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The gut microbiome of the wild great tit (Parus major): drivers and fitness consequences

Thesis presented by

Shane Edmond Somers

BA (Zoology), Trinity College Dublin, 2015

MSc (Bioinformatics), University College Cork, 2018

ORCID: 0000-0001-5985-2396

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University College Cork

School of Biological, Earth, and Environmental Sciences

Head of School: Prof. Astrid Wingler

Supervisors: Prof. John Quinn, Prof. Paul Ross & Prof. Catherine Stanton

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Table of Contents

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Signed: 

Shane Somers, 11/7/2023

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Abstract

The gut microbiome plays a vital role in its host's ecology. Clinical studies have shown gut microbes increase host health and fitness by providing digestive and immune functions, as well as aiding development. Natural variation in the microbiome is widely believed to affect host fitness in the wild but we are lacking experimental studies to test this. The microbiome varies with both host and environmental factors but most studies to date have focussed on individual factors and not adequately addressed the multiple overlapping and hierarchical drivers of microbiome variation working at environmental, host and microbial scales. This thesis investigates the role of the gut microbiota in host fitness, and how this is affected by and varies across contexts. Additionally, we address sources of variation in the gut microbiota at a host and environmental level, accounting for host ecology and drivers at different scales. We find that the host's weight is correlated with microbiome diversity during development but that the direction of this relationship is context dependent. This shows that the microbiome interacts with the environment to determine host fitness and is important because it helps explain the contradictory findings linking diversity to weight. We also show that the interaction between the host, its microbiome and environment change with developmental stage. Specifically, we found that the microbiome of developed individuals is remarkably resilient to environmental perturbation, while developing individuals are much more sensitive, with important implications for future experiments. We developed a novel method for experimentally perturbing the microbiome that will allow microbiome researchers to begin testing hypotheses linking the microbiome to host ecology and evolution in natural settings. Finally, we show that welfare measures, such as environmental enrichment may interact with the gut microbiota to impact on host health and behaviour. In summary, I show that variation in the microbiome is linked to host ecology and that this variation is linked to host fitness.

Chapter 1: General introduction

1.1 Host-microbiome evolution

The community of micro-organisms living within and on an organism, known as the microbiome, plays a vital role in the ecology and evolution of its host (Amato, 2013; Evans et al., 2017; McFall-Ngai et al., 2013). Natural variation in gut microbiome diversity and composition have been correlated with host health and fitness in the wild (Amato et al., 2015; Suzuki, 2017; Teyssier, Lens, et al., 2018). As our knowledge of host-microbiome interactions grows it is becoming apparent that we cannot understand the natural history of organisms without understanding their microbiota. All animal life has evolved from, and with, microbes, so it should not be surprising that there are many feedbacks and dependencies between these diverse branches of life. The host and their microbes can be viewed as a metacommunity, with multiple species interacting at different scales within a dynamic host 'environment', which itself is subject to ecological forces at a micro- and macro- scale (Miller et al., 2018). The gut microbiome, in particular, has a great capacity to interact with its host because of the proximity of the microbes to the host's circulatory system and other host tissues. However, it is important to recognise that microbes can be beneficial, commensal or parasitic (Dale & Moran, 2006; A. E. Douglas, 2008), with effects that are context dependent and can vary within a single host species (Marchetto & Power, 2018). Furthermore, it is often ignored in microbiome research that not all animals require or benefit from microbial symbionts, and animals likely span a continuum of reliance on microbes (Hammer et al., 2019).

The symbiosis between Eukarya and Prokarya is ancient and complex, and has led to specific adaptations and dependencies in both hosts and microbes (K. R. Foster et al., 2017; Hooper et al., 2001; Margulis, 1993). Gut microbes depend on their hosts to provide an environment and food, either indirectly when they feed on material consumed by the host or directly in the case of glycans produced for the microbes by

the host (Cronin et al., 2021; Hooper & Gordon, 2001). Meanwhile, hosts have evolved reliance on their gut microbes for different roles, but particularly immunity and digestion, as hosts have outsourced various functions to their microbiome. Microbes help prevent pathogens from colonising the gut (reviewed by Buffie and Pamer (2013)) and increase the food resources the host can exploit or otherwise aiding their ability to extract energy from novel sources (Delsuc et al., 2013; Kohl et al., 2014; Ley et al., 2008; McFall-Ngai et al., 2013; Zhu et al., 2011). In some cases, hosts are completely reliant on their microbes for survival: for example, ruminants are totally dependent on their gut bacteria to digest plant fibres (Krehbiel, 2014). The microbiome likely played a key role in mammalian evolution by facilitating mammals expansion from carnivory to herbivory (Moeller & Sanders, 2020). Though despite the hyperbolic claims of many microbiome papers- animals from a diverse range of lineages, particularly invertebrates, display little or no reliance on symbionts (reviewed in Hammer et al., 2019).

The three components of natural selection, namely variation, inheritance and differential success must be present for natural selection to occur. The gut microbiome displays huge intra- and inter-specific variation across diverse vertebrate lineages (Amato et al., 2016; Bunker et al., 2022; Dewar et al., 2013; Kropáčková et al., 2017; Ley et al., 2008). Variation is the raw material of natural selection, and so determining sources of variation in host traits is an important goal of evolutionary ecology. There are a wide variety of factors that play a role in microbiome variation which must be determined if we are to understand the functional, and hence evolutionary, significance of host-microbe interactions. To understand this relationship in an evolutionary context we must determine the identity of microbes, sources of variation in the microbial community, the forces that shape that variation and the functional/adaptive implications of that variation.

1.2 Sources of variation in the microbiota

Determining the sources of variation in the microbiome is not trivial as there are multiple physiological and environmental drivers of microbial variation acting simultaneously at different scales. Many microbiome studies fail to adequately describe variation in microbiomes because they do not account for "drivers above the scale of the individual" i.e. they fail to take the ecology of both the host and the microbial community into account (Miller et al., 2018), but see Baniel, Petrullo et al. (2021). These drivers include the host's ecological network, which includes its local environment (e.g. woodland site) which is consistent with the idea that transmission between hosts, the environment and conspecifics could be an important driver of individual variation (Rothschild et al., 2018). The host and their microbiota are affected by both intrinsic physiological and extrinsic non-physiological processes. The gut microbiome in particular is linked to host homeostasis and the immune system, and challenges to either system, for example the stimulation of the stress response system or an immune challenge, has knock on effects on the gut microbiome (Hooper et al., 2012; Petrullo et al., 2022). While many of these stimuli may ultimately originate in the hosts external environment, for example an interaction with a predator or pathogen, the proximate cause lies with the host's internal systems, like the hypothalamic-pituitary-adrenal (HPA) axis or the immune system. Meanwhile, more direct external factors may cause variation in the gut microbiota through exposure, such as a seasonal change in food source or exposure to novel bacteria during migration (Baniel, Amato, et al., 2021; Lewis et al., 2017). In order to understand how the microbiome varies it is necessary to take an ecological approach, which this thesis aimed to do.

1.2.1 Intrinsic sources of variation

Host genetics are a driver of microbial variation, and patterns of microbial variation are tightly linked to host phylogeny, particularly in mammals (Ley et al., 2008; Youngblut et al., 2019) but the precise role of host genetics in structuring the microbiome is poorly understood, and mainly limited to humans and lab rodents (Cahana & Iraqi, 2020). Studies in humans suggest that host genetics are a modest driver of microbiome variation, which act through genes related to diet and immunity

(Goodrich et al., 2016). The immune system is a major driver of variation in the gut microbiome and one of the principal methods by which the host interacts with its gut bacteria in an effort to avoid pathological reaction to the guts enormous bacterial load and maintain the presence of beneficial bacteria (Hooper et al., 2012; J. Zheng et al., 2020). This balance is maintained in the gut by the secretion of a layer of mucus between the gut tissue and the bacteria-rich gut lumen, as well as host production of antibacterial proteins. The mucus layer serves to protect the host tissue from exposure to bacteria and prevent chronic inflammation (a response to infection) and protect the commensal bacteria from host defences (Macpherson et al., 2005). Mcfall-Ngai (2007) suggests that the adaptive immune system developed in order to recognise and manage vertebrates' communities of beneficial microbes.

The gut microbiota is sensitive to internal homeostatic regulation, in particular-stress. Increases in stress hormones (e.g. glucocorticoids) can lead to reductions in gut microbiota diversity (Noguera et al., 2018), though the precise influence of glucocorticoids on the gut microbiota is dependent on the ecological context (MacLeod et al., 2022; Petrullo et al., 2022). Stress, and the associated activity from the HPA axis influences the composition of the gut microbiota, through the secretion of glucocorticoids, in lab based model organisms (Cryan & Dinan, 2012) and in natural populations (Noguera et al., 2018; Petrullo et al., 2022; Stothart et al., 2019). Hormonal changes, as a result of stress, can lead to changes in the amount of mucous secreted in the gastrointestinal tract. This mucous can be used as a source of food by some microorganisms, so a reduction in the mucous can lead to changes in the microbiome composition (Tannock & Savage, 1974).

1.2.2 Extrinsic sources of variation

The external environment of the host is a major source of variation in the microbiome, which changes with geography (Ren et al., 2017), diet (G. L. Davidson, Wiley, et al., 2020; Teyssier et al., 2020), and environmental stressors such as land-use change (Candela et al., 2012; San Juan et al., 2020) and urbanisation (Teyssier,

Rouffaer, et al., 2018). Diet is likely the most important external factor driving variation in the microbiota (Delsuc et al., 2013; Hird et al., 2015; Youngblut et al., 2019) but it is intimately linked to other environmental factors. In wild systems, habitat determines food availability (Naef-Daenzer, 2000; Shutt et al., 2018), and habitat structure is linked to microbiome variation (Goossens et al., 2022). Food availability, and other factors such as rainfall and temperature, change with the season, and many animals' microbiomes show distinct seasonal patterns (Amato et al., 2015; Baniel, Amato, et al., 2021; Hicks et al., 2018; Maurice et al., 2015; Ren et al., 2017). Diet likely shapes the microbiome largely through exposure to certain pools of microbes (Jones et al., 2022) but it also provides specific substrates for gut microbes to subsist on. If the host changes its diet the microbiome will respond, like any ecological community, by increasing the number of those members which favour that substrate.

Cross-fostering experiments indicate that, in birds, the nest environment has a greater influence on community composition than genetic relatedness, with divergence among true siblings and convergence among foster siblings (Teyssier, Lens, et al., 2018). Observational studies also indicate a greater influence of the environment on bird microbiome composition compared to (non-flying) mammals- where diet and phylogeny are the key drivers of microbiome assembly (Grond et al., 2019; Knowles et al., 2019; Song et al., 2020; Youngblut et al., 2019). The drivers of mammalian and avian gut microbiomes likely differ due to the physiological adaptations to flight including a shorter gut, which is supported by the evidence of microbiome community convergence between birds and bats (Song et al., 2020).

1.2.3 Extrinsic vs intrinsic sources

Drawing clear distinctions between internal and external sources of variation in the microbiota are misleading though, as while the external environment can cause changes in the gut microbiota directly, for example via exposure to novel microbes through diet, external stimuli can provoke internal host responses. The effect of the

environment in structuring the gut microbiota will therefore vary with intrinsic host factors like age, sex and immune ability. Adult microbiomes typically show greater diversity and stability than those of developing individuals (Barbosa et al., 2016; Lozupone et al., 2012; van Dongen et al., 2013) and hence mature individuals are expected to show less variation in gut microbiota across environmental variables. Differences in host traits, like sex, should manifest during the breeding season due to differences in sex hormones (Org et al., 2016; Valeri & Endres, 2021) and asymmetric parental energy investment across sexes as the burden gestation (or egg production) is squarely on the female. While there is strong support for a correlation between sex and the gut microbiome in lab studies (Org et al., 2016; Valeri & Endres, 2021), there is more mixed support in wild studies (Capunitan et al., 2020; Drobniak et al., 2021) probably due to unmeasured life history variables.

1.3 Development of microbiome

For most organisms that depend on microbial symbionts, the microbiome is at least partially vertically heritable. Neonates hatch or are born with little to no microbes present in their guts and are rapidly colonised (Borghi et al., 2019; Grond et al., 2017; Videvall et al., 2019) through vertical transmission from parents and horizontal transmission from their environment (Campos-Cerda & Bohannan, 2020; Zilber-Rosenberg & Rosenberg, 2008). The initial colonisers of a vertebrate's gut are likely to be those microbial inoculants it is exposed to in the birth canal, for viviparous animals, or the bacteria present in the nest for oviparous/nesting animals (Campos-Cerda & Bohannan, 2020; S. Wang et al., 2020). Mammals and birds differ in how they are initially inoculated as mammals experience live birth in which they are exposed to a mothers microbes in the birth canal. Mammalian mothers also provide their young with milk which has a major effect on shaping the gut microbiome, and in humans in particular, is adapted to encourage the establishment of specific microbes (Chichlowski et al., 2011). Birds' guts are virtually sterile when they hatch (Grond et al., 2017) (but see Trevelline et al. (2018)), though they are immediately

exposed to a range of microbial sources from the nest and their diet (Campos-Cerda & Bohannan, 2020; Teyssier, Lens, et al., 2018).

Patterns of microbial colonisation differ across species, probably due to differences in life history. Some species (e.g. great tits (Parus major)) undergo a period of rapid colonisation followed by a winnowing period and stabilisation (Teyssier, Lens, et al., 2018), that may correspond with the activation of the host immune system. Other species appear to experience a rapid increase and then stabilisation (Grond et al., 2017; Palmer et al., 2007; Videvall et al., 2019). Regardless, most species experience a rapid increase in diversity during the first few days of development and reach a relatively stable state in adulthood. Changes in the microbiome during development reflect diverse processes including microbial succession, host immune maturation and the transition from yolk to solid food in birds (Grond et al., 2017; Kinross et al., 2011; Videvall et al., 2019; D. Zheng et al., 2020). The taxonomic changes that occur likely provide important information on the underlying functional changes in the microbiota, as discussed below.

1.4 Functional implications of variation

1.4.1 Host development

The microbiome can affect host phenotype and hence host fitness. The gut microbiota are essential for the healthy development of a number of host species, where the timing of microbial exposure and colonisation during specific developmental windows affects adult phenotypes (Cox et al., 2014; Hansen et al., 2012). These patterns of microbial exposure may serve as important signals shaping host development, as microbes can serve as an environmental cue or indicator (Metcalf et al., 2019; Moeller & Sanders, 2020). The microbiome is also much more sensitive to perturbation during development than in adulthood, as developed individuals typically contain more complex and hence resilient microbial communities (Lozupone et al., 2012; Wall et al., 2009). So understanding the timing and patterns of microbial colonisation during development is key to our

understanding of the host and host fitness (Campos-Cerda & Bohannan, 2020; Metcalf et al., 2019). Disruption of the microbiota during development can impact host fitness, altering metabolism, physiology and immunity (Knutie et al., 2017; Warne et al., 2019).

The timing of microbial colonisation is critical to normal immune development in a range of vertebrate hosts. Germ free mice exposed to conventional microbiota can be protected from abnormal development if exposed as neonates but not as adults (Olszak et al., 2012; Simon et al., 2016). Delayed colonisation of the gut by microbes during development has permanent effects on immune phenotype, and can lead to over-reactive inflammatory immune reactions which are deleterious to host health (Hansen et al., 2012), as predicted by the hygiene hypothesis which suggests lack of exposure to microbes during development can lead to poor immune tolerance (Musso et al., 2010; Strachan, 1989). In wild settings, healthy microbial colonisation can be disrupted by a pathogenic infection, by limited exposure to conspecifics in endangered species with limited population size (reduced social contact) or by living in degraded habitats (Hauffe & Barelli, 2019).

Clinical studies have shown microbes can shape physical structures during development by inducing host gene expression and signalling to host cells, regulating vascularisation, cell differentiation and maturation of the intestine (J. M. Bates et al., 2006; Stappenbeck et al., 2002). Microbial colonisation can also modulate brain development and subsequent adult behaviour, particularly anxiety-like behaviour (Heijtz et al., 2011; Ntranos & Casaccia, 2018). The microbiota also affect body composition in developed individuals, including bone mass and fat deposition (Bäckhed et al., 2004; Sjögren et al., 2012; Suzuki et al., 2020).

1.4.2 Role in mature individuals

In developed individuals, the microbiota can play a vital role in immune system functioning where symbiotic and commensal bacteria can protect the host from

pathogenic bacteria directly by attacking invaders (with bacteriocins), or by outcompeting them for nutrients and space, as well as indirectly by enhancing the host's immune response (reviewed by Pickard et al. (2017)). Antibiotic treatments which kill off beneficial and harmful bacteria alike can make the host vulnerable to infection, e.g. C. difficile, highlighting the importance of commensal bacteria in resisting infection.

1.4.3 Role in plasticity

The microbiome may additionally increase host fitness due to its plastic nature, by allowing the host to adapt to its environment (Alberdi et al., 2016). The gut microbiota can acclimate rapidly to environmental change (Candela et al., 2012; Fontaine & Kohl, 2020) which can help the host adapt to the it's environment. For example, the microbiome may allow its host to exploit novel food resources, adapt to changing temperatures or can buffer the effects of seasonal reductions in food availability (Amato et al., 2015; Fontaine & Kohl, 2020; Lindsay et al., 2020) though these relationships may change with increasing anthropogenic pressures such as urbanisation and land use change (San Juan et al., 2020; Teyssier, Rouffaer, et al., 2018). Another mechanism for acclimation may be the programming of the HPA axis which modulates homeostasis through the release of glucocorticoids in response to physical, psychological or immunological stressors (Lupien et al., 2009). The gut microbiome is an important factor in the programming of the HPA axis (Sudo et al., 2004), which determines the stress reactivity of an individual in pre- and post-natal stages of development and has effects on brain development and behaviour (Lupien et al., 2009). The microbiome can therefore 'tune' an individual to its environment through its effect on the HPA axis by influencing how reactive an individual is to stress. Importantly, the gut microbiota can facilitate these host changes on an intra-generational time scale facilitating the rapid acclimation or adaptation of its host to its environment (Alberdi et al., 2016) but these changes are likely dependent on the host's initial community (Bletz et al., 2016; Walker et al., 2011). On an evolutionary (inter-generational) scale, the microbiota may shape host responses to selection by

shifting the mean host phenotype or by changing the variance of the host phenotype in the population (Henry et al., 2021).

1.5 State of microbiome research

Except for humans and a few model species, microbiome research is largely at the biogeographic stage of the discipline: what microbial species live where, why- and why not? Natural variation in gut microbiome diversity and composition have been correlated with host health and fitness in the wild (Amato et al., 2015; Davidson et al., 2021/Chapter 2; Suzuki, 2017; Teyssier, Lens, et al., 2018). These observational studies supply the foundation for our understanding of the microbes' role in the ecology and evolution of their hosts, but we are lacking in experimental studies to explicitly test these correlations. The majority of the microbiome literature to date has been on the human microbiome and its effects on human health, as well as related studies in model organisms- particularly rodents (Colston & Jackson, 2016; Grond et al., 2018; Pascoe et al., 2017). However, if we want our understanding of the microbiota to go beyond that of a host trait indicative of health or disease and access the microbiome's potential for the study of host ecology and evolution, we need to expand our study of the microbiome from the lab to the wild, with all the variation and randomness that entails. Unsurprisingly, due to the relatively high cost of microbiome surveying methods, studies in wild animals have lagged behind human and rodent studies but these are rapidly increasing in number (Colston & Jackson, 2016; Pascoe et al., 2017), and with it our understanding of animal ecology and evolution.

Commercial and laboratory species have limited genetic variability and experience consistent environmental conditions, making any findings difficult to generalise to wild species. Similarly, captive animals typically have less diverse microbiome communities than their wild counterparts (Clayton et al., 2016; Kohl & Dearing, 2014) though this can differ across species (McKenzie et al., 2017). Capturing wild animals

and bringing them into captivity typically leads to reductions in gut diversity and changes in the community (Kohl & Dearing, 2014; Weitzman et al., 2022). Captivity induced microbiome changes have knock on effects on the host's immune response (Kueneman et al., 2016; Rosshart et al., 2017) meaning lab findings may not be generalisable to wild hosts.

Increasingly, experimental methods are being used to investigate the gut microbiome of wild hosts but remain limited by the available methods of microbiome perturbation. Experimental studies have typically manipulated the environment (Jacob et al., 2015), taken advantage of natural experiments (Hird et al., 2014; San Juan et al., 2020; Teyssier, Rouffaer, et al., 2018) or used cross fostering of young (Teyssier, Lens, et al., 2018). While these methods have produced valuable insights, they do not directly experimentally perturb the microbiota, which is ultimately needed to gain a mechanistic understanding of how microbes influence host ecology and evolution. Thus far, a limited number of methods have been used to directly manipulate the microbiome of wild species.

While antibiotics have shown clear efficacy in experimentally disrupting the gut microbiome they have broad spectrum effects, including immune effects, which can complicate the interpretation of results (Motiei et al., 2020; Willing et al., 2011). A more targeted approach using specific bacteria may be necessary to elucidate the effect of gut microbes on fitness indicators such as growth and survival. Beneficial bacterial strains are likely to be adapted to a specific host (Shapira, 2016; Ward et al., 2019). Non-host adapted strains may not interact with the host tissues and transit through the gut more quickly. *Lactobacillus* species are often identified as having beneficial effects on the host and so are commonly used in probiotic treatments (Angelakis & Raoult, 2010; Drissi et al., 2017; Grond et al., 2019; Servin, 2004). Dietary supplementation of domestic pigeons and chickens with commercial *Lactobacillus* probiotics have been shown to be effective in changing the gut microbiome composition of hosts (Forte et al., 2018; Grond et al., 2019) and have been associated

with weight change in both humans and domesticated animals (Angelakis & Raoult, 2010; Forte et al., 2018; Million et al., 2012). *Lactobacillus* have also been associated with better health in wild avian hosts (Davidson et al., 2021/Chapter 2; Lewis et al., 2017) and so represent a promising tool for the study of wild microbiomes.

A majorly under-explored aspect of the microbiome is in cases where animals do not reply on microbes, as is the case for a number of animal species. Hammer et al. (2019) highlight the overstated claims in this field regarding the ubiquity of animal microbiomes and points out that the importance of microbes has not been established for the vast majority of animal species. Hammer et al. (2019) suggest that microbiome research should proceed with the null hypothesis that the potential host species in question does not rely on microbes or host a microbiome. Recognising the limitations of DNA-sequencing technology, including the potential for contamination, is important for correctly characterising the presence and composition of microbiomes.

1.6 Challenges to microbiome investigation

Investigators of the gut microbiome face a variety of challenges, particularly for those studying the gut microbiomes of wild species. One of the main challenges is obtaining samples, as this requires individuals be caught which is a difficult task when the subjects are free roaming. Captive wild animals in zoo settings offer an easier alternative but as I have outlined above these may not be representative of their wild counterparts. After capturing individuals investigators must obtain samples from the gut. The most comprehensive sampling method involves euthanising and excising portions of the gut from the animals however this faces strong ethical constraints and removes the possibility of longitudinal sampling. A promising alternative for this is preserving samples from individuals being caught for other purposes e.g. as museum specimens (Hird et al., 2015). If obtaining post-mortem gut samples is not possible or convenient for ethical or other reasons- faecal samples, cloacal swabs or

oral swabs offer good alternatives. However these methods are representative of only a small portion of the gastrointestinal community, according to a study on ostriches (Videvall et al., 2017). Faecal samples are the least invasive of these options, and there exists simple and effective apparatus for collecting these in a manner that minimises contamination in the field, for birds and small mammals at least, (Knutie et al., 2018). Though precisely how representative faecal samples are of the guts of small birds is unclear due to a lack of data, a consistent finding is that faecal samples are more representative of the gut community at the lower reaches of the gastro-intestinal tract (Videvall et al., 2017; Yan et al., 2019), though this may not be as important in birds adapted to flight which have shorter guts with lower food retention times (Caviedes-Vidal et al., 2007) which likely results in less distinct communities between the regions of the gut.

The exponential increase in microbiome studies in the last two decades was made possible by the development of 16S rRNA sequencing (Woese & Fox, 1977). However, this only allows taxonomic identification of bacterial species. Other methods exist for surveying different micro-organisms such as fungi but as bacteria are far more abundant in the gut they have remained the focus of most research to date (Huffnagle & Noverr, 2013). The drawbacks to 16S sequencing include its somewhat limited ability to resolve taxonomy due to conservation of the target gene, its reliance on taxonomic databases to identify species, which limits its ability to identify strains from poorly characterised hosts, and the fact that we can only infer the functional potential of the identified taxa. More expensive and labour intensive sequencing techniques, such as shotgun sequencing, allow us to sequence all the genetic information in a sample and therefore gain a much more accurate picture of the functional capacity of the microbiome. However, these techniques remain prohibitively expensive for most ecologists and remain the purview of better funded clinical researchers.

A lack of standardised methods constrains microbiome studies, as it makes comparison between studies difficult (Schloss, 2018). There are many steps involved in sequencing the microbiome, including sampling, storage, sequencing and bioinformatics processing- all of which affect the final data product (Bokulich et al., 2013; Johnson et al., 2019; Song et al., 2016; Videvall et al., 2017). The Human Microbiome Project and more recently the Earth Microbiome Project have offered standardised methods for the collection and sequencing of microbiome samples. However bioinformatics tools and resources, like taxonomic databases, are updated regularly complicating comparisons between studies (Schloss, 2018).

1.7 Avian life history

Birds (Aves) are a monophyletic vertebrate class including over 10,000 species with a global distribution (Beletsky, 2009). Birds display a wide range of life history strategies that are likely to have an impact on their microbiome. One common aspect of birds' life histories is that they produce eggs that require incubation and usually require some kind of nest structure for incubation. Birds can have precocial or altricial young, and these modes of development have important consequences for their life histories (Ducatez & Field, 2021). Altricial young typically stay within the nest until fledging, while precocial young are capable of moving around and feeding themselves very early in development (Starck & Ricklefs, 2000). The timing of breeding is an important determinant for breeding success (Grüebler & Naef-Daenzer, 2010; Naef-Daenzer et al., 2001) and parents typically try to match their chicks' hatching with the peak of their preferred food abundance which they must balance against predation pressure (Naef-Daenzer & Grüebler, 2016). Provisioning behaviour differs across precocial and altricial species, with altricial birds requiring much more care (Starck & Ricklefs, 2000). The number and condition of chicks, and whether they are recruited into the breeding population, is largely determined by food abundance and predation pressure (Naef-Daenzer & Grüebler, 2016). Environmental conditions during development affect fitness in later life (Lindström, 1999).

1.8 Great tits as model system

This study investigated the gut microbiome of great tits (*Parus major*), a cavity nesting passerine bird. Great tits are tractable across development as they are altricial (remain in their nest until near-independence) and easily monitored in nest boxes. This may buffer the nestlings from environmental effects. Altricial birds are the fastest growing terrestrial vertebrates (Case, 1978) which likely amplifies host level effects and fitness consequences, particularly regarding growth. Therefore, great tits are a powerful system for addressing microbiome links with host development and growth within a relatively short time frame of weeks rather than months or years. The nestling stage is a key time in a bird's development when they may be most sensitive to microbiome effects from a nutrition and immune point of view. Growth rate is an important factor in both nestling and parental fitness. Greater mass increases post-fledging recruitment into breeding population (Monrós et al., 2002), thus increasing parental fitness. The populations studied here have low local recruitment of fledgling birds, probably due to the relatively small area of the sites, so post-fledging success could not be examined and survival to fledging was looked at instead.

1.9 Thesis overview

In this thesis, I use two observational field-based studies, a field-based experimental manipulation and an aviary-based experiment on wild-caught hosts to investigate the relationship between the gut microbiome and the host. I carry out this work using the great tit (*Parus major),* a common ecological model. In chapter 2, I investigate how the microbiome affects host health and fitness linking microbiome diversity to weight gain (a fitness proxy) in nestlings. I also investigate which specific bacterial taxa are associated with survival and non-survival. In chapter 3, I describe sources of variation in the microbiome, from an ecological perspective. I examine how microbiome's sensitivity to both intrinsic host factors and extrinsic environmental

factors changes with life stage. In chapter 4, I experimentally test the effect of the microbiota on host health with a novel method of microbiome perturbation. In chapter 5, I investigate how captivity induced stress affects the microbiome and host health, and whether the effects of captivity induced stress on the host can be ameliorated by environmental enrichment or by probiotic administration.

Chapter 2: A time-lagged association between the gut microbiome, nestling weight and nestling survival in wild great tits

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Author contributions
SES and GLD are joint first authors. GLD, CS, RPR and JLQ conceived the ideas and designed the methodology. GLD and MSR collected the field data. GLD, NW and CNJ carried out the DNA extraction and library prep. SES analysed the data. GLD and SES wrote the manuscript with input from all authors. All authors gave final approval for publication.

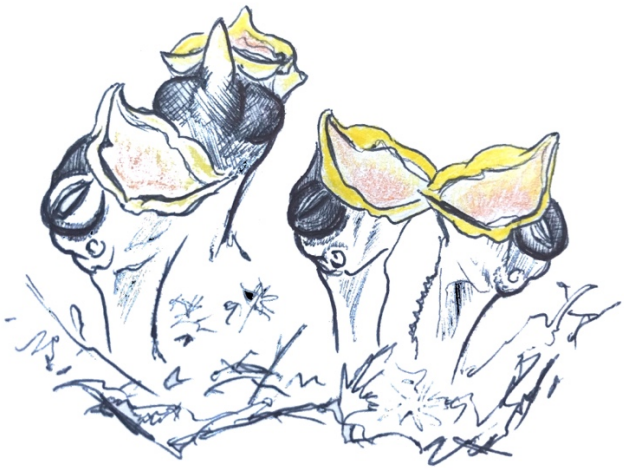

Illustration by Dr. Caroline McKeon

Abstract

Natal body mass is a key predictor of viability and fitness in many animals. While variation in body mass and therefore viability of juveniles may be explained by genetic and environmental factors, emerging evidence points to the gut microbiota as an important factor influencing host health. The gut microbiota is known to change during development, but it remains unclear whether the microbiome predicts fitness, and if it does, at which developmental stage it affects fitness traits. We collected data on two traits associated with fitness in wild nestling great tits (*Parus major*): weight and survival to fledging. We characterised the gut microbiome using 16S rRNA sequencing from nestling faeces and investigated temporal associations between the gut microbiome and fitness traits across development at Day-8 (D8) and Day-15 (D15) post-hatching. We also explored whether particular microbial taxa were 'indicator species' that reflected whether nestlings survived or not. There was no link between mass and microbial diversity on D8 or D15. However, we detected a time-lagged relationship where weight at D15 was negatively associated with the microbial diversity at D8, while controlling for weight at D8, therefore reflecting weight gain over the intervening period. Indicator species analysis revealed that specificity values were high and fidelity values were low, suggesting that indicator taxa were primarily detected within either the survived or not survived groups, but not always detected in birds that either survived or died. Therefore these indicator taxa may be sufficient, but not necessary for determining either survival or mortality, perhaps owing to functional overlap in microbiota. We highlight that measuring microbiome-fitness relationships at just one time point may be misleading, especially early in life. Instead, microbial-host fitness effects may be best investigated longitudinally to detect critical development windows for key microbiota and host traits associated with nestling weight. Our findings should inform future hypothesis testing to pinpoint which features of the gut microbial community impact on host fitness, and when during development this occurs. Such confirmatory research will shed light on population level processes and could have the potential to support conservation.

2.1 Introduction

Understanding the numerous factors that drive fitness has long been the focus of research in ecology and evolution. This has primarily included behavioural factors, such as foraging (Patrick & Weimerskirch, 2014) and parental care (Schwagmeyer & Mock, 2008), and ecological factors, for example, food availability (Van Noordwijk et al., 1995), predation (Götmark, 2002), and more recently climate change (Blomberg et al., 2014). Proximate factors such as genetics (e.g. Merila (1996), Norris (1993)), stress and natal environment effects (Keller & Van Noordwijk, 1994; Pickett et al., 2013) have also been studied intensively. More recently, a growing body of research is beginning to show the gut microbiome - the microbial community in the gut and the host environment - as a powerful proximate mechanism influencing host health and fitness among animals (Kinross et al., 2011; Lloyd-Price et al., 2016; Rosshart et al., 2017). Emerging evidence has highlighted that the gut microbiota varies substantially within and between individuals (David et al., 2014; Maurice et al., 2015), and that microbe-host interactions are likely to be taxonomically widespread and central to many behavioural, ecological and evolutionary processes (Alberdi et al., 2016; G. L. Davidson, Wiley, et al., 2020; Sherwin et al., 2019). However, the role of the gut microbiome in determining fitness in natural populations, and whether there are critical developmental windows whereby it affects future weight and survival, remains poorly understood.

Evidence suggests that the microbiome is associated with many aspects of survival and fitness. In humans, the maternal vaginal microbiota is associated with pre-term birth (Fettweis et al., 2019) and sudden infant death syndrome (Fettweis et al., 2019; Highet et al., 2014), and the gut microbiome is associated with longevity (Biagi et al., 2016). Laboratory-bred mice, which have substantially different gut microbiota than their wild counterparts, were more likely to survive an influenza infection if they had received a gut microbiota transplant from wild donors (Rosshart et al., 2017). Notably, wild mice had higher relative abundances of Bacteroidetes and Proteobacteria, and lower relative abundances of Firmicutes, suggesting these taxa

may play a role in regulating host health. The potential for the gut microbiome to impact animal fitness in natural systems has gained much attention in recent years (Amato, 2013; Suzuki, 2017; Trevelline et al., 2019; West et al., 2019). However, the empirical evidence to support such hypotheses is lacking, perhaps owing to the logistical constraints associated with collecting longitudinal data on both the gut microbiome and indicators of fitness such as survival in these systems.

Research on laboratory model species has shown that the microbial community in the gut can confer specific health benefits to the host (Kinross et al., 2011), and the timing of gut colonization is critical for normal development (Cox et al., 2014; Hansen et al., 2012). In particular, gut microbiota are important for the development and regulation of gut immunity by maintaining intestinal barrier function (Hooper et al., 2012; O'Hara & Shanahan, 2006) and aiding digestive capabilities through the metabolism of dietary components (Rowland et al., 2018). Gut bacteria can induce expression of host genes that, for example, code for proteins in the mucosal layer of the intestines (O'Hara & Shanahan, 2006). Moreover, bacterial genomes can produce enzymes that are absent in the host, yet are necessary for specific digestive functions such as cellulose digestion in termites (*Nasutitermes* spp.) and giant pandas (*Ailuropoda melanoleuca*) (Warnecke et al., 2007; Zhu et al., 2011) and nitrogen metabolism in birds (Vispo & Karasov, 1997). Germ-free mice devoid of any microbes have depressed immune function and lower nutrient assimilation efficiency, which can only be improved by introducing intestinal microbes from normal mice donors (Hansen et al., 2012; Olszak et al., 2012). In many cases, the timing of microbial colonization is critical and microbiome restoration must occur within an early developmental window in order to affect host phenotypes that are observed later in life (Cox et al., 2014; Hansen et al., 2012). Therefore, variation in host microbiota can have important consequences for digestive capability and body condition, and act as a mechanism underlying developmental plasticity (Alberdi et al., 2016). Importantly, effects may be dependent on the timing of microbial colonization if microbial effects on host traits may have immediate effects (i.e. contemporary associations), or delayed effects (i.e. time-lagged associations).

In many vertebrate taxa, natal body size (Bowen et al., 2015; Ringsby et al., 1998; Stige et al., 2019), natal body mass (Both et al., 1999; Monrós et al., 2002), or growth rate (Bergenius et al., 2002; T. E. Martin, 2015) can be key predictors of juvenile viability and recruitment into the breeding population. The association between the gut microbiome and weight has been studied extensively in clinical studies for application in human obesity (Bäckhed et al., 2004; Turnbaugh et al., 2006), and in agricultural studies to increase animal productivity (Coates et al., 1981; Ford & Coates, 1971; Torok et al., 2011). While having a more diverse microbiome may intuitively suggest a healthier gut microbiome (Lozupone et al., 2012), the effects of microbial community structure are likely more complex (Zaneveld et al., 2017). In humans, lower diversity of gut microbiota is associated with high body mass index and high amounts of visceral fat (Beaumont et al., 2016; Le Chatelier et al., 2013). Specific taxa, such as *Lactobacillus* spp. can influence fat storage and weight change, however, the effects of such bacteria are strain-specific (Drissi et al., 2017). In poultry, beneficial bacteria can be selectively enriched (e.g. Patterson et al., (1997)), and the use of antibiotics reduce gut bacterial load, decrease microbial diversity, and improve feed conversion ratios (Banerjee et al., 2018). Weight gain associated with antibiotic administration and decreased bacterial diversity has also been shown in house sparrows (*Passer domesticus*) (Kohl et al., 2018), *Daphnia magna* (Motiei et al., 2020) and magellanic penguins (*Spheniscus magellanicus*) (Potti et al., 2002). By contrast, microbial diversity has been positively associated with body condition and growth rate in wild nestling birds (Teyssier, Lens, et al., 2018).

The great tit (*Parus major*) provides a powerful natural system for addressing microbiome related effects on host phenotypes. It is an altricial species that readily breeds in nest boxes, so that the developmental period can be tracked easily in the wild. We investigated the relationship between natural variation in the gut microbiome and direct and indirect measures of fitness-related traits in nestling great tits when they were 8 and 15 days old. The neonatal stage is a critical time for gut microbiome colonization (Koenig et al., 2011) and the microbial-programming of

adult phenotypes (Cox et al., 2014; Hansen et al., 2012; Sudo et al., 2004). Nestling great tits undergo substantial shifts in gut microbiota between day 8 and day 15 of development(Teyssier, Lens, et al., 2018), a period when we predicted the microbiome would have a detectable effect on body weight and the probability of survival to fledging. We tested whether diversity of the microbiome, or the relative abundances of specific microbes, were associated with nestling weight and survival to fledging. Given the mixed reports of microbial links with host fitness in the existing literature (see above), we had no a priori predictions regarding the direction of such potential relationships. Nevertheless, we proposed that if diversity was positively related to measures of host fitness, then a rich and/or evenly distributed microbial community would be important for healthy development. By contrast, if the relationship was negative, then the host may benefit from the presence or absence of specific microbial taxa, which should be observed in differences in the relative abundance of these specific microbes associated with host fitness. Moreover, because early developmental windows have been reported to be important for microbial influences on host traits (Cox et al., 2014; Hansen et al., 2012), we sampled the gut microbiome from chicks at two developmental stages to test whether microbiome effects on host fitness traits were contemporary or time-lagged. In other words, we asked whether the gut microbiome at D8 or D15 was a better predictor of mass and survival.

2.2 Methods

2.2.1 Field monitoring and microbiome sampling

Birds were sampled from nine nest box populations across Co. Cork, Ireland, five of these were mixed/deciduous habitats and four were coniferous habitats (see O'Shea et al., (2018)). We collected 204 faecal samples from 150 nestling great tits from 54 nests (see below for the number of samples that were successfully sequenced and sample size per developmental stage) for 16S rRNA gene sequencing in order to map the gastrointestinal microbial communities. During April-June 2016, nest boxes were monitored to determine lay dates, hatching dates and nestling survival. Nestlings

aged 8 days (+/- 1 day) (D8) and 15 days (D15) were placed into sterile holding bags inside a heated holding case. Coffee filters were used to line the bags in order to soak up uric acid from the faeces, as uric acid has the potential to affect downstream sequencing (Khan et al., 1991). Samples were collected from nestlings that defaecated naturally within 15-20 minutes of being out of the nest before being returned to the nest. Although the aim was to have repeated samples for all individuals, not all birds survived, and not all birds produced samples within this time limit. Faecal sacks were opened using a sterile inoculation loop to release the faecal matter and place in a microcentrifuge tube containing 500uL of 100% ethanol. Samples were stored at -20^{o}C within 8 hours of collection until DNA extraction. D8 birds were weighed, and uniquely individually identified by clipping the tip of one of their nails, avoiding the blood vessel (commonly known as the “quick”). D15 nestlings were weighed and ringed with a unique identifiable metal ring (British Trust for Ornithology) and matched against their unique nail clipping. Nestlings for which we had repeated samples (i.e. for both D8 and D15) are referred to as ‘repeat samples’.

2.2.2DNA Extractions

Briefly, DNA was extracted from the dried faecal contents of all birds using the Qiagen QIAamp DNA Stool Kit, following the "Isolation of DNA from Stool for Pathogen Detection" protocol (June 2012 edition), with modifications described in Shutt et al. (2020) to accommodate dried avian faeces. A 0.10 - 0.20 g aliquot of each faecal sample was added to the kit, alongside two negative controls which were carried through to sequencing. The negative controls had low sequence reads and were not included in any subsequent analyses. We note that no decontamination methods were used, though they can be beneficial for samples with low biomass (Eisenhofer et al., 2019). Therefore, we recognise that our sequence data may contain some Amplicon Sequence Variants (ASVs) from the external environment, rather than the gut microbiome, although this should not have systematically biased our results. DNA was stored at -20^{o}C. Full extraction methods are described in Supporting Information.

2.2.3 Illumina MiSeq sequencing

Full library preparation details are described in Supporting Information and in Davidson et al. (2020). Briefly, the V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 300 cycle kit, following standard Illumina sequencing protocols.

2.2.4 Bioinformatics and statistical analysis

The DADA2 pipeline (Callahan, McMurdie, et al., 2016) was used to process the raw sequencing data in R version 3.5 (R Core Team, 2019). Sequence quality was visually inspected. Sequences were trimmed to remove adapters and lower quality reads (median quality scores below 25-30 threshold) at the extremities of the sequence and filtered to remove sequences with higher than expected errors. Read errors were estimated before dereplication. Forward and reverse reads were merged to construct 'contig' sequences, these were used to construct a sequence table of ASVs, which counts the number of times each unique sequence is detected. The previous steps were performed for each run separately. Then the separate sequence tables were merged and chimeras removed using the 'consensus' method. Taxonomy was assigned to each ASV by RDP's Naive Bayes Classifier (Q. Wang et al., 2007) against the Silva reference database (version 132) (Quast et al., 2012). This is at 100% sequence identity in contrast to the lower resolution OTU method which groups sequences at 97% identity. ASVs allow greater sensitivity and specificity, better discrimination of ecological patterns than OTU's and are reusable across studies (Callahan et al., 2017).

The DADA2 outputs were assembled into a single Phyloseq object (McMurdie & Holmes, 2013). Sequences identified as mitochondrial or chloroplast were removed. Further filtering of samples and ASVs took place in R. Sample completeness curves were plotted using vegan (Oksanen et al., 2019) and helped determine the lower cut-

off for sample reads at 10,000 reads. Low read samples (<10000 reads, 9 samples) were removed leaving 195 (Day-8=81, Day-15=114, repeat samples=41) samples for the analysis. Alpha diversity (both Shannon and Chao1 diversity) was calculated using the '*estimate_richness'* function from the phyloseq package on the filtered dataset. Shannon diversity (Shannon, 1948) measured richness weighted by abundance (the evenness of a community) and Chao1 (Chao, 1984) measured richness, specifically estimating taxa abundance and rare taxa missed from under sampling.

2.2.5 Statistical analysis

Nestling weight: Alpha diversity and phylum-level relative abundance

Linear mixed models (LMMs) and generalised linear mixed models (GLMMs) were used to examine the association between measures of nestling weight and two measures of alpha diversity: Shannon's index and Chao1 index. Models were fit using the lme4 package (D. Bates et al., 2015) on each data subset. Numeric variables were centered and scaled. Model diagnostics were assessed using the DHARMa package (Hartig, 2019). Raw weight (g) was used as the response variable instead of change in weight (delta-weight) as this allowed us to control for D8 weight in time-lagged models, as well as to differentiate between, for example, very light D8 nestlings achieving normal D15 weight and heavy D8 nestlings achieving heavy D15 weight. We also tested whether alpha diversity at D8 was correlated with alpha diversity at D15 (for repeated samples within individuals) by calculating Pearson's correlation coefficient with the 'cor.test' function in R.

We examined whether nestling body weight was influenced by the gut microbiome in three different ways: (1) whether the microbiome at an early developmental stage predicted future host weight (henceforth 'time-lagged weight'), while controlling for weight at D8, (2) whether the microbiome at the time of sampling was associated with weight at the same developmental stage (henceforth 'contemporary weight') at D8, and (3) contemporary weight at D15. Due to collinearity (alpha diversity) and non-independence (Phylum-level relative abundance), we performed separate analyses for four different measures of microbiome as explanatory variables: alpha

diversity (Shannon diversity and Chao1 index), and phylum-level relative abundance (Proteobacteria and Firmicutes). These two phyla were selected for our analyses because they were the only two taxa that were prevalent across all birds (except for three birds).

For the time-lagged weight analyses, we ran a linear mixed model (LMM) using the lmer function in R with D15 weight as the response variable, and alpha diversity at D8 as a fixed variable. We included D8 weight as a covariable to control for variation in starting weight. We also included habitat, lay date of first egg and brood size as fixed factors as these may influence nestling weight. Site and nest ID were used as nested random effects, as well as sequence plate as a separate random effect. Only birds that had repeat samples were included in this analysis (n= 41).

For the D8 contemporary weight analyses (all D8 samples, n= 81), we ran a LMM as described above, with D8 as the response variable and alpha diversity at D8 as a fixed factor. We included the same fixed effects and random effects as described above. The D15 contemporary analyses (all D15 samples, n = 114) were the same as described for D8, using D15 data as opposed to D8 data. D15 weight was squared in the contemporary D15 model as the model did not converge with raw weight as the response.

Nestling survival: Alpha diversity and phylum-level relative abundance

We tested whether microbiome diversity at D8 predicted nestling survival. Here we define survival as surviving to fledgling. Birds that were previously alive at D8, but were either dead in the nest, or absent (e.g. eviction from the parents) at D15 were scored as not surviving. Nests were checked again approximately 25 days after hatching, when birds were expected to have fledged. Birds found dead in the nest were scored as not surviving, and those that were absent were assumed to have fledged because chicks were too large to be evicted, and we did not see any evidence of predation (woodpeckers are absent in our woodlands, and predation from stoats

always led to the entire nest failing, and was isolated to a single woodland site not included in the current study). We ran a non-parametric cox proportional hazards survival model using the Coxme package (Therneau, 2020), and included weight (at D8), lay date and brood size (at D8) as covariables. We included three ranks of survival times (i.e. died before D15, died after D15 or fledged) as the ranks of survival times provide additional temporal information beyond a binary outcome, i.e. died/survived for the model to estimate mortality risk. The habitat variable was excluded from the survival models as it caused premature model convergence due to a lack of variation in the variable (i.e. all coniferous birds survived). Because all but one bird that survived to D15 survived to fledging, we only performed this analysis for birds that were sampled for gut microbiome at D8. We repeated this analysis with Proteobacteria relative abundance as the predictor variable, and again with Firmicutes relative abundances as the predictor variables.

Nestling survival: Beta diversity

We investigated whether microbial community structure differed between birds that did or did not survive to fledgling, using the adonis function (PERMANOVA) from the vegan package (Oksanen et al., 2019). Beta diversity at D8 was measured using Bray-Curtis distance (weighted by ASV abundance) and Jaccard (presence/absence of ASVs). We ran separate analyses for each beta diversity measure in which D8 weight and survival (Yes/No) were fixed factors. Adonis fits each term sequentially, therefore we used the model formula *beta-diversity~D8-weight + survival*, allowing us to estimate the variation in microbiome community composition associated with survival status after accounting for body weight, as weight was associated with survival in our population (see results, Table 2). Permutations were constrained to within nest with the 'strata' option to control for pseudo-replication of samples from the same nest. Adonis results were very similar with and without this strata option.

The beta diversity dataset (D8 samples) was filtered by prevalence prior to distance calculation, where taxa with less than 1 copy in 5% of samples were removed

following Knowles et al. (2019). This resulted in a dataset containing 1254 ASVs. The relative abundance of each phylum was calculated per sample using 'total sum scaling' (TSS) by dividing each feature count by the total library size (McKnight et al., 2019). Bray Curtis and Jaccard distances were calculated from our TSS normalized data. An assumption of the adonis test is that groups have homogeneity of variance. The dispersion of the groups was checked for homogeneity of variance using the Betadisper and permutest functions from the vegan package.

The beta diversity (Bray-Curtis) of D8 nestlings was visualised using a Principle Coordinate Analysis (PCoA) plot, created using the plot_ordination command in the phyloseq package. This plot compared individuals that survived to those that did not survive (see Figure S2). All graphs were created using ggplot2 (Wickham, 2016), unless otherwise indicated.

Indicator ASVs for nestling survival

We carried out indicator species analysis to explore whether ASVs were representative of birds that were alive at D8 (n=81) and survived to fledgling (n=64), or did not survive to fledgling (n=17). This analysis used the D8 data subset, which was not filtered by prevalence and contained 29709 ASVs. Indicator analysis identified "indicator species" if they reflected the state of the environment (i.e. likelihood the bird will survive, or not survive). We used the multipatt function from the IndicSpecies package (De Cáceres & Legendre, 2009), which assigns a test statistic, or "indicator value" to each ASV for each group, in our case two groups: Survival-Yes, Survival-No. Taxa with an indicator value of > 0.4 and $p \leq 0.05$ were considered indicator species. We also report the "specificity value" and the "fidelity value". The former is the estimate of the probability that an ASV is detected in a particular group. For example, a value of 1.0 would indicate that the ASV is only detected in that group and not another group. The latter is the probability estimate of finding the species in sites (i.e. birds) belonging to the group. For example, a value of 1.0 would indicate that the ASV is recorded in all birds within that group (De

Cáceres, 2020). We did not adjust for multiple comparisons for three reasons. First, our analysis did not include any overlap, and thus multiple analyses, across different combinations of groups (De Cáceres & Legendre, 2009). Second, the purpose of this analysis was exploratory in order to generate, rather than confirm hypotheses (Bender & Lange, 2001; Ranstam, 2019). As such, we report ASVs as potential indicator species, as opposed to reporting a quantitative, total number of taxa associated with survival/non-survival (De Cáceres et al., 2010). Third, this analysis served to generate a posteriori predictions regarding the relationship between indicator taxa and weight gain (see below), although we note that independent studies will be necessary to confirm these exploratory analyses (Ranstam, 2019). We generated a heatmap of indicator species for survival and non-survival using the heatmap function from the phyloseq package (McMurdie & Holmes, 2013).

A posteriori analysis of indicator species

Indicator species from our analysis may reflect whether a bird survives or not. We hypothesized that indicator species may reflect host survival because such indicator species may also predict time-lagged, relative weight gain. We performed a posteriori LMMs with D15 weight as the response variable and D8 relative abundance (of the indicator species) as a fixed variable. D8 weight, habitat, brood size and lay date were also included as fixed variables. Site and nest were included as nested random terms (i.e. 1|site/nest). We restricted this a posteriori analysis to one indicator species that had the highest test statistic from each group (Survival-Yes: Lactobacillus (ASV46) and Survival-No: Rahnella (ASV38)). Because there were a high number of zeros in relative abundance of these taxa, and because our data were proportions and therefore could not be fit using a zero-inflated (count data) model, we included only birds where the taxa were present and the birds with 0 abundance for that taxa were excluded (ASV46: n=21/41; ASV38: n=8/41).

2.3 Results

2.3.1 Sequencing results

There were 18,537,795 high quality reads, clustered into 49,655 ASVs before removing control samples, mitochondria and chloroplast sequences. Mean reads per sample was 88,698 and individual samples ranged from 61 to 579,864 reads. Samples with less than 10,000 reads were discarded before further analysis (n=9). There were 15,183,675 total high-quality reads, clustered into 47,400 ASVs after removing control samples as well as mitochondria and chloroplast sequences. Samples ranged from 10,220 to 557,336 reads and mean reads per sample was 77,865.

The most prominent phyla across all samples (D8 and D15) were as follows (mean percentage relative abundance ±SE): Firmicutes (43.0%±2.5); Proteobacteria (35.2%±2.2); Tenericutes (9.7%±1.8); Bacteroidetes (4.2%±0.4) and Actinobacteria (2.2%±0.2). Relative abundance of the major phyla at D8 (at least ≥10% relative abundance in at least 1 sample) per sample are plotted in Figure 1.

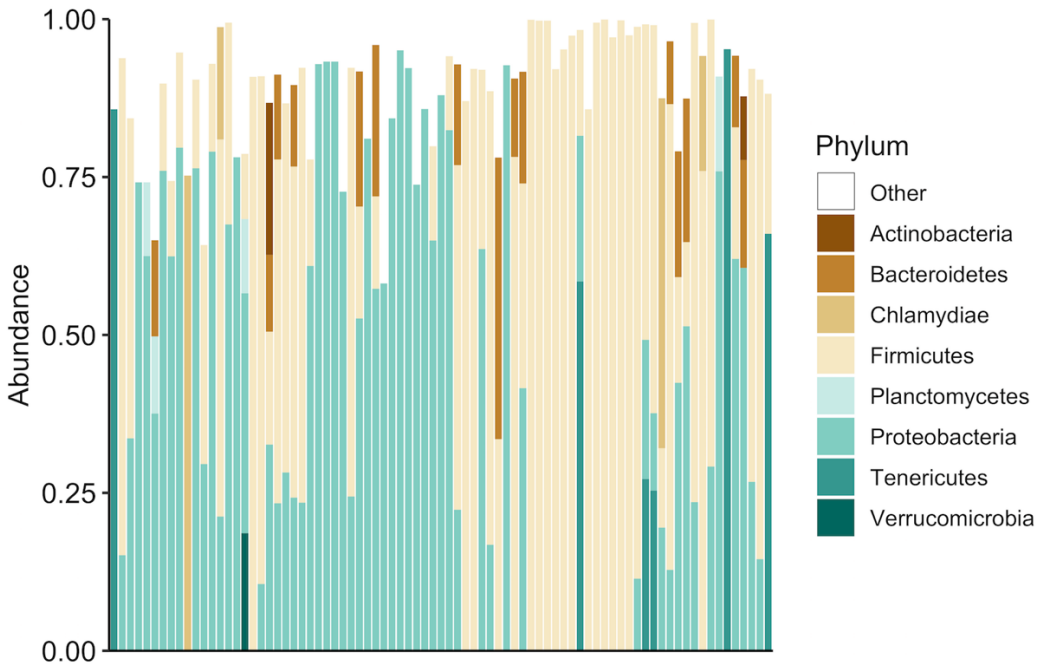

Figure 1. Relative abundance of the 8 most prominent phyla (≥10% relative abundance). Each vertical bar corresponds to a nestling at Day 8. "Other" group represents all low abundance phyla as white space.

2.3.2 Nestling weight

The time-lagged analysis indicated that weight at D15 was negatively related to alpha diversity at D8 (Figure 2a, Table 1, Table 4), controlling for weight at D8 (Table 1). Neither habitat, lay date or brood size had detectable influence in this analysis (Table 1). Cross-sectional analyses indicated no relationship between contemporary weight and diversity at either D8 or D15 (Figure 2b and 2c, Table 4, Table S1). Brood size was negatively related to weight at D8. There was no evidence for an effect of habitat on weight in D15 birds. The relative abundances of Proteobacteria and Firmicutes did not predict weight in any of these analyses.

Table 1. GLMM output from time-lagged (Day-15) weight analyses. The effect of alpha diversity is reported for (a) Shannon diversity and (b) Chao1 diversity. * p < 0.05. *Note: Correlation tests indicate both Shannon (cor=0.33, t=2.17, df=39, p= 0.036) and Chao1 (cor=0.36, t=2.38, df=39, p=0.022) diversity measures were positively correlated within pairs.*

Dependent~Independent variable	Estimate	Std. Error	df	Test statistic	*p*
(a) Nestling weight (time-lagged)					
(Intercept)	16.798	0.525	21.052	31.977	<0.001 *
Shannon (Day-8)	-0.593	0.167	23.749	-3.544	0.002 *
Habitat (deciduous)	0.247	0.630	22.081	0.392	0.699
Weight (Day-8)	0.700	0.173	24.672	4.035	<0.001 *
Lay Date (first egg)	0.252	0.296	21.226	0.851	0.404
Brood size (Day-8)	0.168	0.273	21.991	0.616	0.544
(b) Nestling weight (time-lagged)					
(Intercept)	16.961	0.475	20.856	35.681	<0.001 *
Chao1 (Day-8)	-0.525	0.184	34.314	-2.855	0.007 *
Habitat (deciduous)	0.011	0.572	21.903	0.019	0.985
Weight (Day-8)	0.528	0.192	32.990	2.756	0.009 *
Lay Date (first egg)	0.257	0.270	21.399	0.949	0.353
Brood size (Day-8)	0.121	0.250	22.841	0.483	0.634

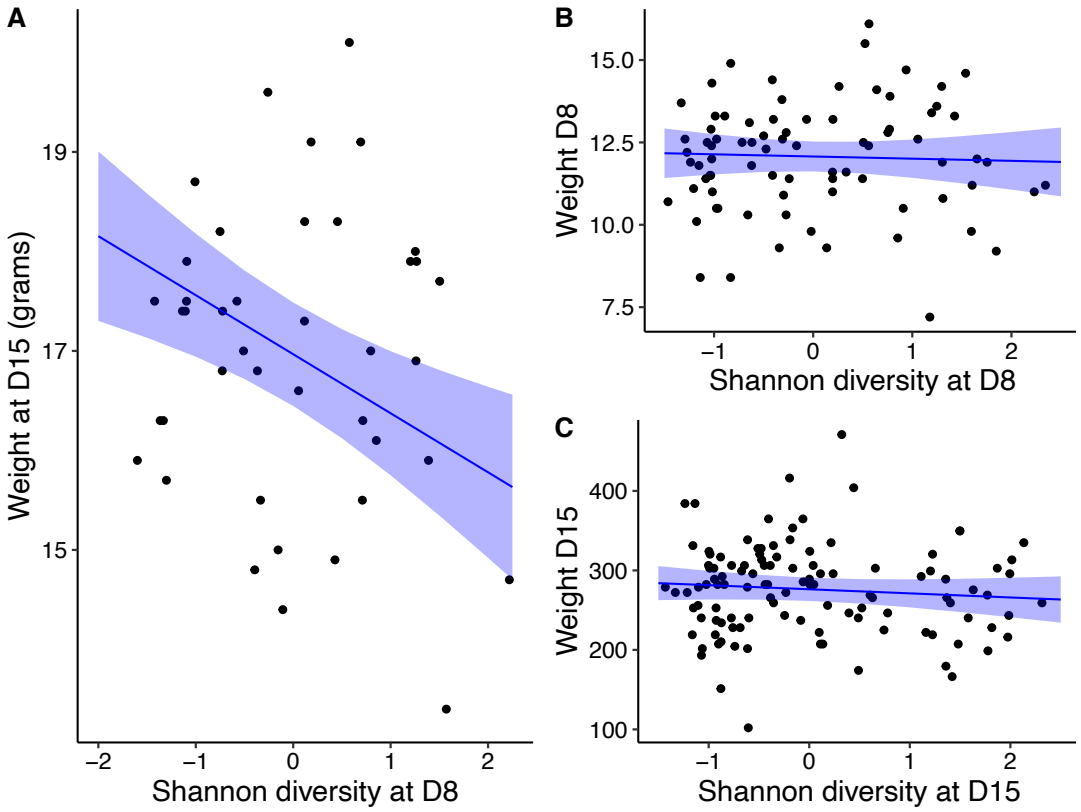

Figure 2. The effect of Shannon diversity on (a) time-lagged weight (i.e. Day 15 (D15)), (b) contemporary weight Day 8 (D8) and (c) contemporary weight D15. Black dots are individual data points, blue line is regression line with 95% CI (shaded blue). Shannon diversity is centred and scaled.

2.3.3 Nestling Survival

Survival was significantly related to weight at D8; the hazard ratio of 0.67 indicated that for every unit increase in weight, mortality risk decreased by a factor of 0.67. Survival was not influenced by lay date or brood size. Nestling survival to fledging was not predicted by Shannon's diversity or Chao1 index at D8 (Table 2, Table 4). The relative abundance of Proteobacteria and Firmicutes at D8 also did not predict survival to fledging.

There was no clear evidence for the violation of the assumption of homogeneity of dispersion for survived/not-survived groups for Jaccard distance (Sum Squares=

0.009, F=3.295, p=0.081) or Bray-Curtis (Sum squares=0.006, F=1.973, p=0.164). Beta diversity did not differ between surviving and non-surviving nestlings for Jaccard (Sum Squares=0.563, F=1.233, p=0.172) or Bray-Curtis (Sum Squares=0.523, F=1.193, p=0.228), Table 4, and see Supporting Information Figure S2, Table S3.

Table 2. Determinants of mortality risk from Cox proportional hazards model. Positive coefficients indicate increased risk of mortality. HR = Hazard ratio, which is the exponentiated coefficients, and gives the ratio of the total number of observed to expected events. Z = test statistic. Note: all numeric covariates are centered and scaled. * p < 0.05.

Dependent~Independent variable	Coefficient	HR	Std. error	z	*p*
Nestling mortality risk					
Shannon D8	0.021	1.021	0.327	0.07	0.95
Weight D8	-0.665	0.514	0.282	-2.36	0.018 *
Lay Date (First egg)	0.304	1.356	0.382	0.80	0.43
Brood Size (Day-8)	0.551	1.735	0.363	1.52	0.13
Nestling mortality risk					
Chao1 D8	0.314	1.368	0.283	1.11	0.27
Weight D8	-0.609	0.544	0.289	-2.11	0.035 *
Lay Date (First egg)	0.297	1.346	0.396	0.75	0.45
Brood Size (Day-8)	0.634	1.885	0.386	1.64	0.1

2.3.4 Indicator species analysis

The indicator species with the highest indicator values associated with survival belonged to ASV46, ASV310 and ASV150 which all belong to the *Lactobacillaceae* family (Figure 3, Table 4, Table S4). The taxa with the highest indicator values associated with non-survival belonged to the ASV38, ASV1034, ASV895 which belong to *Enterobacteriaceae*, *Methylopilaceae* and *Acidothermaceae* families, respectively. The taxa indicative of non-survival and survival (p value ≤0.05 and test statistic ≥0.4)

were plotted on a heatmap (Figure 3). Overall, specificity values were high (mean = 0.94±0.008 standard error) and fidelity values were low (0.21±0.008), suggesting that indicator species were typically only detected within a given group (i.e. survive/non-survive), but that not all birds within a group were recorded to have a given indicator species. All indicator species and test statistics (specificity values and indicator values) are reported in Supporting Information Table S4. A posteriori analyses showed that a higher relative abundance of ASV46 (a *Lactobacillus* spp., and the taxa most strongly associated with survival) at D8 predicted a higher weight at D15. Relative abundance of ASV38 (a *Rahnella* sp., and the taxa most strongly associated with non-survival) was not associated with weight at D15 (Table 3, Table 4).

Table 3. A posteriori GLMM output from time-lagged weight analyses. The effect of indicator species on weight at D15 are reported for a) *Lactobacillus* sp. (ASV46) and b) *Rahnella* sp. (ASV38). * p <0.05

Dependent/Independent variable	Estimate	Std. Error	df	Test statistic	*p*
(a) D15 Weight (time-lagged)					
(Intercept)	17.673	0.739	4.679	23.906	<0.001 *
Relative Abundance ***Lactobacillus* sp. (ASV46)**	1.042	0.367	8.601	2.839	0.02 *
Habitat (deciduous)	-1.946	1.007	5.126	-1.933	0.11
Weight (Day-8)	0.835	0.340	11.184	2.460	0.031 *
Brood Size (Day-15)	-0.459	0.352	11.573	-1.303	0.218
Lay Date (First egg)	-0.457	0.389	7.245	-1.175	0.277
(b) D15 Weight (time-lagged)					
(Intercept)	16.473	0.656	2.000	25.111	0.002 *
Relative Abundance ***Rahnella* sp. (ASV38)**	0.040	0.388	2.000	0.103	0.927
Habitat (deciduous)	2.908	1.320	2.000	2.203	0.158
Weight (Day-8)	5.398	1.579	2.000	3.419	0.076
Brood Size (Day-15)	1.690	0.660	2.000	2.558	0.125
Lay Date (First Egg)	-1.069	1.113	2.000	-0.960	0.438

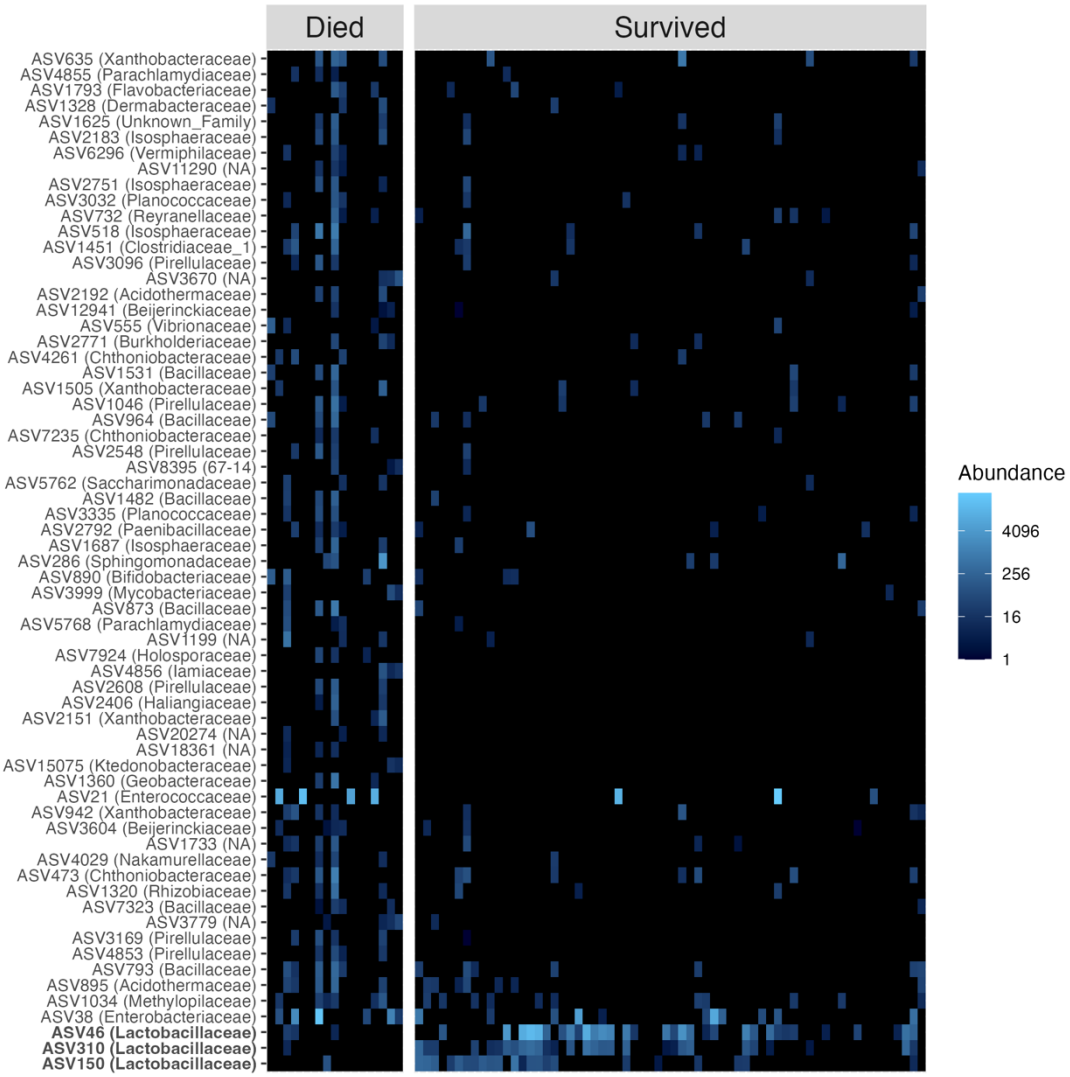

Figure 3. Heatmap of relative abundance of indicator species ASVs for survival. Individual birds are columns with separate panels for individuals that either died or survived. ASVs are in rows where taxa in brackets refer to the ASVs family-level taxonomic assignment, or NA if not resolved to family-level. Taxa associated with survival as opposed to non-survival are in bold. Lighter colours indicate greater relative abundance.

Table 4. Summary of the relationship between microbiota and host performance. Significant relationships are shown as either positive (+), negative (-) or non-significant (NS).

Microbiome metric	Survival	Non-survival	Weight (time-lagged)	Weight (contemporary)
Alpha diversity	NS	NS	(-)	NS
Beta diversity	NS	NS		
Phylum-level relative abundance	NS	NS	NS	NS
Indicator species	(+)	(+)		
Lactobacillus sp. (ASV 46)			(+)	NS
Rahnella sp. (ASV38)			NS	NS

2.4 Discussion

Overall summary

We demonstrate that the gut microbiota correlates with nestling performance in a population of wild passerine birds. We found a negative association between weight at D15 and alpha diversity at D8. In other words, individuals with a low microbial diversity at D8 were heavier than expected at D15, given their weight at D8. Although alpha diversity at D8 was correlated with alpha diversity at D15, neither microbial alpha diversity nor phylum-level relative abundance predicted contemporary weight. Our exploratory analysis indicated that eight-day old nestlings with a greater relative abundance of specific *Lactobacillus* taxa were more likely to fledge, and our a posteriori analysis showed that for at least one of these taxa, greater relative abundance was specifically associated with increased host weight (relative to weight at D8). Notably, although we present potential links between gut microbiota and survival, and a proxy for survival (i.e. weight), the direction of these relationships or the microbial metrics that predict such relationships are variable within our own study (Table 4) and contrast with some, but not other studies. A lack of consensus on what aspects of microbial taxonomic composition predict host traits is prominent in the microbiome literature (Zaneveld et al., 2017). This may be due to broad statistical approaches that are correlative in nature, and therefore require manipulative studies

to confirm relationships found here. Nevertheless, we discuss below how methodological approaches, critical developmental windows, environmental matching and the Anna Karenina Hypothesis may explain such differential findings across studies. Moreover, we suggest hypotheses worthy of future confirmatory testing to understand the effects of gut microbiota on the development of host phenotypes.

We show a negative relationship between alpha diversity and relative weight gain, over one nesting season. Experimentally reducing diversity of the microbiome through antibiotic administration has been shown to improve host growth (Banerjee et al., 2018; Kohl et al., 2018; Lan et al., 2005; Potti et al., 2002), possibly because antibiotics reduce bacterial load, diverting resources towards growth, as opposed to immune function, in a classic life-history trade-off (Rauw, 2012). Our study demonstrates that this negative relationship is present in the absence of microbiome manipulation, although we can only speculate as to the mechanisms responsible for limiting relative weight gain in nestlings with high gut microbial diversity. For example, a conventional gut microbiome compared to a germ-free one lowers absorption capacity of glucose and B-vitamins by increasing the thickness of the gut epithelium (Ford & Coates, 1971), and non-mutualistic microbes may create greater competition for nutrients (Wasielewski et al., 2016). While the diversity of the microbial community may be important for regulating the host's future weight, some taxa may have disproportionate effects. At least one ASV, *Lactobacillus* sp., with the strongest potential association with survival, showed a positive, time-lagged association with host weight (relative to weight at D8). Several *Lactobacillus* spp. have beneficial, probiotic effects on the host (Angelakis & Raoult, 2010; Banerjee et al., 2018; Gomes et al., 2012; Vásquez et al., 2012), though this is certainly not the case for all *Lactobacillus* spp. Nevertheless, we can speculate as to why Lactobacillus may confer benefits to great tit nestlings and encourage confirmatory research to test such hypotheses. For example, some strains of *Lactobacillus sakei* (ASV150) are known to produce antimicrobial peptides which inhibit a known pathogen (Gomes et al., 2012). Therefore, we hypothesise that the presence of *Lactobacillus sakei,* which

was associated with survival, may serve as an anti-pathogen in great tit nestlings. More generally, *Lactobacillus* spp. produce metabolic byproducts such as short chain fatty acids that can improve host energy metabolism(LeBlanc et al., 2017) and modulate immune response (Ratajczak et al., 2019). For example, the SCFA butyrate acts as an anti-inflammation agent, enhances intestinal barrier function and modulates the production of mucosal immune products in mammals (see review: Liu et al. (2018)). Future studies should look to shotgun sequencing to identify specific bacterial strains and genes for functional profiling and for developing probiotic treatments. This could be followed up by manipulative studies to identify the causal relationships between ASV and host traits, such as isolating and culturing ASV46 to test its potential probiotic effects when administered to nestlings (G. L. Davidson, Raulo, et al., 2020; Gatesoupe, 1999; Holzapfel & Schillinger, 2002).

Although a growing number of studies have demonstrated that the microbiome is an important predictor of host weight, the microbial profiles and the direction of this relationship vary across species and studies (Kohl et al., 2018; Potti et al., 2002; Teyssier, Lens, et al., 2018; Videvall et al., 2019). For example, a negative relationship between alpha diversity and future weight gain was found in juvenile ostriches (Videvall et al., 2019), whereas a positive relationship between alpha diversity and contemporary body condition was reported in a different population of great tit nestlings (Teyssier, Lens, et al., 2018). The contrasting results reported in these studies and our own may be due to variations in the microbiome sampling method and storage solutions (i.e. cloacal versus faecal, see Videvall et al. (2017); storing solutions see Vargas-Pellicer et al. (2019)). Moreover, our study and that of others are limited to a single nesting season, and any general patterns of the gut microbiota on host traits may differ across years according to local conditions such as food availability (see below for discussion of Predictive Adaptive Response hypothesis). Therefore it is likely that differences in local environments (Gillingham et al., 2019; Teyssier, Rouffaer, et al., 2018; Youngblut et al., 2019) and the temporal resolution of microbial and host traits dictate when and how microbiota impacts host phenotypes in birds (Videvall et al., 2019).

Time-lagged associations between the gut microbiota and host traits are expected to be especially important during developmental periods when large shifts in the establishing microbial community coincide with the programming of host biology (Cox et al., 2014; Hansen et al., 2012; Sudo et al., 2004), and in systems that are likely to experience abrupt changes in environmental inputs associated with gut microbiome, such as diet. The latter may be especially relevant in birds as their microbiomes are tightly linked with environmental variation, in contrast to mammals where the microbiome is primarily linked to phylogeny (Song et al., 2020). In ostriches, microbiome diversity predicted future weight gain, but the direction of this relationship switched after week 1 post-hatching, a time when ostriches no longer rely on an internal yolk as sustenance (Videvall et al., 2019). In the current study, alpha diversity at D8 predicted future weight at D15, whereas cross-sectional analyses were not sufficient to detect such a relationship. This time-lagged association may be owing to a critical developmental period in which the microbiome impacts on future phenotypes, a phenomenon that is prominent in clinical microbiome literature (Cox et al., 2014; Hansen et al., 2012; Sudo et al., 2004). The gut microbiome may also act as a mechanism for adaptive phenotypic change through its sensitivity to environmental variability, and its connection to host biological processes (Alberdi et al., 2016). Such a mechanism would also be in line with the 'Predictive Adaptive Response hypothesis', which posits that conditions during development cause phenotypic changes that serve to match individuals to their environments (Bateson et al., 2014; Crino & Breuner, 2015; Weber et al., 2018). For example, unpredictable food supply during the development stage can influence adult physiological and behavioral phenotypes (Spencer et al., 2009; Zimmer et al., 2013). Therefore, microbiome-mediated developmental plasticity may be adaptive in species where offspring benefit from a gut microbiome that primes the host for dietary shifts. Dietary shifts are common in tits where the relative frequency of insect taxa can change throughout the provisioning period (Arnold et al., 2007; García-Navas et al., 2012). Although Belgian great tit nestlings had the greatest improvement in body condition if they maintained a stable microbial diversity

throughout development (Teyssier, Lens, et al., 2018), perhaps this may be because the environmental conditions at the time were also stable. In our study, it is difficult to differentiate between whether the gut microbiome is responding to dietary shifts as opposed to priming the host. Pinpointing if and when early life bacterial diversity programs host digestive and/or metabolic efficiency in wild animals, and the extent to which environmental variation, such as food availability and diet, impacts the developing microbiome will progress our understanding of microbial-mediated developmental mechanisms.

The Anna Karenina Hypothesis states that changes in an unhealthy or so-called "dysbiotic" microbiome can be stochastic rather than deterministic, making them difficult to detect statistically (Zaneveld et al., 2017). In other words, unhealthy individuals can have greater variation in their microbial community composition than healthy individuals, and rather than displaying a specific 'unhealthy' state, the microbiomes of diseased or stressed individuals can all look very different due to a breakdown in regulation. Although our study did not examine diseased individuals, as the original Anne Karenina Hypothesis was intended, this phenomenon may nevertheless explain why nestling survival was not associated with either alpha or beta diversity. In contrast to the Anne Karenina Hypothesis and our own study, disease-causing mortality in juvenile ostriches was associated with low alpha diversity, and beta diversity differed between diseased and non-diseased birds (Videvall et al., 2020). In the present study, although several indicator species were identified to be associated with birds that did or did not survive, many of these had low prevalence across nestlings. This is reflected in the high specificity values and low fidelity values, suggesting that although the presence of an indicator species in a nestling's gut microbiome reflects a high probability of survival or non-survival, the same indicator species is not present across all surviving/non-surviving nestlings in our sample population. This distinction is important in, for example, conservation practices whereby microbes are used as markers for population viability (Trevelline et al., 2019; West et al., 2019). Our analysis suggests that in the case of non-survival, there are no microbes that are universally indicative of non-survival across birds, but

rather the presence of one of several candidate microbes predicts a higher probability of mortality, which is consistent with the Anna Karenina Hypothesis. Alternatively, the gut microbiome does not predict survival in great tit nestlings at all, and the indicator species flagged in our study are an artifact of multiple testing without a correction due to the exploratory nature of this analysis. Therefore, whether these same taxa affect survival in other populations and/or other species of birds should be examined in independent confirmatory studies. For example, we hypothesise that ASVs reported for non-survival may be pathogenic to great tits, although we found no evidence in the literature to support this. Nevertheless, ASV1320, ASV2151, ASV3604 and ASV942 are members of the Rhizobiales order which contains genera that are well known human pathogens, and ASV5768 and ASV4855 are members of the Chlamydiales order which also contain pathogenic genera, though these genera were not detected in our study.

We also encourage further investigations into whether indicator taxa are the cause or consequence of host growth, or indeed any trait that may be associated with survival, such as glucocorticoids (Weber et al., 2018) and immune function (Hõrak et al., 1999). Manipulation of the gut microbiome alongside metabolomics or metagenomics will be the next step in providing insight into the causal direction and functional mechanisms of microbial variation and its relationship with host fitness. The latter is particularly important for identifying whether there is functional overlap between microbial taxa and the mechanisms by which these taxa affect host traits (Heintz-Buschart & Wilmes, 2018). Given the temporal importance of host-microbial relationships identified in this study and elsewhere, we anticipate the maturing microbiome will have important consequences on long-term fitness effects. More generally, our results highlight the importance of the gut microbiome in evolutionary processes as it has the potential to mediate rapid adaptation to environmental change and may also support conservation efforts as an indicator of individual and population health.

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Data availability

Our metadata is deposited at Dryad https://doi.org/10.5061/dryad. bk3j9kd9g. Sequence data are available in the European Nucleotide13 Archive under access number PRJEB42330, and ERS5506097 - ERS5506338.

Research and Animal Ethics:

This study was conducted under licences from the Health Products Regulatory Authority (AE19130_P017), The National Parks and Wildlife Services (010/2016) and the British Trust for Ornithology, and permission from Coillte Forestry and private landowners. The research project received ethical approval from the Animal Welfare Body at University College Cork, and was in accordance with the ASAB (Association for the Study of Animal Behaviour) Guidelines for the Treatment of Animals in Behavioural Research and Teaching.

2.5 Appendix 1A: Supplementary results

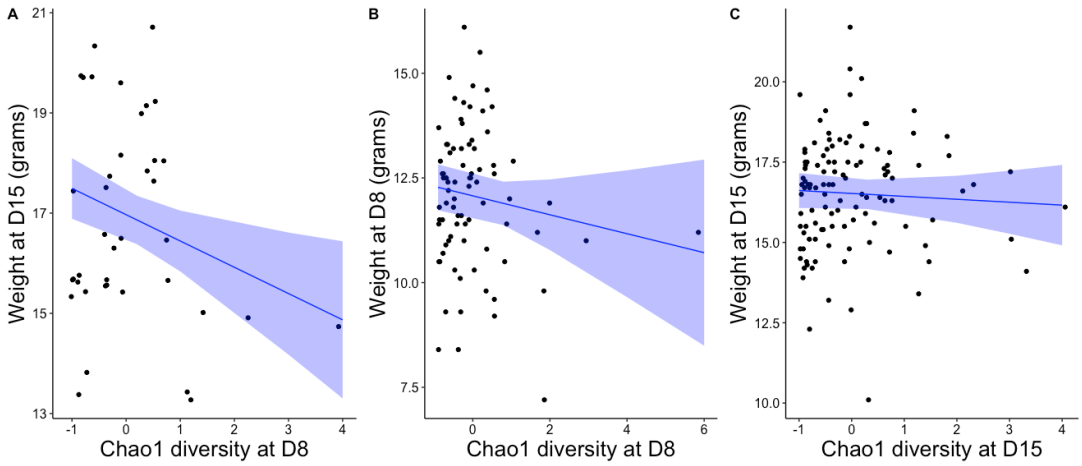

Figure S1. The effect of Chao1 diversity on (a) time-lagged weight (i.e. Day 15 (D15)), (b) contemporary weight D8 and (c) contemporary weight D15. Black dots are individual data points, blue line is regression line with 95% CI (shaded blue). Chao1 diversity is centred and scaled.

Table S1. GLMM outputs from contemporary weight analyses for (a) D8 and (b) D15. Alpha diversity is reported for (i) Shannon, and (ii) Chao1.* D15 weight was squared in order to allow model convergence. * p < 0.05

Dependent/Independent variable	Estimate	Std. Error	df	Test statistic	*p*
(a,i) Day-8 weight (contemporary)					
(Intercept)	12.666	0.507	30.714	24.985	<0.001 *
Shannon	-0.066	0.194	73.965	-0.341	0.734
Habitat	-0.737	0.570	30.528	-1.293	0.206
Lay date (of first egg)	-0.031	0.240	27.262	-0.130	0.897
Brood size (when sampled)	-0.465	0.234	31.274	-1.982	0.056
(a, ii) Day-8 weight (contemporary)					
(Intercept)	12.823	0.501	32.018	25.610	<0.001 *
Chao1	0.000	0.000	74.167	-1.237	0.22

Habitat	-0.680	0.548	29.877	-1.240	0.225
Lay date (of first egg)	-0.024	0.229	26.155	-0.104	0.918
Brood size (when sampled)	-0.478	0.225	30.174	-2.125	0.042 *
(b, i) Day-15 weight* (contemporary)					
(Intercept)	304.157	15.979	39.296	19.035	<0.001 *
Shannon	-5.148	5.148	107.825	-1.000	0.32
Habitat	-36.543	18.708	39.183	-1.953	0.058
Lay date (of first egg)	4.627	7.612	40.062	0.608	0.547
Brood size (when sampled)	-5.932	7.492	46.477	-0.792	0.433
(b, ii) Day-15 weight (contemporary)					
(Intercept)	17.490	0.525	47.490	33.315	<0.001 *
Chao1	0.000	0.000	104.772	-0.612	0.542
Habitat	-1.146	0.576	39.283	-1.989	0.054
Lay date (of first egg)	0.105	0.234	39.904	0.450	0.655
Brood size (when sampled)	-0.181	0.230	46.436	-0.785	0.437

Table S2. PERMANOVA results from nestling beta diversity analysis. Models effect on (a) Jaccard and (b) Bray-Curtis distances, of D8 weight and survival status. * p < 0.05

Dependent/Independent variable	Df	Sum Of Sqs	R2	F	*p*
(a) Jaccard					
D8 Weight	1	0.495	0.014	1.085	0.329
Survive	1	0.563	0.016	1.233	0.172
(b) Bray-Curtis					
D8 Weight	1	0.495	0.014	1.085	0.329
Survive	1	0.563	0.016	1.233	0.172

Table S3. Effect on weight of phyla-level relative abundance for top 2 most abundant phyla, Proteobacteria and Firmicutes. (a-b) time-lagged weight, (c-d) D8 contemporary weight and (e-f) D15 contemporary weight. * p < 0.05

Dependent/Independent variable	Estimate	Std. Error	df	Test statistic	*p*
(a) Nestling weight (time-lagged)					
(Intercept)	17.093	0.506	20.181	33.777	<0.001 *
Proteobacteria Abundance	0.033	0.178	23.541	0.185	0.855
Habitat	-0.179	0.609	21.275	-0.294	0.771
Weight (D8)	0.650	0.216	33.602	3.005	0.005 *
Lay Date First	0.178	0.286	20.597	0.623	0.54
Brood Size (D15)	0.087	0.271	23.469	0.320	0.752
(b) Nestling weight (time-lagged)					
(Intercept)	17.075	0.520	19.833	32.855	<0.001 *
Firmicutes Abundance	0.327	0.194	26.286	1.690	0.103
Habitat	-0.174	0.624	20.792	-0.279	0.783
Weight (D8)	0.568	0.204	30.418	2.788	0.009 *

Lay date (of first egg)	0.108	0.297	20.824	0.363	0.72
Brood Size (D15)	0.220	0.284	23.839	0.775	0.446
(c) Day-8 weight (contemporary)					
(Intercept)	17.491	0.540	42.498	32.390	<0.001 *
Proteobacteria Abundance	-0.001	0.005	4220.445	-0.105	0.916
Habitat	-1.166	0.584	42.561	-1.997	0.052
Lay date (of first egg)	0.023	0.057	42.621	0.404	0.688
Brood Size (D8)	-0.031	0.037	43.477	-0.843	0.404
(d) Day-8 weight (contemporary)					
(Intercept)	12.790	0.519	37.065	24.624	<0.001 *
Firmicutes Abundance	0.167	0.132	220.964	1.261	0.209
Habitat	-0.709	0.568	32.462	-1.248	0.221
Lay date (of first egg)	-0.048	0.159	29.880	-0.301	0.765
Brood Size (D8)	-0.540	0.285	28.342	-1.895	0.068
(e) Day-15 weight (contemporary)					
(Intercept)	17.491	0.540	42.498	32.390	<0.001 *
Proteobacteria Abundance	-0.001	0.005	4220.445	-0.105	0.916
Habitat	-1.166	0.584	42.561	-1.997	0.052
Lay date (of first egg)	0.023	0.057	42.621	0.404	0.688
Brood Size (D8)	-0.031	0.037	43.477	-0.843	0.404
(f) Day-15 weight (contemporary)					
(Intercept)	12.790	0.519	37.065	24.624	<0.001 *
Firmicutes Abundance	0.167	0.132	220.964	1.261	0.209
Habitat	-0.709	0.568	32.462	-1.248	0.221

Lay date (of first egg)	-0.048	0.159	29.880	-0.301	0.765
Brood Size (D8)	-0.540	0.285	28.342	-1.895	0.068

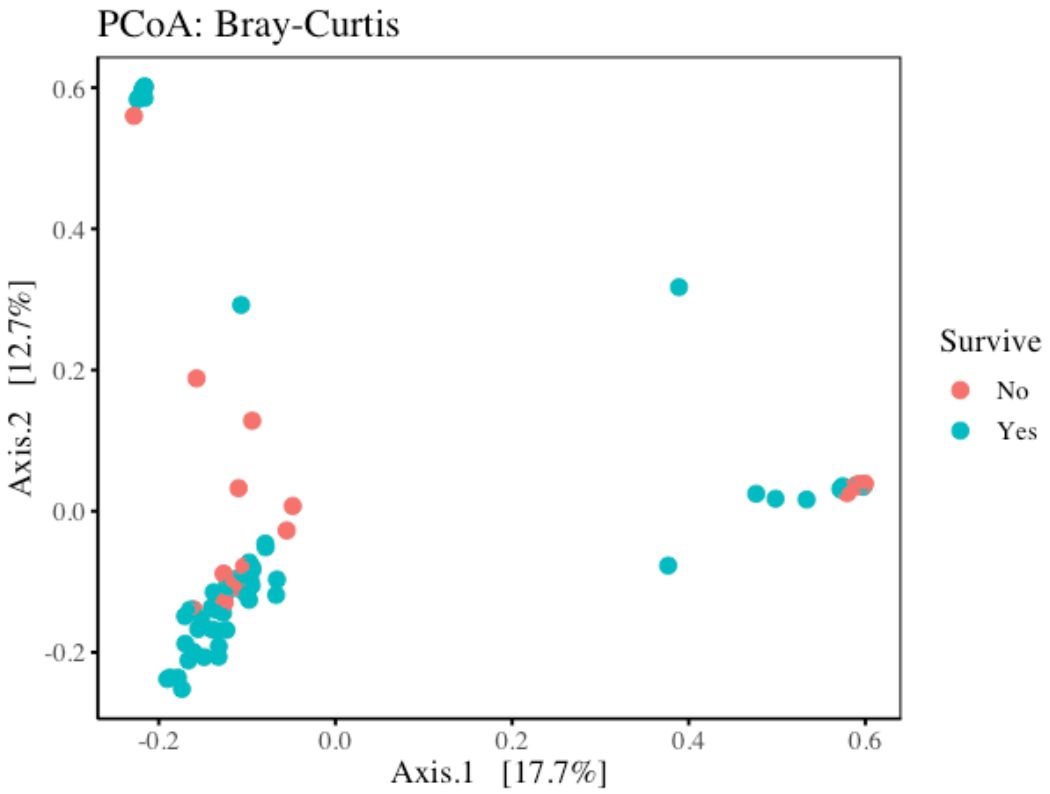

Figure S2. Principal Coordinate Analysis of Bray-Curtis distance matrix of Day-8 nestling samples, samples coloured by survival outcome.

Table S4. Indicator species results.

ASV	Survival	Indicator Value	Specificity Value	Fidelity Value	p value	Phylum	Class	Order	Family	Genus	Species
ASV1034	N	0.532	0.801	0.353	0.05	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylopilaceae	Hansschlegelia	NA
ASV1046	N	0.41	0.952	0.176	0.05	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Pir4_lineage	NA
ASV11290	N	0.407	0.938	0.176	0.015	Actinobacteria	Acidimicrobiia	Microtrichales	NA	NA	NA
ASV1199	N	0.419	0.994	0.176	0.005	Patescibacteria	Saccharimonadia	Saccharimonadales	NA	NA	NA
ASV12941	N	0.408	0.945	0.176	0.015	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Roseiarcus	NA
ASV1320	N	0.474	0.954	0.235	0.025	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Mesorhizobium	NA
ASV1328	N	0.405	0.928	0.176	0.015	Actinobacteria	Actinobacteria	Micrococcales	Dermabacteraceae	Brachybacterium	massiliense
ASV1360	N	0.42	1	0.176	0.005	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter	NA
ASV1451	N	0.408	0.943	0.176	0.04	Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	Clostridium_sensu_stricto_1	disporicum
ASV1482	N	0.413	0.968	0.176	0.03	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
ASV150	Y	0.549	0.877	0.344	0.05	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	sakei
ASV1505	N	0.41	0.952	0.176	0.03	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	NA	NA
ASV15075	N	0.42	1	0.176	0.01	Chloroflexi	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	NA	NA
ASV1531	N	0.409	0.949	0.176	0.015	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	niacini
ASV1625	N	0.405	0.928	0.176	0.025	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria_Incertae_Sedis	Unknown_Family	Acidibacter	NA

ASV1687	N	0.415	0.975	0.176	0.02	Planctomycetes	Planctomycetacia	Isosphaerales	Isosphaeraceae	Singulisphaera	NA
ASV1733	N	0.463	0.912	0.235	0.035	Proteobacteria	Alphaproteobacteria	Elsterales	NA	NA	NA
ASV1793	N	0.404	0.926	0.176	0.02	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Myroides	NA
ASV18361	N	0.42	1	0.176	0.025	Patescibacteria	Saccharimonadia	Saccharimonadales	NA	NA	NA
ASV20274	N	0.42	1	0.176	0.005	Patescibacteria	Saccharimonadia	Saccharimonadales	NA	NA	NA
ASV21	N	0.423	0.759	0.235	0.03	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Catellicoccus	NA
ASV2151	N	0.42	1	0.176	0.02	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	NA	NA
ASV2183	N	0.407	0.937	0.176	0.03	Planctomycetes	Planctomycetacia	Isosphaerales	Isosphaeraceae	Singulisphaera	NA
ASV2192	N	0.408	0.945	0.176	0.015	Actinobacteria	Actinobacteria	Frankiales	Acidothermaceae	Acidothermus	NA
ASV2406	N	0.42	1	0.176	0.015	Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	Haliangium	NA
ASV2548	N	0.41	0.954	0.176	0.045	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	NA	NA
ASV2608	N	0.42	1	0.176	0.015	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Pir4_lineage	NA
ASV2751	N	0.407	0.939	0.176	0.025	Planctomycetes	Planctomycetacia	Isosphaerales	Isosphaeraceae	Aquisphaera	NA
ASV2771	N	0.409	0.947	0.176	0.03	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Xylophilus	NA
ASV2792	N	0.414	0.728	0.235	0.035	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	xylanilyticus
ASV286	N	0.415	0.976	0.176	0.03	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	NA
ASV3032	N	0.407	0.939	0.176	0.025	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	NA

ASV3096	N	0.408	0.943	0.176	0.05	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	NA	NA
ASV310	Y	0.67	0.992	0.453	0.01	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
ASV3169	N	0.485	0.998	0.235	0.01	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	NA	NA
ASV3335	N	0.414	0.971	0.176	0.025	Firmicutes	Bacilli	Bacillales	Planococcaceae	Chungangia	NA
ASV3604	N	0.441	0.825	0.235	0.04	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium	NA
ASV3670	N	0.408	0.944	0.176	0.01	Cyanobacteria	Oxyphotobacteria	NA	NA	NA	NA
ASV3779	N	0.478	0.969	0.235	0.005	NA	NA	NA	NA	NA	NA
ASV38	N	0.565	0.904	0.353	0.045	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Rahnella	NA
ASV3999	N	0.416	0.981	0.176	0.01	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	Mycobacterium	NA
ASV4029	N	0.467	0.927	0.235	0.01	Actinobacteria	Actinobacteria	Frankiales	Nakamurellaceae	Nakamurella	NA
ASV4261	N	0.409	0.949	0.176	0.01	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Candidatus_Udaeobacter	NA
ASV46	Y	0.706	0.997	0.5	0.025	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
ASV473	N	0.468	0.932	0.235	0.05	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Candidatus_Udaeobacter	NA
ASV4853	N	0.485	1	0.235	0.005	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	NA	NA
ASV4855	N	0.404	0.923	0.176	0.05	Chlamydiae	Chlamydiae	Chlamydiales	Parachlamydiaceae	Neochlamydia	NA
ASV4856	N	0.42	1	0.176	0.01	Actinobacteria	Acidimicrobiia	Microtrichales	Iamiaceae	Iamia	NA
ASV518	N	0.408	0.941	0.176	0.05	Planctomycetes	Planctomycetacia	Isosphaerales	Isosphaeraceae	Aquisphaera	NA

ASV555	N	0.408	0.946	0.176	0.04	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	NA
ASV5762	N	0.412	0.962	0.176	0.005	Patescibacteria	Saccharimonadia	Saccharimonadales	Saccharimonadaceae	NA	NA
ASV5768	N	0.418	0.989	0.176	0.02	Chlamydiae	Chlamydiae	Chlamydiales	Parachlamydiaceae	Neochlamydia	NA
ASV6296	N	0.407	0.938	0.176	0.02	Dependentiae	Babeliae	Babeliales	Vermiphilaceae	NA	NA
ASV635	N	0.402	0.687	0.235	0.025	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Afipia	NA
ASV7235	N	0.41	0.954	0.176	0.005	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacter	NA
ASV732	N	0.407	0.941	0.176	0.05	Proteobacteria	Alphaproteobacteria	Reyranellales	Reyranellaceae	Reyranella	NA
ASV7323	N	0.477	0.966	0.235	0.01	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
ASV7924	N	0.42	1	0.176	0.005	Proteobacteria	Alphaproteobacteria	Holosporales	Holosporaceae	NA	NA
ASV793	N	0.514	0.897	0.294	0.025	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
ASV8395	N	0.411	0.958	0.176	0.02	Actinobacteria	Thermoleophilia	Solirubrobacterales	67-14	NA	NA
ASV873	N	0.416	0.983	0.176	0.025	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
ASV890	N	0.415	0.976	0.176	0.015	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	bifidum
ASV895	N	0.522	0.926	0.294	0.035	Actinobacteria	Actinobacteria	Frankiales	Acidothermaceae	Acidothermus	NA
ASV942	N	0.434	0.802	0.235	0.045	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	NA	NA
ASV964	N	0.41	0.953	0.176	0.035	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA

2.6 Appendix 1B: Supplementary methods

2.6.1 DNA Extractions

Prior to extraction, the samples were centrifuged at 13,000 rpm for 3 minutes, and the ethanol was removed using a pipette. Samples were then allowed to dry in a sterile fume hood for 2-3 hours, or until visibly dry. Total DNA was extracted from the dried faecal contents of all birds using the Qiagen QIAamp DNA Stool Kit, following the "Isolation of DNA from Stool for Pathogen Detection" protocol (June 2012 edition), with some modifications following Zeale et al. (2011) and custom modifications to accommodate dried avian faeces. Briefly, a 0.10 - 0.20 g aliquot of faecal sample was added to 1.4 ml of buffer ASL supplied in the kit together with 0.4g of zirconia beads. The samples were homogenised for 1 minute using the Biospec Minibeadbeater. The suspension was then heated for 30 minutes at 70˚C following the addition of Proteinase K. Following centrifugation, 1 InhibitEx tablet was added to 1.2 ml of the supernatant and the suspension was incubated for 1 minute at room temperature to allow inhibitors to absorb to the InhibitEx matrix. The samples were centrifuged twice and 20 µl of proteinase K was added to 400 µl of the supernatant along with 400 µl of AL. The suspension was homogenised and incubated at 70˚C for 15 minutes. 400 µl of ethanol (96-100\%) was added to the lysate and mixed well by vortexing. Subsequent removal of RNA, protein and purification of the DNA was completed according to the manufacturer's instructions. DNA was then stored at -20˚C.

2.6.2 Illumina MiSeq sequencing

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGA- GACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAA- GAGACA GGACTACHVGGGTATCTAATCC). Each PCR reaction contained 23 µl DNA template, 1

µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 25 µl 2X Kapa HiFi Hotstart ready mix (Roche, Ireland), to a final volume of 50 µl. PCR amplification was carried out as follows: heated lid 110°, 95°C x 3mins, 30 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, then 72°C x 5mins and held at 4°C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5\% agarose, 100V). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5µl) to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx), 25µl 2x Kapa HiFi Hot Start Ready mix, 10µl PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 30. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit (Bio-Sciences, Dublin, Ireland), along with the broad range DNA quantification assay kit (Bio-Sciences) and samples were then pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 300 cycle kit, following standard Illumina sequencing protocols.

Chapter 3: Individual variation in the avian gut microbiota: the influence of host state and environmental heterogeneity

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Author contributions
SES and GLD are joint first authors. GLD, CS, RPR and JLQ conceived of the project. SES, GLD, CS, RPR and JLQ designed the methodology. GLD, JMSC and MSR collected the field data. GLD, CNJ carried out the DNA extraction and library preparation. SES analysed the data. SES, GLD and JLQ wrote the manuscript with input from all authors. All authors gave final approval for publication.

Illustration by Dr. Caroline McKeon

Abstract

The gut microbiota have important consequences for host biological processes and there is some evidence that they also affect fitness. However, the complex, interactive nature of ecological factors that influence the gut microbiota has scarcely been investigated in natural populations. We sampled the gut microbiota of wild great tits (*Parus major*) at different life stages allowing us to evaluate how microbiota varied with respect to a diverse range of key ecological factors of two broad types: (1) host state, namely age and sex, and the life history variables, timing of breeding, fecundity and reproductive success; and (2) the environment, including habitat type, the distance of the nest to the woodland edge, and the general nest and woodland site environments. The gut microbiota varied with life history and the environment in many ways that were largely dependent on age. Nestlings were far more sensitive to environmental variation than adults, pointing to a high degree of flexibility at an important time in development. As nestlings developed their microbiota from one to two weeks of life, they retained consistent (i.e. repeatable) among-individual differences. However these apparent individual differences were driven entirely by the effect of sharing the same nest. Our findings point to important early windows during development in which the gut microbiota are most sensitive to a variety of environmental drivers at multiple scales, and suggest reproductive timing, and hence potentially parental quality or food availability, are linked with the microbiota. Identifying and explicating the various ecological sources that shape an individual's gut bacteria is of vital importance for understanding the gut microbiota's role in animal fitness.

3.1 Introduction

The gut microbiome—the enteric microbial community and their genes—is strongly influenced by the environment (Rothschild et al., 2018) and plays a major role in host ecology and evolution (Dethlefsen et al., 2007; Koskella et al., 2017). Although much progress has been made in understanding the interplay between gut microbiota and host phenotypes, research in this field has been restricted primarily to lab-based model organisms (Colston & Jackson, 2016; G. L. Davidson, Raulo, et al., 2020). In nature, animal's microbiota are far more variable and diverse than their captive counterparts because they are exposed to a wider variety of conditions (Hird, 2017; McKenzie et al., 2017) and because wild hosts are more genetically, and therefore phenotypically diverse than lab-reared animals. Exploring how gut microbiota are acquired, their stability across life stages, and their interactions with extrinsic and intrinsic factors in nature are important for understanding what drives host phenotypic responses to gut microbiota variation.

The gut microbiota can directly affect the development of key physiological and biological traits of the host (Cox et al., 2014; Hansen et al., 2012; Sudo et al., 2004). Although most of our understanding of these processes comes from mammalian germ-free lab models, disruption to microbial gut colonisation at critical developmental periods can affect host immunity, metabolism and physical development in other systems such as bees, birds and frogs, (Knutie et al., 2017; Schwarz et al., 2016; Simon et al., 2016; Warne et al., 2019), thus representing a broader taxonomic scope with more ecologically valid microbiota than traditional model organisms (Colston & Jackson, 2016; Pascoe et al., 2017). Cavity nesting birds provide an ideal system for microbiota studies in a natural setting as nestlings can be sampled and monitored easily throughout the early stages of development. Previously we showed in a wild-bird population that nestling gut microbiota diversity

predicts nestling weight (Davidson et al., 2021/Chapter 2), itself a key predictor of offspring survival and recruitment in great tits (*Parus major*) (Both et al., 1999; Monrós et al., 2002) and in many vertebrates generally (Bowen et al., 2015; Ringsby et al., 1998; Stige et al., 2019). It is well documented that the gut microbiota changes in early development across taxa (Derrien et al., 2019; Grond et al., 2017; Kohl et al., 2019; Robertson et al., 2019; W. Z. Stephens et al., 2016). In humans it is assumed that greater gut microbiota variation in infant populations compared to adult populations reflect a shift towards greater microbiota stability (Lozupone et al., 2012), yet the role that ecology plays in this process remains poorly understood.

Many key drivers of ecological variation have the potential to affect the gut microbiota. Exposure to different environmental pools of microbes are likely important, such as those in the air, soil or diet (Grond et al., 2017; Kartzinel et al., 2019; Liddicoat et al., 2020; Ren et al., 2017). The link with diet is especially likely to explain why gut microbiota can vary with habitat (Drobniak et al., 2021; Goossens et al., 2022; Teyssier, Rouffaer, et al., 2018). Vegetation and diet can also vary dramatically within habitats, for example with respect to edge effects (J. Chen et al., 1992; Wilkin et al., 2007), although it is unknown whether individual microbial communities are affected by these fine-scale processes. Between-seasonal dietary change has been linked to temporal variation in the gut microbiota (Baniel, Amato, et al., 2021; Hicks et al., 2018; Maurice et al., 2015; Ren et al., 2017). Within-season variation in the gut microbiota is also likely important because in many animals reproductive timing is critical to ensure breeding coincides with seasonal peaks in food abundance (Brommer et al., 2002; Grüebler & Naef-Daenzer, 2010; Rodríguez et al., 2016; Rubenstein & Wikelski, 2003). Although never tested in the wild, the timing of reproduction could therefore dictate, not just the parent's own microbiota, but also that of their nestlings' through dietary microbes.

Similarly, indirect effects mediated by the host, for example chronic stress, may have knock-on effects on gut microbiota diversity or composition (Noguera et al., 2018; Stothart et al., 2019). Exercise intensity (Mailing et al., 2019) and energetics (reviewed in Lindsay et al. (2020)) are associated with the microbiota, while stress and the microbiota are bidirectionally linked (Sudo, 2014; Sudo et al., 2004). In adults, brood size is an indicator of parental effort (Pettifor et al., 2001) and alters glucocorticoid concentrations (Bonier et al., 2011). While in nestlings, larger brood sizes lead to increased competition for food among siblings (Smith et al., 1989) and higher glucocorticoid hormone concentrations (Greggor et al., 2017; H. G. Smith et al., 1988). Thus brood size could influence the microbiota of the parents and their offspring through energetics or stress, though no link has been found to date (Liukkonen et al., 2022, preprint). Additionally, the gut microbiota in adults is mediated by a variety of sex hormones (Mallott et al., 2020), and can lead to sex-specific associations between the gut microbiota and environmental sources of variation (Org et al., 2016).

Thus, the composition of gut microbes are expected to vary across time in response to environmental variation and with respect to a whole variety of individual state variables. Nevertheless, individuality and stability in microbiota can also be important, as observed in studies of human health (L. Chen et al., 2021; Fassarella et al., 2021). Consistent among-individual differences indicate limited flexibility, that is, constraints caused by an underlying causal factor—for example, genetic, epigenetic, or early environment effects (Rajilić-Stojanović et al., 2013; Zoetendal et al., 2001). Typically stability in phenotypic variation among individuals is quantified by taking multiple measures from the same individual over time, and estimating repeatability in a mixed model framework (Nakagawa & Schielzeth, 2010). Mixed models also enable an examination of whether individual differences are independent of other factors that can be included as random or fixed effects. Despite its widespread use in animal personality and cognition literature (A. M. Bell et al., 2009; Cauchoix et al.,

2018), to our knowledge this approach has only been applied once to identify the sources of individual level variation in the gut microbiota (Risely et al., 2022). Repeatability analysis represents a novel technique that will allow microbiome researchers a greater understanding of microbiome individuality than has been achieved to date (Mallott, 2022).

We explored how the gut microbiota varied with the environment, development, and intrinsic factors using a generalist passerine bird species breeding in nest boxes across heterogenous and fragmented woodlands. The great tit is a widely used model organism in ecological and behavioural studies (Cole & Quinn, 2012; Drent et al., 2003; O'Shea et al., 2018; Tinbergen & Boerlijst, 1990). Given the gut microbiota is correlated with the fitness of great tits in our population (Davidson et al., 2021/Chapter 2), understanding how the microbiota varies with their ecology is an important next step in this system. Within a single breeding season, we simultaneously investigated how the gut microbiota were related to host state (age, sex, reproductive effort) and a range of environmental factors (habitat type, distance to forest edge, number of siblings in the nest), and whether these explained consistent between-individual differences in the gut microbiota. We had clear *a priori* reasons to expect environmental and life history traits to covary with gut microbiota variation, and to do so differentially across life stages because the developing gut microbiota in young animals are generally more sensitive to environmental variation than in the more established adult gut microbiota (Derrien et al., 2019; Grond et al., 2017). However, we had no *a priori* predictions about the direction of such effects in the context of gut microbiota community metrics (i.e. high vs low diversity and relative taxonomic abundance), owing to the complexity of host-microbe interactions (A. E. Douglas, 2018; K. R. Foster et al., 2017; Zaneveld et al., 2017). Finally, we conducted a range of repeatability analyses at the individual, the nest, and the woodland site levels to determine whether individual nestlings differed consistently over a short but critical period of their development, from 8 to 15 days old. We further examined whether

any consistency at the individual-level was driven by intrinsic differences among individuals, or instead by shared nest or woodland effects, or indeed a range of other fixed effects as discussed above. Overall, we hypothesise there may be diverse and complex relationships between an individual, their gut microbiota and the environment. Our analyses aim to resolve these interactions to inform future experimental work and our understanding of how the microbiota mediates ecological effects on host adaptation.

3.2 Methods

3.2.1 Field monitoring and microbiota sampling

Birds were sampled from nine small woodland fragments across Co. Cork, Ireland, five of which were mixed/deciduous and four coniferous woodlands (see O'Shea et al. (2018)). We collected 262 faecal samples from 204 great tits from 63 nests (see Table S1 for final sample sizes post bioinformatic processing) for 16S rRNA gene sequencing. Nest boxes were monitored during April-June 2016 to determine lay dates, hatching dates and nestling survival. Typically, all individuals in a nest were sampled except for cases where nestlings did not produce a sample within 10-15 minutes of being placed in sampling apparatus and risked becoming chilled or weak. Individual nestlings were sampled when they were 8 days old (+/- 1 day), and again, if they survived, when they were 15 days old (D8 and D15 birds respectively), at which point parents were also sampled. Birds were placed individually into sterile holding bags inside a heated holding case and naturally-produced faecal samples were collected. Urea has the potential to affect downstream sequencing (Khan et al., 1991)and was removed through absorption by coffee filters placed as lining in the sampling bags. The faecal matter was collected within 15-20 minutes of placing birds in the sampling bag, after which birds were returned to the nest. Faecal sacks were ruptured immediately using a sterile inoculation loop and placed in a microcentrifuge tube containing 500uL of 100% ethanol. Samples were stored at -20^{o}C within 8 hours

of collection until DNA extraction. D8 nestlings were weighed, and individually identified by clipping the tip of one of their nails, taking care to avoid the blood vessel. Nestlings were again weighed at D15 and ringed with a unique identifiable metal ring (British Trust for Ornithology). We had 'repeat samples' from 41 individual nestlings on D8 and D15. Both parents were trapped on the nest where possible and, if not already ringed, were fitted with a British Trust for Ornithology ring, weighed, and aged as either 'immature' (first year breeding) or 'mature' (second year/+ breeding) and sexed using plumage indicators.

3.2.2 DNA Extractions

DNA was extracted from the dried faecal contents of all birds using the Qiagen QIAamp DNA Stool Kit, following the 'Isolation of DNA from Stool for Pathogen Detection' protocol (June 2012 edition), with modifications described in Shutt et al. (2020) to accommodate dried avian faeces. DNA was stored at -20^{o}C. Full extraction methods are described in the Supporting Information of Davidson et al. (2021) or Appendix 1B.

3.2.3 Illumina MiSeq sequencing

Full library preparation details are described in Supporting Information of Davidson et al. (2021) or Appendix 1B. Briefly, the V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 300 cycle kit, following standard Illumina sequencing protocols. Four negative controls were carried through to sequencing, two extraction controls (Qubit results after extraction: too low to read) and two PCR controls. These samples were subsequently excluded from any analyses following bioinformatic processing.

These negative controls indicated there was a small number of potentially contaminant ASV's (<0.01% of ASV's) detected by the decontam package using the frequency method (Davis et al., 2018). Therefore, our sequence data may contain some ASV's from the external environment though this should not have systematically biased our results as samples were randomized across plates.

3.2.4 Bioinformatics

The DADA2 pipeline was used to process the raw sequencing data (Callahan, McMurdie, et al., 2016) in R version 3.5 (R Core Team, 2019). Sequence quality was visually inspected. Sequences were trimmed to remove adapters and lower quality reads (median quality scores below 25-30 threshold) at the extremities of the sequence and filtered to remove sequences with expected errors above 1. Read errors were estimated before dereplication. Forward and reverse reads were merged to construct 'contig' sequences, which were used to construct a sequence Table of Amplicon Sequence Variants (ASV's), which in turn counts the number of times each unique sequence is detected. The previous steps were performed for each run separately. Then the separate sequence tables were merged and chimeras removed using the 'consensus' method. Taxonomy was assigned to each ASV by RDP's Naive Bayes Classifier (Q. Wang et al., 2007) against the Silva reference database (version 132) (Quast et al., 2012).

The DADA2 outputs were assembled into a single Phyloseq object (McMurdie & Holmes, 2013). Sequences identified as mitochondrial or chloroplast were removed. Sample completeness curves were plotted using vegan (Oksanen et al., 2019) and helped determine the lower cut-off for sample reads at 10,000 reads. Low read samples (<10,000 reads, 11 samples) were removed leaving 246 (adult=51, Day-8=81, Day-15=114) samples for the analysis. Alpha diversity (both Shannon and Chao1

diversity) was calculated using the '*estimate_richness'* function from the phyloseq package on the filtered dataset.

After removing low read samples, chloroplast sequences and mitochondria sequences, there were 18,890,006 total reads clustered into 54,343 ASV's in 246 samples (see Table S1 for sample breakdown). Reads ranged from 10,220 to 557,336, with a mean of 76,789 reads per sample. For the analyses presented here the reads were not rarefied prior to alpha diversity calculation or relative abundance analyses (McMurdie & Holmes, 2014; Willis, 2019). In case alpha diversity estimates were affected by library size, the alpha diversity analyses were rerun with a dataset rarefied to 10220 reads (the minimum library size after removing small libraries) and there was no change in the results.

3.2.5 Statistical analysis

Datasets

The analyses detailed below were conducted on one of two subsets of the data, depending on the questions being explored. The first subset, referred to as the *all birds* subset, contained birds of all developmental ages (8 day-old nestlings (D8); 15 day-old nestlings (D15); adults), and was used to examine the relationship between the gut microbiota, developmental age, and the other life history and environmental variables. The second subset of data contained *adults only*, and focussed on sex and its interaction with other life history and environmental variables. All analyses were conducted in R version 3.6.3 (R Core Team, 2019).

Alpha diversity

Linear mixed models (LMM) were used to test the effect of host and environmental factors on alpha diversity. Shannon diversity (Shannon, 1948) measured richness weighted by abundance (the evenness of a community), and Chao1 (Chao, 1984) measured richness, specifically estimating taxa abundance and rare taxa missed from under sampling. Models were fit using the lme4 package (Bates et al., 2015) on each data subset. Significance was determined using Satterwaite's degrees of freedom method (Satterthwaite, 1946) implemented using the lmerTest package (Kuznetsova et al., 2017). Initially the distribution of each alpha diversity metric was assessed graphically and, when obviously skewed, was transformed towards normality as appropriate. Residuals from all models were assessed using DHARMa (Hartig, 2019) (see below for details), and the suitability of the response variable transformations verified based on the normality of the model residuals. The transformations applied were as follows: *all birds* Shannon model, logged; *all birds* Chao1 model, logged; adult birds Shannon model, cube-root; adult birds Chao1 model, logged.

In the *all birds* global models, the following variables were included as main effects: age (D8, D15, adult); habitat-type (coniferous, mixed-deciduous); distance to edge, i.e. distance from the nest to the nearest woodland edge; maximum brood size of nest; first-egg lay date of nest. All pairwise interactions between these main effects were included, except for the lay date × brood size which was not expected a priori to be related to the microbiota. Sex was excluded because it was unavailable for nestlings. Mature and immature adult individuals were grouped into a single age bracket 'adult' because of convergence issues in the *all birds* models but not the *adults only* models. The *adults only* global models were similar to the *all birds* models with the addition of a sex variable, and its pairwise interactions with the other variables, and the removal of any habitat or age interactions, due to an imbalance in these factors in the dataset (Table S1). Backwards difference coding was used, instead of the default dummy coding, when fitting the age variable which allowed us

to compare each age group to the previous age group sequentially, rather than to a single reference level. This contrast scheme gives sum contrasts, so coefficients reflect main effects rather than marginal effects. Woodland site, nest ID and individual ID were fit to the model as nested random intercepts (in the form woodland site/nest ID/individual ID), to control for non-independence. Bird ID was dropped from the *all birds* Chao1 model and woodland site dropped from both adult diversity models due to singular fit warnings, though this had no effect on the model estimates.

Phylum-level-Relative-Abundance-GLMMs

The two most abundant phyla (Proteobacteria, mean ± SE: 41.6% ± 2.1%; Firmicutes, mean ± SE: 36.6% ± 2.2%) were modelled separately to determine which variables correlated with changes in the phyla relative abundance, in order to develop a broad sense of how the microbial community changed with varying ecological factors. Binomial Generalised Linear Mixed Models (GLMMs) were used, where the binomial response variable was 'phyla abundance' per sample. Each sequence read in a sample was scored a '1'if the sequence was from the phylum in question, or scored a '0' if the sequence was from a different phylum. The 'phyla abundance' was therefore the summed score divided and weighted by the total number of sequence reads per sample to account for different library sizes. These data were also subset into *all birds* and *adults only* subsets, and we used the same model formulas as the alpha diversity models above. The *all birds* global models did not converge so simplified models were refit only including pairwise interactions with age. The age variable was coded using backwards difference coding, as in the alpha diversity models.

Model averaging

Model averaging was performed on the alpha diversity models and the phylum level relative abundance models using the MumIn package (Bartoń, 2020), as outlined by

Grueber et al. (2011). Initially full models were fit using *lmer* or *glmer*, variables were rescaled using the standardize function in the arm package (Gelman et al., 2021), centring all regression inputs on zero and dividing by 2SDs. Then a model set was generated using the *dredge* command. If multiple models were within the 2AICc cut-off, these were averaged; otherwise the single top model was reported. We also tested model averaging with a less conservative AICc cut-off of 7 (Burnham et al., 2011). This returned averaged models with more retained terms, but they were all non-significant. Moreover, the estimates and significance of the significant terms identified with the 2AICc cut-off did not change, so we only report (conditional) averaged model results from the 2AICc cut-off.

Model checking and plotting

Model diagnostics for the alpha diversity and phylum level relative abundance models were checked using DHARMa (Hartig, 2019). DHARMa simulates data based on the model provided and is a better validator than simple residual vs fitted data plots for mixed effects models. Simulated residual plots were made for each of the models in the top model set. In cases where the simulated residuals showed a clear pattern, models were interpreted cautiously. The *adults only* phyla level models showed overdispersion, which was resolved by refitting the model with an observation level random term following Harrison (2015).

All plots were created using ggplot2 (Wickham, 2016). Alpha diversity and phylum-level results were plotted using the JTools and Interaction (Long, 2019, 2022) packages, which are based on ggplot2. The model estimates for the top model in each model set were plotted, not the model averaged results. These plots used partial residuals, which allow the plotting of data accounting for the variables other than the predictor variable of interest and can be better at showing relationships

between variables in multivariate mixed models than plotting the raw data, which agreed with the patterns shown in plots of the raw data (not presented).

Beta-diversity

Beta-diversity measures the dis-similarity between two or more communities, and in our analyses consisted of the pairwise distances between all samples. To address the issue of pseudo-replication in the beta-diversity analyses, due to repeat samples which could not be controlled for using an individual level random effect, the *all birds* analyses were split into a D8-adult model and a D15-adult model. For the *adults only* dataset a single model was run. The beta diversity models used the same variables as the equivalent alpha diversity model but only the non-interacting main effects, as these models cannot estimate the main effect and interaction effect at the same time. Beta-diversity was analysed using a compositional approach, which accounts for the proportional nature of high-throughput sequencing data (Gloor et al., 2016; Gloor & Reid, 2016). Low prevalence taxa, i.e. those with less than one copy in 5% of samples, were excluded to reduce possible contaminants and sequencing artefacts (Bokulich et al., 2013). The filtered dataset was centre-log ratio transformed and then the Euclidean (or Aitchison) distance between samples was calculated (Aitchison, 1983). The beta-diversity of samples was tested using PERMANOVA (*adonis2* function from the vegan package (Oksanen et al., 2019)) after checking variables for homogeneity of dispersion. Models were fit using the 'margin' option which tests the marginal effect of each variable while accounting for the other variables in the model. Nest was used as a blocking factor to control for the non-independence of samples and sequence plate was included as a fixed effect to account for batch effects. Predictor variables were centred and scaled. Some variables in the *all birds* and *adults only* models had non-homogenous dispersions (Table S2 and S3), so significant PERMANOVA results could reflect differences in group variance rather than differences in group means, or could reflect differences in both group variance and group means (Anderson & Walsh, 2013). Beta diversity was visualised using PCA

plots of the (pairwise) Euclidian distance between samples, and plotted samples according to their first and second principal component values. Group dispersion was also plotted to visualise differences in group variance, as well as group means.

Repeatability

Repeatability analyses were conducted on nestlings, for which repeat measures were available across D8 and D15; nestlings with only single measurements were also included to improve estimates (J. G. A. Martin et al., 2011). The rptR package in R (Stoffel et al., 2019) was used to estimate the repeatabilities of individual, nest, and woodland random terms, both on their own, and in combination, to determine the main components of variance underlying microbiota metrics among nestlings. rptR was also used to estimate adjusted repeatabilities, which test whether the components of variance in the random terms were confounded by fixed effects (Nakagawa & Schielzeth, 2010). Thus, repeatabilities for Shannon diversity, Chao1 diversity, phyla-level relative abundance and for each of the first two principal components of beta diversity (using the Euclidian distance matrix as described above), were estimated in the following ways: (i) repeatability for woodland site; (ii) repeatability for nest; (iii) repeatability for individual; (iv) repeatability for individual, adjusted for significant fixed effects; (v) repeatabilities for individual and nest in combination, adjusted for significant fixed effects; and (vi) repeatabilities of individual, nest and woodland site, adjusted for significant fixed effects. The significant fixed effects included in (iv)-(vi) for Shannon and Chao1 diversity are shown in Table S4, for phyla level in Table S5, and for beta diversity in Table S6.

3.3 Results

3.3.1 Alpha diversity

There was no relationship between Shannon diversity and age (Table S7a). Chao1 diversity was lower in D15 than in D8 nestlings (- 0.35 ± 0.15, p = 0.02; Table S7b), and there was no difference between D15 nestlings and adult birds (- 0.02 ± 0.18, p = 0.91; Table S7b, Figure 1). The effect of habitat on alpha diversity depended on age - the decrease in Chao1 diversity from D8 to D15 was more pronounced in mixed/deciduous than in coniferous habitats (habitat × age: - 0.73 ± 0.32, p = 0.025; Table S7b, Figure 1). As nest-box distance from the woodland edge increased, so did both Shannon and Chao1 diversity, regardless of age (Shannon: 0.21 ± 0.1, p = 0.035; Chao1: 0.45 ± 0.2, p = 0.023; Table S7, Figure 2). Neither brood size nor lay date predicted alpha diversity (Table S7). None of the variables (age, sex, habitat, distance to edge, brood size or lay date) predicted alpha diversity when adults were analysed separately (Table S8).

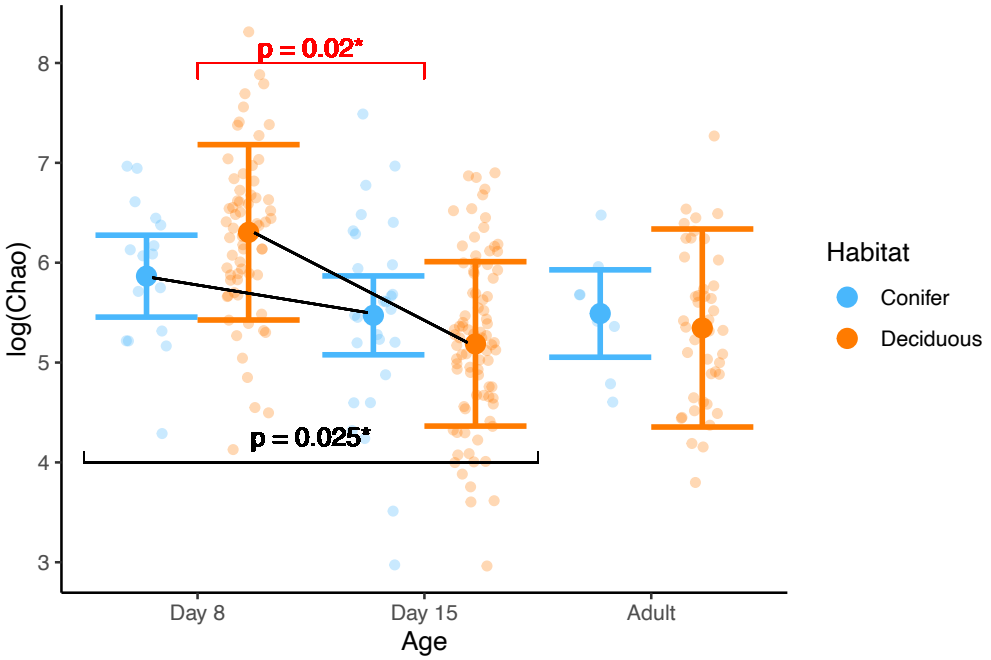

Figure 1. Alpha diversity varies across life-stages and between habitats. Partial residuals plot for log(Chao1) diversity regressed on age across habitats (all birds model), means with 95% confidence intervals, shaded circles denote individual samples (n = 246). Red bracket indicates significant difference between D8 and D15 means. Black lines and bracket indicate significant difference between slopes across age and habitat (significant interaction effect).

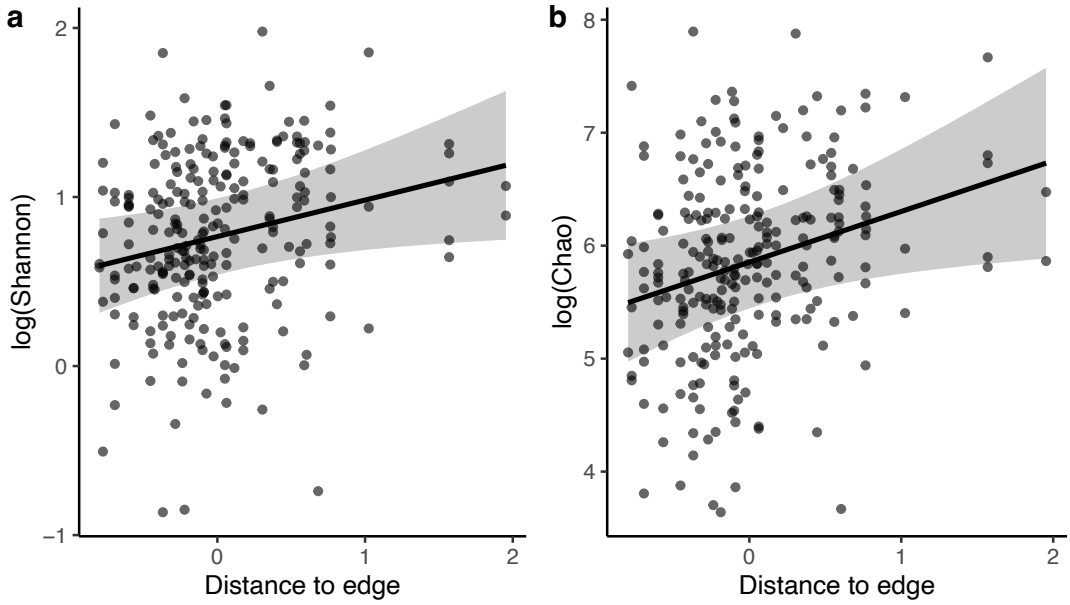

Figure 2. Alpha diversity increases with distance to edge. Shaded circles denote individual samples (n = 246). Fitted line on partial residuals plot with 95% confidence intervals for logged diversity regressed on distance to edge from the *all birds* (a) Shannon (0.21, p = 0.035) and (b) Chao1 (0.45, p = 0.023) diversity models. Note: 41 nestlings were sampled at both D8 and D15, and both measurements are included in the analysis. Distance to edge variable was rescaled so some values are below zero.

3.3.2 Beta diversity

Beta diversity differed across age (Table 1a, 1b) where community composition became more similar among adult individuals compared with nestlings (Figure 3a, 3d). In the D8-Adult analysis dispersion tests and plots indicate this age result may be due to a difference in group dispersions rather than group means (p = 0.069; Table S2; Figure 3a, 3b). Beta diversity varied with habitat, only when D8 nestlings were included in the analysis (Table 1a, 1b, 1c). Beta diversity varied with lay date and brood size (Table 1a, 1b), and there was also a tendency for beta diversity to vary with distance to edge (Table 1b), possibly due to a difference in dispersions rather than means (p = <0.001, Table S2). When adults were analysed separately, there was no difference in beta diversity between mature and immature birds or between sexes (Table 1c), and brood size was the only factor to predict beta diversity (Table 1).

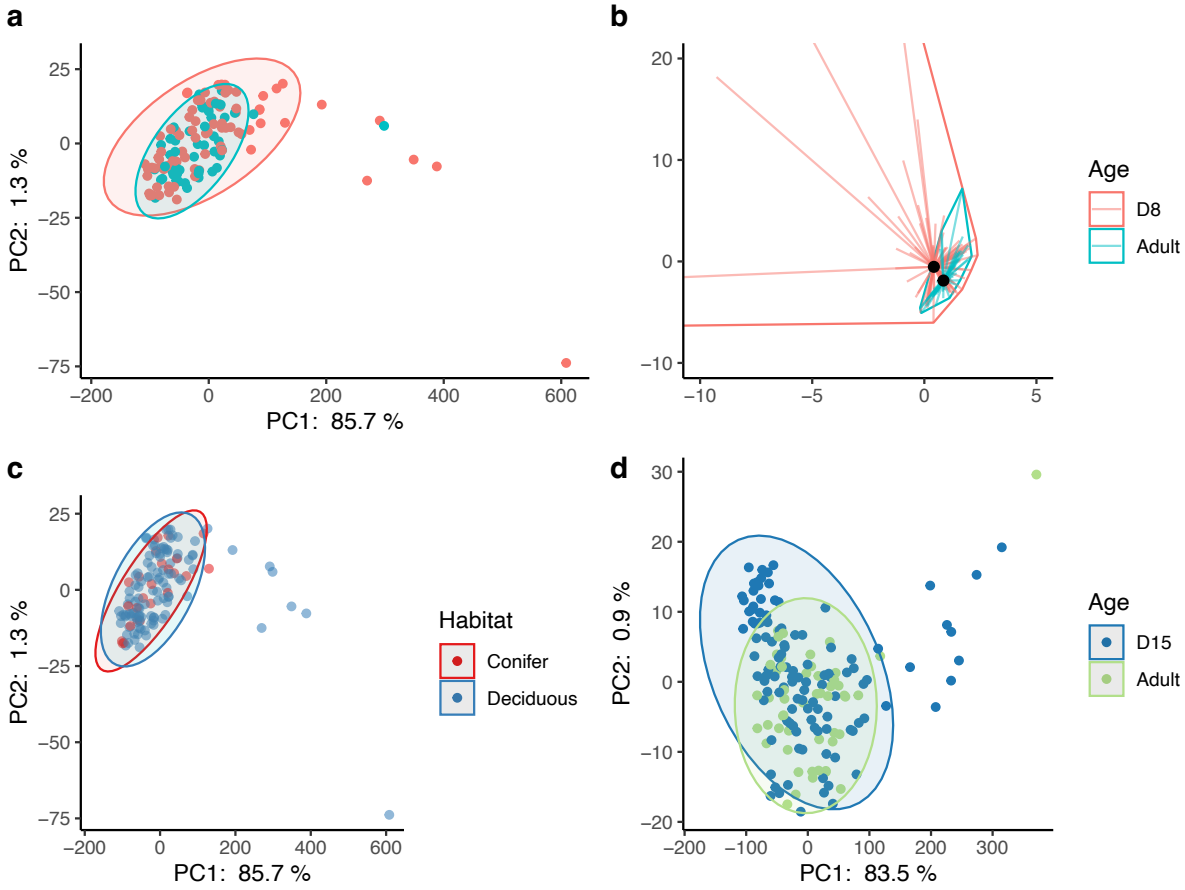

Figure 3. Community composition differs across ages and habitat types: (a) PCA plot of Euclidean distance with ellipses around age groups for D8-adult beta diversity, (PERMANOVA: $p < 0.001$; $n = 132$); (b) Dispersion plots for D8-Adult age groups, showing similar group centroids (black dots are means) but difference in group dispersions (Beta-disper: $p = 0.069$; $n = 132$); (c) PCA plot of Euclidian distance with ellipses around habitat groups for D8-adult beta diversity (PERMANOVA: $p < 0.001$; $n = 132$); (d) PCA plot of Euclidean distance with ellipses around age groups for D15-adult beta diversity (PERMANOVA: $p < 0.001$; $n = 165$). *Note axes are limited in (b) to improve readability, so some extreme values are cut off. Percentage values on axes refer to amount of variation explained by that component. (a), (c), (d) Shaded circles denote individual samples.*

Table 1. Beta-diversity PERMANOVA results using the Euclidian distance between samples with centre-log ratio transformed ASV counts. Results are marginal effects and significance is calculated from 999 permutations. (a) D8 and adult birds model, (b) D15 and adult birds model and (c) *adults only* model. Analyses were split for D8 and D15 nestlings to avoid pseudo-replication due to repeat samples. Cells blank where variable not tested. R^2 values are rounded to nearest two decimal places.

	(a) D8-Adult				(b) D15-Adult				(c) Adults only			
Independent variables	**SS**	**R^2**	**F**	***p***	**SS**	**R^2**	**F**	***p***	**SS**	**R^2**	**F**	***p***
Sex									212.96	0.02	0.82	0.83
Age	1236	0.01	1.66	<0.001	1097	0.01	1.91	<0.001	271.04	0.02	1.05	0.63
Habitat	740	0.01	0.99	<0.001	739	0.01	1.29	0.48	290.32	0.02	1.12	0.44
Lay date	843	0.01	1.13	0.002	883	0.01	1.54	0.006	323.59	0.02	1.25	0.13
Brood size	818	0.01	1.1	0.011	632	0.01	1.11	0.014	223.30	0.02	0.86	0.004
Distance to edge	1119	0.01	1.5	0.35	830	0.01	1.45	0.083	327.04	0.02	1.26	0.34
Sequence plate	5804	0.06	1.95	<0.001	4968	0.05	2.17	<0.001	1462.09	0.11	1.88	0.01

3.3.3 Phylum level relative abundance

D8 nestlings had higher Proteobacteria relative abundance than D15 nestlings, who in turn had lower Proteobacteria relative abundance than adults (Table 2a and Figure 4a). The main effect of age also interacted with habitat. In both habitats, Proteobacteria decreased with nestling age (i.e. between D8 and D15), but this effect was stronger for birds developing in mixed/deciduous compared to coniferous habitats, mirroring the pattern above for alpha diversity (Table 2; Figure 4a, 4b). Adult birds had greater relative abundances of Proteobacteria than D15 birds, and this effect was especially pronounced in mixed/deciduous woodlands compared to conifer woodlands (Table 2b; Figure 4a). We note that although the age-habitat interactions are supported by the model, they are not strongly supported by plots (Figure 4a, 4b) and should be interpreted with caution.

Brood size was negatively related to the relative abundance of Proteobacteria and positively to the relative abundance of Firmicutes in nestlings, especially for D15 nestlings, but there was no relationship with either phylum for adults (Table 2a, 2b; Figure 4c, 4d). There was no effect of distance to edge on phyla level abundance. Proteobacteria relative abundance was negatively related to lay date in D8 nestlings, and positively in D15 nestlings and in adults. All of the patterns described for Proteobacteria were found for Firmicutes in the inverse directions (Figure 4; Table 2). Only lay date predicted phyla level relative abundance in the *adults only* models. Adult's Proteobacteria increased with lay date (1.5 ± 0.5, $p = 0.006$), but did not relate to Firmicutes (- 0.7 ± 0.6, $p = 0.223$; Table S9).

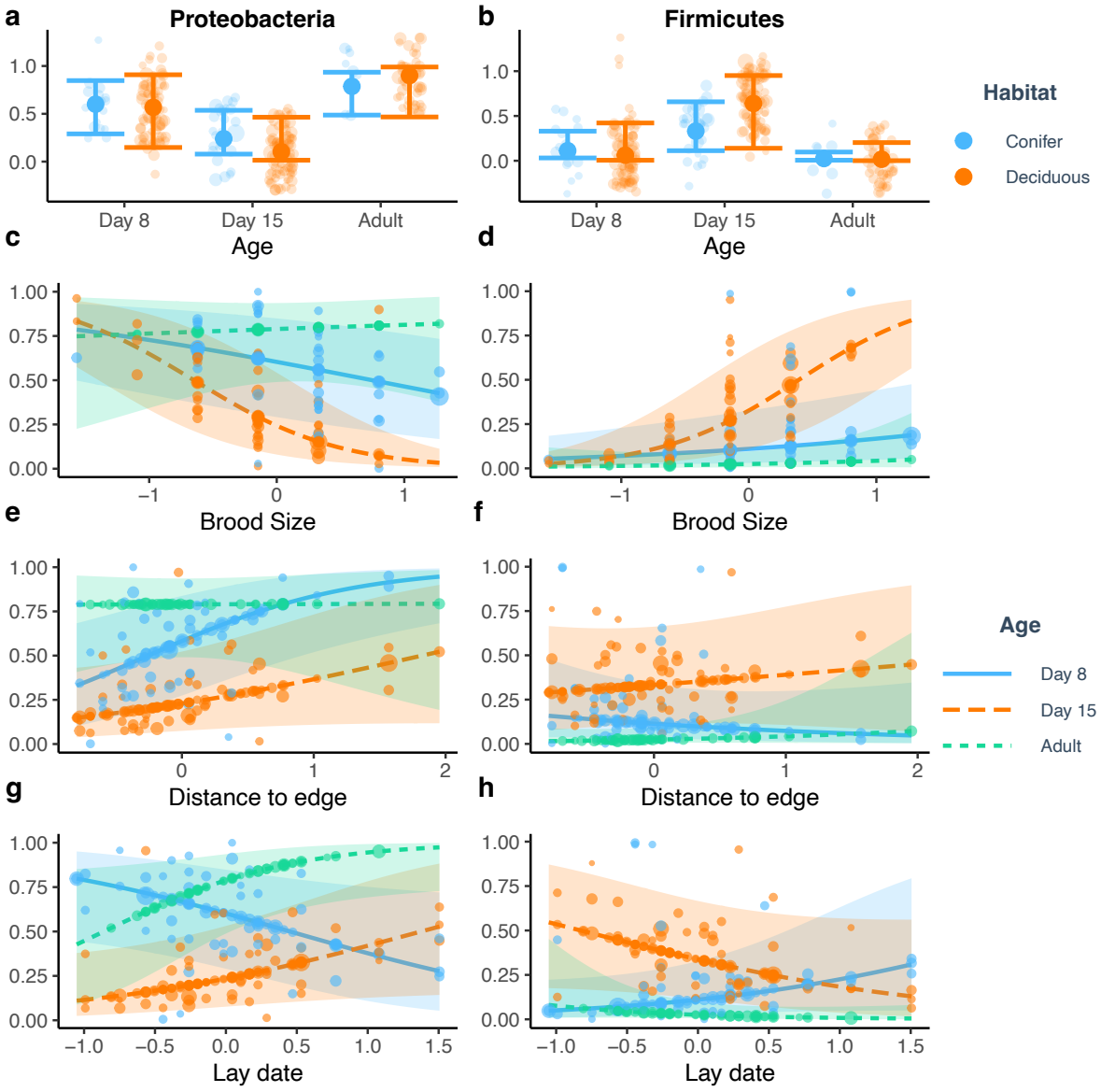

Figure 4. Phyla relative abundance changes with multiple interacting host and environmental factors. Shaded circles denote individual samples (n = 246). Partial residual plots for relative abundance of the two most abundant phyla. Proteobacteria (left column) and Firmicutes (right column) for the *all birds* phyla-level models showing means with 95% confidence intervals. Point size reflects the weight of that observation, i.e. the 'number of sequence reads' of the sample. Some points may appear out of range due to 'jitter' function which separates overlapping points.

Table 2. Phylum level relative abundance, binomial model results for the *all birds* (nestlings and adults) subset. Only a single model was retained in the top model sets (<2AICc) so results are not model averaged. The reference level for habitat is 'coniferous' which is compared here to 'mixed/deciduous'. The age variable is backwards difference coded (see methods for explanation).

	(a) Proteobacteria				**(b) Firmicutes**			
Independent variables	**Estimate**	**S.E.**	**z**	***p***	**Estimate**	**S.E.**	**z**	***p***
(Intercept)	0.16	0.67	0.24	0.81	-2.17	0.70	-3.12	0.002
Age (D15/D8)	-1.52	0.00	-342.63	<0.001	1.30	0.00	315.88	<0.001
Age (Adult/D15)	2.45	0.31	7.88	<0.001	-2.94	0.35	-8.47	<0.001
Habitat	-0.08	0.72	-0.12	0.91	0.08	0.89	0.09	0.93
Brood size	-0.72	0.20	-3.58	<0.001	0.97	0.22	4.36	<0.001
Distance to edge	0.66	0.41	1.62	0.11	0.12	0.48	0.25	0.81
Lay date	0.49	0.44	1.11	0.27	-0.38	0.53	-0.70	0.48
Age (D15/D8) × Habitat	-0.87	0.01	-129.32	<0.001	1.92	0.01	302.68	<0.001
Age (Adult/D15) × Habitat	1.90	0.84	2.26	0.024	-1.67	0.91	-1.84	0.07
Age (D15/D8) × Brood size	-1.19	0.01	-174.16	<0.001	1.36	0.01	211.54	<0.001
Age (Adult/D15) × Brood size	1.90	0.61	3.14	0.002	-1.28	0.66	-1.93	0.054
Age (Day15/Day8) × Distance to edge	-0.63	0.01	-115.05	<0.001	0.73	0.01	129.92	<0.001
Age (Adult/D15) × Distance to edge	-0.66	0.65	-1.01	0.31	0.34	0.71	0.47	0.64
Age (D15/D8) × Lay date	1.77	0.01	319.66	<0.001	-1.67	0.01	-309.36	<0.001
Age (Adult/D15) × Lay date	0.67	0.79	0.85	0.40	-0.34	0.84	-0.40	0.69

3.3.4 Nestling, nest and woodland site repeatability

There were no consistent (repeatable) differences in nestling microbiota among sites (model 1's, Table S10) but there were among nests for Shannon, Chao1, Proteobacteria and Firmicutes relative abundance, and beta diversity PC1 and PC2 (R values from model 2's, respectively: 0.399; 0.421; 0.191; 0.261; 0.377; 0.484. See Table S10 for confidence intervals and further details). Individual repeatability was not significant for Shannon diversity or PC1, but was significant for Chao1 diversity, Proteobacteria relative abundance, Firmicutes relative abundance and PC2, when unadjusted for any other effects (R values from model 3s, respectively: 0.378, n.s.; 0.274, n.s.; 0.46; 0.174; 0.248; and 0.461). After controlling for fixed effects, individual repeatabilities for Shannon diversity, Chao1 diversity and PC1, but not for Proteobacteria or Firmicutes relative abundance, remained significant (R values from model 4's in Table S10 respectively: 0.403; 0.50; 0.242; 0.144, n.s.; 0.165, n.s.).

However, individual repeatabilities for all six gut microbiota metrics approached zero when controlling for nest as a random effect, which itself remained repeatable in all six model 5's (see R values from model 5's for both individual and nest in Table S10), indicating that the majority of microbiota variability in nestlings was driven by nest identity, not individual differences. The addition of woodland site as a random effect suggested that up to a quarter of the nest site repeatability may have been driven by differences among sites (Table S10), though the credibility intervals for woodland site repeatability overlapped zero in all six model 6's. Figure 5 includes repeatability estimates and confidence intervals for all six model 6's (i.e. models including site, nest and bird ID, controlling for fixed effects).

Given these repeatability results, we decided to carry out post-hoc analyses of how parent traits correlated with offspring traits. We found that nestling microbiota diversity was predicted only by the mother's Shannon diversity (Table S11a-d).

Specifically, female Shannon diversity negatively predicted offspring Shannon diversity (Figure S1). None of the mother's Chao1, Proteobacteria relative abundance or Firmicutes relative abundance, or any of the father's microbiota traits predicted the corresponding nestling trait (Table S11e-h). However a post-hoc analysis of mean Euclidian distance of each mother from all offspring vs mean distance of each mother from all non-offspring did not show any difference between community compositions (paired t-test, t = - 0.649, p = 0.52; Figure S3).

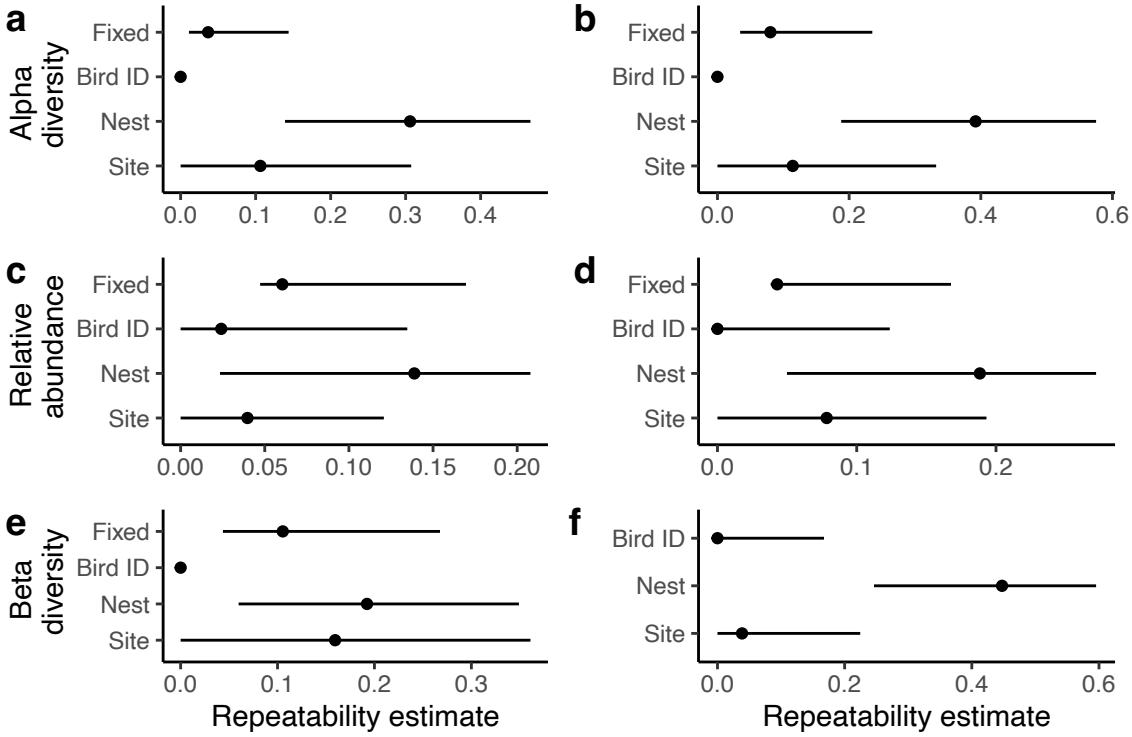

Figure 5. Individual repeatability collapses to zero when accounting for nest (n = 154 individual nestlings with 41 individuals with repeat measurements). Repeatability estimates with confidence intervals for model 6's (model 6's include site, nest and individual level random effects while controlling for relevant fixed effects; Table S10) for each of the six microbiome traits: (a) Shannon diversity; (b) Chao1 diversity; (c) Proteobacteria relative abundance; (d) Firmicutes relative abundance; (e) PC1 and (f) PC2. No fixed effects were included in PC2 analysis, as there were no significant fixed effects in the PC2 nestling only model (Table S6).

Table 3. Summary of results. Arrows show the direction of the effect on the microbiome trait (not applicable for β diversity as effects do not have directionality). Paired arrows indicate the direction of effect for Proteobacteria and Firmicutes, respectively, with "n.s" referring to the corresponding result being non-significant. Asterisks show significant results for beta diversity. 'Coniferous' is the reference level for habitat and bracketed terms with 'Age' indicates age levels which are being compared (second age level is reference level). An effect with a p value < 0.05 is considered significant.

	α diversity	β diversity		Proteobacteria-Firmicutes diversity	
	All birds	All birds	Adults	All birds	Adults
Age (D15-D8)	↓			↓ ↑	
Age (Adult-D8)		*			
Age (Adult-D15)		*		↑ ↓	
Distance	↑				
Lay date		*			↑ n.s.
Habitat		*			
Brood size		*	*	↓ ↑	
Age (D15) x Habitat	↓			↓ ↑	
Age (Adult) x Habitat				↑ ↓	
Age (D15) x Brood size				↓ ↑	
Age (Adult) x Brood size				↑ n.s.	
Age (D15) x Distance				↓ ↑	
Age (D15) x Lay date				↑ ↓	

3.4 Discussion

3.4.1 Overall summary

We found that environmental links with the gut microbiota were dependent on age, suggesting differential sensitivity of gut microbiota across life stages in response to habitat type, local environmental gradients and life history traits (Table 3), although experimental perturbations would be necessary to definitively draw this conclusion. The finding that the gut microbiota community stabilises later in life is consistent with human observational studies (Claesson et al., 2011; Lozupone et al., 2012). Notably, life history, but not environmental effects predicted gut microbiota variation in adults; whereas nestling gut microbiota were predicted by a range of both environmental and life history variations (Table 3). Repeated measures showed that although nestlings appeared to show consistent individual differences in gut microbiota diversity and phylum-level relative abundance, this was explained entirely by the nest in which they developed, though there was some evidence for site level effects too. The post-hoc parent-offspring analyses found the gut microbiota traits of nestlings were not typically correlated with their parents' traits, although nestlings' Shannon diversity was negatively predicted by their mother's Shannon diversity.

3.4.2 Environment and age

Only nestling's, not adult's, gut microbiota varied in response to woodland type (Table 3). This is not surprising given microbial community assembly theory (Costello et al., 2012; Coyte et al., 2021) where source microbes vary according to the external environment (e.g. diet, nesting material, habitat) (C.-Y. Chen et al., 2020; Goodenough et al., 2016; Goossens et al., 2022; Koenig et al., 2011) and compete for available niches as the host physiological environment develops (Costello et al., 2012). Given that nestlings immune and digestive systems are likely still developing (Caviedes-Vidal & Karasov, 2001; Stambaugh et al., 2011) and their microbiota is still

being established, it is likely that the early microbiota contain transient environmental strains that may be replaced by more stable resident bacteria as the birds mature, similar to the distinct stages found in human infant microbiota development (Stewart et al., 2018). The more variable gut community in nestlings compared to adults is apparent in the alternative beta-diversity plots (Figure S2). A cross-fostering experiment found nestling great tits' gut communities were more similar to their foster siblings than their true siblings (Teyssier, Lens, et al., 2018), indicating a strong sensitivity of the microbiota to the immediate environment. Due to low recruitment in our study population it was not possible to investigate whether nest effects persisted into maturity.

The alpha diversity of the microbiota was higher for birds in nests further from the edge of the woodland (Figure 2), with a trend for the strength of this association to diminish with age (Table S7b), which we hypothesise could be due to differences in the exposure to microbes via nesting materials (Goodenough et al., 2016) and diet (G. Davidson, Wiley, et al., 2020) during a time when the microbiota are particularly sensitive to environmental variation. Similarly, D8 nestlings were especially sensitive to the woodland habitat (mixed/deciduous or coniferous), whereas the effect of woodland on alpha diversity was not present in adults, nor did it differ between adults and D15 nestlings, suggesting that the effects of habitat variation on alpha diversity stabilised early in development. However, for phylum-level relative abundance, this stability was not observed until adulthood. Caterpillars are the main food source for nestling great tits and differ in overall abundance, as well as peak abundance, between deciduous and coniferous woodlands (Balen, 1973; Massa et al., 2004), which may encourage great tits in coniferous woodlands to seek a wider variety of prey (Massa et al., 2004). Future studies should collect microbiota community data and with contemporary DNA metabarcoding data across environmental gradients to determine whether the microbiota covaries with diet across habitats.

3.4.3 Life history and age

Lay date was positively correlated to Proteobacteria in D15 nestlings and in adults, but negatively in D8 nestlings, while the reverse was true for Firmicutes relative abundance. Lay date also correlated with variation in community composition (beta diversity) when nestlings and adults were analysed together. The abundance of caterpillars, the primary food source for developing great tits, peaks mid breeding season, the precise timing of which could affect the gut microbiota directly or indirectly through the effects of food availability on stress, immunity or growth (Hooper et al., 2012; Potti et al., 2002; Stothart et al., 2019). Indeed, nestlings with an early or late lay-date may experience stress from lower food availability and higher predation pressure (Naef-Daenzer et al., 2001), and experimentally manipulated glucocorticoids have been reported to affect nestling bird gut microbiota diversity (Noguera et al., 2018). Furthermore, the effect of lay date on stress may depend on nestling age because of the increasing nutritional demands of older nestlings, so that young nestlings from early-nesting parents could face similar conditions to old nestlings from late-nesting parents. If, hypothetically, the positive correlation observed between adult Proteobacteria and lay date was driven by stress, this could explain why the effect of lay date on phylum-level relative abundance was in opposing directions for D8 and D15 nestlings: D8 nestlings from early nests may have been equally stressed to D15 nestlings from late nests (Figure 4g).

Brood size also correlated with microbiota variation, which we suggest is likely due to larger brood sizes leading to higher stress as a consequence of increased sibling competition (Neuenschwander et al., 2003) and lower food availability (H. G. Smith et al., 1988). The effect of brood size on microbiota was especially pronounced for D15 birds, when demand for food is highest and sibling-sibling conflict is greatest. It is possible that larger brood sizes simply have a different reservoir of bacteria due to higher levels of provisioning. Cross fostering experiments that manipulate the size of

broods, and compares broods with nestlings from multiple source nests against broods with nestlings from a single source, would test the relative importance of these reservoir and competition hypotheses. Just one other study, to our knowledge, has tested the link between brood size and the gut microbiota in great tits but found no evidence for an effect (Liukkonen et al., 2022, preprint). The disparity between these results likely shows how relationships between host and environmental variables and the microbiota may change in different settings or contexts, which will not be apparent in studies taking place during a single season over a relatively small geographic area.

Contrary to our predictions, in the *adults only* dataset, we did not detect any effects of lay date on gut microbiota, nor any sex-dependent interactions, despite lay date being strongly determined by female condition (Brown & Brown, 1999). Lay date could also potentially affect the adult gut microbiota indirectly, via increased glucocorticoids related to provisioning effort (Bonier et al., 2011), because provisioning the young is more challenging outside of the peak of food abundance. Although stress hormones have been reported to alter gut microbiota in reproductive female lizards (*Sceloporus undulatus*), these effects were only observed at specific reproductive stages (MacLeod et al., 2022). In the current study, adults were sampled for gut microbiota near the end of their reproductive cycle (i.e. when chicks were near fledging), and therefore it is possible we missed key reproductive time points where gut microbiota were sensitive to physiological influences associated with lay date. Also, this study uses data from just one breeding season and the relationships found here may vary annually depending on environmental conditions. Sampling across multiple years should therefore be an avenue of future research. Nevertheless, brood size, which may also reflect parental quality (Pettifor et al., 2001) and/or higher glucocorticoid demands associated with provisioning (Bonier et al., 2011), predicted gut microbiota community composition in adults of both sexes. Overall, these findings highlight a potentially exciting area of research on reproductive-dependent physiology and parental care, and its interaction with the

function of the gut microbiota. Brood size manipulation in conjunction with glucocorticoid measurements would determine whether the effect of brood size on the microbiota was related to parental quality or glucocorticoid levels.

3.4.4 Repeatability

Our repeatability analyses examined components of gut microbiota variation at individual, local environment and landscape environmental levels, for the first time. These analyses revealed the importance of the immediate nest environment in structuring the gut microbiota of nestling birds, whereas there was at most only weak evidence for any larger-scale effect of woodland site. Repeated sampling of adult birds in future studies could reveal whether these patterns are maintained in adult birds and represents an understudied aspect of microbiome research (Mallott, 2022). The importance of the nest in determining gut microbiota diversity and structure has been highlighted in our study species previously and in others (Campos-Cerda & Bohannan, 2020; Teyssier, Lens, et al., 2018), but has not been quantified relative to individual and landscape-level variation before. Our study site consisted of nine independent sites across a fragmented landscape but it was notable that this landscape level of environmental variation did not have a significant effect on the gut microbiota. We did not investigate whether the effect of nest on the microbiota persists beyond fledging, which would be indicated by continued repeatability, as most nestlings dispersed out of the woodland fragments.

Once nest and site effects were accounted for, there was no detectable effect of individual nestling identity on the gut microbiota. If host genetics had been an important driver of the gut microbiota in nestlings, we should have seen some level of individual repeatability, since siblings share only half of their DNA on average. If the nestling's gut microbiota were directly determined by their parents' microbiota, then we should have seen a positive correlation between parent and offspring gut microbiota. Similarly, mothers and offspring are expected to share more similar ASV's than non-offspring, as has been found in other studies (Diez-Méndez et al., 2022,

preprint; Grosser et al., 2019). However we did not see this in our data, lending support to the dominant influence of local environmental factors in nestlings (Teyssier, Lens, et al., 2018), which we suggest is most likely linked to local food availability. In line with this interpretation, experimental disruption of the microbiota in nestling great tits and blue tits did not have any lasting effect (Diez-Méndez et al., 2022, preprint); the authors provide indirect evidence to suggest that this was most likely due to the parents replenishing the microbiota through feeding and continuous recolonisation from the nest environment. We note that our results do not preclude microbial transfer between parents and nestlings, just that the community and diversity metrics measured here are not comparable between parents and offspring. While we did not detect an effect of host genetics on the nestlings microbiota it is possible that these effects may only become evident at lower taxonomic resolutions or with a larger sample of individual birds. Furthermore these results concern nestlings only and may not hold in adult birds, when the immune system is fully developed- immunogenetic variation has been shown to shape the microbiota of other wild species (Davies et al., 2022; Montero et al., 2021).

Despite lack of evidence for direct transfer of microbiota from the parents themselves (rather than through their provisioned food) to their offspring, we did find a weak negative correlation for the mother's Shannon diversity and that of her offspring. This raises the possibility of a maternal effect on the gut microbiota, which has been demonstrated in wild North American red squirrels (*Tamiasciurus hudsonicus*) (Ren et al., 2017) and barn swallows (Kreisinger et al., 2017). One possibility for the negative association is that mothers with high gut microbiota diversity prime their offspring to minimise gut microbiota diversity, akin to parental infections priming offspring to have increased immunity against parasites (Lorenz & Koella, 2011). Our previous work found a negative correlation between gut microbiota diversity and nestling's weight gain, a key proxy for fitness, (Davidson et al., 2021/Chapter 2), suggesting higher microbiota diversity presents a fitness disadvantage to developing birds. In which case we may expect a selective pressure

on mothers to ensure their young do not experience deleteriously high microbial diversity. Maternal investments in egg immunity is also influenced by the bacterial load and diversity of the local environment, affecting nestling development (Jacob et al., 2015; van Veelen et al., 2022).

3.4.5 Conclusions

Studies of environmental associations with the gut microbiota in natural systems typically focus on a single ecological feature (e.g. habitat type, season), when in fact multiple interacting ecological factors likely shape the gut microbiota simultaneously. Our results point to links between different aspects of the gut microbiota and multiple aspects of the environment and life history variation: the local nest, sibling interactions, location within woodland plots, the woodland plots themselves, and potentially changes in food availability during the season. We found that environmental effects were important in structuring the gut microbiota, confirming previous work but provide novel evidence for the relative influence of the environment being greater in nestling birds than in adult birds, while life-history traits such as lay date and reproductive effort (brood size) were more important for adults. These effects are to be expected since many of the factors involved are fundamental drivers of ecological processes within populations. Notably, it was the natal as opposed to the adult gut microbiota that was most sensitive to ecological variation, and there was little evidence that the parent microbiota influenced that of their offspring, pointing to a high degree of flexibility during development. Unpicking the mechanisms, causes and consequences of gut microbiota variation among wild populations is an exciting avenue of future research that remains largely unexplored.

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Data accessibility statement:

The sample metadata are available at datadryad.org (Davidson et al., 2020: https://doi.org/10.5061/dryad.bk3j9kd9g), this data frame contains variables describing each individual bird sample in this study, their associated environmental variables and microbiome summary statistics (alpha diversity measures and phylum level relative abundance). Tables of ASV abundances by sample and ASV taxonomy are available at also available at datadryad.org (Somers et al., 2022: https://doi.org/10.5061/dryad.fttdz08x2). Sequence data are available in the European Nucleotide Archive under access number PRJEB42330, and ERS5506097 - ERS5506338. Analysis code is available on GitHub at https://github.com/shan-e-s/HostAndEnv.

3.5 Appendix 2A: Supplemental results

Table S1. Breakdown of sampling. Fewer coniferous birds were sampled due to lower population density of conifer compared to mixed deciduous forests. 'Repeats' refers individuals sampled at Day-8 and Day-15.

	Day-8	**Day-15**	**Adult**
Conifer	16	27 (repeats = 13)	8 (Female = 3; Male = 5)
Deciduous	65	87 (repeats = 28)	43 (Female = 21; Male = 22)

Table S2. Betadisper results for each variable in the *D8-adult* and *D15-adult* PERMANOVA models. Significant results indicate violation of homogeneity of variance assumption of PERMANOVA.

	D8-adult			**D15-adult**		
	Sum Sq.	**Mean Sq.**	***p***	**Sum Sq.**	**Mean Sq.**	***p***
Age	374	374	0.069	15	15	0.652
Habitat	150	150	0.3	34	34	0.482
Lay date	5019	186	0.051	2211	85	0.245
Brood size	1709	285	0.61	2348	391	<0.001
Distance to edge	10756	192.1	<0.001	7586	135	<0.001
Sequence plate	1028	257	0.072	1418	355	<0.001

Table S3. Betadisper results for each variable in the *adults only* PERMANOVA models. Significant results indicate violation of homogeneity of variance assumption of PERMANOVA.

	Sum Sq.	**Mean Sq.**	***p***
Age	3.18	3.18	0.562
Sex	13.81	13.8	0.278
Habitat	6.93	6.93	0.4196
Lay date	1328	60.4	<0.001
Brood size	407.7	81.5	<0.001
Distance to edge	1559.5	42.15	<0.001
Sequence plate	142.8	47.6	0.002

Table S4. Alpha diversity, model averaged results for the *nestling only* data subset, for the a) Shannon index and b) Chao1 index. These results informed the fixed effects used in the repeatability analyses. †Adjusted standard errors.

	(a) Shannon				(b) Chao1			
	Estimate	**SE†**	**z value**	***p***	**Estimate**	**SE†**	**z value**	***p***
(Intercept)	0.794	0.145	5.475	<0.001	0.794	0.145	5.475	<0.001
Age	-0.146	0.090	1.627	0.10	-0.146	0.090	1.627	0.10
Distance to edge	0.236	0.130	1.819	0.07	0.236	0.130	1.819	0.07
Habitat	0.112	0.215	0.522	0.60	0.112	0.215	0.522	0.60
Age x habitat	-0.387	0.191	2.028	0.04	-0.387	0.191	2.028	0.04
Habitat x distance to edge	0.393	0.263	1.494	0.14	0.393	0.263	1.494	0.14
Lay date	0.144	0.145	0.989	0.32	0.144	0.145	0.989	0.32
Brood size	-0.104	0.120	0.869	0.38	-0.104	0.120	0.869	0.38
Age x distance to edge	-0.074	0.165	0.449	0.65	-0.074	0.165	0.449	0.65

Table S5. Binomial model results for phylum level relative abundance for the *nestling only* data subset. These results informed the fixed effects used in the repeatability analyses. † Adjusted standard errors.

Independent variables	Estimate	SE†	z	*p*	Estimate	SE†	z	*p*
	(a) Proteobacteria				(b) Firmicutes			
(Intercept)	-0.80	0.76	1.05	0.292	-0.63	0.85	-0.74	0.46
Age	-1.24	0.01	264.95	<0.001	0.97	0.00	225.74	<0.001
Habitat	1.14	1.12	1.02	0.307	-2.74	1.43	-1.91	0.056
Brood size	0.27	0.01	22.18	<0.001	-1.45	0.01	-100.06	<0.001
Distance to edge	0.41	0.56	0.73	0.463	1.20	0.78	1.54	0.125
Lay date	0.40	0.66	0.62	0.538	-0.59	0.91	-0.65	0.515
Age x habitat	-2.63	0.01	242.53	<0.001	5.01	0.01	476.80	<0.001
Age x brood size	-0.92	0.01	116.50	<0.001	1.91	0.01	249.46	<0.001
Age x distance to edge	1.35	0.01	110.43	<0.001	-3.75	0.01	-281.08	<0.001
Age x lay date	2.06	0.01	331.83	<0.001	-1.86	0.01	-308.61	<0.001
Habitat x brood size	-8.32	0.05	162.79	<0.001	14.98	0.06	234.90	<0.001
Habitat x distance	3.15	1.25	2.52	0.012	-4.83	1.62	-2.99	0.003
Brood size x distance to edge	2.22	0.04	63.62	<0.001	-5.16	0.05	-112.53	<0.001
Distance to edge x lay date	1.32	1.63	0.81	0.42				
Habitat x lay date	-2.15	2.37	0.91	0.365				

Table S6. Model averaged results for (a) PC1 and (b) PC2 for the *nestling only* data subset. These results informed the fixed effects used in the repeatability analyses. † Adjusted standard errors.

	(a) PC1				(b) PC2			
Independent variables	**Estimate**	**SE†**	**z**	***p***	**Estimate**	**SE†**	**z**	***p***
(Intercept)	8.52	10.89	0.78	0.434	-1.43	2.57	0.56	0.58
Age	-11.45	5.79	1.97	0.048				
Habitat	13.06	14.87	0.87	0.383	3.96	3.08	1.28	0.2
Distance to edge	20.54	7.58	2.69	0.007	-1.49	2.07	0.72	0.47
Lay date					-2.37	2.32	1.02	0.3
Brood size					-0.79	1.53	0.513	0.608
Age x habitat	-24.94	12.66	1.97	0.049				
Habitat x Distance to edge	8.53	16.04	0.532	0.595				

Table S7. Alpha diversity, model averaged results for the all birds data subset, for the a) Shannon index and b) Chao1 index. Cells are blank where the variable was not retained following the model selection procedure. † Adjusted standard errors.

	(a) Shannon				(b) Chao1			
Independent variables	**Estimate**	**SE†**	**z value**	***p***	**Estimate**	**SE†**	**z value**	***p***
(Intercept)	0.77	0.12	6.65	<0.001	5.61	0.19	29.85	<0.001
Age (D15/D8)					-0.35	0.15	2.33	0.02
Age (Adult/D15)					-0.02	0.18	0.12	0.91
Distance to edge	0.21	0.10	2.11	0.035	0.45	0.20	2.27	0.023
Lay date	0.08	0.11	0.73	0.46				
Age(D15-D8) × Distance-to- edge					-0.46	0.28	1.68	0.09
Age(Adult-D15) × Distance-to-edge					-0.09	0.33	0.28	0.78
Brood size					0.15	0.18	0.83	0.41
Habitat					0.00	0.32	0.01	0.99
Age(D15/D8) × Habitat					-0.73	0.32	2.24	0.025
Age (Adult/D15) × Habitat					0.14	0.42	0.34	0.74

Table S8. Alpha diversity linear model results for the *adults only* data subset, for a) Shannon index and b) Chao1 index. Results are model averaged mixed model values for retained variables. †Adjusted SE

	(a) Shannon				(b) Chao1			
Independent variables	**Estimate**	**SE†**	**z value**	***p***	**Estimate**	**SE†**	**z value**	***p***
(Intercept)	1.31	0.05	26.24	<0.001	5.45	0.19	28.22	<0.001
Brood size	0.07	0.07	0.95	0.34	0.34	0.24	1.41	0.16
Habitat	-0.07	0.09	0.76	0.45				
Sex	-0.04	0.05	0.70	0.48				
Distance to edge					0.24	0.23	1.02	0.31
Age(Mature- Juvenile)					-0.36	0.34	1.09	0.28
Lay date					-0.16	0.24	0.66	0.51

Table S9. Phylum level results for the *adults only* data subset for a) Proteobacteria and c) Firmicutes. Results are model averaged binomial mixed model values for retained variables. †Adjusted SE

	(a) Proteobacteria				(b) Firmicutes			
Independent variables	**Estimate**	**SE†**	**z value**	***p***	**Estimate**	**SE†**	**z value**	***p***
(Intercept)	1.137	0.273	4.169	<0.001	-3.865	0.679	5.692	<0.001
Age (Mature- Juvenile)	-0.779	0.736	1.059	0.289				
Distance to edge					0.689	0.603	1.142	0.253
Lay date	1.458	0.525	2.775	0.006	-0.743	0.609	1.219	0.223
Brood size					0.531	0.641	0.828	0.408

Table S10. Adjusted and unadjusted values of individual and group level repeatability of (a) nestling alpha diversity (logged), with confidence intervals in brackets. Fixed effects, where included, were: Age + Habitat + Age × Habitat; (b) (logit link scale approximation) of nestling phylum level relative abundance. Six models were run for each phyla with stepwise addition of fixed and random effects. Fixed effects where included were: Age + Habitat + Lay date + Brood size + Distance to edge + Age × Habitat + Age × Lay date + Age × Brood size + Age × Distance to edge + Brood size × Habitat + Brood size × Distance to edge; (c) nestling PC1 and PC2 from a Euclidian distance matrix based on community composition. Fixed effects, where included, were: Age + Habitat + Distance +Age × Habitat. No fixed effects were included in PC2 analysis, as there were no significant fixed effects in the PC2 nestling only model (Table S6).

	Repeatability (CI)			
	Site (woodland)	**Nest**	**Individual**	**Fixed**
(a)				
Shannon				
1	0.146 (0, 0.345)			No
2		0.399 (0.185, 0.543)		No
3			0. 378 (0, 0.423)	No
4			0.403 (0.298, 0.455)	Yes
5		0.423 (0.229, 0.562)	0 (0, 0.227)	Yes
6	0.11 (0, 0.33)	0.318 (0.139, 0.493)	0 (0, 0)	Yes
Chao				
1	0.127 (0, 0.298)			No
2		0.421 (0.186, 0.632)		No
3			0.46 (0.399, 0.48)	No
4			0.498 (0.476, 0.515)	Yes
5		0.499 (0.314, 0.641)	0 (0, 0.207)	Yes
6	0.114 (0, 0.365)	0.392 (0.194, 0.576)	0 (0, 0)	Yes
(b)				
Proteobacteria				
1	0.067 (0, 0.145)			No
2		0.191 (0.091, 0.284)		No
3			0.174 (0.27, 0.286)	No
4			0.144 (0, 0.254)	Yes
5		0.178 (0.046, 0.238)	0.026 (0, 0.14)	Yes
6	0.04 (0, 0.112)	0.139 (0.022, 0.202)	0.024 (0, 0.145)	Yes
Firmicutes				
1	0.104 (0, 0.198)			No
2		0.261 (0.138, 0.358)		No
3			0.248 (0.085, 0.391)	No
4			0.165 (0, 0.315)	Yes
5		0.267 (0.099, 0.338)	0.037 (0, 0.126)	Yes
6	0.078 (0, 0.181)	0.188 (0.057, 0.271)	0 (0, 0.124)	Yes
(c)				
Beta diversity (PC1)				
1	0.156 (0.005, 0.333)			No

2		0.377 (0.199, 0.515)		No
3			0.274 (0, 0.504)	No
4			0.242 (0.011, 0.483)	Yes
5		0.34 (0.172, 0.488)	0 (0, 0)	Yes
6	0.159 (0, 0.377)	0.192 (0.058, 0.35)	0 (0, 0)	Yes
Beta diversity (PC2)				
1	0.144 (0, 0.318)			No
2		0.484 (0.31, 0.623)		No
3			0.461 (0.23, 0.65)	No
4				
5		0.484 (0.302, 0.61)	0 (0, 0.177)	No
6	0.039 (0, 0.232)	0.448 (0.237, 0.576)	0 (0, 0.178)	No

Table S11. Parent offspring analysis. Offspring microbiome traits regressed on parents (a-d: mother; e-h: father) corresponding microbiome trait, controlling for fixed effects and the random effect of nest. Observations from 57 nestlings from 21 nests (a-d) and 59 nestlings from 22 nests (e-h).

Independent variables	Estimate	Std. Error	t value	*p*
(a) Shannon diversity				
Shannon (female)	-1.005	0.408	-2.463	0.025
Age	-0.879	0.328	-2.681	0.009
Habitat	1.215	0.603	2.017	0.08
Brood size	-0.477	0.399	-1.196	0.24
Lay date	-0.342	0.700	-0.488	0.631
(b) Chao diversity				
Chao1 (female)	-0.226	0.464	-0.486	0.632
Age	-0.719	0.269	-2.673	0.009
Habitat	0.654	0.654	0.999	0.341
Brood size	0.170	0.362	0.469	0.641
Lay date	0.001	0.719	0.002	0.999
(c) Proteobacteria relative abundance				
Proteobacteria (female)	0.316	0.897	0.353	0.724
Age	-0.515	0.005	-98.828	<0.001
Habitat	0.340	1.367	0.249	0.804
Brood size	-0.907	0.006	-158.429	<0.001
Lay date	-2.104	1.454	-1.447	0.148
(d) Firmicutes relative abundance				
Firmicutes (female)	1.496	0.855	1.749	0.08
Age	-0.515	0.005	-98.831	<0.001
Habitat	-0.218	1.452	-0.150	0.881
Brood size	-0.907	0.006	-158.433	<0.001

Independent variables	Estimate	Std. Error	t value	*p*
Lay date	-2.325	1.346	-1.728	0.084
(e) Shannon diversity				
Shannon (male)	0.765	0.564	1.355	0.189
Age	-0.295	0.349	-0.844	0.401
Habitat	0.547	0.965	0.567	0.596
Brood size	-0.172	0.440	-0.391	0.697
Lay date	-0.191	0.562	-0.340	0.742
(f) Chao diversity				
Chao1 (male)	0.400	0.336	1.193	0.242
Age	-0.233	0.298	-0.781	0.437
Habitat	0.040	0.730	0.054	0.958
Brood size	0.247	0.374	0.660	0.512
Lay date	0.008	0.494	0.015	0.988
(g) Proteobacteria relative abundance				
Proteobacteria (male)	-0.840	0.894	-0.940	0.347
Age	1.253	0.006	227.282	<0.001
Habitat	0.314	1.146	0.274	0.784
Brood size	0.629	0.006	111.184	<0.001
Lay date	1.019	1.159	0.879	0.379
(h) Firmicutes relative abundance				
Firmicutes (male)	0.998	0.810	1.231	0.218
Age	1.185	0.004	300.861	<0.001
Habitat	-0.080	0.963	-0.083	0.934
Brood size	0.628	0.004	158.484	<0.001
Lay date	0.774	1.009	0.767	0.443

Table S12. Beta-diversity PERMANOVA results with nest as a fixed effect, using the Euclidian distance between samples with centre-log ratio transformed ASV counts. Results are marginal effects and significance is calculated from 999 permutations. (a) model with D8 and adult birds and (b) model with D15 and adult birds. Analyses were split for D8 and D15 nestlings to avoid pseudo-replication due to repeat samples of same nestlings. Some cells are blank where value could not be estimated. R^2 values are rounded to nearest second decimal place.

	(a) D8-Adult				**(b) D15-Adult**			
Independent variables	**SS**	**R^2**	**F**	***p***	**SS**	**R^2**	**F**	***p***
Age	889	<0.01	1.32	0.039	795	<0.01	1.55	0.002
Habitat	0	0	-Inf		0	0	-Inf	
Lay date	0	0	Inf		514	<0.01	1	0.366
Brood size	564	<0.01	0.83	0.538	560	<0.01	1.09	0.294
Distance to edge	0	0	Inf		0	0	-Inf	
Sequence plate	4200	0.04	1.55	0.001	3438	0.04	1.67	0.001
Nest	44037	0.44	1.23	0.025	36687	0.37	1.32	0.001

Figure S1. Female Shannon diversity regressed on offspring diversity.

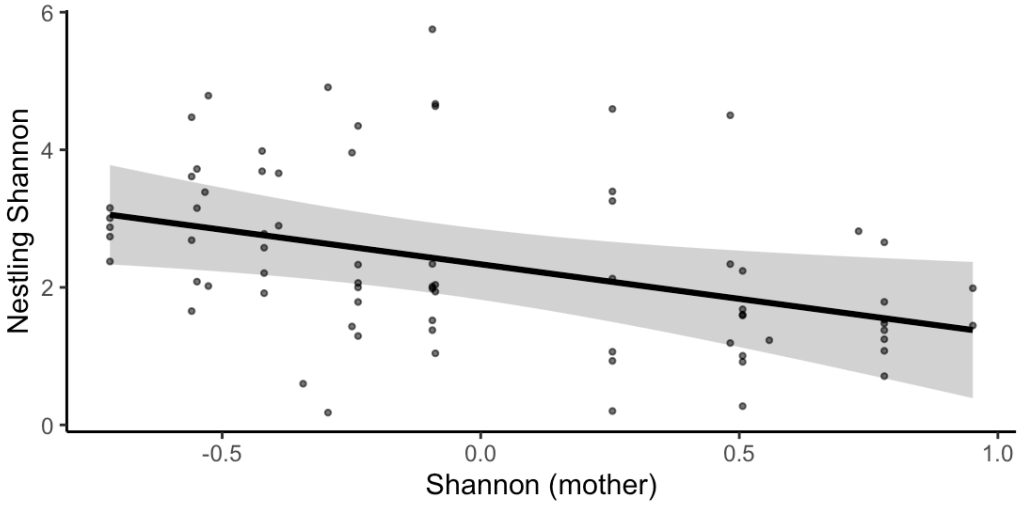


Figure S2. Beta diversity PCA plots of nestlings and adults using alternative ordinations: (a) Bray-Curtis plot of D8-adult birds coloured by age; (b) Jaccard plot of D8-adult birds coloured by age; (c) Bray-Curtis plot of D15-adult birds coloured by age; (d) Jaccard plot of D15-adult birds coloured by age.

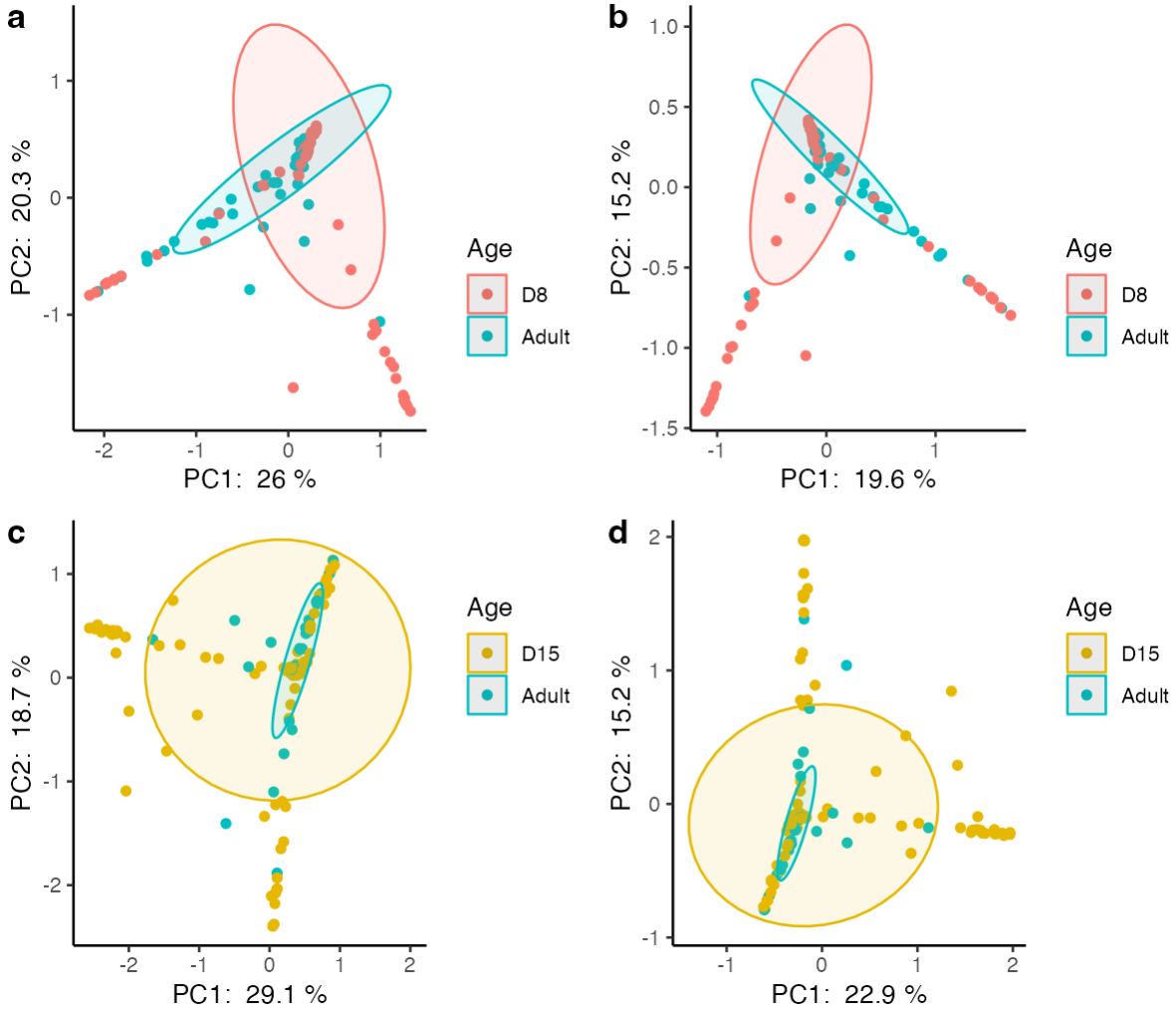
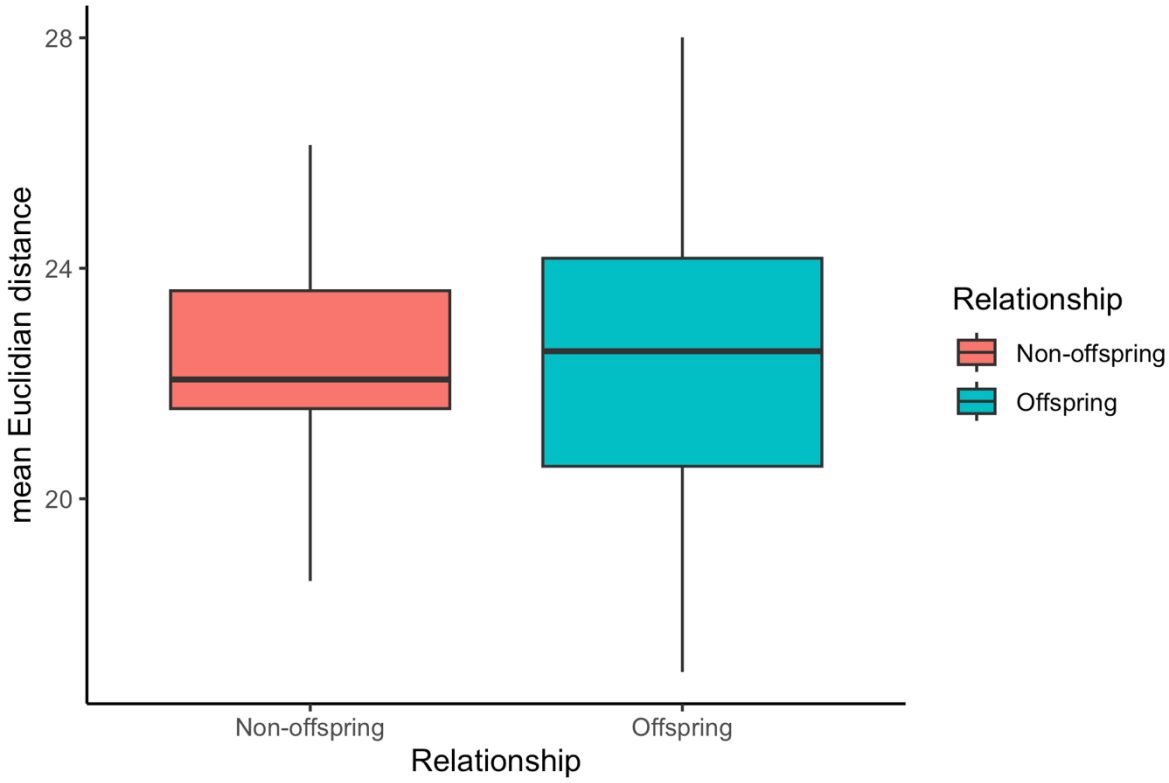


Figure S3. Comparison of mean Euclidian distance values between mothers and offspring vs mothers and non-offspring (n= 24 mothers).



Chapter 4: Experimental gut microbiome perturbation with a host-native strain in a wild bird population

Shane Somers, Gabrielle Davidson, Philiswa Mbandlwa, Caroline McKeon, Catherine Stanton, R. Paul Ross and John L. Quinn

Please note: Philiswa Mbandlwa isolated and characterised the strain L. kimchicus which was used in the following experiment. The supplementary file 'IsolationAndCharacterisation.pdf' is a portion of an unpublished chapter from Philiswa's PhD thesis and is reproduced here with her permission.

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Abstract

Empirical studies from laboratory systems and humans show that the gut microbiota is linked to host health. Similar evidence from nature is rare, not least because experimentally disrupting the gut microbiota in an effective, non-invasive manner among free-living individuals is extremely challenging. We isolated a bacterial strain *Lactobacillus kimchicus* (also known as *Secundilactobacillus kimchicus)*, from a wild population of great tits (*Parus major)*, and provided it as a dietary supplement at the nest, allowing the parents to consume it themselves and to feed it to their offspring without the direct intervention of the experimenters. We assessed the impact of our treatment on the host microbiota, and tested how this treatment impacted the link between the microbiota and host phenotype, i.e. weight- as an indicator of health. Our approach dramatically increased *L. kimchicus* abundance in the treatment birds' microbiota samples and manipulated the gut microbiota as a whole by increasing alpha diversity. This effect was strongest in the youngest birds, validating observational findings pointing to an early, brief developmental window when the gut microbiota are sensitive to environmental drivers. We found that the relationship between diversity and host phenotype was affected by the treatment. Specifically, there was a positive time-lagged relationship between diversity and weight in control birds but not in the treatment birds, where the treatment neutralised the link between diversity and weight- possibly by aiding the growth of low diversity individuals. We discuss how this may demonstrate the importance of the host's ecological context in determining the role of the microbiota on host phenotype. To our knowledge this is the first time this approach has been taken in a wild population and provides direct evidence for the role of the microbiota in the ecology and evolution of natural populations.

4.1 Introduction

Many studies have shown that the gut microbiota can affect host phenotype and health by influencing a variety of processes (McFall-Ngai et al., 2013; Suzuki, 2017). These processes include stress regulation (Sudo et al., 2004), cognitive function (G. L. Davidson et al., 2018), metabolism (Chevalier et al., 2015; Cox et al., 2014; Sommer et al., 2016) and the immunity (Knutie et al., 2017; Warne et al., 2019). The gut microbiota is also highly variable within individuals, a characteristic that is thought to enable them rapidly adapt to environmental variation (Alberdi et al., 2016). For example, flexible gut microbiota allows animals to cope with seasonal variation in food quantity and diet (Amato et al., 2015; Baniel, Amato, et al., 2021), and to detoxify dietary/environmental contaminants (Kohl et al., 2014; Teyssier, Rouffaer, et al., 2018). This may be particularly important during the host's development, when the microbiota is most sensitive to the environment (Somers et al., 2023/Chapter 3) and the host can be readily affected by the microbiota (Cox et al., 2014; Knutie et al., 2017; Warne et al., 2019). However, we are lacking experimental evidence from wild systems that the microbiota can affect host phenotypes generally, and health in particular.

To date, the vast majority of experimental microbiota manipulations have involved commercially important agricultural species or lab model hosts (Pascoe et al., 2017). These have limited genetic variation and experience consistent environmental conditions, making any findings difficult to generalise to wild populations. They also lack much of the microbial and environmental variation observed in wild populations (Amato, 2013; Hird, 2017). In addition to using host species that may not be good model organisms for ecology, many studies do not manipulate the microbiota directly and instead take advantage of natural experiments (Hird et al., 2014; San Juan et al., 2020; Teyssier, Rouffaer, et al., 2018), change the environment (Jacob et al., 2015), diet (G. L. Davidson, Wiley, et al., 2020), or use a cross-fostering approach (Teyssier, Lens, et al., 2018). The use of antibiotics and off the shelf probiotics also

have limitations. Antibiotics can manipulate the gut microbiota considerably but their effects tend to be broad spectrum, which can complicate the interpretation of results (Motiei et al., 2020; Willing et al., 2011). Probiotics can provide a more targeted way to change the microbiome and improve animal health in agricultural species (J. M. Smith, 2014). However, they are not always effective (Kristensen et al., 2016; J. M. Smith, 2014), potentially because the microbes used are not adapted to the host species and do not interact with host tissues (Duar et al., 2017; Shapira, 2016; Ward et al., 2019).

Several studies have shown an association between naturally occurring strains of bacteria and indicators of health or fitness (Davidson et al., 2021/Chapter 2; Moreno et al., 2003; Rosshart et al., 2017; Vásquez et al., 2012). Naturally occurring *Lactobacillus* have been associated with greater weight in wild avian hosts (Davidson et al., 2021/Chapter 2; Lewis et al., 2017). *Lactobacillus* spp. are often identified as having beneficial effects on human model organisms and so are commonly used in probiotic treatments (Angelakis & Raoult, 2010; Drissi et al., 2017; Grond et al., 2019; Servin, 2004). *Lactobacillus* can affect the microbiome by encouraging or inhibiting the growth of other bacteria by altering the pH of the environment and through the production of antimicrobials (including bacteriocins) (reviewed by Drissi et al. (2017)). Dietary supplementation of domestic pigeons and chickens with commercial Lactobacillus probiotics have been shown to be effective in changing the gut microbiome composition of hosts (Forte et al., 2018; Grond et al., 2019), and have been associated with weight change in humans and domesticated animals (Angelakis & Raoult, 2010; Forte et al., 2018; Million et al., 2012). However, host adapted strains of bacteria are rarely if ever used in domestic settings (Gadde et al., 2017; Ward et al., 2019) and never, to our knowledge, in the wild, despite evidence that host adapted strains are more effective than non-specific commercially available strains (Ward et al., 2019).

Here, we use a novel method of directly experimentally perturbing the gut microbiota of a wild, free-living bird (Great tits, *Parus major)* to investigate the role of the microbiota in host health. The aim of this study was to build on observational results linking gut diversity to host weight gain (Davidson et al., 2021/Chapter 2), a key proxy for fitness in nestling birds (Both et al., 1999; Monrós et al., 2002), by perturbing the gut microbiota with a host-adapted Lactobacillus species. We isolated and cultured a Lactobacillus strain directly from the host, after a previous study on this population identified several Lactobacillus strains linked to survival and weight gain (Davidson et al., 2021/Chapter 2). The experiment was conducted in a natural setting which minimised researcher intervention by taking advantage of natural parental provisioning behaviour. We expected the addition of a large volume of the isolated bacterial strain would increase the abundance of this strain in the gut, likely at the expense of other gut bacteria. We expected this would disrupt the gut microbiota diversity, community structure and function, and thereby affect host health, with minimal effects on other processes of host homeostasis, particularly stress, which would be caused by frequent handling and exposure to humans, and immunity, which would be disrupted by antibiotic treatment. Given the importance of the microbiota to host ecology (Amato, 2013) and conservation (Hauffe & Barelli, 2019; Song et al., 2019; Trevelline et al., 2019), this is a timely study offering an experimental method for investigating links between the microbiota and the host, in natural settings.

4.2 Methods

4.2.1 Obtaining and culturing host-native strain

In June 2020 faecal samples were taken from great tit nestlings at Dukes Wood, Co. Cork. These samples were inoculated into BD Difco *Lactobacillus* broth (De Man et al., 1960) (MRS; Difco Laboratories, Detroit, MI). This was incubated anaerobically at 37°C overnight and serially diluted using phosphate-buffered saline (PBS), then spread onto *Lactobacillus* Selective (LBS) agar (Difco Laboratories, Detroit, MI) plates and incubated under different conditions, i.e., anaerobically at 30°C and 37°C, and

aerobically at 30°C and 37°C for two days. Colonies with different morphologies were streaked and re-streaked on LBS to obtain pure cultures. The pure LAB cultures were subsequently kept in LBS broth supplemented with 35% (v/v) glycerol and frozen at −80°C until further analysis. Genomic DNA was extracted, the 16S rRNA gene amplified using Polymerase Chain Reaction (PCR) and sent for sequencing to Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom). The resulting sequences were compared to existing genomic data using the Basic local alignment search tool (BLAST) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The identity of the isolates was determined based on the highest scores (≥ 98%). Subsequently the entire genome of a sample was sequenced and the strain was screened for probiotic characteristics. The strain was freeze-dried and concentrated in powder form using 10% (w/v) trehalose (Sigma Aldrich, Wicklow, Ireland). The viability of the freeze dried powder was confirmed with a 6 week stability trial which demonstrated the strain could still grow well after rehydration. See supplementary file 'IsolationandCharacterisation.pdf for more details.

4.2.2 Dosing

In order to disturb the birds as little as possible during the nesting period, nestlings were fed the powder indirectly by providing mealworms (*Tenebrio molitor*) soaked in a solution in sterilised plastic pots (pot volume = 125 ml) under the front entrance of each nest box. The parents took the supplementary mealworms into the nest and fed them to the young. This provisioning behaviour was confirmed visually on a sample of 5 nests. We observed adults consuming the worms themselves and bringing them into the nest. The first nest to hatch at a site was randomly assigned to a treatment and subsequent nests were alternately assigned to treatment or control. Given the limited adherence of the powder to each worm we decided to simply provide the maximum dose of powder as we could afford given our production capacity. Each treatment nest was provided with approximately 0.07g of *L. kimchicus* per chick per day mixed with 10 mealworms (~ 1.2g) per chick per day. This quantity of worms represents approximately 30-40% of their daily nutritional needs between the ages of D3-D7 and 15-20% between the ages of D8-D15 (Seress et al., 2020). We

aimed to provide enough supplementary food that each chick in the nest would receive some worms, as providing too few worms could lead to only the most competitive and strongest chicks receiving the treatment worms, but not so much that parents did not forage and hence prevent the nestlings from being colonised by 'normal' environmental microbes. Supplementary feeding started on day-0 (day of hatching) and stopped after day-14. Control nests were provided with mealworms soaked in trehalose (0.007g trehalose per chick). Each dose of *L. kimchicus* and trehalose powders were rehydrated with 1mls of distilled water before application to worms.

4.2.3 Nest monitoring, trapping, tagging and faecal sampling

Nests were checked following the schedule in O'Shea et al. (2018). Nestling weight was recorded on day-8 and day-15 post hatching and faecal samples taken. If nestlings were large enough they were given individual ID rings (BTO). Adults were trapped on the nest on day-12 when they were fitted with a BTO ring if they didn't have one already, and had their measurements and faecal samples taken. Nests were checked a final time at the end of the season to determine whether any nestlings failed to fledge.

Faecal samples were taken using an adapted version of Knutie et al.'s (2018) sampling apparatus. Briefly, nestlings were placed on sterilised PVC trays in clean paper bags. This sampling apparatus was placed on a hot water bottle while waiting for nestlings to produce a sample; day-8 nestlings were given 5 minutes to produce a sample and day-15's were given 10 minutes. Nestling body temperature and activity was monitored to ensure they remained warm. If nestlings didn't produce a sample they were placed back in the nest to warm up while their siblings were sampled and if possible a second sampling attempt was made. When sampling adult birds a sterilised grid made of coated fencing was placed over the PVC tray in order to prevent adults contaminating the sample, as adults were much more active than nestlings in the sampling bag. Faecal samples were transferred to a sample tube using

a sterile inoculation loop and preserved with 95% ethanol. Sample tubes were transferred to a -80°C freezer at the end of the day.

Table 1. Sample size breakdown of experimental treatment by age. Includes some birds sampled twice (i.e. at D8 and D15).

	Day-8	**Day-15**	**Adult**
Control	39	44	17
L. kimchicus	49	40	12

4.2.4 DNA extraction & Library preparation

Prior to DNA extraction the ethanol was removed from the samples using a MiVac centrifuge, which spins and heats samples at very low pressure (<100 mbar). Open sample tubes were placed in the Genevac miVac Centrifugal Concentrator (Fisher Scientific) and spun for 2 hours at 45° C and 1465 rpm. Typically, this removed all the ethanol and dried out the samples. Any remaining ethanol was removed using a pipette. DNA was extracted from the faecal samples using the PowerFecal Pro kit (Qiagen, cat no. 51804). Some alterations were made to the kit protocol (May, 2019 version), following Trevelline et al. (2018).

The V3-V4 variable region of the 16S rRNA gene was amplified from the extracted DNA using the 16S metagenomic sequencing library protocol (Illumina: 16s-metagenomic-library-prep-guide-15044223-b) with some modifications. Samples were split across 3 PCR plates. DNA samples from the PCR plates were normalised to 10nM and pooled. Samples were sequenced on the MiSeq sequencing platform (Azenta Life Sciences/Genewiz, Germany), using a 2 × 300 cycle kit, following standard Illumina sequencing protocols. Negative controls using sterile filtered water (Sigma-Aldrich) were included at the extraction, evaporation and amplicon PCR steps, and brought through to sequencing in order to detect experimental or environmental contaminants. See supplementary for more in-depth methods.

4.2.5 Bioinformatics analyses

After sequencing samples were processed using the DADA2 pipeline in R, following the dada2 tutorial v1.16 (Callahan, McMurdie, et al., 2016). Sequences were trimmed and truncated to remove adapters and low quality reads, then filtered to remove sequences with expected errors >2. Errors were estimated and the core sample inference algorithm applied to the filtered and trimmed sequence data. Forward and reverse reads were merged to obtain full denoised sequences. A sequence table of Amplicon Sequence Variants (ASV) was constructed containing counts of the unique sequences by sample. Chimeric sequences were removed using the default 'consensus' method. A taxonomy table was generated using the naïve Bayesian classifier method and the Silva (v138.1) reference database. The dada2 outputs were combined into a single Phyloseq object (McMurdie & Holmes, 2013) in R before further filtering of samples. ASV's identified as chloroplasts, archaea, eukarya or mitochondria were removed. Duplicates, controls and potentially contaminated samples were removed. Sample completeness curves were plotted using the *rarecurve* function from the vegan package (Oksanen et al., 2019). Sample completeness occurred at about 7500 reads so all samples with less than 7500 reads were removed before further analysis. Contaminant ASV's were identified using the prevalence method from the decontam package (Davis et al., 2018). Shannon and Chao1 diversity were estimated with phyloseq's *estimate_richness* function.

Phylogenetic diversity was also calculated in the form of Faith's PD (Faith, 1992). A GTR+G+I (Generalized time-reversible with Gamma rate variation) maximum likelihood tree was constructed using a neighbour-joining tree as a starting point with the Phangorn package (Schliep, 2011) following the Bioconductor workflow (Callahan, Sankaran, et al., 2016). Faith's PD and Chao1 values were very similar for each sample (figure S1). We expected these diversity measures to be related because phylogenetic diversity is positively correlated with richness but considering there is so little difference this suggests that there is very little phylogenetic signal at all, or in other words the taxa are not closely related.

4.2.6 Statistical analyses

Alpha diversity

Variation in alpha diversity (log-Chao1 and Shannon diversity) across treatment groups was assessed using Bartlett's test (Snedecor & Cochran, 1989). Linear mixed models were used to investigate the effect of treatment on alpha diversity across age groups, using the lme4 package (D. Bates et al., 2015). Models included the alpha diversity term (log-Chao1 or Shannon diversity) as the response, and treatment (control, *L. kimchicus*), life-stage (day-8, day-15, adult) and the interaction treatment × life-stage as predictor variables. Woodland site, nest ID and bird ID were used as nested random effects to control for non-independence in the data. Weighted effects coding (*wec*) (Nieuwenhuis et al., 2017) of the life stage variable was used instead of the default dummy coding as we were interested in the effect of treatment at each life-stage, and how treatment affected diversity compared to the overall mean of diversity, rather than compared to a specific reference level. Estimates for age in these *wec* models represent the deviation of each level from the sample mean across all levels, where the sample mean is weighted by the number of observations at each level. In *wec* models the intercept refers to the weighted sample mean rather than the average value for the chosen reference level, and estimates are for the deviation from this sample mean. In weighted effect coding, interactions represent the additional effects over and above the main effects obtained from the model without these interactions (Te Grotenhuis et al., 2017). Interaction estimates are orthogonal to the main effects meaning the main effects are interpretable. Model residuals were checked using DHARMa (Hartig, 2019). The effect of treatment on phylogenetic diversity, namely Faith's PD (Faith, 1992), was also investigated however the results were virtually identical to the results for the Chao1 model (table S1) so these results are not discussed further.

4.2.7 Beta diversity

Before modelling the community composition, we removed low prevalence taxa (<5%). Taxa counts were centre-log transformed and the Aitchison distance between

samples calculated (Gloor et al., 2016; Gloor & Reid, 2016). The PERMANOVA+ function (Anderson et al., 2008) from the Primer (v.7) package (Clarke & Gorley, 2015) was used to determine the between group variation of samples according to treatment, controlling for the random effects of site and nest ID, as well as the effect of age category as a fixed effect. The type III (partial) sum of squares were calculated. The principal components of the data were calculated and the first two components plotted to visualise differences between treatment groups. The dispersion (within-group variance) of samples by treatment group and life-stage were calculated, using PERMDISP function, as PERMANOVA models assume homogeneity of variance.

4.2.8 Detecting L. kimchicus in samples

No sequence was identified as *L. kimchicus* (or *Secundilactobacillus kimchicus*) by taxonomic assignment so we compared all the filtered bacterial sequences detected in the birds with the *L. kimchicus* genome obtained from whole genome sequencing, using BLAST from the rBLAST package (Hahsler & Nagar, 2019). BLAST created alignments between each bacterial taxa and *L. kimchicus* and the alignments were ranked according to bit score, which measures similarities of alignments. The 20 alignments with the highest bit scores and greatest overlap in length were investigated further to verify whether they were the treatment strain. Only ASV27 was present in more than five individuals. A chi-square test was used to test whether ASV27 presence was dependent on treatment.

4.2.9 Relative abundance of Firmicutes

The treatment strain, *L. kimchicus*, is part of the Firmicutes phylum and might have interacted with other microbiota in this phylum. The addition of a Lactobacillus could have (a) promoted the growth of other Lactobacillus species by modifying the environment, or (b) reduced the growth of other Lactobacillus through competition. We used a binomial model from the lme4 package (D. Bates et al., 2015) to test the effect of treatment on Firmicutes relative abundance, controlling for life-stage (which was weighted effects coded) as a fixed effect and the nested random effects

of site, nest and bird ID. The response, proportion of reads that were Firmicutes, was weighted by the total number of reads in the sample.

4.2.10 Treatment effect on weight

A previous study found a time-lagged effect of alpha diversity at D8 on weight at D15 (Davidson et al., 2021/Chapter 2). We first tested the effect of treatment on weight across all birds (nestlings and adults), accounting for age, brood size and microbiota diversity (Shannon, log-Chao1). The age variable was backwards difference coded, meaning each age group is compared to the previous age group i.e. D15 compared to D8, adult compared to D15, as in this case the sequential comparison was of more interest than making a comparison with an arbitrary reference level or to the overall life stage mean. We included the interactions treatment × age, and treatment × alpha-diversity, as we expected the treatment to affect alpha diversity and we thought the treatment might affect the age groups differently considering the differential sensitivity of developing individuals microbiota (Somers et al., 2023/Chapter 3).

We performed a second analysis of weight which repeated the analysis as found in Davidson et al. (2021)/Chapter 2, namely testing the effect of alpha diversity at D8 on weight at D15 (nestlings only), while accounting for D8 weight, lay date and brood size as fixed effects and woodland site and nest ID as nested random effects, but we included an interaction between alpha diversity and treatment to examine whether the treatment affected this relationship. Here we expected that the treatment would have an effect on alpha diversity which would itself affect weight. Model residuals were checked using DHARMa (Hartig, 2019). The life stage variable uses default dummy coding in this model, with D8 as the reference level.

4.2.11 Inferred function and differential abundance analysis

Picrust2 (G. M. Douglas et al., 2020) was used to infer the abundance of functionally relevant KEGG functional orthologs (KO) and MetaCyc pathways. We used

Microbiome Multivariate Association (MaAsLin2) to test for differentially abundant KO and MetaCyc pathways across treatments, accounting for age as a fixed effect and including site and nest ID as random terms. P-values were FDR-corrected using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). We describe functions according to the BRITE hierarchies database. We also tested for differentially abundant ASVs using the same methods.

4.3 Results

4.3.1 L. kimchicus presence and relative abundance was detected in line with experimental treatment.

All 16S sequences retained after filtering were aligned using BLAST with the *L. kimchicus* whole genome and found that ASV27 (*Secundilactobacillus sp.,* also known as *Lactobacillus kimchicus* (Liang et al., 2011; J. Zheng et al., 2020)) aligned with 100% similarity, had the greatest alignment length (442bp) with 0 mismatches or gaps and the highest bit score (817 bits). This ASV was present in 44/101 treatment bird samples and 6/100 control bird samples, and was therefore dependent on treatment (χ^2= 35.9, df = 1, p<0.001). About 200 other ASV's also matched with 100% similarity but with shorter alignment lengths. Of the ASV's with the top 20 bit scores (18 *Secundilactobacillus* sp. and 2 *Latilactobacillus)* all but ASV27 are present in 5 or less individuals and most are present in only a single treatment bird. We also confirmed that ASV27 was significantly more relatively abundant in treated birds (coef = 2.09, BH-correct *p* =0.02). The only other differentially abundant ASVs were two Actinobacteria, ASV163 (*Williamsia sp.:* coef = 1.19, BH-corrected *p* =0.03) and ASV676 (*Conexibacter sp.:* coef = 0.81, BH-corrected *p* = 0.046).

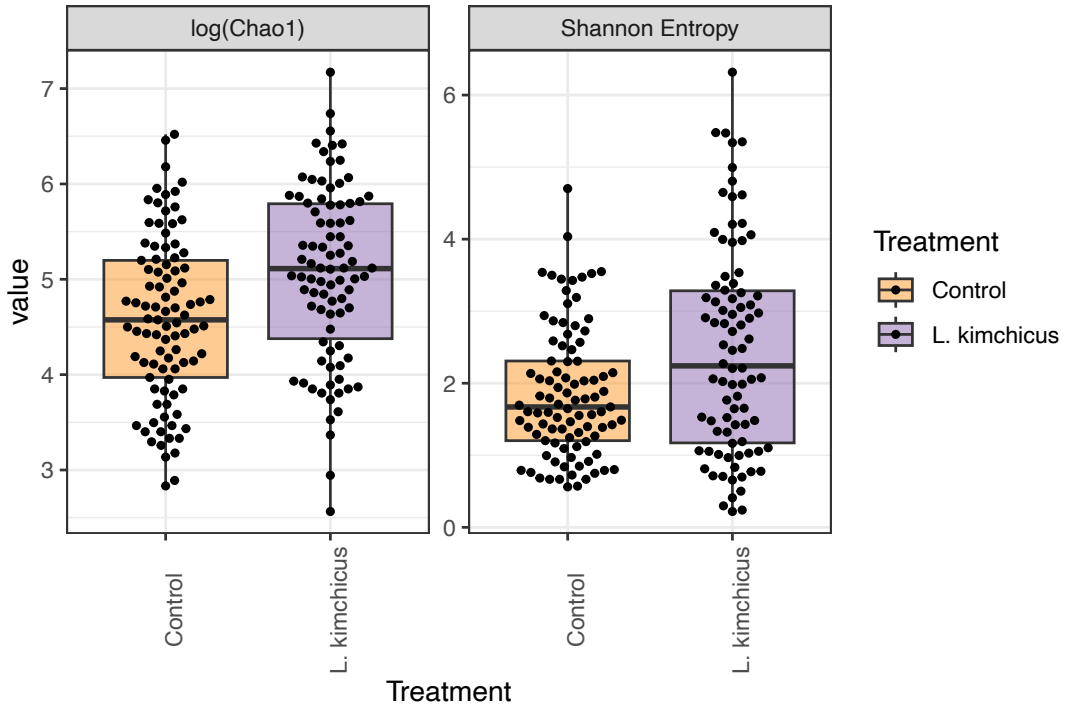

Figure 1. Raw alpha diversity plots comparing *L. kimchicus* treated birds with controls. Excludes huge Shannon outlier to allow clearer visualisation.

4.3.2 *L. kimchicus* treatment increases alpha diversity but does not alter community composition.

L. kimchicus treatment increased variation in Shannon diversity (Bartlett's K-squared: 15.68, p<0.001) but not log-Chao1 diversity (0.146, p=0.7). Across all age groups, *L. kimchicus* treatment increased both log-Chao1 (table 2, figure 1) and Shannon diversity (table S2, treatment: 0.313, p=0.0431; figure 1). This effect was strongest in D8 nestlings (table 2, figure 2) and diminished in later life stages. Community composition was not affected by treatment and differed instead across sites (table 3; figure 3). Treatment (p=0.24) and life stage (p=0.39) had homogeneity of variance when calculated for all age groups together. However treatment was associated with differences in dispersion for D8 nestlings showed (p=0.024) but not D15 (p=0.22) or adult birds (p=0.63) when calculated for each age group separately. There was some support for treatment to affect beta diversity differently across sites (table 3; figure S2). There was weak evidence that Firmicute relative abundance was lower in treated birds compared to controls (table S3; -0.97, p = 0.065), controlling for life stage.

Table 2. Effect of *L. kimchicus* treatment on log(Chao1) diversity, across age groups, N = 201. The model uses weighted effects coding (wec) for the age variable so estimates for age represent the deviation of each level from the sample mean across all levels, where the sample mean is weighted by the number of observations at each level. In wec models interactions represent the additional effects over and above the main effects obtained from the model without these interactions, and interaction estimates are orthogonal to main effects so main effects are interpretable.

Independent variables	Estimate	Std. Error	df	t value	p
(Intercept)	4.970	0.125	3.523	39.852	<0.001 *
Treatment (*L. kimchicus*)	0.248	0.104	25.752	2.373	0.025 *
Age (D8)	0.132	0.063	177.273	2.093	0.038 *
Age (D15)	-0.052	0.066	179.182	-0.786	0.433
Age (Adult)	-0.252	0.135	176.575	-1.864	0.064
Treatment : Age (D8)	0.114	0.056	177.471	2.023	0.045 *
Treatment : Age (D15)	-0.046	0.069	179.150	-0.667	0.505
Treatment : Age (Adult)	-0.311	0.160	176.524	-1.942	0.054 .

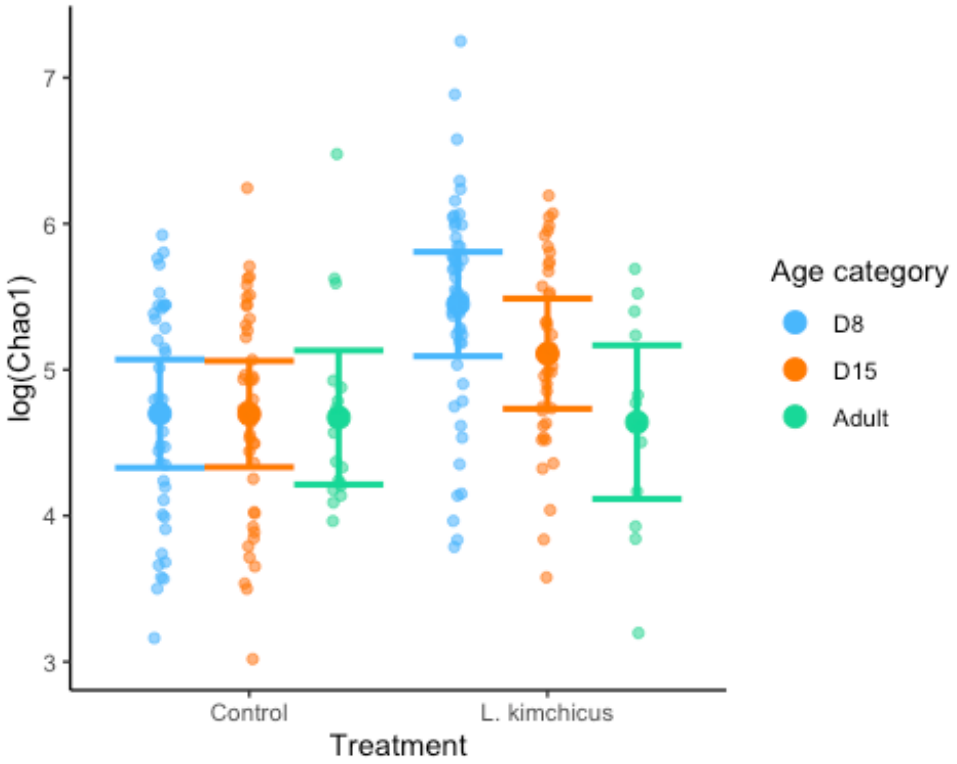

Figure 2. Effect of treatment on log(Chao1) diversity across age groups, N = 201. Partial residual plot of Chao1 diversity by treatment split by age category, with confidence intervals (table 2).

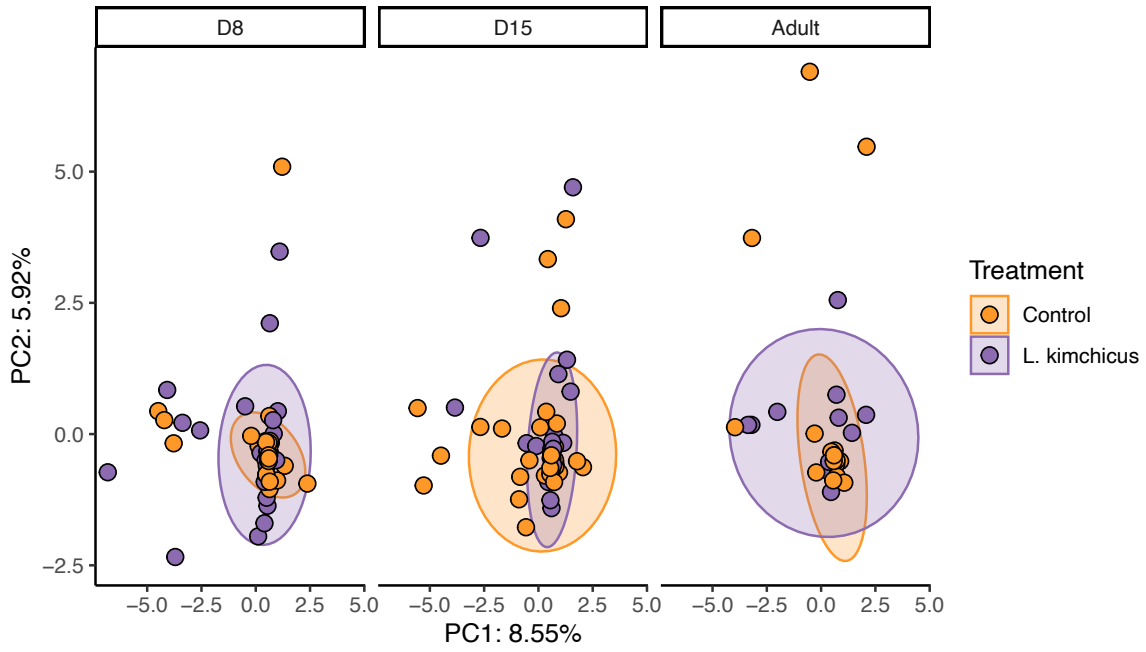

Figure 3. PCA plot of Aitchison distances between samples, N = 139 (repeats removed). Ellipses coloured according to experimental treatment group and each panel represents a different age category.

Table 3. Beta-diversity model results. Community composition is not affected by treatment, N = 139 (repeat samples removed). PERMANOVA+ model using Aitchison distance. Treatment and life stage are main effects. Site and nest ID are nested random terms.

Variable	df	SS	MS	Pseudo-F	p
Treatment	1	31.707	31.707	0.784	0.813
Site	3	141.19	47.064	1.42	0.009*
Life stage	2	81.844	40.922	1.116	0.262
Treatment × Site	3	124.62	41.541	1.255	0.069
Treatment × Life stage	2	67.776	33.888	0.91	0.635
Site × Life stage	6	228.98	38.164	1.264	0.082
Nest (Site × Treatment)	21	697.67	33.222	1.169	0.067
Treatment × Site × Life stage	6	233.37	38.895	1.288	0.074
Nest (Site × Treatment)) × Life stage	26	787.35	30.283	1.066	0.274

4.3.3 L. kimchicus neutralises microbial diversity's link with future weight gain.

There was no effect of treatment or alpha diversity on contemporary weight, at any age (table 4, S3). In the time lagged models, there was a significant effect of treatment on weight, with L. kimchicus treated birds having higher weight at day-15 when controlling for log(Chao) diversity at day-8. There was a positive relationship between log(Chao1) at D8 and weight at D15 in control birds but this effect was negated by the L. kimchicus treatment (log(Chao1) (D8) × treatment interaction, table 5, figure 4). There was no time-lagged effect for Shannon diversity (table S5).

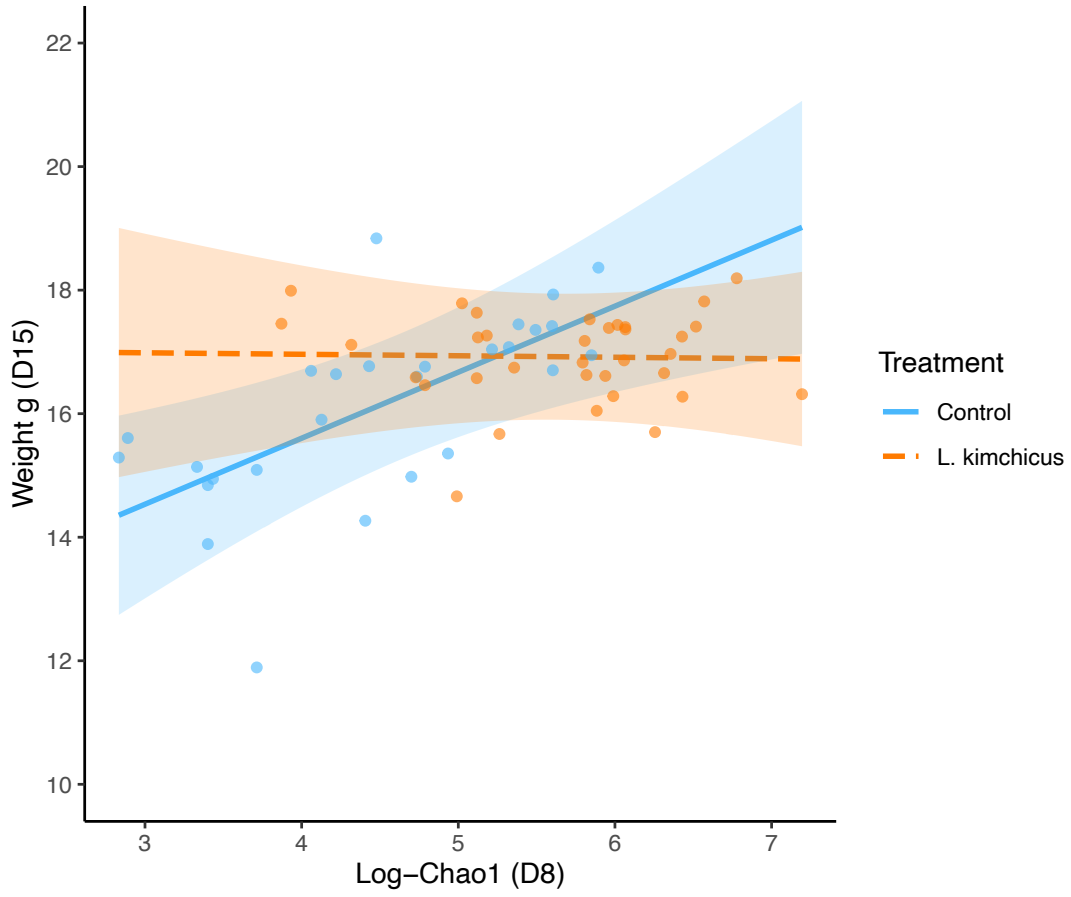

Figure 4. Effect of D8 log(Chao1) diversity on D15 weight in grams, across treatments, N = 61. Partial residual plot with separate lines and 95% confidence intervals for each treatment group.

Table 4. Weight changes according to treatment and log(Chao1) diversity across age groups, controlling for timing of reproduction and reproductive effort, N = 197. Linear model including all birds, modelling treatment and other predictors against bird weight. The age variable is backwards difference coded where each level of the variable is compared to the preceding level, so D15 is relative to D8 and adult is relative to D15, rather than being compared to a single reference level. Site, nest ID and bird ID are included as nested random terms.

Independent variables	Estimate	Std. Error	df	t	p
(Intercept)	0.217	0.111	25.085	1.959	0.061
Treatment (L. kimchicus)	0.007	0.159	27.593	0.047	0.963
log(Chao1)	0.020	0.061	185.088	0.329	0.742
Age (D15-D8)	1.353	0.102	101.074	13.294	<0.001 *
Age (Adult-D15)	0.521	0.143	161.527	3.652	<0.001 *
First egg lay date	-0.067	0.079	25.249	-0.852	0.402
Brood size (D8)	-0.056	0.072	28.133	-0.777	0.444
Treatment (L. kimchicus) : log(Chao1)	-0.024	0.086	179.737	-0.274	0.785
Treatment (L. kimchicus) : Age (D15)	0.142	0.143	97.102	0.994	0.323
Treatment (L. kimchicus) : Age (Adult)	-0.149	0.222	160.729	-0.674	0.501

Table 5. Time-lagged weight model, investigating the effect of log(Chao1) diversity at D8, across treatments, on weight at D15, controlling for weight at D8, timing of reproduction and brood size, N = 61. Site and nest are included as nested random terms.

Independent variables	Estimate	Std. Error	df	t	p
(Intercept)	10.924	2.859	28.970	3.821	0.001 *
Treatment (L. kimchicus)	5.730	2.441	51.177	2.348	0.023 *
log(Chao1) (D8)	1.069	0.345	52.080	3.095	0.003 *
Weight g (D8)	1.386	0.260	51.971	5.333	<0.001 *
First egg lay date	0.002	0.037	16.961	0.067	0.948

Independent variables	Estimate	Std. Error	df	t	p
Brood size (D8)	-0.042	0.342	21.038	-0.122	0.904
Treatment (L. kimchicus) : Chao1 (D8)	-1.093	0.458	47.647	-2.385	0.021 *

4.3.4 *L. kimchicus* gene function associated with carbohydrate, amino acid and protein metabolism.

The complete genome of *L. kimchicus* 5.1 consists of 2,535,859 bp and has no plasmids or transposable elements. 2730 coding sequences were found, including 71 RNAs and 955 protein-coding open reading frames (ORFs) divided into 27 subsystem groups. The genome includes a sequence encoding the bacteriocin Leucocin A. The majority of the genes identified were associated with carbohydrate metabolism (19.6%), amino acid (18.7%) and protein metabolism (14.3%) (figure S3). Further description of the *L. kimchicus* metagenome is described in supplementary (results section of the supplementary file: IsolationAndCharacterisation.pdf).

4.3.5 Functional differential abundance analysis

21 Kegg Orthologs were differentially abundant, all of which were expressed more abundantly in the treated birds. These KOs mapped to carbohydrate metabolism, protein metabolism, lipid metabolism and terpenoids and polyketides metabolism (table S6, S7). No MetaCyc pathways were detected as differentially abundant.

4.4 Discussion

4.4.1 Overall summary

We show that the addition of a host-adapted Lactobacillus strain significantly affected both the gut microbiota and phenotype of a wild bird. The addition of L. kimchicus in the diet affected nestling weight gain and the relationship between microbiota diversity and nestling weight gain. The treatment eliminated the link between gut diversity variation and host weight in nestlings, possibly by providing

additional metabolic functionality that was beneficial to low diversity individuals but not high diversity individuals. We used novel method for directly experimentally perturbing the gut microbiota of wild birds in the field without the use of antibiotics or regular handling. This technique offers an important new tool for experimentally studying the gut microbiome in a wild host without the confounding immune effects associated with antibiotic treatment or stress effects associated with regular handling. The addition of *L. kimchicus* in the diet increased the diversity and changed the predicted functional profile of host's microbiota. Specifically, host's Chao1 and Shannon diversity increased with the addition of the *L. kimchicus* compared to control birds. Additionally, we find further support for the importance of bacteria from the local environment/diet in structuring the microbiome of the young, while adults maintain more stable microbiomes in the face of environmental sources of perturbation.

4.4.2 Treatment alters gut microbiota

The treatment increased the presence and relative abundance of *L. kimchicus* in treatment birds compared to controls. It is not clear whether the strain became permanently established in the birds following the cessation of the treatment because our site's negligible recruitment of nestlings into the local breeding population precluded extensive follow-up sampling. Regardless of its establishment, the presence of the strain did alter the gut microbiota in an unexpected manner. Gut diversity increased and Shannon diversity became more variable in birds in the experimental treatment, compared to control birds, though the gut communities did not change. This result was surprising, as we expected that by providing a large dose of a single strain, that the strain would get a competitive advantage and hence lower the overall gut microbiota diversity, and the variance in the diversity, of experimental birds. *Lactobacillus* can modify their environment by producing anti-microbials that inhibit the growth of other bacteria (Drissi et al., 2017). Whole genome sequencing indicates our strain contains a gene encoding the bacteriocin Leucocin A, which inhibits a broad range of other lactic acid bacteria and some known (non-*Lactobacillus*) pathogens (Hastings & Stiles, 1991). Our analysis provided some

evidence that Firmicutes relative abundance decreased, which may reflect *L. kimchicus* suppressing potential competitors, either directly through competition for resources or with *L. kimchicus* produced anti-microbials, or indirectly by modifying the environment. It is unclear how the treatment increased diversity. *L. kimchicus* may have suppressed another dominant strain or strains that were in turn suppressing other bacteria. Alternatively, the treatment may have simply upset the community dynamics of the gut and allowed very low abundance resident taxa to increase to detectable levels or novel environmental microbes to colonise the gut. Another possibility is that the addition of L. kimchicus could have simulated an infection and stimulated an immune response in the host with a knock-on effect on the microbial community.

The treatment affected the dispersion of D8 birds' communities but not D15 or adult communities. This means the treatment affected the within group compositions rather than between group compositions. Specifically, the treatment increased the within group dispersion compared to the control group. Differences in dispersion are often associated with diseased or unhealthy individuals (Zaneveld et al., 2017) which supports our hypothesis that the treatment could have simulated an infection. This disruption was not maintained in older birds possibly because they became better at exerting control over their microbiome as their homeostatic systems (e.g. immune system) matured.

4.4.3 Early developmental windows

Our results show that younger birds were more sensitive to the experimental treatment, with the effect of *L. kimchicus* treatment on alpha diversity diminishing with age. This provides experimental support for our previous observational results highlighting the presence of early developmental windows during which the microbiota are particularly sensitive to environmental variation (Somers et al., 2023/Chapter 3). This differential sensitivity may be due to older birds having more developed immune systems and more established gut communities which are more

resistant to invasion by novel microbes (Hooper et al., 2012; Segura Munoz et al., 2022). Future experiments could investigate this hypothesis by disrupting the immune system of the host in conjunction with the addition of a native strain to the diet. Similarly to our previous study (Davidson et al., 2021/Chapter 2), there was no contemporary effect of diversity on weight- suggesting any effect of the microbiota on weight takes some time to manifest.

4.4.4 Microbiota diversity and host weight

Alpha diversity was positively correlated with host weight, but this effect was neutralised by the treatment and we speculate as to why this likely occurred by comparing the effects with contrasting results from a previous study in this population. Davidson et al. (2021) /Chapter 2 found that weight at D15 correlated negatively with D8 diversity in the same population of great tits, which it was suggested could have arisen due to the host competing with their gut microbiota for nutrients under natural conditions when nutrition is often constraining (Davidson et al. 2021/Chapter 2). In contrast, in the current study weight at D15 was positively correlated with Chao1 diversity at D8 in the control birds, and there was no correlation at all in the *L. kimchicus* treatment. Given that nutrition was unlikely to be limiting due to the supplementary food provided in the current experiment, but may well have been constraining in the Davidson et al. (2021)/Chapter 2 study, we suggest three mechanisms may explain these effects. The first is that greater diversity increases weight gain but only when food is not limiting. In other words, the potential costs of high microbial diversity, as supported by observations in Davidson et al. (2021)/Chapter 2 and Krams et al. (2017), may be outweighed by the benefits provided by the microbes— aiding digestion, providing useful metabolites, and preventing the colonisation of pathogenic microbes (Buffie & Pamer, 2013; Rowland et al., 2018; Servin, 2004; Sommer et al., 2016) — when there are surplus nutrients, as suggested in the current study. Though this relationship may change as the birds' microbiome adapts to different seasonally available food (Bodawatta et al., 2021). The second is that the lack of a correlation between D15 weight and alpha diversity in the treatment birds arose because the *L. kimchicus* somehow dampens the effect

of microbiota diversity variation on weight. The *L. kimchicus* treatment appears to have counteracted the link between low diversity birds lower weight and we can only speculate as to how this positive effect on low might arise. Perhaps by providing some metabolic functionality absent in low diversity birds but this effect only benefited birds up to a certain threshold and higher levels of diversity did not confer any extra benefit. The third is that diversity varies positively with weight in the control birds, with the reverse causality, where heavier birds have more diverse microbiota because they are heavier. This could be driven by these control birds having greater and more diverse diets than lighter birds for example. The treatment birds, meanwhile, have had their microbiota disrupted to such an extent that there is no link between diversity and weight, and the heavier birds diet was not sufficient to affect the diversity of their guts in the presence of the treatment strain. In short, this suggests there is no causal effect of diversity on weight, but there is an effect of weight on diversity, at least when nutrients are not limiting.

The diet of the birds during the treatment is likely important for determining the effect of the treatment (Grond et al., 2019), as if the supplemental strain aids feed conversion/nutrition it will be effective only if fed a sufficient amount of the nutrient that the microbe engages with. For example if a microbe primarily aids in digestion of carbohydrates, it is unlikely we will see an effect while supplementary feeding with worms, which are high in protein and fat (Mariod, 2020). Studies with a greater number of nests available, could include a control with no supplementary feeding and treatments with different supplementary nutrient profiles, as well as different treatment strains, in addition to the two treatments used here. This would help untangle the effects of the bacteria on host weight from the supplementary feeding and would help determine whether *L. kimchicus* has specific effects or whether any microbiota perturbation could give rise to the patterns described above. Though there may be no straightforward relationship between a simple microbiota metric, such as diversity, and health (Z. S. Ma et al., 2019), where any relationship is likely context dependent. We note that the results here refer to only a single breeding

season, and sampling across multiple years are needed to determine whether these patterns hold across different years.

4.4.5 Predicted effect of treatment on function

Whole genome sequencing of *L. kimchicus* found that the majority of the genes identified were associated with carbohydrate metabolism (19.6%) as well as amino acid (18.7%) and protein metabolism (14.3%) (see supplementary file IsolationandCharacterisation.pdf). Differential abundance analyses of the predicted function in the gut microbiota as a whole found taxa with genes associated with a number of metabolic pathways, including carbohydrate and protein metabolism, became enriched in treatment birds. This may explain the limited benefit from a diverse microbiota in treated birds. Nestling great tits are primarily fed a diet of insects, with caterpillars being the preferred prey, and the birds in this experiment were additionally fed supplementary mealworms. This diet is rich in protein and amino acids (Mariod, 2020; Perrins, 1991; Ramsay & Houston, 2003) but low in carbohydrates (G. P. Bell, 1990)- so the treatment may have provided some useful protein metabolism functionality in low diversity birds but was unnecessary in high diversity birds which already had a microbiota with this functionality.

4.4.6 Conclusions

To our knowledge this is the first attempt to isolate a bacterial strain from wild birds and supply them to another experimental cohort, with the aim of experimentally perturbing the host microbiota to investigate effects on birds health and fitness indicators in the wild. There is a large amount of literature on the use of probiotics and antibiotics in agricultural species (Pan et al., 2014) and bacterial pathogens of wild birds have been investigated (reviewed in Benskin et al. (2009)) but studies in the wild are limited. This study therefore provides a novel method of perturbing the microbiota of a wild, free-living animal that can be used to investigate the relationships between hosts, their gut bacteria and ecology. We found support for the role of the gut microbiota in promoting host health during development and for the greater sensitivity of juveniles' microbiota to environmental sources of variation.

Our results suggest the effect of the microbiota on a fitness proxy (weight) likely depends on the environmental context, specifically nutrient availability. Identifying microbes that are important for health in wild hosts, as well as when and how they act, could inform future mitigative actions to help wildlife adapt to rapidly changing environments (Hauffe & Barelli, 2019; Song et al., 2019). This may be particularly important for improving the health of animals living in isolated or fragmented populations as well for successful reintroduction of animals to the wild, in cases where populations have lost key microbes due to impeded inter-individual transmission, by using microbiome transplants or probiotics. This would be most relevant to animals with clear, fundamental dependencies on symbiotic microbes, e.g. if you were to re-introducing a ruminant to an environment where it required a specific microbiome to digest food that was not present in its captive environment.

4.5 Appendix 3A: Supplementary results

Table S1. Effect of *L. kimchicus* treatment on Faith's Phylogenetic diversity, across age groups, N = 201. The model uses weighted effects coding (wec) for the age variable.

Independent variables	Estimate	Std. Error	df	t value	p
(Intercept)	2.919	0.117	3.559	24.993	<0.001 *
Treatment (L. kimchicus)	0.231	0.098	25.063	2.362	0.026 *
Age (D8)	0.149	0.059	113.546	2.516	0.013 *
Age (D15)	-0.051	0.062	111.386	-0.833	0.407
Age (Adult)	-0.303	0.127	163.333	-2.381	0.018 *
Treatment : Age (D8)	0.106	0.053	113.806	2.008	0.047 *
Treatment : Age (D15)	-0.047	0.064	111.750	-0.730	0.467
Treatment : Age (Adult)	-0.275	0.151	163.408	-1.817	0.071

Table S2. Effect of *L. kimchicus* treatment on Shannon diversity, across age groups, N = 201. The model uses weighted effects coding (wec) for the age variable.

Independent variables	Estimate	Std. Error	df	t value	p
(Intercept)	2.381	0.197	3.355	12.099	0.001 *
Treatment (*L. kimchicus*)	0.313	0.149	26.923	2.106	0.045 *
Age (D8)	0.043	0.088	177.515	0.490	0.625
Age (D15)	-0.046	0.092	179.316	-0.503	0.615
Age (Adult)	0.003	0.189	176.933	0.016	0.987
Treatment : Age (D8)	0.092	0.079	177.732	1.168	0.245
Treatment : Age (D15)	-0.071	0.096	179.313	-0.735	0.463
Treatment : Age (Adult)	-0.139	0.224	176.893	-0.619	0.537

Table S3. Relative abundance of Firmicutes shows non-significant negative trend. Model uses weighted effects coding so intercept reflects grand mean across all levels of age and estimates are deviation from the grand mean.

Independent variables	Estimate	Std. Error	z value	p
(Intercept)	2.416	0.544	4.442	<0.001 *
Treatment (*L. kimchicus*)	-0.972	0.527	-1.846	0.065
Age (D8)	0.128	0.063	2.032	0.042 *
Age (D15)	0.326	0.063	5.162	<0.001 *
Age (Adult)	-1.333	0.374	-3.566	<0.001 *

Table S4. Weight changes according to treatment and Shannon diversity across age groups, controlling for timing of reproduction and reproductive effort, N = 197. Linear model including all birds, modelling treatment and other predictors against bird weight. The age variable is backwards difference coded where each level of the variable is compared to the preceding level, so D15 is relative to D8 and adult is relative to D15, rather than being compared to a single reference level. Site, nest ID and bird ID are included as nested random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(Intercept)	0.211	0.111	25.029	1.905	0.068
Treatment (*L. kimchicus*)	0.019	0.158	27.483	0.122	0.904
Shannon	-0.004	0.081	184.413	-0.047	0.962
Age (D15-D8)	1.355	0.102	103.889	13.284	<0.001 *
Age (Adult-D15)	0.521	0.143	162.226	3.649	<0.001 *
First egg lay date	-0.058	0.079	25.804	-0.737	0.468
Brood size (D8)	-0.056	0.072	27.644	-0.773	0.446
Treatment (*L. kimchicus*) : Shannon	-0.030	0.094	182.376	-0.321	0.748
Treatment (*L. kimchicus*) : Age (D15)	0.133	0.141	96.660	0.943	0.348
Treatment (*L. kimchicus*) : Age (Adult)	-0.147	0.221	160.252	-0.666	0.507

Table S5. Time-lagged weight model, investigating the effect of Shannon diversity at D8, across treatments, on weight at D15, controlling for weight at D8, timing of reproduction and brood size, N = 61. Site and nest are included as nested random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(Intercept)	16.206	2.521	17.668	6.428	<0.001 *
Treatment (*L. kimchicus*)	0.603	0.782	20.438	0.771	0.449
Shannon (D8)	0.004	0.594	50.607	0.007	0.994
Weight g (D8)	1.248	0.286	53.335	4.357	<0.001 *
First egg lay date	-0.002	0.040	17.540	-0.058	0.954
Brood size (D8)	-0.034	0.361	21.309	-0.095	0.925
Treatment (*L. kimchicus*) : Shannon (D8)	0.076	0.632	52.435	0.120	0.905

Table S6. Significant KO numbers and their pathways. All associated with *L. kimchicus* treatment. 'Present in' refers to number of individuals that the feature occurs in.

Feature	Coefficient	St. derr	N	Present in	p	Q	KO name
K11528	1.880247	0.519144	201	76	0.001178	0.030864	UDP-N-acetylglucosamine pyrophosphorylase [EC:2.7.7.23]
K15864	1.005323	0.301289	201	47	0.002255	0.041775	nitrite reductase (NO-forming) / hydroxylamine reductase [EC:1.7.2.1 1.7.99.1]
K05578	1.437731	0.43887	201	88	0.003113	0.048741	NAD(P)H-quinone oxidoreductase subunit 6 [EC:7.1.1.2]
K19246	1.017551	0.297163	201	30	0.00256	0.044633	arachidonate 15-lipoxygenase [EC:1.13.11.33]
K00435	2.454875	0.690285	201	125	0.001449	0.033788	hydrogen peroxide-dependent heme synthase [EC:1.3.98.5]
K13316	0.683275	0.197862	201	24	0.000678	0.024292	NDP-hexose 5-epimerase [EC:5.1.3.-]
K13315	1.331516	0.368378	201	53	0.001311	0.032507	NDP-hexose C3-ketoreductase / dTDP-4-oxo-2-deoxy-alpha-D-pentos-2-ene 2,3-reductase [EC:1.1.1.-]
K16016	0.968089	0.299313	201	29	0.001431	0.033698	3-amino-5-hydroxybenzoate synthase [EC:4.2.1.144 2.6.1.-]
K15937	0.709758	0.22626	201	21	0.001969	0.038992	glycosyltransferase
K16015	0.639681	0.205661	201	26	0.002146	0.040854	oxidoreductase [EC:1.1.1.-]
K16018	0.66564	0.221573	201	33	0.003011	0.048159	kanosamine 6-kinase [EC:2.7.1.179]
K16021	0.66564	0.221573	201	33	0.003011	0.048159	5-deoxy-5-amino-3-dehydroquinate dehydratase
K05873	2.390952	0.67405	201	166	0.001718	0.036427	adenylate cyclase, class 2 [EC:4.6.1.1]
K11434	1.455543	0.390077	201	58	0.001208	0.031274	type I protein arginine methyltransferase [EC:2.1.1.319]
K09012	1.04324	0.307314	201	64	0.002398	0.043101	DeoR family transcriptional regulator, suf operon transcriptional repressor
K16922	2.798146	0.742065	201	158	0.000881	0.027444	putative peptide zinc metalloprotease protein
K15387	0.592182	0.192047	201	22	0.002341	0.04281	FAD-dependent monooxygenase
K05956	1.796571	0.531747	201	84	0.002522	0.044425	geranylgeranyl transferase type-2 subunit beta [EC:2.5.1.60]
K09143	1.516108	0.442863	201	50	0.002239	0.041628	uncharacterized protein
K18700	1.739025	0.464709	201	83	0.000989	0.028472	fluoroacetyl-CoA thioesterase [EC:3.1.2.29]
K07239	2.800919	0.753825	201	145	0.000998	0.028475	heavy-metal exporter, HME family

Table S7. Significant KO numbers and their Brite names. All associated with *L. kimchicus* treatment.

Feature	BriteName
K11528	Carbohydrate metabolism
K15864	Energy metabolism
K05578	Energy metabolism
K19246	Lipid metabolism
K00435	Metabolism of cofactors and vitamins
K13316	Metabolism of terpenoids and polyketides
K13315	Metabolism of terpenoids and polyketides
K16016	Metabolism of terpenoids and polyketides
K15937	Metabolism of terpenoids and polyketides
K16015	Metabolism of terpenoids and polyketides
K16018	Metabolism of terpenoids and polyketides
K16021	Metabolism of terpenoids and polyketides
K05873	Nucleotide metabolism
K11434	Protein families: genetic information processing
K09012	Protein families: genetic information processing
K16922	Protein families: metabolism
K15387	Protein families: metabolism
K05956	Protein families: metabolism
K09143	uncharacterized protein
K18700	Unclassified: metabolism
K07239	Unclassified: signaling and cellular processes

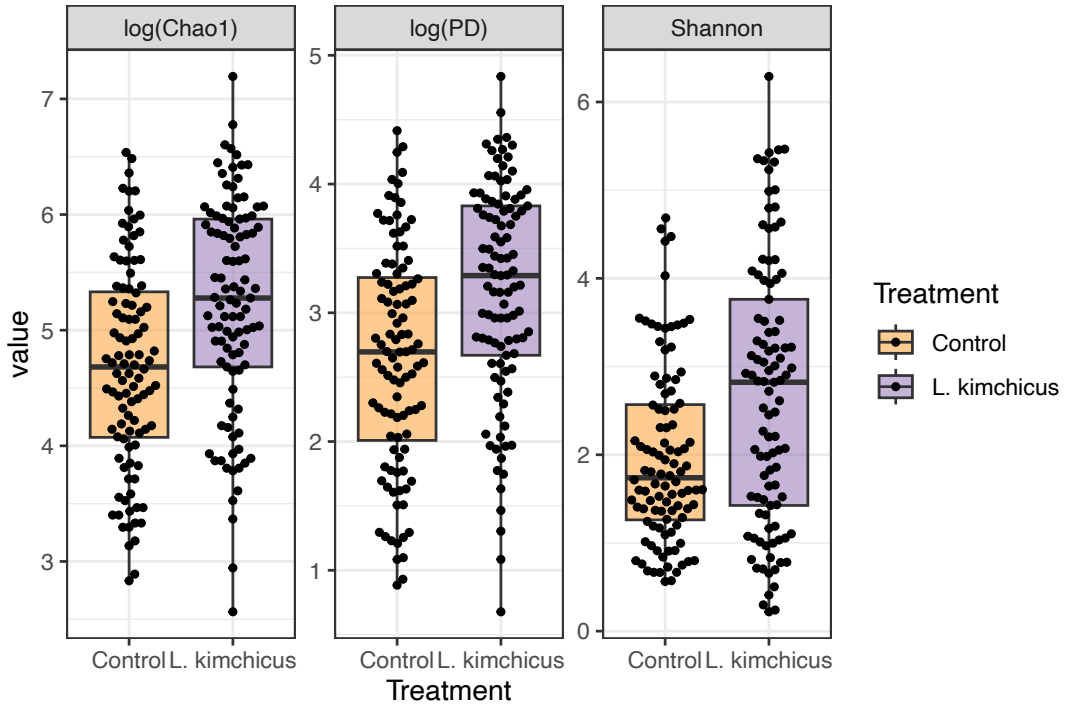

Figure S1. Alpha diversity by treatment including the log of Faith's PD for comparison.

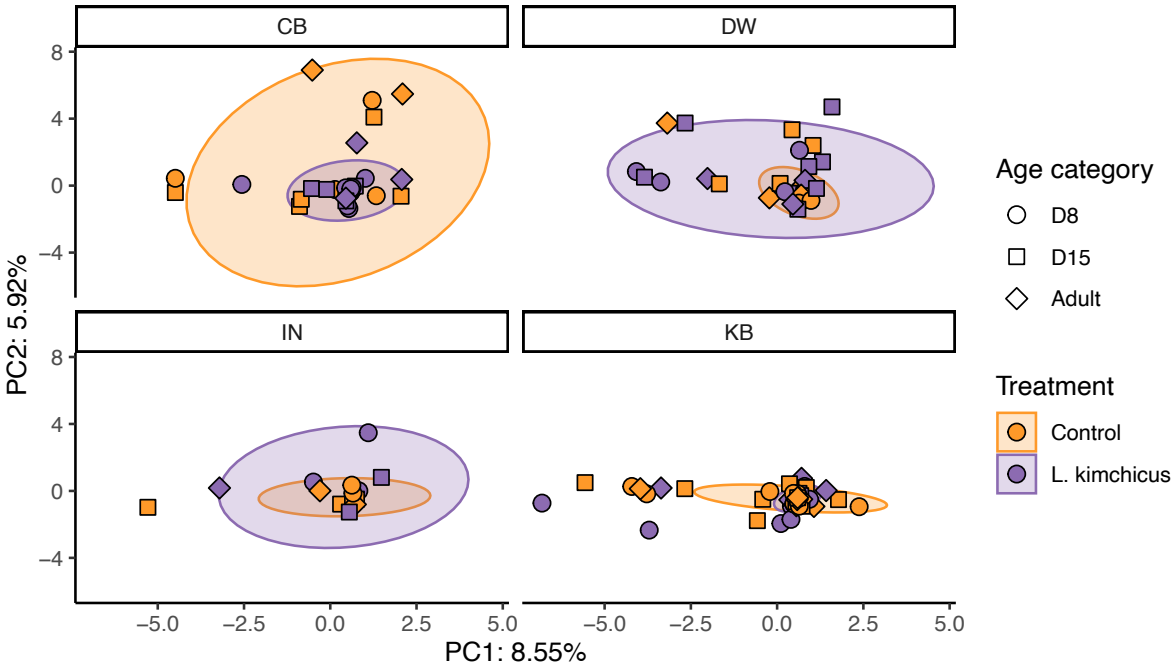

Figure S2. PCA plot of Aitchison distances between samples, split by site, N = 139 (repeats removed). Ellipses coloured according to experimental treatment group and points shape differs by age category. 'CB' = Castle Bernard, 'DW' = Dukes Wood, 'IN' = Innishannon, 'KB' = Kilbrittain.

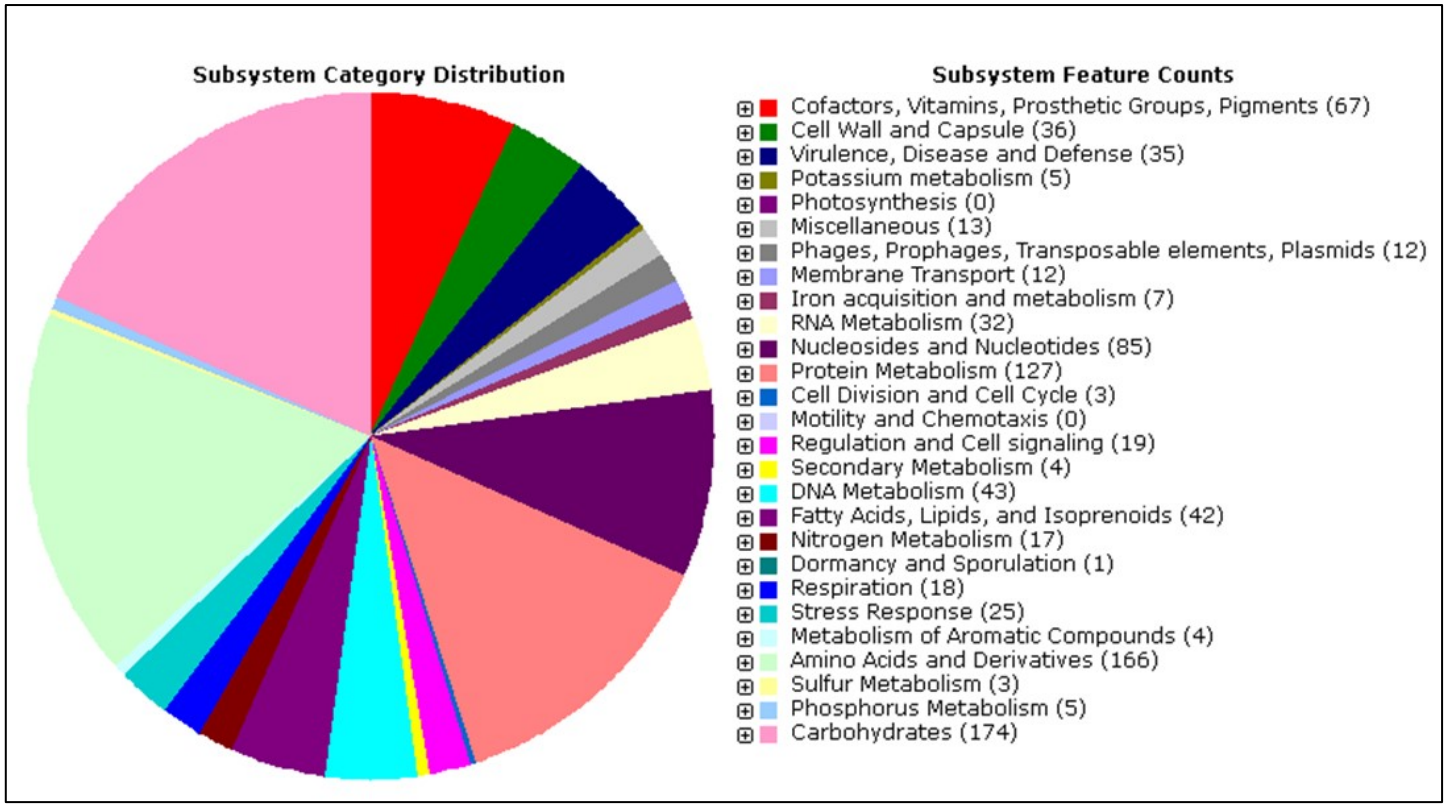

Figure S3. Pie graph of the gene subsystems for different functions in *L. kimchicus* 5.1. See supplementary file IsolationAndCharacterisation.pdf for details.

4.6 Appendix 3B: Supplementary methods

DNA Extraction (modifications)

We extracted genomic DNA from faecal samples that had been stored in 95% ethanol and frozen at -80°C for approximately 1 year. Ethanol was removed from the samples using a Genevac miVac Centrifugal Concentrator (Fisher Scientific) and DNA extracted using the QIAamp PowerFecal Pro DNA kit (Qiagen; 51804) generally following the suppliers (May 2019) protocol. We made some changes to the protocol in order to increase DNA yield given that avian faeces are generally regarded to be difficult to extract DNA from. The modifications to the extraction protocol, using Trevelline et al. (2018) as a guide, were as follows:

Step 2. Vortex bead tube for 10 minutes as normal. Heat samples in a heatblock at 65°C for 10 mins. Repeat this heating and vortexing step again. Finally, vortex for 15 minutes and heat samples in heat block overnight for 12 hours (timer).

Step 5. Repeat step 5 (CD2 solution) a second time to remove any potential inhibitors and contaminants.

Step 13. Repeat C5 wash a second time.

Step 16-17. Warm C6 in 50°C water bath prior to use. Elute with 60uL of C6 by pipetting elution solution onto membrane and incubating at room temperature for 5 minutes before centrifuging. Then pipette elutant back onto the centre of the filter. Incubate for 5 minutes and centrifuge again before storing elutant at -20°C.

Library preparation

The extracted DNA was amplified using Polymerase chain reaction (PCR) with 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 341R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) primers (Sigma-Aldrich), which include the Illumina overhang adapter sequences, targeting the V3-V4 region of the 16S chromosome.

Extracted DNA's concentration was quantified using the Qubit (Bio-Sciences, Dublin, Ireland) and normalised to 5ng/uL (double check correct units) if DNA concentration exceeded 5 ng/uL. If concentration was below 5 ng/uL then template DNA was added to the PCR reaction neat. 3 uL of template DNA was added to a PCR plate with 6 uL of forward primer (0.2 µM), 6 uL of reverse primer (0.2 µM) and 15 uL of Phusion High Fidelity PCR master mix with HF buffer (Thermofisher). Amplicon PCR conditions were as follows:

PCR Conditions (amplicon)

Initialisation	98°C - 30 s	
Denaturation	98°C - 10 s	
Annealing	55°C - 15 s	x 35 cycles
Extension	72°C - 20 s	
Final extension	72°C - 5 min	
Final hold	4°C - ∞	

The resulting amplicon was the samples were purified using AMPure XP protocol (Agencourt, Beckmann-Coulter) as found in the Illumina 16s-metagenomic library preparation guide (Illumina; 15044223-b). A second PCR reaction was run using Illumina index primers (Set A and D). Phusion High fidelity PCR mix was used for this reaction as well, instead of the KAPA HiFi HotStart Readymix. This 50 uL reaction included 25 uL of Phusion, 10 uL sterile PCR water (Sigma-Aldrich), 5 uL of template DNA and 5 uL of primer from each of the forward and reverse Illumina index primers. Set A was used for the first PCR plate, set D for the second plate and the N7xx primers from set A and the S5xx primers from set D for the third plate thereby giving each sample unique indexes and allowing pooling for a single MiSeq run.

PCR Conditions (index)

Initialisation	98°C - 30 s	
Denaturation	98°C - 10 s	
Annealing	55°C - 15 s	x 8 cycles
Extension	72°C - 20 s	
Final extension	72°C - 5 min	
Final hold	4°C - ∞	

Following the index PCR, the samples were purified using AMPure XP protocol (Agencourt, Beckmann-Coulter) following the Illumina protocol and frozen before the normalisation and pooling stage. For normalisation and pooling, samples were defrosted and centrifuged briefly before quantifying the DNA concentration with a Qubit. Samples concentration was normalised to 10 nM using Buffer AE (Qiagen: 10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0). 5 uL of each sample was pooled in a single sample tube and frozen before shipping to the Genewiz GmbH facility (Leipzig, Germany) sequencing facility. The pooled libraries were loaded onto a MiSeq flow cell and sequenced (2x300 paired end reads) with a 5% Phi-X spike-in. De-multiplexing of reads was performed on instrument.

Chapter 5: The effect of environmental enrichment and probiotic supplementation on captivity-induced stress indicators in a wild bird

Shane E. Somers, Camille A. Troisi, Gabrielle L. Davidson, Ruth M. O'Connell, Catherine Stanton, R. Paul Ross, John L. Quinn

Photo taken under NPWS licence

Abstract

The gut microbiota is linked to host fitness, allowing rapid adaptation to a changing environment and buffering the effects of environmental stressors. Prolonged exposure to a stressor can have negative effects on health, while even acute exposure can cause changes in the diversity and community composition of an individual's gut microbiota. Many research paradigms involve taking wild birds into captivity which, in effect, mimic environmental stress by exposing captured individuals to a change in their external environment, diet and freedom of movement, as well as greater exposure to humans, all of which can induce a stress response and negatively impact animal's welfare. Environmental enrichment and probiotic supplementation are predicted to have a positive effect on bird health and welfare in captivity, by encouraging naturalistic behaviours and the proliferation of beneficial bacteria, respectively. This study investigated whether the effects of captivity induced stress could be ameliorated by environmental enrichment or probiotic supplementation. Additionally, we sought to determine if the effects of captivity on host gut microbiota, activity and behaviour changed with enrichment and/or probiotic treatments. We used a commercially available probiotic species, *L. sakei*, which a previous study found to be associated with increased survival in this population. We found some support for the negative physiological effects of captivity, specifically weight loss, to be ameliorated by environmental enrichment however this was negated by the probiotic treatment. Enrichment was correlated with a reduction in overall activity. We found that the microbiota was strongly affected by the probiotic intervention on a much shorter time scale than anticipated, where birds alpha diversity showed a dramatic decrease by the midpoint of the experiment (day 6) which returned to normal by the end of the experiment (day 12), suggesting a large degree of resilience in the gut microbiota. In summary, the microbiota was affected by the treatments on different time scales, with weak support for an effect on overall welfare possibly acting through the effect of enrichment on activity levels.

5.1 Introduction

The gut microbiota provides a broad range of benefits and functions to its host. Gut bacteria help breakdown otherwise indigestible or toxic foodstuffs (Kohl et al., 2014), improve intestinal barrier function (Hooper et al., 2012) and some species, particularly Lactobacillus species, can inhibit or suppress pathogens (Messaoudi et al., 2013). The microbiota can also increase host plasticity in response to rapidly changing external environments (Alberdi et al., 2016; Kolodny & Schulenburg, 2020), for example by increasing dietary flexibility (Lindsay et al., 2020) or mediating stress reactivity (Sudo et al., 2004). However, the microbiota may impose a cost on the host by competing for nutrition and requiring regulation by the hosts immune system in order to maintain a healthy balance (Davidson et al., 2021/Chapter 2; Krams et al., 2017). Activation of the stress response, specifically glucocorticoid production, can result in a reduction of bacterial gut diversity (Noguera et al., 2018; Petrullo et al., 2022) with negative effects on growth (Noguera et al., 2018), likely to allow the hosts to devote greater energy resources to more pressing issues such as mounting an escape or an immune reaction. Host stress or disease can drive stochastic change in gut microbiota communities with negative outcomes for the host (Zaneveld et al., 2017). Both psychological and physical stress can result in the dysregulation of the microbiome, and provoke anxiety-related disorders, such as IBS, and anxiety-like behaviour (reviewed by Dinan and Cryan (2017)).

Wild animals experience stress as a response to capture and captivity (Fischer & Romero, 2019; Madden et al., 2022). Both wild caught and captive-born animals experience reduced dietary diversity, restricted movement and greater exposure to humans (a potential predator), all of which induce stress (Cockrem & Silverin, 2002; Morgan & Tromborg, 2007). Thus, captivity can result in poor health and deleterious repetitive behaviours in some species (Mason et al., 2013), resulting in an overall reduction in welfare. Captivity induced stress can have a variety of physiological and behavioural consequences including the reduction of muscle and heart density, the

increase of fat deposits and an increase in anxiety-related behaviours (Fischer et al., 2018; Lattin et al., 2017). A key regulator in this process is the gut microbiota, which interacts with the host via the microbiota-gut-brain axis in a bidirectional manner to mediate changes in the host's physiology, including hypothalamic-pituitary-adrenal (HPA) axis activation (Collins & Bercik, 2009; J. A. Foster et al., 2017; Rea et al., 2016). Greater levels of stress, lower dietary diversity and lower environmental heterogeneity may contribute to the common pattern in captive animals of less diverse gut microbiota (Clayton et al., 2016; Koziol et al., 2022; Kueneman et al., 2016) though this pattern is not consistent across all species (Alberdi et al., 2021; McKenzie et al., 2017). Wild caught birds exposed to stressors in a captive environment experienced significant changes in their (cloacal) microbiota communities compared to controls, including the loss of potentially beneficial bacteria (including Lactobacillus sp.) and an increase in endotoxin producing bacteria- which can cause damaging inflammation (Madden et al., 2022).

Behavioural responses to stress include faster cognition, decreased feeding behaviour and suppression of breeding behaviour (Sapolsky et al., 2000) as well as repetitive actions without obvious function (Mason, 1991). Environmental enrichment offers a method of ameliorating some of the negative effects of captivity induced stress by encouraging the performance of naturalistic behaviours and increasing engagement with the environment (Bryan Jones & Waddington, 1992). Environmental enrichment can be broadly defined as any technique that improves the biological functioning of a captive animal by modifying their environment (Newberry, 1995). Enrichment can lead to an increase in some behaviours, such as play, (Kells et al., 2001; Monte & Pape, 1997) as well as reduce stereotypical behaviour (Damasceno et al., 2017). Studies of the effects of environmental enrichment on the microbiota are rare, though there are a handful of studies on agricultural animals that show enrichment increases the abundance of beneficial bacteria (Colombino et al., 2021; Wen et al., 2021). Though environmental enrichment with natural elements (soil, moss and stones) did not prevent the loss of wild-like microbiota in two small mammals brought into captivity, it did alter the

trajectory of change of the community composition suggesting enrichment can affect the microbiota in captivity (Koziol et al., 2022) so enrichment may also indirectly improve animals welfare via effects on their microbiota. There is experimental evidence that the benefits of environmental enrichment on the host are at least partially driven by the microbiome (Lupori et al., 2022). Therefore it is important to investigate the bidirectional nature of the relationship between the gut microbiota and stress in captive animals as a means to improve their welfare (Williams et al., 2020).

Probiotics are *"live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"* (Hill et al., 2014; WHO & FAO, 2001). Thus they offer a potential method for ameliorating the loss of key bacteria from the guts of animals during the course of captivity (Kueneman et al., 2016). Lactobacillus species, in particular, often display probiotic properties (J. A. Foster et al., 2017), such as modulating inflammation (Cristofori et al., 2021) and are associated with weight gain (Drissi et al., 2017; Million et al., 2012) a key indicator of health. Probiotics may also alleviate stress and anxiety by modulating gut microbiota related neuroactive compounds (T. Ma et al., 2021) in addition to replacing, or preventing the loss of, beneficial bacteria due to stress. Probiotics are being increasingly studied in agriculture as an alternative to antibiotics, as the negative effects of wide spread antibiotic use in agricultural animals has come to light (Al-Shawi et al., 2020; WHO, 2015). Studies of probiotics in wild animals are rare though the potential for using probiotics to aid species adapt to climate change and ecosystem damage, particularly in corals, has been highlighted (Peixoto et al., 2022; Song et al., 2019). In a previous study on a neighbouring population we found a number of Lactobacillus species were associated with survival in nestling birds, suggesting they may have probiotic properties (Davidson et al., 2021/Chapter 2).

Here we used a two factorial experiment to examine the effect of a potentially probiotic bacteria *Latilactobacillus sakei* (previously known as *Lactobacillus sakei*)

and environmental enrichment on how wild caught birds respond to captivity. Specifically we examined how the probiotic and enrichment, independently and in combination, influenced: (i) *Latilactobacillus* abundance itself, primarily to check if the probiotic was taken up by animals effectively; (ii) the microbiota as a whole, as we expected that the stress induced by captivity would result in a decrease in gut microbiota diversity, similar to Noguera et al. (2018) though the impact of stress on the microbiota has had mixed results in wild populations; (iii) behavioural indicators of stress, including reduced feeding behaviour (Sapolsky et al., 2000), reduced exploratory behaviour (Garcia-Marquez & Armario, 1987) and increased hiding (Carlstead et al., 1993) assessed by measuring activity budgets; (iv) three measures of cognitive performance: a problem solving task where they had to extract visible, but difficult to access food, foraging ability, as measured by how quickly they found hidden food and a food handling task, which measures individuals time efficiency; and (iv) body weight, a key indicator of health in captive animals and predictor of survival in the wild. *L. sakei* is naturally occurring in great tits and was previously linked with survival in a wild population (Davidson et al., 2021/Chapter 2).

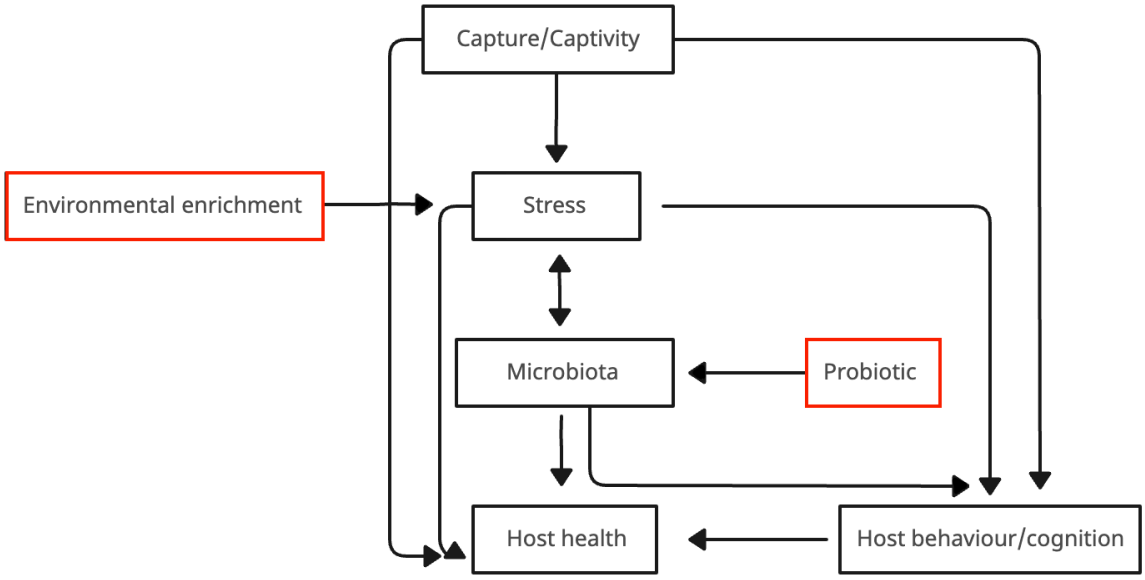

Figure 1. Hypothesis diagram illustrating known and potential links of interest between host traits and the environment (not exhaustive), with interventions highlighted in red. This experiment investigated the effects of environmental enrichment and a probiotic on the microbiota and host health/behaviour, and whether they moderated the effects of stress.

5.2 Methods

5.2.1 Trapping and housing birds

Birds were trapped using mist-nets in three locations in and around Cork city (Distillery Fields, Environmental Research Institute and Bon Secours care home) over two consecutive winter periods, between December 2020-February 2021 and November-December 2021. The age (first winter = 'immature'; second winter and older = 'mature') and sex of the birds were determined using plumage indicators. Weight, tarsus length and wing length were recorded and the birds given unique tags (British Trust for Ornithology) before being transported to the aviary facility in UCC in clean cloth bird bags. The aviary consisted of two individual housing rooms, each containing 10 boxes that had independent ventilation and access to a larger 'exploration room'. Birds were randomly assigned to "cages", with dimensions of 57 x 56 x 46 cm (L x H x W), which were boxes made from solid board that had port-hole windows at the sides allowing the birds to see conspecifics from their perches, and that were lit on a 12 hour light-dark cycle.

5.2.2 Treatments

The experiment lasted for 12 days in most cases (N=64), with the day of capture referred to here as day-0. Birds were typically released on day-12 but the experimental period was reduced or lengthened by 1-2 days in some cases for logistical reasons (N=25). Each experimental group consisted of birds caught within 1-3 days of each other, housed individually in cages in the same room and released on the same day. Birds were randomly allocated to a 2 x 2 factorial experimental design for probiotic (*L. sakei*, control) and enrichment (standard, high). Individuals were allocated to only one of the four treatments. Sample sizes were 22 birds for three of the four treatment combinations and 23 for the standard enrichment/no-probiotic treatment. The probiotic treatment consisted of the bacteria *Latilactobacillus sakei* (starterculteres.eu link) in powder form. A previous study of ours found a naturally occurring strain of *L. sakei* to be positively associated with survival in great tits from part of our wider study area (Davidson et al., 2021/Chapter 2). We mixed 0.21g of the probiotic powder, which contained approximately $1x10^9$ colony forming units (CFU), with 0.2 ml of distilled water and added this to their morning food, daily. Control (no probiotic) birds received 0.2ml of distilled water only. The CFU of the powder was determined by counting the colonies present following serial dilution and anaerobic incubation at 37° C for 48 hours on LBS.

Birds were allocated to one of two enrichment treatments: standard or high. Bird song was played through a speaker all day for all birds. All birds were given water ad-libitum and fed a food mixture of sunflower seeds, peanuts, several mealworms and a wax-moth larvae, twice daily. The standard enrichment treatment birds received their sunflower seeds and peanuts crushed up to make feeding relatively easy. High enrichment birds received the same food but it was served whole which they had to manipulate more before ingestion, thereby increasing the stimulation birds might gain from feeding (Coulton et al., 1997; Fangmeier et al., 2020). Standard enrichment cages featured a cuttlefish-bone tied with string as well as two perches (Appendix 4B: figure S5). High enrichment cages additionally featured: a small plastic container (kinder egg) with hidden food accessible via small holes in either end and replenished

daily; a 10 x10 cm cork mat; a short length of hose pipe; sterilised sheep's wool and bird feathers; and a small refuge of dimensions 17 x 6 x 6 cm (L x H x W) made of plastic drain pipe secured to the roof of the cage, mimicking a natural cavity where birds naturally hid for safety (Appendix 4B: figure S6, S7). Finally, standard enrichment birds were released into a larger room (4.5 x 2.6 x 3.1 m; L x H x W) with multiple perches for them to explore for a period of 40 minutes on one occasion during their time in captivity. High enrichment birds were released into the larger room three times during captivity.

5.2.3 Behavioural assays

Our first assay was a problem-solving task which ran over night, twice, on the two days prior to release (day-10 and day-11 usually). Problem solving is a compound trait linked to several cognitive processes in the great tit, including selective attention and associative learning (Cooke et al., 2021; Morand-Ferron et al., 2015), and performance in this task is predicted by the gut microbiota in wild-caught great tits (G. L. Davidson, Wiley, et al., 2020). This task consisted of obtaining a food reward (one freshly killed waxmoth larva and mealworm) from a transparent tubular device (4cm inner diameter, 5cm outer diameter, 17 cm length; after Cooke et al. (2021); Appendix 4B: figure S8) via one of three options that accommodated different motor skills and biases: (a) pulling a string from the top of the tube to access worms tied to the end of the string, (b) pushing aside a small door in the lower half side of the tube and retrieving worms on a platform inside the tube, or (c) pulling a pin protruding from below the small side door which would cause the platform and worm rewards to drop out of the end of the device. Birds were presented with this task, one hour before lights out, and the device was retrieved the following morning two hours after lights-on, and the solving method recorded. The device, without rewards, was placed in birds cages and left overnight, on the day before the task, to habituate the birds to the object. An extra food reward (1 waxmoth larvae) was affixed to the outside of the device to encourage the birds to approach it, when the task was running. Problem solving was measured as a binary term (solved/not solved). Birds were considered to be solvers if they used any of the methods to access a reward. Birds were not food

deprived for this task because the reward was a preferred prey item (waxmoth larvae) and because the birds had plenty of time to solve the task. The experimental protocol was slightly adapted to encourage birds participation in the task, so individuals from groups prior to the protocol adaptation were excluded from the analyses (55/89 individuals included in analysis). The adaptation was implemented after the initial experimental groups did not engage with the task, and involved providing an extra food reward on the device to encourage the birds to approach it.

Our second assay measured time spent active (moving around) and feeding in the cage, and we assumed that certain behaviours, specifically higher levels of non-food directed activity, would indicate higher levels of stress (Breuner et al., 1998; Fischer et al., 2018) and higher levels of food directed activity would indicate lower stress (Sapolsky et al., 2000). Video recordings of the birds behaviour were taken between 12am-1pm, one day before release, using cameras built into the cage roofs. A 10 minute segment, starting at 12:20, for each of these videos was then scored with the ethogram described below, using the video scoring software JWatcher (Blumstein et al., n.d.), to determine the activity of each individual. Only individuals from the first winter period were included in this assay (N=53). Behavioural states were: *feeding,* any activity associated with food, beginning when the bird is at the food bowl and ending when they leave the food bowl or finish interacting with any food taken from the bowl; *non-feeding activity,* any movement that is not associated with feeding; *out of view,* described when the bird was out of view of the camera, the uncertain nature of which led to it being excluded from analyses.

Our third assay measured foraging success, an important determinant of fitness (D. W. Stephens & Krebs, 1986), and was conducted two days before release (typically day 10). Birds were trained during the preceding three days to search a 24 well tray for mealworms hidden under sand. The training tasks required birds to find worms that were progressively more hidden. Individuals had to pass each training task before progressing to the next task. If birds failed to complete a training task twice

they were presented with the proceeding phase. The training is described in more detail in the supplementary methods. When an individual passed each of the four training tasks their training was completed (75/89 birds passed the training phase). Birds were not food deprived before the training tasks. On the day of the task proper, each bird was presented with a tray containing 10 freshly dead mealworms concealed under sand. The number of worms found and consumed in 10 minutes was recorded. Birds were food deprived for 60 minutes prior to the task to standardise motivation.

Our final assay on the final day tested food handling efficiency, an important determinant of foraging success (D. W. Stephens & Krebs, 1986). Initially birds were food deprived for 60 minutes and then were given a bowl of whole sunflower seeds and were video recorded for 5 minutes. The number of seeds opened and consumed was recorded, and videos were later scored manually. Only individuals from the first winter period were included in this assay (N=53). The mean handling time it took for each seed to be opened and consumed was recorded. Following the task, food was restored with extra wax moth larvae to provide extra nutrition prior to the birds' release.

5.2.4 Faecal sampling and DNA extraction

Faecal samples were taken on the initial day of capture (day-0), at the midpoint (day-6 or 7) and on the final day (day-12, 13 or 14). This involved placing a sterilised (washed with 10% bleach solution and rinsed in water) Plexiglas sheet on the floor of each cage until birds had produced a sample. Trays were then removed and the samples transferred to a sample tube using a sterile inoculation loop and placed in a -80°C freezer within 30 minutes. Subsequently, DNA extraction was carried out on defrosted samples with the PowerFecal Pro kit (Qiagen, cat no. 51804). Some alterations were made to the kit protocol (May, 2019 version), including alternately vortexing (with 24 tube vortex adapter) and heating samples (65°C) for 10 minutes twice, then vortexing for a further 10 minutes and finally heating overnight following

Trevelline et al.'s (2018) Supplemental Molecular Protocols in order to lyse cells more efficiently. Additionally the CD2 step, to remove contaminants and inhibitors, and the C5 wash step were repeated. Eluted DNA was pipetted back onto the filter membrane and centrifuged a second time to increase DNA yield. See supplementary methods for a more detailed methodology. For simplicity, all day-0 samples are referred to as initial samples, day 6 and 7 samples are referred to as midpoint samples and all day 12, 13 and 14 samples are referred to as final day samples.

5.2.5 Library preparation and Sequencing

The extracted DNA was amplified using Polymerase chain reaction with 341F and 341R v3-v4 16S primers (Sigma-Aldrich). The resulting amplicon was purified with AMPure XP protocol (Agencourt, Beckmann-Coulter) as found in the Illumina 16s-metagenomic library preparation guide (Illumina; 15044223-b). A second PCR reaction was run using Illumina index primers (Set A and D). Following the index PCR, the samples were purified using AMPure XP protocol (Agencourt, Beckmann-Coulter) following the Illumina protocol and frozen before the normalisation and pooling stage. For normalisation samples concentration was normalised to 10 nM using Buffer AE (Qiagen: 10 mM Tris-Cl, 0.5 mM EDTA). Samples were frozen and shipped on ice to the Genewiz GmbH facility (Leipzig, Germany) for sequencing. See the extended supplementary protocol for the full details. Samples were sequenced on the MiSeq sequencing platform (Azenta Life Sciences/Genewiz, Germany), using a 2 x 300 cycle kit, following standard Illumina sequencing protocols. Negative controls using PCR grade water (Sigma-Aldrich, sterile filtered water for molecular biology) were included at the extraction, evaporation and amplicon PCR steps, and brought through to sequencing in order to detect environmental contaminants.

5.2.6 Bioinformatics

Sequencing data were processed using the DADA2 pipeline in R, following the dada2 tutorial v1.16 (Callahan, McMurdie, et al., 2016). Trimming and truncation parameters were chosen based on FastQC quality plots (Andrews, 2010) and the

sequences were filtered to remove sequences with expected errors greater than 2. Errors were estimated and the core sample inference algorithm applied to the filtered and trimmed sequence data for each sequencing run separately. Forward and reverse reads were merged to obtain full denoised sequences. A sequence table of Amplicon Sequence Variants (ASV) was constructed containing counts of the unique sequences by sample for each run. The two sequence tables were merged prior to removing chimeric sequences using the default 'consensus' method. A taxonomy table was generated using the naïve Bayesian classifier method and the Silva (v138.1) reference database.

The ASV and taxonomy tables were combined into a single Phyloseq object (McMurdie & Holmes, 2013) in R before further filtering of samples. Sequences identified as chloroplasts, Archaea, Eukarya or mitochondria were removed. Duplicate samples, blank controls and any samples thought to be contaminated during lab work were removed. Sample completeness curves were plotted using the rarecurve function from the vegan package (Oksanen et al., 2019). Sample completeness occurred at approximately 7500 reads for almost all samples so samples with less reads than this threshold were removed before further analysis. Potentially contaminant taxa were identified using the prevalence method from the decontam package (Davis et al., 2018), with a threshold of 0.5. Chao1, Shannon diversity and inverse Simpson's index were estimated with phyloseq's estimate_richness function.

5.2.7 Statistical analysis

Detect L. sakei in samples

No ASV was identified as *Latilactobacillus sakei* (or *Lactobacillus sakei*) so to determine whether *L. sakei* was present in the birds the taxonomy table was checked for any taxa in the genus *Latilactobacillus*. The abundance of the most common of these were plotted to check if the taxa displayed different patterns of abundance across the probiotic treatment groups over time as we would expect the probiotic

strain to show an extreme increase in abundance between day 0 and later sampling points. Chi-square tests were used to determine if any of the potential probiotic taxas' presence were more correlated with probiotic samples than control samples. It is not unexpected that the strain was not identified to species level because of the limited resolution of short read 16S sequencing, had we sequenced the full 16S gene it is likely that we would have easily identified the strain as longer sequences are better at resolving taxonomic differences (Johnson et al., 2019).

Treatments effect on microbiota

We investigated how the microbiota changed over the course of the captive period and whether the treatments affected these changes. Linear mixed effect models (LMMs), from the lme4 package (D. Bates et al., 2015), were used to examine the change in alpha diversity over time. The effect of each treatment (probiotic vs control; high vs standard enrichment) was interacted with sampling day (D0, D6, D12) to determine whether the effect of the treatments changed during the experiment, while accounting for the variables sex, age as fixed effects and aviary cage, sequencing plate, release date and capture site as random terms to control for their potential effects on alpha diversity. Three LMMs were run, one for each measure of alpha diversity: Chao1, Shannon diversity and inverse Simpson's diversity. Model residuals were assessed using the DHARMa package (Hartig, 2019) and response variables were transformed as necessary: Chao1 and inverse Simpsons were log-transformed.

The change in beta diversity, or community composition, over time was investigated using pairwise.adonis2 (Martinez Arbizu, 2020), which allowed us to make pairwise comparisons between each sampling day, accounting for probiotic treatment and enrichment. We adjusted for multiple comparisons using the "holm" method from the p.adjust function though this did not qualitatively affect the results. Pairwise.adonis2 is based on the adonis2 (permutational ANOVA) function from the vegan package (Oksanen et al., 2019) which partitions distance matrices among

sources of variation. Before calculating distances, taxa with prevalence below 5% were removed, to reduce possible contaminants and sequencing artefacts. Then the Aitchison distance between samples was calculated, as this accounts for the compositional nature of sequencing data (Gloor et al., 2016; Gloor & Reid, 2016). The adonis2 test assumes predictor variables have homogeneity of variance which was confirmed using the betadisper function from the vegan package.

We then tested whether the treatments had an effect on the birds final microbiota diversity, while controlling for the initial microbiota diversity (D0), using LMMs. Models used one of three diversity measures for each bird on day 12 as the response (Chao1, Shannon and inverse Simpson's index) and included probiotic application, enrichment treatment and their interaction as fixed effects. Models included the variables sex, age and diversity on day 0 as fixed effects and aviary cage, sequencing plate, release date and capture site as random terms to control for their potential effects on alpha diversity. The variance of gut diversity at day 12 according to probiotic treatment group, enrichment treatment group and the combined treatment groups (i.e. probiotic-standard enrichment, probiotic-high enrichment etc.) were each tested using Bartlett's test for homogeneity of variances. Alpha diversity's variance was investigated as the probiotic might not have a consistent effect on diversity and could cause a change in dispersion rather than a change of mean.

The beta diversity of the bird's gut communities on the final day of the experiment was investigated. The effect of each treatment was tested using adonis2 from the vegan package (Oksanen et al., 2019), by calculating the Aitchison distance between final day samples. The variance in distance according to probiotic and enrichment treatment, and their interaction, as well as age and sex, was calculated using the 'margin' option in adonis2. The homogeneity of dispersion of the groups was confirmed using the betadisper function from the vegan package. Dispersion tests indicate that all five variables had homogenous dispersions (Betadisper: p=0.754,

0.92, 0.86 , 0.66 and 0.59 and respectively). PCA plots were made with the first two principal components.

Treatment's effect on behaviour/cognition

Results from the behavioural assay conducted using JWatcher were analysed using linear mixed models. Two separate models were used to determine how either probiotic or enrichment treatments or their interaction affected each behavioural state (i.e. feeding and non-feeding activity), while controlling for age and sex as fixed effects, and cage, sequencing plate, experimental group and capture site random effects, as above. For the problem solving analysis, the response was a binary 'solve-not solve', where birds that successfully accessed worms via any method over two trials were deemed to have solved the task (model did not converge). For the seed handling task the response variable was the log of the mean time it took the birds to successfully open seeds (time spent handling seeds, whether or not seeds were successfully opened, divided by the number of seeds opened). For the foraging ability analysis a generalised LMM Poisson model was used to determine whether success, measured as the number of worms consumed in ten minutes, was related to either treatment. The number of training trials was included as a fixed effect, to account for the number of times an individual was exposed to a similar task.

Weight change and treatment's effect on weight

LMMs were used to investigate the interactive effect of the probiotic and enrichment treatments on final weight, while controlling for weight at day-0, sex, age and gut microbiota alpha diversity as fixed effects and cage, sequencing plate, group and capture site as random effects. Three models were run to investigate the effect of treatment on final weight, each using a different diversity term (Chao1 richness, Shannon diversity and inverse Simpson's diversity), as we have previously found that diversity affects weight in nestling birds (Davidson et al., 2021/Chapter 2; Chapter 4). Model residuals were assessed using simulated residuals from the DHARMa package (Hartig, 2019).

5.3 Results

5.3.1 *L. sakei* detection in samples

L. sakei was not taxonomically identified to species level; however, ASV1 was identified as *Latilactobacillus* sp., the genus that contains *L. sakei*. This ASV was present in low amounts in most birds, regardless of treatment, on day-0 but increased dramatically in abundance by day-6 in probiotic treated birds (figure S1), so we are confident that this is the probiotic species. The presence of *L. sakei* was not associated with probiotic treatment at day 0 (Chi-square test: X=2.3, p=0.129) but it was dependent on treatment at day 12 (X=7.15, p=0.008). Four (two probiotic, two control) birds were opportunistically resampled when retrapped post-release while mist netting for new birds. *L. sakei* was detected in one of the probiotic treated re-trapped birds (two days post release), showing a reduction from 3633/30385 reads (approximately 12%) to 101/23173 reads (<1%). *L. sakei* was not present in the second probiotic treated retrap (eight months post release). *L. sakei* was present in small amounts in the two re-trapped control birds, 9-11 months after release; *L. sakei* was also present in both of these birds at the beginning of the experiment, so they appear to contain that taxa naturally. These transient effects indicate that the treatment strain probably does not become established and is not self-sustaining. This may be due to its lack of shared evolutionary history with the hosts and hence lack of adaptation to the birds' gut environment. However, the fact that the strain does not become established does not mean that the strain might not impact the host (during the captive period) on some level as, for example, the strain may disrupt the microbiome community with knock on effects on the host.

5.3.2 Treatments effect on microbiota

Alpha diversity remained stable in the control treatment for Shannon and inverse Simpson's diversity (table S1) while there was some support for Chao1 diversity to be lower in birds on the final day (-0.336, p = 0.095). All three diversity measures were lower midway through the experiment in the probiotic treatment (figure 2; table 1, S1), where probiotic treated birds exhibited a major reduction in diversity

that appears to recover by the end of the captive period. This effect was much greater on Shannon and Simpsons diversities, which account for evenness rather than absolute richness described by Chao1. There was some support for inverse Simpson's diversity to remain affected by the treatment by the final day (-0.476, p = 0.061). The alpha diversity of birds at the start of the experiment does not appear to have influenced their response to the probiotic treatment (figure S2). Beta diversity changed during captivity irrespective of treatment (table 2, figure 3). Beta diversity also showed a reduction in community variability for probiotic treated birds (i.e. individuals communities became more similar) at the midpoint and partially recovered by the end of the experiment (table 2, figure 3). Notably, the 'recovered' communities of the probiotic treated birds were different to the control birds on the final day of the experiment (table S4, figure 3), but more similar to controls than they were at the midpoint (figure 3). There was no effect of enrichment on the alpha (table 1, S1) or beta diversity (table 2) over the course of the experiment.

Table 1. Change in Chao1 alpha diversity at different sampling time points (start, mid and final) in captivity across probiotic and enrichment treatments, N=89. Response variable was log transformed. Age (immature, mature) and sex (female, male) were included as control variables. Model includes cage number, PCR plate, release date and catching site as random terms. Shannon and inverse Simpson results in supplementary (table 1a, 1b).

Independent variables	Estimate	Std. Error	df	t value	p
log(Chao1)					
(Intercept)	4.378	0.196	12.382	22.366	<0.001 *
Probiotic (*L. sakei*)	0.258	0.160	235.421	1.610	0.109
Sample day (mid)	-0.051	0.198	235.775	-0.258	0.796
Sample day (final)	-0.336	0.201	235.966	-1.677	0.095 .
Enrichment (High)	0.011	0.163	237.049	0.065	0.949
Sex (M)	0.014	0.096	235.448	0.148	0.883
Age (Mature)	-0.113	0.110	236.721	-1.033	0.303
Probiotic : Sample (mid)	-0.587	0.232	235.354	-2.533	0.012 *
Probiotic : Sample (final)	-0.108	0.227	235.132	-0.474	0.636
Sample (mid) : Enrichment	-0.023	0.232	235.331	-0.098	0.922
Sample(final): Enrichment	0.163	0.230	236.233	0.710	0.478

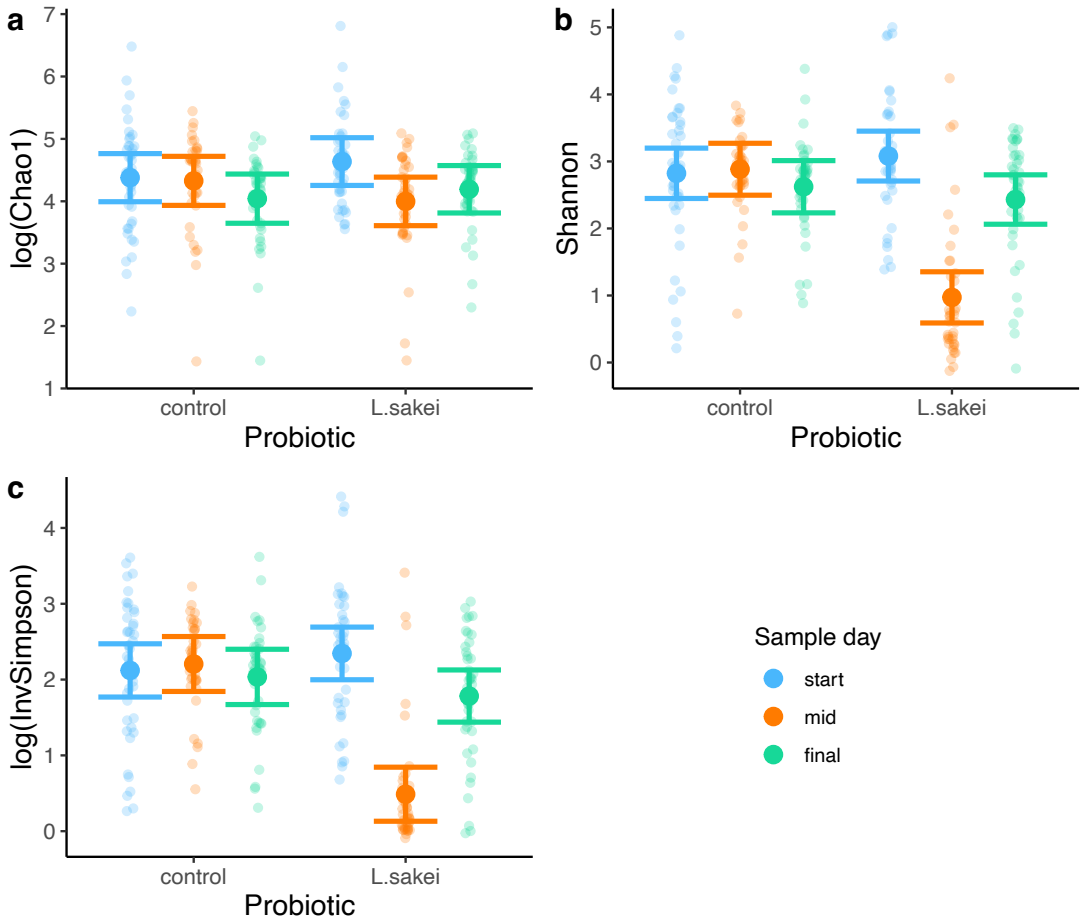

Figure 2. Effect of probiotic treatment on diversity at each sampling point, N=89. Estimates from LMM's with confidence intervals and partial residuals for (a) Chao, (b) Shannon and (c) inverse Simpsons diversity. Response variables were transformed as appropriate to improve model fit.

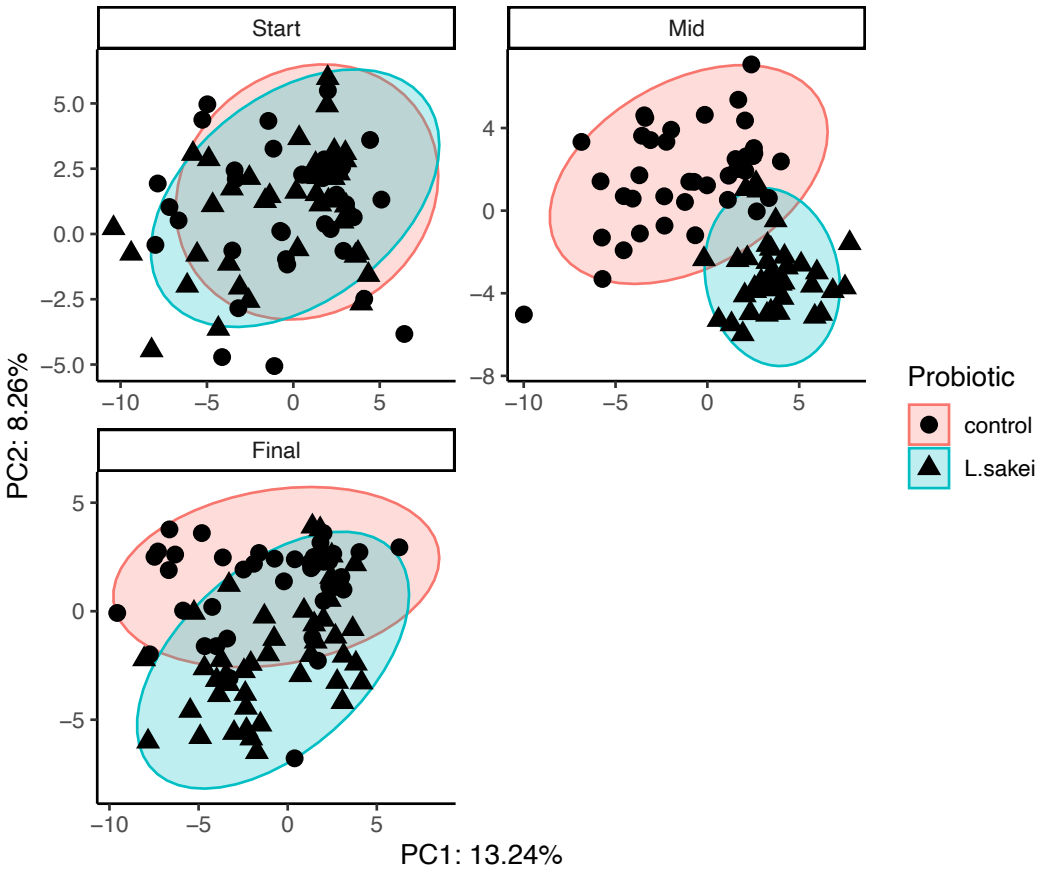

Figure 3. Beta diversity (community composition) changes according to probiotic treatment. Community composition more dissimilar with probiotic addition at mid-point than final day. PCA plot separated by sampling day, with ellipses for probiotic treatment groups, N= 86, 79 and 84, respectively. Sample sizes vary slightly due to sample loss at PCR stage or low read sample removal.

Table 2. Pairwise comparison of beta diversity between sampling days and across treatments, N=89. Pairwise PERMANOVA model partitioning variance in Aitchison distances, with multiple test correction.

Independent variables	DF	Sum Of Sqs	R^2	F	*p*
(a) Start vs Mid					
Sample day	1	393.423	0.023	4.017	0.003 *
Probiotic	1	514.899	0.031	5.257	0.003 *
Enrichment	1	66.764	0.004	0.682	0.938
(b) Mid vs Final					
Sample day	1	355.431	0.020	3.459	0.003 *
Probiotic	1	991.341	0.056	9.649	0.003 *
Enrichment	1	91.907	0.005	0.895	0.641
(c) Start vs Final					
Sample day	1	266.208	0.015	2.553	0.003 *
Probiotic	1	221.676	0.012	2.126	0.006 *
Enrichment	1	74.891	0.004	0.718	0.93

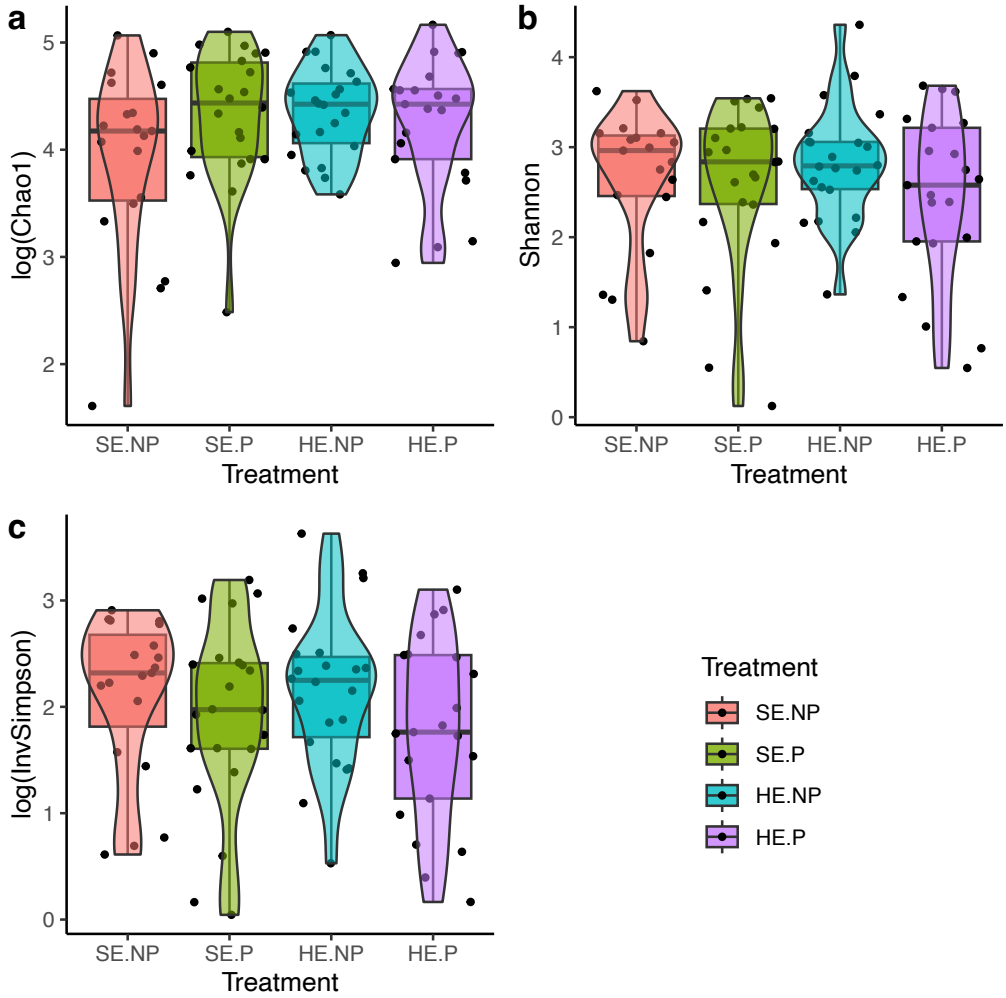

Figure 4. Boxplots and violin plots of final day alpha diversity means and dispersions across treatments, N=84. (a) log Chao; (b) Shannon and (c) log inverse Simpsons diversity. 'SE' = Standard enrichment; 'HE' = High enrichment; 'NP' = No probiotic (i.e. control); 'P' = Probiotic.

Final day Shannon and inverse Simpson's diversity were not affected by the probiotic or enrichment treatments (figure 4; table 3, S2), though there was some weak support for enriched birds to have higher Chao1 diversity than non-enriched birds, when controlling for Chao1 diversity at the start (0.383, p = 0.05; figure S3, table 3). Additionally, the dispersion of Chao1 diversity was affected by the enrichment treatment according to Bartlett's test (Bartlett's K-squared = 5.4, p = 0.019; table S3a) with enriched birds showing less variable Chao1 diversity values (figure 4a). The dispersion of Shannon and inverse Simpson's diversity was not affected by treatment (figure 4b, 4c; table S3b, S3c). Beta diversity at day 12 did differ according to probiotic

application (PERMANOVA: p=0.001) but not enrichment (PERMANOVA: p=0.708; figure 3, S4), age, sex or the interaction between treatments (table S4).

Table 3. Change in final day Chao1 alpha diversity across probiotic and enrichment treatments, controlling for day-0 diversity, age (immature, mature) and sex (female, male), N=81. Response variable is log transformed. Model includes cage number, PCR plate, release date and catching site as random terms. Shannon diversity and inverse Simpson's diversity results in supplementary (table S2a, S2b).

Independent variables	Estimate	Std. Error	df	t value	p
log(Chao1) ~ Treatment					
(Intercept)	3.944	0.224	9.541	17.617	<0.001 *
Probiotic (*L. sakei*)	0.317	0.197	71.180	1.606	0.113
Enrichment (High)	0.383	0.192	68.671	1.995	0.05 .
Sex (M)	0.057	0.137	66.696	0.417	0.678
Age (Mature)	0.040	0.160	70.161	0.252	0.802
Chao1 (Day-0)	0.000	0.000	71.233	-0.285	0.776
Probiotic : Enrichment	-0.347	0.270	70.839	-1.285	0.203

5.3.3 Treatment effect on behaviour/cognition

Birds spent an average of 256/600 seconds (SD = 143) in non-food directed activity and 71/600 seconds feeding (SD = 56), irrespective of treatment. Enriched birds were less active than non-enriched birds (-0.671, p = 0.031; table S5), regardless of the probiotic treatment (-0.193, p = 0.517). There was no effect of either enrichment (-0.546, p = 0.161) or probiotic (-0.099, p = 0.792) treatment on the time spent feeding. 21/55 individuals successfully solved the problem solving task, though there was no support for either probiotic or enrichment treatment affecting this ability (table S6). The mean number of food items found during the foraging task was 4.4 (SD = 2). There was no support for either probiotic or enrichment treatment affecting foraging ability (table S8). Birds spent on average 92 seconds (SD = 63) handling seeds per

success. There was some limited support for interaction between probiotic and enrichment treatments to affect seed handling (table S7, figure S5).

5.3.4 Weight change and treatment's effect on weight

Most individuals lost weight during the captive period (mean change in weight = -0.59 g; t test, t = -7.12, p <0.001). There was support for final weight being higher in the high enrichment compared to the standard enrichment treatment, when controlling for Chao1 (figure 5; 4) as well as Shannon (table S9a) and inverse Simpson's diversity (table S9b). Also, there was weak-moderate support for an interaction between probiotic treatment and enrichment (table 4), suggesting the slightly greater weight in enriched birds was not present when they were fed the probiotic. This weak interaction effect was also present when controlling for Shannon diversity or inverse Simpson's diversity (table S9a, S9b). Additional control variables included age, sex and starting weight (table 4).

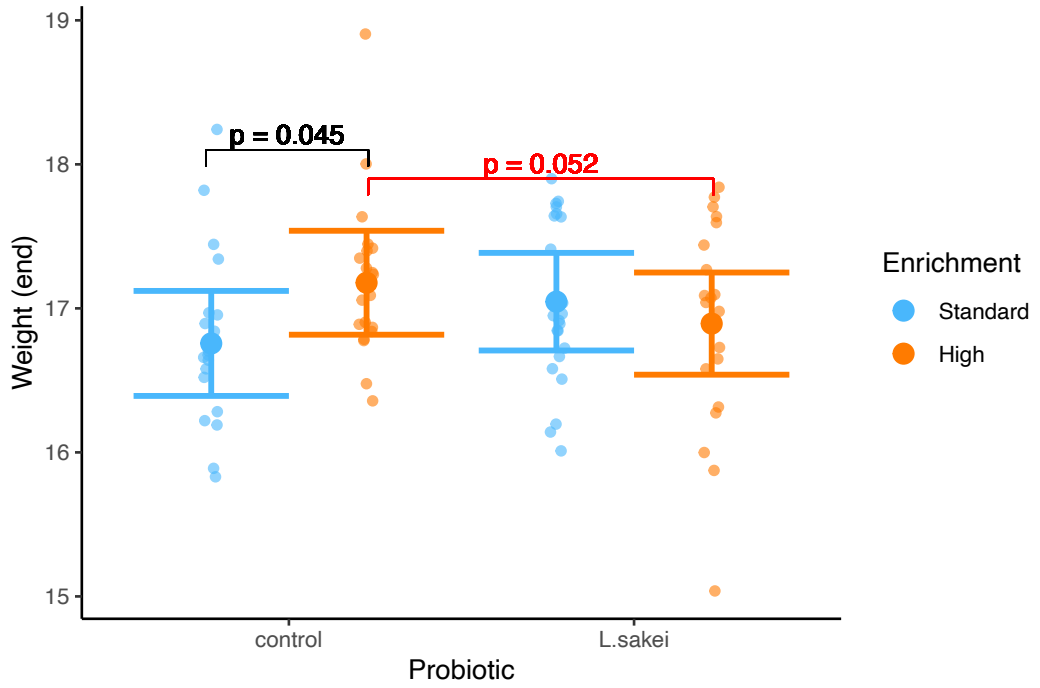

Figure 5. The effect of probiotic supplementation and environmental enrichment on final weight, accounting for Chao1 diversity, N=83.

Table 4. Change in bird's final weight according to probiotic and enrichment treatments and their interaction, controlling for diversity at day 12, weight at day 0, age (immature, mature) and sex (female, male), N=83. Numeric predictor variables are centred and scaled. Model includes cage number, PCR plate, release date and catching site as random terms. Shannon and inverse Simpsons model results in supplementary (table S9a, S9b).

Independent variables	Estimate	Std. Error	df	t value	p
Weight (final) ~ Treatment + Chao1					
(Intercept)	16.758	0.182	59.311	91.838	<0.001 *
Probiotic (*L. sakei*)	0.290	0.206	70.176	1.405	0.164
Enrichment (High)	0.421	0.206	68.992	2.047	0.045 *
Weight (D0)	0.560	0.079	67.865	7.116	<0.001 *
Sex (M)	0.605	0.158	71.463	3.836	<0.001 *
Age (Mature)	-0.085	0.167	73.165	-0.507	0.614
Chao1 (D12)	-0.096	0.077	73.387	-1.237	0.22
Probiotic : Enrichment	-0.574	0.290	71.712	-1.977	0.052 .

5.4 Discussion

5.4.1 Overall summary

We found the application of a bacteria previously identified as a candidate probiotic (Davidson et al., 2021/Chapter 2; Gomes et al., 2012), *Latilactobacillus sakei,* strongly affected host microbiota diversity and community composition. Community composition, and some measures of alpha diversity, changed during captivity in control birds. Probiotic treated hosts experienced a dramatic decrease in microbial community evenness, and to a lesser degree richness, as well as changes in community composition by the mid-point of the experiment. Though alpha diversity rapidly returned to pre-treatment levels and was similar to that of the control (no probiotic, irrespective of enrichment) birds by the final day, their gut microbiota composition (beta diversity) was significantly altered by the final day in captivity

compared to the controls. The gut microbiota was not strongly affected by the enrichment treatment, though there was weak support for enriched birds to have greater Chao1 diversity on the final day. We found that behaviour was affected by the enrichment treatment but not the probiotic, as enriched birds spent less time in non-food directed activity than non-enriched birds. While enrichment positively affected weight, suggesting an improvement in welfare, if anything the probiotic treatment had a negative effect on weight, possibly by reducing feeding efficiency.

5.4.2 Captivity

Gut diversity did not substantially change in captivity for control birds, though there was some evidence for a decrease in Chao1 diversity by the final day. Studies of captive species suggest that gut diversity is lower in captive individuals compared to wild conspecifics (Clayton et al., 2016; Koziol et al., 2022; Kueneman et al., 2016), possibly due to reductions in dietary diversity or increased stress (Madden et al., 2022; Martínez-Mota et al., 2020), but this is not consistent across all species (McKenzie et al., 2017). It is possible that captivity did have such a small effect on diversity because any microbial taxa lost during captivity were simply replaced by another from the environment or diet. Community composition did change over time. The change in community composition could be due to new microbes introduced through the captive diet, novel environmental microbes associated with the cage or aviary, internal host regulation of the microbiota related to stress or a combination of all these factors. These results are consistent with a similar study in house sparrows, which found the birds cloacal microbiota changed in community composition but not diversity during captivity (Madden et al., 2022). We expected that the stress induced by captivity would result in a decrease in gut microbiota diversity, similar to Noguera et al. (2018), though the impact of stress on the microbiota has had mixed results in wild populations (Noguera et al., 2018; Petrullo et al., 2022; Stothart et al., 2019). A similar study in house sparrows (*Passer domesticus*) found the birds cloacal microbiotas changed in captivity but kept the birds for a much longer period of at least 8 weeks (Kelly et al., 2022), so it is likely a longer experimental period would have shown a stronger effect on diversity. Another

study on a house sparrows found that captivity-induced stress caused an increase in a stress hormone (CORT) over a one week period which then decreased (Fischer et al., 2018), so we might expect any stress related microbiota effects to become apparent over this time frame however it may be the case that a hormone induced change takes longer to manifest in the microbiota.

5.4.3 Probiotic

The treatment strain was previously identified as a naturally occurring and potentially probiotic species in this population, and was associated with survival in nestling birds (Davidson et al., 2021/Chapter 2). Though the precise nature of its benefit in these birds was unclear, *L. sakei* used in this experiment is used as a preservative in some foods because of its antimicrobial activity, particularly against known pathogens (Bredholt et al., 1999; Gomes et al., 2012). Metagenomic whole genome sequencing would be necessary to decipher whether the genetic functionality of the commercial *L. sakei* matches that of the naturally occurring strain we detected in these birds.

The alpha diversity and community composition of the hosts were affected by the application of a dietary probiotic, *Latilactobacillus sakei*. Importantly, the change in the microbiota was most apparent at the midpoint compared to the endpoint of the experiment. Control birds communities remain quite similar between the start and mid sampling points, while the probiotic treated group shows a drastic reduction in community variability and becomes quite dissimilar to the control birds by the midpoint. The communities show greater convergence by the final sampling point compared to the midpoint but show significantly less overlap than they did at the start of the experiment. The birds' microbiota therefore seem to partially recover from the probiotic disruption by the end of the experiment, despite the birds consuming it continuously (i.e. daily) throughout the experiment. This demonstrates the resilience of the gut microbiota to perturbation. Figure S1 indicates that the abundance of ASV1 remains high in the final day probiotic treated birds'

communities, suggesting that the recovery of the gut communities is related to tolerance of the treatment strain rather than resistance. The timing of the microbiota recovery was similar in an experiment that tested the effect of an artificial versus a natural diet on the microbiota in captivity (Martínez-Mota et al., 2020). The gut microbiota hence appear to demonstrate a natural resilience to the introduction of novel dietary microbes and may help the host adjust this in a natural setting. Our results also highlight the importance of multiple sampling points, as sampling at only the beginning and end of the experiment would have suggested that the probiotic had a much smaller effect on the microbiota. Additionally, calculating multiple diversity metrics which describe different aspects of alpha diversity is important. Here we found that richness (Chao1: number of bacterial taxa) was not strongly affected by the probiotic application but the richness weighted by the relative abundance of taxa was much more dramatically affected (Shannon and inverse Simpsons diversity), indicating that few taxa were completely eliminated from the gut communities but some taxa changed drastically in relative abundance (evenness). Considering the beta diversity of probiotic treated individuals differed from control individuals on the final day some bacterial taxa must have been replaced by new taxa leading to different communities by the end of the experiment. This may have occurred naturally through exposure to novel bacteria in the aviary environment but may have been accelerated by the probiotic perturbation which could have created opportunities for new bacteria to establish by reducing the relative abundance of native bacteria through its antimicrobial activities.

The probiotic treatment did not affect behaviour in terms of time spent active or feeding but may have effected foraging efficiency with regard to handling time as there was some support for the probiotic treatment to increase seed handling time for high enrichment birds. The precise mechanism for this greater handling time is unclear. Probiotics have been found to affect anxiety-related behaviour in rodent lab models though these effects are often strain dependent (Cryan & O'Mahony, 2011). The strain used here has not previously been linked to any behaviour so we did not expect it to directly influence behaviour though we hypothesised that it could

perturb the gut microbiota and hence have an indirect effect on behaviour. Previous studies on both wild-caught and captive birds in the laboratory have found links between the gut microbiota communities and performance on cognitive tasks (G. L. Davidson, Wiley, et al., 2020; Slevin et al., 2020). The lack of a stronger effect on behaviour in probiotic treated birds may be due to testing birds too late in the treatment, by which time their perturbed microbiota had recovered to a similar state to the controls.

5.4.4 Enrichment

There was some support for the enrichment treatment to increase richness (Chao1 diversity) but not evenness of microbes (Shannon and inverse Simpson's diversity). This may indicate there was little difference in stress levels between birds in the opposing treatments, possibly because the source of stress was not well addressed by the enrichment provided or the enrichment treatment used was not sufficient to lower stress. A follow up experiment could expose one group to an additional stressor, e.g. human handling, to investigate whether increasing the magnitude of the stress was sufficient to cause a knock-on effect on the microbiota. A common failure of enrichment is providing stimuli that have no functional relevance to the animals (Newberry, 1995). Some of the items we provided did encourage engagement and natural seeming behaviours, notably the corkboard mat which birds often flipped over in a similar manner to which they might flip over a leaf while foraging. Future experiments could provide 'foraging trays' for the birds to interact with- these might feature several sterilised mats or leaves concealing food items like mealworms, which would encourage naturalistic foraging. Other items, such as the sheep's wool and bird feathers, were less successful- probably because the birds were not interested in nesting. These items might be more interesting to the birds if the experiment took place during the breeding season when nesting behaviour occurs.

In an ideal world the experiment would have included a control group with very low stress however this would be extremely difficult to achieve in reality. Capture and captivity are inherently stressful, particularly due to birds increased proximity to humans (Mason et al., 2013). One potential improvement to the experiment would be to reduce birds exposure to humans throughout the captive period by using automated feeders and a less invasive cleaning routine. Cleaning and feeding times were clearly stressful for all the birds, though high enrichment individuals had the refuge pipe to hide in and the refuge was often used by these birds at this time.

There was some good support for the enrichment treatment to affect birds activity levels where enriched birds spent less time in non-food directed activity. This suggests that enriched birds had lower stress- considering other studies found activity increased with the levels of the stress hormone CORT (Breuner et al., 1998; Fischer et al., 2018). Therefore the enrichment treatment appears to have succeeded in lowering birds stress as measured at a behavioural level. Measuring stress related hormonal changes was beyond the scope of this study, as we deemed regular handling and bleeding of birds to be an extreme stressor that would likely drown out any amelioration of the stress response that could be induced by the environmental enrichment. Other potential measures of stress hormones, that would be applicable to the time scale of our experiment, i.e. faecal CORT, are insufficiently validated (Karen Spencer, personal communication). The other behavioural measures were not affected by enrichment treatment.

5.4.5 Behaviour and health

Birds weight decreased over the captive period, across all treatments. There was support for a positive effect of enrichment on the bird's final weight. This may be related to birds activity levels, which were lower in general in enriched conditions, according to the behavioural states analysis, suggesting enriched birds may have experienced lower decrease in weight because of lower energy expenditure and suggests enrichment may have indirectly reduced weight loss in captivity by

preventing hyper-activity. There was a weak support for the interaction between probiotic and enrichment on weight, indicating probiotic treated birds obtained less benefit of enrichment on weight. This negative trend suggests the probiotic may have disrupted birds metabolism or energy harvesting but also may be related to the reduced foraging efficiency (i.e. increased food handling time) of probiotic treated birds in the enriched treatment. Weight loss is a symptom of chronic exposure to stress (Fischer & Romero, 2019) and considering birds were provided with a high volume of both seeds and insects in this experiment, the weight loss detected here is unlikely to be because of insufficient nutrition and may be due to stress induced changes in body composition such as a decrease in dense muscle tissue and an increase in less dense fat tissue (Lattin et al., 2017). Probiotics may only show beneficial effects on health in disease states (Moloney et al., 2014), so there may have been insufficient scope for the bacteria to improve host health given the birds were generally healthy.

5.4.6 Conclusions

Understanding the interaction between stress and the microbiota is key to elucidating the adaptive role of the microbiota in animal ecology, as well as understanding the role of the microbiota in animal welfare. The stress response itself is a complex series of biochemical feedback loops, and understanding how this interacts with both the microbiota and an organism's ecology is a difficult task. Captivity may be a good proxy for human-induced rapid environmental change, where the wild caught captive animal may experience challenges analogous to deforestation, habitat change and greater human proximity (Mason et al., 2013). Captive animals are removed from their familiar surroundings and often placed in very simplified physical and social environments, as well as being constantly exposed to humans- which has many parallels with human driven processes like urbanisation. This study attempted to address the negative impacts of captivity induced stress using a well-studied technique of environmental enrichment in addition to a more novel probiotic intervention. We found evidence for the negative physiological effects of captivity (weight loss) to be ameliorated by environmental enrichment but

not probiotic treatment. We also found that the probiotic treatment sometimes negated the benefit of enrichment on the birds welfare, suggesting some effects of captivity may be mediated by the gut microbiota in wild caught captive great tits, though generalising these results must be done cautiously given the variability in gut community responses to captivity (Diaz & Reese, 2021). Additionally, the microbiota was strongly affected by the probiotic intervention on a much shorter time scale than anticipated, suggesting a large degree of resilience in the gut microbiota. This highlights the importance of conducting investigations at the correct temporal scale and helps create a framework for future studies which may attempt to address these issues.

5.5 Appendix 4A: Supplementary results

Table S1. Change in alpha diversity at different sampling time points (start, mid and end) in captivity across probiotic and enrichment treatments, N=89. Response variables were transformed as appropriate to improve model fit. Age (immature, mature) and sex (female, male) were included as control variables. Model includes cage number, PCR plate, release date and catching site as random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(a) Shannon					
(Intercept)	2.823	0.191	47.311	14.804	<0.001 *
Probiotic (L. sakei)	0.257	0.193	236.138	1.329	0.185
Sample day (mid)	0.060	0.239	237.170	0.253	0.8
Sample day (end)	-0.201	0.242	237.415	-0.830	0.407
Enrichment (High)	-0.154	0.196	237.332	-0.785	0.433
Sex (M)	0.186	0.116	236.278	1.606	0.11
Age (Mature)	-0.006	0.132	238.000	-0.049	0.961
Probiotic : Sample (mid)	-2.168	0.280	236.125	-7.755	<0.001 *
Probiotic : Sample (end)	-0.448	0.274	235.326	-1.632	0.104
Sample (mid) : Enrichment	0.122	0.279	236.041	0.436	0.663
Sample (end) : Enrichment	0.160	0.276	237.914	0.580	0.562
(b) Inv. Simpson					
(Intercept)	2.121	0.178	53.155	11.897	<0.001 *
Probiotic (L. sakei)	0.224	0.182	235.092	1.232	0.219
Sample day (mid)	0.085	0.220	158.236	0.385	0.7
Sample day (end)	-0.086	0.223	160.772	-0.385	0.701
Enrichment (High)	-0.181	0.184	235.351	-0.980	0.328
Sex (M)	0.181	0.113	78.315	1.598	0.114
Age (Mature)	0.037	0.129	78.469	0.284	0.777

Independent variables	Estimate	Std. Error	df	t value	p
Probiotic : Sample (mid)	-1.942	0.258	157.665	-7.532	<0.001 *
Probiotic : Sample (end)	-0.476	0.253	153.668	-1.885	0.061 .
Sample (mid) : Enrichment	0.133	0.258	157.542	0.517	0.606
Sample (end) : Enrichment	0.128	0.255	156.634	0.504	0.615

Table S2. Change in final day alpha diversity across probiotic and enrichment treatments, controlling for day-0 diversity, age (immature, mature) and sex (female, male), N=81. Response variables are transformed as appropriate. Model includes cage number, PCR plate, release date and catching site as random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(a) Shannon					
(Intercept)	2.479	0.399	26.874	6.212	<0.001 *
Probiotic (L. sakei)	-0.168	0.258	69.827	-0.652	0.516
Enrichment (High)	0.165	0.257	67.102	0.643	0.522
Sex (M)	0.103	0.180	70.975	0.575	0.567
Age (Mature)	0.042	0.207	70.599	0.205	0.838
Shannon (D0)	0.054	0.092	68.377	0.583	0.562
Probiotic : Enrichment	-0.111	0.359	71.669	-0.310	0.757
(b) log(Inv. Simpson)					
(Intercept)	2.070	0.262	9.236	7.889	<0.001 *
Probiotic (L. sakei)	-0.305	0.259	71.136	-1.179	0.242
Enrichment (High)	0.008	0.252	68.223	0.034	0.973
Sex (M)	0.129	0.179	71.434	0.720	0.474
Age (Mature)	0.013	0.212	71.167	0.060	0.952
Inv. Simpson (D0)	0.002	0.006	73.000	0.431	0.668
Probiotic : Enrichment	0.007	0.354	72.664	0.020	0.984

Table S3. Bartlett's test for equal variance on alpha diversity measures for probiotic treatment, enrichment treatment and their combination (each treatment combination tested separately). Response variables transformed as appropriate.

	Bartlett's K-squared	df	p
(a) log(Chao1)			
Probiotic (*L. sakei*)	0.514	1	0.474
Enrichment (High)	5.426	1	0.019 *
Combined treatments	10.05	3	0.018 *
(b) Shannon^2			
Probiotic (*L. sakei*)	0.261	1	0.6
Enrichment (High)	0.429	1	0.512
Combined treatments	0.569	3	0.903
(c) log(Inv. Simpson)			
Probiotic (*L. sakei*)	1.188	1	0.276
Enrichment (High)	<0.001	1	0.985
Combined treatments	1.117	3	0.773

Table S4. Changes in final day beta diversity (community composition) across probiotic and enrichment treatments, and their interaction, N = 84. Controlling for age and sex. PERMANOVA model performed on Aitchison distances.

Independent variables	Df	Sum Of Sqs	R2	F	p
Probiotic	1	339.547	0.036	3.059	0.001 *
Enrichment	1	96.298	0.010	0.867	0.666
Sex	1	105.313	0.011	0.949	0.535
Age	1	83.104	0.009	0.749	0.882
Probiotic : Enrichment	1	113.326	0.012	1.021	0.387

Table S5. Changes in (a) non-food directed movement and (b) food directed behaviour as measured by the behavioural assay, according to probiotic and enrichment treatments, and their interaction, N = 53. Response variables were log transformed to improve model fit. Age (immature, mature) and sex (female, male) were included as control variables. Model includes cage number, PCR plate, release date and catching site as random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(a) log(Activity)					
(Intercept)	12.423	0.315	2.941	39.427	<0.001 *
Probiotic (*L. sakei*)	-0.193	0.294	36.848	-0.655	0.517
Enrichment (High)	-0.671	0.301	44.170	-2.231	0.031 *
Sex (M)	0.230	0.208	37.228	1.104	0.277
Age (Mature)	-0.127	0.233	39.216	-0.545	0.589
Probiotic : Enrichment	0.545	0.424	43.375	1.285	0.206
(b) log(Feeding)					
(Intercept)	11.205	0.321	29.880	34.913	<0.001 *
Probiotic (*L. sakei*)	-0.099	0.375	43.258	-0.265	0.792
Enrichment (High)	-0.546	0.381	39.058	-1.430	0.161
Sex (M)	0.200	0.270	41.725	0.740	0.463
Age (Mature)	-0.932	0.295	35.765	-3.160	0.003 *
Probiotic : Enrichment	0.517	0.536	43.254	0.964	0.34

Table S6. LMM results for problem solving task. Problem solving ability is not affected by either treatment. Birds given problem solving task twice over two nights. Model did not converge.

Independent variables	Estimate	Std. Error	z value	p
(Intercept)	-1.572	0.888	-1.770	0.077 .
Probiotic (*L. sakei*)	1.244	0.878	1.417	0.157
Enrichment (High)	0.265	0.909	0.291	0.771
Sex (M)	0.305	0.650	0.470	0.638
Age (Mature)	0.476	0.719	0.662	0.508
Probiotic : Enrichment	-0.700	1.258	-0.556	0.578

Table S7. Effect on mean seed handling time of probiotic and enrichment treatments, and their interaction. Response is the sum of all time spent handling seeds divided by the number of successfully opened seeds. Birds given five minutes in total to participate in task after a one hour food deprivation. Response variables were transformed as appropriate to improve model fit. Age (immature, mature) and sex (female, male) were included as control variables. Model includes cage number, PCR plate, release date and catching site as random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(Intercept)	4.645	0.257	4.480	18.105	<0.001 *
Probiotic (*L. sakei*)	-0.186	0.240	35.339	-0.773	0.445
Enrichment (High)	-0.307	0.231	35.687	-1.330	0.192
Sex (M)	-0.146	0.176	35.621	-0.828	0.413
Age (Mature)	-0.132	0.197	35.004	-0.670	0.507
Probiotic : Enrichment	0.550	0.331	34.611	1.662	0.106

Table S8. Effect on foraging ability (sand foraging task) of probiotic and enrichment treatments, and their interaction. Response is the number of buried mealworms found and consumed, out of ten possible worms, in ten minutes after a one hour food deprivation period. Age (immature, mature) and sex (female, male), as well as the number of training tasks taken to progress to the main task were included as control variables. Model includes cage number, PCR plate, release date and catching site as random terms.

Independent variables	Estimate	Std. Error	z value	p
(Intercept)	2.024	0.269	7.531	<0.001 *
Probiotic (*L. sakei*)	0.036	0.166	0.218	0.828
Enrichment (High)	-0.157	0.176	-0.891	0.373
Number of training tasks	-0.085	0.027	-3.101	0.002 *
Sex (M)	0.137	0.117	1.163	0.245
Age (Mature)	0.210	0.130	1.611	0.107
Probiotic : Enrichment	0.183	0.234	0.785	0.433

Table S9. Change in bird's final weight according to probiotic and enrichment treatments and their interaction, controlling for diversity at day 12, weight at day 0, age (immature, mature) and sex (female, male), N=83. Numeric predictor variables are centred and scaled. Model includes cage number, PCR plate, release date and catching site as random terms.

Independent variables	Estimate	Std. Error	df	t value	p
(a) Weight (final) ~ Treatment + Shannon					
(Intercept)	16.804	0.179	61.550	93.954	<0.001 *
Probiotic (*L. sakei*)	0.260	0.207	72.315	1.257	0.213
Enrichment (High)	0.387	0.207	70.658	1.867	0.066 .
Weight (D0)	0.595	0.081	68.464	7.327	<0.001 *
Sex (M)	0.550	0.162	72.169	3.397	0.001 *
Age (Mature)	-0.082	0.168	73.726	-0.488	0.627
Shannon (D12)	0.041	0.079	74.290	0.517	0.607
Probiotic : Enrichment	-0.537	0.293	72.692	-1.831	0.071 .
(b) Weight (final) ~ Treatment + Inv. Simpson					
(Intercept)	16.801	0.178	60.564	94.139	<0.001 *
Probiotic (*L. sakei*)	0.262	0.207	72.262	1.270	0.208
Enrichment (High)	0.386	0.207	70.753	1.869	0.066 .
Weight (D0)	0.591	0.079	69.626	7.491	<0.001 *
Sex (M)	0.553	0.160	72.073	3.461	0.001 *
Age (Mature)	-0.076	0.168	73.756	-0.451	0.654
Inv. Simpson (D12)	0.045	0.076	72.309	0.592	0.555
Probiotic : Enrichment	-0.537	0.293	72.663	-1.834	0.071 .

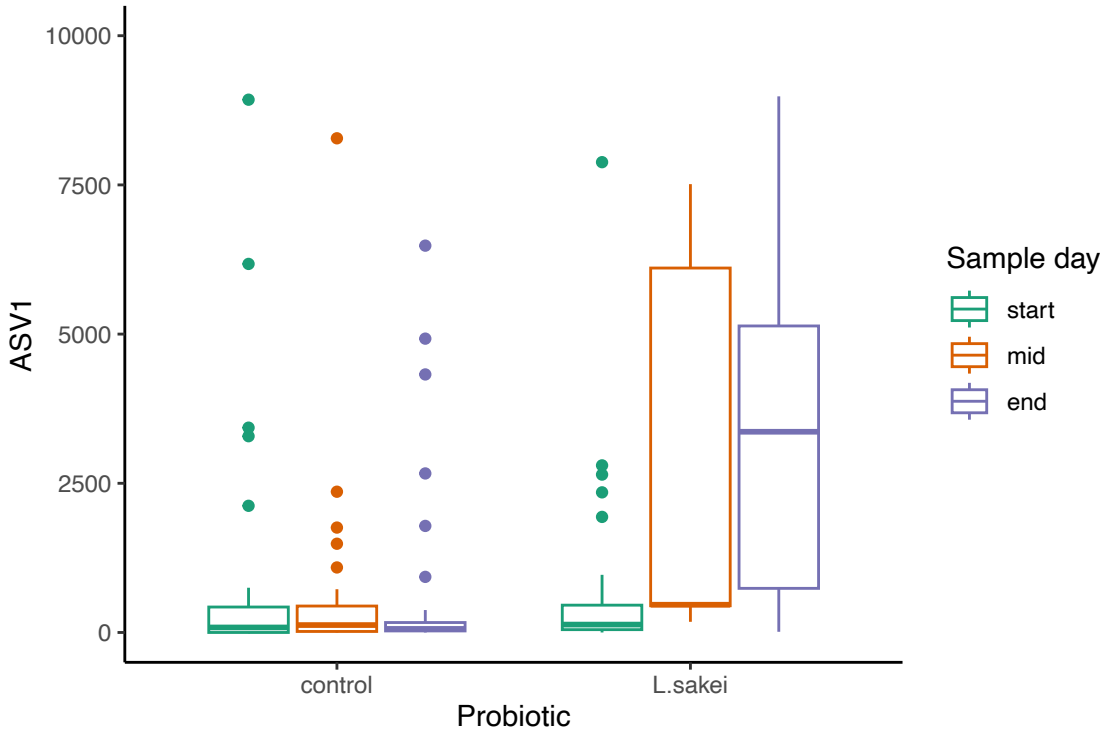

Figure S1. Abundance of ASV1 (*Latilactobacillus sakei*) over time separated by probiotic treatment.

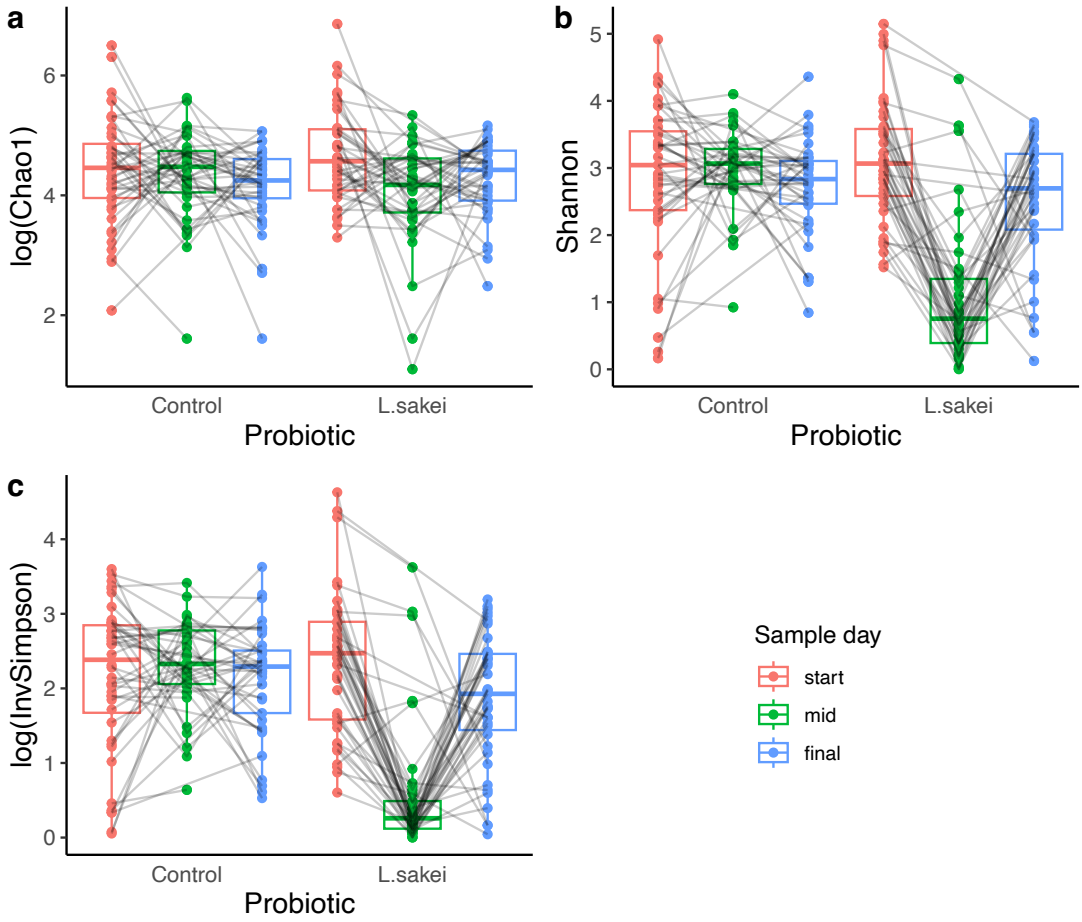

Figure S2. Effect of probiotic treatment on diversity at each sampling point with individual bird's linked by lines, N=89.

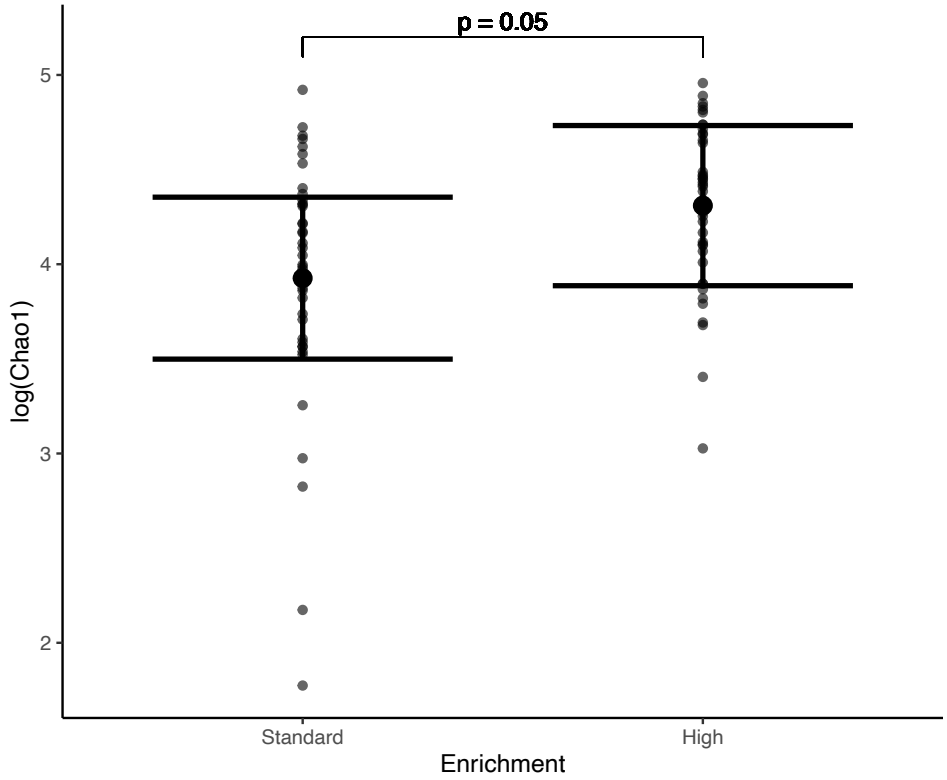

Figure S3. Effect of enrichment treatment on final day log(Chao1) diversity. Partial residual plot with confidence intervals based on model reported in table 3.

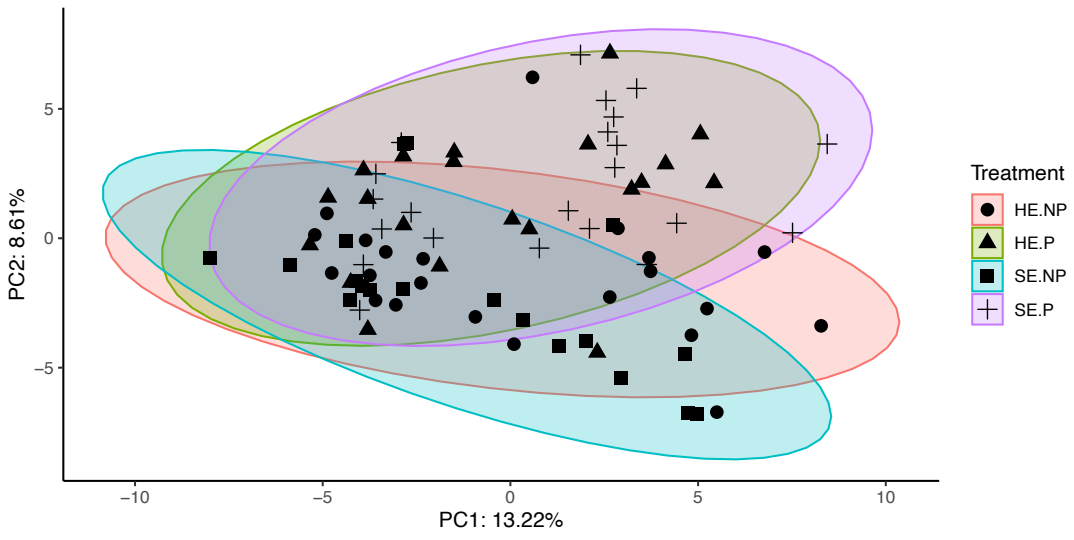

Figure S4. PCA plot of Aitchison distances between final day samples with ellipses for each treatment. Beta diversity (community composition) of day 12 birds differ with probiotic but not enrichment treatment. Sample shapes differ according to treatment. 'HE' = High enrichment; 'SE' = Standard enrichment; 'P' = Probiotic; 'NP' = No probiotic i.e. control.

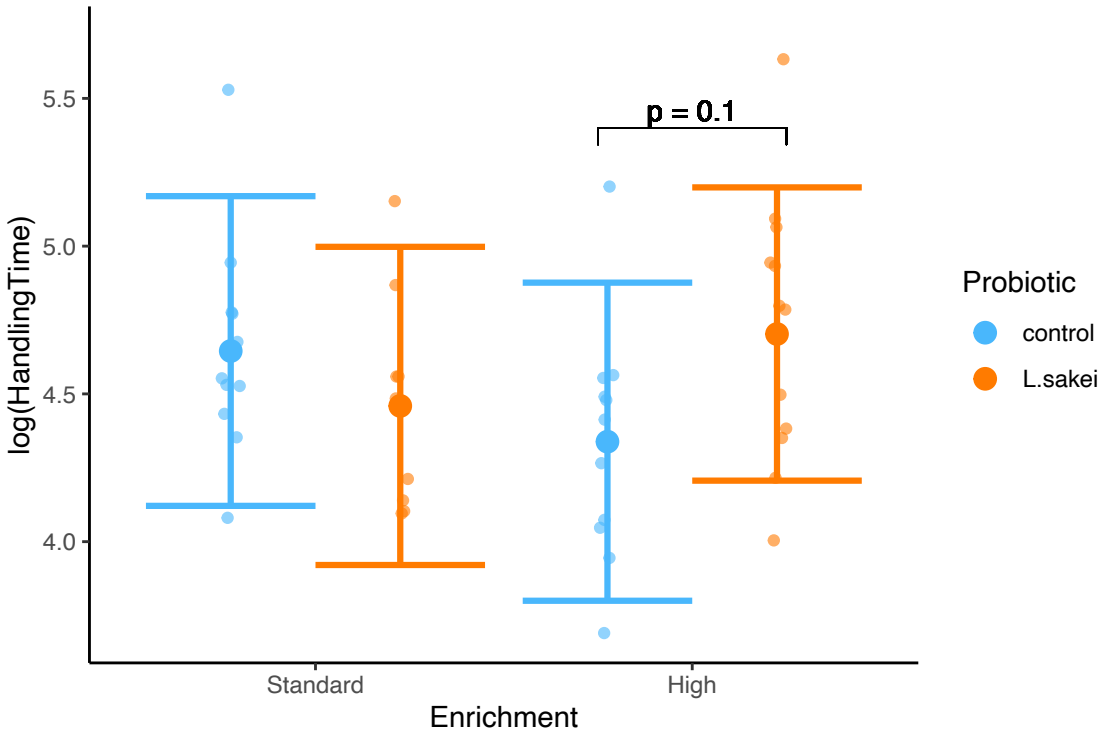

Figure S5. Probiotic and enrichment treatment effect on seed handling time. Some support for probiotic x enrichment interaction effect.

5.6 Appendix 4B: Supplementary methods

Library preparation and sequencing extended methods

Library preparation

The extracted DNA was amplified using Polymerase chain reaction (PCR) with 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 341R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) primers (Sigma-Aldrich), which include the Illumina overhang adapter sequences, targeting the V3-V4 region of the 16S chromosome.

Extracted DNA's concentration was quantified using the Qubit (Bio-Sciences, Dublin, Ireland) and normalised to 5ng/uL if DNA concentration exceeded 5 ng/uL. If concentration was below 5 ng/uL then template DNA was added to the PCR reaction neat. 5 uL of template DNA was added to a PCR plate with 5 uL of forward primer (1.2 µM), 5 uL of reverse primer (1.2 µM) where the final primer concentration was 0.2 µM for both the forward and reverse primer. 15 uL of Phusion High Fidelity PCR master mix with HF buffer (Thermofisher). Amplicon PCR conditions were as follows:

PCR Conditions (amplicon)

Initialisation	98°C - 30 s	
Denaturation	98°C - 10 s	
Annealing	55°C - 15 s	x 35 cycles
Extension	72°C - 20 s	
Final extension	72°C - 5 min	
Final hold	4°C - ∞	

The resulting amplicon was the samples were purified using AMPure XP protocol (Agencourt, Beckmann-Coulter) as found in the Illumina 16s-metagenomic library preparation guide (Illumina; 15044223-b). A second PCR reaction was run using

Illumina index primers (Set A and D). Phusion High fidelity PCR mix was used for this reaction as well, instead of the KAPA HiFi HotStart Readymix. This 50 uL reaction included 25 uL of Phusion, 10 uL sterile PCR water (Sigma-Aldrich), 5 uL of template DNA and 5 uL of primer from each of the forward and reverse Illumina index primers. Set A was used for the first PCR plate, set D for the second plate and the N7xx primers from set A and the S5xx primers from set D for the third plate thereby giving each sample unique indexes and allowing pooling for a single MiSeq run.

PCR Conditions (index)

Initialisation	98°C - 30 s	
Denaturation	98°C - 10 s	
Annealing	55°C - 15 s	x 8 cycles
Extension	72°C - 20 s	
Final extension	72°C - 5 min	
Final hold	4°C - ∞	

Following the index PCR, the samples were purified using AMPure XP protocol (Agencourt, Beckmann-Coulter) following the Illumina protocol and frozen before the normalisation and pooling stage. For normalisation and pooling, samples were defrosted and centrifuged briefly before quantifying the DNA concentration with a Qubit. Samples concentration was normalised to 10 nM using Buffer AE (Qiagen: 10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) 5 uL of each sample from PCR plates 1 and 2 were pooled in a single sample tube, and 5ul of sample from plates 3 and 4 were pooled into a separate tube. Samples tubes were frozen before shipping to the Genewiz GmbH facility (Leipzig, Germany) sequencing facility on ice. The pooled libraries were loaded onto a MiSeq flow cell and sequenced (2x300 paired end reads) with a 5% Phi-X spike-in. De-multiplexing of reads was performed on instrument.

Sand foraging training

Birds were given a foraging efficiency task on day-10, after training on days-7, 8 and 9. A foraging tray (24 well ice-cube tray) was placed in each birds cage on day-6 to habituate them to the object. The task involved presenting birds with a tray containing freshly killed mealworms in the wells which were concealed under sand, which they had to forage for. Birds had to progress through 4 training phases before being given the test task. Training phase 1 presented birds with a tray with 6 visible mealworms and no sand, birds had to consume at least 3/6 mealworms to move onto next phase. Phase 2 presented birds with 6 mealworms partially covered in sand where birds had to consume 3/6 mealworms to move on. Phase 3 presented birds with a tray completely covered in sand where each well contained a worm hidden under sand, with 2 visible worms on top of the sand, birds had to consume 5/24 hidden worms to move on to the final training phase. Final training phase had 10 worms hidden under sand and 2 worms exposed. If birds failed to progress past a training phase twice they were presented with the proceeding phase. Each training task lasted 10 minutes and there was at least 30 minutes between each training task. Birds were not food deprived before the training tasks.

Experimental set-up

Figure S5. Refuge on roof of high enrichment bird cage.

Figure S6. Low enrichment bird cage setup including cuttlefish bone and 2 perches, as well as food and water bowls. Clear sterile plastic sampling tray on the floor of the cage was used for collecting faecal samples on days 0, 6, 12. Access pipe to exploration room is seen at the rear of the cage.

Figure 7. High enrichment bird cage setup. Included cuttlefish bone, kinder egg with food, cork mat, hose pipe with sheep's wool, feathers, cubby hole and 2 perches, as well as food and water bowls. Clear sterile plastic sampling tray on the floor of the cage was used for collecting faecal samples on days 0, 6, 12.

Figure S8. Problem solving device. Device can be solved by removing string through the top, opening the side door or removing the trigger to drop the platform.

Chapter 6: General discussion

6.1 Summary

This thesis examined the effect of the microbiota on the host and the effect of the host, and host's environment, on the microbiota. Specifically, I found that the diversity of the gut microbiota is linked to an important proxy for host fitness, weight. However, I found that the direction of this effect varied across years, and the effect likely reflects the ecological context of the host and may change according to food availability or presence of pathogens. I also found that the microbiota varies with host ecology, and that the sensitivity of the microbiota to host ecology changes with age. In short, the microbiota of developing individuals are more sensitive to environmental factors, like habitat type and distance to edge, while the microbiota of developed individuals is more sensitive to host life history traits, like reproductive effort. While developed individuals do not show a direct effect of gut diversity on weight, I present evidence linking microbiome perturbation in developed individuals to physiological and behavioural changes with implications for host health and welfare, particularly in captivity. However, the evidence for the link between host phenotype and microbiota variation is much more apparent during development which likely reflect the presence of early developmental windows when the microbiota can more easily affect host phenotype.

6.2 Circular relationship

There is a circular relationship between the microbiota and host phenotype, where the microbiota influences the host's phenotype, e.g. weight or immune ability, and the host's phenotype influences the microbiota in turn. Phenotype is the result of interaction between the genotype and the environment, this thesis shows that the microbiota also plays a role in this interaction and that the environment also shapes the microbiota. In this sense the microbiota is a host trait, a sort of extended phenotype similar to a nest (Dawkins, 2016), though this could also work with the

reverse view where the host is an extended phenotype of the microbiota analogous to Dawkin's (2016) examples of parasites manipulating their hosts as part of their lifecycle. The bidirectional/circular nature of the microbiome-host relationship makes untangling correlation and causation difficult without manipulative experiments. In chapter two, I found a correlation between diversity and weight gain, however this could have reflected causation in either direction- the birds may have gained less weight because they had a diverse microbiota or they may have had a diverse microbiota because they were in worse condition and less able to exert control over their gut bacteria. Chapter four aimed to elucidate this relationship, but found a conflicting result, where the microbiota was positively correlated with host weight, thereby highlighting that this is likely a more complex relationship than I had originally thought and may be responding to the host's ecological context which I will discuss in more detail below.

Chapter three investigated sources of variation in the microbiota, finding that both environmental and host traits influenced microbiota diversity and composition, at different scales (individual, nest i.e. family, local area i.e. woodland site). Although complex- simultaneously examining these multiple dimensions of gut microbiota composition provides important context for future work aiming to understand the consequences of gut microbiota variation. Causality is difficult to determine without experimental manipulation so we cannot make causal inference using the observational data analysed in chapters two and three, however we can use these data to generate hypothesis driven questions to determine causality and consequences. Future studies could combine data on the nest and dietary microbiomes, in addition to the microbiome of the host, to determine the source of environmental microbes as understanding which microbes are obtained horizontally, from the environment, and vertically, from the parents, will be an important part of understanding which microbes might be subject to selection.

Experimental brood size manipulation, in conjunction with glucocorticoid measurements, would determine whether brood size effects on the microbiota are related to parental investment (which would reduce with increasing brood size), exposure (smaller brood means lower exposure to horizontally transmitted microbes; if individuals moved from another nest this should introduce a new pool of microbes to be shared among nestmates) or hormonal changes (increased brood size should lead to lower food availability, greater inter-sibling competition and higher stress hormone levels).

6.3 Microbiome costs and benefits

The microbiome has well known benefits, outlined in chapter one and the various introductory sections above, as well as costs, investigated by the bulk of research prior to the recent adoption of Next-Generation Sequencing, and reviewed by Benskin et al. (2009) and Evans et al. (2017). The results of chapters two and four, which described conflicting links between microbiota diversity and nestling weight, may reflect a trade-off between the metabolic opportunities offered by gut bacteria and the immune burden of the same. Greater dietary diversity can increase microbiome diversity with a concomitant immune cost, in an invertebrate host (Krams et al., 2017). This raises the question as to whether individuals who suffer from reduced growth with a diverse microbiota are trading off growth during the nestling stage with an improved immune system post-fledging. Individuals who are exposed to a greater number of bacteria during development are likely to have better calibrated immune responses, in line with the hygiene hypothesis (Musso et al., 2010; Strachan, 1989). The chapter two study could be extended to investigate this, in a population with greater juvenile recruitment, by resampling individuals in subsequent years and measuring their indices of immunity, i.e. microbial killing ability and antibody response which measures innate and adaptive immunity respectively, in a similar manner to Evans et al. (2016) which investigated the effect of nest bacterial load on immunity. In this experiment we would expect birds that had more diverse guts during the nestling stage to have 'better calibrated' immune systems, and to react more quickly and efficiently than individuals that had less

diverse microbiomes. An alternative hypothesis may be that the more diverse individuals will show greater immune tolerance, and hence a more muted immune response. The timing of microbial exposure is important, as there is evidence for a 'critical developmental window' when a host's immune system can learn tolerance (Metcalf et al., 2022).

That any given animal hosts, and depends upon, a microbiome- is often taken for granted in microbiome studies (Hammer et al., 2019). In order to avoid misinterpreting microbiome data it is important to recognise that not all animals host symbiotic bacteria or rely on bacteria to carry out functions necessary for survival. A number of articles, including those within this thesis, have demonstrated that great tits host gut bacteria (Bodawatta et al., 2021; Diez-Méndez et al., 2022; Teyssier et al., 2020; Teyssier, Lens, et al., 2018) but no study to date has found strong experimental evidence for gut bacteria to be necessary for great tit survival. An important aspect of this thesis has therefore been providing evidence for a role of the microbiome in great tit fitness, and has been recognising the potential costs of hosting gut bacteria, as was supported in Chapter 2, though this view is complicated by the evidence presented in Chapter 4 which indicates specific bacteria may benefit the host in some scenarios. Ultimately, microbiome researchers should not assume that the microbiome plays an important role in determining host phenotype and should proceed with the null hypothesis that the microbiome is not important for the host, or host trait, in question. Though I have shown here that it is important to consider the gut microbiome of great tits, if we are to more fully understand their ecology and evolution.

6.4 Early developmental windows and environmental matching

This thesis builds on earlier experimental work which found the presence of early developmental windows during which the microbiome shapes the metabolism and immune system of amphibian hosts (Knutie et al., 2017; Warne et al., 2019). I found that variation in the microbiota early in development is linked to future host weight

gain, in chapter two and four. Importantly, I also found that the sensitivity of the microbiome to environmental variation changes with life stage, and that the microbiomes of older individuals are less sensitive to environmental variation. This makes intuitive sense, that the microbiome has a greater effect on host phenotype early in development while the host's body is changing, and that developed individuals experience less or no phenotypic change as their bodies are changing less dramatically meaning there is less opportunity for the microbiota (or environment) to impact on the host. The increased sensitivity of the microbiome to the environment during ontogeny is an interesting and complementary phenomenon in light of the evidence for 'critical developmental windows' as it suggests *both* the host and the microbiota are more sensitive to environmental variation during development. This has important implications for 'Environmental matching', where environmental conditions during early development help adapt an individual's phenotype to its future environment (Monaghan, 2007). Future work could investigate how the predictability or stability of the environment affects the microbiome- we might expect that less predictable environments would select for more diverse microbiota (Alberdi et al., 2016). In chapter four, where we experimentally perturbed the microbiota, I ran a preliminary investigation into this relationship using an analysis of predicted function. These results did suggest treated individuals gained some functionality via their microbiome compared to control individuals, which may have allowed them to better exploit their food resources as appeared to be the case for low diversity treatment individuals, but this analysis looked at predicted rather than actual function so should be interpreted cautiously. Subsequent studies could build on the chapter four experiment, using a strain with unnecessary metabolic function, e.g. carbohydrate metabolism in a system that requires only protein and fat metabolism, and investigate the metabolome of treatment individuals to see if their metabolic function has been shifted towards a state reflecting functions from the treatment strain rather than those of the untreated microbiome and whether there was a concomitant decrease in the hosts metabolic capacity- perhaps by testing the ability of the host to extract energy from the diet using bomb calorimetry of faeces. The microbiome likely underpins metabolic plasticity and experimentally disrupting the gut microbiota, and creating a

mismatch between the microbiota and the diet would provide strong evidence for the causal role of the microbiome in host energetics and metabolism (Lindsay et al., 2020).

The increased sensitivity of the microbiome during development to environmental variation suggests this would be an optimum time to experiment with microbiome interventions and their effect on host fitness. Chapter two found several bacterial species that were associated with both increased and decreased survival to fledging. Where chapter four sought to isolate and culture any of the Lactobacillus that were linked to increased survival, the effort was limited due to pandemic restrictions which meant only individuals from a single nest were sampled. A greater sampling effort could yield the specific bacteria (e.g. a host adapted *L. sakei*) that have been linked to host fitness. Using the methodology from chapter four, the strength of these associations could be tested experimentally to determine which bacterial species are truly important to their host's survival. The experiment would have to be expanded, perhaps by further -omics data, to determine precisely how these bacteria benefit their host. Individual taxa have previously been found to affect hosts ability to adapt to challenging conditions through metabolism (Chevalier et al., 2015).

6.5 Ecological context

The ecological context determines the effect of the microbiome on the host. There is conflicting evidence regarding how microbiota diversity affects host health, even within birds (Kohl et al., 2018; Teyssier, Lens, et al., 2018; Videvall et al., 2019). I show in chapter two, an observational study, that diversity is negatively correlated with nestling weight at D15, an important proxy for fitness, but in an experimental study using birds from the same population in a different year I find an opposing trend (chapter four) where diversity correlates positively with weight. While these studies may not be directly comparable as they take place in different years, taken together they provide strong evidence that ecological context dictates how the microbiota varies with host phenotype. Specifically, in this thesis, nutrient availability/dietary

context may have been relevant to the microbiome links to host phenotype. In chapter two birds were not supplementally fed and were therefore likely nutrient limited; it may have been that a more diverse microbiota imposed a growth cost, while in chapter four, where birds received supplemental feeding, a diverse microbiota improved growth, possibly by providing a greater number of metabolic functions than were available to less diverse individuals. Further investigation of the effect of supplemental feeding and its interaction with the microbiome, by varying the amount of food available between nests, is necessary to fully understand these dynamics.

An alternative, but not mutually exclusive, explanation for the contrasting effect of diversity on weight could reflect yearly variation in pathogenic loads, due to environmental conditions like increased temperature variability or precipitation (Dallas & Drake, 2016; Delavaux et al., 2021). In a scenario where 2016 (sampling year for chapter two) had a greater pathogen abundance than 2021 (sampling year for chapter four), greater diversity could reflect this greater environmental pathogen load while lower diversity individuals might have experienced less exposure to pathogens and hence avoided costly immune reactions. The dynamic interaction between the host, its microbiome, its environment and a pathogen is reviewed by Bernardo-Cravo et al. (2020) in the context of a single fungal pathogen-*Batrachochytrium dendrobatidis,* highlighting the potential for interaction between these different aspects of host ecology. Knutie (2020) found that increased food availability was negatively related to host parasite abundance, and this may have been mediated by the gut microbiome. Knutie's (2020) experiment, which manipulated food abundance and parasite load, could be extended by using the host native strain (chapter four) protocol to perturb the microbiome and determine whether the gut microbiota does indeed mediate the relationship between food availability and parasite abundance. Of course, in any ecological system there are going to be many different contexts at play. The social environment, and the number of social interactions an individual engages in, will determine which horizontally transmitted microbes an individual might host (Gillingham et al., 2019; Tung et al.,

2015). Habitat characteristics will determine which environmental microbes an individual is exposed to (Goossens et al., 2022). While climatic conditions can influence microbial abundance, likely by acting on the host (Chevalier et al., 2015; Huus & Ley, 2021; Sommer et al., 2016).

6.6 Our system

Our system allowed us to sample the microbiota of both nestling and adult birds, and hence examine the contribution of host and environmental factors in shaping the microbiota at different ages. Recent evidence indicates that the avian gut microbiota is more sensitive to environmental factors than terrestrial mammals (Song et al., 2020; Youngblut et al., 2019). The nest represents a unique system as nestlings have only been exposed to a single environment while adults can move freely between different habitats; therefore, I expect nestlings' microbiota may exhibit especially strong correlations with their local habitat type. Host genetics are likely to play a role in determining microbiota variation (Davies et al., 2022; Goodrich et al., 2014). Chapter three found that the nest was the dominant factor driving microbiota repeatability but this could be confounded with host genetics, which could be investigated using a cross-fostering approach, though such an experiment by Teyssier et al. (2018) suggests that it is largely the nest environment driving the microbiota community over genetic affects- at least in great tits. However, heritability may only be apparent when comparing the parents with their adult offspring, after the tumultuous period of microbiome assembly has stabilised and the mature phenotype has been reached. Untangling the relative contribution of intrinsic and extrinsic factors is key to understanding the causality of microbiota variation. The dominance of environmental/extrinsic factors (particularly habitat type and distance to edge) may support the role of the microbiome as an adaptive plastic trait (Alberdi et al., 2016) while the dominance of host factors (particularly reproductive investment and host age) would indicate the microbiome reflects the state of the individual (hormonally, nutritionally, immunologically). Our results support a large role of the environment in structuring avian microbiota, with edge effects acting on the microbiota at multiple levels. The nest environment in particular is a key driver of

microbiota variation, which was the salient message of the repeatability analyses. However, host factors such as age moderate the effect of environmental variables. Hence, sampling multiple individuals within a nest is necessary to determine which factors are important and at what life stage they have an effect. These data will have inherent hierarchies that must be accounted for in the modelling procedures (e.g. with random terms) or important effects may be missed. Experimental work is necessary to determine how exactly these factors cause microbiota variation.

6.7 Multiple sampling points

The importance of longitudinal sampling is a key lesson from this thesis. In chapter two, I describe a time lagged effect of the microbiome on host phenotype and, importantly, no contemporary effect- so if nestlings were only sampled once we would not have detected this effect. Both chapters three and four suggest that individuals' microbiomes become more stable and resilient to environmental perturbation, as both studies found that younger birds were more strongly affected by environmental variables (such as distance to edge and habitat type) and the application of a host-adapted strain, respectively. However, the results from chapter five suggest that this might reflect the timing of sampling relative to the treatment, at least in part, as in the aviary experiment developed individuals did show a strong effect of the treatment on the microbiota at the midpoint (approximately day 6) of the experiment but this effect rapidly diminished over time as the microbiome community appeared to adapt to the presence of the probiotic by the end of the experiment (approx. day 12). Therefore, the lack of an effect on adult birds in the chapter four experiment may, in part, reflect the fact that these birds' microbiome communities have adapted to the presence of the treatment strain over time. In chapter four, the adult birds were sampled on day 12 while nestlings were sampled on day 8 and day 15, so the lack of an effect on adult birds may simply be due to the adult's microbial communities having recovered from the perturbation, if it followed a similar pattern to the chapter five experiment. Though notably, the day 15 nestlings in chapter four showed a greater effect of the treatment than adults. So, the lessened effect of the treatment on the adult birds is not only a function of time but also some

other factor- likely the resilience of their mature gut communities. In other words, if the smaller effect of treatment on the adult bird's microbiota compared to day 8 birds was only due to the length of time since the treatment began then we should have seen an even smaller effect on the day 15 nestlings. This result would also not be apparent without multiple sampling points.

6.8 Conclusion

The gut microbiota can play a key role in the ecology of their host, though the presence of a microbiome cannot be taken for granted (Hammer et al., 2019). Natural variation in the microbiota affects host health and is linked to host survival (Suzuki, 2017). However, the microbiota is not simply a subordinate community of tame microbes but an unruly mob of competing interests that can sicken and kill its host if the conditions are right (Cash et al., 2006; Sorci, 2013). This thesis investigated the role of the microbiota in the ecology of a wild free-living vertebrate, the great tit (Parus major), from the perspective of the host and that of the microbiota, in order to progress our understanding of the ecological costs and benefits of hosting this community of micro-organisms. Most microbiome studies to date have investigated captive model species (rats and mice) or agricultural species (Colston & Jackson, 2016; Pascoe et al., 2017) because of their medical and economic value. However, understanding the microbiome is important for ecology and evolution as well (Hird, 2017). I combine observational findings with a novel experimental manipulation of the wild microbiome to investigate the microbiome's role in host ecology. Linking the gut microbiome to fitness has important implications for evolutionary ecology because the microbiome exhibits high inter-individual variability, so is a potential target for selection (Hird et al., 2014; van Veelen et al., 2017), and it is vertically transmitted and heritable in birds (Banks et al., 2008; Dewar et al., 2013; Ruiz-Rodríguez et al., 2009). Microbes have played a key role in the evolutionary history of many organisms (Ley et al., 2008) and on shorter time scales, the microbiome may also allow hosts to acclimate to rapid environmental changes (Alberdi et al., 2016). As I have shown here, the microbiome, particularly during development, is sensitive to the environment. These microbiome changes may reflect differences in exposure

or host reactions to environmental variation but as I have also shown- the microbiota may benefit or disadvantage their hosts depending on the ecological context. This thesis describes the nuanced, context dependent relationship between the microbiota and their wild hosts, highlighting the benefits of its study in ecologically realistic contexts for progress towards a functional understanding of the microbiome.

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