIO Laboratories	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads	Average of 51,176 reads/sample	PRJNA398630	Macke *et al.*, 2017[4]	selected
3	*D. magna*	NIES 3 pooled adult Daphnia per relicate per sample	Oxytetracycline exposure	Whole animal	Tsukuba, Japan	Laboratory	515F-806R	V4	Quick Genomic DNA prep	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads	Average of 96,833 reads/sample	PRJNA413666	Callens *et al.*, 2018[5]	selected
4	D. magna	G2, G6, G9 10 *Daphnia* guts were collected from each recipient population	Effect of microbial source	Gut	OHZ, Heverlee, Belgium,	Laboratory	515F-806R	V4	Power soil DNA isolation kit, MOBIO Laboratories	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads	Average of 27,766 reads per sample	PRJNA498431	Macke *et al.*, 2020[6]	selected
5	*D. magna*	G1, G2, G3, G4, G5, G6, G7, G8, G9 10 *Daphnia* guts were	Effect of diet (Scendesmus vs. Microcystis)	Gut	OHZ, Knokke, Heverlee, Belgium, Bysjön Lake,Swede	Laboratory	515F-806R	V4	Power soil DNA isolation kit, MOBIO Laboratories	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt	Average 29,427 reads per sample	PRJNA498417	Macke *et al.*, 2020[6]	selected

		collected from each recipient population			n					paired-end reads				
6	*D. magna*	BSW7,F clone, KNO, OM2, NIES guts of three individuals were dissected and pooled per replicate per clone	Composition of environmental bacteria	Gut	Bysjön Lake, Sweden, Knokke, Heverlee, Belgium, Tsukuba Japan	Laboratory	515F-806R	V4	Phenol-Chloroform-Isoamylalcohol Extraction	v2 PE500 kit, Illumina Miseq platform-2 × 250-nt paired-end reads	Average 38 688 reads per sample		Callens *et al*., 2020 [7]	selected
7	*D. magna*	8A *D. magna* from each treatment were pooled in sets of 10 in 1.5-ml microcentrifuge tubes for DNA extraction and processing ($n = 4$ per treatment).	Sulfamethoxazole, Erythromycin, Aztreonam exposure	Whole animal	Leitholm UK	Laboratory	515F-806R	V4	DNeasy Blood and TissueKit (Qiagen)	v2 PE500 kit, Illumina Miseq platform-2 × 250-nt paired-end reads		PRJNA543842	R Cooper *et al.*, 2020 [8]	selected
8	*D. magna*	BH, ZWE, L, K, OM2, T8, 10 individuals pooled per replicate per clone for recipients gut isolation and 25 individuals per donor	Role of local microbiome on exposure to toxic Microcystis	Gut	Kortrijk, Leuven, OHZ, Heverlee Belgium	Laboratory	515F-806R	V4	Power soil DNA isolation kit, MOBIO Laboratories	v2 PE500 kit, Illumina Miseq platform-2 × 250-nt paired-end reads	Average 24,536.38 reads per sample	PRJNA690081	Houwenhuyse *et al.*, 2021 [9]	selected

		region × diet combination were randomly isolated for gut dissection for donor population												
9	*D. magna*	DM1, DM2, DM3, DM4, DM5, DM15, DM18, DM19, DM20, DM22, DM23 20-25 guts from each sample pooled together per genotype	Lab microbiome vs natural microbiome	Gut	Bysjön Lake Sweden	Laboratory and natural	515F-806R	V4	DNeasy Power Soil ProDNA kit (Qiagen)	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads		PRJNA748877	Hegg *et al*., 2021 [10]	selected
10	*D. magna*	8A sets of five individuals for DNA extraction and microbiome sequencing (n = 4 per treatment per generation)	Multigenerational effects of antibiotic exposure	Whole animal	Leitholm UK	Laboratory	515F-806R	V4	DNeasy Power Soil ProDNA kit (Qiagen)	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads		PRJNA703930	R Cooper *et al.*, 2021 [11]	selected
11	*D. magna*	MS, KP, KNO, OM2, T8 Guts from 6 daphnids per replicate were pooled	The role of microbiome on parasite re-exposure	Gut	OHZ, Knokke, Heverlee Belgium	Laboratory	515F-806R	V4	Power soil DNA isolation kit, MOBIO Laboratories	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads	Average of 31,883,25 reads per sample	PRJNA688519	Bulteel *et al.*, 2021 [12]	selected

		together												
12	*D. magna*, *D. dentifera* DM were pooled in sets of 5 individuals (n = 10 samples per time point) & DD in sets of 10 individuals (n = 10 samples per time point) for DNA extraction	DM, DD	Effect of daily feeding rythm	Whole animal	Nebraska USA	Laboratory	515F-806R	V4	Qiagen DNeasy Blood and Tissue Kit	v2 PE500 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads		PRJNA715454	Pfenning butterworth *et al.*, 2022 [13]	selected
13	*D. magna*	Klon 5 5 animals per replicate per pooled and individual?	Effect of trimethoprim treatment	Whole animal	Berlin, Germany	Laboratory	fD1-926R	V1	10% chelex extraction	96-capillary ABI3730 XL DNA analyzer	Good's coverage (~85-95%)	KM603394 - KM603467	Gorokhova *et al.*, 2015 [14]	Not selected
14	*D. magna*	KNO15.04 15 *Daphnia* pooled for whole microbiome samples and dissected guts of 15 daphnids were pooled for gut microbiome analysis	Variation in food availability	Gut, Whole animal	Knokke, Belgium	Laboratory	343F-798R	V3-V4	Phenol-chloroform extraction	454 pyrosequencing		LN869908-LN869916	Callens *et al.*, 2016 [15]	Not selected
15	*D. magna*	MN	Ephippia-	Ephipp	Munich,		341F, 785R	V3	Power soil	Illumina	Rarefaction	PRJEB20984	Mushegian	Not selected

		DNA extracted from individual ephippia	associated microbiota	ium	Germany				DNA isolation kit, MOBIO Laboratories	MiSeq (reagent kit v3, 300 bp paired-end reads).	depth: 26,307 reads		*et al.*, 2017 [16]	
16	*D. magna*	FI−N−47−20 RU-RM1-009 RU-BOL1-1 RU-KOR1-1 FI-FHS2-11-8 FI-FUT1-2-1 FR-C1-1 IT-ISR1-8 GB-EL75-69 SE-EL75-69 SE-G1-9 BY-G1-9 IL-M1-8 FI-FSP1-16-2 BE-OM-2 CH-H-149 DE-K35-Mu11 RU-YAK1-1 IR-GG1-7 Individual daphnids taken from each sample per clone	Combined effect of temperatura and host clonal line	Whole animal	Finland, Russia, France, Italy,UK, Sweden, Belarus, Israel, Belgium, Switzerland, Iran, Germany	Laboratory	positions 327–969bp	V3-V5	CTAB-based isolation	454 LibL sequencing technology (Roche, Switzerland)	Rarefaction depth: 2511 reads per sample		Sullam K *et al.*, 2017 [17]	Not selected
17	*D. magna*	CH.H.149 Individual daphnids extracted per clone per sample	Temporal dynamics of microbiota before and after host death	Whole animal	Switzerland	Laboratory	341F, 785R	V3-V4	CTAB-based isolation	300-bp paired-end run, Illumina MiSeq (440nt)	Rarefaction: 15,390 reads each	PRJEB26643 ERS2473695–ERS2473793	Preiswerk D *et al.*, 2018 [18]	Not selected
18	*D. magna*	BE-OHZ-M5,	Role of gut microbiome	Whole animal	Belgium, Germany,	Laboratory	F (5′-ACACGGY	V3-V5	DNeasy Blood and	454 GS-FLX	Rarefaction: 3893 sequence	PRJNA40785	Sison-magnus *et*	Not selected

		BE-OHZ-M10, QTL-IXF-1, FI-Xinb3, DE-K35-Iinb1, HU-HO-2, IR-GG1-1, IL-M1-1 Two daphnids each per genotype	in parasitic tolerance (*P. ramosa*		Finland, Hungary, Iran, Jerusalem, Switzerland		CCARACTCCTAC-3′) and R (5′-TTGCWTCGAATTAAWCCAC-3′)		Tissue Kit (Qiagen, Netherlands)	instrument with LibL Titanium chemistry (Roche, USA)	depth	8	*al*., 2018 [19]	
19	*D. magna*	F2.82, F2.918, BE.OHZ.T10, BE.WE.G59, CZ.N1.1, CZ.N2.6, DE.K35.Mu10,DE.KA.F28, ES.DO1.1, I-F1, NO.V.7, TR.EG.1 DNA extracted from single animals per clone	Exposure to natural desiments	Whole animal	Belgium, Germany, Turkey, Norway, Czech Republic, Spain	Laboratory	341F, 785R	V3-V4	CTAB based isolation	Illumina MiSeq platform	Minimum 5,000 total reads per sample	PRJEB30308	Mushegian *et al.*, 2019 [20]	Not selected
20	*D. magna*	CAY, DE-K35-Iinb1, FI-Xinb3 8-10 individuals per replicate per sample per clone	Mercury stress	Whole animal	USA, Germany, Finland	Laboratory	515F-806R	V4	DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD)	MiSeq Illumina sequencing platform	Maximum depth of 12030 sequences	PRJNA421566	JC Fong *et al.*, 2019 [21]	Awaiting submission of sequences, no response from author
21	*D. magna*	FA, FB, FC,	Temp	Whole	Finland,	Laboratory	341F-785R	V3-V4	Zymo	V3	Rarefaction		JF Bricker	Not selected

		IA, IB, IC, GA, GB, GC Individual animals used for DNA extraction	dependent effects in lab-reared vs native pops.	animal	Israel, Germany				Research Fungal/Bacterial DNA Microprep kit (#D6007)	PE600 kit, Illumina Miseq platform- 2 × 250-nt paired-end reads	depth: 15,000 reads per sample		*et al.*, 2020 [22]	
22	*D. magna*	Clone 5 4 individuals pooled per replicate	Ciprofloxacin exposure	Gut	Berlin, Germany	Laboratory	341F, 805R	V3-V4	10% Chelex extraction	MiSeq V3 reagent kit (600-cycles) Illumina MiSeq platform	Zhang Huang's index : (99.8 ± 0.001%, mean ± SD)	PRJNA560134	Motiei *et al.*, 2020 [23]	Not selected
23	*D. pulex*	LD33 30 individuals per treatment	Multiple stressor exposure: Copper/Predator cues, antibiotics (Amp, Kanamycin sulphate)	Gut	UK	Laboratory		16S rDNA sequencing (RTL Genomics, Texas, USA)					SA Sadeq *et al.*, 2020 [24]	No response from author, Not selected
24	*D. magna*	LRV3.5_15, LRV13.5_1, LRV13_2 & P-IT 20 individuals pooled per genotype	Effect of glyphosate	Gut	Denmark,Verbania Italy	Laboratory		V1 region	Power Soil DNA Isolation Kit MoBio (Thermo Fisher Scientific)	250 bp sequencing, Illumina MiSeq platform	Rarefaction depth: 13,439 reads per sample	PRJNA606209-	A Suppa *et al.*, 2020 [25]	Not selected
25	*D. magna*	Strauss 15 individuals per replicate per treatment	Effect of poor vs rich diet and, antibiotic exposure (tetracycline HCl)	Gut	China	Laboratory	338F, 806R	V3-V4	E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.)	MiSeq V3 reagent kit (600-cycles) Illumina MiSeq platform		PRJNA561047	S. Akbar *et al.*, 2020 [26]	Not selected
26	*D. magna*	NIES	Exposure to dragonfruit		Tsukuba Japan							PRJNA597416	Sangkuanun *et al.*, 2020	No response from

		5 individuals per treatment	oligosaccharides										[27]	author,Not selected
27	*D. magna*	Laboratory reared clones 20-25 guts were isolated for each sample	Effect of predator kairomone exposure	Gut	China	Laboratory	27F-1492R	16S rRNA	HiPure Soil DNA Kits (Magen, Guangzhou, China)	PacBio Sequel platform		Pre-print	Liu *et al*., 2021 [28]	Not selected
28	*D. magna*	260 guts of *D. magna* from each experimental group were extracted	Exposure to nutrient imbalanced algal diet	Gut	Hong Kong	Laboratory	341F, 787R	V3-V4	PureLink Genomic DNA kit (Invitrogen)	Illumina Hiseq 2500 System- 2× 250 bp paired-end reads	26,418 to 47,344 reads across all samples	PRJNA597965	Ying Dong Li *et al*., 2021 [29]	Not selected
29	*D. magna*	CH 15-18 guts were isolated for each sample in triplicate	Elevated temp, *Microcystis* exposure	Gut	China	Laboratory	338F, 806R	V3-V4	E.Z.N.A.® soil DNA Kit (Omega Bio-tek)	MiSeq V3 reagent kit (600-cycles) Illumina MiSeq platform		PRJNA660107	S Akbar *et al*., 2021 [30]	Not selected
30	*D. magna*	G1, G2 20 guts were isolated per replicate treatment for microbiome analysis	Diet and médium associated microbes	Gut	China	Laboratory	338F, 806R	V3-V4	E.Z.N.A.® soil DNA Kit (Omega Bio-tek)	MiSeq V3 reagent kit (600-cycles) ,Illumina MiSeq platform		PRJNA737109	S Akbar *et al*., 2021 [31]	Not selected
31	*D. magna*	Strauss 7 to 10 guts/sample	Gut microbiome link to host development growth and neutral communication	Gut	Berlin, Germany	Laboratory	341F, 805R	V3-V4	DNeasy PowerBiofilm extraction kit (Qiagen)	Illumina NovaSeq 6000 platform (2 × 250 bp paired-end)	Mean read depth per sample of 43,192 sequences	PRJNA694094	RE Shehawy *et al*., 2021 [32]	Not selected
32	*D. galeata*	GR_020, GR_023, GR_024, GR_025,	Effect of host genotype on host	Gut and whole animal	Greifensee Switzerland	Laboratory	341F, 806R	V3-V4	Qiagen Blood & Tissue kit	Novogene UK	Rarefaction depth: 55,000 reads	https://doi.org/10.25678/0005DS	A Rajarajan *et al*., 2023 [33]	Not selected

		GR_052, GR_053, GR_054, GR_055 Twenty adult *Daphnia* per replicate per clone	associated bacterial communities											
33	*D. magna*	C3, B, D, H	Effect of pesticide exposure	Whole animal	Belgium	Laboratory	F9-R19	V3-V4	NucleoSpin Tissue Kit MacheryNagel	MiSeq V3 reagent kit (600-cycles), Illumina MiSeq platform		Unpublished study from the lab		Not selected
34	*D. magna*	UKw	Effect of pesticide tolerance	Gut	Belgium	Laboratory	F9-R19	V3-V4	NucleoSpin Tissue Kit MacheryNagel	MiSeq V3 reagent kit (600-cycles)2, Illumina MiSeq platform		Unpublished study from the lab		Not selected

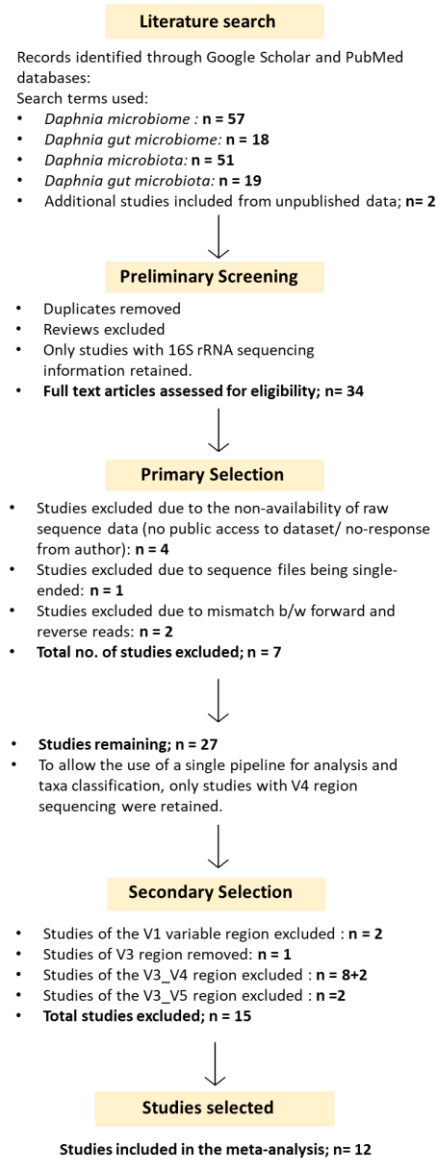

Figure S1: Schematic diagram of the workflow of the literature search and screening criteria for the selection of *Daphnia* microbiome studies for our analysis on core microbiome (adaptation of the PRISMA diagram, based on Moher et al. [34]). This analysis resulted in the selection of 12 studies that included a total of 656 samples originating from five countries, involving the gut as well as whole-body microbiomes of host animals directly collected from their source habitat as well as cultured in the laboratory. All studies involved the same host species, *D. magna*.

1. Core microbiome in the combined dataset: all gut samples of laboratory and field origin

A total of 423 *Daphnia* gut microbiomes comprising both laboratory-cultured and field-grown animals were analyzed. This dataset comprised a total of 14,743,383 read counts representing 116,500 OTUs belonging to 957 genera and 29 phyla. From a higher-level analysis conducted at the "phylum" and "order" levels, four phyla were detected in 90% of these samples: Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. At the order level, these were Burkholderiales, Chitinophagales, Flavobacteriales, Micrococcales, Rhizobiales and Bacillales. The same groups also met the double criterion of 10^{-4} relative abundances and 90% prevalence. The more stringent thresholds of 10^{-3} and 90% prevalence were met by the phyla Proteobacteria, Bacteroidetes and Actinobacteria and the orders Burkholderiales, Rhizobiales, and Micrococcales. Only the phylum Proteobacteria and order Burkholderiales met the 10^{-2} relative abundance threshold at 90% prevalence. No taxa were detected on the basis of the relative abundance criteria alone in 99% of the samples.

At the OTU level, no taxa were detected at a 99% prevalence. *Limnohabitans planktonicus* (OTU1) was detected in the gut samples and was by far the most prevalent group in the digestive tract of *Daphnia magna* (90% prevalence). Other

prevalent bacterial groups, identified at 80% prevalence, included *Ideonella paludis* and *Variovorax sp*. (OTU4) (both belonging to the family Comamonadaceae), *Staphylococcus* sp. (OTU41), unclassified *Microbacteriaceae* (OTU34) and *Escherichia/Shigella* sp. (OTU13). The results from the combined prevalence and relative abundance-based thresholds were quite aligned with the prevalence-defined thresholds. Only one OTU, *Limnohabitans planktonicus* (OTU 1), met the relative abundance threshold of 10^{-3} in 90% of the samples. Other members identified at 50% prevalence for a detection threshold of 10^{-4} included *Formosimonas* sp., *Ideonella paludis*, *Variovorax* sp., *Pseudomonas* sp., *Escherichia/Shigella* sp., *Shinella* sp., *Emticicia* sp., *Streptococcus* sp., *Staphylococcus* sp. and unclassified *Chitinophagaceae*, *Rhodobacteriaceae* and *Comamonadaceae*. At a detection threshold of 10^{-3}, *Formosimonas* sp., *Variovorax* sp., *Ideonella paludis*, *Escherichia/Shigella* sp. and unclassified *Chitinophagaceae* were detected in 50% of the samples. For an assigned threshold of 10^{-2}, *Limnohabitans planktonicus* was identified only at 50% prevalence.

2. Microbial community composition

To gain an overview of the microbial composition at the different levels of categorization, namely based on countries, sample type and origin, stacked barplots were constructed using the packages *microbiome*, *phyloseq* and *MicroViz* in R. Taxonomic aggregation was carried out at the "Phylum" level to ease visualization and summarization of the top 5 phyla in the community. The top 5 phyla were selected based on their total count abundance in the dataset. To effectively ensure visualization of within-group compositional variation, individual samples are presented without averaging, allowing all samples in each group to be displayed. Additionally, samples were sorted by similarity based on the Bray Curtis distance measure.

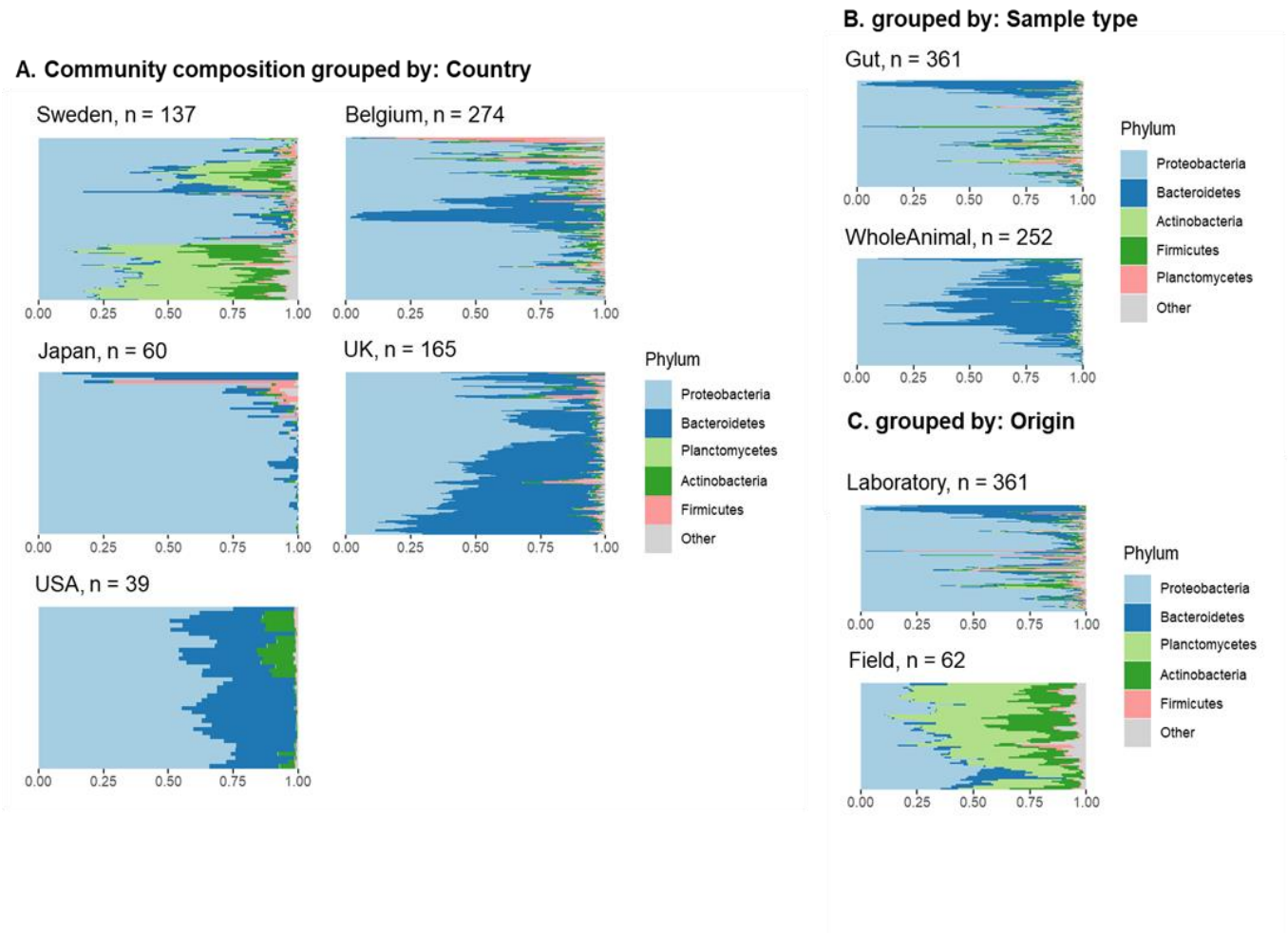

Figure S2: Stacked bar plots representing within-group variation in microbiome composition at the "Phylum" level for the following categorization. A) grouped by Country B) Sample type i.e., gut microbiome and whole microbiome (of laboratory reared animals only) C) Origin i.e., laboratory cultured or field collected samples (for gut samples only).

3. Comparison of microbiome composition of laboratory and field samples originating from Lake Bysjön (Sweden)

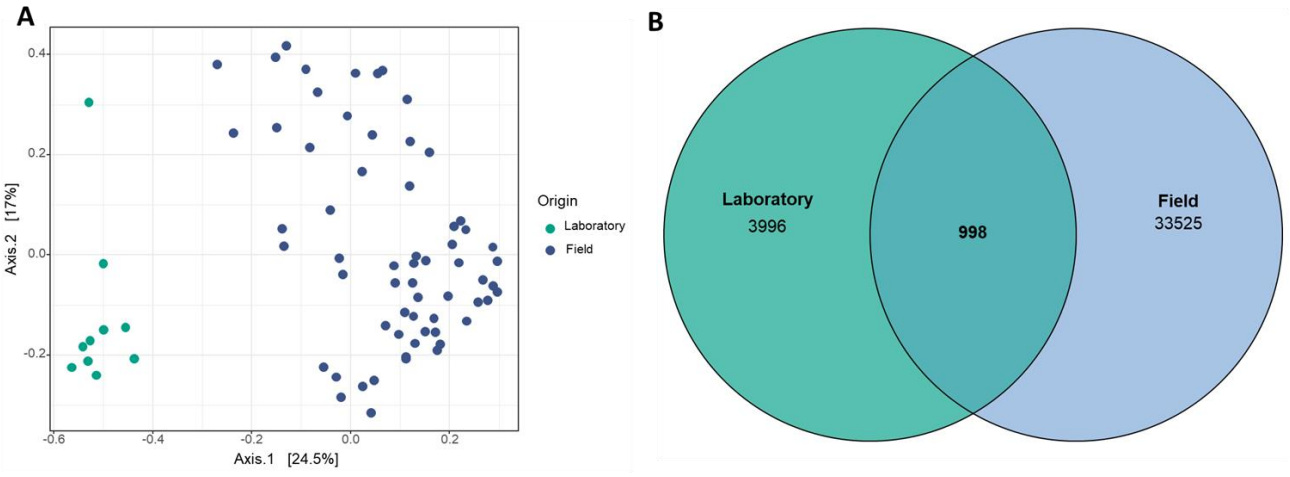

Figure S3: Comparison of laboratory-cultured samples with that of field-grown samples of the Sweden dataset (A) MDS plot highlighting the microbiome community composition of laboratory-cultured clones versus field-grown clones all derived from the same lake (Lake Bysjön, Sweden) (B) Venn diagram representing shared membership between the two sources of origin. From a total of 39,810 OTUs, there occurred an overlap of 998 OTUs exhibiting a 2.5% shared membership between these environments.

Table S3: List of core taxa detected in the subgroups of the dataset at different core quantification metrices.

Combined dataset, both gut and whole animal samples n= 675							
% of samples	**Prevalence-based core**	**Relative abundance and prevalence based core**			**Relative abundance core**		
	>0	**>0.0001**	**>0.001**	**>0.01**	**>0.0001**	**>0.001**	**>0.01**
100%	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected
90%	*Limnohabitans planktonicus (1)*	*Limnohabitans planktonicus*	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA
80%	***Limnohabitans planktonicus(1)*** *Variovorax sp (4)* *Chitinophagaceae_ unclassified(6)* *Ideonella paludis(8)* *Escherichia/Shigella(13)* *Staphylococcus*	***Limnohabitans planktonicus***	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA
50%	***Limnohabitans planktonicus (1)*** ***Formosimonas (2)*** *Acinetobacter* ***Variovorax sp (4)*** ***Ideonella paludis (8)*** *Chitinophagaceae_unclassified (6)* *Psudomonas (10)* *Rhodobacteraceae_unclassified(11)* ***Escherichia/Shigella*** *Comamonadaceae_unclassified(19, 29, 69, 164, 293)* *Shinella (23)* *Polynucleobacter(24)* *Flavobacterium(14,26)* *Emticicia(28),* *Streptococcus(32)* *Microbacteriaceae_unclassified(34)* *Staphylococcus(41)* *Hydrogenophaga(50)* *Bosea(78)* *Brevundimonas (81)* *Sphingomonas (126)*	***Limnohabitans planktonicus (1)*** ***Formosimonas (2)*** ***Variovorax sp*** *(4)* *Chitinophagaceae_unclassified (6)* ***Ideonella paludis (8)*** *Pseudomonas(10)* *Rhodobacteraceae_unclassifie d(11)* ***Escherichia/Shigella(13)*** *Comamonadaceae_unclassifie d (19)* *Shinella* *Emticicia(28)* *Streptococcus(32)* *Staphylococcus(41)*	***Limnohabitans planktonicus (1) Formosimonas(2)*** ***Variovorax sp*** *(4)* *Chitinophagaceae_unclassifi ed (6)* ***Ideonella paludis (8)*** ***Escherichia/Shigella (13)***	***Limnohabitans planktonicus***	-NA	-NA	-NA
Gut samples; n=423							
100%	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected
90%	*Limnohabitans planktonicus*	*Limnohabitans planktonicus*	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA
80%	***Limnohabitans planktonicus*** *Variovorax sp,* *Chitinophagaceae_unclassified* ***Ideonella paludis*** *Escherichia/Shigella* *Staphylococcus(41)*	***Limnohabitans planktonicus***	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA
50%	***Limnohabitans planktonicus***	***Limnohabitans planktonicus***	***Limnohabitans planktonicus***	***Limnohabitans***	-NA	-NA	-NA

	Formosimonas ***Acinetobacter*** ***Variovorax sp,*** ***Chitinophagaceae_unclassified(6)*** ***Ideonella paludis*** ***Pseudomonas(10)*** *Rhodobacteriaceae_unclassified(11)* ***Escherichia/Shigella(13)*** *Flavobacterium(14)* *Comamonadaceae_unclassified(19)* *Shinella* *Polynucleobacter* *Flavobacterium(26)* *Emticicia(28)* *Comamonadaceae_unclassified(29)* *Streptococcus(32)* *Microbacteriaceae_unclassified(34)* ***Staphylococcus(41)*** *Hydrogenophaga (50)* *Comamonadaceae_unclassified(69)* *Bosea (78)* *Brevundimonas (81)* *Sphingomonas (126)* *Comamonadaceae_unclassified(164)* *Comamonadaceae_unclassified(293)*	***Formosimonas*** ***Ideonella paludis*** ***Variovorax sp,*** ***Chitinophagaceae_unclassifie d (6)*** ***Pseudomonas*** *Rhodobacteriaceae_unclassifie d (11)* ***Escherichia/Shigella(13)*** *Comamonadaceae_unclassifie d (19)* *Shinella* *Emticicia (28)* *Streptococcus (32)* ***Staphylococcus (41)***	***Formosimonas*** ***Variovorax sp,*** ***Unclassified*** ***Chitinophagaceae*** ***Ideonella paludis*** ***Escherichia/Shigella***	***planktonicus***			
Gut samples: laboratory origin; n= 361							
100%	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected
95%	***Limnohabitans planktonicus(1)***	***Limnohabitans planktonicus (1)***	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA
90%	***Limnohabitans planktonicus (1)*** *Ideonella paludis(8)* ***Escherichia/Shigella(13)***	***Limnohabitans planktonicus (1)*** ***Escherichia/Shigella(13)***	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA
80%	***Limnohabitans planktonicus (1)*** ***Variovorax sp(4)*** ***Ideonella paludis(8)*** *Chitinophagaceae_unclassified(6)* *Pseudomonas (10)* ***Escherichia/Shigella(13)*** *Polynucleobacter (24)* *Streptococcus (32)* *Microbacteriaceae_unclassified (34)* ***Staphylococcus (41)***	***Limnohabitans planktonicus(1)*** ***Ideonella paludis (8)*** ***Escherichia/Shigella(13)*** ***Staphylococcus (41)***	***Limnohabitans planktonicus***	***Limnohabitans planktonicus***	-NA	-NA	-NA
50%	***Limnohabitans planktonicus*** ***Formosimonas*** ***Acinetobacter (3)***	***Limnohabitans planktonicus*** ***Formosimonas*** ***Acinetobacter***	***Limnohabitans planktonicus*** ***Formosimonas*** ***Variovorax sp,***	***Limnohabitans planktonicus***	-NA	-NA	-NA

	Variovorax sp, *Unclassified Microbacteriaceae (5)* ***Ideonella paludis*** ***Chitiniphagaceae_unclassified(6)*** ***Flavobacterium(9)*** ***Pseudomonas(10)*** ***Rhodobacteriaceae_unclassified (11)*** ***Deefgea(12)*** ***Escherichia/Shigella(13)*** ***Aeromonas(15)*** *Enterobacteriaceae_unclassified(16)* ***Comamonadaceae_unclassified(19)*** *Rhodanobacter (22)* *Shinella (23)* ***Polynucleobacter (24)*** *Flavobacterium(26)* *Emticicia(28)* *Comamonadaceae_unclassified(29)* ***Streptococcus(32)*** ***Microbacteriaceae_unclassified(34)*** ***Staphylococcus(41)*** *Lacihabitans(44)* *Micrococcales_unclassified(45)* *Hydrogenophaga(50)* *Comamonadaceae_unclassified (84)* *Streptococcus (86)* *Sphaerotilus (118)* *Comamonadaceae_unclassified(164)* *Haemophilus (205)* *Cutibacterium(266)* *Comamonadaceae_unclassified(293)*	***Variovorax sp,*** ***Unclassified Microbacteriaceae*** ***Chitinophagaceae_unclassified*** ***Ideonella paludis*** ***Flavobacterium (9)*** ***Pseudomonas (10)*** ***Rhodobacteraceae_unclassified (11)*** ***Deefgea (12)*** ***Escherichia/Shigella (13)*** ***Aeromonas (15)*** ***Comamonadaceae_unclassified(19)*** *Rhodanobacter (22)* *Shinella (23)* ***Polynucleobacter (24)*** *Flavobacterium(26)* *Emticicia(28)* *Comamonadaceae_unclassified(29)* ***Streptococcus(32)*** ***Microbacteriaceae_unclassified(34)*** ***Staphylococcus(41)*** *Micrococcales_unclassified(45)* *Comamonadaceae_unclassified(293)*	***Chitinophagaceae_unclassified*** ***Ideonella paludis*** ***Pseudomonas (10)*** ***Escherichia/Shigella (13)*** ***Polynucleobacter (24)*** ***Microbacteriaceae_unclassified(34)*** ***Staphylococcus(41)***				
Gut samples: field origin; n=62							
100%	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected
99%	***Pirellulales_unclassified*** ***Rhodobacteraceae_unclassified*** ***Solirubrobacterales_unclassified*** *Methylocystis* *Roseimaritima* ***Pirellulales_unclassified*** *Rhodobacteraceae_unclassified* *Mycobacterium* *Rhodobacteraceae_unclassified* ***Pirellulales_unclassified*** *Hyphomicrobiaceae_unclassified* *Rhizobiales_unclassified* ***Pirellulaceae_unclassified***	***Pirellulales_unclassified (7)*** ***Rhodobacteraceae_unclassified (11)*** ***Solirubrobacterales_unclassified (21)*** ***Pirellulales_unclassified(43)*** *Rhodobacteraceae_unclassified (71)* *Mycobacterium (74)* *Rhodobacteriaceae_unclassified (75)* ***Pirellulales_unclassified (87)***	***Pirellulales_unclassified (7)*** ***Rhodobacteraceae_unclassified (11)*** ***Solirubrobacterales_unclassified (21)*** ***Pirellulales_unclassified(43)***	***Pirellulales_unclassified***	-NA	-NA	-NA

	Ilumatobacter *Actinobacteria_unclassified* *Acidimicrobiales_unclassified* *Rhodobacteraceae_unclassified* *Actinobacteria_unclassified* *Isosphaeraceae_unclassified* ***Pirellulales_unclassified*** *Nocardioides* ***Pirellulales_unclassified*** ***Pirellulales_unclassified*** *Aquihabitans* *Bacteria_unclassified* ***Pirellulales_unclassified*** ***Pirellulales_unclassified***	*Rhizobiales_unclassified (98)* ***Pirellulaceae_unclassified (127)*** *Ilumatobacter (134)* *Actinobacter_unclassified (147)* *Acidimicrobiales_unclassiified (182)* *Actinobacter_unclassified (197)* ***Pirellulales_unclassified (336)***					
80%	153 taxa Link to table	*50 taxa* Link to table	*Variovorax sp.* *Pirellulales_unclassified (7)* *Rhodobacteraceae_unclassified (11)* *Verrucomicrobia_unclassified (17)* *Solirubrobacterales_unclassified (21)* *Pirellulales_unclassified (43)* *Isosphaeraceae_unclassified (67)* *Rhodobacteraceae_unclassified (71)* *Pirellulales_unclassified (87)* *Hyphomicrobiaceae_unclassified (97)* *Rhizobiales_unclassified (98)* *Acidimicrobiales_unclassified (182)* *Actinobacteria_unclassified (197)*	*Pirellulales_unclassified (7)* *Rhodobacteraceae_unclassified (11)* *Solirubrobacterales_unclassified (21)*	-NA	-NA	-NA
50%	527 taxa Link to table	141 taxa Link to table	*Variovorax sp* ***Pirellulales_unclassified*** *Rhodobacteraceae_unclassified (11)* *Verrucomicrobia_unclassified (17)* *Solirubrobacterales_unclassified (21)* *Methylocystis (39)* *Roseimaritima (40)* ***Pirellulales_unclassified (43)*** *Arenimonas (51)*	*Variovorax sp* *Pirellulales_unclassified (7)* *Rhodobacteraceae_unclassified (11)* *Solirubrobacterales_unclassified (21)* *Roseimaritima (40)* ***Pirellulales_unclassified (43)*** *Isosphaeraceae_unclassified*	-NA	-NA	-NA

			Pirellulaceae_unclassified (60) *Porphyrobacter (65)* *Isosphaeraceae_unclassified (67)* *Rhodobacteraceae_unclassified (71)* *Mycobacterium (74)* *Rhodobacteraceae_unclassified (75)* ***Pirellulales_unclassified (87)*** *Hyphomicrobiaceae_unclassified (97)* *Rhizobiales_unclassified (98)* ***Pirellulaceae_unclassified (127)*** *Ilumatobacter (134)* *Actinobacteria_unclassified (147)* *Betaproteobacteria_unclassified (176)* *Acidimicrobiales_unclassified (182)* *Rhodobacteraceae_unclassified (186)* *Actinobacteria_unclassified (197)* ***Pirellulales_unclassified (199)*** *Isosphaeraceae_unclassified (217)* ***Pirellulales_unclassified (225)*** *Leucobacter (263)* *Alphaproteobacteria_unclassified (296)*	*(67)* *Rhodobacteraceae_unclassified (71)*			
Whole animal samples; n = 252							
100%	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected	No taxa detected
95%	***Limnohabitans planktonicus*** ***Formosimonas*** ***Chitinophagaceae_unclassified*** ***Flavobacterium (20)***	***Limnohabitans planktonicus***	No taxa detected	No taxa detected	-NA	-NA	-NA
90%	***Limnohabitans planktonicus*** ***Formosimonas*** ***Chitinophagaceae_unclassified*** ***Flavobacterium***	***Limnohabitans planktonicus*** ***Formosimonas*** ***Chitinophagaceae_unclassified***	***Limnohabitans planktonicus***	No taxa detected	-NA	-NA	-NA

	Emticicia (28)						
80%	***Limnohabitans planktonicus*** ***Formosimonas*** ***Chitinophagaceae_unclassified*** ***Rhodobacteraceae_unclassified*** *Escherichia/Shigella* ***Comamonadaceae_unclassified (19)*** ***Flavobacterium (20)*** ***Shinella (23)*** ***Emticicia (28)*** *Bosea(78)* ***Brevundimonas (81)***	***Limnohabitans planktonicus*** ***Formosimonas*** ***Chitinophagaceae_unclassifie d*** ***Flavobacterium*** ***Emticicia***	***Limnohabitans planktonicus***	***Limnohabitans planktonicus***	-NA	-NA	-NA
50%	Link to table	Link to table	***Limnohabitans planktonicus*** ***Formosimonas*** ***Variovorax sp.*** ***Chitinophagaceae_unclassifi ed*** ***Rhodobacteraceae_unclassif ied*** ***Flavobacterium*** ***Shinella*** ***Emticicia*** *Limnobacter* *Hydrogenophaga* *Sphingopyxis* ***Brevundimonas (54)*** *Blastomonas* *Bosea* ***Brevundimonas (81)*** *Unclassified Rhizobiales (85)*	***Limnohabitans planktonicus*** ***Formosimonas*** ***Chitinophagaceae_ unclassified*** ***Flavobacterium***	-NA	-NA	-NA

References

1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project. Nature. 2007;449:804–10.

2. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res. 2009;19:1141–52.

3. Risely A. Applying the core microbiome to understand host–microbe systems. J Anim Ecol. 2020;89:1549–58.

4. Macke E, Callens M, De Meester L, Decaestecker E. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat Commun. 2017;8:1608.

5. Callens M, Watanabe H, Kato Y, Miura J, Decaestecker E. Microbiota inoculum composition affects holobiont assembly and host growth in Daphnia. Microbiome. 2018;6:56.

6. Macke E, Callens M, Massol F, Vanoverberghe I, De Meester L, Decaestecker E. Diet and Genotype of an Aquatic Invertebrate Affect the Composition of Free-Living Microbial Communities. Front Microbiol. 2020; 11:380

7. Callens M, De Meester L, Muylaert K, Mukherjee S, Decaestecker E. The bacterioplankton community composition and a host genotype dependent occurrence of taxa shape the Daphnia magna gut bacterial community. FEMS Microbiol Ecol. 2020;96:fiaa128.

8. Cooper RO, Vavra JM, Cressler CE. Targeted Manipulation of Abundant and Rare Taxa in the Daphnia magna Microbiota with Antibiotics Impacts Host Fitness Differentially. mSystems. 2021;6:10.1128/msystems.00916-20.

9. Houwenhuyse S, Stoks R, Mukherjee S, Decaestecker E. Locally adapted gut microbiomes mediate host stress tolerance. ISME J. 2021;15:2401–14.

10. Hegg A, Radersma R, Uller T. A field experiment reveals seasonal variation in the Daphnia gut microbiome. Oikos. 2021;130:2191–201.

11. Cooper RO, Tjards S, Rischling J, Nguyen DT, Cressler CE. Multiple generations of antibiotic exposure and isolation influence host fitness and the microbiome in a model zooplankton species. FEMS Microbiol Ecol. 2022; 10: p.fiac082

12. Bulteel L, Houwenhuyse S, Declerck SAJ, Decaestecker E. The Role of Microbiome and Genotype in Daphnia magna upon Parasite Re-Exposure. Genes. 2021;12:70.

13. Pfenning-Butterworth A, Cooper RO, Cressler CE. Daily feeding rhythm linked to microbiome composition in two zooplankton species. PLOS ONE. 2022;17:e0263538.

14. Gorokhova E, Rivetti C, Furuhagen S, Edlund A, Ek K, Breitholtz M. Bacteria-Mediated Effects of Antibiotics on Daphnia Nutrition. Environ Sci Technol. 2015;49:5779–87.

15. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, et al. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. ISME J.2016; 10:911-20.

16. Mushegian AA, Walser J-C, Sullam KE, Ebert D. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. J Anim Ecol. 2018;87:400–13.

17. Sullam KE, Pichon S, Schaer TMM, Ebert D. The Combined Effect of Temperature and Host Clonal Line on the Microbiota of a Planktonic Crustacean. Microb Ecol. 2018;76:506–17.

18. Preiswerk D, Walser J-C, Ebert D. Temporal dynamics of microbiota before and after host death. ISME J.

19. Sison-Mangus MP, Metzger CMJA, Ebert D. Host genotype-specific microbiota do not influence the susceptibility of D. magna to a bacterial pathogen. Sci Rep. 2018;8:9407.

20. Mushegian AA, Arbore R, Walser J-C, Ebert D. Environmental Sources of Bacteria and Genetic Variation in Behavior Influence Host-Associated Microbiota. Appl Environ Microbiol. 2019;85:e01547-18.

21. Fong JC, De Guzman BE, Lamborg CH, Sison-Mangus MP. The Mercury-Tolerant Microbiota of the Zooplankton Daphnia Aids in Host Survival and Maintains Fecundity under Mercury Stress. Environ Sci Technol. 2019;53:14688–99.

22. Frankel-Bricker J, Song MJ, Benner MJ, Schaack S. Variation in the Microbiota Associated with Daphnia magna Across Genotypes, Populations, and Temperature. Microb Ecol. 2020;79:731–42.

23. Motiei A, Brindefalk B, Ogonowski M, El-Shehawy R, Pastuszek P, Ek K, et al. Disparate effects of antibiotic-induced microbiome change and enhanced fitness in Daphnia magna. PLoS One. 2020;15:e0214833.

24. Sadeq SA, Mills RIL, Beckerman AP. The microbiome mediates the interaction between predation and heavy metals. Science of The Total Environment. 2021;775:145144.

25. Suppa A, Kvist J, Li X, Dhandapani V, Almulla H, Tian AY, et al. Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota functionality in non-target species. Microbiome. 2020;8:170.

26. Akbar S, Gu L, Sun Y, Zhou Q, Zhang L, Lyu K, et al. Changes in the life history traits of *Daphnia magna* are associated with the gut microbiota composition shaped by diet and antibiotics. Sci Tot Environ. 2020;705:135827.

27. Sangkuanun T, Wichienchot S, Kato Y, Watanabe H, Peerakietkhajorn S. Oligosaccharides derived from dragon fruit modulate gut microbiota, reduce oxidative stress and stimulate toll-pathway related gene expression in freshwater crustacean Daphnia magna. Fish Shellfish Immunol. 2020;103:126–34.

28. Liu Q, Akbar S, Ding Z, Zhou Q, Gu L, Sun Y, et al. Anti-predation Defense Traits of Daphnia are Associated With the Gut Microbiota Composition Shaped by Fish Kairomone [Internet]. Research Square; 2021 [cited 2025 Jan 22]. Available from: https://www.researchsquare.com/article/rs-757585/v1

29. Li Y, Xu Z, Liu H. Nutrient-imbalanced conditions shift the interplay between zooplankton and gut microbiota. BMC Genomics. 2021;22:37.

30. Akbar S, Huang J, Zhou Q, Gu L, Sun Y, Zhang L, et al. Elevated temperature and toxic *Microcystis* reduce *Daphnia* fitness and modulate gut microbiota. Environmental Pollution. 2021;271:116409.

31. Akbar S, Li X, Ding Z, Liu Q, Huang J, Zhou Q, et al. Disentangling Diet- and Medium-Associated Microbes in Shaping Daphnia Gut Microbiome. Microb Ecol. 2022;84:911–21.

32. El-Shehawy R, Luecke-Johansson S, Ribbenstedt A, Gorokhova E. Microbiota-Dependent and -Independent Production of l-Dopa in the Gut of Daphnia magna. mSystems. 2021;6:e00892-21.

33. Rajarajan A, Wolinska J, Walser J-C, Dennis SR, Spaak P. Host-Associated Bacterial Communities Vary Between Daphnia galeata Genotypes but Not by Host Genetic Distance. Microb Ecol. 2023;85:1578–89.

34. Moher D, Liberati A, Tetzlaff J, Altman DG, PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009;6:e1000097.

CHAPTER 2:

Strain- 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 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.

Keywords: *microbiome community assembly, host-microbiome interactions, microbiome-mediated fitness, time-lag experiments.*

Introduction

Gut microbiome composition has been shown to influence a broad array of host traits, including digestion, physiology, metabolism, immunity and behaviour (1–5). Alterations in gut community structure and composition have also been implicated in health in humans (6,7), animals (8,9) and in responses to environmental stressors in free-living systems (10–12). It is therefore crucial to understand the determinants of gut microbiome assembly. Although host diet (13,14), genotype (12,13,15), lifestyle (16) and antibiotic use (17) 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% (18,19). 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 (20–24). 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 species suppresses later arriving species. Such priority effects are expected to be strong when niche overlap is high and differences in fitness low (23,25). In such cases, if the first arriving species changes the environment to its own benefit, these effects may persist and become permanent (24). Conversely, niche modification by early colonists can also facilitate the establishment and growth of late-arriving immigrants, resulting in facilitative priority effects (21,26,27).

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. (19) 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. (28,29), 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 (30,31). 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 (32).

The role of priority effects in microbiome assembly has been shown through manipulative experiments in the phyllosphere of *Arabidopsis* (33), in the microbiome of green macroalga *Ulva australis* (34) and in nectar-inhabiting yeast communities of *Mimulus aurantiacus* (35,36). However, there remains only limited experimental evidence for the occurrence of priority effects in gut microbiomes (19,37). 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 (38). 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 (39). 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 (40). The species *D. magna* is an excellent model system for experimental manipulation of inoculation order in the gut. The species is known to harbour abundant bacteria, both on its surface and in its gut (41). Controlled inoculation of bacterial species into the gut at different times is possible because one can sterilize the eggs (42) to produce germ-free animals. *D. magna* are non-selective filter feeders that readily take up bacteria from their surrounding waters (12), 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 (42–44). 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 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 (12,42,45,46) 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 (Figure 1A) either simultaneously, with a time-lag of 48h, or in isolation (Figure 1B). The gut microbiome composition and four host life history traits were determined after 8 and 12 days (Figure 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, i.e., depend on the identity of the inoculated bacterial strains, 3) or host genotype-dependent, i.e., 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 Figure 1. We use *Daphnia 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 (Figure 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 12 of the experiment. Given that the average maturation time of healthy *D. magna* at 20°C is approximately 8 days when kept under favourable laboratory conditions and that egg development time in the brood pouch is approximately 3 days at 20°C, 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. 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 48h time-lag, and (4) reciprocal time-lag inoculation where the previous order was reversed. This 48h time-frame (day 3) thus represents a sufficiently early period of inoculation in *Daphnia's* life span, early enough to effectively impact the microbiome assembly process within the host's life span while also allowing enough time for the 1st arriving species to grow and gain numerical advantage before the arrival of the 2nd species. 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, totalling 3240 *Daphnia* individuals.

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

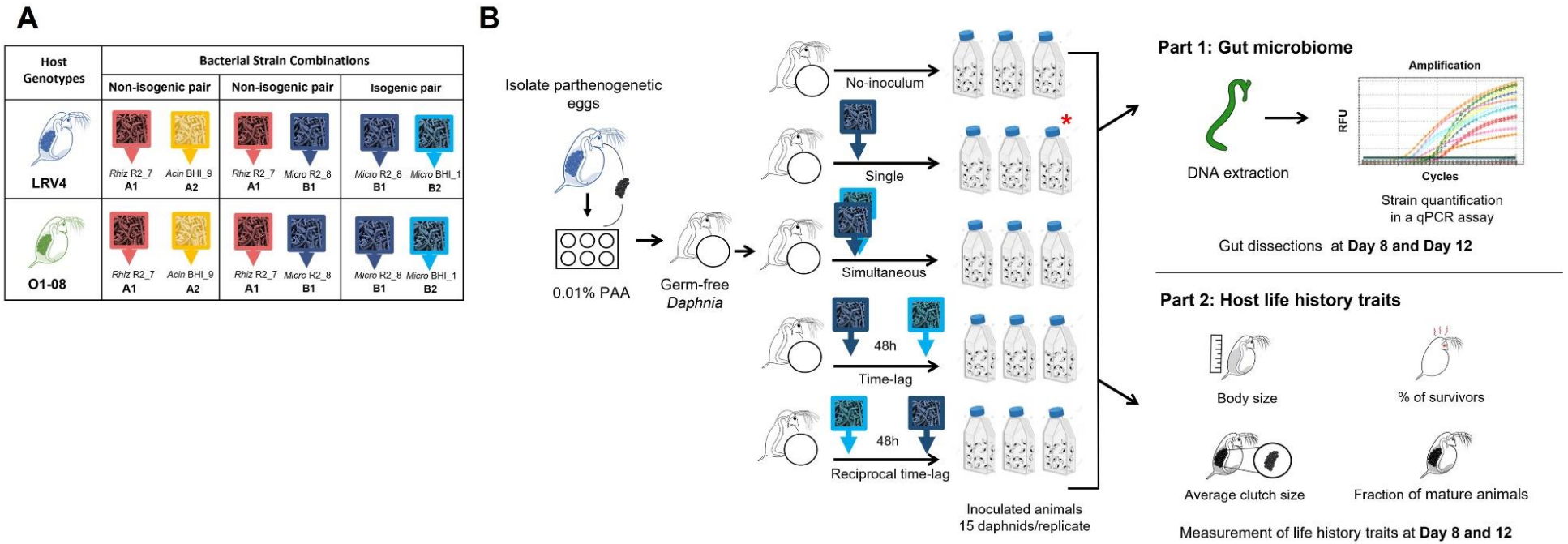

Figure 1. A) Summary of *Daphnia* genotypes and gut bacterial strain combinations used in the priority effects experiment. B) Schematic representation of the experimental design. Germ-free host individuals were generated by treating parthenogenetic eggs with 0.01% peracetic acid (PAA). The axenic hatchlings (< 24h 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 48h. This experiment was run full factorial for each bacterial strain pair combination in two host genotypes and two time points, with three replicates per treatment consisting each of 15 *Daphnia* individuals. Gut dissections were conducted at two time points (day 8 and 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 12 (see text)

Selection of bacterial strains

Selection of the specific strains was based on the following considerations. Firstly, 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. Secondly, both heterogeneric and cogeneric strain pairs were established, since cogeneric bacterial strains are expected to occupy a more similar niche in comparison to heterogeneric strains and possibly display stronger priority effects through niche pre-emption (23). Thirdly, strain pairs were chosen such that their 16S rRNA gene sequences shared a sufficiently similar ±250bp 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 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 (Figure S1, see Supporting Information): *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 (Figure 1). Detailed information on each of these steps is given in full in Supporting Information, section 4-8. In short, *Daphnia* eggs were isolated from non-sterile mothers and sterilized using 0.01% peracetic acid (47). The sterile hatchlings were then transferred into 50 ml falcon tubes (15 hatchlings per replicate unit) containing 30 ml sterile ADaM medium and $0.5x10^5$ sterile algae cells/ml. The appropriate bacterial strain combination was then inoculated to each experimental unit at a standardized total concentration of 10^6 cells/ml. The second exposure in the (reciprocal) time-lag treatments was executed 48h after the first inoculation, again at $0.5x10^6$ cells/ml.

After 96 hours 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. All experimental units were incubated at 20°C with a 16:8h 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 days when kept under favourable laboratory conditions and that egg development time in the brood pouch is approximately 3 days at 20°C (48,49), 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 7 guts per experimental unit were collected for DNA extraction. DNA extraction was performed using the DNeasy® PowerSoil® Pro-Kit (Qiagen, Hilden, Germany). 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 *Daphnia 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') (50) and a custom reverse primer R206 (5'- CGGAAATGGGGTGGTTG - 3') specifically designed to maintain an amplicon size of 200bp, 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 200bp 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), *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) labelled with FAM or HEX dyes (PrimeTime qPCR probes) were purchased from Integrated DNA Technologies (IDT, Leuven, Belgium). For probe design and testing we refer to Supporting Information, section 7.2.

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

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 minutes followed by 40 cycles of denaturation and a combined annealing and extension step at 95°C (15 seconds) and 61°C (30 seconds), 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 (20µM 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, Hilden, Germany) 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 ultra-pure 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 4 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:

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

Quantity = $10^{(Cq-b/m)}$

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 analysed, 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 analysed. 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 Figure S7). In both cases, a linear mixed model was constructed with the *lmer* function of the *lme4* package (51). 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 (52) and significance tested with Type III sum of squares using the *Anova* function of the *car* package (53). Tukey correction was performed for pairwise posthoc comparisons using the *emmeans* package in R for significant interactions (54), 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 analysed separately following the same scheme as the bacterial densities (see Figure S8). Normality of residuals and homogeneity of variance was checked and formally tested with the Shapiro–Wilk test and the Levene test, respectively (53).

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 heterogeneric 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 (Figure 2A). In line with these observations, growth in all pair-wise 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 (Figure 2A, see ANOVA Table S9 in Supporting Information). 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 (Figure 2A). However, no significant differences in densities were exhibited between the time-lag and reciprocal time-lag inoculation for *Rhizobium* R2_7.

In the heterogeneric pair *Rhizobium* R2_7 (A1) and *Microbacterium* R2_8 (B1), a different pattern was detected (Figure 2B). The single inoculation treatments show that both strains grow 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 (Figure 2B). In both host genotypes, we observed significant differences in bacterial density between time-lag and reciprocal time-lag inoculations, in addition to 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) (Figure 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 cogeneric 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 (Figure 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).

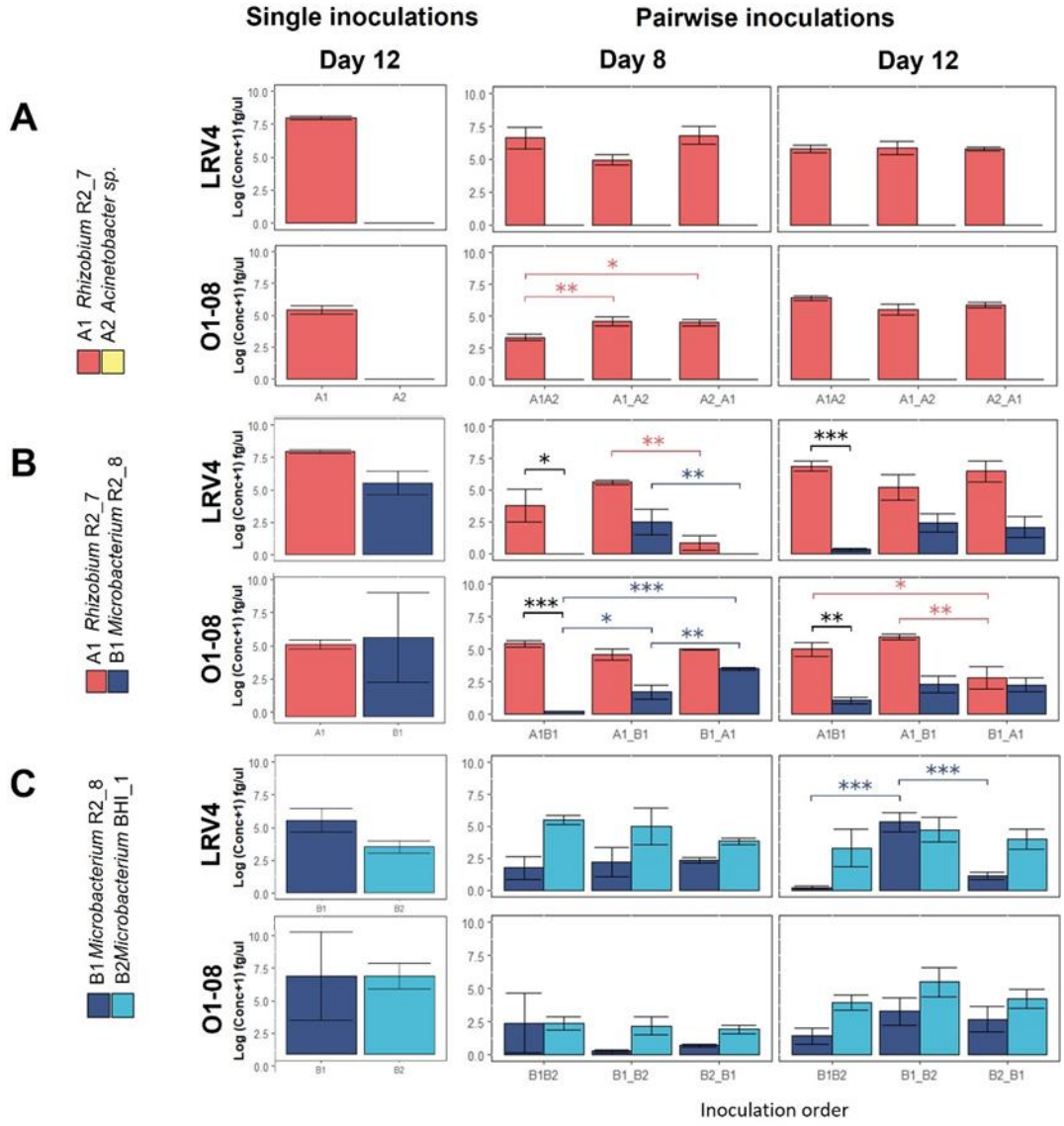

Figure 2. Mean bacterial strain densities (per *Daphnia* gut) plotted against treatment for A) heterogeneric strain combination *Rhizobium* R2_7 (A1) and *Acinetobacter* BHI_9 (A2), B) heterogeneric strain combination *Rhizobium* R2_7 (A1) and *Microbacterium* R2_8 (B1) and C) cogeneric strain combination *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 days; right: 12 days). 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 48h before X2 (time-lag inoculation); X2_X1 = X2 inoculated 48h before X1 (reciprocal time-lag inoculation). N=3. *p < 0.05, **p < 0.01, ***p < 0.001.

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 (Figure 3, 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 (Figure 3, ANOVA table S10). For the cogeneric strain pair *Microbacterium* R2_8 – *Microbacterium* BHI_1, significant differences in reproductive traits i.e., fraction of mature animals and average clutch size were recorded at day 12 for genotype LRV4 (Figure 3, 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 ANOVA table, S10). For the heterogeneric species pair *Rhizobium* R2_7 - *Microbacterium* R2_8, significant differences in body size at day 8 were observed for host genotype O1-08 (Figure 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 cogeneric 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; 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 (Figure 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.

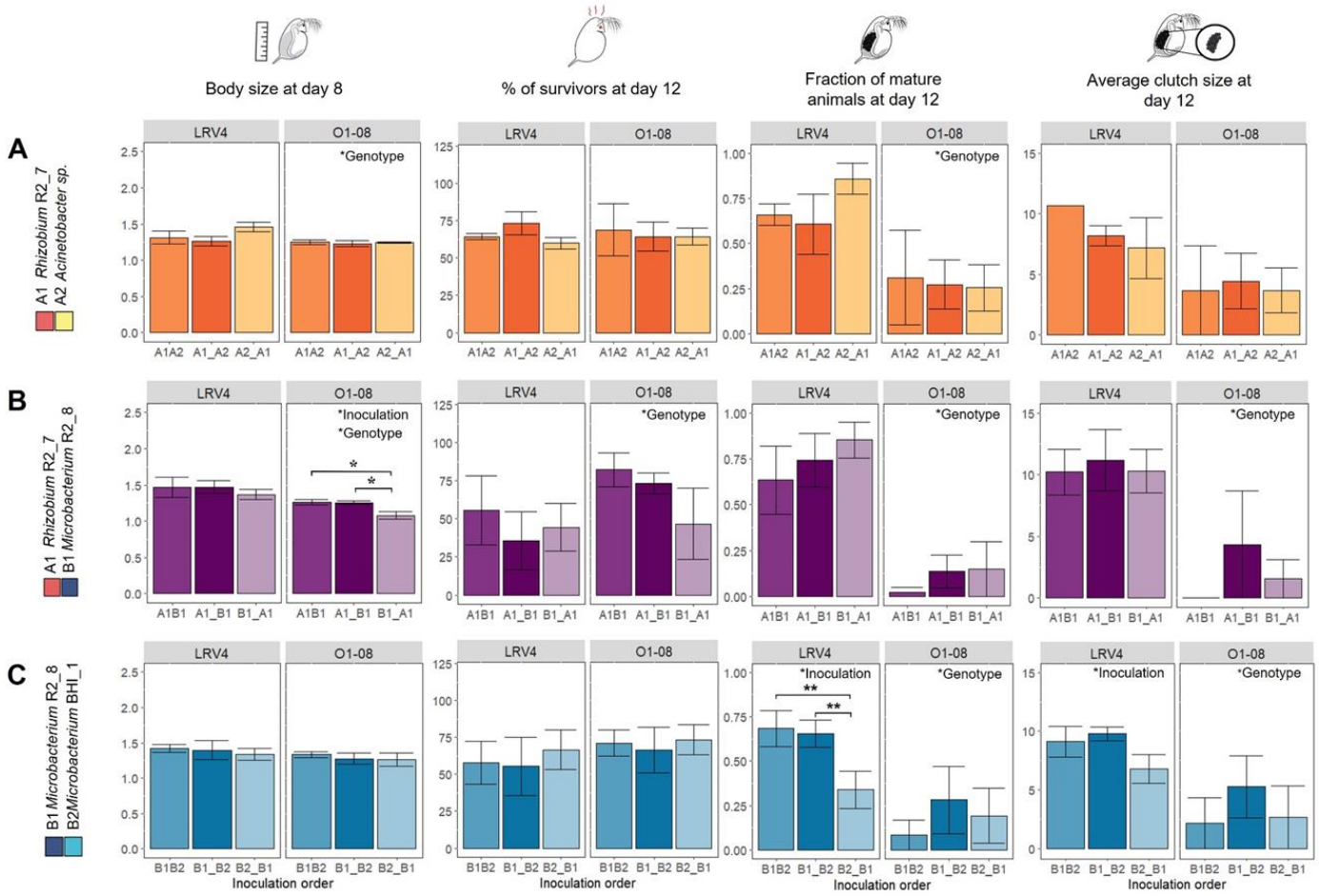

Figure 3. Mean values ± one standard error of host body size at day 8 (mm), survival at day 12 (percentage of survivors at day 12), fraction of mature animals at day 12 (fraction of survivors that is mature) and clutch size (number of eggs per female at day 12), for two host (*D. magna*) genotypes (LRV4 and O1-08) in the two-strain bacterial inoculations (simultaneous, time-lag and reciprocal-time-lag) of A) heterogeneric strain combination *Rhizobium* R2_7 - *Acinetobacter* BHI_9; B) heterogeneric strain combination *Rhizobium* R2_7 - *Microbacterium* R2_8; and C) cogeneric strain combination *Microbacterium* R2_8 - *Microbacterium* BHI_1. X1X2 = simultaneous inoculations: X1_X2 = X1 inoculated 48h before X2 (time-lag inoculation); X2_X1 = X2 inoculated 48h 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 Fig S7.

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 non-significant 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 Figure 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.

	Strain combination	***Daphnia* genotype**	**Time point**	**Bacterial abundance**			**Host trait**			
				Treatment comparison	**Mean diff**	**Effect size**	**Treatment comparison**	**Trait**	**Mean diff**	**Effect size**
A1A2	*Rhiz*_R27-*Acin*_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	*Rhiz*_R27-*Micro*_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	*Micro*_R28-*Micro*_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**	**-0.34**	**-1.91**

			B1: B1_B2-B2_B1	**-4.20**	**-2.40**	**B1_B2-B2_B1**	**animals 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

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 (55). 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.(19) 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 (56–60), 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 (58,61)] 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 (23,24). Most priority effects reported in literature are indeed inhibitory in nature (35,61), with a few studies demonstrating facilitative effects (62,63). 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 (23) or evolutionary adaptation of the early colonist (24,64). For *Microbacterium* R2_8 in these same settings, however, a facilitative priority effect (23) 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 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. (19) 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 cogeneric 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. (19), where priority effects were demonstrated in the gut independent of host genotype. Our results align with the findings of Leopold et al. (65), 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.* Notably, the facilitative priority effects observed in the fungal assemblages were found to be dependent on the specific genotype of the host (63). Additionally, 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 Figure S11 in Supporting Information). Second, we plotted total bacterial densities against body size of our experimental animals and did not observe a significant positive correlation (Figure S10)

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 (66), there remain certain species within this group that demonstrate strain-specific nutrient preferences (67). 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 (68), 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 (69–72). Microbiome-mediated stress tolerance in *Daphnia* has been demonstrated for exposure to cyanobacterial toxins (12) and mercury (73). Likewise, previous studies have shown that germ-free *Daphnia* demonstrate reduced fitness and high mortality (42,43), which can be restored in the presence of specific bacterial strains, such as *Limnohabitans* and *Aeromonas* sp. (43,74,75). 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- and host genotype-dependent responses might reflect a combination of priority effects in bacterial abundances, context-dependent microbe-microbe interactions (76) 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 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 (Figure S9, Supporting Information), 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 Figure S9). 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 (77–79) 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 (80). Given the importance of gut microbiome composition on plant, animal, human functioning and health (1,69,71), a better understanding of priority effects also has important implications in an applied context, for instance in the context of micro-organism-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 (81) and thus foster better health for humans, animals and plants. Furthermore, the success of micro-organism-based interventions such as pre-biotics, pro-biotics, faecal 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 (82) and the multifaceted relationship between gut microbiome composition and host functioning and fitness (73,77,83,46) 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 (80).

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 et al 2018). 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 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.

Author contributions

LDM and SM designed the study; SM, MD and AG performed the experiments; AG and SM analysed data with input of CS and LDM; AG wrote the first drafts with input of CS and LDM; all authors contributed to refining the text.

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References

1. Lee WJ, Hase K. Gut microbiota–generated metabolites in animal health and disease. Nat Chem Biol. 2014 Jun;10(6):416–24.

2. Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol. 2012 May;10(5):323–35.

3. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. Science. 2016 Apr 29;352(6285):539–44.

4. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol. 2013 Nov;13(11):790–801.

5. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The Central Nervous System and the Gut Microbiome. Cell. 2016 Nov 3;167(4):915–32.

6. Martinez C, Antolin M, Santos J, Torrejon A, Casellas F, Borruel N, et al. Unstable Composition of the Fecal Microbiota in Ulcerative Colitis During Clinical Remission. Am J Gastroenterol. 2008 Mar;103(3):643–8.

7. Sharma S, Tripathi P. Gut microbiome and type 2 diabetes: where we are and where to go? J Nutr Biochem. 2019 Jan 1;63:101–8.

8. Xu Q, Qiao Q, Gao Y, Hou J, Hu M, Du Y, et al. Gut Microbiota and Their Role in Health and Metabolic Disease of Dairy Cow. Front nutr. 2021;8:701511.

9. Fouhse JM, Zijlstra RT, Willing BP. The role of gut microbiota in the health and disease of pigs. Anim Front. 2016 Jul 1;6(3):30–6.

10. Pita L, Rix L, Slaby BM, Franke A, Hentschel U. The sponge holobiont in a changing ocean: from microbes to ecosystems. Microbiome. 2018 Mar 9;6(1):46.

11. Ziegler M, Seneca FO, Yum LK, Palumbi SR, Voolstra CR. Bacterial community dynamics are linked to patterns of coral heat tolerance. Nat Commun. 2017 Feb 10;8(1):14213.

12. Macke E, Callens M, De Meester L, Decaestecker E. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat Commun. 2017 Nov 20;8(1):1608.

13. Smith CC, Snowberg LK, Gregory Caporaso J, Knight R, Bolnick DI. Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. ISME J. 2015 Nov;9(11):2515–26.

14. Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C, Stalder G, et al. Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. Nat Commun. 2019 May 16;10(1):2200.

15. Fan P, Bian B, Teng L, Nelson CD, Driver J, Elzo MA, et al. Host genetic effects upon the early gut microbiota in a bovine model with graduated spectrum of genetic variation. ISME J. 2020 Jan;14(1):302–17.

16. Campbell TP, Sun X, Patel VH, Sanz C, Morgan D, Dantas G. The microbiome and resistome of chimpanzees, gorillas, and humans across host lifestyle and geography. ISME J. 2020 Jun;14(6):1584–99.

17. Pérez-Cobas AE, Gosalbes MJ, Friedrichs A, Knecht H, Artacho A, Eismann K, et al. Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut. 2013 Nov 1;62(11):1591–601.

18. Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, et al. Population-level analysis of gut microbiome variation. Science. 2016 Apr 29;352(6285):560–4.

19. Martínez I, Maldonado-Gomez MX, Gomes-Neto JC, Kittana H, Ding H, Schmaltz R, et al. Experimental evaluation of the importance of colonization history in early-life gut microbiota assembly. eLife. 2018;7:e36521.

20. Alford RA, Wilbur HM. Priority effects in experimental pond communities: competition between Bufo and Rana. Ecology. 1985;66(4):1097–105.

21. Connell JH, Slatyer RO. Mechanisms of succession in natural communities and their role in community stability and organization. The American naturalist. 1977;111(982):1119–44.

22. De Meester L, Gómez A, Okamura B, Schwenk K. The Monopolization Hypothesis and the dispersal–gene flow paradox in aquatic organisms. Acta oecologica. 2002;23(3):121–35.

23. Fukami T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. Annu Rev Ecol Evol Syst. 2015 Dec 4;46:1–23.

24. De Meester L, Vanoverbeke J, Kilsdonk LJ, Urban MC. Evolving Perspectives on Monopolization and Priority Effects. Trends Ecol Evol. 2016 Feb 1;31(2):136–46.

25. Fukami T, Mordecai EA, Ostling A. A framework for priority effects. J Veg Sci. 2016;27(4):655–7.

26. Poza-Carrion C, Suslow T, Lindow S. Resident bacteria on leaves enhance survival of immigrant cells of Salmonella enterica. Phytopathology. 2013;103(4):341–51.

27. Monier JM, Lindow SE. Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. Microbial Ecology. 2005;49(3):343–52.

28. Solís G, de los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe. 2010 Jun 1;16(3):307–10.

29. Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, et al. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci USA. 2008 Dec 2;105(48):18964–9.

30. Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, et al. Bacteroides in the Infant Gut Consume Milk Oligosaccharides via Mucus-Utilization Pathways. Cell Host Microbe. 2011 Nov 17;10(5):507–14.

31. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci USA. 2011 Mar 15;108(supplement_1):4578–85.

32. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature. 2013;502(7469):96–9.

33. Carlström CI, Field CM, Bortfeld-Miller M, Müller B, Sunagawa S, Vorholt JA. Synthetic microbiota reveal priority effects and keystone strains in the Arabidopsis phyllosphere. Nat Ecol Evol. 2019 Oct;3(10):1445–54.

34. Nappi J, Goncalves P, Khan T, Majzoub ME, Grobler AS, Marzinelli EM, et al. Differential priority effects impact taxonomy and functionality of host-associated microbiomes. Molecular Ecology. 2022;

35. Peay KG, Belisle M, Fukami T. Phylogenetic relatedness predicts priority effects in nectar yeast communities. Proc R Soc B: Biol Sci. 2012 Feb 22;279(1729):749–58.

36. Dhami MK, Hartwig T, Fukami T. Genetic basis of priority effects: insights from nectar yeast. Proc R Soc B: Biol Sci. 2016 Oct 12;283(1840):20161455.

37. Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat Rev Gastroenterol Hepatol. 2018 Apr;15(4):197–205.

38. Boyle JA, Simonsen AK, Frederickson ME, Stinchcombe JR. Priority effects alter interaction outcomes in a legume–rhizobium mutualism. Proc R Soc B: Biol Sci. 2021 Mar 10;288(1946):20202753.

39. Hoverman JT, Hoye BJ, Johnson PTJ. Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. Oecologia. 2013 Dec 1;173(4):1471–80.

40. Miner BE, De Meester L, Pfrender ME, Lampert W, Hairston NG. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc R Soc B: Biol Sci. 2012 May 22;279(1735):1873–82.

41. Qi W, Nong G, Preston JF, Ben-Ami F, Ebert D. Comparative metagenomics of Daphnia symbionts. BMC Genomics. 2009 Apr 21;10(1):172.

42. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, et al. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. ISME J. 2016 Apr;10(4):911–20.

43. Sison-Mangus MP, Mushegian AA, Ebert D. Water fleas require microbiota for survival, growth and reproduction. ISME J. 2015 Jan;9(1):59–67.

44. Mushegian AA, Ebert D. Presence of microbiota reverses the relative performance of Daphnia on two experimental diets. Zoology. 2017 Dec 1;125:29–31.

45. Akbar S, Gu L, Sun Y, Zhou Q, Zhang L, Lyu K, et al. Changes in the life history traits of *Daphnia magna* are associated with the gut microbiota composition shaped by diet and antibiotics. Sci Tot Environ. 2020 Feb 25;705:135827.

46. Bulteel L, Houwenhuyse S, Declerck SAJ, Decaestecker E. The Role of Microbiome and Genotype in Daphnia magna upon Parasite Re-Exposure. Genes. 2021 Jan 7;12(1):70.

47. Callens M, De Meester L, Muylaert K, Mukherjee S, Decaestecker E. The bacterioplankton community composition and a host genotype dependent occurrence of taxa shape the Daphnia magna gut bacterial community. FEMS Microbiol Ecol. 2020 Jun 23;96(8):fiaa128.

48. De Meester L. Life history characteristics of Daphnia magna clones differing in phototactic behaviour. Hydrobiologia. 1995 Jul 1;307(1):167–75.

49. Van Doorslaer W, Stoks R, Duvivier C, Bednarska A, De Meester L. Population dynamics determine genetic adaptation to temperature in Daphnia. Evolution. 2009 Jul 1;63(7):1867–78.

50. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991 Jan;173(2):697–703.

51. Bates D, Kliegl R, Vasishth S, Baayen H. Parsimonious mixed models. arXiv preprint arXiv:150604967. 2015;

52. Singmann H, Bolker B, Westfall J, Aust F, Ben-Shachar MS. afex: Analysis of factorial experiments. R package version 013–145. 2015;

53. Fox J, Weisberg S. Using car functions in other functions. 2019;

54. Lenth R, Lenth MR. Package 'lsmeans.' Am Stat. 2018;34(4):216–21.

55. Stollewerk A. The water flea Daphnia--a "new" model system for ecology and evolution? Journal of biology. 2010/01/13 ed. 2010;9(2):21.

56. Nappi J, Goncalves P, Khan T, Majzoub ME, Grobler AS, Marzinelli EM, et al. Differential priority effects impact taxonomy and functionality of host-associated microbiomes. Mol Ecol. 32(23):6278–93.

57. Fukami T, Beaumont HJE, Zhang XX, Rainey PB. Immigration history controls diversification in experimental adaptive radiation. Nature. 2007 Mar 22;446(7134):436–9.

58. van Gremberghe I, Vanormelingen P, Van der Gucht K, Souffreau C, Vyverman W, De Meester L. Priority effects in experimental populations of the cyanobacterium Microcystis. Environ Microbiol. 2009 Oct;11(10):2564–73.

59. Knope ML, Forde SE, Fukami T. Evolutionary history, immigration history, and the extent of diversification in community assembly. Front Microbiol. 2011;2:273.

60. Tucker CM, Fukami T. Environmental variability counteracts priority effects to facilitate species coexistence: evidence from nectar microbes. Proc R Soc B: Biol Sci. 2014 Mar 7;281(1778):20132637.

61. Devevey G, Dang T, Graves CJ, Murray S, Brisson D. First arrived takes all: inhibitory priority effects dominate competition between co-infecting Borrelia burgdorferi strains. BMC Microbiol. 2015 Mar 7;15(1):61.

62. Alford RA, Wilbur HM. Priority Effects in Experimental Pond Communities: Competition between Bufo and Rana. Ecology. 1985;66(4):1097–105.

63. Halliday FW, Penczykowski RM, Barrès B, Eck JL, Numminen E, Laine AL. Facilitative priority effects drive parasite assembly under coinfection. Nat Ecol Evol. 2020 Nov;4(11):1510–21.

64. Scanlan PD. Microbial evolution and ecological opportunity in the gut environment. Proc R Soc B: Biol Sci. 2019 Nov 20;286(1915):20191964.

65. Leopold DR, Busby PE. Host Genotype and Colonist Arrival Order Jointly Govern Plant Microbiome Composition and Function. Curr Biol. 2020 Aug 17;30(16):3260-3266.e5.

66. Towner K. The Genus Acinetobacter. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, editors. The Prokaryotes: A Handbook on the Biology of Bacteria Volume 6: Proteobacteria: Gamma Subclass [Internet]. New York, NY: Springer; 2006 [cited 2024 Feb 26]. p. 746–58. Available from: https://doi.org/10.1007/0-387-30746-X_25

67. Glover JS, Browning BD, Ticer TD, Engevik AC, Engevik MA. Acinetobacter calcoaceticus is Well Adapted to Withstand Intestinal Stressors and Modulate the Gut Epithelium. Front Physiol. 2022 May 24;13:880024.

68. Kehe J, Ortiz A, Kulesa A, Gore J, Blainey PC, Friedman J. Positive interactions are common among culturable bacteria. Sci Adv. 2021 Nov 5;7(45):eabi7159.

69. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. Trends Plant Sci. 2012 Aug 1;17(8):478–86.

70. Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytol. 2015;206(4):1196–206.

71. Shreiner AB, Kao JY, Young VB. The gut microbiome in health and in disease. Curr Opin Gastroenterol. 2015 Jan;31(1):69–75.

72. Bahrndorff S, Alemu T, Alemneh T, Lund Nielsen J. The Microbiome of Animals: Implications for Conservation Biology. Int J Genomics. 2016 Apr 18;2016:e5304028.

73. Fong JC, De Guzman BE, Lamborg CH, Sison-Mangus MP. The Mercury-Tolerant Microbiota of the Zooplankton Daphnia Aids in Host Survival and Maintains Fecundity under Mercury Stress. Environ Sci Technol. 2019 Dec 17;53(24):14688–99.

74. Peerakietkhajorn S, Tsukada K, Kato Y, Matsuura T, Watanabe H. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, Daphnia magna. Environ Microbiol Rep. 2015 Apr;7(2):364–72.

75. Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. Betaproteobacteria Limnohabitans strains increase fecundity in the crustacean Daphnia magna: symbiotic relationship between major bacterioplankton and zooplankton in freshwater ecosystem. Environ Microbiol. 2016 Sep;18(8):2366–74.

76. Rao C, Coyte KZ, Bainter W, Geha RS, Martin CR, Rakoff-Nahoum S. Multi-kingdom ecological drivers of microbiota assembly in preterm infants. Nature. 2021 Mar;591(7851):633–8.

77. Houwenhuyse S, Stoks R, Mukherjee S, Decaestecker E. Locally adapted gut microbiomes mediate host stress tolerance. ISME J. 2021 Aug;15(8):2401–14.

78. O'Brien AM, Harrison TL. Host match improves root microbiome growth. Nat Microbiol. 2021 Sep;6(9):1103–4.

79. Jackrel SL, Yang JW, Schmidt KC, Denef VJ. Host specificity of microbiome assembly and its fitness effects in phytoplankton. ISME J. 2021 Mar;15(3):774–88.

80. Decaestecker E, Van de Moortel B, Mukherjee S, Gurung A, Stoks R, De Meester L. Hierarchical Eco-Evo Dynamics Mediated by the Gut Microbiome. Trends Ecol Evol. In press;39/2.

81. Weidlich EWA, Nelson CR, Maron JL, Callaway RM, Delory BM, Temperton VM. Priority effects and ecological restoration. Restor Ecol. 2021;29(1):e13317.

82. Leibold MA, Govaert L, Loeuille N, De Meester L, Urban MC. Evolution and Community Assembly Across Spatial Scales. Annu Rev Ecol Evol Sys. 2022;53(1):299–326.

83. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. Cell. 2017 Nov 16;171(5):1015-1028.e13.

Supporting Information

1. Daphnia *genotypes: origin and culturing*

Two *Daphnia magna* genotypes were used in our study. Genotype LRV4 was sampled in June 2018 from Langerodevijver pond, Huldenberg, Belgium (x-50.828413, y-4.639922). Genotype LRV4 is the genotype from which all bacterial strains used in this study were isolated. Genotype O1-08 was sampled from a pond near Wulfhoek 2 in Nevele, Belgium (x-51.048305, y-3.549410) also in June 2018. Genotype O1-08 is thus derived from another pond than both clone LRV4 and the bacterial strains.

To avoid interference from (grand)maternal effects, replicate cultures of each host genotype were established and cultured separately for at least two generations under standardized conditions (three individuals in a 500 ml jar filled with 24h-aged, dechlorinated tap water; 20°C, 14:10h L:D photoperiod). The water in the jars was refreshed every two days (80% fresh water, 20% old medium) and 1 x 10^5 cells ml^{-1} of the green alga *Acutodesmus obliquus* was added to the medium. Eggs of 12 to 16h from the second to third clutch were collected for the experiment (i.e. for the sterilization step).

2. *Bacterial strains: isolation, identification and selection*

For isolation of *Daphnia* gut bacterial strains, a gravid female, directly collected from the field, was dissected under sterile conditions to isolate the gut. The dissected gut was homogenized in sterile PBS and plated onto five different media (ranging in nutrient composition): Reasoner's 2A agar (R2A), Nutrient agar (NA), Tryptic Soy Agar (TSA), Brain Heart Infusion (BHI), and Mac Conckey agar (MC). The plates were incubated at 25°C until visible growth appeared. Single colonies were picked and streaked on their corresponding medium until pure cultures were obtained. A total of 76 pure cultures were obtained. DNA was extracted from these cultures using the DNeasy® UltraClean® Microbial DNA extraction Kit (Qiagen, Hilden, Germany). The DNA samples were stored at -20°C. For each of the 76 strains, the full length of the 16S rRNA gene was amplified (15s at 95°C, 15s at 57°C, 40s at 72°C, and 72°C for 5 mins, 40 cycles) with primers 27F (10µM) and 1492R (10 µM) (1) using Platinum™ SuperFi™ DNA Polymerase (Invitrogen™, ThermoFisher Scientific, Waltham, USA). PCR products were purified using AMPure beads (CleanPCR kit, CleanNA, Waddinxveen, The Netherlands) and libraries were prepared for Sanger sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific, Waltham, USA). The samples were sequenced on the 3500xL Genetic Analyzer at the Genomics core, KU Leuven.

To construct a phylogenetic tree from the obtained sequences, the data were uploaded to the ACT (Alignment, Classification and TREE) service available at *www.arb-silva.de*. Using the SILVA ribosomal RNA database, the sequences were taxonomically classified. The preliminary tree generated through this platform was subsequently refined and visualized using iTOL (Interactive Tree if Life; *www.itol.embl.de*). The final phylogenetic tree comprising both the obtained strains and their closest reference matches from the SILVA databased in presented below in Figure S1. The gut microbiome composition comprised of six main bacterial classes, namely, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes, Firmicutes and Actinobacteria.

Of the 76 isolated strains, four strains were selected for the experiment.: *Rhizobium* R2_7, *Acinetobacter* BHI_9, *Microbacterium* R2_8, and *Microbacterium* BHI_1 (selected from the three most predominant bacterial classes). Using these four strains, three strain pairs were constructed such that they could be differentiated with qPCR and colony morphology: heterogeneric strain pairs *Rhizobium* R2_7 & *Acinetobacter* BHI_9 and *Rhizobium* R2_7 & *Microbacterium* R2_8, and cogeneric strain pair *Microbacterium* R2_8 & *Microbacterium* BHI_1. While these strains were originally isolated on different growth media, we established that all grew optimally on R2A medium.

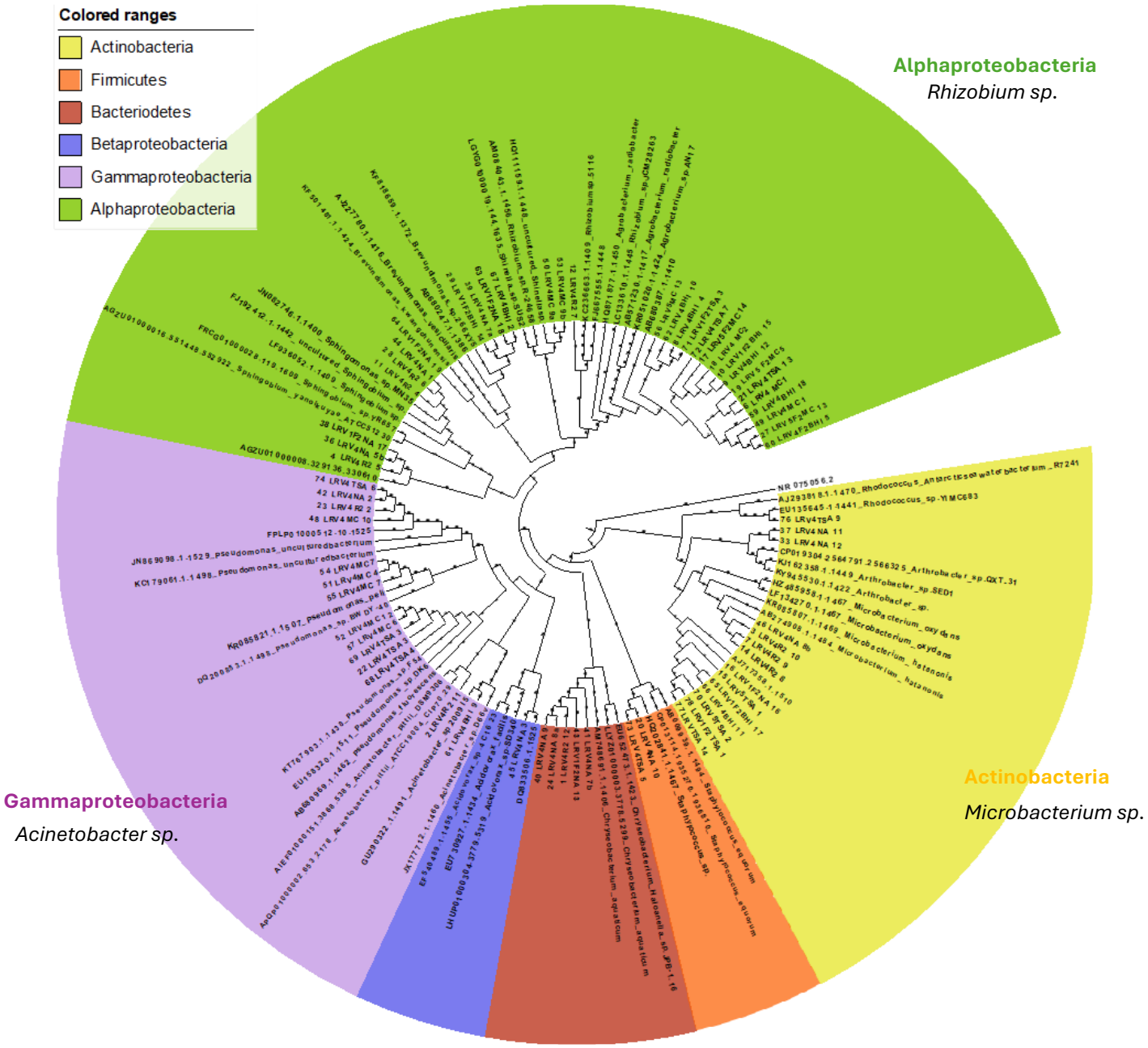

Figure S1: Phylogenetic tree of obtained isolates from the gut of *Daphnia magna*, clone LRV4 (Langerodevijver pond, Leuven) and their closest reference matches from the SILVA database.

3. *Culturing of sterile* Acutodesmus obliquus

Axenic green algae (*Acutodesmus obliquus*) was cultured as a sterile feed for *Daphnia* during the entire duration of the experiment. The sterile starting (stock) culture was obtained from the Culture Collection of Algae and Protozoa (CCAP 276/3A). ES-Walne was used as the growth medium for algae culture. This medium was prepared by adding three media components (ES, Walne and EPA; for their composition see Tables S1-S5) into 0.45 µm filtered sterile water. Each medium was prepared separately and autoclaved prior to use. For every 1L of the ES-Walne medium, 10 mL of Provasoli's ES medium (2–4) and 5 mL of Walne medium (5) was added respectively. EPA components were added as 1 mL/L each.

All media and equipment were autoclaved prior to use. 10 ml of the original sterile stock culture (CCAP 276/3A) was inoculated into 450 ml of the ES-Walne medium and allowed to grow at ±20.5°C under constant light and aeration. The inlet and outlet of the aeration tubes were provided with 0.22 µm filters to prevent bacterial entry into the culture. Batches were renewed every week; each inoculum batch was checked for sterility by plating them on NA and R2A plates. The algae concentration of each batch was measured using a flow cytometer (Attune® acoustic focusing cytometer, Applied Biosystems).

Table S1: Components of the ES medium (2). ES and Walne can be stored at 4˚C for a maximum of 3 weeks. The EPA stock bottles can be stored for a maximum of 2 months.

Product name	**Weight per L MilliQ**	**Comment**
$NaNO_3$	3,5 g	
Na-glycerophosphate	0,5 g	
Tris buffer	5 g	
Metal mix*	25mL	
Fe solution**	25mL	
Cyanobalamine (Vit. B12)	0,1 mg	Added **after** autoclaving
Thiamine (Vit. B1)	5 mg	Added **after** autoclaving
Biotine (Vit. H)	50 µg	Added **after** autoclaving

Table S2: *Metal mix (amounts for 1 L Metal mix stock, freeze in portions of 25 mL for addition in 1 L of ES)

Product name	**Weight per L MilliQ**
H_3BO_3	11,4 g
$FeCl_3.6H_2O$	0,490 g
$MnSO_4.4H_2O$	1,640 g
$ZnSO_4.7H_2O$	0,22 g
$CoSO_4.7H_2O$	48 mg
Na_2-EDTA	10 g

Table S3: **Fe-solution (amounts for 1 L Fe-solution stock, freeze in portions of 25 mL for addition in 1 L of ES)

Product name	**Weight per L MilliQ**
$Fe(NH_4)_2(SO_4)_2.6H_2O$	702 mg
Na_2-EDTA	660 mg

Table S4: Components of the Walne medium.

Product name	**Weight per L MilliQ**
$FeSO_4.7H_2O$	0,278 g
$NaH_2PO_4.2H_2O$	5 g

$NaNO_3$	30 g
$MnCl_2.4H_2O$	0,47 g

Table S5: Components of the EPA medium. EPA (stock made separate per component in 500 mL)

Product name	**Weight per 500mL MilliQ**	**Product nr.**	**Comment**
$NaHCO_3$	48 g	324	
$CaSO_4.2H_2O$	30 g	72	Will not dissolve completely
$MgSO_4$	30 g	215	
KCl	2 g	275	

4. *Isolation and sterilization of* Daphnia *eggs*

For egg collection of the host genotypes, non-sterile mothers carrying 12–16 hour old eggs (characterised by their smooth round edges and black colour) were selected for dissection. Taking a 50% hatching success into account, double the number of eggs required for the experiment were collected. Dissection of the mothers were carried out under a stereo-microscope (OLYMPUS SZX16, Japan) using sterile dissection needles. The brood pouch of the mothers were torn apart to release the eggs. These eggs were then transferred into sterile petri dishes containing 15-20 ml sterile ADaM medium (6).

Egg sterilization was carried out under a laminar air flow following the protocol of Callens et al. (7). 6-well plates were prepared to contain 10 ml of 0.01% peracetic acid (PAA, Sigma-Aldrich, Munich, Germany) in the first column of wells and sterile ADaM in the next two columns. Sterilization was carried out in batches of 50 eggs, each time using a different well plate. Using a sterile Pasteur pipette, the eggs were first transferred to the wells containing PAA. Following a 10-minute exposure with gentle agitation, the eggs were rinsed consecutively in the next two wells with sterile ADaM to remove residual PAA. After washing, each batch of eggs was transferred to a petri dish containing sterile ADaM. This dish was sealed with parafilm and incubated at ±20.5°C with a 14:10h photoperiod for 48-72h. Germ-free *Daphnia* that hatched were subsequently used in the experiment. Since the required number of eggs could not be obtained in a single day, a randomized staggered design was implemented to allow egg isolation and hence experimental replicates to be carried out in multiple batches. Two independent batches for the two time points (namely day 8 and day 12) were set up for gut extraction.

4.1. Sterility check

To assess axenity and sterility, checks were conducted at two levels. Firstly, *Daphnia* hatchlings from 0.01% PAA-exposed eggs were homogenized and plated onto five different media types ranging in nutrient complexity (NA, BHI, TSA, MC, R2A) and monitored for bacterial growth. No bacterial growth was observed for the hatchlings.

Secondly, DNA was extracted from sterile hatchlings and subjected to a qPCR assay to assess axenity. Likewise, for the no-inoculum treatments, DNA extracted from dissected daphnid guts was screened for contamination using a qPCR assay. A SYBR Green qPCR assay was used to detect the presence of all potential bacteria in these treatments. Because SYBR green is an intercalating dye, it binds to all double stranded DNA that is amplified by the 16S rRNA primer, thus allowing the detection of all bacterial contamination present in the samples.

Given that *Daphnia* were fed unicellular green algae during the experiment, a SYBR green qPCR assay on the water or the gut would result in positive results due to the presence of mitochondrial DNA of both algae and gut cells as well as chloroplast DNA of the algae cells fed. A melt curve analysis was thus performed to check for the formation of distinct peaks corresponding to melting temperatures (T_m) specific to each amplicon. The melt curves revealed two distinct peaks: one at 83°C and the other more prominent peak at 90°C (Fig S2) indicating non-specific amplification occurring from the presence of multiple DNA species. The curve at 83°C corresponds to the melting of PCR products amplified from bacterial 16S rRNA genes using the primer pairs, while the dominant peaks at 87-90°C likely correspond to PCR products from DNA of higher GC content, such as chloroplast DNA and mitochondrial DNA. Due to the presence of multiple curves from the melt curve analysis, the results from the SYBR green assay could not be used.

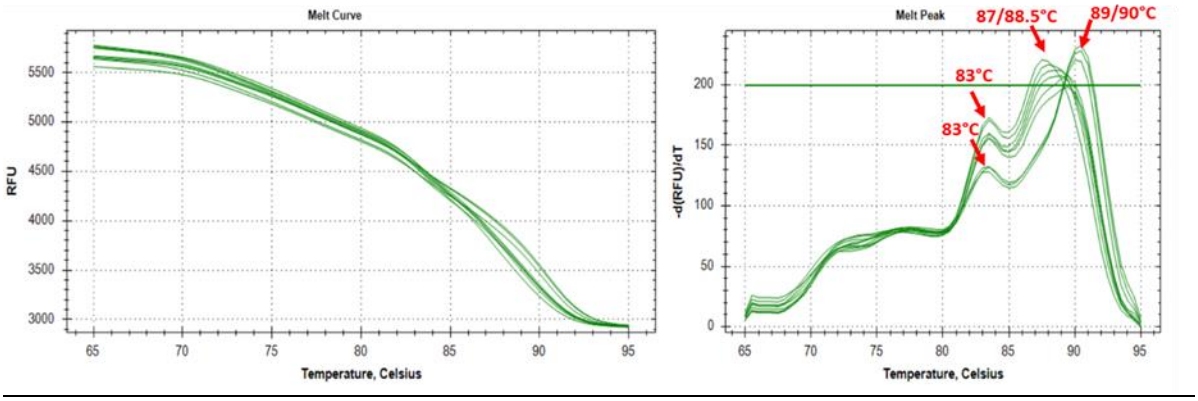

Figure S2: Melt curve analysis of the no-inoculum treatments using a SYBR green qPCR assay.

While the results from the SYBR green qPCR assay were not definite to confirm axenity, the poor fitness of the *Daphnia* in the no-inoculum treatment testifies to the fact that they either were axenic or had minimal bacteria that were insufficient to make them perform better. In line with expectations for axenic animals, we observed that by day 12 significant mortality was observed in animals of the no-inoculum treatments. For Genotype LRV4, only two replicate units remained, while for Genotype O1-08, only one replicate unit remained. In both cases, all 15 individuals in each of these units had died. These findings were in alignment with previous findings indicating high mortality among germ-free animals (8) thereby suggesting a high state of sterility for the control units.

5. *Microbial inoculations, and incubation*

The bacterial strains used for inoculation were grown in 50 ml of sterile R2A broth at RT for 72h. The culture was pelleted down by centrifugation at 4000rpm for 15 minutes. The pellet so obtained was resuspended in sterile PBS. A second washing step with sterile PBS was performed to obtain the final pellet. A concentration of 10^6 bacterial cells/ml was maintained as inoculation dose for each strain. This concentration was established based on a previous estimation of bacterial cells from multiple pond water samples, to mimic the natural environment of *Daphnia*. Bacterial density was measured through DAPI staining followed by flow cytometric quantification (Attune® acoustic focusing cytometer, Applied Biosystems).

To investigate the effect of inoculation order on gut community assembly, the following treatment groups 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 48h time-lag and 4) reciprocal time-lag inoculation where the previous order was reversed. In addition, a no-inoculum treatment was maintained as control to assess *Daphnia* life history traits in the absence of any added inoculum. For each of these inoculation treatments, 15 sterile hatchlings (per replicate per treatment group) were transferred to a 50 ml falcon tube containing 30 ml sterile ADaM and $0.5x10^5$ sterile algae cells/ml separately for the two time points. The appropriate strain/strain combination was inoculated to each experimental unit at a standardized total concentration of 10^6 cells/ml. This implies that for the simultaneous and time lag inoculations, the density of each individual strain was set to be $0.5x10^6$ cells/ml, while this was $1x10^6$ cells/ml per strain for the single inoculation treatments. The second exposure in the (reciprocal)time lag treatments was executed 48h after the first inoculation, again at $0.5x10^6$ cells/ml. After 96 hours, 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. All experimental units were placed in an incubator at 20°C with a 16:8h L:D photoperiod, with their positions randomized. On day nine, the time point 2 samples were refreshed by transferring the animals into new culture flasks with fresh sterile media and algae, in identical settings as mentioned above. All procedures requiring sterile conditions were carried out under a laminar air flow chamber.

6. *Gut dissection and DNA extraction*

Gut dissections were performed on day 8 and day 12. On day 8 and 12, the guts were sampled from *Daphnia* in the strain combination treatments (simultaneous, time lag and reciprocal time lag treatments) and no-inoculation treatment. Gut dissections for single strain inoculations were only performed on day 12. Sampling of single strain inoculation at two time points was deemed unnecessary as there could not be changes in species composition in these treatments. For gut dissections, sterile needles were used to dissect the *Daphnia* and pull out the gut. A minimum of 7 guts per experimental unit were transferred into sterile Eppendorf tubes filled with 20µl Milli-Q® water and stored for DNA extraction. However, for a few units, due to the lack of sufficient surviving individuals, the total number of guts collected was reduced from 7 to a range of 2 to 5 guts depending on the number of remaining individuals.

DNA extraction from the gut samples was performed using the DNeasy® PowerSoil® Pro-Kit (Qiagen, Hilden, Germany) and quantified using Quant-iT™ PicoGreen™ dsDNA Assay (Invitrogen™ , Life Technologies, Eugene, USA) and a microplate reader (Infinite® M Nano +, Tecan).

7. *Development of the qPCR assay*

7.1 Primer design

The primers used for amplification comprised the 16S forward primer 27F (8) and a reverse R206 primer, which was designed to maintain an amplicon size of approximately 200bp to ensure high amplification efficiency (table S6). A multiple sequence alignment of all 4 strains was performed using CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to identify regions in their 16S gene where they display the highest identity (shown in figure S3). A wobble code was implemented to cover the nucleotide differences. For our designed primer, a wobble code was introduced at positions 4 (T or A =W) and 10 (C or T =Y). Additionally, the T_m and annealing temperature was calculated using NEB T_m calculator v.1.11.0 which bases T_m calculation on the GC content of the primer sequence. This was done to maintain as much similarity with the T_m of the forward primer (maximum difference of 5°C).



Figure S3: Multiple sequence alignment of 16S rRNA code extracted via Sanger sequencing. Order of sequences: B1, B2, A1, A2. Red: region on which the R206 primer is based.

Table S6: Codes for forward and reverse primers 3'-5'

Type	Primer	5'-3' sequence with wobble	RC with wobble 5'-3'
Forward	F27	AGAGTTTGATCMTGGCTCAG	AGAGTTTGATCMTGGCTCAG
Reverse	R206	CGGAAATGGGGTGGTTG	GCCWTTACCYCACCAAC

To test the amplification efficiency of the primers, gDNA of each of the selected strains was amplified in a qPCR reaction with the following conditions: 40 cycles at 95°C for 15s and 57°C for 30s, followed with a melt curve analysis from 65°C to 95°C, with an increment of 0.5°C. The master mix was prepared using the SYBR® Green I Dye (Bio-Rad, California, USA) following the protocol of Weisburg et al. (1). The products were then run on an agarose gel (1%) to check for DNA integrity. The PCR products showed quite intense bands for all four strains. Following this, a gradient qPCR (95°C-15s, gradient 53.0/61.0°C-30s, 72°C-40s for 30 cycles) using a dilution series (1:100, 1:200, 1:400, 1:2000, 1:10000, NTC) of all four strains (pure culture gDNA) was prepared to define the ideal annealing temperature in which all strains amplified best. The annealing temperatures tested were 61.0°C, 59.7°C, 56.2°C, and 53.0°C. Based on the amplification efficiency of the lowest dilution, 61°C was chosen as the annealing temperature.

7.2 Probe design and testing

To quantify the inoculated strain combinations from the gut extracts, four strain-specific hydrolysis probes were designed, the details of which can be found in table S7. A multiple sequence alignment of all isolates was executed using CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to identify regions in their 16S rRNA gene where all the four strains showed significant nucleotide differences (Fig. S4). Based on the results from CLUSTAL Omega, all four strains displayed considerable variation in the 110-120bp region of their 16S gene (Fig. S4). This region was thereby selected to identify unique targets for probe design. Additionally, a pairwise alignment of the strain combinations (*Rhizobium-Acinetobacter, Rhizobium-Microbacterium, Microbacterium-Microbacterium*) was also conducted to evaluate sufficient pairwise nucleotide differences to avoid cross-amplification (Fig. S5). The selected sequences (as highlighted in figure S4) were analysed in the OligoAnalyzer tool (IDT, Integrated DNA Technologies, Leuven, Belgium) to determine the ideal requirements for probe efficiency and specificity. These included an average length of 20-28 nucleotides, GC% 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) labelled with FAM or HEX dyes (PrimeTime qPCR probes) were purchased from Integrated DNA Technologies (IDT, Leuven, Belgium). In total, four strain-specific hydrolysis probes targeting regions in the first 200bp region of the bacterial 16S rRNA gene were designed to accurately detect the following strains: *Rhizobium* R2_7 (probe A1; FAM), *Acinetobacter* BHI_9 (probe A2; HEX), *Microbacterium* R_28 (probe B1; HEX), *Microbacterium* BHI_1 (probe B2; FAM).

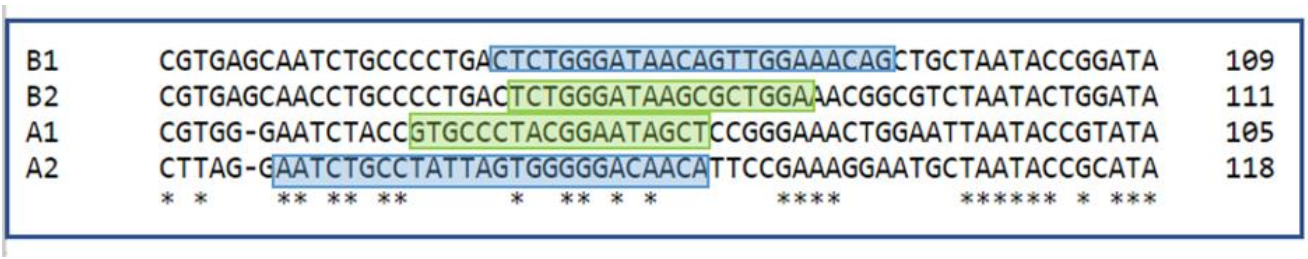

Figure S4: Multiple sequence alignment of the 16S rRNA region of all 4 isolates using CLUSTAL Omega. The highlighted regions represent the target sequences based on which the probes are designed.



Figure S5: Pairwise sequence alignments 1) A1A2 2) B1B2 3) A1B1

Table S7: Specifications of our designed probes based on a final reaction composition of 50mM KCl, 3mM $MgCl_2$, and 0.8mM dNTPs.

No	**Target species**	**Target sequence** 5'-3'	**Probe sequence** 5'-3'	**Length**	**GC%**	**T_m ºC**
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**	**62.6**
B2	*Microbacterium* BHI_1	CTCTGGGATAACAGTTGGAAACAG	TCCAGCGCTTATCCCAGA	**24**	**45.8**	**62.9**

7.3 Validation of qPCR assay: Amplification efficiency and linearity

To validate the performance of the multiplex assay, a serial dilution of the target strains was run in a multiplex reaction alongside their individual assays to ensure equivalent execution. Singleplex and multiplex assays for all targets were run on the same plate and the quantification cycle, C_q values achieved per probe from varying target DNA concentrations were assessed. The values for a given target in the singleplex and multiplex assays should not be significantly different. If they do differ, there is a need to optimize the reactions (mainly by varying reaction components). From our study, although the endpoint fluorescence signal was slightly reduced in the multiplex assay (as is expected, since reagents are consumed much faster), the C_q values obtained for the target genes in both multiplex and singleplex reactions were quite similar (Table S8, figure S6).

To determine the efficiency and linearity of each reaction, a series of template dilutions (dilution factor of 10) for each target were run and their obtained C_q values were used to generate a standard curve. The standard curves were constructed by plotting the log of the dilution factor of the target template against the C_q value achieved during the amplification of each dilution. The amplification efficiency (E) was calculated for each probe from the slopes of the standard curve using the formula: $E=10^{-1/slope}$; E (% efficiency) = (E-1) x 100% while the r or the R^2 value of the standard curve was used to determine the linearity of the data.

Mean C_q values were calculated for each hydrolysis probe in both singleplex (single template) and multiplex setups (strain combinations) and compared (Table S8). Linear correlations for C_q values and DNA concentrations (dilutions) were observed for all the four hydrolysis probes in singleplex and multiplex assays yielding high correlation coefficient values of $R^2 > 0.993$ and amplification efficiencies (E) in the range of 87.5-93.9%. Since the average C_q values of singleplex and multiplex reactions were highly similar, it indicated optimum performance of our multiplex assay with minimum interference effects from possible primer-probe interactions.

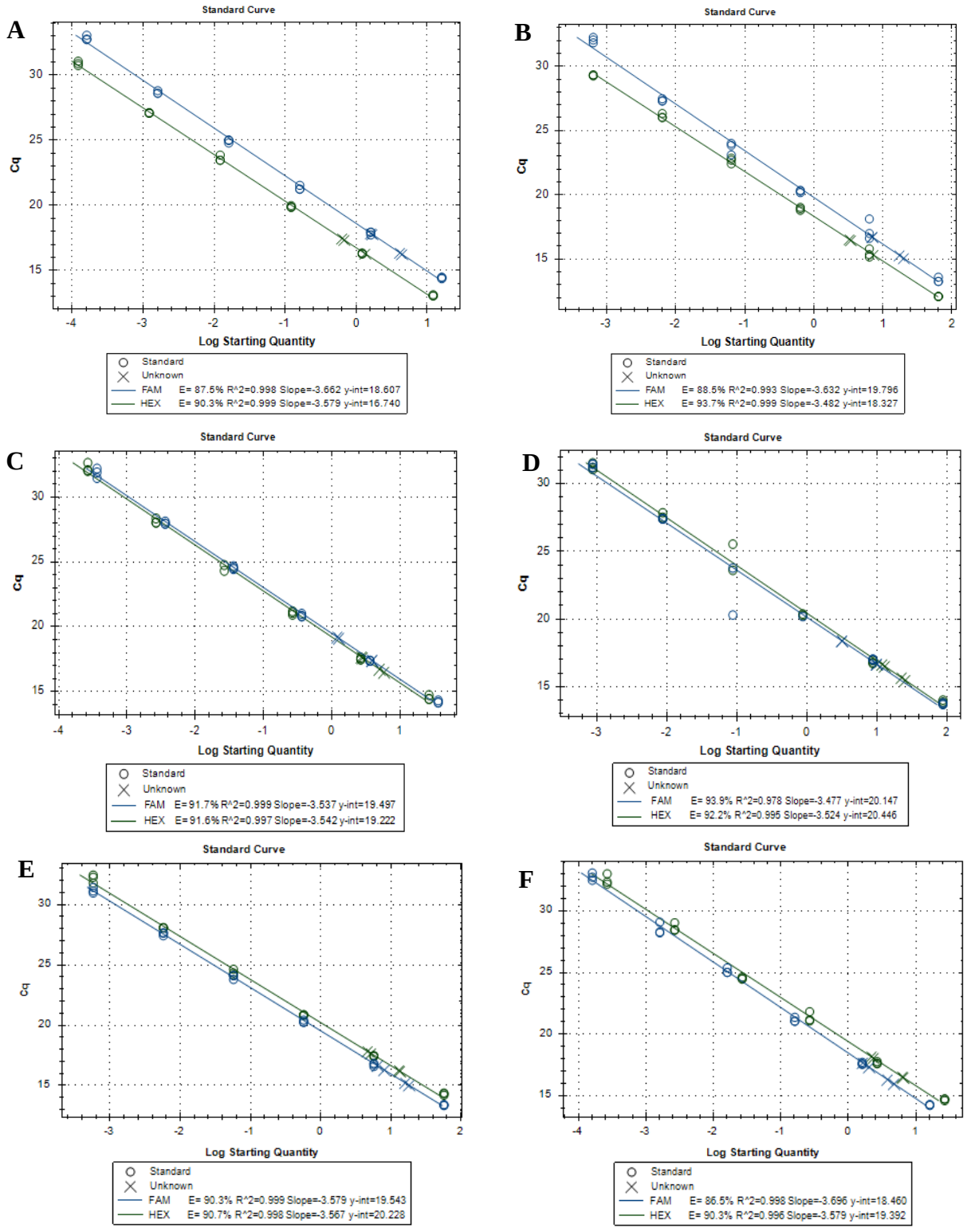

Figure S6: Standard curve of singleplex vs multiplex reactions and their amplification efficiencies.
A: A1/A2 singleplex, B: A1A2 multiplex, C: B1/B2 singleplex, D: B1B2 multiplex, E: A1/B1 singleplex, F: A1B1 multiplex

Table S8: Correlation of C_q values from singleplex and multiplex reactions.

Probe	Dilution	Conc	Singleplex		Multiplex	
A1			**Mean C_q values**	**Efficiency/ R^2**	**Mean C_q values**	**Efficiency/ R^2**
	1:10	16.04	13.68	87.5% 0.998	13.82	88.5% 0.993
	1:100	2.08	17.11		17.68	
	1:1000	0.32	20.53		20.68	
	1:10000	0.11	24.06		23.96	
	1:100000	0.08	27.84		27.77	
	1:1000000	0.074	31.68		32.65	
A2	1:10	12.15	12.5	90.3% 0.999	12.44	93.7% 0.999
	1:100	1.51	15.75		15.86	
	1:1000	0.23	19.31		19.26	
	1:10000	0.03	23.08		23.06	
	1:100000	0.105	26.5		26.49	
	1:1000000	0.105	30.26		29.72	
B1	1:10	26.66	14.53	91.6% 0.997	13.87	92.2% 0.995
	1:100	2.685	17.53		16.82	
	1:1000	0.423	21.07		20.33	
	1:10000	0.136	24.54		24.58	
	1:100000	0.078	28.15		27.61	
	1:1000000	0.091	32.25		31.27	
B2	1:10	36.39	14.22	91.7% 0.999	13.75	93.9% 0.978
	1:100	4.139	17.38		17.02	
	1:1000	0.670	20.88		20.19	
	1:10000	0.140	24.56		22.04	
	1:100000	0.083	28.01		27.42	
	1:1000000	0.193	31.87		31.38	
A1	1:10	16.04	13.81	86.5% 0.998	13.97	90.3% 0.999
	1:100	2.08	17.14		17.25	
	1:1000	0.32	20.64		20.86	
	1:10000	0.11	24.61		24.46	
	1:100000	0.08	27.99		28.15	
	1:1000000	0.074	32		31.84	
B1	1:10	26.66	14.61	90.3% 0.996	14.55	90.7% 0.998
	1:100	2.685	17.60		17.71	
	1:1000	0.423	21.29		21.10	
	1:10000	0.136	24.47		24.63	
	1:100000	0.078	28.58		28.31	
	1:1000000	0.091	32.44		32.50	

8. Quantification of Daphnia *life history traits*

Four life history traits were quantified for *Daphnia magna* in all treatments. For units of both time points (day 8 and 12), body sizes of three random *Daphnia* individuals per unit were measured using an Olympus SZX16 microscope and Image J software (9). Measurements were taken from the eye to the base of the spine. Per experimental unit, the average of these three values was taken as the estimate of body size. The total number of individuals that were alive at the end of the experiment was used to calculate percentage of surviving individuals. Additionally, the units were also checked for mature animals. As none of the experimental units possessed any mature animals before day 8, their reproductive traits such as fraction of mature animals and clutch size were not calculated. For the units of day 12 however, from day 8 to twelve, the number of individuals with eggs in their brood pouch (1st brood) was counted to assess the fraction of animals that matured among the surviving individuals. In addition, the average clutch size per experimental unit was assessed by counting the eggs of three random mature animals.

9. Statistical analysis

9.1 Bacterial abundances

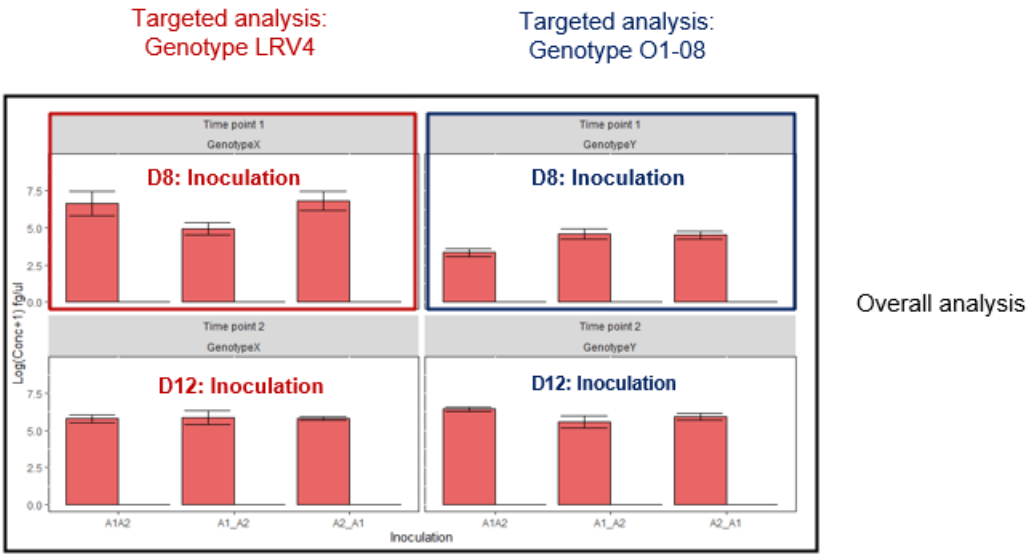

Figure S7: Scheme depicting the overall and targeted analysis for bacterial abundances.
The direct and indirect effects of inoculation order, genotype, and time point on *Daphnia* gut microbiome assembly were assessed using a type III ANOVA model. Tests were carried out at two levels, namely 1) an overall analysis to test for differences between all three treatment groups differing in their inoculation order for both genotypes and time points 2) targeted analyses assessing the three treatment groups individually for each genotype and time point.

Table S9: Summary output of the linear mixed model assessing the effects of 1) Inoculation order 2) Genotype 3) Time point and 4) interaction effects on bacterial stra

	A1A2					A1B1								B1B2							
			A1		A2			A1				B1				B1				B2	
	F	**Df**	**Df.res**	***P* value**		**F**	**Df**	**Df.res**	***P* value**	**F**	**Df**	**Df.res**	***P* value**	**F**	**Df**	**Df.res**	***P* value**	**F**	**Df**	**Df.res**	***P* value**
Overall analysis: Main effects																					
Inoculation	1.87	2	74.31	0.159	NA	**4.107**	**2**	**68.63**	**0.020**	**8.222**	**2**	**45.51**	**0.0008**	1.996	2	60.97	0.144	0.772	2	70.99	0.465
Genotype	**7.14**	**1**	**6.749**	**0.032**	NA	0.505	1	72.88	0.479	2.909	1	52.35	0.094	0.358	1	63.30	0.551	3.908	1	71.90	0.051
Time point	**11.7**	**1**	**76.49**	**0.0009**	NA	**13.76**	**1**	**80.00**	**0.0003**	**4.605**	**1**	**68.00**	**0.035**	1.643	1	71.86	0.204	1.575	1	71.48	0.189
Overall analysis: Interaction effects																					
Inoc: Gen	1.86	2	74.31	0.161	NA	0.772	2	68.63	0.465	**4.377**	**2**	**45.51**	**0.018**	2.332	2	71.40	0.104	0.101	2	71.82	0.903
Inoc: Time	1.73	2	76.49	0.182	NA	2.341	2	80.00	0.102	0.944	2	68.00	0.394	**4.958**	**2**	**71.21**	**0.009**	0.877	2	71.42	0.420
Gen: Time	**23.7**	**1**	**76.49**	**5.83e-06**	NA	**12.83**	**1**	**80.00**	**0.0005**	0.703	1	68.00	0.404	1.115	1	71.86	0.294	6.679	1	71.48	0.011
Inoc:Gen: Time	**6.68**	**2**	**76.49**	**0.002**	NA	**10.10**	**2**	**80.00**	**0.0001**	3.053	2	68.00	0.053	0.801	2	71.21	0.452	0.174	2	71.42	0.840
Targeted analysis: Genotype LRV4 at day 8																					
Inoculation	2.54	2	4.946	0.174	NA	**8.951**	**2**	**7.940**	**0.009**	**9.531**	**2**	**6.963**	**0.010**	0.049	2	4.542	0.951	1.029	2	4.916	0.423
Targeted analysis: Genotype LRV4 at day 12																					
Inoculation	0.01	2	22.863	0.987	NA	0.850	2	13.364	0.449	1.647	2	13.372	0.229	**32.46**	**2**	**23.26**	**1.85e-07**	0.190	2	23.08	0.827
Targeted analysis: Genotype O1-08 at day 8																					
Inoculation	**5.92**	**2**	-	**0.008**	NA	2.103	2	-	0.143	23.32	2	-	**1.33e-05**	1.122	2	17.35	0.347	0.198	2	20.52	0.821
Targeted analysis: Genotype O1-08 at day 12																					
Inoculation	2.24	2	-	0.127	NA	**7.008**	**2**	-	**0.004**	1.735	2	-	0.198	2.027	2	23.62	0.153	1.318	2	21.26	0.288

9.2 Daphnia *life history traits*

For the analysis of life history traits, the datasets were separated for the two time points, namely Day 8 and Day 12, since both time points focus on different traits (reproduction only on day 12). Thus, for each time point an overall analysis was conducted to test the effect of inoculation order, genotype and their interaction effects on host traits. A targeted analysis comprising only the 3 inoculation order treatments per genotype per time point were also tested.

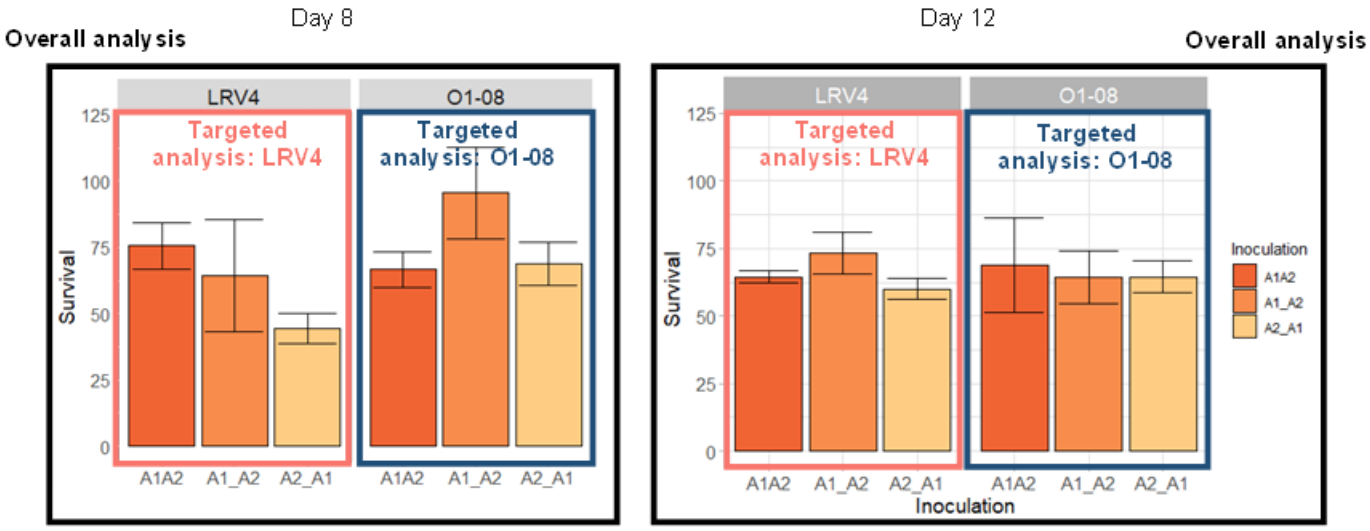

Figure S8: Scheme depicting the overall and targeted analysis for *Daphnia* Life history traits

Table S10: Results from the linear mixed model used to test for the effect of inoculation order and genotype on *Daphnia* fitness traits namely 1) Percentage of survivors on day 8 2) Average Body Size at day 8 3) Average Clutch size at day 12 4) Fraction of mature animals at day 12. Significant *p* values have been indicated in bold.

Host trait	Time point		A1A2				A1B1				B1B2			
			Sum Sq	Df	F-value	Pr (>F)	Sum Sq	Df	F-value	Pr (>F)	Sum Sq	Df	F-value	Pr (>F)
Survival														
Overall analysis: Main and interaction effects														
	Day 8	Inoculation	1664	2	1.710	0.222	104	2	0.073	0.9294	1664	2	4.320	**0.0386**
		Genotype	1089	1	2.238	0.160	121	1	0.171	0.6857	484	1	2.512	0.1389
		Inoc: Genotype	1378	2	1.416	0.280	953	2	0.677	0.5264	242	2	0.628	0.5502
Targeted analysis: Genotype LRV4														
		Inoculation	2.486	2		0.2885	751	2	0.520	0.6187	1.925	2		0.3818
Targeted analysis: Genotype O1-08														
		Inoculation	1551	2	1.891	0.2307	306	2	0.223	0.8064	11.83	2		**0.0026**
	Day 12	Inoculation	138	2	0.271	0.766	1664	2	0.910	0.4283	242	2	0.2016	0.8201
		Genotype	0	1	0.000	1.000	2222	1	2.432	0.1448	484	1	0.8066	0.3868
		Inoc: Genotype	178	2	0.349	0.7120	993	2	0.543	0.5945	35	2	0.0288	0.9717
Targeted analysis: Genotype LRV4														
		Inoculation	277	2	1.75	0.2519	0.162	2		0.9218	1.879	2		0.3908
Targeted analysis: Genotype O1-08														
		Inoculation	40	2	0.046	0.9554	2054	2	1.434	0.3096	0.1885	2		0.9101
Body size														
	Day 8	Inoculation	0.036	2	1.862	0.197	0.079	2	2.151	0.1590	0.0166	2	0.345	0.7150
		Genotype	0.049	1	5.011	**0.0449**	0.252	1	13.71	**0.0030**	0.0352	1	1.467	0.2511
		Inoc: Genotype	0.028	2	1.441	0.2749	0.006	2	0.167	0.8475	0.0022	2	0.046	0.9545
Targeted analysis: Genotype LRV4														
		Inoculation	0.0008	2	0.134	0.8769	1.569	2		0.4562	4.813	2		0.0900
Targeted analysis: Genotype O1-08														
		Inoculation	0.063	2	1.931	0.2252	0.064	2	7.320	**0.0245**	0.4169	2		0.8118
	Day 12	Inoculation	0.199	2	1.626	0.2406	0.068	2	0.574	0.5792	0.004	2	0.034	0.9659
		Genotype	0.996	1	16.28	**0.0019**	1.504	1	25.23	**0.0003**	1.078	1	17.49	**0.0012**
		Inoc: Genotype	0.074	2	0.605	0.5629	0.025	2	0.211	0.8124	0.020	2	0.165	0.8497
Targeted analysis: Genotype LRV4														
		Inoculation	277	2	1.75	0.2519	0.088	2	1.562	0.2842	0.019	2	0.284	0.7623

Host trait	Time point		A1A2				A1B1				B1B2			
			Sum Sq	Df	F-value	Pr (>F)	Sum Sq	Df	F-value	Pr (>F)	Sum Sq	Df	F-value	Pr (>F)
Targeted analysis: Genotype O1-08														
		Inoculation	0.223	2	1.107	0.3998	0.005	2	0.027	0.973	0.0056	2	0.031	0.9696
Clutch size														
	Day 12	Inoculation	5.45	2	0.206	0.8185	15.75	2	0.679	0.5308	22.58	2	1.169	0.3463
		Genotype	63.88	1	4.827	0.0640	265.8	1	22.95	**0.0009**	111.6	1	11.56	**0.0059**
		Inoc: Genotype	6.02	2	0.227	0.8021	6.27	2	0.270	0.7689	6.71	2	0.347	0.7140
Targeted analysis: Genotype LRV4														
		Inoculation	8.20	2	0.590	0.6287	0.429	2		0.8069	6.001	2		**0.0497**
Targeted analysis: Genotype O1-08														
		Inoculation	1.134	2	0.036	0.9648	19.41	2	0.745	0.5306	1.708	2		0.4255
Fraction of mature animals														
	Day 12	Inoculation	0.584	2		0.7465	1.028	2		0.5981	0.123	2	1.302	0.3077
		Genotype	11.59	1		**0.0006**	51.59	1		**6.82e-13**	0.627	1	13.23	**0.0034**
		Inoc: Genotype	0.966	2		0.6166	0.415	2		0.8123	0.153	2	1.621	0.2381
Targeted analysis: Genotype LRV4														
		Inoculation	3.209	2		0.2009	0.083	2		0.657	72.21	2		**< 2.2e-16**
Targeted analysis: Genotype O1-08														
		Inoculation	0.005	2	0.024	0.976	0.019	2	0.523	0.617	0.058	2	0.437	0.6647

10. Comparison of Daphnia *life history traits between no-inoculum treatment, pairwise bacterial inoculations and natural microbiota*

To determine if the low diversity of the microbiome has a limited effect on life history traits compared to what can be achieved with more diverse, natural microbiomes, we here assess the full spectrum of life history variation, by including a comparison of life history trait values of *Daphnia* in the no-inoculum treatment, pairwise inoculation treatments and *Daphnia* with their natural microbiota in Figure S9. For trait values of *Daphnia* with their natural microbiota, we carried out an additional set-up where we have reared daphnids (of both genotypes) under the same experimental conditions of our study, however under non-sterile conditions. For this, eggs of both genotypes were isolated, with the sterilization step with 0.01% peracetic acid being skipped and allowed to grow at 20°C while being fed non-sterile *Acutodesmus obliquus*.

We note that this experiment was not carried out simultaneously with the other treatments, so any comparison we provide is for illustrative reasons only – to roughly indicate what the position is of animals that were only exposed to our experimental bacterial strains rather than to complete microbiota. As expected, the differences are substantial. The results are indeed in alignment with studies in literature (8–10) that indicate that *Daphnia* suffer from the lack of or low diversity of bacteria in their microbiome and exhibit sub-optimal performance.

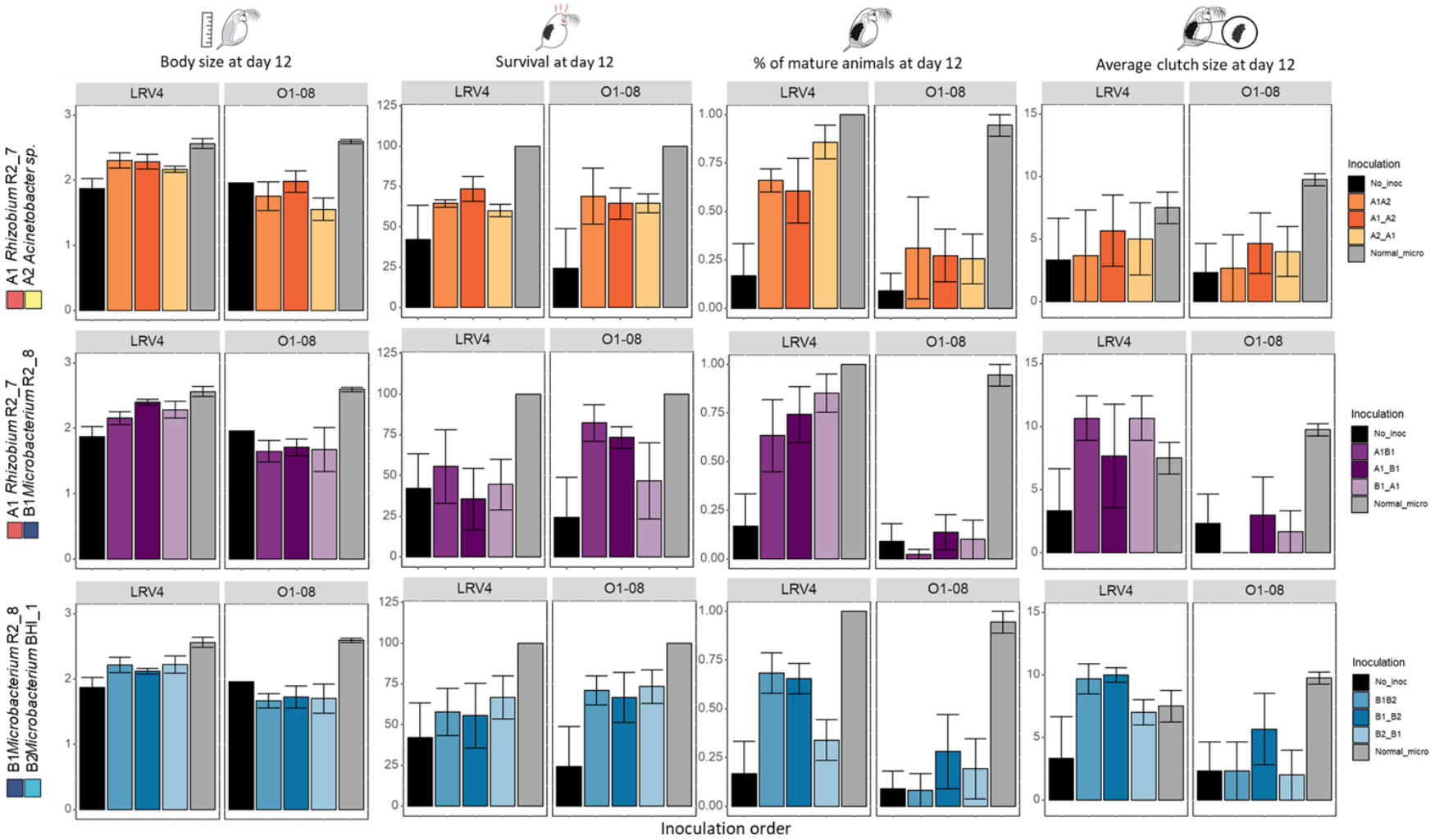

Figure S9: Comparison of life history traits between *Daphnia* of the no-inoculum treatment (black bars), all pairwise inoculations (colored bars) tested in the current study, and *Daphnia* grown without removing their microbiome (i.e., with a fully developed microbiome; gray bars). The data of the *Daphnia* with their complete microbiome were obtained in a separate experiment from those of the negative control and the inoculation order treatments. Additionally, due to the fatalities in the no-inoculum treatment, the number of remaining units fell below three. Hence, the data are here shown for illustrative purposes only, and no statistics were performed.

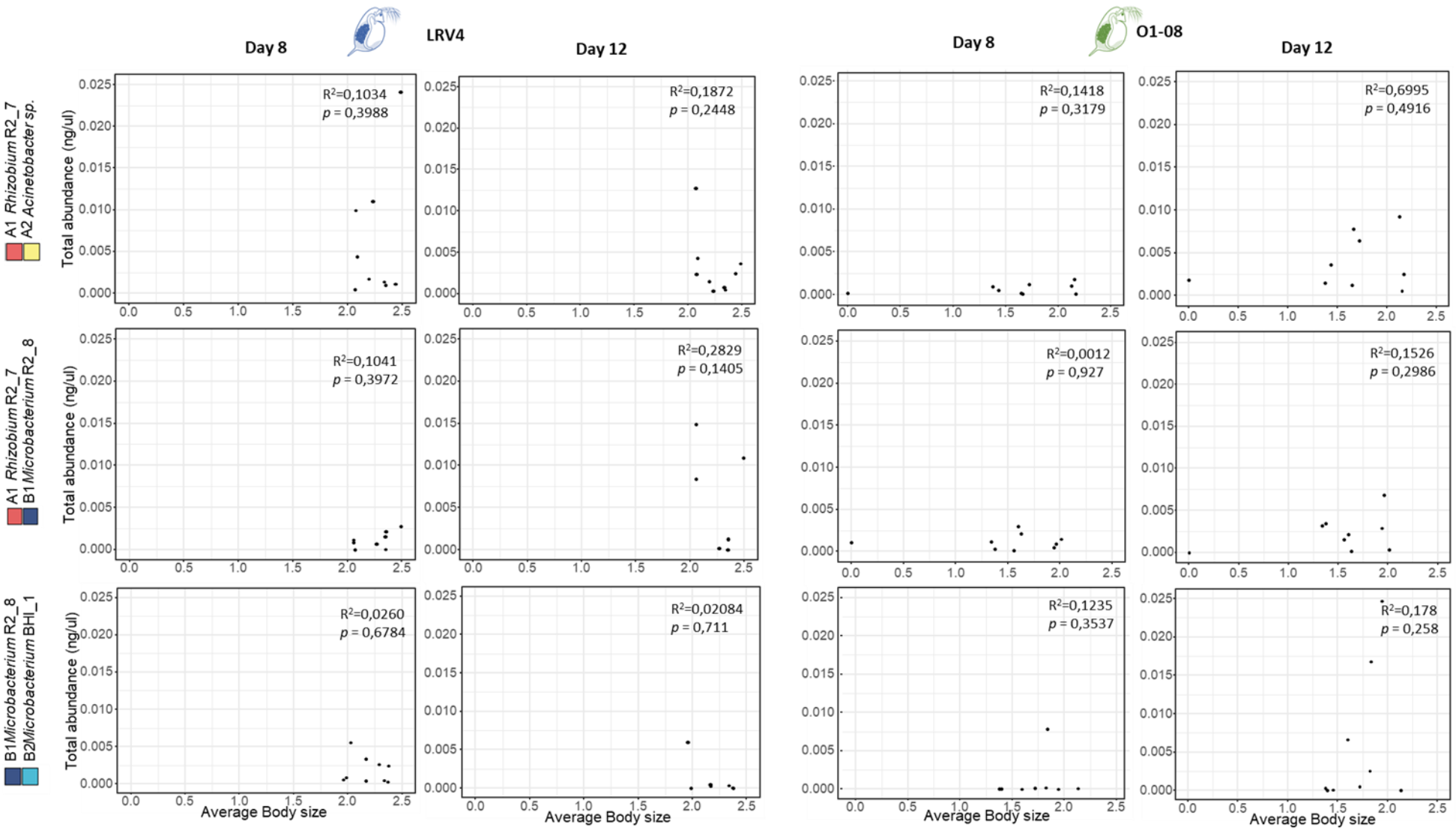

Figure S10: Comparison of total bacterial densities against average body size in *Daphnia* for the three bacterial strain pair combinations, in the two genotypes (LRV4 and O1-08) at two time points (Day 8 and Day 12). None of the relationships are significant.

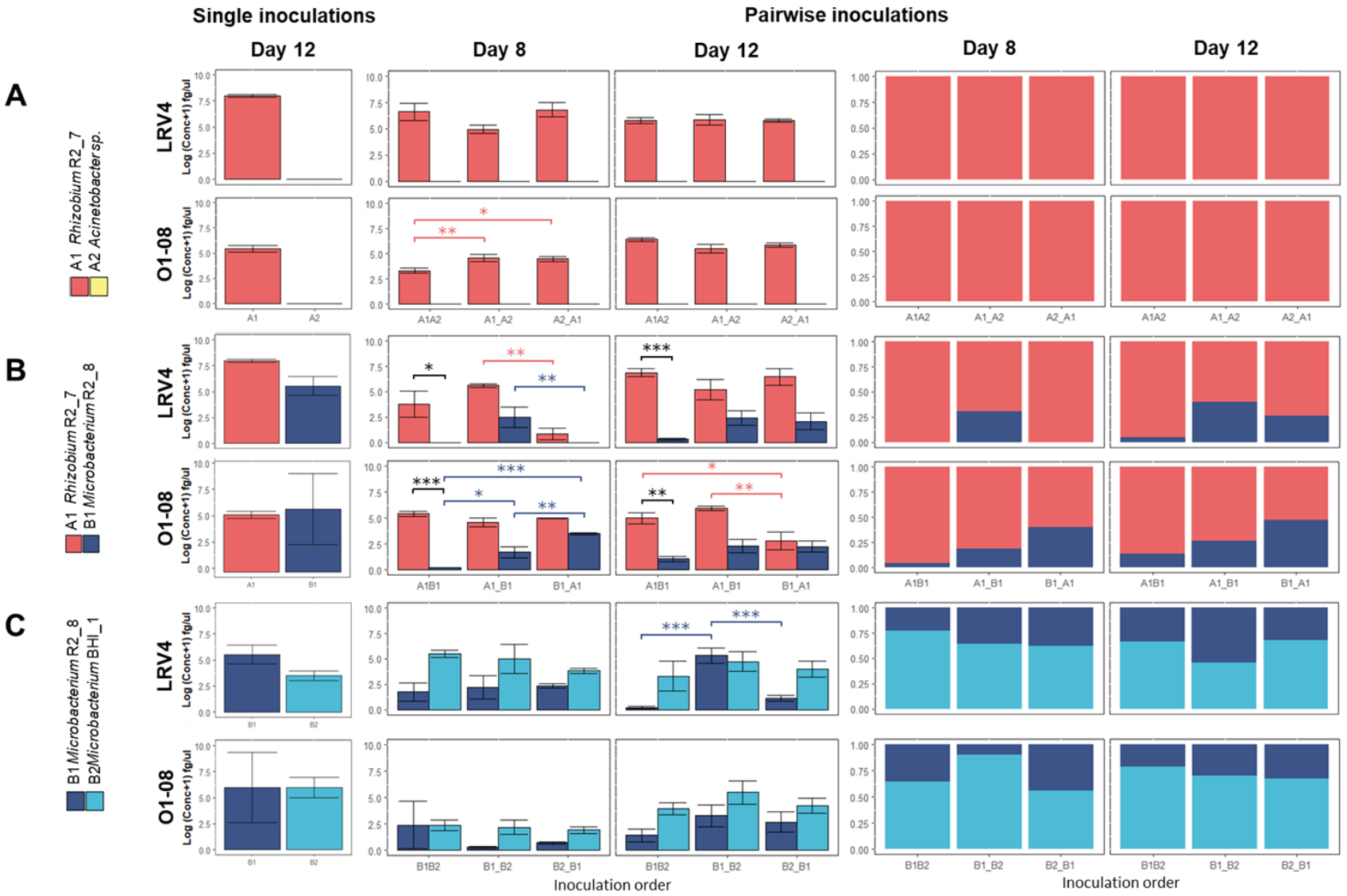

Figure S11: Comparison of absolute abundances and relative abundances of the bacterial strain pairs established in the gut.

References

1. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991 Jan;173(2):697–703.

2. Provasoli L, McLaughlin JJA, Droop MR. The development of artificial media for marine algae. Archiv Mikrobiol. 1957 Dec 1;25(4):392–428.

3. Provasoli L. Nutrition and Ecology of Protozoa and Algae. Annu Rev Microbiol. 1958;12(1):279–308.

4. Littler DS, Hellebust JA, America PS of, Littler MM, Craigie JS. Handbook of Phycological Methods: Culture methods and growth measurements, edited by J. R. Stein. Cambridge University Press; 1973. 536 p.

5. De Pauw N, De Leenheer L, Verlet H, Dochy M. Culturing of marine microscopic algae. ICES, Marie Comm F. 1979;54:185–95.

6. Klüttgen B, Dülmer U, Engels M, Ratte HT. ADaM, an artificial freshwater for the culture of zooplankton. Water Research. 1994 Mar 1;28(3):743–6.

7. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, et al. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. ISME J. 2016 Apr;10(4):911–20.

8. Sison-Mangus MP, Mushegian AA, Ebert D. Water fleas require microbiota for survival, growth and reproduction. ISME J. 2015 Jan;9(1):59–67.

9. Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. Betaproteobacteria Limnohabitans strains increase fecundity in the crustacean Daphnia magna: symbiotic relationship between major bacterioplankton and zooplankton in freshwater ecosystem. Environ Microbiol. 2016 Sep;18(8):2366–74.

10. Mushegian AA, Ebert D. Presence of microbiota reverses the relative performance of Daphnia on two experimental diets. Zoology. 2017 Dec 1;125:29–31.

CHAPTER 3:

Evolution mediated priority effects in the gut microbiome assembly of *Daphnia magna*

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Abstract

Given the fundamental role of the gut microbiome in host health, there has been extensive research focusing on the ecological aspects shaping gut community assembly and composition. Yet, the evolutionary responses that may contribute to community assembly of host-associated microbiomes are much less understood. This includes understanding how bacterial strains adapt over time to the gut environment within individual hosts and how these evolutionary processes impact strain fitness and influence gut community composition and structure. In this study, we investigated whether short-term adaptation of gut bacterial strains to a new host genotype affects their fitness and influences priority effects during gut community assembly. Bacterial strains of the genera *Rhizobium* and *Microbacterium* originally isolated from a specific genotype of the water flea *Daphnia magna* were experimentally evolved for 12 days to the gut environment of another host genotype of the same species to obtain pre-exposed (evolved) populations. The fitness of these pre-exposed populations was then evaluated in the guts of the two host genotypes and compared with that of the ancestral strains. Subsequently, the impact of pre-exposure on priority effects was assessed by manipulating the order of introduction of ancestral and pre-exposed populations in the gut of germ-free host animals in pairwise combinations. Our results show that the brief period of pre-exposure did not produce significant fitness improvements in the pre-exposed populations of both strains in their new host environment. For the pre-exposed *Microbacterium* population, however, a marked reduction in fitness was observed upon re-introduction into the original host genotype, indicating a rapid loss of ancestral adaptation to the original host genotype. Furthermore, manipulating inoculation order did not reveal a clear influence of evolution on priority effects. Instead, *Rhizobium* strains were observed to dominate and suppress the growth of *Microbacterium* regardless of pre-exposure status or arrival order.

Keywords: experimental evolution, evolution-mediated priority effects, *Daphnia magna, microbiome-host genotype co-adaptation*

Introduction

The gut microbiome with its diverse selection of micro-organisms is a dynamic ecosystem that plays an important role in host health and fitness. The composition of the gut microbiome has been shown to depend on factors such as host diet, genotype, age, environment and the use of antibiotics (1,2). Gut microbiota thus inhabit a complex environment that is shaped by interactions with their host and other members of the community (3). Given the fundamental role of the gut microbiome in host health, there has been extensive research focusing on the ecological aspects shaping gut community assembly and composition. Yet, the degree to which evolutionary processes shape gut microbiome assembly is much less understood. This encompasses the ways in which bacterial strains evolve to adapt to the gut environment over time and how these evolutionary processes function within individual hosts to influence strain fitness ultimately affecting gut microbiome assembly and composition. Given the inherent features of microbial populations such as large population sizes, short generation times, relatively high mutation rates and ability to undergo horizontal gene transfer, the idea that microbes undergo rapid evolution while in their host, gradually adapting to the specific features of the host is intuitive (4).

Vellend (2010, 2016) (5,6) emphasized four key processes that interact to shape ecological communities: dispersal, environmental sorting, drift and novelty, with environmental sorting encompassing both abiotic filtering and biotic interactions. In the initial stages of community assembly, the arrival order of species may have a profound influence on community assembly trajectories through priority effects (7). Priority effects are a form of historical contingency in community assembly, arising from differences in arrival times (linked to dispersal) and changes in environmental sorting caused by early colonizers and their descendants. In the case of inhibitory priority effects, early arriving species may reduce the success of late immigrants through resource consumption, by reducing the capacity of newly arriving species or genotypes to grow in density (niche pre-emption; (7)) or by altering the environment in ways that either favor their own growth or reduce growth of newly arriving species (niche modification, (7)). Priority effects can also be facilitative, for instance, if niche modification is negative for the resident community and promotes the establishment and growth of new colonists. Such ecological priority effects, are well documented in various biological settings (8).

Evolution can influence community assembly through multiple pathways, including dispersal (9–11), responses to the abiotic environment (12) and interactions with other species (13). As such, community assembly involves an interaction of ecological and evolutionary forces (14), emphasizing the need for an eco-evolutionary perspective to better understand dynamics in community composition. Evolution-mediated priority effects serve as one such example (15) where local genetic adaptation of the early colonists to the habitat may enhance their fitness and thus result in or strengthen priority effects by reducing establishment success of late arriving immigrants (15–17). For instance, Nadeau et al (2021) (18) experimentally demonstrated the impact of evolution on priority effects using two archaeal species, namely *H. volcanii* and *H. mediterranei.* Following 135 days of adaptation to high temperatures, early arriving populations showed improved performance in high temperature environments, suppressing the growth of late immigrants by 63% compared to non-adapted controls. Similarly, in a phyllosphere system, a 6 week experimental evolution of *P. dispersa* in tomato seedlings led to the evolution of this population allowing them to exploit a new niche on the plant. By expanding its niche, the evolved *P. dispersa* population demonstrated strong priority effects when introduced first into the plant, limiting the success of subsequent colonizers (19).

Given the evidence for evolution in the assembly of microbial communities in both free-living organisms and host-associated systems (20–22,17), it is likely that such effects also play an important role in gut microbiome assembly, with potentially significant implications for the host (23). Increasing evidence further suggests that microbial populations undergo local adaptation within the host gut (24–26). For instance, previous studies in murine models and humans have reported the occurrence of within-host evolutionary changes in gut communities spanning from fluctuations in single nucleotide variants (SNV) frequencies to horizontal gene transfers over time scales of years, months, weeks and even a few days (27–30). Likewise, the potential for gut microbial populations to undergo rapid evolutionary change in the gut environment and thus influence host adaptive potential is increasingly being explored (28,31,32). Local adaptation to the host individual might be particularly relevant during the early life stages of the host, when the gut environment represents a relatively open and unoccupied habitat that is still open to colonization events. Although recent studies have begun to bring to light the importance of priority effects in gut community assembly (33–35), the role of microbial evolution in shaping these dynamics remains, however, relatively understudied. The extent to which the variability in gut microbiome composition among host individuals is driven by initial colonization events versus subsequent eco-evolutionary processes, such as in situ bacterial adaptation, selective survival of the species in the host and competition with immigrant and potentially better adapted strains remains an open question (36).

In recent years, water fleas of the genus *Daphnia* has gained traction as a model system for the study of host–microbiome interactions. This is largely due to their short life cycle, ease of laboratory cultivation and the feasibility of performing gut microbiome transplants. Additionally, *Daphnia* can be maintained as clonal lineages, which enables the standardization of host genetic background (37,38). The use of genetically identical clones thus facilitates controlled experimental designs that allow to quantify the relative contribution of host genotype, environmental microbial sources and environmental conditions on community assembly of the gut microbiome (39) and assess microbiome-mediated trait change across different host genotypes (40). Beyond these advantages, *Daphnia* offers unique advantages for experimentally manipulating gut microbiome assembly. Their eggs can be sterilized to generate germ-free animals (41), which mimic empty habitat patches ideal for targeted microbial manipulation of the (gut) microbiome composition. Furthermore, *Daphnia* are generalist non-selective filter feeders that readily ingest bacteria from their environment (39), thereby enabling the controlled introduction of microbial strains into the gut, including a sequential exposure to different strains or communities, essential for the study of priority effects. Importantly, the gut microbiome has been shown to influence *Daphnia* fitness (42–44), further underscoring the relevance of *Daphnia* in microbiome research.

Building on these strengths, this study investigates whether bacterial strains can adapt to a new host genotype within the life cycle of a host individual and quantifies the extent to which this evolution contributes to priority effects in gut community assembly. To this end, we conducted an experimental evolution trial followed by a community assembly experiment in which we sequentially exposed germ-free *Daphnia* hosts to two strains with a time-lag, using both ancestral and evolved populations. Specifically, the two bacterial strains used in this study (*Rhizobium* R2_7 and *Microbacterium* R2_8, (35)) originally isolated from the gut of one *Daphnia* host genotype (LRV4) were allowed to grow and adapt for 12 days in the gut environment of a second *Daphnia* host genotype (O1-08) to allow for within-host adaptation. We then quantified the growth of the ancestral and evolved populations of both strains in the two host genotypes, inoculating them as monocultures. In a second follow-up experiment, we quantified community composition in settings in which we exposed the new *Daphnia* genotype (O1-08) to the two strains with a time lag, testing for priority effects. By carrying out this experiment with both ancestral and evolved populations of both strains, we also assess whether evolution influences ecological priority effects thereby testing for the occurrence of evolution-mediated priority effects in gut community assembly.

Materials and methods

Selection of bacterial strains

The bacterial strains used in the experiment were originally isolated from the gut of *Daphnia magna* genotype LRV4, isolated from Langerodevijver pond (LRV, Huldenberg, Belgium) in June 2018. The selection of strain pairs for this study was based primarily on results of a previous experiment (35), where strong priority effects were observed for this particular bacterial strain pair combination. These strains included *Rhizobium* R2_7 and *Microbacterium* R2_8.

Daphnia culture and rearing

Two clones of *Daphnia magna* were used in this study, namely clone LRV4, sampled from Langerodevijver pond in Huldenberg, Belgium and clone O1-08, sampled from a pond near Wulfhoek, Nevele, Belgium. Clone LRV4 was the original host genotype from which the bacterial strains were isolated while clone O1-08 was derived from a pond distinct from both clone LRV4 and the bacterial strains. A separate clone was chosen for the experiment to allow the evolution of bacterial strains to a novel gut environment.

Independent replicate clonal cultures were raised under controlled laboratory conditions for two generations to purge (grand)maternal effects. Four adult daphnids were maintained per 500 mL jar filled with aged and dechlorinated tap water at 20°C ± 1°C and a 14:10h L:D photoperiod. The water in the jar was refreshed every alternate day (20% old medium, 80% fresh water) and fed with 1 mgC/mL of the green algae *Acutodesmus obliquus*. Eggs of 12-16h from the second to the fourth clutch were collected for the generation of germ-free animals for the experiment.

Generation of germ-free animals

Eggs were dissected from gravid females using sterile dissection needles under the microscope SZX16 (Olympus, Japan) and subsequently treated with 0.01% peracetic acid following the protocol of Callens et al (41). The sterilized eggs were then transferred into sterile Petri dishes containing autoclaved tap water and sealed with parafilm. These Petri dishes were incubated at 20°C ± 1°C and a 14:10h L:D photoperiod for 48h to allow hatching. After 48h, the axenic

hatchlings were collected for the experiment. Axenity was assessed by plating whole animals, homogenized animals and the water from the Petri dish onto Reasoners 2A Agar (R2A) plates. For the whole animals, 4 individual hatchlings were directly plated onto solid R2A agar plates. For the homogenized animals, 4 individual hatchlings were transferred into 1.5 mL Eppendorf tubes containing 100 µL of 1X PBS. These animals were homogenized in the Eppendorf® tubes using sterile plastic micropestles and the homogenate was plated onto R2A agar. For the water samples, 100 µL of water from the Petri dish where the eggs were hatched was directly plated onto R2A solid agar. All plates were incubated at 24°C for 48h and monitored for visible colony growth.

The sterilization protocol was performed in batches to ensure a sufficient number of animals for the experiment. This resulted in a staggered design of the experiment, with inoculations conducted in batches, each batch corresponding to a replicate. To account for this, axenity was also verified separately for each batch of sterilization.

Overview of the experimental design

To investigate the role of evolution in shaping priority effects in the gut, a 12-day experimental evolution trial was first carried out to allow the evolution of both bacterial strains (*Rhizobium* R2_7 and *Microbacterium* R2_8) to the gut environment of *Daphnia* genotype O1-08. Next, we tested to what extent pre-exposure affects the growth of these bacterial strains in both *Daphnia* host genotypes (*Daphnia* genotype LRV4 and O1-08). In a second follow-up experiment, we tested for priority effects using both the ancestral and pre-exposed populations of the two bacterial strains. Specifically, the inoculation order of the bacterial strains (pre-exposed versus ancestral) into the gut was manipulated to test to what extent a period of 12 days of pre-exposure to the new host genotype influences the outcome of priority effects in the gut during colonization.

Experimental evolution trial

In the experimental evolution trail, the two strains (*Rhizobium* R2_7 and *Microbacterium* R2_8), originally isolated from *Daphnia* genotype LRV4 were allowed to grow and adapt individually to the local gut environment of *D. magna* genotype O1-08, for a total duration of 12 days, which corresponds to the generation time of *Daphnia*. For this set-up, six replicate units were maintained per strain, with each replicate housing 5 germ-free hatchlings in sterile 40 mL culture flasks, filled with autoclaved, aged and dechlorinated tap water (Figure 1). To obtain pre-exposed (evolved) strains, each replicate unit was inoculated with 100 µL of the bacterial inoculum (either *Rhizobium* R2_7 or *Microbacterium* R2_8) at an optical density (OD) of 0.7. This OD was selected based on a previous measurement of bacterial densities in multiple ponds, to mimic the bacterial concentrations in natural waters. Two units with no bacterial inoculum were also maintained as negative controls. Each unit was fed with live sterile green algae, *Acutodesmus obliquus* (obtained from CCAP 276/3A) at a concentration of 1mgC/mL. Following 48h of incubation, the daphnids were transferred into 500 mL jars filled with 450 mL autoclaved, aged and dechlorinated tap water and fed with sterile live algae at a concentration of 1mgC/L. These units were incubated at 20°C ± 1°C and a 14:10h L:D photoperiod for a total duration of 12 days. After 12 days of incubation, the guts from daphnids were dissected and pooled together per replicate unit. The guts were homogenized using a sterile micro pestle to allow the breakdown of gut cells and the release of bacteria. The gut homogenate was then plated on R2A agar plates to allow the re-isolation of the inoculated bacterial strains. From the visible colony growth obtained, 10 random colonies were picked up from each plate to create a pre-exposed population (further called 'evolved'). The evolved population thus comprised a combination of 10 strains per replicate to reduce chance effects of picking a colony that would deviate from the mean in growth rate.

Comparing population growth of evolved versus ancestral populations

To assess whether the pre-exposed populations had evolved in ways that affected their establishment success and growth in the gut in comparison to the ancestral strains, we exposed germ-free neonates of the two host genotypes to monocultures of either the ancestral or evolved populations of *Rhizobium* and *Microbacterium* and assessed their densities in the gut at day 14. While a competition experiment would be an ideal way to test relative fitness of the ancestral and evolved populations, we did not have markers to differentiate these two populations in a mixed sample. Each experimental unit consisted of 5 germ-free *Daphnia* hatchlings in sterile 40 mL culture flasks filled with autoclaved, aged and dechlorinated tap water. Each flask was inoculated with 100µL of either the ancestral or the pre-exposed population at an OD of 0.7. For each bacterial inoculum treatment, 6 replicates were maintained, resulting in an experiment consisting of 2 strains × 2 populations (ancestral and evolved) x 2 host genotypes x 6 replicates = 48 units). Each unit was fed with sterile live algae at a concentration of 1mgC/mL and maintained at 20°C ± 1°C and a 14:10h L:D photoperiod. The position of the flasks was randomized daily to prevent micro-climatic effects. Following 48h of

incubation, the water and the *Daphnia* from the 40 mL culture flasks were transferred into 720 L jars containing 650 mL of fresh autoclaved aged dechlorinated tap water and they were fed daily with 1mgC/mL of algae. After 14 days of incubation, the animals from each flask were isolated and dissected to extract the guts. The guts were pooled for each experimental unit and used to extract DNA for strain quantification in the gut.

Quantifying the role of evolution in mediating priority effects in gut community assembly

To quantify whether evolution plays a role in mediating priority effects in gut community assembly, a follow-up experiment was performed, in which the inoculation order of the two strains in the gut of *D. magna* was manipulated, using both ancestral and evolved populations. The inoculation orders mainly comprised (1) single inoculations, wherein single strains were inoculated in isolation to understand how the strain performed in the gut in the absence of competition (2) simultaneous inoculations, wherein both strains were introduced simultaneously into the gut at equal inoculum densities to assess competitive ability and relative fitness in the gut environment of the two host genotypes; (3) a time-lag inoculation, in which one strain was introduced first, followed by the introduction of a second strain after a time-lag of 48h; and (4) a reciprocal time-lag inoculation, wherein the order of the time-lag was reversed, i.e., the strain that was added first earlier was now added second. A negative control treatment was also set up in parallel where germ-free host individuals were not exposed to any bacterial strains (Figure 2). In the simultaneous and time-lag treatments, we always worked with pairwise combinations of strains that involved one *Rhizobium* and one *Microbacterium* population, as we have probes that allow us to differentiate between the two strains to reconstruct community composition via qPCR (see further). To assess whether evolution affects community assembly and priority effects, we combined ancestral *Rhizobium* and ancestral *Microbacterium*, evolved *Rhizobium* and evolved *Microbacterium*, and the two combinations of one ancestral and one evolved population. The experimental set-up was generally similar to that during the experimental evolution trial, comprising 6 replicate units per treatment each with 10 germ-free *Daphnia* hatchlings in 40 mL jars for bacterial inoculation, following their transfer to 720 mL jars after 96 hours. Unlike the experimental evolution trial, we here used autoclaved green algae (*Acutodesmus obliquus*) at a concentration of 2mgC/mL (a higher concentration to compensate for the lack of live cells). Culture units were maintained at 20°C ± 1°C and a 14:10h L:D photoperiod. The positions of the experimental units was randomized daily to account for micro-climatic effects.

The bacterial strains used for inoculation were grown in 40 mL of sterile R2A broth at 20°C for 72h. The culture was pelleted down by centrifugation at 4000 rpm for 15 minutes. The pellet so obtained was resuspended in sterile PBS to obtain the desired optical densities. A total optical density of 0.7 was standardized as total inoculation dose per treatment. The OD measurements were carried out in 96-well plates using a multiplate microplate reader (BioRad). The second exposure in the (reciprocal) time-lag inoculations was performed 48h after the first inoculation. Gut extractions were performed on day 14, for which the guts dissected from all animals within a replicate were pooled and stored in 100 µL of 1X PBS for DNA extraction.

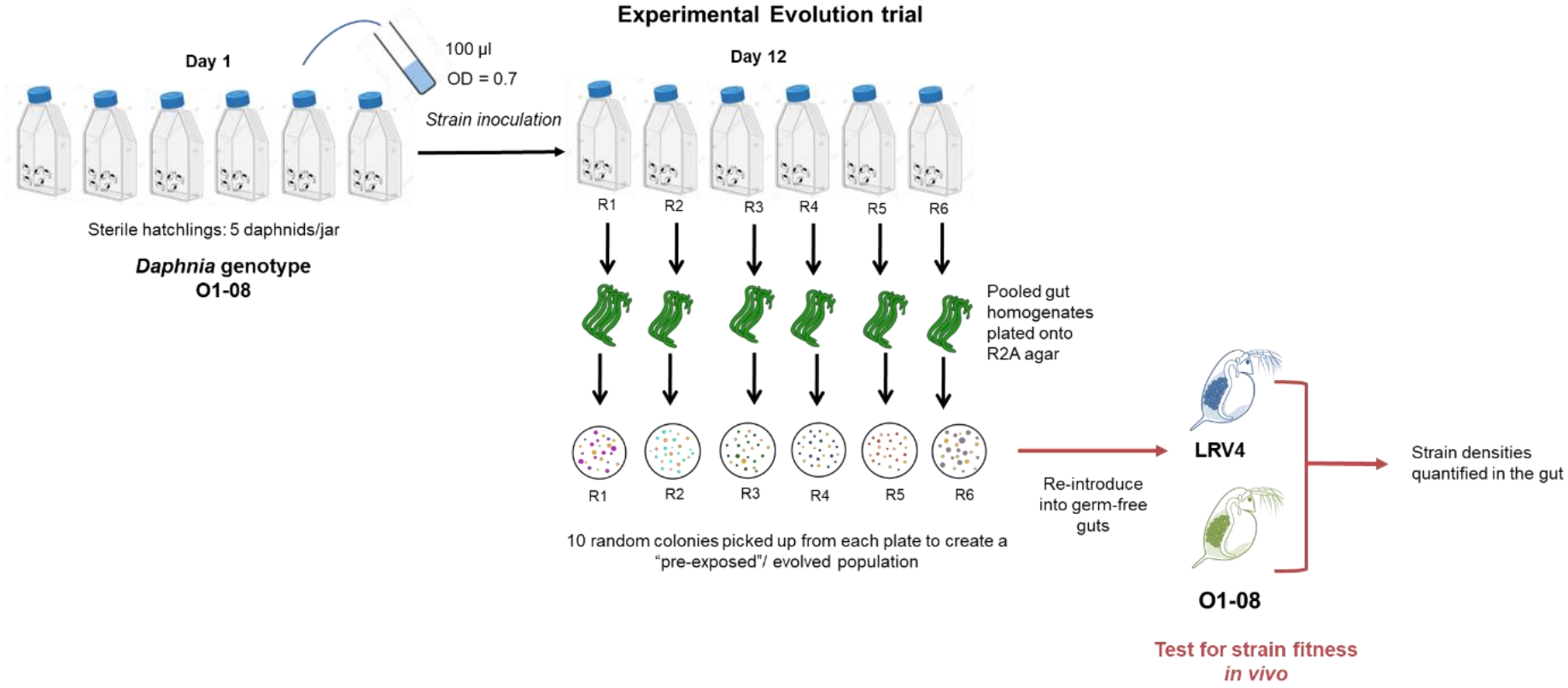

Figure 1: Schematic representation of experimental evolution trial and quantification of growth rates of evolved and ancestral strains. Ancestral strains of *Rhizobium* and *Microbacterium* were inoculated separately into the guts of germ-free *Daphnia magna* genotype O1-08 at an OD of 0.7 and allowed to grow and adapt to the gut environment for 12 days. Following 12 days of inoculation, the guts of the animals were dissected, pooled together, homogenized and plated onto R2A agar. From the visible colony growth obtained, 10 random colonies were picked up per replicate unit to create six independent pre-exposed populations used in the follow-up experiment. To compare fitness effects between ancestral strains and pre-exposed (evolved) isolates, these strains were re-introduced into the germ-free guts of their original and new host genotype and their densities in the gut quantified using strain-specific qPCR assay.

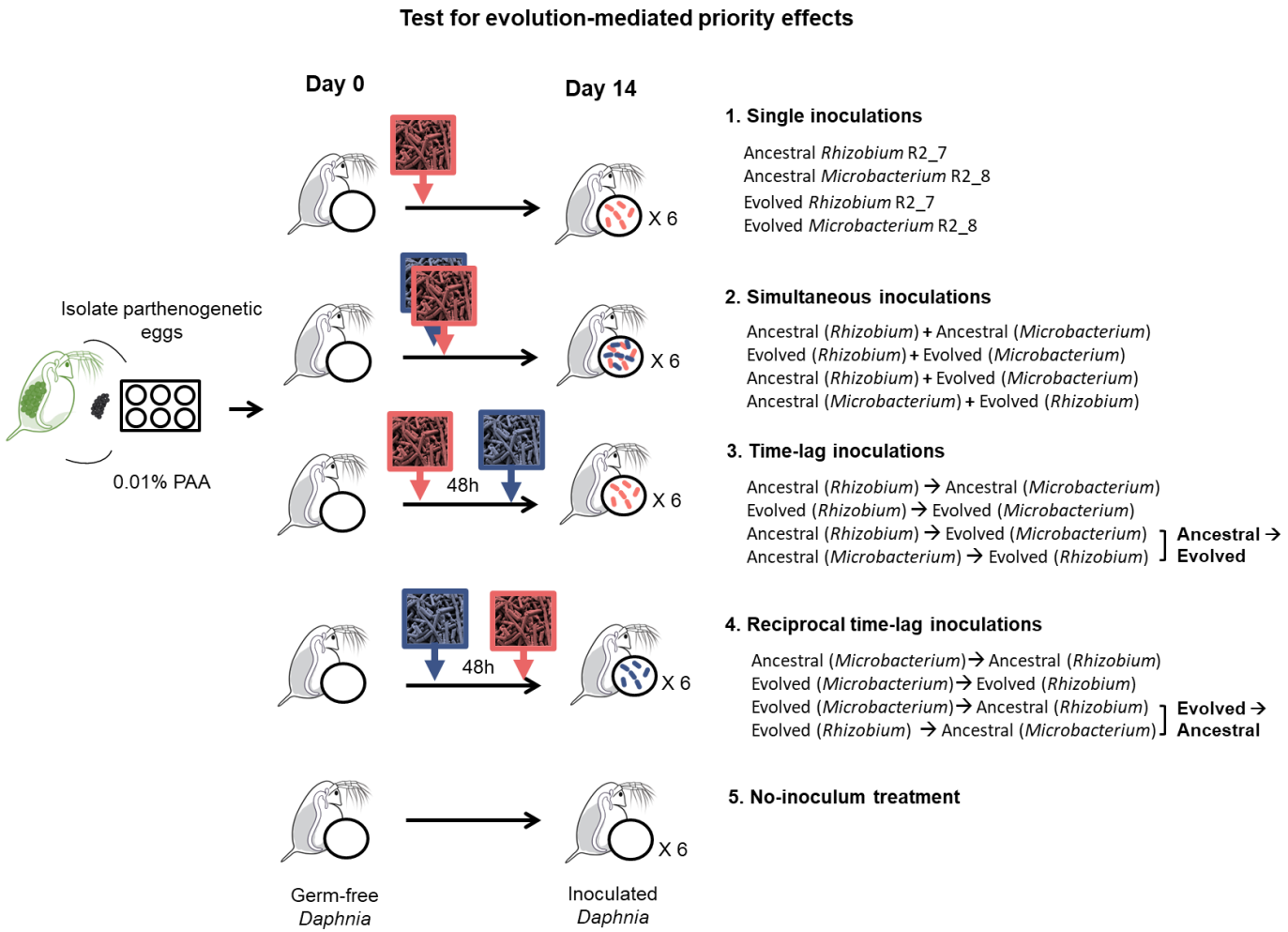

Figure 2: Schematic representation of the priority effects experiment wherein the inoculation order of ancestral and pre-exposed (evolved) strains into the guts of germ-free animals was manipulated. Sterile germ-free animals were generated by sterilizing parthenogenetic eggs with 0.01% peracetic acid. The sterile hatchlings so obtained were then inoculated with the bacterial strains in the following orders: single inoculations (to test for performance of each strain separately, quantifying evolution) and simultaneous, time-lag and reciprocal time-lag inoculations (to test competitive interactions and priority effects). Six replicate units were maintained per inoculation order treatment. A no-inoculum treatment, wherein no bacterial inoculum was added was maintained as negative control.

DNA extraction and strain quantification using qPCR

The gut homogenate prepared from the pooled gut samples per replicate treatment was used for DNA extraction. DNA extraction was performed using the NucleoSpin Tissue mini spin kit (Macherey-Nagel, Germany). For integrity check, the extracted DNA was run on 1% agarose gel and visualized using Midori Green Advance DNA Stain (Nippon Genetics Co., Japan). The DNA concentration was determined using the Quant-iT™ Picogreen™ dsDNA Assay (Invitrogen, USA) and a microplate reader (Infinite® M Nano +, Tecan). For bacterial quantification in the gut, a strain-specific qPCR assay was performed. The DNA concentration of all samples were standardized to 1 ng/µL to ensure uniform template input across reactions. The primers used for quantification comprised the 16S forward primer F27 (45) (Biolegio, The Netherlands) and a customized reverse primer R206 (Biolegio, The Netherlands), designed to maintain an amplicon size of 200bp to ensure high amplification efficiency (35). Likewise the probes consisted of custom probes designed specifically for the detection of the two strains (35).

Quantification of the strains in the gut was conducted employing the CFX96 Touch Real-Time PCR detection system (BioRad Laboratories NV) and CFX Maestro™ software. A singleplex reaction was performed independently for *Rhizobium* and *Microbacterium* for the single inoculations. To enable accurate quantification, a ten fold dilution series for each target strain was prepared to generate standard curves for absolute quantification. For the standard curves, 10 µL of the sample was diluted in 90 µL of PBS. The dilution was performed up to a 1:1000000 dilution. This comprised a total reaction mixture of 20 µL comprising 10 µL PrimeTime® Gene Expression master mix (Integrated DNA technologies, Leuven), 0.5 µL of each primer primer (20 µM F27/R206), 1 µL of probe (3 µM; PrimeTime® custom qPCR probes, IDT Leuven) and 1 µL of template DNA. The remaining volume was made up with ultra-pure distilled water (Qiagen, Hilden, Germany). The same reaction mix and conditions were applied for quantifying the unknown treatment samples to ensure comparability with the standard curves. For quantification of the pairwise inoculations, a multiplex reaction was set up. Unlike the singleplex reaction, two strains were quantified in the samples using a 20 µL reaction mixture comprising 10 µl PrimeTime® Gene Expression master mix (Integrated DNA technologies, Leuven), 0.5 µL primer (20µM F27/R206), 1 µL each of the two strain probes (3µM; PrimeTime custom qPCR probes, IDT Leuven) and 2 µl of template. Ultra-pure distilled water (Qiagen, Hilden, Germany) 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 ultra-pure distilled water as template. For both set-ups, an initial denaturation step at 95°C was maintained for 3 minutes followed by 40 cycles of denaturation and a combined annealing and extension step at 95°C (15 seconds) and 61°C (30 seconds), respectively. Concentrations of the strains in the gut were calculated from the obtained C_q values.

Given the large number of samples distributed across multiple qPCR runs, an inter-run calibration was conducted to account for variability among runs using a 'gene-maximization' set-up (qbase+, (35)). Strain concentrations were determined based on the C_q value obtained from the qPCR assay. For each treatment, the mean C_q values from three technical replicates was calculated and used to estimate 16S rRNA concentrations as a proxy for bacterial cell densities.

Statistical analysis

To understand differences in fitness between the ancestral strain and the pre-exposed (evolved) population, the densities of the ancestral strains were compared with that of the pre-exposed population in the gut. A linear mixed-effects model was constructed with the *lmer* function of the *lme4* package (46) with replicate number added as a random factor for each strain separately (*Rhizobium* and *Microbacterium*). For models with singular random effects (near zero variance), the replicate was removed as random factor and fixed-effects linear models were used instead. Significant main effects of adaptation status, genotype and their interaction effects were tested with Type III sum of squares using the *Anova* function of the *car* package (47). Tukey correction was performed for pairwise comparisons using the *emmeans* package in R for significant interactions (48).

Likewise for the pairwise inoculations, the main effect of inoculation order was tested with a linear mixed-effects model integrating the replicate number as the random factor. For singular models, the random factor was removed and instead a fixed-effects linear model was used. Significance was tested with Type II sum of squares

using the *Anova* function of the *car* package. Similar to the previous model, significant pairwise differences between inoculation order treatments was determined using the *emmeans* package in R.

All statistical analyses were performed in R version 4.0.2.

Results

Densities of ancestral strains vs evolved populations in host guts

For *Rhizobium* strain R2_7, the densities of the evolved populations in the host guts at day 14, were not significantly different from that of the ancestral population, both in the original host genotype (LRV4) and the new genotype (O1-08). Additionally, no host genotype effect was observed. Both the ancestral strains and the evolved population displayed similar densities in both host genotypes (Figure 3A).

For *Microbacterium* R2_8 strain, the pattern was different in the new genotype in comparison to the original host genotype (Figure 3B). The evolved population did not significantly differ in densities from the ancestral strain in the new host genotype (O1-08). However, when the evolved population was introduced back into its original host genotype (LRV4), it showed a substantial fitness loss compared to the ancestral strain, with a significant reduction in densities at day 14. All evolved populations of *Microbacterium* R2_8 exhibited a consistent reduction in densities across replicates when introduced into their original host genotype (Figure 3D).

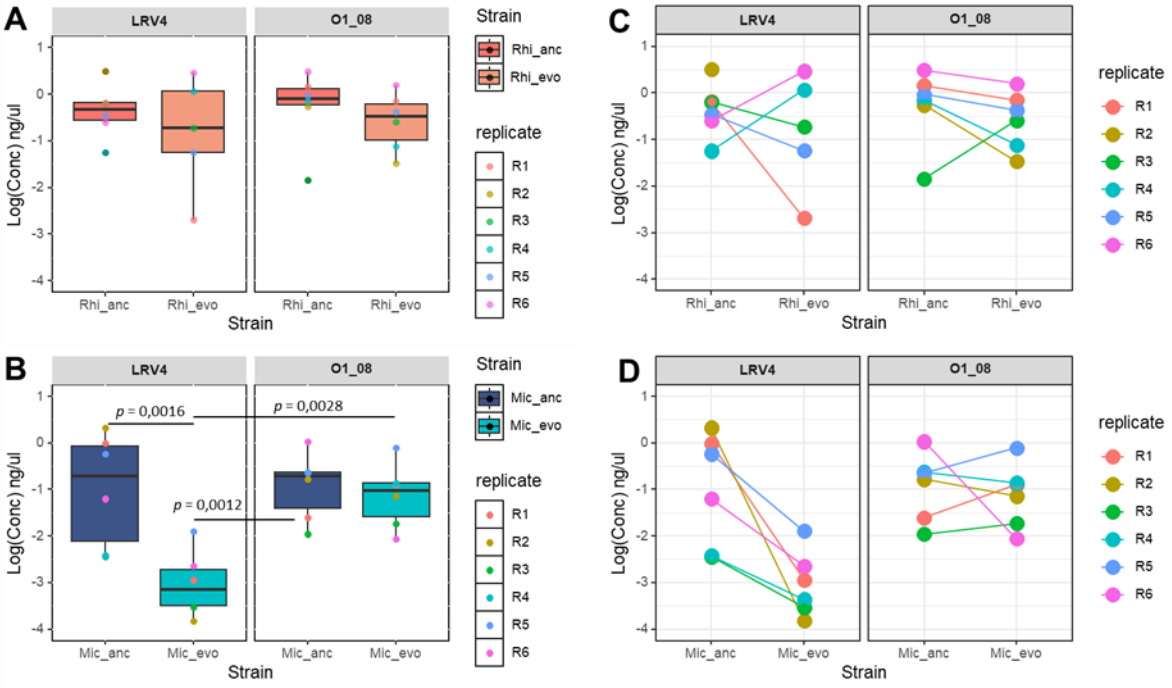

Figure 3: Median strain densities of ancestral ("anc") and evolved ("evo") populations for (A) *Rhizobium* R2_8 and (B) *Microbacterium* R2_8 *in vivo* in the guts of the original (LRV4) and new host genotype (O1-08). Reaction norms depict the densities of *Rhizobium* R2_7 (C) and *Microbacterium* R2_8 (D) in each replicate, which consisted of an independent set of 10 colony isolates from the microbiome homogenate of experimental animals at the end of the evolution trial.

Table 2: Results of type III ANOVA assessing the effect of pre-exposure to a new host genotype, O1-08; (ancestral versus evolved population), host genotype and their interaction effects on bacterial densities of the two strains (*Rhizobium* R2_7 and *Microbacterium* R2_8) in the gut when grown in single-species inoculations. Linear mixed-effects models with replicate as random factor were employed, where the variance from random factors could be estimated. For models with singular random effects (near zero variance), replicate was removed as random factor and fixed-effects linear models were used instead. Test statistics are reported as Chi Sq for mixed-effects models and F-statistics for linear models. Significant effects are indicated in bold.

Type	**Test effect**	***Rhizobium* R2_8**				***Microbacterium* R2_8**			
		Sum Sq	**Df**	**F-value**	**Pr**	**Chi Sq**	**Df**	**F-value**	**Pr**
Single inoculations	Pre-exposure	0.047	1	0.0576	0.8128	21.739	1	NA	**3.123e-06 *****
	Genotype	0.024	1	0.0304	0.8634	0.0225	1	NA	0.880783
	Pre-exposure: Genotype	0.052	1	0.0640	0.8029	8.8374	1	NA	**0.002951 ****
	Residuals	16.400	20	NA	NA	NA	NA	NA	NA

Test for evolution-mediated priority effects

In all single species inoculation treatments, it was observed that *Rhizobium* R2_7 consistently displayed higher densities in the gut in contrast to *Microbacterium* R2_8, irrespective of the adaptation status (Figure 4).

Overall, neither the strain adaptation status nor the inoculation order had a significant effect on strain densities in the gut of host genotype O1-08. Instead, across all treatments, *Rhizobium* R2_7 consistently achieved higher densities than *Microbacterium* R2_8, and no significant pairwise differences between inoculation orders were noted. In the ancestral-ancestral setting, inoculation order is near significant (p = 0.055).

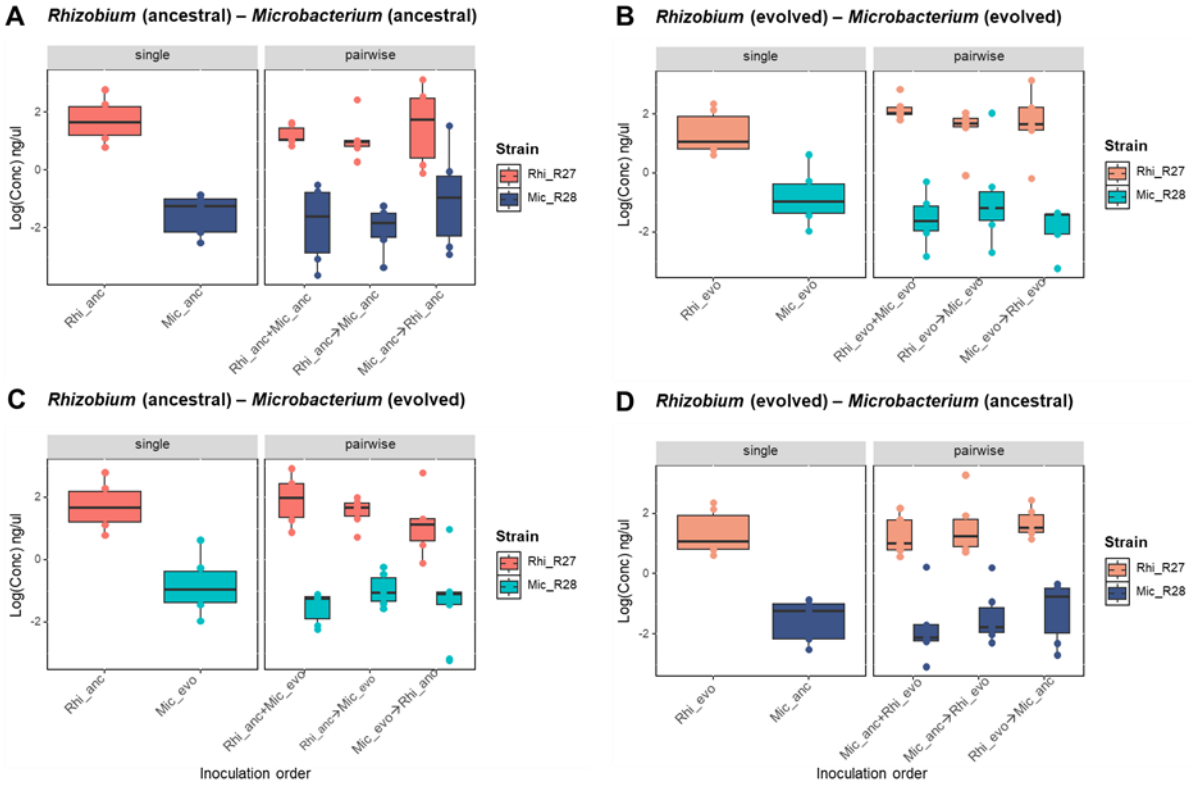

Figure 4: Mean bacterial strain densities in the gut of *D. magna* genotype O1-08 plotted against inoculation order treatments (X anc/ X evo: single, X1 + X2 = simultaneous, X1→X2: time-lag and X2→X1: reciprocal time-lag inoculations) for (A) both ancestral combinations: *Rhizobium* R2_7 (ancestral) and *Microbacterium* R2_8 (ancestral); (B) both evolved combinations: *Rhizobium* R2_7 (evolved) and *Microbacterium* R2_8 (evolved); (C) *Rhizobium* R2_7 (ancestral) and *Microbacterium* R2_8 (evolved); and (D) *Rhizobium* R2_7 (evolved) and *Microbacterium* R2_8 (ancestral) combination. Absolute strain concentrations in the single inoculations (left panel) and absolute strain concentrations in pairwise inoculations (right panel).

Table 3: Results of type II ANOVA assessing the effect of inoculation order (simultaneous, time-lag and reciprocal time-lag) on densities of *Rhizobium* R2_7 and *Microbacterium* R2_8 in the gut in the pairwise inoculations. Linear mixed-effects models with replicate as random factor were employed, in which the variance from random factors could be estimated. For models with singular random effects (near zero variance), the replicate was removed as random factor and fixed-effects linear models were used instead. Test statistics are reported as Chi Sq for mixed-effects models and F-statistics for linear models. Strain pairs represent the bacterial combinations, namely ancestral-ancestral, evolved-evolved and two combinations of ancestral-evolved.

Bacterial combination	**Strain pair**	**Main effect**	***Rhizobium* R2_8**				***Microbacterium* R2_8**			
			Chi Sq	**Df**	**F-value**	**Pr**	**Chi Sq**	**Df**	**F-value**	**Pr**
Ancestral-ancesral	*Rhi*_anc – *Mic*_anc	Inoculation order	0.869	2	NA	0.647	5.783	2	NA	0.055
Evolved-evolved	*Rhi*_evo – *Mic*_evo	Inoculation order	4.435	2	NA	0.108	3.041 (Sum Sq)	2	1.110	0.356
Ancestral-evolved	*Rhi*_anc – *Mic*_evo	Inoculation order	1.888 (Sum Sq)	2	1.587	0.236				
Ancestral-evolved	*Rhi*_evo – *Mic*_anc	Inoculation order	1.137	2	NA	0.566	1.090	2	NA	0.579

Discussion

In this study, we aimed to explore the role of within-host bacterial evolution in fostering growth rates and in mediating priority effects during gut microbiome assembly. We first performed an experimental evolution trial, allowing two bacterial strains originally isolated from the gut of *D. magna* genotype LRV4 to adapt for 12 days to the gut environment of a new host genotype (*D. magna* genotype O1-08), to obtain evolved populations. The ancestral and evolved population were then used in two experiments: single-strain inoculations to compare growth rate changes between ancestral and evolved populations and a priority effects experiment where the inoculation order of the two strains into the host gut was manipulated using both ancestral and evolved populations. Our results reveal that the 12-day period of within-host evolution was insufficient to elicit significant improvements in strain fitness to the gut environment of the new host genotype. We do, however, observe a rapid loss of adaptation to the original host genotype in the evolved population of *Microbacterium* R2_8. This suggests that, when grown in another host genotype, the strain rapidly lost traits that are relevant for growth in its original host genotype. Manipulating the inoculation order of the two strains in the gut using both ancestral and evolved populations did not yield detectable changes in priority effects in gut community assembly. Regardless of the selection history of the populations or inoculation order, *Rhizobium* consistently achieved higher densities in the gut compared to *Microbacterium*, which is in line with the higher population densities that they also reached in single species inoculations.

Historical adaptation of commensal bacteria to their host environments has been documented (49,50) implying that evolutionary interactions have significantly contributed to shaping and maintaining host-specific microbial associations (30,31). Although the impact of these long-term co-evolutionary processes are well recognized (30), recent studies now indicate that gut commensals are also capable of undergoing evolutionary change

within time-scales relevant to a single host's lifespan. For instance, a genetic adaptation in the *lrp* gene, which encodes a global regulator of amino acid metabolism and enhances *E. coli's* competitive ability for amino acids, was repeatedly selected within two weeks after mono-colonization in germ-free mice (51). Likewise, Dapa T et al (2022) (3) demonstrated rapid evolutionary changes in *B. thetaiotaomicron* within the mouse gut in response to a Western style diet that promoted their consumption of mucus-derived host glycans (52). In humans, native gut bacterial populations have been shown to accumulate changes in both community composition and gene frequencies over a 6 month time scale through a combination of within-host evolution and replacement by distantly related strains (53). Additionally, experimental evolution trials conducted in controlled settings have provided direct evidence for rapid microbial adaptation to host-gut like conditions. For instance, Zhao et al (2019) demonstrated how *E. coli* populations quickly evolved enhanced colonization ability and competitive fitness within a synthetic gut environment over just a few dozen generations (28).

In our study, we implemented a 12-day evolution experiment to allow two *Daphnia* gut bacterial strains to adapt to the gut environment of a new *Daphnia* genotype. The adaptation period of 12 days was chosen as this corresponds to the generation time of *Daphnia*. While *Daphnia* can live for more than 100 days (54,55) it would be most compelling if we could detect significant effects of evolution within a single egg-to-egg generation, as this would highlight the relevance of microbial evolutionary dynamics within the host and their potential for transfer to offspring. Given an approximate generation time of six hours for these strains, this adaptation period thus corresponds to roughly 48 generations. We observed no significant improvement in fitness of the evolved populations compared to their ancestral counterparts. This may reflect that the time period during which we allowed the strain to grow in the new environment was too short to allow substantial adaptation. It may also be that adaptation did occur but did not translate in enhanced population growth rates. Previous work has shown that adaptation of bacterial strains in the host can indeed occur over short time scales, often leading to subtle but measurable fitness advantages such as antibiotic resistance, tolerance, immune evasion and metabolic adaptations (28,56–58). It is possible that the magnitude of fitness gains in our study were subtle, as also stated by Dapa et al 2024, who report typically less than 10% change in growth rate by physiological standards (3). To more fully capture such fine scale evolutionary shifts, future studies should allow for longer adaptation periods and supplement the analyses with whole-genome sequencing to compare ancestral strains to evolved isolates to identify potential mutations in the genome associated with key physiological and metabolic adaptations to the new gut environment.

Our finding that *Microbacterium* R2_8 strain exhibited a marked decline in fitness when re-introduced into its original host genotype after evolution in a new host suggests that even short term within-host evolution can lead to loss of traits essential for survival in previous environments. This pattern aligns with the principle of antagonistic pleiotropy, where adaptations beneficial in one environment can compromise fitness in another environment (59–61). Or the loss of fitness may simply reflect a mere absence of selection pressures by the original host genotype. In both cases, our results strongly indicate that different genotypes of the same host species can exert differential selection pressures on microbial strains in the gut microbiome. In other words, even though we did not detect adaptation to the new genotype, the loss of adaptation to the ancestral genotype provides evidence that such genotype-specific adaptation is likely important. These findings highlight the need for longer exposure times in experimental evolution trials in future studies, for instance through serial transfers and across multiple generations. The rapid loss of adaptation also suggests a cost of adaptation when confronted with a new environment. Similar patterns have been documented in other studies involving experimental evolution trials, where microbial strains adapted to specific host genotypes or conditions exhibit reduced versatility or colonization efficiency in alternate environments (24,62). For instance, the acquisition of antibiotic resistance in bacterial populations have often been associated with a fitness cost in antibiotic-free environments often due to the genetic burden essential for resistance (63,64). Likewise, the evolution of social amoeba, *D. discoideum* in isolation resulted in the loss of ability to limit the growth of extracellular symbiont *Paraburkholderia* in complex symbiotic contexts (65). Alternatively, the observed decline in fitness of this strain may be attributed to plasmid loss which can render the strain less adapted to its former host environment. For instance, evidence from Bharathan et al (2019) (66) demonstrates that carbapenem resistant *E. coli*, following plasmid curing or plasmid removal (achieved by treating these strains with pectin-capped platinum nanoparticles), not only lost their antibiotic resistance but also exhibited slower growth, smaller colony

morphology and a 50% reduction in biofilm formation both *in vitro* and *in vivo*. Re-introducing the plasmid from the wild-type strain restored antibiotic resistance in the cured strain (66) which suggests that host-adaptative traits can be linked to plasmid gain and loss. These results underscore the rapid and potentially asymmetric nature of host-specific adaptation, where evolution toward a new host context may come at the cost of performance in the original environmental context. It is noteworthy that all replicate evolved populations exhibited a similar reduction in fitness to the original host (cf reaction norms in Figure 3D); the result was thus repeatable even though in all replicates a different random set of 10 colony isolates was used.

For the priority effects experiment, in which the order of exposure of the host to ancestral and evolved strains was manipulated, we considered two possible mechanisms by which priority effects would emerge. First, under the hypothesis of evolution-mediated priority effects, we expected that the evolved populations, having undergone a period of adaptation to the new host genotype, would display higher fitness than their ancestral counterparts. In this scenario, introducing the evolved strains first would allow them to monopolize the gut environment more efficiently, leading them to exclude late arriving ancestral population of the other strain from establishing through a niche pre-emption mechanism. Conversely, if introduced later, the evolved populations might still invade and displace ancestral residents of the other strain if adaptation results in competitive superiority in absolute terms. Given the intrinsic differences in population growth and competitive ability (35); Figure 3 and Figure 4 of the present study), we did not expect a reversal of priority effects, as the priority effects observed in our previous study were quite asymmetric (*Rhizobium* always being the dominant strain but *Microbacterium* having higher relative densities when inoculated first than when inoculated later. Instead, we expected the evolved *Rhizobium* to dominate more than the ancestral *Rhizobium* strain when inoculated first. Conversely, we expected a reduced dominance of *Rhizobium* when the evolved *Microbacterium* strain, rather than the ancestral strain, was inoculated first. We did not observe this. In contrast, we observed no significant changes in relative abundances of the strains in the competition and priority effect experiments between settings in which we used ancestral or evolved populations. This is surprising, given that in an earlier study we did observe a significant effect of inoculation order on the relative abundance of the two strains. In this context, it is an interesting observation that the one near-significant effect of inoculation order we observed was in the setting using both ancestral *Rhizobium* and ancestral *Microbacterium*. While not significant, the pattern observed in this setting is similar to the observations of Gurung et al (2024) (35). One reason for a less pronounced pattern might lie in the fact that the animals in the current experiment were for technical reasons fed autoclaved and thus dead algae, whereas in the experiment of Gurung et al (2024) the animals were fed with sterile live algae.

Our study suffered from some methodological limitations that need to be considered when interpreting the results. First, our experimental evolution trial ran over a relatively short time scale of 12 days. This limited time frame may have been insufficient for substantial adaptive changes to occur. As such, our study likely underestimates both the capacity of microbiome members to genetically adapt to a host genotype and the extent to which evolutionary changes influence community assembly and priority effects in gut microbiomes. While our study suggests that evolution-mediated priority effects (15,67) may not play a major role in the gut community assembly of individual *Daphnia* over short timescales, this does not preclude more important contributions of evolution in settings that allow for longer periods of adaptation. Future studies could address this limitation by implementing serial passage over longer time-scales and across multiple host generations to allow more robust and sustained adaptation, potentially allowing the detection of clearer and measurable fitness advantages in the host. In the context of taxa such as *Daphnia*, in which populations during the growing season consist of assemblages of clones, adaptation to specific genotype across multiple generations would still be ecologically highly relevant. Second limitation of our study is that we only tested for influences on priority effects in the new host genotype and not in the ancestral host genotype. Future studies might also quantify this in the original host genotype, given that we did observe a loss of adaptation to this genotype. Another important limitation of our study is that the experimental evolution was conducted using single-strain inoculations, which may not fully capture the community context of a natural thriving microbiome. By inoculating strains in isolation, there is a risk of traits that are advantageous in competitive dynamics. This may interfere with a proper interpretation of the effect of evolution on community assembly. Lawrence et al. (2012) (13) reported the rapid loss of competitive ability in bacteria when kept for longer periods in monospecific cultures. Such dynamics may be of key importance when designing eco-evolutionary experiments involving community assembly (68).

Loss of competitive ability was also observed by Vanvelk et al (2024) (69) when *Daphnia* populations were allowed to adapt to warming in monocultures to then be used in community assembly experiments. Together, these findings point towards the need to design evolutionary adaptation experiments to novel environments in a proper community context. However, this can be very challenging, as it requires standardizing the community composition while avoiding strong species sorting or even species exclusion during this selection period. For example, in the study of Vanvelk et al (2024) (69), selection in the presence of a community was initially planned, but failed due to the rapid dominance of a single species in all communities.

While our study did not detect an impact of evolution on priority effects in gut microbiome assembly, it provides valuable insights into the evolutionary dynamics of bacterial strains within the host gut environment. Although several studies demonstrate contemporary evolution over short time-scales in host-associated microbiomes (28,53), our study found a rapid loss of adaptation to the original host genotype with no significant increase in fitness in the new host environment. This suggests that loss of adaptation can be faster than gain in fitness. This has important implications for the design of experiments on gut microbiome assembly as well as for applications such as gut microbiome engineering and live microbiome biotherapeutics. Our results stress that there is a need to consider a rapid loss of adaptation during pre-conditioning and the need to pre-adapt strains to specific clinical environments, including conditions that are specific to the host individual and genotype.

Data availability statement

All data required to evaluate the conclusions of this manuscript are included in supporting information.

Author contribution statement

LDM, AG and CS designed the study; AG and CC performed the experiments; AG and CC analysed data with input of CS and LDM; AG wrote the first drafts with input of CS and LDM; all authors contributed to refining the text.

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References

1. Hasan N, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ. 2019;7:e7502.

2. Kurilshikov A, Medina-Gomez C, Bacigalupe R, Radjabzadeh D, Wang J, Demirkan A, et al. Large-scale association analyses identify host factors influencing human gut microbiome composition. Nat Genet. 2021 Feb;53(2):156–65.

3. Dapa T, Wong DPGH, Vasquez KS, Xavier KB, Huang KC, Good BH. Within-host evolution of the gut microbiome. Curr Opin Microbiol. 2023 Feb;71:102258.

4. Scanlan PD. Microbial evolution and ecological opportunity in the gut environment. Proc Biol Sci. 2019 Nov 20;286(1915):20191964.

5. Vellend M. Conceptual Synthesis in Community Ecology. The Quarterly Review of Biology. 2010 Jun;85(2):183–206.

6. Vellend M. The theory of ecological communities. InThe theory of ecological communities 2016 Aug 23. Princeton University Press.

7. Fukami T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. Annual Review of Ecology, Evolution, and Systematics. 2015 Dec 4;46(Volume 46, 2015):1–23.

8. Stroud JT, Delory BM, Barnes EM, Chase JM, De Meester L, Dieskau J, et al. Priority effects transcend scales and disciplines in biology. Trends Ecol & Evol. 2024 Jul 1;39(7):677–88.

9. Williams JL, Kendall BE, Levine JM. Rapid evolution accelerates plant population spread in fragmented experimental landscapes. Science. 2016 Jul 29;353(6298):482–5.

10. Zilio G, Deshpande JN, Duncan AB, Fronhofer EA, Kaltz O. Dispersal evolution and eco-evolutionary dynamics in antagonistic species interactions. Trends Ecol & Evol. 2024 Jul 1;39(7):666–76.

11. Lustenhouwer N, Moerman F, Altermatt F, Bassar RD, Bocedi G, Bonte D, et al. Experimental evolution of dispersal: Unifying theory, experiments and natural systems. J Anim Ecol. 2023;92(6):1113–23.

12. Pantel JH, Duvivier C, Meester LD. Rapid local adaptation mediates zooplankton community assembly in experimental mesocosms. Ecol Lett. 2015;18(10):992–1000.

13. Lawrence D, Fiegna F, Behrends V, Bundy JG, Phillimore AB, Bell T, et al. Species Interactions Alter Evolutionary Responses to a Novel Environment. PLOS Biology. 2012 May 15;10(5):e1001330.

14. Leibold MA, Govaert L, Loeuille N, De Meester L, Urban MC. Evolution and Community Assembly Across Spatial Scales. Annu Rev Ecol Evol Sys. 2022;53(1):299–326.

15. De Meester L, Vanoverbeke J, Kilsdonk LJ, Urban MC. Evolving Perspectives on Monopolization and Priority Effects. Trends Ecol & Evol. 2016 Feb;31(2):136–46.

16. Urban MC, De Meester L. Community monopolization: local adaptation enhances priority effects in an evolving metacommunity. Proc R Soc B: Biol Sci. 2009 Sep 9;276(1676):4129–38.

17. Leibold MA, Govaert L, Loeuille N, De Meester L, Urban MC. Evolution and Community Assembly Across Spatial Scales. Annu Rev Ecol Evol Syst. 2022 Nov 2;53(1):299–326.

18. Nadeau CP, Farkas TE, Makkay AM, Papke RT, Urban MC. Adaptation reduces competitive dominance and alters community assembly. Proc R Soc B: Biol Sci. 2021 Feb 17;288(1945):20203133.

19. Debray R, Conover A, Zhang X, Dewald-Wang EA, Koskella B. Within-host adaptation alters priority effects within the tomato phyllosphere microbiome. Nat Ecol Evol. 2023 May;7(5):725–31.

20. Terhorst CP. Experimental evolution of protozoan traits in response to interspecific competition. J Evol Biol. 2011 Jan 1;24(1):36–46.

21. terHorst CP, Lennon JT, Lau JA. The relative importance of rapid evolution for plant-microbe interactions depends on ecological context. Proc R Soc B: Biol Sci. 2014 Jun 22;281(1785):20140028.

22. terHorst CP, Zee PC, Heath KD, Miller TE, Pastore AI, Patel S, et al. Evolution in a Community Context: Trait Responses to Multiple Species Interactions. Am Nat. 2018 Mar;191(3):368–80.

23. Decaestecker E, Van de Moortel B, Mukherjee S, Gurung A, Stoks R, De Meester L. Hierarchical Eco-Evo Dynamics Mediated by the Gut Microbiome. Trends Ecol Evol. In press;39/2.

24. Barroso-Batista J, Sousa A, Lourenço M, Bergman ML, Sobral D, Demengeot J, et al. The first steps of adaptation of Escherichia coli to the gut are dominated by soft sweeps. PLoS Genet. 2014 Mar;10(3):e1004182.

25. Wu M, McNulty NP, Rodionov DA, Khoroshkin MS, Griffin NW, Cheng J, et al. Genetic determinants of in vivo fitness and diet responsiveness in multiple human gut Bacteroides. Science. 2015 Oct 2;350(6256):aac5992.

26. Lescat M, Launay A, Ghalayini M, Magnan M, Glodt J, Pintard C, et al. Using long-term experimental evolution to uncover the patterns and determinants of molecular evolution of an Escherichia coli natural isolate in the streptomycin-treated mouse gut. Mol Ecol. 2017 Apr;26(7):1802–17.

27. Ghalayini M, Launay A, Bridier-Nahmias A, Clermont O, Denamur E, Lescat M, et al. Evolution of a Dominant Natural Isolate of Escherichia coli in the Human Gut over the Course of a Year Suggests a Neutral Evolution with Reduced Effective Population Size. Appl Environ Microbiol. 2018 Mar 1;84(6):e02377-17.

28. Zhao S, Lieberman TD, Poyet M, Kauffman KM, Gibbons SM, Groussin M, et al. Adaptive Evolution within Gut Microbiomes of Healthy People. Cell Host Microbe. 2019 May 8;25(5):656-667.e8.

29. Yaffe E, Relman DA. Tracking microbial evolution in the human gut using Hi-C reveals extensive horizontal gene transfer, persistence and adaptation. Nat Microbiol. 2020 Feb;5(2):343–53.

30. Groussin M, Poyet M, Sistiaga A, Kearney SM, Moniz K, Noel M, et al. Elevated rates of horizontal gene transfer in the industrialized human microbiome. Cell. 2021 Apr 15;184(8):2053-2067.e18.

31. Suzuki TA, Ley RE. The role of the microbiota in human genetic adaptation. Science. 2020 Dec 4;370(6521):eaaz6827.

32. Henry LP, Bruijning M, Forsberg SKG, Ayroles JF. The microbiome extends host evolutionary potential. Nat Commun. 2021 Aug 26;12(1):5141.

33. Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat Rev Gastroenterol Hepatol. 2018 Apr;15(4):197–205.

34. Debray R, Herbert RA, Jaffe AL, Crits-Christoph A, Power ME, Koskella B. Priority effects in microbiome assembly. Nat Rev Microbiol. 2022 Feb;20(2):109–21.

35. Gurung A, Mukherjee S, Declercq M, Souffreau C, De Meester L. Strain-dependent and host genotype–dependent priority effects in gut microbiome assembly affect host fitness in Daphnia. L&O. 2024;69(8):1782–96.

36. Hildebrand F, Gossmann TI, Frioux C, Özkurt E, Myers PN, Ferretti P, et al. Dispersal strategies shape persistence and evolution of human gut bacteria. Cell Host Microbe. 2021 Jul 14;29(7):1167-1176.e9.

37. Ebert D. Daphnia as a versatile model system in ecology and evolution. EvoDevo. 2022 Dec;13(1):1–13.

38. Akbar S, Gu L, Sun Y, Zhang L, Lyu K, Huang Y, et al. Understanding host-microbiome-environment interactions: Insights from *Daphnia* as a model organism. Sci Tot Environ. 2022 Feb 20;808:152093.

39. Miner BE, De Meester L, Pfrender ME, Lampert W, Hairston NG. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc R Soc B: Biol Sci. 2012 May 22;279(1735):1873–82.

40. Macke E, Callens M, De Meester L, Decaestecker E. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat Commun. 2017 Nov 20;8(1):1608.

41. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, et al. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. ISME J. 2016 Apr;10(4):911–20.

42. Sison-Mangus MP, Mushegian AA, Ebert D. Water fleas require microbiota for survival, growth and reproduction. ISME J. 2015 Jan;9(1):59–67.

43. Peerakietkhajorn S, Tsukada K, Kato Y, Matsuura T, Watanabe H. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, Daphnia magna. Environ Microbiol Rep. 2015 Apr;7(2):364–72.

44. Mushegian AA, Ebert D. Presence of microbiota reverses the relative performance of Daphnia on two experimental diets. Zoology. 2017 Dec 1;125:29–31.

45. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991 Jan;173(2):697–703.

46. Bates D, Kliegl R, Vasishth S, Baayen H. Parsimonious mixed models. arXiv preprint arXiv:150604967. 2015;

47. Fox J, Weisberg S. Using car functions in other functions. 2019;

48. Lenth R, Lenth MR. Package 'lsmeans.' Am Stat. 2018;34(4):216–21.

49. Bradley PH, Nayfach S, Pollard KS. Phylogeny-corrected identification of microbial gene families relevant to human gut colonization. PLOS Comput Biol. 2018 Aug 9;14(8):e1006242.

50. Matamouros S, Hayden HS, Hager KR, Brittnacher MJ, Lachance K, Weiss EJ, et al. Adaptation of commensal proliferating Escherichia coli to the intestinal tract of young children with cystic fibrosis. PNAS. 2018 Feb 13;115(7):1605–10.

51. Barroso-Batista J, Pedro MF, Sales-Dias J, Pinto CJG, Thompson JA, Pereira H, et al. Specific Eco-evolutionary Contexts in the Mouse Gut Reveal Escherichia coli Metabolic Versatility. Current Biology. 2020 Mar 23;30(6):1049-1062.e7.

52. Dapa T, Ramiro RS, Pedro MF, Gordo I, Xavier KB. Diet leaves a genetic signature in a keystone member of the gut microbiota. Cell Host & Microbe. 2022 Feb 9;30(2):183-199.e10.

53. Garud NR, Good BH, Hallatschek O, Pollard KS. Evolutionary dynamics of bacteria in the gut microbiome within and across hosts. PLOS Biology. 2019 Jan 23;17(1):e3000102.

54. Dudycha JL, Tessier AJ. Natural Genetic Variation of Life Span, Reproduction, and Juvenile Growth in Daphnia. Evolution. 1999;53(6):1744–56.

55. Pietrzak B, Bednarska A, Grzesiuk M. Longevity of Daphnia magna males and females. Hydrobiologia. 2010 Apr 1;643(1):71–5.

56. Somerville GA, Proctor RA. At the crossroads of bacterial metabolism and virulence factor synthesis in Staphylococci. Microbiol Mol Biol Rev. 2009 Jun;73(2):233–48.

57. Snitkin ES, Zelazny AM, Gupta J, Program NCS, Palmore TN, Murray PR, et al. Genomic insights into the fate of colistin resistance and Acinetobacter baumannii during patient treatment. Genome Res. 2013 Jul 1;23(7):1155–62.

58. Lieberman TD, Michel JB, Aingaran M, Potter-Bynoe G, Roux D, Davis MR, et al. Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. Nat Genet. 2011 Nov 13;43(12):1275–80.

59. Remold SK, Lenski RE. Contribution of individual random mutations to genotype-by-environment interactions in Escherichia coli. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11388–93.

60. Elena SF, Lenski RE. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat Rev Genet. 2003 Jun;4(6):457–69.

61. Le Gac M, Brazas MD, Bertrand M, Tyerman JG, Spencer CC, Hancock REW, et al. Metabolic Changes Associated With Adaptive Diversification in Escherichia coli. Genetics. 2008 Feb 1;178(2):1049–60.

62. Lourenço M, Ramiro RS, Güleresi D, Barroso-Batista J, Xavier KB, Gordo I, et al. A Mutational Hotspot and Strong Selection Contribute to the Order of Mutations Selected for during Escherichia coli Adaptation to the Gut. PLOS Genetics. 2016 Nov 3;12(11):e1006420.

63. Andersson DI, Levin BR. The biological cost of antibiotic resistance. Curr Opin Microbiol. 1999 Oct;2(5):489–93.

64. Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev. 2013 Apr;26(2):185–230.

65. Larsen T, Jefferson C, Bartley A, Strassmann JE, Queller DC. Inference of symbiotic adaptations in nature using experimental evolution. Evolution. 2021 Apr 1;75(4):945–55.

66. Bharathan S, Sundaramoorthy NS, Chandrasekaran H, Rangappa G, ArunKumar G, Subramaniyan SB, et al. Sub lethal levels of platinum nanoparticle cures plasmid and in combination with carbapenem, curtails carbapenem resistant Escherichia coli. Sci Rep. 2019 Mar 28;9(1):5305.

67. De Meester L, Gómez A, Okamura B, Schwenk K. The Monopolization Hypothesis and the dispersal–gene flow paradox in aquatic organisms. Acta Oecol. 2002 Jun 1;23(3):121–35.

68. De Meester L, Brans KI, Govaert L, Souffreau C, Mukherjee S, Vanvelk H, et al. Analysing eco-evolutionary dynamics—The challenging complexity of the real world. Funct Ecol. 2019;33(1):43–59.

69. Vanvelk H, Govaert L, van den Berg EM, De Meester L. Eco-Evolutionary Interactions With Multiple Evolving Species Reveal Both Antagonistic and Additive Effects. Ecol Lett. 2024;27(11):e70006.

CHAPTER 4:

Reciprocal priority effects between hosts and associated microbial communities

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To be submitted

Abstract

The arrival order of species into a habitat is often key to shaping community structure and assembly, a phenomenon termed priority effects. While such effects have been demonstrated across multiple systems, the role of the host-associated microbiome in mediating such interactions during community assembly remains largely unexplored. Here, we focus on the two-way interaction between hosts and their associated microbial communities in determining priority effects in an aquatic model system involving two species of the water flea *Daphnia*. Specifically, we test whether host-conditioned medium mediates priority effects in host community assembly and, conversely, whether priority effects at the level of the host influences the assembly and composition of the microbial community in the environment. We performed inoculation order manipulations with two *Daphnia* species, *D. magna* and *D. pulicaria*, and crossed these treatments with a supplementation regime comprising host-conditioned medium derived from one or the other species, or non-conditioned medium. We quantified whether adding host-conditioned medium of the first or second colonizing species affects the outcome of host community assembly, and also tested whether host priority effects affects community composition of the microbial communities in the medium. Strong priority effects were recorded at the host level, where host communities were always dominated by the species inoculated first. Supplementation with host-conditioned medium in some treatments affected the relative abundance of host species but did not reverse dominance of the first inoculated species. Conversely, host inoculation order and thus priority effects at the host level significantly influenced bacterioplankton composition in the medium, with the identity of the first arriving host species emerging as the main driver of microbial community divergence. Our findings suggest that priority effects at the host level are not primarily microbiome-mediated, but they can nonetheless exert cascading downstream effects on the composition of free-living bacterioplankton communities.

Keywords: priority effects, community assembly, host-mediated effects, microbiome-mediated effects, *Daphnia,* host-conditioned medium.

Introduction

A fundamental goal in community ecology is to determine how ecological communities assemble, and which processes, whether deterministic or stochastic, drive patterns in community composition. Despite a century of research, the field is still divided between those positing that assembly is a deterministic process that can be predicted versus those that emphasize the importance of stochasticity (1). Order of arrival, timing of arrival and historical legacies hold an interesting position in this debate. While the sequence and timing of species arrival into a habitat introduce elements of stochasticity, due to the inherently random nature of individual immigration events, they often produce deterministic and predictable outcomes in community assembly. Arrival order can impact species assembly when the initial colonization of species alters the subsequent establishment and dominance patterns within the community, a process termed priority effects (2). The occurrence of priority effects has been reported across a wide range of taxa and ecosystems spanning multiple basic and applied fields of biology, yet is insufficiently recognized as an important factor in community assembly (3). In restoration ecology, priority effects are increasingly recognized as a tool for the management of desirable species over invasive and non-desirable species, by manipulating the order of species introductions into habitats (4). In the context of disease biology, parasitology and microbial ecology, the significance of species arrival order has often been reported, demonstrating how founder microbes can alter the colonization trajectory of other symbionts, parasites or pathogens in host-associated systems (5,3). Priority effects have also been explored in the light of eco-evolutionary dynamics, more specifically when ecological priority effects are further strengthened by evolution and assume a more permanent form when the primary colonizers adapt to local conditions and thereby gain an additional advantage over late-arriving immigrants (6–8).

The impact of ecological priority effects is attributed to a numerical advantage, wherein the first arriving species gain priority to the untapped space and resources in the habitat, thereby exhibiting rapid population growth in an environment devoid of competitors (7). This advantage in numbers poses a challenge for late arriving immigrants, as the carrying capacity of the habitat is saturated, limiting opportunities for them to successfully establish (9). This phenomenon, termed niche pre-emption, has been categorized as one of the mechanisms underlying inhibitory priority effects, where first arriving species suppress the colonization success of late arrivers by depleting space and resources, thereby establishing dominance (9). Another mechanism that can drive priority effects is niche modification, where the primary colonists are capable of altering their environment physically or chemically (through the release of metabolic by-products) that may subsequently either suppress (through niche-inhibitory modification) or promote (niche-facilitatory modification) the growth and proliferation of late arriving immigrants (9,10). Evolution of early arriving species can affect niche pre-emption by allowing a higher population growth through adaptation (7) and can potentially affect niche modification, although this has been less studied.

Here, we propose that the host-specific microbiome may also play a role in determining the strength of priority effects. With increasing evidence on the fundamental role of the host-associated microbiome towards host fitness, physiology, metabolism, immune system development, and adaptation to environmental stressors and pathogens (11–16), its role in shaping species coexistence and competition becomes a promising avenue for exploration. For instance, investigations have revealed that alterations in the gut microbiome composition in response to factors such as diet, antibiotics and stressors can influence host life history traits such as body size, reproductive success, and resistance to pathogens and pollutants (17–19). Studies on zooplankton, particularly *Daphnia* species, have identified key microbial taxa within the gut microbiome, such as *Limnohabitans* sp., *Pigmentiphaga* sp., *Pedobacter ruber* and *Polaromonas* sp. (20), whose genomes harbor genes that are potentially beneficial to the host, suggesting a potential role in shaping host performance and ecological interactions. Given this link between host microbiome and host fitness, it is plausible that microbiome-mediated mechanisms could indirectly influence host community assembly dynamics and priority effects. This may occur through niche pre-emption, as microbiome-mediated conditioning of the environment might enhance the fitness and population growth rate of the first colonizing species. Conversely, microbiome-mediated conditioning might also suppress growth rate of early colonizers and thus reduce the strength of numerical priority effects, for instance in case the microbiome contains species that harm the fitness of the host. The mechanism may also

involve niche modification, when early arrivers, through the release of their associated microbiome members, change the environment to either promote or suppress later arriving species.

While the host-associated microbiome might play a role in mediating priority effects and shaping host community assembly, the reciprocal interaction, where host priority effects mediate the assembly process of the host associated microbial community, is also worth investigating. Different host species may release distinct microbial communities into their environment through defecation, respiration, epithelial cell turn-over, infections etc. (i.e. host-specific "*microbial shedding*") (21,22). The excretory products of host species can modify the chemical environment in ways that alter nutrient availability, thereby impacting microbial community composition (23). Additionally, host physiological processes and behaviors such as preferential feeding of bacteria can further condition the environment in ways that selectively favor the enrichment of certain microbial taxa over others (24,25). It is thus plausible that the order and timing of host colonization and the resulting priority effects may influence the composition of free-living microbial communities in the environment, resulting in a cascading effect of host-level priority effects on surrounding microbial community assembly. Here again, the mechanisms might involve both niche pre-emption and niche modification.

In this study, we explore the two-way interaction between host and microbiomes via priority effects, testing for both the degree to which microbiome-mediated effects can impact priority effects at the host community level and to what extent priority effects at the host community level can mediate community assembly in associated microbial communities. First, we test the hypothesis that priority effects are microbiome-mediated, where the species that arrives first conditions the surrounding medium with species-specific microbial taxa, which subsequently enhance the fitness of its host species while suppressing that of competitors. Second, we assess the reciprocal interaction, where host arrival order dictates the community assembly pattern of microbial communities in the medium. We employ an experimental community assembly approach using two freshwater zooplankton species, namely, *D. magna* and *D. pulicaria*. In a first experiment, we first test whether host-conditioned medium influences competitive outcomes by introducing both species simultaneously into media either conditioned by their own species, by the competing species, or non- conditioned medium. In a second experiment, we test for an impact on priority effects by sequentially manipulating the inoculation order of the two host species to investigate how species arrival order impacts zooplankton community assembly. To assess the influence of host-conditioned medium on these dynamics, these inoculation order treatments are crossed with a supplementation regime (at the time of introduction of the second species), with host-conditioned medium originating from their own species, the competing species, or non-conditioned tap water. In addition, we test for different fractions of the host-conditioned medium (metabolic fraction, bacterial fraction, combination of both) to further dissect which component of the host-conditioned medium has a (larger) influence on priority effects. In this experiment, we specifically assess (i) if arrival order alone imposes priority effects between the two species, (ii) whether altering the identity of the host-conditioned medium influences the strength and direction of the priority effects, and if so, (iii) which fraction of the conditioned medium has the largest effect (the microbial component, the chemical/metabolic fraction, or a combination of both). To explore whether priority effects at the host level influence microbial community assembly, we also analyze microbial community composition of all experimental units at the end of the experiment, directly testing whether host priority effects or medium conditioning by the host are main drivers of microbial community composition.

Material and methods

Daphnia species and clones

Two species of *Daphnia* were used in this study, namely *Daphnia magna* and *Daphnia pulicaria*. These species were isolated in April 2023 from two separate ponds, namely Langerodevijver in Huldenberg (50.7885995, 4.5880687), Belgium (LRV) and Bellefroid-North in Leuven (50.910860, 4.703303), Belgium (BF_N), respectively. The two species were sourced from two different ponds to ensure distinct microbiomes corresponding to their original environmental conditions. Two clones for each of these species were used in the experiment. For *D. magna*, the clones used were LRV4-DM11 and LRV4-DM12. For *D. pulicaria*, the clones were BF-N-DP4 and BF-N-DP6.

Daphnia growth and culture conditions

Prior to the start of the experiment, the two *Daphnia* clones from each species were reared as independent lines in the laboratory for three successive generations to prevent interference from (grand-)maternal effects. Second clutch neonates were used to start each generation. Multiple replicate cultures were established for each clone by housing four daphnids in 720 mL jars filled with filtered, aged and de-chlorinated tap water. The jars were maintained at an ambient temperature of 20 ± 1°C with a 14:10h day-night cycle. 80% of the medium in the jars was refreshed three times a week, each time adding the unicellular green algae *Acutodesmus obliquus* as feed to each unit at a final concentration of 1mgC/L. To avoid micro-climatic effects caused by differences in light and airflow, the positions of the jars were randomly adjusted on the days that refreshing was carried out. After purging (grand-)maternal effects, the number of animals per unit was scaled up to maintain 15 daphnids per 720 mL water to obtain sufficient animals for the experimental set-up.

Preparation of conditioned medium

For the preparation of host-specific conditioned medium for supplementation during the experiment, monocultures were established for each clone used in the experiment (Figure 1A). Four replicates per clone were set up using 2 L aquaria filled with 1.5 L filtered, aged and de-chlorinated tap water. A total of 10 animals were added in each aquarium for the *D. magna* clones; for *D. pulicaria* a total of 30 animals were added per aquarium, to compensate for biomass differences between the two species (26,27). The light, temperature and feeding regime was maintained as mentioned above. The animals were allowed to condition their medium for 8 days, so as to allow the daphnids to grow and release their microbiome specific taxa as well as other compounds into the surrounding water. To obtain conditioned medium specific to each species, water from the two clones of each species was combined. This species-specific conditioned medium was used for supplementation during the experiment (see further). Water collected from each replicate of the monoculture was exclusively used to supplement the corresponding replicate in the treatment units of both experiments (see further). After harvesting the conditioned medium the monocultures were re-filled with fresh water and left to be conditioned for another 8 days before being harvested for the next supplementation.

Experiment 1: Assessing effects of host-conditioned medium on competitive outcomes

To study the impact of conditioned medium on competition strength, the two species were added simultaneously to 2 L aquaria at standardized biomass densities (10 8-day old individuals of *D. magna*: 5 individuals of clone DM11 + 5 individuals of clone DM12; and 30 individuals of *D. pulicaria*: 15 individuals of clone DP4 + 15 individuals of clone DP6). Three treatments were implemented, namely (1) a no-inoculum treatment, where the aquaria were filled with de-chlorinated tap water without any added conditioned medium; (2) a *D. magna* conditioned medium treatment, where the aquaria were supplemented with medium conditioned by the *D. magna* clones; and (3) a *D. pulicaria* conditioned medium treatment, where the aquaria were supplemented with medium conditioned by the *D. pulicaria* clones. The host-specific conditioned medium was added to the aquarium at the start of the experiment (i.e., in week 0). Supplementation was imposed 3 times at weekly intervals for the first three weeks. The conditioned medium replaced 25% of the volume of the 1.5 L aquarium. These 3 weeks were followed by a recovery period of another 3 weeks during which the microbiota was maintained undisturbed with no additional supplementation (Figure 1C). At the end of week 6, all *Daphnia* were isolated using a 125 µm mesh filter and transferred to 43 mL jars with 40 mL water and 3 mL formaldehyde (40%) for analysis of community composition. The competition trial thus consisted of 3 conditioned medium treatments × 4 replicates, resulting in 12 experimental units in total, each comprising 40 *Daphnia* individuals at the start.

Experiment 2: Assessing the bidirectional nature of priority effects in zooplankton and bacterioplankton community assembly

To introduce priority effects, the arrival order of the two *Daphnia* species in the aquaria was manipulated, resulting in two combinations of entry, namely (1) *D. magna* first, where *D. magna* was inoculated first into the system, followed by the inoculation of *D. pulicaria*, two weeks later and (2) *D. pulicaria* first, where *D. pulicaria* was inoculated first followed by *D. magna* (Figure 1D). A time-lag of 14 days was maintained to allow

sufficient time for the growth and proliferation of the first added species before the introduction of the second species (28). For each of these treatments, four replicate units were set-up consisting of 2 L aquaria filled with 1.5 L filtered, aged and dechlorinated tap-water. The number of animals added per species were standardized by biomass, with 10 animals in total being added for *D. magna* (5 animals of clone DM11 + 5 animals for clone DM12) and 30 animals in total for *D. pulicaria* (15 animals of clone DP4 + 15 animals for clone DP6). The aquaria were maintained at 20 ± 1°C with a 14:10h photoperiod and fed daily with *Acutodesmus obliquus* at concentration of 1.5 $mgCL^{-1}$. The positions of the aquaria were randomized three times a week to avoid interference from differences in micro-climatic effects.

To assess whether or not the driving component of a potential priority effect was related to the bacteria or the metabolites present in the inoculum or a combination of both, the inoculation order treatments were crossed with a supplementation regime of conditioned medium coming from either the first or the second inoculated species. In summary, the animals in the inoculation order treatments were exposed to eight different conditioned media types (Figure 1B). There were two control treatments, namely (1) a no-inoculum treatment, where de-chlorinated tap water was used as a supplement instead of conditioned medium (2) an own-inoculum treatment, wherein water of the aquaria was maintained from the start to the end of the experiment with no additional supplementation with conditioned medium. There were three treatments wherein the animals were exposed to the conditioned medium of *D. magna*: (1) combined medium that was conditioned by *D. magna* clones, to mimic settings comprising both bacterial and metabolic components released by *D. magna*; 2) metabolic fraction, wherein all the bacteria from the conditioned medium were filtered out using a 0.22 µm filter (Millipore®, 0.22 µm, White GSWP, 47 mm) to retain only the medium containing metabolites and by-products of *D. magna*; and 3) bacterial fraction, where all the bacteria that were earlier filtered out from the conditioned medium were resuspended in sterile water and added to the aquaria. Likewise, the same three treatments were set up for *D. pulicaria* conditioned medium. Each treatment consisted of four replicates and each replicate unit received conditioned medium from their corresponding monoculture replicate. This resulted in a total of 8 microbiome supplementation types (2 controls, 3 *magna* conditioned medium treatments, 3 *pulicaria* conditioned medium treatments, Figure 1B) for the two inoculation order treatments (*magna* first and *pulicaria* first) (Figure 1D). With four replicates per treatment, this resulted in a total of 64 aquaria. Each aquarium was inoculated with 40 common-garden grown daphnids (10 *D. magna* and 30 *D. pulicaria*).

The first supplementation of conditioned medium was carried out at the time of the introduction of the second species, i.e. after the time-lag of 2 weeks. We hypothesized that the conditioned medium would either facilitate or inhibit the late arriving species from overcoming the numerical advantage of the first arriving species, based on whether the conditioned medium came from its own species or rather from a different species. Supplementation was imposed 3 times, every 5 days during a period of 15 days, and consisted of a replacement of 25% of the total medium volume with differentially conditioned medium. We thus did not fully replace the medium.

The supplementation period of 2 weeks was followed by a recovery period of 2 weeks to allow the added microbiota to stabilize and impose their effects. At the end of the experiment, i.e., after a total duration of 6 weeks, all the animals per replicate were sieved out using a mesh filter (125 µm mesh size) and transferred to 45 mL jars containing 40 mL water and 3 mL formaldehyde (40 %) for fixation, to enable preservation until quantification was carried out. In addition, 45 mL of medium per replicate was collected for microbial characterization (see below).

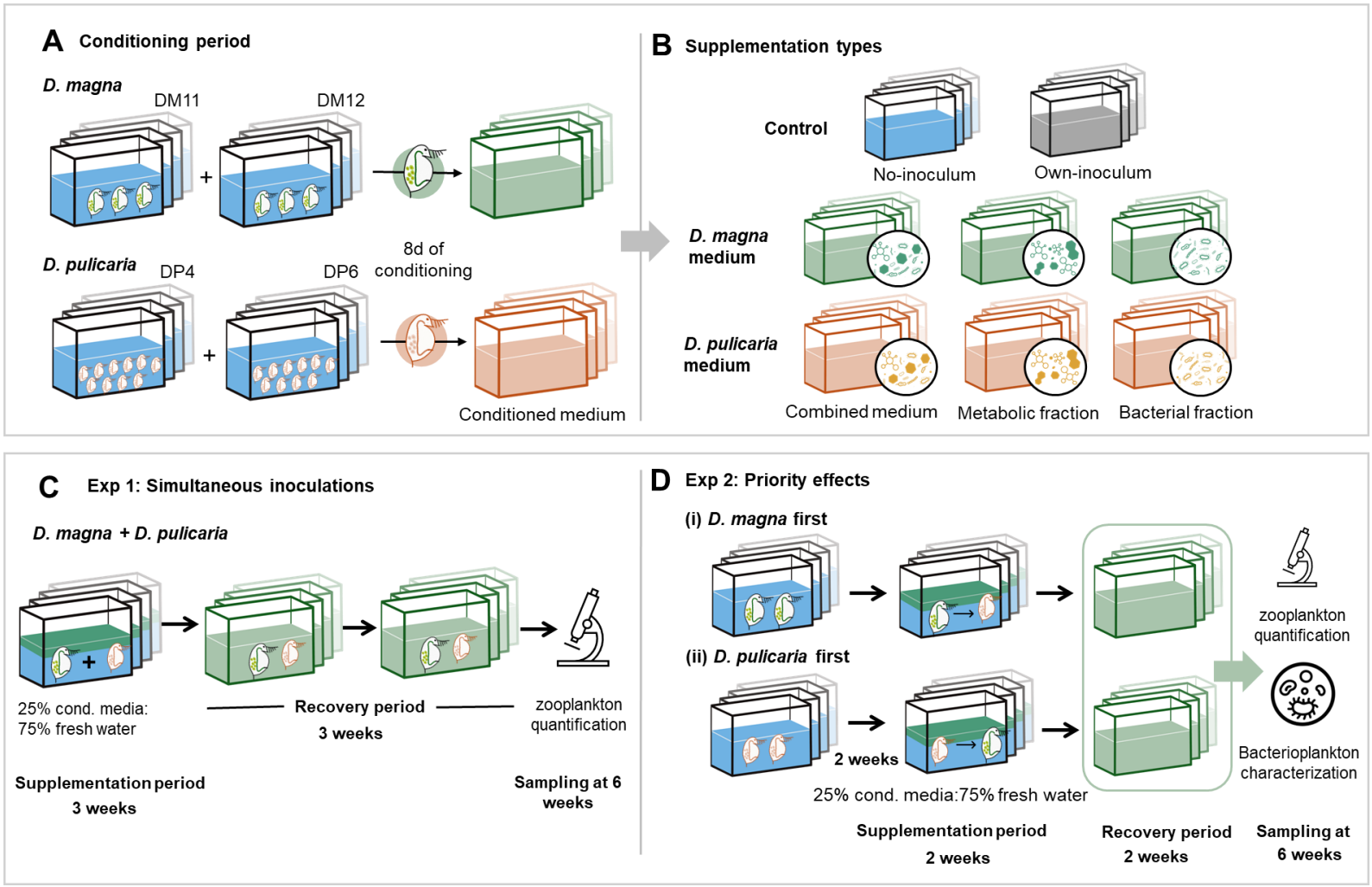

Figure 1: Overview of experimental set-up. A) Preparation of conditioned medium: the two clones of each species were grown independently in 2 L aquaria and allowed to condition their water for 8 days. After 8 days of conditioning, the water was harvested from the aquaria of the two clones and mixed together to obtain the conditioned medium of that species. B) supplementation types: the different types of conditioned medium inoculum used for treatment in experiment 2. C) Time line for the assessment of microbiome-mediated effects on competition dynamics (experiment 1) and D) on priority effects (experiment 2).

Assessment of community composition

Species identification and counting was carried out using an Olympus SZX16 (Japan) microscope. Identification of species was made primarily based on the morphology of the post-abdomen and head. *D. magna* is easily distinguished from *D. pulicaria* by the presence of an undulation at the posterior side of the post abdomen, a flatter head structure and the presence of a lateral expansion on their head (29,30). In comparison, *D. pulicaria* were identified by the absence of a dent on their post abdomen, spines on the base of the post-abdominal claw and their "S"-shaped head structure (29,30). In addition to differentiation among species, during counting, a distinction was made between juveniles, adult females and males of the two species. The individuals carrying eggs or being larger than >1.3 mm were registered as adults, while the animals smaller than this threshold were considered juveniles. Males were distinguished from the females by their smaller size, larger antennules and the presence of a hook-like structure on their front legs. For species quantification, subsamples were drawn from the total volume (43 mL) of each treatment. Subsample were counted in full until the cumulative total animals counted reached 250 daphnids. To standardize the counts across all samples, the number of animals per 1mL volume was calculated.

Body size of 10 individuals per experimental unit in the monocultures of each clone was measured using the ImageJ software (31). With this, the average body length of each clone was determined. For *D. magna* species, the body length was determined based on the average body length of the two clones used (10 individuals per replicate per clone, 10 (individuals) × 4 (replicates) × 2 (clones) =80 individuals). With this, the average body length of each clone was determined. For *D. magna* species, the body length was determined based on the average body length of the two clones used (10 individuals per replicate per clone, 10 (individuals) × 2 (clones) × 4 (replicates) = 80 individuals). Likewise, the same was carried out to determine the average body length for *D. pulicaria.* The body length was then used to carry out biomass conversions using the equations of *D. magna* and *D. pulex* (used as proxy for *D. pulicaria*) from Bottrel et al 1976 (26). Total zooplankton biomass values were determined for each experimental unit by summing up the biomass values of *D. magna* and *D. pulicaria.*

Bacterioplankton characterization: DNA extraction, library preparation and sequencing

To characterize the community composition of the free-living bacterioplankton in the medium of the different experimental units, we sampled 45 mL of medium from each aquarium at the end of the experiment (6 weeks). The medium from the four replicate units per treatment were pooled to have one sample per treatment (total volume 180 mL). This medium was filtered using Millipore® 0.22 µm White GSWP, 47 mm filters to retain the microbial communities. The filters were stored in 1X PBS for DNA extraction. Microbial DNA was extracted from these filters using the Macherey Nagel NucleoSpin® Tissue kit (filterService, Düren, Germany).

For metabarcoding, we first carried out a nested PCR, in which the full length (1500bp) of the prokaryotic 16S ribosomal RNA (16S rRNA) gene was amplified (5 ng template DNA) with the primer pair EUB8F and 1492R using the MyTaq™ HS mix (GC Biotech, Waddinxveen, The Netherlands) for 25 cycles: 15s at 95°C, 15s at 55°C and 10s at 72°C. The PCR products from this amplification were purified with CleanPCR beads (CleanNA, Waddinxveen, The Netherlands). A second round of PCR amplification was performed on the purified products to specifically amplify the V3-V4 hypervariable region (460bp) of the 16S rRNA gene using the primer pair 16s-IllumTS-F and 16s-IllumTS-R primers (32). Both primers were labelled with an Illumina adapter and an 8-nucleotide barcode at the 5'-end. A template concentration of 20 ng was used per sample and amplified using the MyTaq™ HS mix (GC Biotech, Waddinxveen, The Netherlands) for 16 cycles: 15s at 95°C, 15s at 51°C and 10s at 72°C. This was followed by a purification step with CleanPCR beads (CleanNA, The Netherlands). The amplicons were quantified using the Quant-iT™ PicoGreen™ kit (ThermoFisher, Belgium) and their integrity checked on 1.2% agarose gel. The purified amplicons were sequenced using a MiSeq Reagent kit v3 (600-cycle) on an Illumina Miseq platform (Genomics core, KU Leuven, Belgium) producing 2 × 300-nt paired end reads.

Sequence analyses were conducted using the DADA2 pipeline (version 1.8; BJ Callahan et al 2016) in R version 4.0.2. First, the read quality profiles of the sequences were investigated and trimmed based on the following parameters: maxN = 0, maxEE = c(2,2), truncLen = (290, 240), truncQ = 2, rm.phix = TRUE. Sequence variants were inferred using the high resolution DADA2 method, which relies on a parameterized model of substitution

errors to distinguish sequencing errors from real biological variation (33). Chimeras were removed from the dataset and taxonomy assigned with the Bayesian classifier method using the Silva nr99 v138.1 training set. ASVs assigned to “Chloroplast” or “Cyanobacteria” were removed from the dataset. Four samples had less than 10 reads and were not incorporated in the analysis. For visualization of β-diversity, a centred-log-ratio (CLR) transformation was performed at the Phylum and Genus levels using the *Microviz* package. Principal component analysis (PCA) plots depicting the top 10 phyla and genera driving group distinctions were generated using the *ord_calc* and *ord_plot* functions of the *Microviz* package. Compositional barplots were made using the *comp_barplot* function of the *Microviz* package.

Statistical analyses

For the competition trial with simultaneous inoculation (experiment 1), the main effect of conditioned medium on the absolute and relative biomass of the two *Daphnia* species was tested using a generalized linear mixed model with a Gamma distribution and a log link function built using the *glmer* function of the *lme4* package (34). To account for the variability arising from the use of conditioned medium from corresponding replicates, the replicate number was added to the model as a random factor. Analyses on species-specific biomasses were performed separately for the two species. Significance was tested with type III sum of squares using the *Anova* function of the *car* package (35). Significant pairwise interactions were identified with pairwise contrasts using the estimated marginal means with Tukey correction (*emmeans* package; (36)).

For the experiment testing for priority effects (experiment 2), the main effects of inoculation order, conditioned medium and their interaction on the absolute and relative biomass of the two *Daphnia* species was first tested using the same model as used for the competition trial. Given the significant interaction effects between inoculation order and conditioned medium, pairwise comparisons between conditioned medium treatments were performed within each inoculation order separately using the estimated marginal means with Tukey correction (*emmeans* package; (36)).

To investigate variability within treatments, the coefficient of variation (CV) of species specific and total biomass was calculated across replicates for each treatment using the *dplyr* package in R (37). To compare the relative variability in biomass across treatments, the mean CV across treatments was calculated by averaging the mean CV values of the *magna* treatments (combined, metabolic and bacterial fraction), *pulicaria* treatments and the control. These averages were computed separately for each inoculation order. Both the mean CV and its standard deviation are reported.

To determine differences in bacterioplankton community composition (β-diversity) between the inoculation order and conditioned medium treatments, Aitchison distances were computed from phylum and genus level taxonomic abundances. The three fractions of the conditioned medium were pooled and analyzed collectively, as a broader category termed species-specific medium. The combined, metabolic, and bacterial fraction treatments were grouped under "*magna* medium" for *D. magna*, while the corresponding fractions for *D. pulicaria* were grouped under "*pulicaria* medium." The "no-inoculum" and "own inoculum" treatments were categorized as "control". Significant differences in community composition between groups were determined with a permutational multivariate analysis of variance (PERMANOVA) using the *adonis2* function of the *vegan* package (38) with 999 permutations. The analysis was conducted with inoculation order and the species medium as the explanatory variables and a seed value (seed = 1234) was set to ensure reproducibility.

All statistical analyses were performed in R version 4.0.2.

Results

Effects of host-conditioned medium on zooplankton competition (experiment 1)

A significant main effect of conditioned medium was observed for absolute biomass values of both *D. magna* (*p*-value < 4.572e-06, Table 1) and *D. pulicaria* (*p*-value=0.04869, Table 1) in the simultaneous inoculation experiment (experiment 1). In the control treatment, i.e., supplementation with only tap water, it could be observed that *D. magna* displayed higher biomass values in comparison to *D. pulicaria*, reflecting that *D. magna* is the superior competitor over *D. pulicaria* in the standardized control treatment. The competitive

advantage of *D. magna* was significantly dampened when supplemented with conditioned medium, either from its own species or from *D. pulicaria*.(Figure 2A, B). For *D. pulicaria*, a significant reduction in its absolute biomass values was noted in the treatment supplemented with its own conditioned medium compared to that of the control treatment (*p*-value = 0.0374, Figure 2A, 2B). Supplementation with *D. magna* conditioned medium did not result in a significant reduction in absolute biomass of *D. pulicaria*. Conditioned medium also significantly impacted the relative biomass of both *D. magna* and *D. pulicaria* (*p*-value = 7.431e-07, *p*-value = 1.459e-07, Table 1).

Microbiome-mediated priority effects in zooplankton community assembly (experiment 2)

A significant main effect of inoculation order on absolute (*D. magna*, *p*-value = 0.048; *D. pulicaria*, *p*-value = 4.680e-12; Table 1; Figure 3A) and relative biomass (*D. magna*, *p*-value = 0.004; *D. pulicaria*, *p*-value = 8.585e-10; Table 1; Figure 3B) was detected for both *D. magna* and *D. pulicaria*. For both inoculation orders (*magna* first or *pulicaria* first), the species inoculated first into the system displayed significantly higher absolute and relative biomass values as compared to the species that was inoculated later.

No main effect of conditioned medium was observed on the absolute or relative biomass of *D. magna* (*p* = 0.173, 0.999; Table 1) in the communities. We did observe, however, a significant interaction effect between conditioned medium and inoculation order on *D. magna* absolute biomass (*p*-value = 0.047; Table 1). Pairwise comparisons between different conditioned media were subsequently performed within each inoculation order treatment. Based on a post-hoc test with Tukey correction, no significant pairwise differences were identified between conditioned medium treatments for *D. magna* absolute biomass in the treatments in which *D. magna* was inoculated first. However, in the treatments in which *D. pulicaria* was inoculated first, we observed a significant difference in *D. magna* absolute biomass between the *D. magna* metabolite fraction and *D. magna* bacterial fraction of the conditioned medium (*p*-value = 0.044, Figure 3A).

A significant main effect of conditioned medium was observed for *D. pulicaria* absolute (*p*-value = 8.238e-05, Table 1) and relative biomass (*p*-value = 0.005, Table 1). A post-hoc test with Tukey correction showed that the *D. pulicaria* absolute biomass in the treatments in which *D. magna* was inoculated first was significantly higher in the no-inoculum control than in the own inoculum treatment (*p*-value = 0.003; SI), *D. pulicaria* metabolite fraction (*p*-value = 0.002) and *D. pulicaria* bacterial fraction (*p*-value = 0.015) (Figure 3A). Additionally, *D. pulicaria* biomass was significantly higher in the *D. magna* metabolite fraction compared to the own total inoculum (*p*-value = 0.009), and the *D. pulicaria* bacterial fraction (*p*-value = 0.038) (Figure 3A). In the treatments in which *D. pulicaria* was inoculated first, no significant pairwise differences in *D. pulicaria* absolute biomass were observed among conditioned medium treatments. Given that both species appeared more impacted by the host-conditioned medium when inoculated as the second species, the mean coefficient of variation (CV) in biomass was assessed for both species in the two inoculation orders (Figure 3D). Results indicated that in the treatments in which *D. magna* was inoculated first, *D. pulicaria* exhibited a higher CV than *D. magna*. Conversely, in treatments in which *D. pulicaria* was inoculated first, *D. magna* displayed higher variability in biomass.

Table 1: Results of *glm* gamma model assessing the main effects of inoculation order, conditioned medium and their interaction effects on absolute and relative biomass of the two *Daphnia* species in Experiment 1 and Experiment 2. Significant *p* values (<0.05) are indicated in bold.

Experiment 1: Test for microbiome effects on competitive ability						
Absolute biomass						
	D. magna			***D. pulicaria***		
Effect	**Chi sq**	**Df**	**Pr**	**Chi sq**	**Df**	**Pr**
Conditioned medium	24.591	2	**4.572e-06**	6.0447	2	**0.04869**
Relative biomass						
Conditioned medium	28.225	2	**7.431e-07**	31.48	2	**1.459e-07**
Experiment 2 : Test for microbiome-mediated priority effects						
Absolute biomass						
	D. magna			***D. pulicaria***		
Effect	**Chi sq**	**Df**	**Pr**	**Chi sq**	**Df**	**Pr**
Inoculation order	3.8811	1	**0.04883**	47.817	1	**4.680e-12**
Conditioned medium	10.271	7	0.17368	30.336	7	**8.238e-05**
Medium × Inoculation order	14.2718	7	**0.04655**	13.431	7	0.06228
Relative biomass						
Inoculation order	8.2462	1	**0.00408**	37.622	1	**8.585e-10**
Conditioned medium	0.1352	7	0.99999	26.032	7	**0.0004971**
Medium × Inoculation order	8.5644	7	0.28546	16.158	7	**0.0237131**

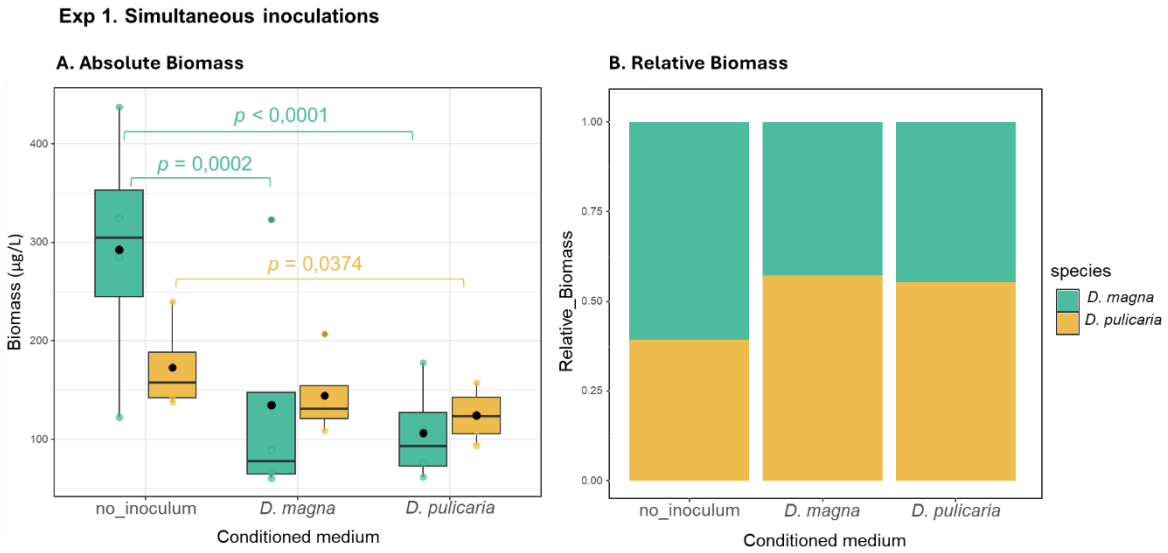

Figure 2: Assessment of effects of host-specific conditioned medium on host fitness in a competition experiment between *D. magna* and *D. pulicaria* (A) Absolute biomass, (B) Relative biomass. The *p*-values indicate significant pairwise differences between the three conditioned medium treatments based on a Tukey post-hoc analysis. Green lines indicate significant pairwise differences for *D. magna* biomass values between conditioned medium treatments; yellow line indicates significant pairwise differences for biomass values of *D. pulicaria*. Green dots indicate data points for *D. magna* biomass and yellow dots represent data points for *D. pulicaria* biomass values. Black dots indicate mean biomass values for each species in each treatment.

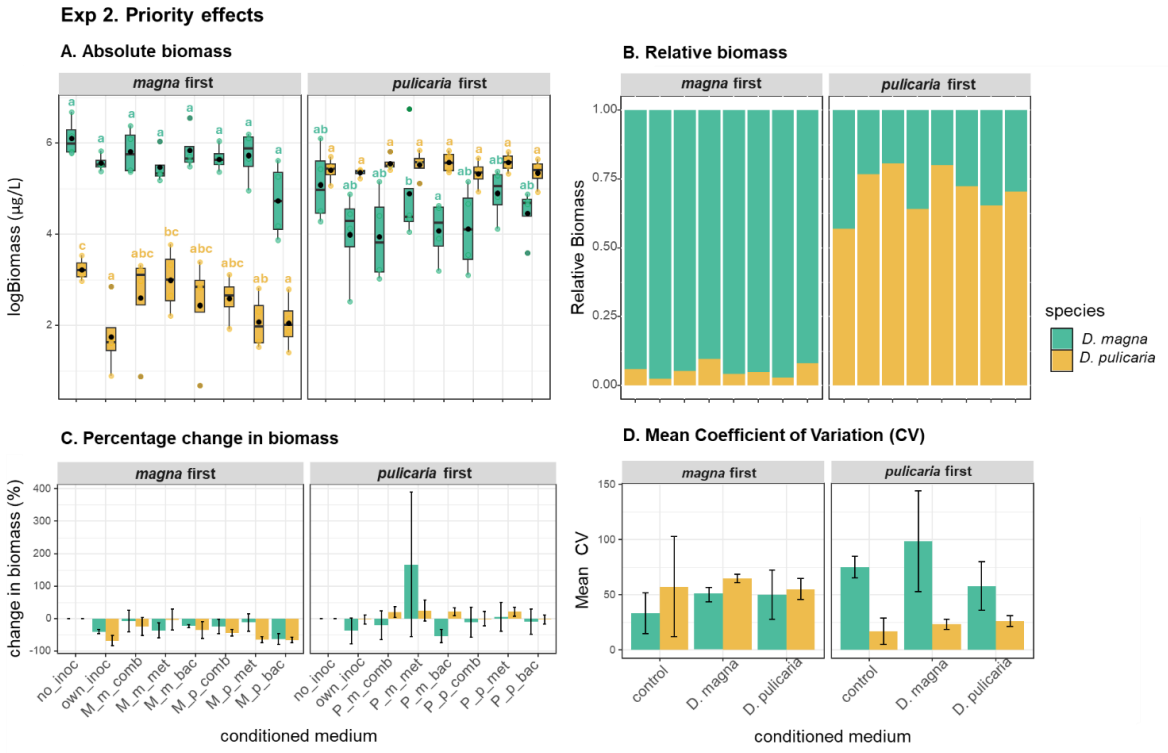

Figure 3: Assessment of effects of host-specific conditioned medium on priority effects. (A) Absolute biomass of the two species; (B) relative biomass of the two species; (C) percentage change in biomass of *D. magna* and *D. pulicaria* per inoculation order treatment (two treatments: *D. magna* first, *D. pulicaria* first) for each of the eight conditioned-medium treatments compared to the no-inoculum control treatment; (D) mean coefficient of

variation (CV) of the two species in each of the six inoculum order × conditioned-medium treatments. In labeling the treatments, the first letter in capital (M/P) points to the species that was inoculated first; the second letter in small m/p represents the source of the conditioned medium, i.e., whether it is conditioned by *D. magna* or *D. pulicaria*; comb/met/bac represent the fraction of the conditioned medium, i.e., whether it is the combined medium, the metabolic fraction or the bacterial fraction, respectively. For the absolute biomass, significant pairwise differences are indicated with different small-case characters.

Host priority effects drive bacterioplankton community assembly (experiment 2)

Beta diversity estimates based on aitchison distances revealed distinct clusters in community composition of the bacterioplankton in the medium of the experimental aquaria based on the inoculation order of the two *Daphnia* species (Figure 4). There was a significant effect of inoculation order and conditioned medium on bacterioplankton community composition (PERMANOVA, Table 2, Figure 4), both at the phylum and genus level. At the phylum level, inoculation order explained 27.16% of the observed variance (pseudo-F = 5.7351, $p < 0.001$), while conditioned medium accounted for 19.05% of total variance (pseudo-F = 2.0111, $p < 0.040$). At the genus level, inoculation order contributed 17.53% of the observed variance (pseudo-F = 2.9948, $p < 0.001$), while 15.39% (pseudo-F = 1.3145, $p < 0.048$) was explained by the species conditioned medium.

For both the inoculation treatments in which either *D. magna* or *D. pulicaria* was inoculated first, the dominant phyla consisted of Proteobacteria, Bacteroidota and Verrucomicrobiota (Figure 4A). The main differences between these treatments were mainly observed among the subdominant phyla, namely Planctomycetota, Acidobacteriota, Actinobacteriota, Gemmatimonadota, Patescibacteria, Bdellovibrionota, Hydrogenedentes, Margulisbacteria, Myxococcota, WPS-2, Desulphobacterota and Spirochaetota. The main phyla driving the variation along the first two principal components (PC1 and PC2) in the treatments in which *D. magna* was inoculated first included Acidobacteria, Spirochaetota, Dependentiae, Chroloflexi, WPS-2, Margulisbacteria and Sumerlaeota, while these included Firmicutes, Desulfobacterota and Patescibacteria in the treatments in which *D. pulicaria* was inoculated first. At the genus level, the top ten genera contributing to the variation among the two inoculation order treatments included *MWH-Ta3, Oligoflexus, Rhodobacteraceae_unclassified and KI89A clade_unclassified* that were more abundant in the treatments that were inoculated first with *D. magna*, while *oc32, Porphyrobacter, Polycyclovorans, Limnobacter, Bryobacter and Micropepsaceae_unclassified* were more abundant in the treatments in which *D. pulicaria* was inoculated first (Figure 4B).

The metabarcoding analysis of the bacterioplankton sampled from the monocultures showed that the two *Daphnia* species had different impacts on the bacterioplankton community in their medium. The community composition of the bacterioplankton in the monocultures did not strongly align with the bacterioplankton community in the mixed cultures, also not if categorized by the species that was inoculated first and therefore dominated the community. Instead, *D. magna* monoculture (DM11) clusters more closely with treatments where D. *magna* conditioned medium was applied while *D. pulicaria* (DP6) is similarly positioned near treatments with *D. pulicaria* conditioned medium.

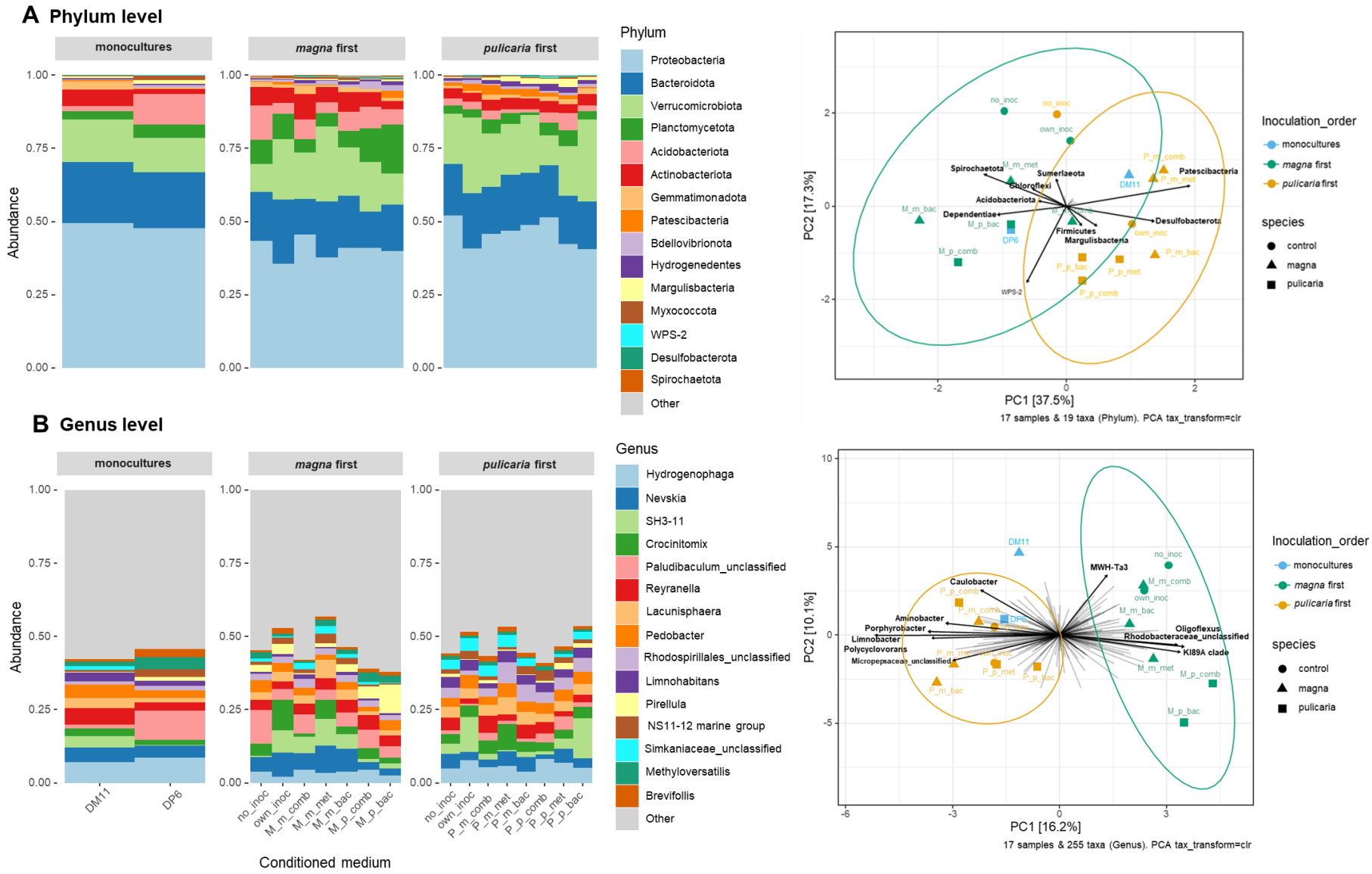
A Phylum level
monocultures
magna first
pulicaria first
Abundance
1.00
0.75
0.50
0.25
0.00
Phylum
Proteobacteria
Bacteroidota
Verrucomicrobiota
Planctomycetota
Acidobacteriota
Actinobacteriota
Gemmatimonadota
Patescibacteria
Bdellovibrionota
Hydrogenedentes
Margulisbacteria
Myxococcota
WPS-2
Desulfobacterota
Spirochaetota
Other
PC2 [17.3%]
PC1 [37.5%]
17 samples & 19 taxa (Phylum). PCA tax_transform=clr
Inoculation_order
monocultures
magna first
pulicaria first
species
control
magna
pulicaria
B Genus level
DM11
DP6
no_inoc
own_inoc
M_m_comb
M_m_met
M_m_bac
M_p_comb
M_p_bac
P_m_comb
P_m_met
P_m_bac
P_p_comb
P_p_met
P_p_bac
Conditioned medium
Genus
Hydrogenophaga
Nevskia
SH3-11
Crocinitomix
Paludibaculum_unclassified
Reyranella
Lacunisphaera
Pedobacter
Rhodospirillales_unclassified
Limnohabitans
Pirellula
NS11-12 marine group
Simkaniaceae_unclassified
Methyloversatilis
Brevifollis
Other
PC2 [10.1%]
PC1 [16.2%]
17 samples & 255 taxa (Genus). PCA tax_transform=clr
Caulobacter
Aminobacter
Porphyrobacter
Limnobacter
Polycyclovorans
Micropepsaceae_unclassified
MWH-Ta3
Oligoflexus
Rhodobacteraceae_unclassified
KI89A clade

Figure 4: Differences in microbial community composition at the (A) Phylum level and (B) Genus level represented in compositional barplots and PCA plots (using CLR transformation) across samples grouped and colored by inoculation order. In the PCA plots, each point represents a sample, with the colors indicating the inoculation order and shapes indicating the type of conditioned medium. The ellipses represent 95% confidence intervals for the community composition of each inoculation order. The black arrows represent the top 10 phyla contributing most to the variation along PC1 and PC2. The direction and length of the arrows reflect the correlation between each phylum and the ordination axes, as calculated using biplot scaling.

Table 2: Summary results of PERMANOVA based on Aitchison distances at the Phylum and Genus levels. The table reports the main effects of inoculation order, conditioned medium and their interaction terms. Monocultures are not included in the analysis. Significant effects ($p < 0.05$) are indicated by asterisks in bold (*p < 0.05, **p < 0.01, ***p < 0.001).

		Df	SumofSqs	R2	F	Pr(>F)
Phylum	Inoculation order	1	96.91	0.27167	5.7351	**0.001*****
	Conditioned medium	2	67.97	0.19053	2.0111	**0.040***
	Inoculation order × cond. medium	2	39.76	0.11146	1.1765	0.274
	Residual	9	152.08	0.42633		
	Total	14	356.73	1.00000		
Genus	Inoculation order	1	1810.8	0.17535	2.9948	**0.001****
	Conditioned medium	2	1589.5	0.15393	1.3145	**0.048***
	Inoculation order × cond. medium	2	1484.7	0.14377	1.2277	0.101
	Residual	9	5441.7	0.52696		
	Total	14	10326.7	1.00000		

Discussion

In this study, we investigate both the degree to which microbial communities influence host species community assembly as well as the degree to which host community composition affects the assembly of the microbial community in their medium, using an experimental zooplankton community comprising two large-bodied cladoceran species, namely *D. magna* and *D. pulicaria*. More specifically, we test how host-specific conditioned medium influence host community assembly dynamics and priority effects, and study how host priority effects impact the assembly and structure of their surrounding microbial community via host conditioning of the medium. In line with earlier work, we observed strong priority effects in community assembly involving the two *Daphnia* species, with the first arriving species displaying significantly higher biomass than the species that arrived later in the community, underscoring the importance of host arrival order on host community assembly. Manipulating the host environment with medium conditioned by either the same species or the competing species moderately influenced community assembly, but did not result in an overruling of priority effects. This leads to the conclusion that the priority effects are not driven by conditioning of the medium by host species, and are rather resilient to strong manipulations of the free-living bacterioplankton community in their medium. Although the priority effects driven by arrival order were not overruled, the host-conditioned medium generally had a negative influence on host population growth and biomass with the late arriving species appearing to be more susceptible to the inhibitory effects of the conditioned medium. While our results do not provide evidence that microbiome composition can overrule priority effects in host communities, we do show that priority effects at the host community level impact community composition of the free-living bacterioplankton in our experimental aquaria.

In line with the results of an earlier study reporting strong priority effects among different species of the family Daphniidae (using *D. magna*, *D. obtusa* and *Simocephalus vetulus*, (28)), we also observed strong inhibitory priority effects between *D. magna* and *D. pulicaria*. In all our experimental aquaria where arrival order was manipulated, the species inoculated first consistently displayed substantially higher biomass than the species introduced two weeks later. Given that the larger species (*D. magna*) is clearly competitively dominant (as observed in (28); and this study (experiment 1)) when both species are introduced simultaneously, the strong priority effects observed are not trivial, as they reflect an overruling of inherent fitness differences. In theory, such priority effects may be the result of both niche pre-emption as well as niche modification (9). The most likely explanation in our case is rapid niche pre-emption, where the early arriving species quickly occupies available space and resources, leaving little room for the second colonist to establish. Here we also explored whether the strong priority effects could partly be explained by microbiome-mediated advantages for the resident population. Specifically, this would require that the first arriving species alters the bacterioplankton community in a way that reinforces competitive advantage over late arriving species. Our experimental test was thus designed to determine whether exposing host communities to medium conditioned by the later arriving species would overrule the priority effect. If so, this would provide strong evidence that microbiome-mediated changes can drive significant shifts in community assembly. However, we did not observe a strong effect of host-conditioned medium. While our results do not rule out the role of microbiome-conditioned medium, they indicate that the effect is rather modest, and insufficient to overcome the numerical advantage of early arrivers. We did observe that manipulating the identity of host species conditioning the medium had some influence on host community composition. However, most of the observed impacts were negative, primarily resulting in reduction in population growth rate rather than an enhancement. For instance, in the treatments in which *D. magna* was inoculated first, there was a significant reduction (~80%) in *D. pulicaria* biomass when supplemented with medium conditioned by *D. pulicaria*, especially in the metabolic and bacterial fractions compared to the no-inoculum control. In contrast, supplementation with *D. magna* conditioned medium had no such effect on *D. pulicaria*. Likewise, in the treatments in which *D. pulicaria* was inoculated first, *D. magna* showed a significant reduction in biomass when supplemented with *D. magna* conditioned medium of the bacterial fraction. Apart from these two clear examples, there is no consistent pattern indicating that one species is more affected by the medium conditioned by monocultures, nor is there a consistent signal of medium conditioned by one species having a stronger effect than medium conditioned by another species (but see Hobaek & Larsson, 1990 (39)). We also found no consistent differences in the effects of the combined conditioned medium, the metabolic fraction and the bacterial fraction on the absolute and relative biomasses of the two *Daphnia* species. There is, however, some indication that the second species added to the culture is more affected by the conditioned medium than the first.

Although the microbiome is known to confer important fitness benefits to *Daphnia* (40,41), our results align with earlier studies on crowding factors in conditioned medium, showing that the microbial communities in the conditioned medium tend to slow down rather than enhance the population growth rate of *Daphnia*. For instance, Matveev (1993) (42) found that pre-conditioning of the medium for 1-2 weeks with *D. carinata* (20-30 daphnids/L) resulted in a strong decline in grazing rates of *Daphnia lumholtz*i, *Moina micrura* and *Diaphanosoma unguiculatum* relative to un-preconditioned controls. *D. carinata* itself appeared to be the most sensitive species, exhibiting the strongest decline in grazing rate. In another example, Burns (2008) (43) highlighted the range of responses in *Daphnia* to chemicals released by crowding, where *Daphnia* at high densities influenced the growth and reproduction of their own and other species by mechanisms that were independent of ambient food depletion. These inhibitory effects were attributed to crowding-induced chemicals which showed suppressive effects on the growth of three large *Daphnia* species, namely, *D. magna*, *D. pulicaria* and *D. pulex* , as also observed by De Meester *et al.*(1999) (44) among four populations of *D. magna*. Several studies have also provided evidence that chemicals released by high densities of zooplankton can depress feeding and grazing rates of their own and other species of zooplankton (42,45–48). For example, the feeding rate of *D. pulex* on *Chlamydomonas reinhardi* was found to be significantly reduced at a density of 270 daphnids/L relative to 30/L (46). Other studies have shown strong negative responses in life-history traits to crowding by conspecifics in the large-bodied species, *D. magna* and *D. carinata* (42,47,49).

While the impact of host-conditioned medium was inconsistent and relatively moderate in the experiment manipulating host arrival order (experiment 2), the effect was much stronger and more consistent in the experiment where the two *Daphnia s*pecies were inoculated simultaneously (experiment 1). In the absence of host-conditioned medium, *D. magna* was clearly the competitively dominant species. However, when conditioned medium was applied, *D. magna* was significantly more affected than *D. pulicaria*. This influence of host-conditioned medium was negative, reducing the growth of *D. magna*, resulting in coexistence between the two species at nearly equal biomass. Interestingly, this

response was species-specific in the sense that only the growth of *D. magna* was suppressed by the addition of conditioned medium. However, it was not species -specific with respect to the source of the conditioned medium, i.e., medium conditioned by *D. magna* and *D. pulicaria* had similar inhibitory effects on *D. magna*. These findings align well with those of Louette et al (2008) (28), who also observed that the competitive dominance of *D. magna* under simultaneous inoculation could be reversed when it was introduced after a competitor.

The design of our experiment testing for the impact of the microbiome on host community assembly suffered from an important methodological limitation. Due to practical constraints, only 25% of the medium was replaced when manipulating the conditioning of the medium. While this constitutes a clear disturbance event, it does not result in a complete change of the characteristics of the medium. Consequently, the microbial community in the medium likely remained dominated by bacteria associated with the dominant, i.e. the first inoculated host species. This limitation is important in interpreting our results for two reasons. First, the moderate changes in host community assembly we observed occurred in a context where only 25% rather than 100% of the medium was replaced by medium conditioned by the competing species. The effects might have been stronger under a complete 100% replacement. Secondly, our observation that priority effects are not driven by microbiome conditioning of the medium should be interpreted with caution, given that we did not test a full replacement of the medium. Future experiments could address this limitation using a 100% supplementation in a flow-through system that would allow for the constant refreshment of conditioned medium, enabling a more robust assessment of microbiome-mediated effects on host community assembly.

Next to the moderate impact of conditioned medium on community assembly of a host community, our results also show that host community composition impacts microbial community composition in the medium. Strikingly, the priority effect observed in the host community extended to the bacterioplankton community composition in the surrounding medium. Specifically, we observed that the host species arriving first played a key role in shaping the bacterioplankton community composition in the treatments where host species arrival was manipulated. In the multivariate analysis of the bacterioplankton communities across all experimental aquaria, we indeed observed that the primary axis of variation in microbial community composition corresponded to the inoculation order of the host species (Figure 4A, B). In principle, the observed effect of host species dominance on the microbial community could result from an enrichment of the bacterioplankton with species-specific host microbiome communities, differential grazing pressure exerted by the host species, or species-specific conditioning of the medium through metabolites that differentially stimulate specific bacterial strains. In a study comparing the microbiome composition of *D. magna* and *D. pulex* in monocultures and co-cultures, the difference in microbiome composition between these two treatments were attributed to variations in species biomass. It was suggested that the species with the higher biomass could alter the bacterioplankton community to a larger extent, potentially through grazing or defecation (23). Our results align with this hypothesis, where the higher biomass achieved by the early arriving species plays a key role in altering bacterioplankton community composition in the medium. This mechanism is further supported by the study of Degans et al (2002) (50) who showed that high *Daphnia* densities (30 daphnids/ L) cause significant shifts in both the morphological and taxonomical composition of bacterioplankton communities through direct grazing. As such, the high *Daphnia* densities achieved as a result of early host arrival likely impacted the surrounding bacterioplankton community via differential grazing, providing a clear link between priority effects in hosts and cascading changes in bacterioplankton composition. Indirect effects of the zooplankton community via differential grazing pressure could likely also have induced changes in the phytoplankton community composition thereby imposing changes in the bacterioplankton community composition (51).

Additionally, a significant effect of host conditioned medium on bacterioplankton community composition was also observed (Table 2). This suggests that the host-specific conditioned medium applied did change the microbial composition. These changes were, however, not sufficient to reverse the effects of arrival order of the host species. Our results thus highlight the potential of zooplankton species composition to influence the structure and composition of bacterioplankton communities. It is important to note that these findings were obtained under highly simplified laboratory conditions, using media with low diversity bacterioplankton communities and standardized phytoplankton communities dominated by a single species. In nature, bacterioplankton community composition is strongly shaped by phytoplankton community composition (52–55) and due to a higher microbial diversity, may exhibit increased resilience to zooplankton-induced changes. Nevertheless, our results indicate that historical contingency (e.g., priority effects) in zooplankton communities can have cascading effects on other species in their ecosystem (here demonstrated for the bacterioplankton community), and thus influence downstream ecosystem processes such as nutrient cycling, organic matter degradation and microbial community resilience to environmental change. In addition, changes in the

bacterioplankton community, serving as a source of the microbiome, may in turn, influence the zooplankton communities themselves. If these interactions are predominantly negative, as observed in the present study, this could enhance diversity of the zooplankton communities by causing dominant species to suppress their own growth more than that of other species. Alternatively, if these interactions are positive, then a dominant species might enhance its own growth through its influence on the bacterioplankton community, potentially leading to alternative stable states driven by the arrival order of the zooplankton species.

References

1. Godoy O, Soler-Toscano F, Portillo JR, Langa JA. The assembly and dynamics of ecological communities in an ever-changing world. Ecol Monographs. 2024;94(4):e1633.

2. Fukami T, Mordecai EA, Ostling A. A framework for priority effects. J Veg Sci. 2016;27(4):655–7.

3. Stroud JT, Delory BM, Barnes EM, Chase JM, De Meester L, Dieskau J, et al. Priority effects transcend scales and disciplines in biology. Trends Ecol Evol. 2024 Jul 1;39(7):677–88.

4. Aoyama L, Shoemaker LG, Gilbert B, Collinge SK, Faist AM, Shackelford N, et al. Application of modern coexistence theory to rare plant restoration provides early indication of restoration trajectories. Ecol Appl. 2022;32(7):e2649.

5. Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat Rev Gastroenterol Hepatol. 2018 Apr;15(4):197–205.

6. De Meester L, Gómez A, Okamura B, Schwenk K. The Monopolization Hypothesis and the dispersal–gene flow paradox in aquatic organisms. Acta Oecol. 2002 Jun 1;23(3):121–35.

7. De Meester L, Vanoverbeke J, Kilsdonk LJ, Urban MC. Evolving Perspectives on Monopolization and Priority Effects. Trends Ecol Evol. 2016 Feb;31(2):136–46.

8. Leibold MA, Govaert L, Loeuille N, De Meester L, Urban MC. Evolution and Community Assembly Across Spatial Scales. Annu Rev Ecol Evol Sys. 2022;53(1):299–326.

9. Fukami T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. Ann Rev Ecol Evol Sys. 2015 Dec 4;46(Volume 46, 2015):1–23.

10. Debray R, Herbert RA, Jaffe AL, Crits-Christoph A, Power ME, Koskella B. Priority effects in microbiome assembly. Nat Rev Microbiol. 2022 Feb;20(2):109–21.

11. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006 Dec;444(7122):1027–31.

12. Brown EM, Clardy J, Xavier RJ. Gut microbiome lipid metabolism and its impact on host physiology. Cell Host Microbe. 2023 Feb 8;31(2):173–86.

13. Yoo JY, Groer M, Dutra SVO, Sarkar A, McSkimming DI. Gut Microbiota and Immune System Interactions. Microorganisms. 2020 Oct;8(10):1587.

14. Suzuki TA, Ley RE. The role of the microbiota in human genetic adaptation. Science. 2020 Dec 4;370(6521):eaaz6827.

15. McLaren MR, Callahan BJ. Pathogen resistance may be the principal evolutionary advantage provided by the microbiome. Phil Trans Royal Soc B: Biol Sci. 2020 Aug 10;375(1808):20190592.

16. Janssens L, Van de Maele M, Delnat V, Theys C, Mukherjee S, De Meester L, et al. Evolution of pesticide tolerance and associated changes in the microbiome in the water flea *Daphnia magna*. Ecotox Environ Safety. 2022 Jul 15;240:113697.

17. Obrestad K, Einum S, Vadstein O. Stochastic variation in gut bacterial community affects reproductive rates in the water flea Daphnia magna. FEMS Microbiol Ecol. 2022 Oct 1;98(10):fiac105.

18. Bulteel L, Houwenhuyse S, Declerck SAJ, Decaestecker E. The Role of Microbiome and Genotype in Daphnia magna upon Parasite Re-Exposure. Genes. 2021 Jan 7;12(1):70.

19. Varg JE, Outomuro D, Kunce W, Kuehrer L, Svanbäck R, Johansson F. Microplastic exposure across trophic levels: effects on the host–microbiota of freshwater organisms. Environ Microbiome. 2022 Jul 6;17(1):36.

20. Cooper RO, Cressler CE. Characterization of key bacterial species in the Daphnia magna microbiota using shotgun metagenomics. Sci Rep. 2020 Jan 20;10(1):652.

21. Kempf F, La Ragione R, Chirullo B, Schouler C, Velge P. Super Shedding in Enteric Pathogens: A Review. Microorganisms. 2022 Nov;10(11):2101.

22. Kim M, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H, Sasakawa C. Bacterial Interactions with the Host Epithelium. Cell Host Microbe. 2010 Jul 22;8(1):20–35.

23. Houwenhuyse S, Massol F, Macke E, De Meester L, Vanoverberghe I, Stoks R, et al. How does co-occurrence of Daphnia species affect their gut microbiome? Oikos. n/a(n/a):e10983.

24. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. PNAS. 2013 Feb 26;110(9):3229–36.

25. Foster KR, Schluter J, Coyte KZ, Rakoff-Nahoum S. The evolution of the host microbiome as an ecosystem on a leash. Nature. 2017 Aug;548(7665):43–51.

26. BOTTRELL H. A review of some problems in zooplankton production studies. Norw J Zool. 1976;24:419–56.

27. Vanvelk H, Govaert L, van den Berg EM, Brans KI, De Meester L. Interspecific differences, plastic, and evolutionary responses to a heat wave in three co-occurring Daphnia species. L&O. 2021;66(4):1201–20.

28. Louette G, De Meester L. Predation and priority effects in experimental zooplankton communities. Oikos. 2007;116(3):419–26.

29. Flössner D. Beitrag zur Kenntnis der Branchiopoden- und Copepodenfauna der Mongolei. Mitteilungen aus dem Museum für Naturkunde in Berlin Zoologisches Museum und Institut für Spezielle Zoologie (Berlin). 1986;62(1):3–40.

30. Benzie JA. Guides to the Identification of the Microinvertebrates of the Continental Waters of the World: Cladocera: the Genus Daphnia (including Daphniopsis). SPB Acad. Publ.; 2005.

31. Rasband W, ImageJ U, National Institutes of Health. Bethesda, Maryland, USA, 1997–2016. ImageJ[Google Scholar]. 2018;

32. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013 Jan 7;41(1):e1.

33. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581–3.

34. Bates D, Kliegl R, Vasishth S, Baayen H. Parsimonious mixed models. arXiv preprint arXiv:150604967. 2015;

35. Fox J, Weisberg S. Using car functions in other functions. 2019;

36. Lenth R, Lenth MR. Package 'lsmeans.' Am Stat. 2018;34(4):216–21.

37. Yarberry W. DPLYR. In: CRAN Recipes [Internet]. Apress, Berkeley, CA; 2021 [cited 2025 Jul 10]. p. 1–58. Available from: https://link.springer.com/chapter/10.1007/978-1-4842-6876-6_1

38. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al. vegan: Community Ecology Package [Internet]. 2024 [cited 2025 Jan 6]. Available from: https://cran.r-project.org/web/packages/vegan/index.html

39. Hobaek A, Larsson P. Sex Determination in Daphnia Magna. Ecology. 1990;71(6):2255–68.

40. Sison-Mangus MP, Mushegian AA, Ebert D. Water fleas require microbiota for survival, growth and reproduction. ISME J. 2015 Jan;9(1):59–67.

41. Peerakietkhajorn S, Tsukada K, Kato Y, Matsuura T, Watanabe H. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, Daphnia magna. Environ Microbiol Rep. 2015 Apr;7(2):364–72.

42. Matveev V. An investigation of allelopathic effects of Daphnia. [cited 2024 Dec 13]; Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2427.1993.tb00748.x

43. Burns CW. Crowding-induced changes in growth, reproduction and morphology of Daphnia. Freshwater Biol. 2000;43(1):19–29.

44. De Meester L, Boersma M, Spaak P. Environmental stress and local adaptation in Daphnia magna. L&O. 1999;44(2):393–402.

45. Seitz A. Are there allelopathic interactions in zooplankton? Laboratory experiments with Daphnia. Oecologia. 1984 Apr;62(1):94–6.

46. Helgen JC. Feeding rate inhibition in crowded Daphnia pulex. Hydrobiologia. 1987 Nov 1;154(1):113–9.

47. Goser B, Ratte HT. Experimental evidence of negative interference in Daphnia magna. Oecologia. 1994 Aug;98(3–4):354–61.

48. Burns CW. Effects of crowding and different food levels on growth and reproductive investment of Daphnia. Oecologia. 1995 Feb 1;101(2):234–44.

49. Guisande C. Reproductive strategy as population density varies in Daphnia magna (Cladocera). Freshwater Biol. 1993;29(3):463–7.

50. Degans H, Zöllner E, Van der Gucht K, De Meester L, Jürgens K. Rapid Daphnia-mediated changes in microbial community structure: an experimental study. FEMS Microbiol Ecol. 2002 Oct 1;42(1):137–49.

51. Verreydt D, De Meester L, Decaestecker E, Villena MJ, Van Der Gucht K, Vannormelingen P, et al. Dispersal-mediated trophic interactions can generate apparent patterns of dispersal limitation in aquatic metacommunities. Ecol Lett. 2012;15(3):218–26.

52. Niu Y, Shen H, Chen J, Xie P, Yang X, Tao M, et al. Phytoplankton community succession shaping bacterioplankton community composition in Lake Taihu, China. Water Research. 2011 Aug 1;45(14):4169–82.

53. Landa M, Blain S, Christaki U, Monchy S, Obernosterer I. Shifts in bacterial community composition associated with increased carbon cycling in a mosaic of phytoplankton blooms. ISME J. 2016 Jan 1;10(1):39–50.

54. Camarena-Gómez MT, Lipsewers T, Piiparinen J, Eronen-Rasimus E, Perez-Quemaliños D, Hoikkala L, et al. Shifts in phytoplankton community structure modify bacterial production, abundance and community composition. Aquatic Microb Ecol. 2018 Mar 21;81:149–70.

55. Richert I, Yager PL, Dinasquet J, Logares R, Riemann L, Wendeberg A, et al. Summer comes to the Southern Ocean: how phytoplankton shape bacterioplankton communities far into the deep dark sea. Ecosphere. 2019;10(3):e02641.

Supporting information

Effects on total biomass

Total biomass was tested using a generalized linear mixed model with a Gamma distribution and a log link function built using the *glmer* function of the *lme4* package (34). To account for the variability arising from the use of conditioned medium from corresponding replicates, the replicate number was added to the model as a random factor. Significance was tested with type III sum of squares using the *Anova* function of the *car* package (35). Significant pairwise interactions were identified with pairwise contrasts using the estimated marginal means with Tukey correction (emmeans package; (36)). For experiment 2, the main effects of inoculation order, conditioned medium and their interaction on total biomass was tested also using the above mentioned model.

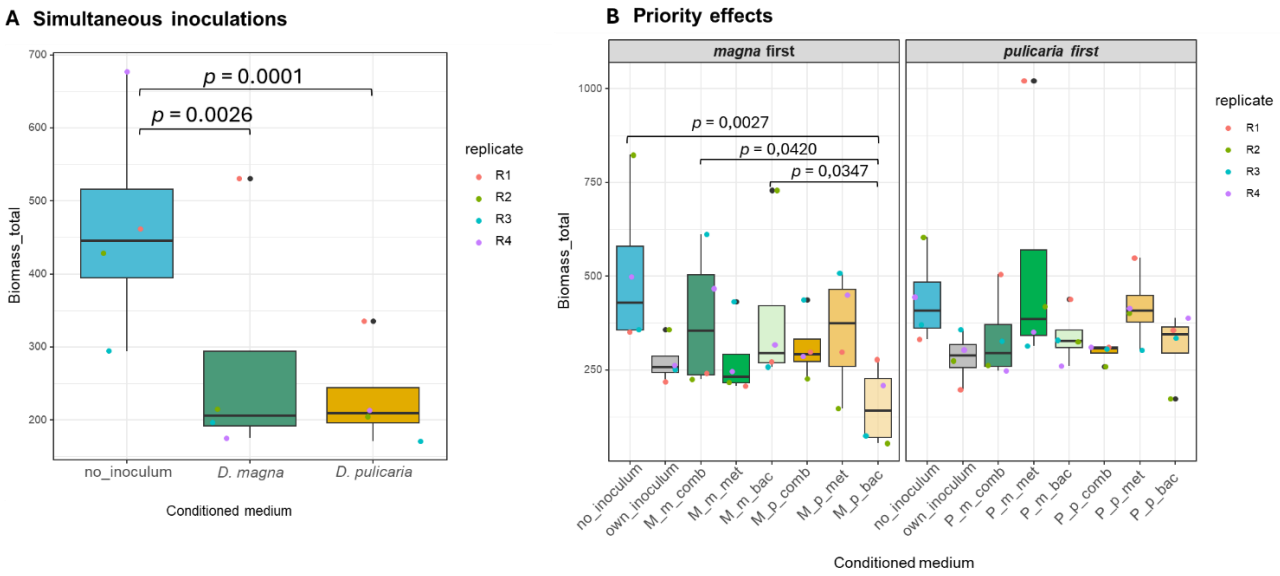

Figure S1: Total zooplankton biomass in (A) simultaneous inoculations (experiment 1) and (B) in the inoculation order treatments (experiment 2) across each of the conditioned-medium treatments compared to the no-inoculum control treatment.

In the test for competition, in the simultaneous inoculations (experiment 1), a significant effect of conditioned medium was observed for the total biomass. In particular, the total biomass was significantly higher in the control treatment in comparison to the *D. magna* (p = 0.0001) and *D. pulicaria* conditioned medium treatment (p = 0.0026) .

In experiment 2, no significant main effect of inoculation order on the total biomass of the communities established (p = 0.6008, Table 1) was observed, i.e., the inoculation order was not sufficient enough to bring about differences in the total biomass of the species established at the experimental end point.

However, there did occur a significant effect of conditioned medium for the total zooplankton biomass (p = 0.005652, Table 1, Figure 3C). In particular, in the *magna* first inoculations, significantly lower total biomass values were observed for the *pulicaria* bacterial fraction in comparison to the no-inoculum treatment (p = 0.0027), *magna* combined (p = 0.0420) and *magna* bacterial (p = 0.0347) treatments.

Table S1: Results of *glm* gamma model assessing the main effects of inoculation order, conditioned medium and their interaction effects on the total biomass of the *Daphnia* community in Experiment 1 and Experiment 2. Significant *p* values (<0.05) are indicated in bold.

Experiment 1: Test for microbiome effects on competitive outcomes			
	Chi sq	**Df**	**Pr**
Conditioned medium	19.856	2	**4.878e-05**
Experiment 2: Test for microbiome-mediated priority effects			
Inoculation order	0.2737	1	0.600892
Conditioned medium	19.9619	7	**0.005652**
Medium × Inoculation order	10.7724	7	0.148859

Impact of host-conditioned medium on bacterioplankton communities

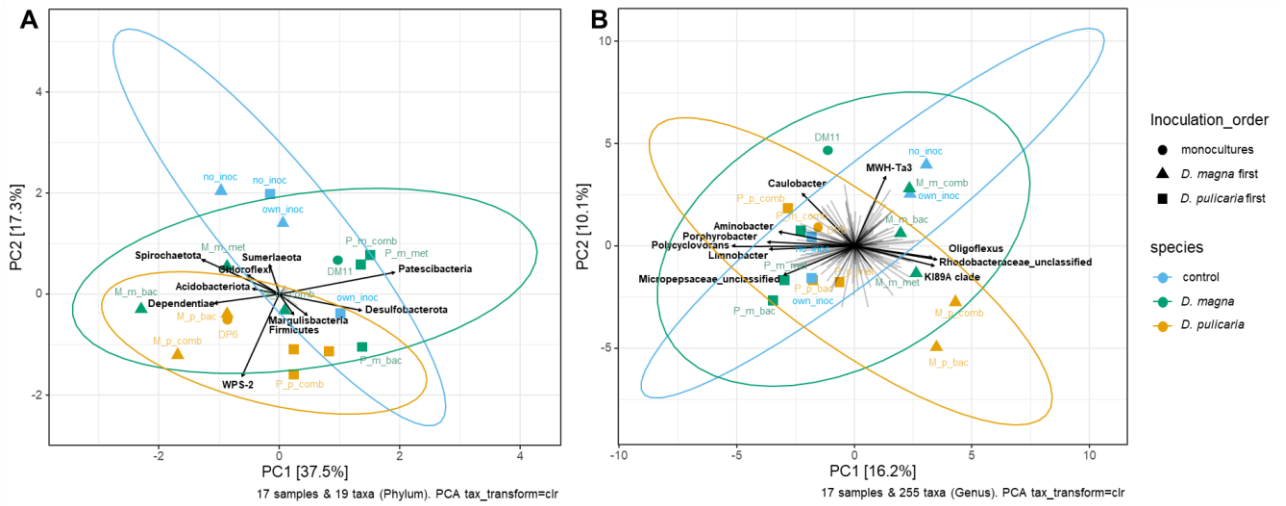

Figure S2: Differences in microbial community composition at the (A) Phylum level and (B) Genus level represented in compositional barplots and PCA plots (using CLR transformation) across samples grouped and colored by type of conditioned medium applied. In the PCA plots, each point represents a sample, with the colors indicating the inoculation order and shapes indicating the type of conditioned medium. The ellipses represent 95% confidence intervals for the community composition of each inoculation order. The black arrows represent the top 10 phyla contributing most to the variation along PC1 and PC2. The direction and length of the arrows reflect the correlation between each phylum and the ordination axes, as calculated using biplot scaling.

GENERAL DISCUSSION

General discussion

In this thesis, I aimed to investigate the role of species arrival order and timing, an event termed priority effects in the community assembly of the gut microbiome. I primarily made used of the freshwater cladoceran species, *Daphnia*, as a study system to explore the contribution of such processes to gut community assembly dynamics and monitored life history traits of this organism to further gain a deeper understanding on how such processes occurring in the gut could impact gut community composition and in turn contribute to fitness consequences in the host. Overall, my results underscore the need to integrate species arrival order and timing as explicit, testable factors into experimental design to better understand and predict the range of possibilities and conditions when gut microbiome assembly is more sensitive or resistant to historical contingency effects.

In the following paragraphs, I synthesize the main findings of my thesis and place them within a broader community assembly framework. The first objective of my thesis was to determine the core microbiome of *Daphnia*, identifying taxa that consistently inhabit this organism across diverse environmental conditions which are of potential relevance for experimental manipulation. Next, to understand how microbiome composition is shaped, I examine the role of species arrival order in gut community assembly. Specifically, I test how priority effects influence gut microbiome assembly by manipulating the introduction of strains known to occur in the *Daphnia* gut microbiome into the guts of germ free *Daphnia* and tracking colonization dynamics. In the next chapter, I allow the strains (used in chapter 2) to evolve to the gut environment of a new host genotype and test how within-host adaptation modifies gut microbiome assembly and priority effects. Lastly, in my fourth chapter, I expand the scope beyond the gut to examine how microbiome-mediated priority effects shape zooplankton community assembly and also explore the reciprocal effects of host arrival order on bacterioplankton community assembly.

Below I present the findings of my thesis in the order of my chapters, focusing on the (1) composition of the *Daphnia* microbiome (2) the role of priority effects in *Daphnia* gut microbiome assembly (3) the impact of evolutionary adaptation on these dynamics and (4) the bidirectional interactions between host and environmental microbial communities. I conclude by discussing the application potential of these findings in microbiome-based interventions and propose a framework for integrating priority effects into experimental design and the design of therapeutics. Lastly, I shed light on uncharted territories where such processes may play a role but have not yet been studied, pointing to future directions for research.

Linking microbiome composition, assembly history and host dynamics in *Daphnia*

The Daphnia microbiome is highly flexible and environmentally derived

With the emerging use of *Daphnia* as a model system for the study of host-microbe interactions, there has been a significant expansion in our understanding of its (gut) microbiome. Despite this advancement, most studies to date have focused on the response of the (gut) microbiome to specific environmental stressors such as temperature, parasite exposure, toxic diet, use of antibiotics etc. (1–5) or its role in the adaptive potential of the host under specific conditions (6–8). As a result, our knowledge of the *Daphnia* (gut) microbiome has primarily centered around condition-specific microbiome shifts and the enrichment of particular taxa that confer fitness benefits to the host in these contexts. In contrast, a fundamental understanding of the baseline microbiome, specifically whether a core set of taxa exists or not for this model system, remains unresolved and highly debated (9). Although an attempt was made by Freese et al (2011)(10) to experimentally characterize the microbial community composition within the digestive tract of *D. magna*, their study was limited to laboratory-reared clones and did not account for broader ecological variation. To address this gap, the first goal of my thesis was aimed at characterizing the core microbiome of *Daphnia* across diverse environmental and experimental contexts using published data. The results revealed that a large portion of the *Daphnia* microbiome was highly flexible, with only a few conserved taxa that consistently occurred across samples. This is in alignment with a previous study by Eckert et al (2021) (11) where they showed that the microbiome of freshwater zooplankton varied strongly with their external environmental conditions. Specifically, for *D. magna*, a rise in temperature explained 14% of the observed microbiome variability, while laboratory culture conditions explained a striking 63% of observed variation. These observations underscore the significance of environmental seeding, highlighting the critical role of environmental bacteria as the primary source of inoculum for *Daphnia*. This idea was further supported by Mushegian et al (2020) (12), who demonstrated that vertical transmission of bacteria from mother to offsprings via resting eggs was insufficient for normal hatchling success. Horizontal transfer of bacteria from the environment proved more beneficial for *Daphnia*. Despite this strong environmental input, the microbiome of *Daphnia*

is not identical to that of its surrounding water, suggesting that some form of filtering mechanism or active selection occurs within the host (10,11,13).

Despite the high variability observed in the *Daphnia* microbiome, *Limnohabitans sp*., belonging to the family *Comamonadaceae*, emerged as the most prevalent and dominant taxon, especially under laboratory conditions. This was in line with the findings of Freese et al (2011) (10), who reported the dominance of Betaproteobacteria in the gut bacterial community of *D. magna*, specifically with a constant presence of *Limnohabitans* sp. This finding also aligns with the results of Eckert et al (2021) (11), who reported *Limnohabitans sp*. as the sole core taxon across diverse freshwater zooplankton (rotifers, copepods and cladocerans). This minimal core in *Daphnia* (under laboratory conditions) is even smaller than that of caterpillars (14) which are typically considered to lack a stable, resident microbiome. However, unlike caterpillars, *Daphnia* appears to benefit from or even depend on the presence of a microbiome as demonstrated by positive effects of microbial associations on *Daphnia* fecundity, mortality and population size (15,16). This is in contrast to caterpillars, which are not dependent on microbial partners. Furthermore, the occurrence of the family *Comamonadaceae* has consistently been observed in *Daphnia* across a wide range of studies (10), which may point towards a potentially symbiotic relationship with the host. For instance, studies show that *Limnohabitans* sp. are capable of increasing fecundity and population size in *Daphnia* (16,17). Likewise, *Limnohabitans* have also been detected in the filtering combs of *Daphnia* (18), where they may benefit from the continuous influx of nutrients and organic compounds generated by *Daphnia's* filter feeding activity. Additionally, the occurrence of this family is not exclusive to *Daphnia*, but has also been detected in rotifers which suggest that the association might not be taxon-specific, but rather driven by shared ecological traits such as filter feeding and gut environments rich in phytoplankton, that create favorable niches for these bacteria. As such, microbial degradation of organic matter in the zooplankton gut may simply mirror that in the surrounding aquatic environment.

In contrast, the limited data in my literature-based survey on animals isolated from the field indicated that the *Daphnia* microbiome under natural conditions exhibited a highly diverse community, comprising a broader range of taxa that were equally prevalent and dominant. In these natural conditions, the prominence of *Limnohabitans* was notably reduced. The vast diversity of the field microbiome of *Daphnia* can largely be attributed to the heterogenous and dynamic conditions in natural lakes and ponds that experience fluctuating environmental conditions (e.g. temperature, nutrient availability, seasonality and microbial species pool) that allow for a wider and richer assortment of bacterioplankton community that can be taken up by *Daphnia*. This contrasts sharply from laboratory settings that instead maintain controlled homogenous abiotic parameters (such as temperature, light intensity, photoperiod, diet, culture medium) that may create a narrower microbial landscape, favoring the dominance of fast growing, opportunistic taxa such as *Limnohabitans* (19). As emphasized in chapter 1, the dominance of *Limnohabitans* in laboratory settings can thus alternatively reflect opportunistic growth under reduced microbial competitive dynamics and lower overall diversity, rather than specific mutualistic relationship with the host.

This observation thus tempers the conclusion that *Limnohabitans* is a universal core taxon in *Daphnia* and highlights an important limitation in my study, namely that the microbiome under controlled laboratory conditions may not reflect actual conditions of the natural environment. Thus conclusions drawn from laboratory studies may not be directly applicable to natural settings and warrants caution in interpretation. This is analogous to the general problem of reliance on *in-vitro* versus *in-vivo* studies in biological research, where the complexity and diversity of biological gradients as they occur in nature is often difficult to replicate in laboratory controlled systems. The limited inclusion of field data (only one field study in my analysis) restricts the generalizability of my findings, as they may reflect site specific conditions. While the diversity of gut microbiome communities and divergence of their composition among individuals varied much more in field-collected than in laboratory-grown animals, my findings may actually be a vast underestimation of the real diversity in gut microbiome composition in nature. Future studies should prioritize expanding sampling across diverse habitats and incorporate experimental designs in natural settings to better capture microbiome dynamics under natural environmental variability. Additionally, with the advancement and integration of multi-omics approaches in *Daphnia* research (20,21), future studies could focus on identifying a functional core microbiome based on the presence of specific genes, gene families and pathways provided by the microbiome and its association with host traits, to provide a deeper understanding of the specific roles of microbial taxa in host functional capacity and overall biological function.

In conclusion, my findings reveal that the microbiome of *Daphnia* is highly flexible, varying with the external conditions of its environment. The surrounding environmental species pool serves as an important inoculum source for *Daphnia* enabling it to acquire a diverse range of microbial taxa via active and passive selection. This is also

exemplified by the low diversity microbiomes observed under controlled laboratory conditions, typically dominated by *Limnohabitans*, in contrast to the high microbial diversity observed in field-grown animals. However, this acquisition is not passive. Despite being a generalist filter feeder, there seems to be a selective uptake or establishment of bacterial taxa in the *Daphnia* gut, and the gut microbiome composition thus reflects an hitherto insufficient studied interaction between the "supply" of microbial taxa in the environment and the selective uptake and establishment success of bacterial taxa in the gut, likely via a combination of processes linked to differential growth of bacteria (due to niche or functional differences), processes driven by the host (e.g., antimicrobial peptides), processes such as priority effects, and chance events.

Priority effects in gut microbiome assembly: Does arrival order really matter?

The role of historical contingency in gut community assembly remains a fairly underexplored part of literature. In general, there is more focus directed towards deterministic patterns and processes in community assembly, because they are more amenable to general predictions (22). If priority effects are important, it leads to a component of randomness in community assembly, even if the processes following colonization events are rather deterministic in nature. Priority effects are therefore difficult to study, often due to lack of information on species arrival history, compounded with the unique nature of historical events, specific to each community setting. This makes it difficult to integrate priority effects within a general predictive framework (22). While studies indicate that such processes may be particularly relevant in shaping gut community assembly, there remain limited empirical data to support this hypothesis (23). To date, we know of only one study where the inoculation order of strains was manipulated specifically in the gut to test for the impact of colonization history on gut microbiome assembly dynamics; this study used mice as an experimental model (24). Strong priority effects were observed in this study, where the community added first was observed to dominate, thereby dictating the trajectory of the final gut composition.

Such strong priority effects observed in the laboratory raise compelling questions about how priority effects may operate under natural settings. In lakes and ponds, *Daphnia* are exposed to temporally dynamic and spatially heterogenous bacterioplankton communities. The identity of early colonizers could vary depending on time in the season, environmental conditions and extreme events such as storms or droughts strongly impacting free-living bacterial communities. Additionally, microbes from the environment do not colonize the host all at once. Different microbial taxa are present in different local and regional abundances and change in abundance over time, as such, colonization events among host individuals might differ strongly, in part due to deterministic processes, in part due to chance events. Acquisition of microbes into the gut microbiome is therefore not limited to early stages, but rather a continuous influx of microbial species that either sequentially or simultaneously arrive in the gut. The order of their arrival and the state of the microbial community at the moment of their arrival can thereby strongly affect their establishment success and contribution to the community. Priority effects can potentially play a significant role in shaping gut microbiome diversity across individuals and populations, with potential consequences for host phenotype, performance and ecological interactions. Moreover, priority effects may affect how hosts respond to changing environmental conditions by affecting the degree to which hosts can acquire a microbiome that is best suited for the prevailing environmental conditions. The overarching goal of the experimental part of my thesis was thus aimed at dissecting the role of species arrival order in host gut community assembly.

To experimentally study priority effects in *Daphnia* gut microbiome assembly, I designed experiments that involve: (1) the use of germ-free animals, namely axenic *Daphnia* neonates that allowed me to mimic empty guts (i.e., habitat patches) suitable for monitoring community assembly from scratch , (2) the manipulation of species arrival order, (3) the characterization of gut community composition in adult animals using metabarcoding, (4) combined with an assessment of host fitness and life history traits.

In the first experiment (Chapter 2 of my thesis), I directly quantify the role of species arrival order and priority effects in *Daphnia* gut microbiome assembly. Given the flexible nature of the *Daphnia* microbiome (see Chapter 1), I selected strains that were directly isolated from the gut of *Daphnia* of a given habitat in the neighborhood of Leuven, Langerodevijver. More specifically, I used strains that were isolated from the gut of field-collected animals of clone LRV4. I made use of 76 strains that were isolated by Dr. Shinjini Mukherjee, with the intention of forming a synthetic mock community representative of the natural microbial members inhabiting this particular *Daphnia* clone. By using naturally co-occurring strains isolated from the host gut, I aimed to capture essential members of *Daphnia's* natural gut microbial community, which I assumed would be superior to using a combination of artificial laboratory strains lacking a historical connection with the host. To investigate priority effects, I employed a pairwise colonization approach using

three strain combinations: two heterogeneric strain pairs (strains belonging to two separate genera) and one cogeneric pair (strains belonging to the same genera). This design allowed me to explore whether priority effects differ among strain combinations and potentially link it to niche differences, using membership of the same genus as a rough proxy. Moreover, the use of controlled pairwise interactions aided the design of strain-specific probes and primers enabling an absolute quantification of bacterial densities in the gut and relate differences in abundances to arrival order. While this simplified and reductionist approach provides a methodologically powerful and robust framework to test for priority effects in the gut with high resolution, it also introduces certain limitations. One limitation of my work is that I focused on pairwise bacterial interactions, which lacks the complexity and community context typical of natural microbiomes. My approach did not attempt to mimic the complexity of microbial exposure sequences experienced by *Daphnia* in their natural environments, but rather aimed to provide proof of concept on the presence, extent and variation of priority effects in gut microbial assembly. While focusing on pairwise combinations of bacteria in germ-free animals enables absolute quantification of bacterial densities in the gut and clarity in identifying specific interaction outcomes, it does not accurately capture the complexity of microbial exposures experienced in nature. Microbial communities in freshwater environments typically consist of dozens to hundreds of taxa arriving simultaneously or in overlapping waves. Whether the priority effects observed under controlled conditions scale up to multi-species introductions, specifically in a community context, and whether they persist over longer time-scales or in fluctuating environmental conditions remains an open question.

The results of my experiment demonstrates that priority effects do indeed affect microbiome community assembly, but that the strength and direction of priority effects are host-genotype dependent and strain-specific. The outcomes of varying inoculation order can differ significantly depending on the identity of the bacterial strains used as well as the identity of the host genotype. These priority effects at the level of gut community assembly also translated into changes in life history trait of the host, although these effects were moderate. Changes in gut composition imposed by species arrival order led to variations in *Daphnia* body size and reproductive output across inoculation order treatments. These findings indicate that species arrival order can indirectly impact host phenotype and fitness by bringing about changes in gut microbiome composition. An interesting observation is that, in general, the genotype from which the microbial strains were isolated profited more from the presence of bacterial strains than the other genotype I tested. Because I only used two host genotypes, this observation remains to some extent anecdotal, but points to an interesting future experiment in which this indication of local co-adaptation of host genotype and microbiome could be tested in a more extensive set of host genotypes.

One the one hand, with priority effects being observed in gut community assembly, my experimental results open up intriguing implications. Yet, given the strength and even direction of priority effects being very dependent on the identity of the strains used and of the host genotype used, this may reflect genotype × genotype interactions. While this is an important observation, it also makes predictions and generalizations difficult, as this does not only show the importance of historical contingency on community composition, but also shows that its nature is very context dependent.

The fact that I observed only moderate effects on host life history traits is likely the result of my approach in selecting bacterial strains. While theoretically this approach to isolate a mock community from field-collected animals was ideal to achieve relevance for my experimental results, a key disadvantage of using strains isolated through culture-dependent methods is that such approaches may not always result in the isolation of the most important species from the community. Culture-based techniques often isolate bacteria that are readily cultivable, and this may not be the taxa that contribute most to host fitness or are central to the development of a healthy microbiome. As a result, while the strains used in my study are arguably more ecologically relevant than artificial communities with no evolutionary history with the host, the taxa I used may still fail to reflect key species that may have a strong functional impact on the host. Given that strains were isolated from field-collected animals, *Limnohabitans* strains, which have been shown to have a strong effect on host fitness (15,17) were not isolated. Its absence during the isolation process may likely occur due to the reliance of *Limnohabitans* on low nutrient conditions and their specific growth requirements that are often difficult to replicate with typical culture-based artificial mediums (25–27). The inclusion of strains such as *Limnohabitans* and *Aeromonas* (16) with demonstrated positive effects on *Daphnia* fitness could be tested in future research. I did however, use the most dominant microbial genera isolated from the *Daphnia* LRV4 clone for my experiments, at least when judged on their representation in cultures, namely *Rhizobium* and *Microbacterium* sp.. Alternatively, the moderate impact of priority effects on host life history traits could also result from insufficient establishment of the inoculated strains in the gut. One notable biological feature of *Daphnia* that can influence microbial colonization in its guts

includes the process of molting. Throughout its lifespan, *Daphnia* molts numerous times, allowing for their growth and development, typically passing through 4 to 6 juvenile instars (28); with adults molting every 72-96 hours and juveniles every 36 hours (29). Each molt involves shedding the entire outer cuticle layer, including the exoskeletal lining of the foregut and hindgut, potentially disrupting or eliminating established bacterial populations attached to these surfaces (30). Further, the periodic shedding of the peritrophic matrix in the midgut can also contribute to a reduction in microbial load (31). Together, these physiological processes likely diminish microbial populations in the gut thereby resulting in only moderate impact of the inoculated strains on *Daphnia* life history traits.

While my chapters focus on early colonization phases, the long-term stability of these effects i.e., whether these effects are transient or long lasting are not yet fully studied. In my study, I assessed community composition at two time points, namely Day 8 and Day 12 of the *Daphnia* life cycle. These represent key phases in the life history of *Daphnia*, i.e. close to maturation and at the transition of the first and second adult instar (i.e. release of the first clutch) (32). While it might be worthwhile to extend the study to longer time spans and especially investigate transgenerational effects, my results do indicate that priority effects early in life can have lasting effects on the gut microbiome in adults.

One potential extension of my work would be to expose adult *Daphnia* with a well-characterized gut microbiome to changes in diet, environmental conditions, and surrounding free-living microbial communities, to test the stability and resilience of the microbiome community that colonized the gut early in life. Research on human guts (33) and mice (34) indicate that microbiome community responds strongly to dietary changes, which is logical given that different microbial taxa may thrive under different nutritional conditions. However, whether the gut microbiome composition shifts in response to changes in the surrounding free-living microbial community without any dietary alteration remains unexplored. More in general, longitudinal designs could further help determine whether early colonizers maintain dominance or are eventually displaced over time. Finally, functional perspectives are needed to complement taxonomic analyses to understand how and under what conditions early colonizers influence community assembly. Functional analyses may also indicate to what extent the underlying processes of priority effects involve modifying the gut environment (niche modification, either physically by producing a biofilm, or chemically, or through modifying host physiology) or pre-empting key resources (niche pre-emption).

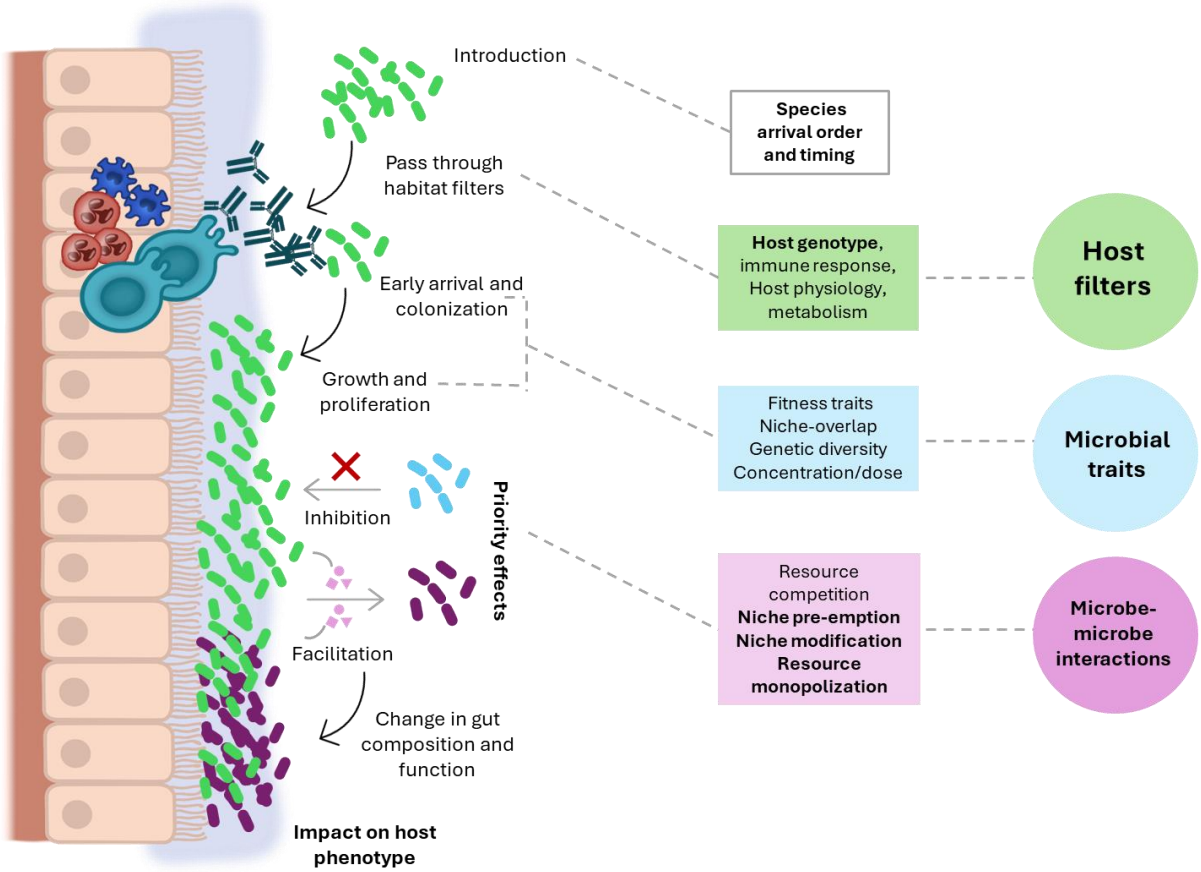

Figure 1: Scheme illustrating the key factors that influence microbial colonization and establishment in the gut: (1) host factors (2) microbial traits and (3) microbe-microbe interactions. Microbial cells must first pass through host filters, which include the host immune response, gut physiology and metabolism, all of which shape the gut environment and can restrict colonization. Microbes that overcome these barriers must possess favorable traits that allow their survival such as high fitness, niche compatibility, genetic adaptability and sufficient inoculation dose. Once inside, colonizing microbes are further shaped by microbe-microbe interactions including competition, mutualism, antagonism etc.

The impact of evolutionary adaptation on priority effects

In Chapter 3, I studied the role of within-host adaptation of microbiome community strains in mediating priority effects during gut microbiome assembly. Given the high capacity for rapid evolution of microbial organisms, owing to their large population sizes, short generation times, rapid mutation rates and their ability to undergo horizontal gene transfer, I wanted to test the possibility that rapid adaptation to a new host environment would affect priority effects. I performed an experimental evolution trial, by introducing bacterial strains isolated from one host genotype (LRV4, the same strains as used in Chapter 2) into the gut of another *Daphni*a host genotype, clone O1-08. Over a 12-day period, these strains were grown in isolation and allowed to adapt to the gut environment of their new host. To test for priority effects, the arrival order of the pre-exposed (evolved) strains and their ancestral counterparts were in a follow-up experiment manipulated in the gut of germ-free animals of both their new host and ancestral host genotype. If rapid adaptation would strengthen inhibitory priority effects of the pre-exposed (evolved) strain or reduce inhibitory priority effects of the competing strain, this would imply that sufficiently long time periods between the first and second colonist of *Daphnia* guts might affect community assembly not only through ecological niche-preemption or niche modification, but also through evolution-mediated effects.

The impact of evolution was ambiguous and deviated from expectations. In alignment with my observations on ecological priority effects (Chapter 2), the impact of evolution on performance of the bacterial strains in the two host genotypes and their competitive strength were strain- and host genotype-specific. For the *Rhizobium* R2_7 strain, no differences in population growth and population densities were observed between the ancestral and pre-exposed (evolved) populations, neither in the original nor in the new host genotype. For *Microbacterium* R2_8, again no effect of evolution on performance of the pre-exposed population was observed in the new host genotype. This suggests that for both strains, a 12 day adaptation period was insufficient to bring about pronounced and detectable evolutionary changes in performance in the new gut environment.

Although there was no evidence for adaptive evolution for any of the pre-exposed (evolved) populations in the new host genotype, a strong effect of evolution was noted for the pre-exposed *Microbacterium* population in the original host genotype. When re-introduced into their original genotype, the pre-exposed *Microbacterium* population exhibited significant decline in density in the gut relative to the ancestral strain. This suggests that during growth and adaptation to the gut environment of the new host, the population lost some traits that were beneficial for colonization and growth in the gut of its original host genotype. So although no adaptive evolution was observed in the new host genotype, potentially due to the exposure time being too short, this loss of adaptation to the original host points to the fact that this bacterial population was actually adapted to its host genotype and lost this adaptation when exposed to a novel environment. This observation also suggests that the loss of evolved fitness benefits to a host can go faster (i.e. can be observed within a period of 12 days) than the genetic adaptation to a new host.

My study has some methodological limitations, which potentially may have affected the outcome. One key limitation is the time that was allowed for adaptation. The adaptation period of 12 days was chosen as this corresponds to the generation time of *Daphnia*, making the results particularly relevant for understanding priority effects that can occur within a single host's lifespan. The lack of evidence for adaptive evolution impacting priority effects is an important insight, as my results indicate that a few days of residence in the gut is insufficient for adaptive evolution to strongly affect community assembly. This implies that evolution-mediated priority effects (35,36) may not be important in the gut assembly of individual *Daphnia*. It would, however, still be highly relevant to investigate the possibility of such effects over longer periods of adaptation. Many organisms have considerable longer generation time than *Daphnia*, allowing for more extensive evolutionary dynamics within their guts. In humans, for instance, evolution over a few months or even years would potentially have a strong effect on gut microbiome composition (37–40). Even in *Daphnia*, evolution over longer time scales can be important, at two levels. First, *Daphnia* reproduce clonally, and individual clones can often persist for an entire growing season or even multiple seasons in permanent habitats that provide refuge

during harsh seasons. To the extent that microbial strains can be transmitted vertically (9) or via the medium (see Chapter 4), evolution might still influence community assembly in later generations of the same clone. More broadly, co-evolutionary dynamics between the *Daphnia* host and microbial communities might still be very important at the population and habitat patch level. There are indeed suggestions that microbiomes might be locally adapted to their host population (6), and our observation that the *Microbacterium* strain lost adaptation to its ancestral genotypes reflects the importance of evolution towards performance of the microbial strains. In future work, one should allow for longer periods of adaptation, which could be achieved through serial passage over multiple generations of the host.

One other limitation of my study is that the conditions offered in the experimental evolution trial were slightly different from those in the priority effects experiment. More specifically, sterile live algae was supplied as food during the experimental evolution trial to avoid interference of bacteria associated with algae with our observations. But during the priority effects experiment, autoclaved (dead) algae was supplied as feed for *Daphnia*. This is an important limitation, because food quality of autoclaved algae for *D. magna* is much lower and suboptimal for their fitness (16,41). A third and likely important methodological limitation of my study particularly with respect to testing of community assembly, competitive strength and priority effects, is that during adaptation to the new host, the bacterial strains were not inoculated in a thriving microbiome but were instead grown in isolation, in sterile *Daphnia*. While this approach ensures a clean experimental design and theoretically allows for the most rapid evolution to the host genotype, it presents two main drawbacks. First, since a microbiome consisting of only one species is undoubtedly suboptimal, this setup may limit the capacity of the bacterial population to thrive in what would be its "normal" habitat, creating an extreme and artificial situation. Second, by inoculating the strains in isolation, we risk the loss of traits that are essential for competitive ability. A strain may rapidly adapt to the new niche potentially at the cost of traits that are important for competitive interactions. As a result, the strain becomes weaker or competitively inferior in experiments involving community assembly and inter-species competition, thereby also affecting priority effects. This is in line with findings by Lawrence et al (2014) (42) who reported the rapid loss of competitive ability in bacteria, and thus highlights a key consideration when designing eco-evolutionary experiments involving community assembly. Vanvelk et al (2024)(43) also observed a similar loss of competitive ability when *Daphnia* populations were allowed to adapt to warming in monocultures. Together, these findings point towards the need to design evolutionary adaptation experiments to novel environments in a proper community context. However, this can be very challenging, as it requires standardizing the community composition while avoiding strong species sorting or even species exclusion during this selection period. For example, in the study of Vanvelk et al (2024) (43), selection in the presence of a community was initially planned, but failed due to the rapid dominance of a single species in all communities.

Beyond the host: cascading priority effects in bacterioplankton communities

While Chapters 2 and 3 of my PhD thesis focus on the role of microbial strain arrival order in shaping the *Daphnia* gut microbiome, my work presented in Chapter 4 expands this perspective by demonstrating that host arrival order itself can generate priority effects that can have cascading effects on the composition of surrounding microbial communities. These findings contribute to a growing recognition that hosts are not passive recipients of microbiota but can also influence microbial landscapes in their immediate environment. In this context, historical contingencies at the level of the host community, such as the order in which host species arrive in a given patch, can influence community assembly of the bacterioplankton community. The priority effect in the host community then "extends" to also influence community composition of an entirely different community ("*cascading priority effects*"). Host species arrival order thus *externally engineers* free-living microbial communities. This might then potentially feed back to influence host fitness and host community structure.

While a feedback of the altered free-living communities on host community assembly is conceivable, the results of the experiment outlined in Chapter 4 do not show that manipulating the free-living bacterioplankton community can alter priority effects at the level of the host community. This leads to two conclusions. First, the priority effects that we observe at the level of the host community and were shown in earlier studies (44) are not driven by conditioning of the medium by host-specific microbiomes as a niche-modification mechanism. Second, it shows that the priority effects between *Daphnia* species are resilient to strong manipulations of the free-living bacterioplankton community in their medium. While priority effects were not reversed, it is noted that the manipulation with conditioned medium, changing either metabolites, the free-living bacterioplankton community or both, did have some effects on competitive interaction between the two host species we studied. In summary, the identity of the free-living bacterioplankton community can

induce changes in competitive interactions between host species, but these are rather subtle and not strong enough to change dominance patterns established through arrival order of species.

One important methodological limitation of my experiment is that I only replaced 25% of the medium with conditioned medium of choice in the conditioned medium treatments. In hindsight, this does not provide us with the strongest possible test, and it would have been more optimal to replace all the medium. For a lack of conditioned medium, this was not possible in the current experiment, but should be done in future work, as it is difficult to assess whether a replacement of 25% of the bacterial community is sufficient to counteract conditioning by the dominant species. Future research should aim to test the effects of host conditioned medium at higher concentrations to assess to what extent microbiome-mediated effects influence community assembly dynamics. Additionally, the experiment could be further repeated with the use of germ-free animals to more directly test for the role of the microbiome in driving these effects.

Integration across experiments

Overall, my results demonstrate strong priority effects in gut community assembly that are specific to both microbial strain identity and host genotype. While my work highlights in several instances the importance of evolution at the level of the host (host genotype effect) and bacteria (indications for host-strain co-adaptation, some influences of microbial evolution on competitive outcomes), I did not find evidence that microbial evolution upon colonization of a host gut influences priority effects. Similarly, I found no evidence that medium conditioning by the microbiome alters priority effects; however, I did observe that medium conditioning can affect host community assembly and competitive abilities. Additionally, I observed that host priority effects driven by host arrival order can cascade down to influence the assembly of free-living microbial communities. My findings suggest that priority effects are highly contingent on specific conditions, but at the same time they also reveal that the ecological priority effects that can structure *Daphnia* communities are quite resilient, as they seem not to be influenced by substantial disturbances in the free-living bacterial communities. As outlined in the paragraphs above, there are several improvements possible to the experiments I performed, and there is much scope for research on priority effects using more complex microbial communities, examining evolution over longer time spans, applying stronger manipulation of bacterial communities in the medium, and elucidating the precise mechanisms leading to priority effects both within the microbiome and at the level of the host community, and how these priority effects on interacting communities can interact with each other (45).

Translational potential

Applications: pre-biotics, probiotics, synbiotics, FMTs and recombinant microbial therapeutics

With the crucial role of the microbiome established in disease and host health, the gut microbiota has become a compelling target for the design of therapeutic strategies and interventions aimed at addressing dysbiotic patterns and restoring homeostasis in the host. This has resulted in the rise of microbiome-based therapeutics, which can generally be categorized into three groups based on their mode of action: additive, subtractive or modulatory therapy (46). As the name suggests, additive therapy involves the addition of beneficial live species into the host microbiota. Subtractive therapy aims at eliminating specific harmful and pathogenic members from the host microbiome. And modulatory therapy involves the use of non-living agents such as prebiotics to alter/modulate the composition and function of the existing microbiome (46). These interventions can result in a range of outcomes, from small temporary alterations in community composition and metabolic output to a more permanent and widespread impact on microbiome structure and functional capacity. Early strategies relied on the oral administration of live microorganisms (46) in the form of pure cultures or mixed communities, also known as probiotics or live biotherapeutics. More advanced approaches include Fecal Microbiota Transplants (FMTs), which involve the complex transfer of stool components from healthy donors into the gut of diseased individuals (47). Additionally, the use of synbiotics, i.e., a combination of probiotics and prebiotics have also gained popularity over the years to enhance the survival of live microbial components in the gastrointestinal tract (48). These interventions have further evolved to next-generation microbiome therapeutics, which involve recombinant probiotics (strains that have been genetically engineered to express therapeutic biomolecules), designed microbial consortia, and selective antimicrobials (46).

Despite substantial evidence supporting the beneficial effects of such interventions for gut health (49), several studies report the failure of such cells to colonize, exhibiting only short-term persistence and minimal impact on resident microbiome composition (46,50). This suggests that the success of these therapeutics is not always guaranteed. Stable colonization and establishment in the gut may not always be a requirement, for example in the case where strains (eg.

Lactococcus lactis) can exert their intended effect during their transit through the gastrointestinal tract (51,52). For other treatments, however, stable establishment into the endogenous microbiota is a strict criterium. The failure of such therapeutics is often attributed to natural colonization resistance conferred by native members of the gut towards incoming immigrant microbes, reducing their establishment success. Colonization resistance in the gut can be driven by typical habitat filters of the host gut environment (e.g., high stomach acidity) and host immune response (53). In addition, failure to establish can also be the result of resistance exerted by the resident microbiome, either because of a strong numerical advantage, allelopathic effects or altered niches (54). Priority effects in microbiomes have been coined the founder hypothesis in microbiome ecology (55) and predicts the failure of incoming microbes to replace existing resident communities in a habitat due to the priority access gained by the founding occupants to growth limiting resources that grant competitive advantage (55). The incoming immigrants are in direct competition for available niches and nutrients with the indigenous resident microbiota, which limits their scope to establish and replicate in the gut to sufficient densities (56). A well known example is that of *Bifidobacterium* spp. that arrive in the human gut soon after birth. Due to their ability to break down a wide range of human milk oligosaccharides (HMOs) found in breastmilk, they deplete the intestinal lumen of these resources, thereby preventing late immigrants from colonizing (23,57,58). Next to this niche-preemption mechanism employed by primary colonists, the resident community may also excrete metabolites and as such create an environment that is less hospitable for immigrants, which translates into a niche modification mechanism (54). Studies have also shown that the indigenous gut microbiota through intrinsic metabolic activities can create conditions that modify the gut environment, inhibiting the growth and virulence expression of invading pathogens. For instance, the production of short chain fatty acids (SCFAs) suppresses the growth of pathogenic *Enterobacteriaceae* such as *Salmonella* spp. and *E. coli* O157 (59). Similarly, native commensals have been shown to produce bacteriocins with bactericidal activity against pathogens such as *Clostridium botulinum*, *C. perfringens*, *Listeria monocytogenes* etc. (60). Given the likelihood of strong priority effects, the timing and frequency of introduction of therapeutics such as pre-biotics, probiotics and synbiotics are critical factors that ought to be considered to optimize their outcomes. A thorough understanding of priority effects is thus warranted to better inform the underlying ecological interactions that can further ensure the success and efficacy of such microorganism-based interventions.

Framework to integrate priority effects in the design of microbiome-based therapeutics

To fully realize the potential of microbiome-based therapeutics in restoring and modulating the gut microbiome, it is essential to consider the role of possible priority effects and the consequences of alternative assembly trajectories. Current approaches largely focus on the colonization success and functional impact of the introduced strains, yet often overlook how the order, timing of introduction, dosage and temporal frequency of introduction may influence colonization outcomes - factors all closely linked to priority effects in microbial community assembly dynamics. Thus, in the following paragraphs, I dissect current intervention protocols and integrate them with the findings of my study to propose a framework to systematically inform the design of more predictable and effective microbiome-based interventions.

I here refer to the framework proposed by Walter et al. (2018) (50) to discuss the principles that need to be studied to increase the success of microbiome therapy. This framework organizes the assembly process into four sequential ecological factors, namely, dispersal, habitat filtering, establishment and functional engagement. This model emphasizes that introduced microbes should first be delivered in sufficient doses in a viable and metabolically active state (dispersal), must overcome host-associated biotic (immune response) and abiotic (acidic environment of the stomach, bile acids, pH) barriers of the host (habitat filtering/ selection), successfully establish in the presence of resident microbes (establishment) and engage metabolically with the host and microbial community to induce functional shifts (functional engagement). I propose that the existing framework be extended by integrating priority effects (PE) as an active ecological filter and embedding it into therapeutic design.

First, in the original framework, the dispersal phase focuses solely on the introduction of metabolically active cells delivered in adequate doses into the gastrointestinal tract. I propose a PE-informed expansion, wherein arrival order and timing could be treated as design variables. For instance, one could manipulate sequential introduction of strains into the gut to determine how order and timing of introduction influences colonization and downstream effects. Additionally, staggered dosing protocols could be optimized to precondition the environment for later strains or disrupt resident competitors.

With respect to the habitat filtering phase, emphasis has previously been placed on trait-based strain selection to select strains that possess properties that enhance survival in the host. For instance, Walter et al (2018) (50) emphasize that strains that share an evolutionary history with the host would have more chances to overcome habitat filters and compete for available resources with resident microbes. This is in alignment with my results of chapter 1, where I demonstrate that the strength and direction of priority effects vary with host genotype, indicating that host factors must be accounted for in designing microbiome interventions. For instance, most commercial probiotic strains (eg *Lactobacillus* and *Bifidobacterium* species) are allochthonous to the human gastrointestinal tract and often lack the traits necessary for stable colonization. In contrast, *B. longum subsp. longum* AH1206, autochthonous to the human core microbiome (61), has been shown to stably establish in a subset of humans for at least 6 months (62). The significance of joint evolutionary history of incoming microbes with the host is further supported by evidence that strains can better colonize germ-free mice when they originate from rodents compared to other hosts (63). This is also supported and extended to the level of within-species variation in host genotypes in my thesis in chapter 1, where the strains isolated from *Daphnia* clone LRV4 demonstrated a more significant impact on host life history traits such as body size and reproductive output when reintroduced into LRV4 than in another host genotype (O1-08), potentially due to a more stable gut colonization. Future probiotics may take the form of a consortium of long-term community residents: a fecal transplant in a capsule (64) to increase the chances of survival of the introduced microbial therapeutics. Additionally, this framework also underscores the need for genotypic and phenotypic diversity and plasticity of the introduced strains to enable rapid adaptation to the new environment (65) to better overcome host habitat filters.

The next step in the framework of Walter et al (2018) (50) is the establishment phase. Here, the framework highlights the need to study how interactions between immigrants and resident members govern the successful integration of new-comers into the gut community. This involves the study of biotic interactions such as competition, parasitism, mutualism, predation etc. Competition of resources is a key mechanism that determine species co-existence (66) and thus the success of colonization. One expectation is that communities with higher diversity and evenness are more resilient to invasions as they exploit resources more efficiently (65). Based on the findings of my thesis, the outcome of biotic interactions can, even in very simple two-strain settings, be very variable and context-dependent. Theory predicts that priority effects are overruled when immigrant species have a clear fitness advantage over resident microbes. In such a case, the more fit immigrant can outcompete the first-arriving species, by more effectively exploiting available resources in that environment. This underscores the need to assess the fitness of microbial strains in the gut environment of the host individual as a critical parameter when designing such therapeutics. To incorporate microbial fitness assessments into therapeutic design, competition experiments between resident species and candidate strains in a mimicked gut environment becomes necessary. Artificial anaerobic bioreactors or simulators of the gut environment such as SHIME® (Simulator of the Human Microbial Ecosystem, Ghent University - ProDigest, Belgium) and SIFR technology (Cryptobiotix, Ghent) could be employed to replicate gut conditions, enabling systematic testing of such dynamics to further assess which species or communities are more likely to establish successfully. When immigrant and resident species display similar fitness, priority effects can be pronounced, with the early colonizers gaining numerical advantage and reaching carrying capacity, thereby limiting the establishment success of newcomers. Overcoming colonization resistance may thus require the intentional introduction of strains with demonstratively higher fitness to ensure desired therapeutic output, or a (partial) removal of the resident gut community prior to the inoculation with the desired gut microbiome.

Niche overlap is another critical factor governing the strength of priority effects (22). Priority effects are expected to be stronger when niche overlap is high, i.e., when the immigrant and resident species compete for the same niche and resources, thereby engaging in exclusionary interactions. Depending on the goal of the therapeutic treatment, therapeutics should therefore be designed with niche information in mind to ensure factors such as niche-overlap and ecological compatibility between introduced and resident species. This ensures that the introduced organisms may potentially still gain access to the unoccupied niches in the gut and increase their probability of successful establishment.

My study demonstrates that priority effects are strain-specific. For instance, as shown in chapter 1, interactions between strains were not uniform, and can result in either an inhibitory or facilitative response depending on the specific type of strain combinations used. This suggests that therapeutic consortia should be carefully selected based not only on the individual strain performances but also on their ecological interactions with one another and with the resident gut community. To promote stable colonization, strain combinations and consortia should encourage cross-feeding and cooperative interactions that support a self-sustaining community, particularly under nutrient limiting conditions.

Simultaneously, strains should ideally also exhibit inhibitory effects against competitors in the resident gut community, while fostering facilitative interactions within their own community. By integrating principles such as microbial trait-based selection involving fitness differences, niche occupancy and overlap, host genotype and strain interactions, the resulting framework can refine the design of microbiome-based therapeutics to ensure more targeted, resilient and effective outcomes.

Lastly, in the functional engraftment step, the original framework emphasizes the need for the introduced microbes to achieve sufficient metabolic activity to influence host functions. I suggest a PE-informed extension, where we examine how sequence of colonization alters functional outputs such as SCFA production, bacteriocin production, pathogen exclusion or immune modulation. Ecological simulation tools could be employed to predict PE-based outcomes before clinical implementation (67) using input data such as microbial community profiles, host genotype, immune status, colonization timing and order and experimentally derived microbial interaction networks.

Priority effects in gut microbiome assembly: uncharted territories

Priority effects can in practice have an impact on any stage in life, because of the continuous influx of new taxa that can or cannot establish in the face of an existing resident community. Yet, priority effects may be particularly relevant during early developmental stages such as birth and weaning, precisely because they can potentially have lasting effects during the rest of life. During the initial stages or immediately after birth, the gut habitat is empty and open to colonization events. For instance, experimental studies in mouse models find that early life colonization order can influence the outcome of community assembly through priority effects (24). Differences in the trajectory of early-life microbe acquisition including mode of delivery, weaning practices, feeding method (breastfeeding vs formula), ICABS (cultural, ethnic and biogeographical differences), siblings, pets in the environment, use of probiotics and antibiotics, may have long term effects on microbiome ecology and shaping of the developing gut microbiota (23,68). A systematic testing of these effects in model systems might reveal generalities that can potentially inform health management. Importantly, while priority effects may hinder therapeutic management of gut microbiome composition, they can also stabilize a shift in community upon a successful therapy.

While priority effects might stabilize the composition of the gut microbiome composition to some extent, there is also the observation that the composition and structure of the gut microbiome is dynamic. The gut microbiome has often been shown to oscillate compositionally, varying from small fluctuations around a relatively stable community to complete shifts in community membership to alternate states over timescales ranging from hours to decades (69). These changes range from those occurring during specific predictable life stages to changes that are seemingly random or triggered by specific disturbance events or changes in, for instance, diet or habitat use (70–73). In these regards, concepts borrowed from community ecology, such as succession and temporal stability in community structure, may provide insights into factors driving the temporal variability within the gut community. Likely these have to be supplemented with dynamics that are to some extent more specific for microbial communities, such as waste networks, cross-feeding and complementarity in resource niches. Identifying how the balance between stability and dynamic responses in microbiomes plays out in host individuals and how it varies among patches and host genotype, is still an open field for research.

The microbiome often undergoes significant changes during transitional events across the life span such as metamorphosis, molting, and reproductive onset. It is plausible that these transitions can create critical windows of opportunity for manipulation of the microbiome (e.g. in the context of therapies), as ecological niches are transiently opened or re-structured, freeing up resources. These provide opportunities for invasion but also additional windows of opportunity for priority effects to manifest, and actively restructure gut community assembly. This mirrors natural disturbance events such as the creation of new habitat patches after calamities (74–76), where community assembly begins anew following the removal of most or all individuals (22). Despite growing recognition of the importance of temporal dynamics in microbiome research, priority effects remain a largely uncharted territory, especially during the later stages of life, which often involve physiological, hormonal, immunological and behavioral transitions that might reset or destabilize the microbiome. For instance, holometabolous organisms that undergo complete metamorphosis (77) experience a dramatic change as they transform from the larval stage to the pupal stage before emerging as adults. During the pupal stage, the insect's body undergoes extensive remodeling, wherein all larval organs are reorganized. One of the most significant reconstructions during this phase includes that of the gut, wherein the foregut and hindgut are rebuilt and their contents often completely purged from microbial taxa (78–80). In species such as *Bombyx mori* and *Drosophila melanogaster*, larval intestinal tissues degenerate through apoptosis and autophagy during pupation (81–84).

Similarly, in mosquitoes (Dipetera: Culicidae), bacterial abundance in the midgut peaks during the fourth larval feeding stage but declines sharply during the pupal and adult stages (85), suggesting a process of gut sterilization. This complete gut renewal and sterilization combined with profound anatomical and physiological transformations, host immune modulation and ontogenic diet shifts (80) open new ecological niches analogous to habitats formed post disturbance, providing ample opportunity for priority effects to influence microbial community assembly during these phases. A comparable process occurs during molting, when arthropods shed their exoskeleton and gut lining, disrupting existing microbial community and creating opportunities for recolonization. In mud crabs (*Scylla paramamosain)*, molting involves the periodic replacement of not just the old exoskeleton but also internal structures such as gills, foregut, the outer membrane of the hindgut and residual intestinal contents resulting in markedly reduced to no gut contents post molt (86). Similarly, crustaceans such as the giant freshwater prawn, *Macrobrachium rosenbergii* (87) shed a major component of their gut and associated microbiome during molting. This can be viewed as a window of opportunity where the order and timing of microbial colonization events may critically influence community assembly. It might also be a moment that can be used for the management of gut microbiome composition, for instance in the context of more efficient, healthy or sustainable aquaculture.

Likewise, in humans, transitional life events such as puberty, pregnancy and menopause represent critical windows during which the gut microbiome is especially vulnerable to physiological and hormonal changes in the host. Unlike classical ecological disturbances that are abrupt, unpredictable and externally induced shocks to the system that result in the wipe out of all or most individuals in the habitat, developmental stages are often interpreted as deterministic processes triggered by host physiology, which rather follow a gradual, predictable and internally programmed structure. In such a situation, the window of opportunity to overcome priority effects and install new ones is likely less strong compared to when external, abrupt disturbances occur. Yet, such developmental transitions in the host may still remodel the habitat enough to allow community reshuffling, which could create opportunistic windows for priority effects to occur and change the trajectory of the community. While developmental stages such as puberty, pregnancy and menopause do not involve gut sterilization or drastic physical remodeling as observed in metamorphosis or molting, priority effects can still arise through more subtle but significant shifts in gut environment. Hormonal changes during these stages often induce dysbiosis that can partially disrupt resident microbial communities. For instance, androgen excess in individuals with polycystic ovary syndrome (PCOS) has been linked to gut dysbiosis and lower bacterial diversity, which result in reduced butyrate production, higher BMI and higher testosterone production. Interestingly, supplementation with phytoestrogen was found to promote gut colonization by specific bacterial species, and a phytoestrogen-rich diet was associated with a lower risk of metabolic syndrome in Asian postmenopausal women (88,89). Beyond compositional shifts, hormonal fluctuations can modify host metabolism and promote shifts in nutrient profiles and resource availability in the gut, potentially giving an advantage to immigrant microbes with specialized metabolic capabilities. For instance, the enrichment of *Clostridia* species, particularly *Ruminococcaceae Faecalibacterium* and *Ruminococcus* during puberty may potentially be attributed to their beta-glucuronidase activity, which allow these species to express enzymes that cleave both estrone and estradiol (90,91). Shifts in host immune tolerance during these transitions (e.g., during pregnancy) may transiently relax selection pressures, allowing newly arriving microbes to monopolize resources and outcompete others. Koren et al (2012) (92) show how women during pregnancy demonstrate a shift in gut microbiome composition toward lower abundances of *Clostridiales* and increased abundances of *Proteobacteria* and *Actinobacteria*. During these critical windows, when the gut environment is reshaped, the order and identity of the microbes that are the first to respond and their degree of adaptation to the novel environment determine the dynamics of community shifts, and may then impose priority effects that allow them to dominate in the system and influence how late life transitions unfold.

Transitional periods in host life might represent “reset points” that can be targeted in future studies to enhance our understanding of microbiome dynamics and the role of priority effects therein, and optimize the timing of interventions aimed at maximizing the effectiveness of microbiome-based therapies across diverse applications such as pest and pathogen control in agriculture and aquaculture, ecosystem restoration and disease ecology. Furthermore, intentional and ontogenetic dietary shifts, antibiotics use, disease onset, infections, surgical interventions can cause microbiome disruption leading to shifts in community composition that warrant further investigation. These investigations should pay attention to priority effects, both because they may provide windows of opportunity for immigrant species as well as result in a regime shift in priority effects. The controlled manipulation of colonization order during these open niches offers valuable opportunities to experimentally test priority effects and unravel how such effects can be leveraged to promote a stable and beneficial gut community at the host individual level, ultimately improving host health and disease prevention.

References

1. Macke E, Callens M, De Meester L, Decaestecker E. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat Commun. 2017 Nov 20;8(1):1608.

2. Akbar S, Huang J, Zhou Q, Gu L, Sun Y, Zhang L, et al. Elevated temperature and toxic *Microcystis* reduce *Daphnia* fitness and modulate gut microbiota. Environ Poll. 2021 Feb 15;271:116409.

3. Frankel-Bricker J, Song MJ, Benner MJ, Schaack S. Variation in the Microbiota Associated with Daphnia magna Across Genotypes, Populations, and Temperature. Microb Ecol. 2020 Apr 1;79(3):731–42.

4. Bulteel L, Houwenhuyse S, Declerck SAJ, Decaestecker E. The Role of Microbiome and Genotype in Daphnia magna upon Parasite Re-Exposure. Genes. 2021 Jan 7;12(1):70.

5. Janssens L, Van de Maele M, Delnat V, Theys C, Mukherjee S, De Meester L, et al. Evolution of pesticide tolerance and associated changes in the microbiome in the water flea *Daphnia magna*. Ecotox Environm Safety. 2022 Jul 15;240:113697.

6. Houwenhuyse S, Stoks R, Mukherjee S, Decaestecker E. Locally adapted gut microbiomes mediate host stress tolerance. ISME J. 2021 Aug;15(8):2401–14.

7. Houwenhuyse S, Massol F, Macke E, De Meester L, Vanoverberghe I, Stoks R, et al. How does co-occurrence of Daphnia species affect their gut microbiome? Oikos.e10983.

8. Coone M, Vanoverberghe I, Houwenhuyse S, Verslype C, Decaestecker E. The effect of hypoxia on Daphnia magna performance and its associated microbial and bacterioplankton community: A scope for phenotypic plasticity and microbiome community interactions upon environmental stress?. Frontiers Ecol Evol. 2023 Apr 20;11:1131203.

9. Akbar S, Gu L, Sun Y, Zhang L, Lyu K, Huang Y, et al. Understanding host-microbiome-environment interactions: Insights from *Daphnia* as a model organism. Sci Tot Environ. 2022 Feb 20;808:152093.

10. Freese HM, Schink B. Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean Daphnia magna. Microb Ecol. 2011 Nov 1;62(4):882–94.

11. Eckert EM, Anicic N, Fontaneto D. Freshwater zooplankton microbiome composition is highly flexible and strongly influenced by the environment. Mol Ecol. 2021;30(6):1545–58.

12. Mushegian AA, Arbore R, Walser JC, Ebert D. Environmental Sources of Bacteria and Genetic Variation in Behavior Influence Host-Associated Microbiota. Appl Environ Microbiol. 2019 Apr 4;85(8):e01547-18.

13. Callens M, De Meester L, Muylaert K, Mukherjee S, Decaestecker E. The bacterioplankton community composition and a host genotype dependent occurrence of taxa shape the Daphnia magna gut bacterial community. FEMS Microbiol Ecol. 2020 Jun 23;96(8):fiaa128.

14. Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP, Fierer N. Caterpillars lack a resident gut microbiome. PNAS. 2017 Sep 5;114(36):9641–6.

15. Peerakietkhajorn S, Tsukada K, Kato Y, Matsuura T, Watanabe H. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean, Daphnia magna. Environ Microbiol Rep. 2015 Apr;7(2):364–72.

16. Sison-Mangus MP, Mushegian AA, Ebert D. Water fleas require microbiota for survival, growth and reproduction. ISME J. 2015 Jan;9(1):59–67.

17. Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. Betaproteobacteria Limnohabitans strains increase fecundity in the crustacean Daphnia magna: symbiotic relationship between major bacterioplankton and zooplankton in freshwater ecosystem. Environ Microbiol. 2016 Sep;18(8):2366–74.

18. Eckert EM, Pernthaler J. Bacterial epibionts of Daphnia: a potential route for the transfer of dissolved organic carbon in freshwater food webs. ISME J. 2014 Sep 1;8(9):1808–19.

19. Jezberová J, Jezbera J, Znachor P, Nedoma J, Kasalický V, Šimek K. The Limnohabitans Genus Harbors Generalistic and Opportunistic Subtypes: Evidence from Spatiotemporal Succession in a Canyon-Shaped Reservoir. Appl Environ Microbiol. 2017 Oct 17;83(21):e01530-17.

20. Jia J, Dong C, Han M, Ma S, Chen W, Dou J, et al. Multi-omics perspective on studying reproductive biology in *Daphnia sinensis*. Genomics. 2022 Mar 1;114(2):110309.

21. Grzesiuk M, Grabska M, Malinowska A, Świderska B, Grzesiuk E, Garbicz D, et al. Daphnia stress response to environmental concentrations of chloramphenicol—multi-omics approach. Environ Sci Pollut Res. 2024 Oct 1;31(49):58876–88.

22. Fukami T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. Ann Rev Ecol Evol Sys. 2015 Dec 4;46(Volume 46, 2015):1–23.

23. Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat Rev Gastroenterol Hepatol. 2018 Apr;15(4):197–205.

24. Martínez I, Maldonado-Gomez MX, Gomes-Neto JC, Kittana H, Ding H, Schmaltz R, et al. Experimental evaluation of the importance of colonization history in early-life gut microbiota assembly. eLife. 2018;7:e36521.

25. Šimek K, Kasalický V, Jezbera J, Jezberová J, Hejzlar J, Hahn MW. Broad Habitat Range of the Phylogenetically Narrow R-BT065 Cluster, Representing a Core Group of the Betaproteobacterial Genus Limnohabitans. Applied and Environmental Microbiology. 2010 Feb;76(3):631–9.

26. Kasalický V, Jezbera J, Hahn MW, Šimek K. The Diversity of the Limnohabitans Genus, an Important Group of Freshwater Bacterioplankton, by Characterization of 35 Isolated Strains. PLOS ONE. 2013 Mar 7;8(3):e58209.

27. Jezberová J, Jezbera J, Znachor P, Nedoma J, Kasalický V, Šimek K. The Limnohabitans Genus Harbors Generalistic and Opportunistic Subtypes: Evidence from Spatiotemporal Succession in a Canyon-Shaped Reservoir. Appl Environ Microbiol. 2017 Oct 17;83(21):e01530-17.

28. Ebert D. Ecology, Epidemiology, and Evolution of Parasitism in Daphnia [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2005. Chapter 2, Introduction to Daphnia Biology. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2042/

29. Izhar R, Gilboa C, Ben-Ami F. Disentangling the steps of the infection process responsible for juvenile disease susceptibility. Func Ecol. 2020;34(8):1551–63.

30. Engel P, Moran NA. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev. 2013 Sep 1;37(5):699–735.

31. Auffan M, Bertin D, Chaurand P, Pailles C, Dominici C, Rose J, et al. Role of molting on the biodistribution of CeO2 nanoparticles within *Daphnia pulex*. Water Research. 2013 Aug 1;47(12):3921–30.

32. Lampert W. Daphnia: model herbivore, predator and prey. P J Ecol. 2006;Vol. 54(nr 4):607–20.

33. Graf D, Di Cagno R, Fåk F, Flint HJ, Nyman M, Saarela M, et al. Contribution of diet to the composition of the human gut microbiota. Microb Ecol Health Disease. 2015 Dec 1;26(1):26164.

34. Ravussin Y, Koren O, Spor A, LeDuc C, Gutman R, Stombaugh J, et al. Responses of Gut Microbiota to Diet Composition and Weight Loss in Lean and Obese Mice. Obesity. 2012;20(4):738–47.

35. De Meester L, Gómez A, Okamura B, Schwenk K. The Monopolization Hypothesis and the dispersal–gene flow paradox in aquatic organisms. Acta Oecol. 2002 Jun 1;23(3):121–35.

36. De Meester L, Vanoverbeke J, Kilsdonk LJ, Urban MC. Evolving Perspectives on Monopolization and Priority Effects. Trends Ecol Evol. 2016 Feb 1;31(2):136–46.

37. Matamouros S, Hayden HS, Hager KR, Brittnacher MJ, Lachance K, Weiss EJ, Pope CE, Imhaus AF, McNally CP, Borenstein E, Hoffman LR. Adaptation of commensal proliferating Escherichia coli to the intestinal tract of young children with cystic fibrosis. PNAS. 2018 Feb 13;115(7):1605-10.

38. Remold SK, Lenski RE. Contribution of individual random mutations to genotype-by-environment interactions in Escherichia coli. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11388–93.

39. Groussin M, Poyet M, Sistiaga A, Kearney SM, Moniz K, Noel M, et al. Elevated rates of horizontal gene transfer in the industrialized human microbiome. Cell. 2021 Apr 15;184(8):2053-2067.e18.

40. Ghalayini M, Launay A, Bridier-Nahmias A, Clermont O, Denamur E, Lescat M, et al. Evolution of a Dominant Natural Isolate of Escherichia coli in the Human Gut over the Course of a Year Suggests a Neutral Evolution with Reduced Effective Population Size. Appl Environ Microbiol. 2018 Mar 1;84(6):e02377-17.

41. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, et al. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. ISME J. 2016 Apr;10(4):911–20.

42. Lawrence D, Fiegna F, Behrends V, Bundy JG, Phillimore AB, Bell T, et al. Species Interactions Alter Evolutionary Responses to a Novel Environment. PLOS Biology. 2012 May 15;10(5):e1001330.

43. Vanvelk H, Govaert L, van den Berg EM, De Meester L. Eco-Evolutionary Interactions With Multiple Evolving Species Reveal Both Antagonistic and Additive Effects. Ecol Lett. 2024;27(11):e70006.

44. Louette G, De Meester L. Predation and priority effects in experimental zooplankton communities. Oikos. 2007;116(3):419–26.

45. Decaestecker E, Van de Moortel B, Mukherjee S, Gurung A, Stoks R, De Meester L. Hierarchical eco-evo dynamics mediated by the gut microbiome. Trends Ecol Evol. 2024 Feb;39(2):165-174. doi: 10.1016/j.tree.2023.09.013. Epub 2023 Oct 18. PMID: 37863775.

46. Mimee M, Citorik RJ, Lu TK. Microbiome therapeutics — Advances and challenges. Adv Drug Delivery Rev. 2016 Oct 1;105:44–54.

47. Wang JW, Kuo CH, Kuo FC, Wang YK, Hsu WH, Yu FJ, et al. Fecal microbiota transplantation: Review and update. J Formosan Medical Assoc. 2019 Mar 1;118:S23–31.

48. Aggeletopoulou I, Konstantakis C, Assimakopoulos SF, Triantos C. The role of the gut microbiota in the treatment of inflammatory bowel diseases. Microbial Pathogenesis. 2019 Dec 1;137:103774.

49. Sorbara MT, Pamer EG. Microbiome-based therapeutics. Nat Rev Microbiol. 2022 Jun;20(6):365–80.

50. Walter J, Maldonado-Gómez MX, Martínez I. To engraft or not to engraft: an ecological framework for gut microbiome modulation with live microbes. Curr Op Biotech. 2018 Feb 1;49:129–39.

51. Takiishi T, Korf H, Van Belle TL, Robert S, Grieco FA, Caluwaerts S, et al. Reversal of autoimmune diabetes by restoration of antigen-specific tolerance using genetically modified Lactococcus lactis in mice. J Clin Invest. 2012 May;122(5):1717–25.

52. Robert S, Gysemans C, Takiishi T, Korf H, Spagnuolo I, Sebastiani G, et al. Oral delivery of glutamic acid decarboxylase (GAD)-65 and IL10 by Lactococcus lactis reverses diabetes in recent-onset NOD mice. Diabetes. 2014 Aug;63(8):2876–87.

53. Chang PV. Chemical Mechanisms of Colonization Resistance by the Gut Microbial Metabolome. ACS Chem Biol. 2020 May 15;15(5):1119–26.

54. Woelfel S, Silva MS, Stecher B. Intestinal colonization resistance in the context of environmental, host, and microbial determinants. Cell Host Microbe. 2024 Jun 12;32(6):820–36.

55. Litvak Y, Bäumler AJ. The founder hypothesis: A basis for microbiota resistance, diversity in taxa carriage, and colonization resistance against pathogens. PLoS Pathog. 2019 Feb 21;15(2):e1007563.

56. Lawley TD, Walker AW. Intestinal colonization resistance. Immunology. 2013;138(1):1–11.

57. Solís G, de los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe. 2010 Jun 1;16(3):307–10.

58. Marcobal A, Sonnenburg JL. Human milk oligosaccharide consumption by intestinal microbiota. Clin Microbiol Infect. 2012 Jul;18 Suppl 4(0 4):12–5.

59. Cherrington CA, Hinton M, Mead GC, Chopra I. Organic acids: chemistry, antibacterial activity and practical applications. Adv microb physiol 1991 Jan 1;32:87-108.

60. Umu ÖCO, Bäuerl C, Oostindjer M, Pope PB, Hernández PE, Pérez-Martínez G, et al. The Potential of Class II Bacteriocins to Modify Gut Microbiota to Improve Host Health. PLOS ONE. 2016 Oct 3;11(10):e0164036.

61. Schloissnig S, Arumugam M, Sunagawa S, Mitreva M, Tap J, Zhu A, et al. Genomic variation landscape of the human gut microbiome. Nature. 2013 Jan;493(7430):45–50.

62. Maldonado-Gómez MX, Martínez I, Bottacini F, O'Callaghan A, Ventura M, van Sinderen D, et al. Stable Engraftment of Bifidobacterium longum AH1206 in the Human Gut Depends on Individualized Features of the Resident Microbiome. Cell Host Microbe. 2016 Oct 12;20(4):515–26.

63. Frese SA, MacKenzie DA, Peterson DA, Schmaltz R, Fangman T, Zhou Y, et al. Molecular Characterization of Host-Specific Biofilm Formation in a Vertebrate Gut Symbiont. PLOS Genetics. 2013 Dec 26;9(12):e1004057.

64. Lemon KP, Armitage GC, Relman DA, Fischbach MA. Microbiota-Targeted Therapies: An Ecological Perspective. Sci Transl Medicine. 2012 Jun 6;4(137):137rv5-137rv5.

65. Mallon CA, Elsas JD van, Salles JF. Microbial Invasions: The Process, Patterns, and Mechanisms. Trends Microbiol. 2015 Nov 1;23(11):719–29.

66. Catford JA, Jansson R, Nilsson C. Reducing redundancy in invasion ecology by integrating hypotheses into a single theoretical framework. Diversity Dist. 2009;15(1):22–40.

67. Xiao Y, Angulo MT, Lao S, Weiss ST, Liu YY. An ecological framework to understand the efficacy of fecal microbiota transplantation. Nat Commun. 2020 Jul 3;11(1):3329.

68. Martin R, Makino H, Yavuz AC, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PLOS ONE. 2016 Jun 30;11(6):e0158498.

69. Gerber GK. The dynamic microbiome. FEBS Letters. 2014 Nov 17;588(22):4131–9.

70. Beam A, Clinger E, Hao L. Effect of Diet and Dietary Components on the Composition of the Gut Microbiota. Nutrients. 2021 Aug;13(8):2795.

71. Teyssier A, Rouffaer LO, Saleh Hudin N, Strubbe D, Matthysen E, Lens L, et al. Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine. Sci Tot Environ. 2018 Jan 15;612:1276–86.

72. San Juan PA, Hendershot JN, Daily GC, Fukami T. Land-use change has host-specific influences on avian gut microbiomes. ISME J. 2020 Jan 1;14(1):318–21.

73. Heni AC, Fackelmann G, Eibner G, Kreinert S, Schmid J, Schwensow NI, et al. Wildlife gut microbiomes of sympatric generalist species respond differently to anthropogenic landscape disturbances. Anim microbiome. 2023 Dec;5(1):1–18.

74. Thornton IWB. Krakatau: The Destruction and Reassembly of an Island Ecosystem. Harvard University Press; 1996. 372 p.

75. Chase JM. Drought mediates the importance of stochastic community assembly. PNAS. 2007 Oct 30;104(44):17430–4.

76. Walker LR, Shiels AB. Landslide Ecology. Cambridge University Press; 2012. 315 p.

77. Grimaldi D, Engel MS. Evolution of the Insects. Cambridge University Press; 2005. 788 p.

78. Rowland IJ, Goodman WG. Magnetic Resonance Imaging of Alimentary Tract Development in Manduca sexta. PLoS One. 2016;11(6):e0157124.

79. González-Serrano F, Pérez-Cobas AE, Rosas T, Baixeras J, Latorre A, Moya A. The Gut Microbiota Composition of the Moth Brithys crini Reflects Insect Metamorphosis. Microb Ecol. 2020 May 1;79(4):960–70.

80. Manthey C, Johnston PR, Nakagawa S, Rolff J. Complete metamorphosis and microbiota turnover in insects. Mol Ecol. 2023 Dec;32(23):6543–51.

81. Takashima S, Younossi-Hartenstein A, Ortiz PA, Hartenstein V. A novel tissue in an established model system: the Drosophila pupal midgut. Dev Genes Evol. 2011 Jun;221(2):69–81.

82. Xu Q, Lu A, Xiao G, Yang B, Zhang J, Li X, et al. Transcriptional Profiling of Midgut Immunity Response and Degeneration in the Wandering Silkworm, Bombyx mori. PLOS ONE. 2012 Aug 24;7(8):e43769.

83. Yang B, Huang W, Zhang J, Xu Q, Zhu S, Zhang Q, et al. Analysis of gene expression in the midgut of Bombyx mori during the larval molting stage. BMC Genomics. 2016 Dec;17(1):1–16.

84. Franzetti E, Casartelli M, D'Antona P, Montali A, Romanelli D, Cappellozza S, et al. Midgut epithelium in molting silkworm: A fine balance among cell growth, differentiation, and survival. Arthropod Structure & Development. 2016 Jul 1;45(4):368–79.

85. Moll RM, Romoser WS, Modrakowski MC, Moncayo AC, Lerdthusnee K. Meconial Peritrophic Membranes and the Fate of Midgut Bacteria During Mosquito (Diptera: Culicidae) Metamorphosis. J Med Entomol. 2001 Jan 1;38(1):29–32.

86. Zhang M, Zhang X, Tran NT, Sun Z, Zhang X, Ye H, et al. Molting Alters the Microbiome, Immune Response, and Digestive Enzyme Activity in Mud Crab (Scylla paramamosain). mSystems. 2021 Oct 12;6(5):10.1128/msystems.00917-21.

87. Mente E, Gannon AT, Nikouli E, Hammer H, Kormas KA. Gut microbial communities associated with the molting stages of the giant freshwater prawn *Macrobrachium rosenbergii*. Aquaculture. 2016 Oct 1;463:181–8.

88. Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. J Transl Medicine. 2012 Dec 21;10(1):253.

89. Chan JL, Kar S, Vanky E, Morin-Papunen L, Piltonen T, Puurunen J, et al. Racial and ethnic differences in the prevalence of metabolic syndrome and its components of metabolic syndrome in women with polycystic ovary syndrome: a regional cross-sectional study. Am J Obstetrics Gynecology. 2017 Aug 1;217(2):189.e1-189.e8.

90. Hollister EB, Riehle K, Luna RA, Weidler EM, Rubio-Gonzales M, Mistretta TA, et al. Structure and function of the healthy pre-adolescent pediatric gut microbiome. Microbiome. 2015 Aug 26;3(1):36.

91. Calcaterra V, Rossi V, Massini G, Regalbuto C, Hruby C, Panelli S, Bandi C, Zuccotti G. Precocious puberty and microbiota: The role of the sex hormone–gut microbiome axis. Frontiers in endocrinology. 2022 Oct 21;13:1000919.

92. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK, et al. Host Remodeling of the Gut Microbiome and Metabolic Changes during Pregnancy. Cell. 2012 Aug 3;150(3):470–80.