A time-lagged association between the gut microbiome, nestling growth and nestling survival in wild great tits

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Abstract:

1. 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.
2. 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.

3. There was no link between mass and microbial diversity on D8 or D15. However, we detected a time-lagged relationship whereby the microbial diversity at D8 was negatively associated with weight at D15, while controlling for weight at D8, therefore reflecting weight gain over the intervening period. Indicator species analysis revealed that while several taxa were unique to birds that either survived or did not survive, there were no universal taxa that were consistently found across all birds within either survival group. This suggests that the presence of particular bacterial taxa may be sufficient, but not necessary for determining future survival, perhaps owing to functional overlap in microbiota.
4. We highlight that measuring microbiome-fitness relationships at just one time point may be misleading, especially early in life. Instead, microbial-host fitness effects should be investigated longitudinally as there may be critical development windows in which key microbiota are established and prime host traits associated with nestling growth. Pinpointing which features of the gut microbial community impact on host fitness, and when during development this occurs, will shed light on population level processes and has the potential to support conservation.

Introduction:

Understanding the numerous factors that drive fitness has long been the focus of research in ecology and evolution (Merila, 1996; Pickett et al., 2013). 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 (Crino & Breuner, 2015; Weber et al., 2018) 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; Davidson 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 growth 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 (Bian et al., 2017). 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 significantly higher abundances of Bacteroidetes and Proteobacteria, and lower 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 taxa, natal body size (Ringsby et al., 1998), natal body mass (Both et al., 1999; Monrós et al., 2002), or growth rate (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, higher 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

129 penguins (*Spheniscus magellanicus*) (Potti et al., 2002). By contrast, microbial diversity has
130 been positively associated with body condition and growth rate in wild nestling birds
131 (Noguera et al., 2018; Teyssier et al., 2018).
132
133 The great tit (*Parus major*) provides a powerful natural system for addressing microbiome
134 related effects on host phenotypes. It is an altricial species that readily breeds in nest boxes,
135 so that the developmental period can be tracked easily in the wild. We investigated the
136 relationship between natural variation in the gut microbiome and direct and indirect
137 measures of fitness in nestling great tits when they were 8 and 15 days old. The nestling
138 stage is a critical time for gut microbiome colonization (Koenig et al., 2010) and the
139 microbial-programming of adult phenotypes (Cox et al., 2014; Hansen et al., 2012; Sudo et
140 al., 2004). Nestling great tits undergo substantial shifts in gut microbiota between day 8 and
141 day 15 of development (Teyssier et al., 2018), a period when we predicted the microbiome
142 would have a detectable effect on body weight and the probability of survival to fledging.
143 We tested whether diversity of the microbiome, or the abundances of specific microbes,
144 were associated with nestling weight and survival to fledging. Given the mixed reports of
145 microbial links with host fitness in the existing literature (see above), we had no a priori
146 predictions regarding the direction of such potential relationships. Nevertheless, we
147 proposed that if diversity was positively related to measures of host fitness, then a rich
148 and/or evenly distributed microbial community would be important for healthy
149 development. By contrast, if the relationship was negative, then the host may benefit from
150 the presence or absence of specific microbial taxa, which should be observed in differences
151 in the relative abundance of these specific microbes associated with host fitness. Moreover,
152 because early developmental windows have been reported to be important for microbial
153 influences on host traits (Cox et al., 2014; Hansen et al., 2012), we sampled the gut
154 microbiome from chicks at two developmental stages to test whether microbiome effects
155 on host fitness traits were contemporary or time-lagged. In other words, we asked whether
156 the gut microbiome at D8 or D15 was a better predictor of mass and survival.
157
158 **Methods:**
159 ***Field monitoring and microbiome sampling***

160 Birds were sampled from nine nest box populations across Co. Cork, Ireland, five of these
161 were mixed/deciduous habitats and four were coniferous habitats (see O'Shea et al. (2018)).
162 We collected 204 faecal samples from 150 nestling great tits from 54 nests (see below for
163 the number of samples that were successfully sequenced and sample size per
164 developmental stage) for 16S rRNA gene sequencing in order to map the gastrointestinal
165 microbial communities. During April-June 2016, nest boxes were monitored to determine
166 lay dates, hatching dates and nestling survival. Nestlings aged 8 days (+/- 1 day) (D8) and 15
167 days (D15) were placed into sterile holding bags inside a heated holding case. Coffee filters
168 were used to line the bags in order to soak up urea from the faeces, as urea has the
169 potential to affect downstream sequencing (Khan et al., 1991). Samples were collected from
170 nestlings that defaecated naturally within 15-20 minutes of being out of the nest before
171 being returned to the nest. Faecal sacks were opened using a sterile inoculation loop to
172 release the faecal matter and place in a microcentrifuge tube containing 500uL of 100%
173 ethanol. Samples were stored at -20°C within 8 hours of collection until DNA extraction. D8
174 birds were weighed, and uniquely individually identified by clipping the tip of one of their
175 nails, avoiding the quick (i.e. blood vessel). D15 nestlings were weighed and ringed with a
176 unique identifiable metal ring (British Trust for Ornithology) and matched against their
177 unique nail clipping. Nestlings for which we had repeated samples (i.e. for both D8 and D15)
178 are referred to as 'repeat samples'.
179
180 ***DNA Extractions***
181 Briefly, DNA was extracted from the dried faecal contents of all birds using the Qiagen
182 QIAamp DNA Stool Kit, following the "Isolation of DNA from Stool for Pathogen Detection"
183 protocol (June 2012 edition), with modifications described in (Shutt et al., 2020) to
184 accommodate dried avian faeces. DNA was stored at -20°C. Full extraction methods are
185 described in Supporting Information.
186
187 ***Illumina MiSeq sequencing***
188 Full library preparation details are described in Supporting Information. Briefly, the V3-V4
189 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S
190 metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers
191 specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina

192 overhang adaptor. Samples were sequenced on the MiSeq sequencing platform (Clinical
193 Microbiomics, Denmark), using a 2 x 300 cycle kit, following standard Illumina sequencing
194 protocols.
195
196 ***Bioinformatics and statistical analysis***
197 The DADA2 pipeline was used to process the raw sequencing data (Callahan et al., 2016) in R
198 version 3.5 (R Core Team, 2019). Sequence quality was visually inspected. Sequences were
199 trimmed to remove adapters and lower quality reads (median quality scores below 25-30
200 threshold) at the extremities of the sequence and filtered to remove sequences with higher
201 than expected errors. Read errors were estimated before dereplication. Forward and
202 reverse reads were merged to construct 'contig' sequences, these were used to construct a
203 sequence table of Amplicon Sequence Variants (ASV's), which counts the number of times
204 each unique sequence is detected. The previous steps were performed for each run
205 separately. Then the separate sequence tables were merged and chimeras removed using
206 the 'consensus' method. Taxonomy was assigned to each ASV by RDP's Naive Bayes
207 Classifier (Wang et al., 2007) against the Silva reference database (version 132) (Quast et
208 al., 2012). This is at 100% sequence identity in contrast to the lower resolution OTU method
209 which groups sequences at 97% identity. ASV's allow greater sensitivity and specificity,
210 better discrimination of ecological patterns than OTU's and are reusable across studies
211 (Callahan et al., 2017).
212
213 The DADA2 outputs were assembled into a single Phyloseq object (McMurdie & Holmes,
214 2013). Sequences identified as mitochondrial or chloroplast were removed. Further filtering
215 of samples and ASV's took place in R. Sample completeness curves were plotted using vegan
216 (Jari Oksanen, 2019) and helped determine the lower cut-off for sample reads at 10,000
217 reads. Low read samples (<10000 reads, 9 samples) were removed leaving 195 (Day-8=81,
218 Day-15=114, repeat samples=41) samples for the analysis. Alpha diversity (both Shannon
219 and Chao1 diversity) was calculated using the '*estimate_richness*' function from the
220 phyloseq package on the filtered dataset. Shannon diversity (Shannon, 1948) measured
221 richness weighted by abundance (the evenness of a community) and Chao1 (Chao, 1984)
222 measuresd richness, specifically estimating taxa abundance and rare taxa missed from
223 under sampling.

224

225 **Statistical analysis**

226 ***Nestling growth: Alpha diversity and phylum-level relative abundance***

227 Linear mixed models (LMMs) and generalised linear mixed models (GLMMs) were used to
228 examine the association between measures of nestling growth and two measures of alpha
229 diversity: Shannon's index and Chao1 index. Models were fit using the lme4 package (Bates
230 et al., 2015) on each data subset. Numeric variables were centered and scaled. Model
231 diagnostics were assessed using the DHARMa package (Hartig, 2019). Raw weight (g) was
232 used as the response variable instead of change in weight (delta-weight) as this allowed us
233 to control for D8 weight in time-lagged models, as well as to differentiate between, for
234 example, very light D8 nestlings achieving normal D15 weight and heavy D8 nestlings
235 achieving heavy D15 weight.

236

237 We examined whether nestling body weight was influenced by the gut microbiome in three
238 different ways: (1) whether the microbiome at an early developmental stage predicted
239 future host weight gain (henceforth 'time-lagged weight'), (2) whether the microbiome at
240 the time of sampling was associated with weight at the same developmental stage
241 (henceforth 'contemporary weight') at D8, and (3) contemporary weight at D15. Due to
242 collinearity (alpha diversity) and non-independence (Phylum-level relative abundance), we
243 performed separate analyses for four different measures of microbiome as explanatory
244 variables: alpha diversity (Shannon diversity and chao1 index), and phylum-level abundance
245 (Proteobacteria and Firmicutes). These two phyla were selected for our analyses because
246 they were the only two taxa that were prevalent across all birds (except for three birds).

247

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

255

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 (i.e. the nestling was not found dead in the nest between D8 and fledging). 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 abundance as the predictor variable, and again with Firmicutes 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 (Jari Oksanen, 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

288 were constrained to within nest with the 'strata' option to control for pseudo-replication of
289 samples from the same nest. Adonis results were very similar with and without this strata
290 option.
291
292 The beta diversity dataset (D8 samples) was filtered by prevalence prior to distance
293 calculation, where taxa with less than 1 copy in 5% of samples were removed following
294 Knowles et al. (2019). This resulted in a dataset containing 1254 ASV's. The relative
295 abundance of each phyla was calculated per sample using 'total sum scaling' (TSS) by
296 dividing each feature count by the total library size (McKnight et al., 2019). Bray Curtis and
297 Jaccard distances were calculated from our TSS normalized data. An assumption of the
298 adonis test is that groups have homogeneity of variance. The dispersion of the groups was
299 checked for homogeneity of variance using the Betadisper and permutest functions from
300 the vegan package.
301
302 ***Indicator ASVs for nestling survival***
303 We carried out indicator species analysis to identify which ASVs were representative of birds
304 that were alive at D8 (n=81) and survived to fledgling (n=64), or did not survive to fledgling
305 (n=17). Indicator analysis identified "indicator species" if they reflected the state of the
306 environment (i.e. likelihood the bird will survive, or not survive). We used the multipatt
307 function from the IndicSpecies package (M. De Caceres, Legendre, P., 2009), which assigns a
308 test statistic, or "indicator value" to each ASV for each group, in our case two groups:
309 Survival-Yes, Survival-No. Taxa with an indicator value of > 0.4 and p <= 0.05 were
310 considered indicator species. We also report the "specificity value" and the "fidelity value".
311 The former is the estimate of the probability that an ASV is detected in a particular group.
312 For example, a value of 1.0 would indicate that the ASV is only detected in that group and
313 not another group. The latter is the probability estimate of finding the species in sites (i.e.
314 birds) belonging to the group. For example, a value of 1.0 would indicate that the ASV is
315 recorded in all birds within that group (De Caceres, 2020). We did not adjust for multiple
316 comparisons for three reasons. First, our analysis did not include any overlap, and thus
317 multiple analyses, across different combinations of groups (De Caceres et al., 2009). Second,
318 the purpose of this analysis was exploratory in order to generate, rather than confirm
319 hypotheses (Bender & Lange, 2001; Ranstam, 2019). As such, we report ASVs as potential

320 indicator species, as opposed to reporting a quantitative, total number of taxa associated
321 with survival/non-survival (De Cáceres et al., 2010). Third, this analysis served to generate a
322 posteriori predictions regarding the relationship between indicator taxa and weight gain
323 (see below), although we note that independent studies will be necessary to confirm these
324 exploratory analyses (Ranstam et al 2019). We generated a heatmap of indicator species for
325 survival and non-survival using the heatmap function from the phyloseq package (McMurdie
326 & Holmes, 2013).
327
328 ***A posteriori analysis of indicator species***
329 Indicator species from our analysis may reflect whether a bird survives or not. We
330 hypothesized that indicator species may reflect host survival because such indicator species
331 may also predict weight gain. We performed a posteriori LMMs with D15 weight as the
332 response variable and D8 relative abundance (of the indicator species) as a fixed variable.
333 D8 weight, habitat, brood size and lay date were also included as fixed variables. Site and
334 nest were included as nested random terms (i.e. 1|site/nest). We restricted this a posteriori
335 analysis to one indicator species that had the highest test statistic from each group
336 (Survival-Yes: Lactobacillus (ASV46) and Survival-No: Rahnella (ASV38)). Because there were
337 a high number of zeros in relative abundance of these taxa, and because our data were
338 proportions and therefore could not be fit using a zero-inflated (count data) model, we
339 included only birds where the taxa were present and the birds with 0 abundance for that
340 taxa were excluded (ASV46: n=21/41; ASV38: n=8/41).
341
342 All graphs were created using using ggplot2 (Wickham, 2016), unless otherwise indicated.
343
344 **Results:**
345 ***Sequencing results***
346 There were 18,537,795 high quality reads, clustered into 49,655 ASV's before removing
347 control samples, mitochondria and chloroplast sequences. Mean reads per sample was
348 88,698 and individual samples ranged from 61 to 579,864 reads. Samples with less than
349 10,000 reads were discarded before further analysis (n=9). There were 15,183,675 total
350 high-quality reads, clustered into 47,400 ASV's after removing control samples as well as

351 mitochondria and chloroplast sequences. Samples ranged from 10,220 to 557,336 reads and
352 mean reads per sample was 77,865.
353
354 The most prominent phyla across all samples (D8 and D15) were as follows (mean
355 percentage relative abundance ±SE): Firmicutes (43.0%±2.5); Proteobacteria (35.2%±2.2);
356 Tenericutes (9.7%±1.8); Bacteroidetes (4.2%±0.4) and Actinobacteria (2.2%±0.2). Relative
357 abundance of the major phyla at D8 (at least >=10% relative abundance in at least 1 sample)
358 per sample are plotted in Figure 1.
359

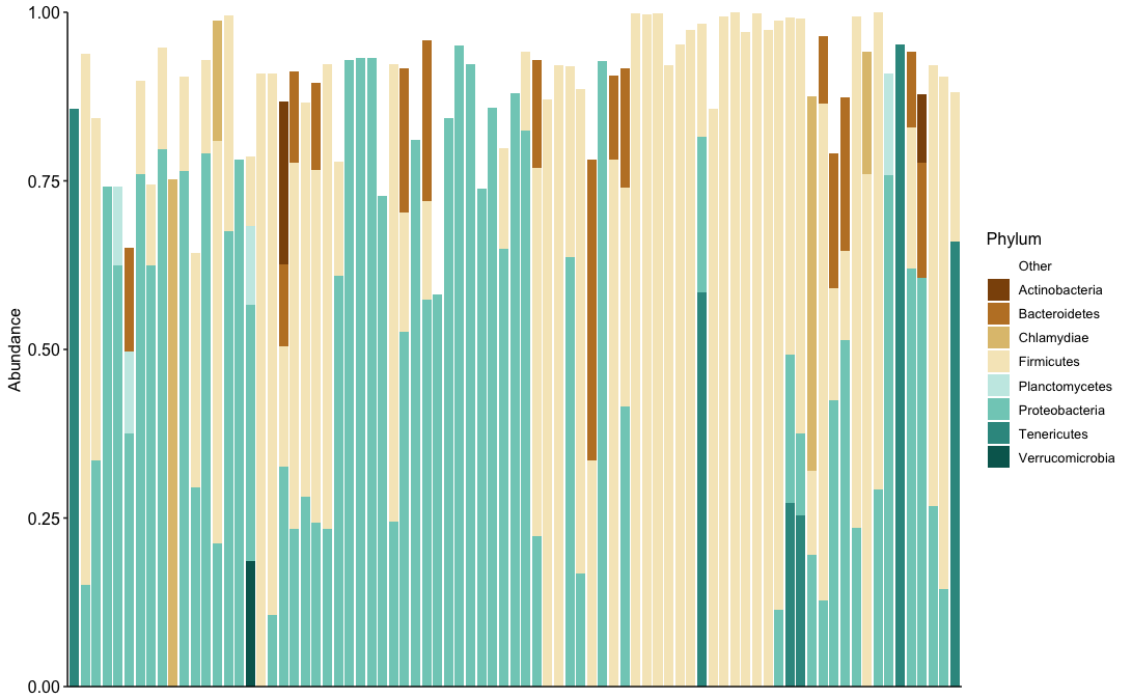

Figure 1. Relative abundance of the 8 most prominent phyla (>=10% relative abundance). Each vertical bar corresponds to a nestling at Day 8. "Other" group represents all low abundance phyla as white space.

360 ***Nestling growth/weight***

361 The time-lagged analysis indicated that weight at D15 was negatively related to alpha
362 diversity at D8 (Figure 2a, Table 1), controlling for weight at D8 (Table 1). Neither habitat,
363 lay date or brood size were significant in this analysis (Table 1). Cross-sectional analyses
364 indicated no relationship between contemporary weight and diversity at either D8 or D15

365 (Figure 2b and 2c, Table S1). Brood size was negatively related to weight at D8. Habitat had
366 a non-significant effect on weight in D15 birds where deciduous birds had lower weight than
367 coniferous birds. The relative abundances of Proteobacteria and Firmicutes did not
368 significantly predict weight in any of these analyses.
369
370
371

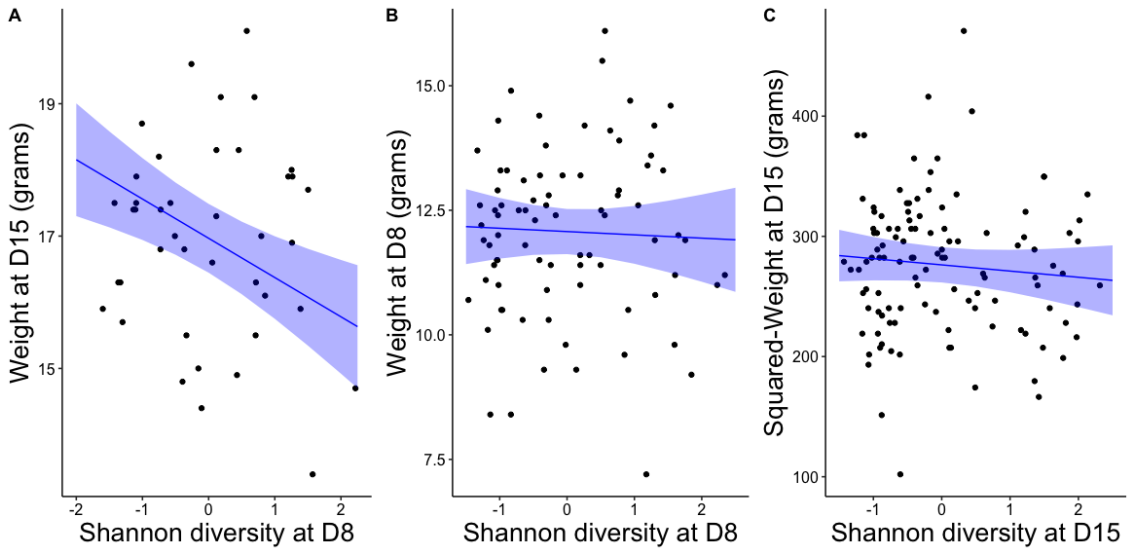

Figure 2. The effect of Shannon diversity on (a) time-lagged weight (i.e. Day 15 (D15)), (b) contemporary weight Day 8 (D8) and (c) contemporary weight D15. Black dots are individual data points, blue line is regression line with 95% CI (shaded blue).

372

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. * $p < 0.05$.

Dependent~Independent variable	Estimate	Std. Error	df	Test statistic	P_estimate
(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

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). The relative abundance of Proteobacteria and Firmicutes at D8 also did not predict survival to fledging.

Tests to verify the assumption of homogeneity of dispersion for survived/not-survived groups were marginally non-significant for Jaccard distance (Sum Squares= 0.009, F=3.295, p=0.081) and non-significant for 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), see Supporting Information 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. Note: all numeric covariates are centered and scaled. * p < 0.05.

Dependent~Independent variable	Coef	HR	se	Test statistic (z)	P_estimate
Nestling mortality risk					
Shannon	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 survival					
Chao1	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

388

389

390 ***Indicator species analysis***

391 The indicator species with the highest indicator values associated with survival belonged to
392 ASV46, ASV310 and ASV150 which all belong to the *Lactobacillaceae* family (Figure 3, Table
393 S4). The taxa with the highest indicator values associated with non-survival belonged to the
394 ASV38, ASV1034, ASV895 which belong to *Enterobacteriaceae*, *Methylopilaceae* and
395 *Acidothermaceae* families, respectively. The taxa indicative of non-survival and survival (p
396 value <=0.05 and test statistic >=0.4) were plotted on a heatmap (Figure 3). Overall,
397 specificity values were high (mean = 0.94±0.008 standard error) and fidelity values were low
398 (0.21±0.008), suggesting that indicator species were typically only detected within a given
399 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).

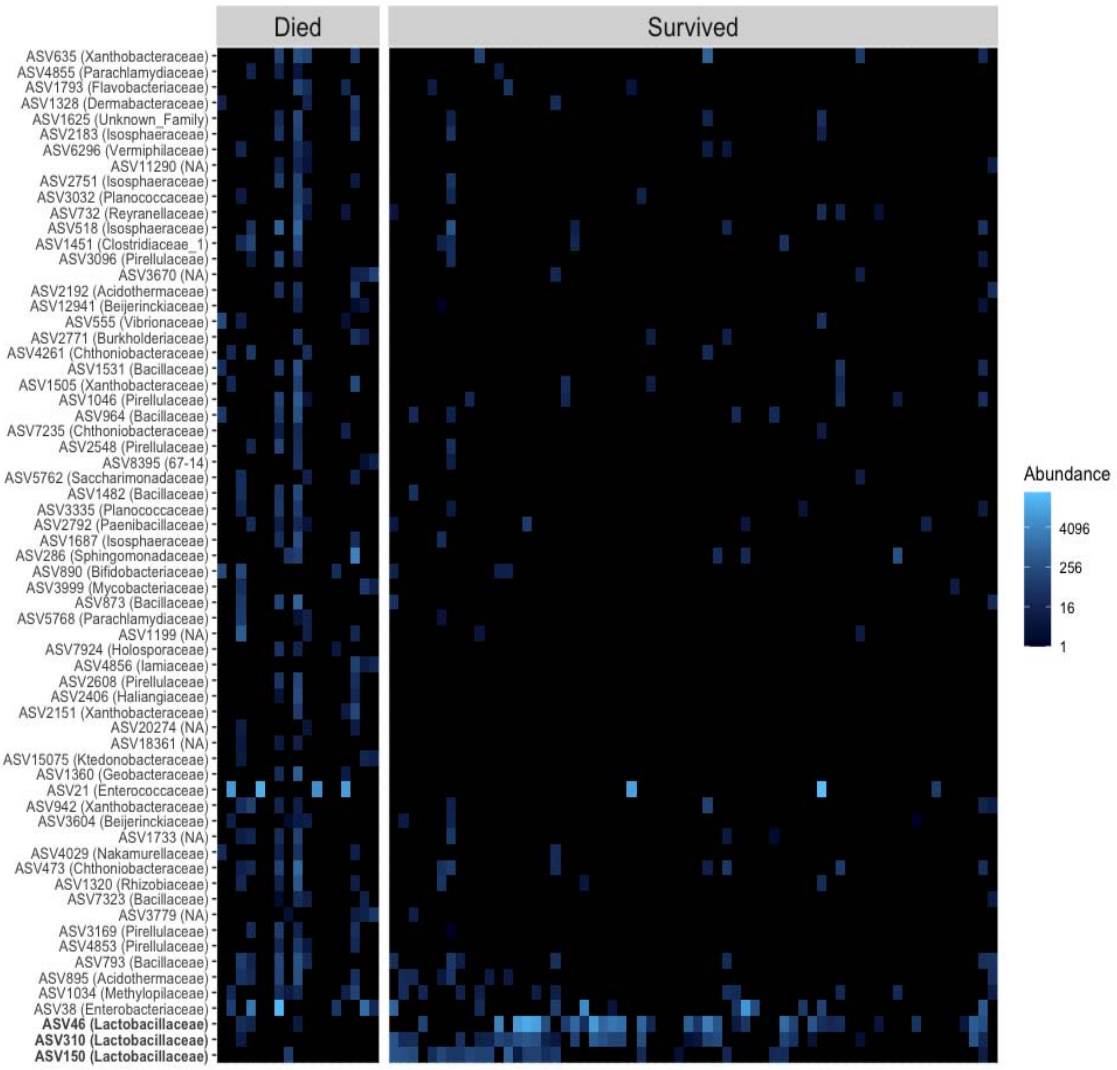

Figure 3. Heatmap of relative abundance of indicator species ASV's for survival. Individual birds are columns with separate panels for individuals that either died or survived. ASVs are in rows where taxa in brackets refer to the ASV's family-level taxonomic assignment, or NA if not resolved to family-level.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

408

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_estimate
(a) D15 Weight (time-lagged)					
(Intercept)	17.673	0.739	4.679	23.906	<0.001 *
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 *
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

Discussion:

Overall summary

We demonstrate that the gut microbiota predicts nestling fitness in a population of wild passerine birds. Neither microbial alpha diversity nor phylum-level abundance predicted contemporary weight, but alpha diversity did predict future weight gain. Moreover, eight-day old nestlings with a greater relative abundance of specific *Lactobacillus* taxa were more likely to fledge, and for at least one of these taxa, greater relative abundance was

specifically associated with increased host weight. Notably, although we show clear links between gut microbiota and 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 (Figure 3) 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). We discuss below how critical developmental windows, environmental matching and the Anna Karenina Hypothesis may explain such differential findings across studies.

We show a negative relationship between alpha diversity and future weight gain. 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 growth 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 host growth, some taxa may have disproportionate effects. At least one ASV, *Lactobacillus* sp., with the strongest potential association with survival, was positively associated with host growth. 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. 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 (Davidson et al., 2020; Gatesoupe, 1999; Holzapfel & Schillinger, 2002).

450 Although a growing number of studies have demonstrated that the microbiome is an
451 important predictor of host weight, the microbial profiles and the direction of this
452 relationship vary across species and studies (Kohl et al., 2018; Potti et al., 2002; Teyssier et
453 al., 2018; Videvall et al., 2019). For example, a negative relationship between alpha diversity
454 and future weight gain was found in juvenile ostriches (Videvall et al., 2019), whereas a
455 positive relationship between alpha diversity and contemporary body condition was
456 reported in a different population of great tit nestlings (Teyssier et al., 2018). The
457 contrasting results reported in these studies and our own may be due to variations in the
458 microbiome sampling method and storage solutions (i.e. cloacal versus faecal, see Videvall
459 et al. (2017); storing solutions see Vargas-Pellicer et al. (2019)). Nevertheless, it is more
460 likely that differences in local environments (Song et al., 2020) and the temporal resolution
461 of microbial and host traits dictate when and how microbiota impacts on host phenotypes
462 in birds (Videvall et al., 2019).
463
464 Time-lagged associations between the gut microbiota and host traits are expected to be
465 especially important during developmental periods when large shifts in the establishing
466 microbial community coincide with the programming of host biology (Cox et al., 2014;
467 Hansen et al., 2012; Sudo et al., 2004), and in systems that are likely to experience abrupt
468 changes in environmental inputs associated with gut microbiome, such as diet. The latter
469 may be especially relevant in birds as their microbiomes are tightly linked with
470 environmental variation, in contrast to mammals where the microbiome is primarily linked
471 to phylogeny (Song et al., 2020). In ostriches, microbiome diversity predicted future weight
472 gain, but the direction of this relationship switched after week 1 post-hatching, a time when
473 ostriches no longer rely on an internal yolk as sustenance (Videvall et al., 2019). In the
474 current study, alpha diversity at D8 predicted future weight at D15, whereas cross-sectional
475 analyses were not sufficient to detect such a relationship. This time-lagged association may
476 be owing to a critical developmental period in which the microbiome impacts on future
477 phenotypes, a phenomenon that is prominent in clinical microbiome literature (Cox et al.,
478 2014; Hansen et al., 2012; Sudo et al., 2004). The gut microbiome may also act as a
479 mechanism for adaptive phenotypic change through its sensitivity to environmental
480 variability, and its connection to host biological processes (Alberdi et al., 2016). Such a
481 mechanism would also be in line with the 'Environmental Matching Hypothesis', which

posits that conditions during development cause phenotypic changes that serve to match individuals to their environments (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 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 on 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. This phenomenon may therefore explain why nestling survival was not associated with either alpha or beta diversity. Moreover, 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 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

514 (Trevelline et al., 2019; West et al., 2019). Our analysis suggests that in the case of non-
515 survival, there are no microbes that are universally indicative of non-survival across birds,
516 but rather the presence of one of several candidate microbes predicts a higher probability
517 of mortality, which is consistent with the Anna Karenina Hypothesis. Whether these same
518 taxa affect survival in other populations and/or other species of birds is unknown; thus
519 these candidate taxa should be examined in independent confirmatory studies. For
520 example, ASVs reported for non-survival may be pathogenic to great tits, although we found
521 no evidence in the literature to support this. Nevertheless, ASV1320, ASV2151, ASV3604
522 and ASV942 are members of the Rhizobiales order which contains genera that are well
523 known human pathogens, and ASV5768 and ASV4855 are members of the Chlamydiales
524 order which also contain pathogenic genera, though these genera were not detected in our
525 study.
526
527 We also encourage further investigations into whether indicator taxa are the cause or
528 consequence of host growth, or indeed any trait that may be associated with survival, such
529 as glucocorticoids (Weber et al., 2018) and immune function (Hõrak et al., 1999).
530 Manipulation of the gut microbiome alongside metabolomics or metagenomics will be the
531 next step in providing insight into the causal direction and functional mechanisms of
532 microbial variation and its relationship with host fitness. The latter is particularly important
533 for identifying whether there is functional overlap between microbial taxa and the
534 mechanisms by which these taxa affect host traits (Heintz-Buschart & Wilmes, 2018). Given
535 the temporal importance of host-microbial relationships identified in this study and
536 elsewhere, we anticipate the maturing microbiome will have important consequences on
537 long-term fitness effects. More generally, our results highlight the importance of the gut
538 microbiome in evolutionary processes as it has the potential to mediate rapid adaptation to
539 environmental change and may also support conservation efforts as an indicator of
540 individual and population health.
541

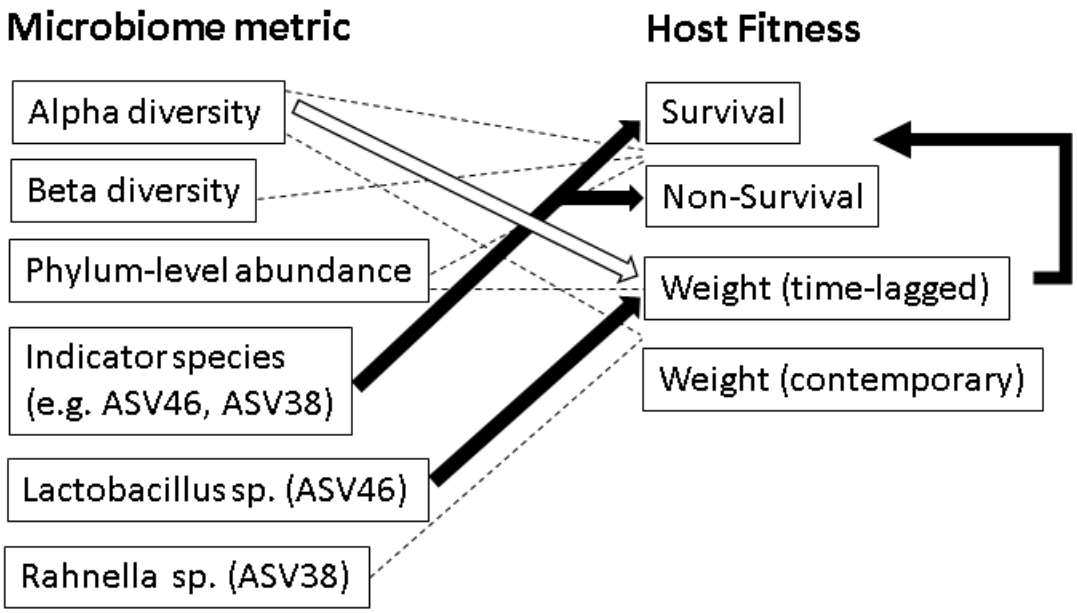

Figure 3. Associations between microbiome metrics and nestling fitness traits. Solid arrows represent positive associations and open arrows represent negative associations. Dashed lines indicate no relationship. For visualization purposes, some lines terminate between two host fitness boxes to indicate that both traits had the same (significant/non-significant) result. Note that the direction of arrows does not suggest causality from our analyses.

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Data accessibility.

Our data will be deposited at Dryad, and sequence data uploaded to a repository such as NCBI Sequence Read Archive, if the manuscript is published.

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.

Author contributions: 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.

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