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Manipulating a host-native microbial strain compensates for low microbial diversity by increasing weight gain in a wild bird population.

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Abstract

Empirical studies from laboratory systems and humans show that the gut microbiota is linked to host health. Similar evidence for effects on traits linked to fitness in nature is rare, not least because experimentally manipulating the gut microbiota is challenging. We demonstrate a novel approach in which we isolated, characterised, and cultured a bacterial strain, *Lactobacillus kimchicus*, directly from a wild bird (the great tit *Parus major*) and provided it as a self-administered dietary supplement. We assessed the impact of the treatment on the host microbiota community, on weight, and further tested if the treatment's effect on weight affected a previous result linking microbiota alpha diversity to the weight in nestlings. The treatment dramatically increased *L. kimchicus'* abundance in the gut microbiota and increased alpha diversity. This effect was strongest in the youngest birds, validating earlier findings pointing to a brief developmental window when the gut microbiota are most sensitive. In time lagged models, nestling weight was higher in the treatment birds suggesting *L. kimchicus* may have probiotic potential. There was also a positive time-lagged relationship between diversity and weight in control birds but not in the treatment birds, suggesting *L. kimchicus* helped birds compensate for low alpha diversity. We discuss why ecological context is likely key when predicting impacts of the microbiome. To our knowledge, this is the first time manipulating the gut microbiota

with a host native strain has been achieved in a wild population and provides direct evidence for the role of the microbiota in the ecology and evolution of natural populations.

Significance Statement

The gut microbiota has been linked to host health in laboratory studies but evidence for similar effects in wild systems is lacking. We use a novel approach to manipulate the microbiota of a wild bird, the great tit (*Parus major*), to explore links between the microbiota and weight, an important phenotypic trait related to survival- and hence biological fitness- in many animals. We isolated a bacterial strain from the host and provided it as a dietary supplement. The treatment changed the microbiota and increased the weight of individuals with low microbiota diversity. This provides direct evidence of the role of the microbiota in the ecology and evolution of natural populations and suggests that the strain we isolated has probiotic potential.

Main Text

Introduction

Many studies have shown that the gut microbiota can affect host phenotype and health by influencing a variety of processes (1, 2). These processes include stress regulation (3), cognitive function (4), sociality (5), metabolism (6–8) and immunity (9, 10). The gut microbiota is highly variable within individuals, which is thought to help host's rapidly adapt to environmental variation (11). For example, a flexible gut microbiota allows animals to cope with seasonal variation in food quantity and diet (12, 13), and to detoxify dietary/environmental contaminants (14, 15). This may be particularly important during development when the microbiota is most sensitive to the environment (16) and the host can be readily affected by the microbiota (7, 9, 10). However, there is a lack of experimental evidence from wild systems that the microbiota causally affects host phenotypes generally and traits linked to fitness in particular.

The vast majority of experimental microbiota manipulations are laboratory based and focused on commercially important agricultural species or model hosts (17). Typically hosts in these systems have limited genetic variation and experience consistent environmental conditions, making it difficult to generalise findings to wild populations that tend to have much higher microbial and environmental variation (18, 19). While conditions experienced by host species in the wild have ecological realism, many wild studies do not manipulate the microbiota directly and instead take advantage of natural observational experiments (15, 20, 21), or indirectly manipulate the gut microbiome by experimentally altering the environment (22, 23) or diet (24). Although more direct manipulations of the gut microbiome are necessary for causal inference—most commonly antibiotics and off the shelf probiotics—these have their limitations. Antibiotic effects tend to be broad spectrum making it difficult to understand the causes underlying any observed effects (25, 26). Single strain probiotic interventions can provide a more targeted way of changing the gut microbiome (27) but their effects often seem to be host specific (27–29) perhaps because the microbes used are not adapted to the host species and do not interact with host tissues (30–32). To our knowledge, the successful use of a probiotic or any direct microbial intervention has yet to be achieved in a wild animal, despite their promising potential for helping to understand ecological and evolutionary processes.

Candidate microbial strains for interventions have been identified through observational association between naturally occurring strains of bacteria and indicators of health and fitness, or traits closely linked to fitness, in wild animals (33–36). Notably, naturally occurring *Lactobacillus* explained weight and survival in wild avian hosts (33, 37). *Lactobacillus* species are commonly used in probiotic treatments because they are often linked to beneficial effects on human model organisms (38–41). These effects include moderating the pH environment and the production of antimicrobials (including bacteriocins), thus encouraging or inhibiting the growth of microbes in the microbiome community (reviewed by Drissi et al. (39)). Commercial *Lactobacillus* probiotics change the gut microbiome of domestic pigeons and chickens (40, 42), improve the feed conversion rate in agriculture (43) and cause weight change in humans and domesticated animals (38, 42, 44). In the wild, body condition is a significant predictor of survival, particularly at the natal life stage (45, 46), yet whether the gut microbiome plays a causal role in determining animal fitness in the wild remains untested. Leveraging host-derived microbial interventions are needed to understand the fitness implications of host-microbiome interactions in nature. However, despite evidence that host adapted strains are more effective than non-specific commercially available strains (32), host adapted strains of bacteria are

rarely if ever used in laboratory or domesticated animals (32, 47) and to our knowledge have never been used in the wild.

In a previous observational study, we reported time-lagged links between gut microbiota, weight gain and survival during development in a model species in avian ecology, the great tit *Parus major* (33). Here, we build on this by experimentally investigating the effect of the gut microbiota using the novel approach of isolating, characterising and culturing a host-derived gut *Lactobacillus* strain from wild, free-living birds in the same population. We facilitated self-administration of this strain, also in the wild, when parents were feeding the nestlings at the nest, thus minimising researcher interference and enhancing ecological validity.

First, we checked that the administration worked and explored what effect artificially increasing the abundance of one strain had on the gut microbiota alpha and beta diversity. We expected that a large dose of a single strain would give that strain a competitive advantage and hence lower the overall gut microbiota diversity, and the variance in the diversity because, for example, *Lactobacillus* can modify their environment by producing anti-microbials that inhibit the growth of other bacteria (39). We then tested whether the manipulation influenced nestling weight. Although the strain we isolated had not previously been linked to weight gain, given that the strain did show some probiotic characteristics and had functionality (see results) that hypothetically could benefit carbohydrate metabolism, and given the widely reported probiotic effects of *Lactobacillus* generally (38–41), we predicted a positive impact of the treatment on nestling weight. At the same time we expected the effect could be influenced by our previous findings of a negative correlation between nestling weight and alpha diversity in this wild system, suggesting that any benefits of our manipulation would most likely benefit nestlings with high alpha diversity. As our results emerged, however, this prediction was superseded by the opposite because alpha diversity and weight were positively, not negatively, correlated in this experiment, which we speculate was caused by supplemental feeding and points to the importance of nutritional status in determining the direction of the effect of the microbiota on host fitness. Our approach of manipulating gut microbiota using a host-derived strain and self-administration in the wild supports the hypothesis of a causal link between the microbiota and host phenotype. This approach is a necessary advancement for determining the role of the gut microbiota in host ecology (18) and is timely given its potential importance in wildlife conservation (48–50).

Results

Lactobacillus kimchicus as a candidate probiotic, with gene function associated with carbohydrate, amino acid and protein metabolism.

The host derived treatment strain, *L. kimchicus* (*Lactobacillus kimchicus* also known as *Secundilactobacillus kimchicus* (51, 52)) passed the isolation and characterisation screening which indicated the strain did not display antibiotic resistance, could survive in the adverse conditions found in the gastrointestinal tract and had potential probiotic properties. 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 S2). Further description of the *L. kimchicus* metagenome is described in Supplementary Information (results of the 'Isolation and characterisation' section).

L. kimchicus-treated birds show increase in microbiota's inferred functional abundance.

The inferred functional analysis, performed using Picrust2, generated 7657 inferred Kegg Orthologue Pathways and 428 inferred Meta Cyc pathways from the full amplicon sequence variant (ASV) dataset with a mean Nearest Sequence Taxon Index (NSTI) score of 0.22 (SE $\pm$ 0.001; median NSTI = 0.16, SE $\pm$ 0.001). 21 inferred Kegg Orthologs were differentially abundant, all of which were expressed more abundantly in the experimentally treated birds. These inferred KOs mapped to carbohydrate metabolism, protein metabolism, lipid metabolism and terpenoids and polyketides metabolism (table S6, S7). No inferred MetaCyc pathways were detected as differentially abundant.

Experimental treatment increased the presence and abundance of *L. kimchicus*.

All 16S rRNA sequences retained after filtering were aligned using BLAST with the *L. kimchicus* whole genome to determine which amplicon sequence variant (ASV) was the experimental treatment

strain. We found that ASV27 aligned with 100% similarity and 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 its presence was therefore highly dependent on treatment (χ^2= 35.9, df = 1, p<0.001). Approximately 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* spp. and 2 *Latilactobacillus* spp.) all but ASV27 were present in 5 or less individuals and most were 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).

***L. kimchicus* treatment increased gut alpha diversity and increased variation in community composition.**

L. kimchicus treatment increased mean gut microbiota diversity for all three alpha diversity measures: log-Chao1 (table 2; figure 1), Shannon diversity (table S1, treatment: 0.288, p=0.024; figure 1) and log-Faith's phylogenetic diversity (table S2, treatment: 0.231, p = 0.026; figure 1). This effect was strongest in D8 nestlings (table 2; figure 2) and diminished in later life stages. *L. kimchicus* treatment did not affect variation in any of our three measures of alpha diversity: log-Chao1 diversity (0.006, p=0.94), Shannon diversity (Bartlett's K-squared: 0.36, p=0.55) or log-Faith's phylogenetic diversity (0.077, p = 0.781).

The community composition of the gut microbiota was not affected by treatment in a consistent manner and differed instead across woodland sites (table 3; figure 3), although there was some support for treatment to affect beta diversity differently across sites (table 3; figure S1). 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 day-8 (D8) (p=0.024) but not day-15 (D15) nestlings (p=0.22) or adult birds (p=0.63) when calculated for each age group separately. There was no evidence that Firmicutes relative abundance was affected in treated birds compared to controls (table S3; -0.6, p = 0.158), controlling for life stage.

***L. kimchicus* treatment affected host weight by neutralising 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, S4), as we found in a previous study (33). However, in the time lagged models there was a significant main effect of treatment on weight, with *L. kimchicus* treated birds having higher weight at day-15 when controlling for either log(Chao) or log(Faith's PD) diversity at day-8 (table 5, S7). The effect of treatment on weight is marginally significant when controlling for Shannon diversity (table S6). There was a positive relationship between all three diversity measures at D8 and weight at D15 in control birds but this effect was negated by the *L. kimchicus* treatment, meaning treatment birds with low diversity had higher weights than control birds with low diversity (table 5, S5, S6; figure 4). In other words, there was a time lagged relationship between diversity and weight in control birds but not in treatment birds.

Discussion

We show that the addition of a host-adapted *Lactobacillus* strain significantly affected both the gut microbiota of a wild bird and its phenotype. The addition of L. kimchicus in the diet increased nestling weight and eliminated the positive link between gut diversity variation and host weight observed in control nestlings. In other words, the ingestion of *L. kimchicus* appeared to compensate for low alpha diversity that would otherwise result in nestlings having low weight, possibly by providing additional metabolic functionality. The addition of *L. kimchicus* in the diet increased the diversity and changed the predicted functional profile of hosts' microbiota. Specifically, hosts' Chao1, Shannon and Faith's phylogenetic diversity increased, as did carbohydrate and protein metabolism 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 microbiota of the young, while adults maintain more stable microbiota in the face of environmental sources of perturbation.

Treatment alters gut microbiota

Our method for direct experimental manipulation of the gut microbiota of wild birds, without the use of antibiotics or regular handling, successfully changed the microbiota of treatment birds. Though the strain was not taxonomically identified in the experimental samples, probably because of the different sources of the database and experimental strains, the BLAST results indicate that ASV27 is almost

certainly the experimental strain. ASV27 shared 100% identity with the experimental strain and was taxonomically identified as the same genus, so we are confident that ASV27 is the experimental strain. 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, fragmented breeding population precluded extensive follow-up sampling. Nevertheless, the presence of the strain did alter the gut microbiota, albeit in the opposite direction to our predictions. 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 (39). Instead, all three measures of alpha diversity increased among birds in the experimental treatment, compared to control birds, though variation in diversity was unchanged across treatment groups. The gut community compositions did not change with treatment in a predictable manner but became more variable in younger nestlings. 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 (53). 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.

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, and the dispersion of the overall community (i.e. beta diversity), diminishing with age. This provides experimental support for our previous observational results highlighting the presence of early developmental windows during which the microbiota of nestling great tits are particularly sensitive to environmental variation (16). This is important because laboratory studies in tadpoles and mice have shown that microbiota variation during development can affect the host's future phenotype (10, 54). 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 (55, 56). 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. Similar to our previous study (33), there was no contemporary effect of diversity on weight, suggesting that any effect of the microbiota on weight takes some time to manifest.

Beneficial effects of L. kimchicus on host weight

There was a positive main effect of the *L. kimchicus* treatment on nestling weight, which to our knowledge is the first such demonstration of a direct link between the gut microbiota and a trait closely linked to fitness in a wild population. Alpha diversity was positively correlated with host weight in the control birds, meaning that nestlings with low alpha diversity were below average weight, and theoretically at higher risk of mortality. The ingestion of *L. kimchicus* appeared to compensate for this effect of low alpha diversity by increasing weight in low diversity nestlings, but there was no difference in weight between the two treatments at high alpha diversity. This pattern means treated birds had higher overall weight and less variation in weight, when accounting for D8 weight. There appears to be an upper limit of diversity, beyond which *L. kimchicus* does not provide any benefit to the host. Whether ASV27 was detected in a treated individual did not affect their weight (table S8), which could be due to a variety of reasons, including: (i) the incompleteness of 16S sequencing means that not all taxa in a sample will be identified, particularly for samples with high overall microbial abundance; and (ii) the fieldwork protocol meant that birds were sampled at different times of day, which could affect the amount of the treatment strain present in their system at the time we took the faecal sample.

Predicted functional analysis suggests that *L. kimchicus* increased metabolic function, which may have compensated for loss of functionality in low diversity birds, although only up to a certain diversity threshold since higher levels of diversity did not confer any extra benefit. 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 information section on 'Isolation and characterisation'). Predicted function in the gut microbiota as a whole also found taxa with genes associated with carbohydrate and protein metabolism, which were enriched in treatment birds. Nestling great tit diet is rich in protein and amino acids (57–59) but low in carbohydrates (60) as they primarily feed on insects, especially caterpillars, while the birds in this experiment were fed supplementary mealworms. The treatment may have provided protein metabolism functionality that was otherwise lacking in low diversity birds, but which

was unnecessary in high diversity birds who already had a microbiota with this functionality. A follow up study that includes a metabolomic analysis of the host's gut microbiome would be an important next step for understanding precisely how the treatment affects weight.

The predicted functional analysis results should be interpreted cautiously, as the metabolic pathways are inferred from existing reference genomes, and therefore may not represent poorly characterised microbial environments. Our NSTI scores were relatively high (suggesting a relatively poor match to existing reference genomes) compared to OTU-based benchmarks (61). However, our NSTI scores are similar or lower than other comparable wildlife studies and may be inflated as they were based on ASV's (100% identity) rather than OTU's (97% identity), which increases variation and consequently decreases reference sequence similarity (62, 63). Despite these limitations, the predicted functional analysis was consistent with our complementary functional analysis of *L. Kimchicus* from metagenomic data, which we interpret as strong support that our microbiome manipulation led to overall changes in gut microbiota function (and consequently host phenotype), though remains to be mechanistically confirmed through metabolomic and metagenomic analyses.

Previously, we reported a negative correlation between D8 alpha diversity and D15 weight in an observational study of the same wild population (33) where food was likely limited. This contrasts to the pattern in this experiment where significant supplemental food was provided daily and we found a positive correlation between D8 alpha diversity and D15 weight. We suggest that greater diversity increases weight gain but only when food is abundant, as was the case during our current study. In other words, the potential costs of high microbial diversity, as supported by observations in Davidson et al. (33) and Krams et al. (64), may be outweighed by the benefits provided by the microbes—aiding digestion, providing useful metabolites, and preventing the colonisation of pathogenic microbes (8, 41, 65, 66) —but only when there are surplus nutrients such as the supplementary food provided in the current experiment. On the other hand, when food is limiting- greater diversity may impose a cost due to the greater immune burden. There may therefore be a threshold of nutrient availability that sees the drain on host resources, due to high diversity, become outweighed by the alternative benefits provided by gut microbes, such as preventing the colonisation of pathogenic bacteria, when hosts experience a sufficient calorie surplus. A future study could help examine this by including a non-supplementary fed control group, alongside a supplementary fed control and supplementary fed experimental group. It follows that the costs and benefits of the relationship between diversity and weight may change as the birds' microbiomes change in response to different seasonally available food (67) or climatic variables such as rainfall (13). Indeed mammalian studies have found that the specific changes in the microbiota may help their hosts adapt to seasonal reductions in food supply (12, 13), possibly at the expense of microbes that aid host immune function (68).

Conflicting links between diversity and host health have also been found in humans. Low diversity during development, which is associated with Caesarean sections (69), has being linked with diarrhoea in infants (70), and obesity and diabetes in later life (71, 72). Meanwhile, formula fed infants have higher diversity than breastfed infants early in development (69) but worse health outcomes (73). These conflicting links suggest that greater diversity is not a positive or negative trait per se, but that the host's context probably plays a major role in determining the links between the microbiota and host health. Comparisons between mammals and birds may shed light on the role of diversity in promoting health or fitness outcomes, though is likely to be confounded by the very different time frames involved and the dominant role of mammalian milk in structuring the microbiota in early life (74).

Conclusions

To our knowledge, this is the first study to demonstrate how direct ingestion of a microbial strain affects host phenotype in a wild, free-living animal. We did this using a microbial strain that was isolated from the host gut microbiome and was self-administered, facilitating an efficacious method appropriate to the host's ecology. Our key findings support the role of the gut microbiota in promoting host health during development and 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. Experimentally identifying microbes that are important for fitness in wild hosts, as well as when and how they act, is important for both our ability to further investigate the functional and evolutionary role of the microbiome, for our

fundamental understanding of its host's ecology, and for the utility of microbe interventions in applied ecology.

Materials and Methods

Obtaining and culturing host-native strain

Faecal samples were taken from great tit nestlings at Dukes Wood, Co. Cork, Ireland in June 2020. These samples were inoculated into BD Difco *Lactobacillus* broth (75) (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 lactic acid bacteria 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 to check for antibiotic resistance, bacteriocin and metabolic genes to understand the strains suitability for experimentation and its' functional capabilities. The strain was screened for probiotic characteristics as we wished to preferentially target beneficial strains for the microbial intervention. A potential probiotic needs to survive the adverse conditions of the gastrointestinal tract (GIT); therefore, in vitro tests that simulated the GIT were conducted. *L. rhamnosus* GG was used as a reference strain. The tests included bile salt and acidity tolerance testing, antimicrobial agent production, and pathogen inhibition, amongst others. 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 information section on 'Isolation and characterisation' for more details on the isolation and testing of the host native strain.

Dosing

In order to disturb the birds as little as possible during the nesting period, nestlings were fed the lyophilised powder indirectly by providing mealworms (*Tenebrio molitor*) soaked in a solution (see below) 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 five nests. We observed adults consuming the worms themselves and bringing them into the nest to feed their chicks, at all five nests. 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 that 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 (76). 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 1ml of distilled water before application to worms.

Nest monitoring, trapping, tagging and faecal sampling

Nests were checked following the schedule in O'Shea et al. (77). Nestling weight was recorded on day-8 and day-15 post hatching and faecal samples taken. If nestlings were large enough on day-8, they were given individual ID rings from the British Trust for Ornithology (BTO). If the nestlings were too small to ring they were marked with a unique pattern by trimming the downy feathers on their heads until they could be ringed at day-15. Adults were trapped on the nest on day-12 when they were fitted with a BTO ring if they had not already been, 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 the sampling apparatus of Knutie et al. (78). 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 did not 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.

DNA extraction & Library preparation

Prior to DNA extraction the ethanol was removed from the samples using a MiVac centrifuge, which centrifuges and heats samples at very low pressure (<100 mbar). Open sample tubes were placed in the Genevac miVac Centrifugal Concentrator (Fisher Scientific) and centrifuges 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. (79).

The V3-V4 variable region of the 16S rRNA gene was amplified using 341F and 341R primers (Sigma-Aldrich) 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 and indexed using Illumina index primers sets A and D. 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 methods section of the supplementary information file for more in-depth laboratory methods.

Bioinformatics analyses

After sequencing samples were processed using the DADA2 pipeline in R (R version 4.2.2; R Core Team, 2022), following the dada2 tutorial v1.16 (81). 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 (82) 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 (83). 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 (84). Shannon and Chao1 diversity were estimated with phyloseq's *estimate_richness* function.

Phylogenetic diversity was also calculated in the form of Faith's PD (85). A '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 (86) following the Bioconductor workflow (87). Faith's PD and Chao1 values were very similar for each sample (figure 1). 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.

Inferred function and differential abundance analysis

Picrust2 (88) 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 (89). We describe functions according to the BRITE hierarchies database. We also tested for differentially abundant ASVs using the MaAsLin2 method, as described above.

Statistical analyses

Alpha diversity

Variation in alpha diversity (log-Chao1, Shannon diversity and Faith's PD) across treatment groups was assessed using Bartlett's test (90). Linear mixed models were used to investigate the effect of treatment on alpha diversity across age groups, using the lme4 package (91). 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*) (92) 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 (93). Interaction estimates are orthogonal to the main effects meaning the main effects are interpretable. Model residuals were checked using DHARMa (94).

Beta diversity

Before modelling the community composition, we removed low prevalence taxa (<5%). Taxa counts were centre-log (CLR) transformed and the Aitchison distance between samples calculated (95, 96). The PERMANOVA+ function (97) from the Primer (v.7) package (98) 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. To visualise differences between treatment groups the ASV count data with low prevalence taxa removed was CLR transformed with imputation to eliminate zero values using the clr_c function from the Tjazi package (99). Imputation was used to avoid issues with zero inflation in principal component analysis (PCA). The principal components of the transformed data were calculated and the first two components plotted. 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.

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 (100). 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. None of these 20 best aligned sequences were present in more than 5 individuals except for ASV27, which was present 50 individuals. A chi-square test was used to test whether ASV27 presence was dependent on treatment.

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 (39). We used a binomial model from the lme4 package (91) 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.

Treatment effect on weight and weight gain

A previous paper from our study system reported a time-lagged effect of alpha diversity at D8 on weight at D15 (33). We first tested the effect of treatment on weight across all birds (nestlings and adults), accounting for age, brood size and contemporary 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 that the treatment might affect the age groups differently considering the differential sensitivity of developing individuals microbiota (16).

We then repeated the time-lagged analysis of weight gain on nestlings alone as in Davidson et al. (33). This modelled weight at D15 against the fixed effects weight at D8, alpha diversity at D8, lay date and brood size, with woodland site and nest ID as nested random effects. We subtracted the minimum scaled value from the diversity term (setting the minimum value to zero) in order to explicitly test the effect of treatment at low diversity. Additionally, we also examine whether the impact of treatment was dependent on alpha diversity by including their interaction. Model residuals were checked using DHARMa (94). We also investigated whether the detection of ASV27 at D8 (True/False) affected the D15 weight of treated birds, while controlling for the same fixed and random effects as above.

Data accessibility

Sequence data are available in the European Nucleotide Archive under access number PRJEB74941, and ERS18960426-ERS18960697. The sample metadata, ASV and taxonomy tables (https://doi.org/10.5281/zenodo.10987294) and analysis code are available (https://doi.org/10.5281/zenodo.11369716) on Zenodo.

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Figure legends

Figure 1. Alpha diversity boxplots comparing the *L. kimchicus* treatment birds with control birds, N = 201. PD refers to Faith's Phylogenetic diversity.

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). Horizontal line indicates the grand mean.

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

Figure 4. Effect of D8 log(Chao1) diversity on D15 weight in grams, across treatment groups, N = 61. Partial residual plot with separate lines and 95% confidence intervals for each treatment group. Chao values (x axis) are scaled to a minimum of zero while weight values (y axis) are not scaled.