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ORIGINAL ARTICLE

Gut microbes limit growth in house sparrow nestlings (*Passer domesticus*) but not through limitations in digestive capacity

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Abstract

Recent research often lauds the services and beneficial effects of host-associated microbes on animals. However, hosting these microbes may come at a cost. For example, germ-free and antibiotic-treated birds generally grow faster than their conventional counterparts. In the wild, juvenile body size is correlated with survival, so hosting a microbiota may incur a fitness cost. Avian altricial nestlings represent an interesting study system in which to investigate these interactions, given that they exhibit the fastest growth rates among vertebrates, and growth is limited by their digestive capacity. We investigated whether reduction and restructuring of the microbiota by antibiotic treatment would: (i) increase growth and food conversion efficiency in nestling house sparrows (*Passer domesticus*); (ii) alter aspects of gut anatomy or function (particularly activities of digestive carbohydrases and their regulation in response to dietary change); and (iii) whether there were correlations between relative abundances of microbial taxa, digestive function and nestling growth. Antibiotic treatment significantly increased growth and food conversion efficiency in nestlings. Antibiotics did not alter aspects of gut anatomy that we considered but depressed intestinal maltase activity. There were no significant correlations between abundances of microbial taxa and aspects of host physiology. Overall, we conclude that microbial-induced growth limitation in developing birds is not driven by interactions with digestive capacity. Rather, decreased energetic and material costs of immune function or beneficial effects from microbes enriched under antibiotic treatment may underlie these effects. Understanding the costs and tradeoffs of hosting gut microbial communities represents an avenue of future research.

Key words: antibiotics, food conversion efficiency, gut microbiota, host-microbe interactions, maltase

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INTRODUCTION

In the past few decades, there has been a great deal of attention focused on the role that symbiotic bacteria play in the development, ecology and evolution of hosts.

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For the most part, these associations are thought to be beneficial for hosts, by enhancing digestion or training the immune system (McFall-Ngai *et al*. 2013; Kohl & Carey 2016). However, there is a lack of research into understanding the potential costs or tradeoffs associated with hosting such communities (Mushegian & Ebert 2016).

One metric where hosting a gut microbiota may come at a cost is in early life growth. Germ-free chickens (those lacking a gut microbiota) grow faster than conventional chicks (Forbes & Park 1959; Coates *et al*. 1963), and similar results have been demonstrated for growing germ-free rodents when under slight food restrictions (Snyder & Wostmann 1987). Similarly, administering antibiotics to penguin chicks in the field enhances growth compared to controls (Potti *et al*. 2002). This microbial-induced growth limitation could have impacts on host fitness, given that growth rates and juvenile body size can be strong predictors of survival later in life (Magrath 1991; Rieger 1996). The mechanisms underlying the microbial reduction in host growth are poorly understood.

Altricial nestling birds represent an excellent study system in which to investigate interactions between gut microbes and host growth. Nestling birds exhibit the fastest growth rates among terrestrial vertebrates: roughly twice the rate of eutherian mammals (Case 1978). This growth is fueled by the ability of nestlings to digest and assimilate nutrients provisioned by their parents (Ricklefs *et al*. 1998; Karasov & Wright 2002). At hatching, nestlings have proportionately larger intestines compared to adults, and intestinal tissue exhibits accelerated growth compared to the rest of the body (Gille *et al*. 1999; Caviedes-Vidal & Karasov 2001; Karasov & Wright 2002). Ingested food material is digested by a series of digestive enzymes (e.g. carbohydrases: maltase and sucrase; peptidases: aminopeptidase-N), which hydrolyze macronutrients into monomers that can be absorbed across the gut lining (Karasov & Martinez del Rio 2007). The activities of carbohydrase enzymes are responsive to changes in diet (Brzęk *et al*. 2009, 2011), presumably allowing nestlings to better digest and absorb nutrients from changing food sources. Despite these digestive adaptations for fueling early-life growth, it is hypothesized that the growth of altricial nestlings is limited by digestive capacity (Ricklefs *et al*. 1998; Karasov & Wright 2002).

Gut microbes may interact with digestive physiology to limit growth in developing birds. Germ-free mice inoculated with the gut microbiota from cold-acclimated mice exhibit longer small intestines with longer villi and microvilli when compared to germ-free mice inoculated with gut microbes from mice held at room temperature (Chevalier *et al*. 2015), demonstrating that microbes can influence gut anatomy. Regarding digestive function, germ-free rats exhibit 2.5× higher maltase activities than conventional rats, and this activity is reduced upon inoculation with a conventional microbiota (Reddy & Wostmann 1966). If the presence of gut microbes alters gut anatomy, digestive enzyme activities or the responsiveness of digestive enzymes to dietary changes, there could be implications for nestling growth.

Here, we designed an experiment to investigate the interactions between gut microbes and aspects of growth and digestive physiology in altricial nestling house sparrows (*Passer domesticus* Linnaeus, 1758). When house sparrows hatch, their parents provide them with protein-rich insects and bring them increasing amounts of carbohydrate-rich plant material (largely seeds; Anderson 2006). Through development, wild nestling house sparrows increase the activities of carbohydrase enzymes, correlating with the increasing amount of starch in their natural diet (Caviedes-Vidal & Karasov 2001). The activities of these digestive enzymes are flexible and responsive to diet (Brzęk *et al*. 2009, 2011). For example, in the laboratory, nestlings upregulate intestinal carbohydrase enzymes within 24 h of a switch to a high carbohydrate diet (Rott *et al*. 2017).

In the current experiment, we reared house sparrow nestlings in captivity on a high protein diet, and switched a group of them to a high-starch diet for 24 h. Subsets of animals in each group were given antibiotics to reduce and restructure the gut microbiota. We predicted that treatment with antibiotics would increase growth and food conversion efficiency in nestling house sparrows, consistent with results in other young birds (Coates *et al*. 1963; Potti *et al*. 2002). It has been hypothesized that the activities of digestive enzymes may be dependent on the abundance and composition of gut microbial communities (Reddy & Wostmann 1966). Therefore, we also hypothesized that antibiotics would alter digestive enzyme activities and influence the responsiveness of carbohydrases to the 24-h diet switch. We acknowledge that in addition to reducing and restructuring the microbiota, antibiotic treatment may have its own direct effects on host physiology. However, there are no established methods for generating germ-free altricial nestlings. We will address our findings in the context of both the microbiota and the direct effects of antibiotics in the Discussion.

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MATERIALS AND METHODS

Animal collection, feeding and growth parameters

Nestlings were collected from natural and artificial nests located on the University of Wisconsin – Madison campus. Nests were checked daily to determine hatch date, and nestlings were marked with indelible marker to track their age. The day nestlings were found was considered day 0. At 3 days of age, nestlings were brought into the laboratory, where they were fed artificial diets. Nestlings were housed individually in round (12 × 9-cm) tissue-lined plastic containers and housed in an environmental chamber under constant conditions of 15:9 h light : dark photoperiod, 35°C, and 40–45% relative humidity using a water bath system. All procedures were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee under protocol #A005514.

Birds were assigned to 1 of these 4 different schedules: (A) 4 days on a high protein (HP) diet ($N = 9$); (B) 3 days on the HP diet and then switched to the high carbohydrate (HC) diet for 24 h ($N = 12$); (C) same as A, with the addition of neomycin mixed into food at a concentration of 0.5 g/L wet food ($N = 9$); or (D) same as B with the addition of neomycin mixed into food at a concentration of 0.5 g/L wet food ($N = 8$). Food composition can be found in Table 1. Nestlings were syringe-fed every hour 15 times per day. Meal sizes were calculated using age-specific energy requirements. Meal sizes for nestlings consuming the HP diet were 0.64, 0.88, 1.04 and 1.20 mL for nestlings aged 3–6 days post-hatch, respectively. Meal sizes were 0.79 mL for nestlings fed the HC diet on day 6 post-hatch. Birds collected from the same nest were placed in different treatments as often as possible, and only group B contained a pair of siblings. After completion of this schedule, animals were euthanized as described below. Dry matter content of food was measured during the feeding trials by collecting a food sample of our artificial diets each day between 1100 and 1600 hours, measuring the wet mass, drying samples overnight at 60°C, and then dividing the dry mass by the wet mass. The amount of wet food consumed at each meal was measured by weighing syringes before and after feeding. This value was converted to dry matter intake using the average dry matter content of food (measured from food samples), and then summed over the 3.5 days in captivity. Nestlings were weighed 3 times daily (0615, 1330 and 2030 hours). Mass gain over the course of the experiment was determined by subtracting the beginning mass (measured at 1330 hours at 3 days old) from the ending mass (measured at 2030 hours at 6 days old). Food conversion efficiency was calculated as the amount of mass gained divided by the amount of dry food consumed during the experiment. Mass gain and food conversion efficiencies were compared with analysis of variance (ANOVA) with type III sum of squares, using diet, antibiotic treatment and an interaction term as the main variables.

Table 1 Composition of diets used in the present study

Diet composition (% dry mass)	High carbohydrate	High protein
Corn starch	38	5
Casein	27	60
Corn oil	8	8
Essential nutrients[†]	10	10
Inert ingredients	17	17
kJ/g dry mass	14.8	14.8

[†]Essential vitamins and minerals.

Sample collection

Nestlings were euthanized using CO_2, and dissected to remove the intestines. We collected contents of the distal portion of the intestine to inventory the "luminal" microbiota (techniques for microbiome analysis described below).

The intestine was flushed with ice-cold avian Ringer solution, blotted dry, weighed, and length was measured with calipers. The distal 2 cm of the intestine were cut and frozen to later inventory the adherent "mucosal" microbiota. The intestine was then cut open longitudinally and laid flat with the mucosa facing up. With a scalpel, a longitudinal strip of approximately 2-mm width was cut from the entire length of the gut, from the proximal to the distal ends of the intestine, and frozen for later enzymatic analysis. Similar longitudinal strips were collected for other purposes not discussed in this manuscript (gene expression, proteomics). All samples were frozen immediately in liquid nitrogen and stored at −80°C.

Enzyme assays

Activities of maltase, sucrase and aminopeptidase-N were measured as described elsewhere (Brzęk *et al*. 2009; Kohl *et al*. 2011). Briefly, tissues were homoge-

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nized and incubated with relevant substrate. Enzymatic products (glucose for the carbohydrase enzymes and p-nitroaniline for the APN assay) were measured spectrophotometrically, and activity was determined using a standard curve.

Enzyme activities were compared across treatments with ANOVA with type III sum of squares, using diet, antibiotic treatment and an interaction term as the main variables, and nest of origin as a random effect. We investigated date of hatch as a covariate but it was insignificant for all factors tested, so was removed from final analyses. In addition, maltase activity is conducted by both the maltase-glucoamylase and sucrase-isomaltase enzymes. To investigate the relationship between these enzymes to measured maltase activity, we investigated the effects of diet, antibiotic treatment and an interaction term on maltase activity, and included sucrase activity as a covariate. The contribution of sucrase-isomaltase to maltase activity can be determined by multiplying the mean sucrase activity for each group by the slope of the relationship between sucrase and maltase activity, and dividing it by the mean maltase activity (Biviano *et al.* 1993).

Microbiome analyses

Total DNA was extracted from samples using the MoBio PowerFecal DNA Isolation Kit. Extracted DNA was sent to Argonne National Laboratory for sequencing. The primers *515F* and *806R* were used to amplify the V4 region of the 16S rRNA gene (Caporaso *et al*. 2012). Amplicons were sequenced on the Illumina MiSeq platform using previously described techniques (Caporaso *et al*. 2012).

Microbial sequences were analyzed using the program QIIME version 1.9.1 (Caporaso *et al*. 2010). We applied standard quality control settings and split sequences into libraries using default parameters in QIIME. Sequences were grouped into operational taxonomic units (OTUs) using the open-reference method (He *et al*. 2015) and minimum sequence identity of 99%. The most abundant sequences within each OTU were designated as a "representative sequence" and aligned against the Greengenes core set (DeSantis, Hugenholtz *et al*. 2006) using PyNAST (Caporaso *et al*. 2009) with default parameters set by QIIME. FastTree (Price *et al*. 2009) was used to generate a phylogenetic tree of representative sequences. Taxonomic classification of OTUs was performed using UCLUST (Edgar 2010). Singleton OTUs and sequences identified as chloroplasts or mitochondria were removed from the analysis.

We calculated several aspects of alpha diversity for each sample. For each alpha diversity metric, we calculated the mean of 20 iterations for a subsampling of 480 sequences for luminal samples and 100 sequences for mucosal samples. These numbers were based on the minimum number of sequences returned from each sample type, and have been shown to be sufficient for differentiating microbial communities (Caporaso *et al*. 2012). First, we measured the number of observed OTUs in the randomly chosen subsamples. We also calculated evenness, a metric measuring how similar the relative abundances of various OTUs are to one another (Rousseau & Van Hecke 1999). In a perfectly even community, all OTUs would be present at similar relative abundances. The Shannon index was calculated, which is a metric that incorporates both richness (the number of observed OTUs) and evenness (Shannon & Weaver 1949). Finally, we calculated Faith's phylogenetic diversity (Faith 1992), which measures the cumulative branch lengths of the randomly chosen subset of sequences on a phylogenetic tree of all OTUs. Alpha diversity metrics were compared within a sample type (lumen or mucosa) using ANOVA with diet, antibiotic treatment and an interaction term as the main variables.

Microbial community membership and structure were compared by conducting principal coordinates analysis (PCoA) on unweighted and weighted UniFrac distances (Lozupone & Knight 2005). Microbial community membership only takes the presence and absence of microbial OTUs into account, while microbial community structure incorporates their relative abundances. Distance matrices and PCoA plots were made using the number of sequences depending on the analysis (see above). The adonis function in R (Ihaka & Gentleman 1995) was used to compare distance matrices with 999 permutations (the default number), using diet, antibiotic treatment and an interaction term as the main variables (Clarke 1993).

Next, we compared the relative abundances of microbial taxa across treatments. Relative abundances of phyla, families and genera in each individual sample were transformed using a variance stabilizing transformation of arcsin (abundance$^{0.5}$) (Shchipkova *et al*. 2010; Kumar *et al*. 2012). Then, we used JMP, Version 12.0 (SAS Institute, Cary, NC, USA) to compare relative abundances of bacterial phyla and genera using the Response Screening function to conduct multiple ANOVAs using diet, antibiotic treatment and an interaction term as the main variables, and corrected *P*-values with the Benja-

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mini–Hochberg false discovery rate (FDR) correction (Benjamini & Hochberg 1995). We conducted similar analyses using host mass gain, food conversion efficiencies or enzyme activities as covariates to investigate connections between abundances of microbial taxa and host measurements

Finally, we were interested in comparing mucosal and luminal communities. Here, we only used samples where we had successful paired microbial inventories from both luminal and mucosal communities in the same individual. To compare alpha diversity metrics, we conducted analysis of covariance (ANCOVA) on mucosal diversity, using diet and antibiotic as main variables and luminal diversity measurements as a covariate. We also conducted linear regressions between luminal and mucosal measurements. To compare abundances of taxa between luminal and mucosal samples, we conducted analyses similar to those presented above, including individual as an additional variable.

RESULTS

Antibiotics enhance nestling growth and depress maltase activity

Nestlings given antibiotics gained roughly 12% more mass over the course of the experiment compared to those without antibiotics, (Fig. 1a; antibiotic effect: $F_{1,34} = 4.57$; $P = 0.039$). There were no effects of diet nor any interaction on mass gain (Diet effect: $F_{1,34} = 0.12$; $P = 0.73$; antibiotic × diet: $F_{1,34} = 0.19$; $P = 0.66$). A graph depicting mass gain over the course of the experiment can be found in Supplementary Figure S1. Nestlings treated with antibiotics exhibited significantly higher feed conversion efficiencies (Fig. 1B, antibiotic effect: $F_{1,34} = 5.25$; $P = 0.03$), while there was no effect of diet or any interaction on this metric (diet effect: $F_{1,34} = 0.13$; $P = 0.72$; antibiotic × diet: $F_{1,34} = 0.37$; $P = 0.55$). There were no significant effects of diet or antibiotic treatment on aspects of gut anatomy (intestinal mass or length) when including body mass as a covariate ($P > 0.05$ for all effects).

Both diet and antibiotics influenced digestive enzyme activities. Mass-specific maltase activities were upregulated by 20% after feeding on the HC diet for 24 h, while antibiotics depressed maltase activities by 9.2% (Fig. 2a, Table 2). There was no significant interaction between diet and antibiotics for maltase activity. Sucrase activities of birds fed the HC diet for 24 h= were 3.2× higher than birds maintained on the HP diet (Fig. 2b, Table 2), with no effects of antibiotics, nor any interaction. Aminopeptidase-N activity was not significantly affected by diet or antibiotics, although activity tended to be slightly lower in birds fed the HC diet and those given antibiotics (Fig. 2c and Table 2). Finally, we investigated the contribution of the enzyme sucrase-isomaltase to overall maltase activity. Using analysis of covariance, we found a significant relationship between sucrase and maltase activity (Fig. 3; slope: 16.06; $F_{1,32} = 22.40$; $P < 0.0001$), a significant effect of antibiotics (fixed-effect coefficient: 1.75; $F_{1,32} = 7.65$; $P = 0.009$), but no significant effect of diet (fixed-effect coefficient: 0.03; $F_{1,32} = 0.0004$; $P = 0.98$). There was a significant antibiotic ×

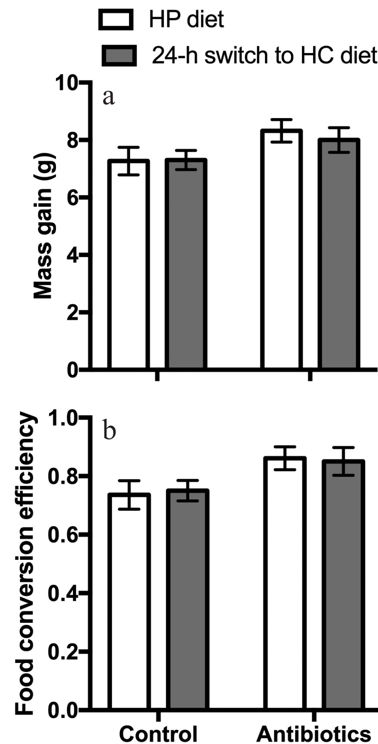

Figure 1 Mean (a) mass gain and (b) food conversion efficiencies for nestlings subjected to different diet and antibiotic treatments. HC, high carbohydrate; HP, high protein. Mass gain over the course of the experiment was determined by subtracting the beginning mass (measured at 1330 hours at 3 days old) from the ending mass (measured at 2030 hours at 6 days old). Food conversion efficiency was calculated as the amount of mass gained divided by the amount of dry food consumed during the experiment. Post-hoc analysis with Tukey's honest significant difference revealed no differences between group means. Bars represent means ± SEM.

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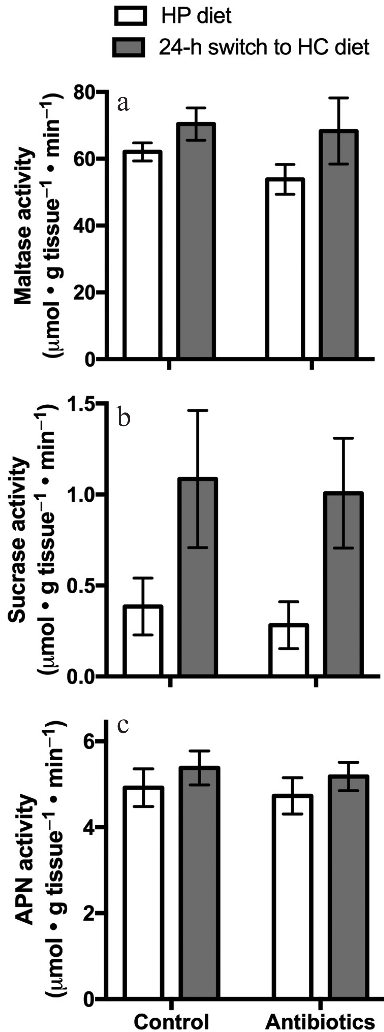

Figure 2 Mass specific activities of (a) maltase, (b) sucrase and (c) aminopeptidase-N from the small intestines of nestlings subjected to 2 diet treatments and antibiotic treatments. Bars represent means ± SEM. HC, high carbohydrate; HP, high protein.

sucrase interaction effect on maltase activity (Fig. 3; interaction coefficient: −5.65; $F_{1,32}$ = 14.80; P = 0.0005), but no diet × sucrase interaction (interaction coefficient: 1.86; $F_{1,32}$ = 0.29; P = 0.59). Using the methods of Biviano *et al.* (1993), we determined that sucrase-isomaltase made a greater contribution to total maltase activity in the antibiotic treated nestlings (32.2% of maltase activity in birds switched to HC diet, 11.4% in birds fed the HP diet) compared to birds not treated with antibiotics (17.2% in birds switched to the HC diet and 6.8% for the HP group).

Table 2 Summary from ANOVA of mass-specific enzyme activities

	Sum of squares	*F*	*P*
Maltase			
Diet	1207.34	34.44	<0.0001
Antibiotics	248.17	7.44	0.011
Interaction	88.47	0.87	0.36
Error	1165.45		
Sucrase			
Diet	4.73	49.42	<0.0001
Antibiotics	0.08	1.04	0.32
Interaction	0.001	0.004	0.95
Error	2.54		
Aminopeptidase-N			
Diet	1.92	1.89	0.18
Antibiotics	0.36	0.09	0.76
Interaction	0.0002	0.009	0.92
Error	53.32		

Nest was included as a random effect. Degrees of freedom are 1,34 for all analyses.

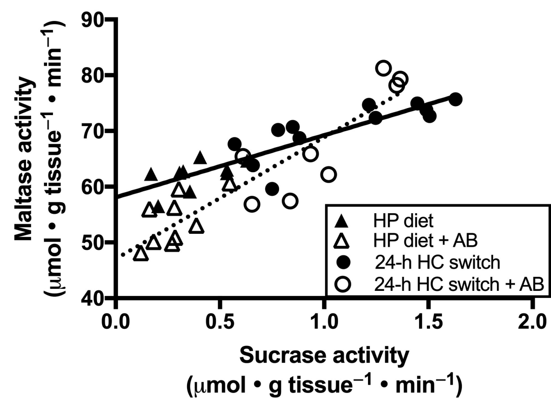

Figure 3 Correlation of mass-specific sucrase and maltase activities from the small intestines of nestling house sparrows. Lines are linear regressions of control or antibiotic-treated individuals (diet groups were combined for this analysis).

Antibiotics alter microbial communities

Sequence return was higher for luminal samples (average: 14 267 sequences/sample; median: 13 233) compared to mucosal samples (average: 2437; median: 328). There were no effects of diet or antibiotics on the number of sequences per sample (P > 0.05 for both effects).

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A number of mucosal samples had low sequence return (less than 100 sequences), and, thus, had to be removed from the analysis. Therefore, the final sample sizes for mucosal samples were as follows: HP diet: 7; HP diet + antibiotics: 6; 24 h HC switch: 9; 24 h HC switch + antibiotics: 5.

There were no significant effects of diet or antibiotics on measurements of alpha diversity of the luminal microbiota ($P > 0.05$ for the Shannon index, Faith's phylogenetic diversity, observed OTUs and evenness). In the mucosal communities, antibiotic treatment resulted in a 1.5× higher measurement of Faith's phylogenetic diversity (Fig. S1, antibiotic effect: $F_{1,23} = 4.95$; $P = 0.036$), while there was no effect of diet nor any interaction (diet effect: $F_{1,23} = 0.38$; $P = 0.54$; antibiotic × diet: $F_{1,23} = 0.08$; $P = 0.78$). There were no effects of antibiotics or diet on the Shannon index, number of observed OTUs or evenness of the mucosal microbiota.

Antibiotic treatment significantly altered the microbial community membership and structure of both the luminal and mucosal microbial communities (Fig. 4, Table 3). There were no microbial phyla that differed sig-

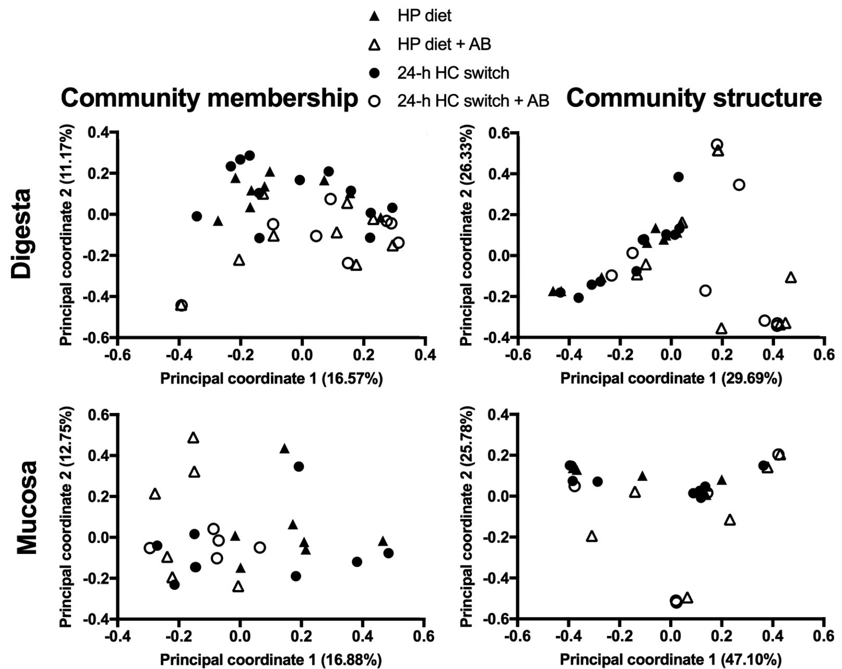

Figure 4 Principal coordinate graphs of microbial communities from the digesta and mucosa of nestling house sparrows. Community membership uses unweighted UniFrac distances, which investigates presence/absence of microbial taxa. Community structure uses weighted UniFrac distances, which takes relative abundances of microbes into account. AB, antibiotics; HC, high carbohydrate; HP, high protein.

Table 3 Summary of adonis results for luminal and mucosal microbial communities

	Community membership				Community structure			
	SSE	*F*	R^2	*P*	SSE	*F*	R^2	*P*
Luminal								
Diet	0.21	0.79	0.021	0.77	0.06	0.33	0.008	0.90
Antibiotics	0.70	2.62	0.069	0.001	1.20	6.28	0.154	0.001
Interaction	0.16	0.61	0.016	0.98	0.04	0.22	0.005	0.95
Mucosal								
Diet	0.31	1.04	0.039	0.37	0.04	0.28	0.011	0.90
Antibiotics	0.55	1.87	0.069	0.009	0.41	2.80	0.106	0.045
Interaction	0.29	0.99	0.037	0.46	0.06	0.41	0.016	0.77

Degrees of freedom are 1,34 for luminal samples and 1,23 for mucosal samples. SSE, sum of squared errors.

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nificantly in relative abundances as a result of diet or antibiotic treatment. A single microbial genus, *Lactococcus*, was significantly more abundant in the luminal communities of birds treated with antibiotics (38.4% ± 9.9% of community) compared to control birds (1.1% ± 0.5%; FDR-corrected P = 0.008). In mucosal communities, the relative abundance of *Lactococcus* exhibited the same pattern (25.4% ± 9.4% of community in antibiotic-treated birds, 0.7% ± 0.2% in control birds), although this result was not significant after correction for multiple tests. No other genera were differentially abundant in mucosal communities. The family Streptococcaceae (which contains *Lactococcus*) was significantly more abundant in the mucosal communities of antibiotic-treated birds (FDR-corrected P = 0.041).

There were no significant correlations between relative abundances of microbial taxa (luminal or mucosal) and host mass gain, food conversion efficiency or digestive enzyme activities.

Luminal and mucosal microbial communities differ significantly

Antibiotics altered the relationship between luminal and mucosal microbial communities. For example, when analyzing paired samples of luminal and mucosal microbial communities, there was a significant correlation between the number of OTUs observed in the lumen and mucosa (Fig. 5; luminal OTUs' effect: slope: 0.41; $F_{1,21}$ = 6.32; P = 0.02). As stated above, neither diet nor antibiotics affected the number of observed OTUs in the mucosa. However, there was a significant antibiotic × luminal OTU effect (interaction coefficient: 0.35; $F_{1,21}$ = 4.48; P = 0.046), such that there was a positive relationship between luminal and mucosal diversity in control animals, but no such relationship in antibiotic-treated animals. The diet × luminal OTU effect was not significant (interaction coefficient: 0.01; $F_{1,21}$ = 0.01; P = 0.93).

These trends were also demonstrated for other diversity metrics. When the data were subsetted by antibiotic treatment, control animals exhibited significant or near-significant correlations between luminal and mucosal diversity for all alpha diversity metrics (Shannon index: Parameter estimate, 0.56, R^2 = 0.38, P = 0.011; Faith's phylogenetic diversity: Parameter estimate, 0.44, R^2 = 0.20, P = 0.08; observed OTUs: Parameter estimate, 0.76, R^2 = 0.58, P = 0.0007; Evenness: Parameter estimate, 0.53, R^2 = 0.33, P = 0.019), while diversity did not correlate in the antibiotic-treated animals (P > 0.75 for all metrics).

The gut lumen, in comparison to the mucosa, was significantly enriched in a single phylum Actinobacteria (luminal community: 6.7% ± 2.4%; mucosal: 1.2% ± 0.6%; FDR-corrected P = 0.007), specifically the genus *Rothia* (luminal community: 6.1% ± 2.3%; mucosal: 0.4% ± 0.2%; FDR-corrected P = 0.008).

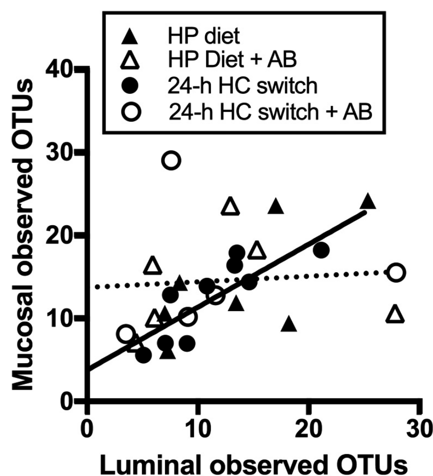

Figure 5 Correlation of the number of observed operational taxonomic units (OTUs) in luminal and mucosal communities of nestling house sparrows. Lines are linear regressions of control or antibiotic treated individuals (diet groups were combined for this analysis). AB, antibiotics; HC, high carbohydrate; HP, high protein.

DISCUSSION

Here, we investigated whether microbial growth limitation in developing birds is driven by microbial effects on digestive physiology. Treating developing house sparrow nestlings with neomycin significantly increased growth and food conversion efficiency. However, antibiotics did not affect aspects of digestive physiology in a manner that would support the idea that the growth effects are mediated through interactions with digestive capacity. Therefore, we hypothesize that enhanced growth under antibiotic treatment is driven by other mechanisms, such as lower energetic costs of immune defenses or enhanced beneficial effects from bacteria enriched under antibiotic treatment. We discuss our findings below.

Treatment with antibiotics increased growth and food conversion efficiency in developing house sparrow nestlings. These results are consistent with what has been demonstrated in growing chickens (Forbes & Park 1959; Coates *et al*. 1963) and penguins (Potti *et al*. 2002). To

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our knowledge, the current study is the first to demonstrate similar antibiotic growth promotion in an altricial or passerine nestling. In poultry, the growth-promoting effects of antibiotics are thought to be driven through effects on the microbiota, given that antibiotics increase growth in conventional chicks but have no growth-promoting effect in germ-free chicks (Forbes & Park 1959; Coates *et al*. 1963).

Overall, we did not find any evidence that microbial-induced growth limitation was driven by interactions with digestive physiology. There were no effects of antibiotics on gut mass or length. Intestinal maltase and sucrase activities were both significantly increased when nestlings were switched to the high-carbohydrate diet for 24 h, consistent with previous studies (Brzęk *et al*. 2009, 2011). There were no significant diet × antibiotic interaction effects on carbohydrase activities, suggesting that microbes do not influence the responsiveness of digestive enzymes to dietary changes. Antibiotic treatment significantly decreased intestinal maltase activities, with no significant effects on sucrase or APN activities. Similar reductions in maltase as a result of antibiotics have been demonstrated in previous studies in rodents (Marano *et al*. 1969; Madge 1970), although other studies in rodents, pigs and chickens have found no effects of antibiotics on maltase activities (Casanovas & Torralba 1977; Thymann *et al*. 2007; Lee *et al*. 2011a,b), or even increases (Lee *et al*. 2011b). One would predict that the reductions in maltase activity should reduce digestion and growth in nestlings, but these nestlings actually exhibit enhanced growth and food conversion efficiency. Thus, we hypothesize that other mechanisms underlie this growth enhancement, which will we discuss below.

Antibiotic treatment differentially affected the carbohydrase activities we investigated, such that maltase activity was decreased but there was no significant effect on sucrase activities. These results, together with the ANCOVA of maltase and sucrase activities, suggest that antibiotics significantly affect the maltase-glucoamylase enzyme, but not the sucrase-isomaltase enzyme. Further research is needed to understand the mechanisms by which these enzymes are differentially inhibited by antibiotics. Treating germ-free mice with antibiotics can alter intestinal gene expression, thus representing a direct effect of antibiotic compounds (Morgun *et al*. 2015). However, maltase-glucoamylase was not found to be differentially expressed in this experiment (Morgun *et al*. 2015).

Antibiotics significantly altered microbial membership and structure. These results are consistent with studies in other systems, such as chickens (Dumonceaux *et al*. 2006; Pedroso *et al*. 2006) and rodents (McCracken *et al*. 2001; Kohl *et al*. 2014), which also demonstrate that antibiotics alter gut microbial community structure. We were unable to detect any significant correlations between abundances of microbial taxa and aspects of host mass gain or food conversion efficiency. Previous studies in chickens have aimed to identify microbial members that might be associated with growth performance. For example, when comparing the microbiota of chickens with high or low food conversion efficiencies, those with high food conversion efficiencies were enriched in the genera *Escherichia*, *Shigella* and *Salmonella* (Singh *et al*. 2014). Similarly, a large-scale study of chickens on different diet treatments found a positive association between the presence of *Escherichia* in the gut of chickens and their food conversion efficiencies (Torok *et al*. 2011). The particular functions or benefits of hosting microbial taxa may vary across host species, which may explain why we did not identify such a connection between the abundance of *Eschericia* and food conversion efficiency in our study. Alternatively, our limited sample sizes may have prevented us from detecting statistical significance. Here, our sample sizes were 5–9 individuals per group, while studies on growing chickens have sample sizes >100 individuals (Torok *et al*. 2011; Singh *et al*. 2014). However, it should be noted that hand-rearing altricial nestlings is much more labor-intensive than raising precocial chicks.

Given our lack of evidence that microbial interactions with digestive capacity underlie the enhanced growth, these differences must be driven by other mechanisms. The potential mechanisms for antibiotic growth promotion have been excellently reviewed elsewhere (Visek 1978; Dibner & Richards 2005). Again, antibiotics do not promote growth in germ free animals (Forbes & Park 1959; Coates *et al*. 1963), suggesting that these effects are mediated through the microbiota. One possibility is that reduction in the microbiota reduces competition for nutrients between the host and gut microbes (Vervaeke *et al*. 1979). In addition, reductions in the gut microbiota may lower the nestlings' energetic costs of defending themselves against microbes, such as lowered immune responses or lessened mucus secretion and epithelial cell shedding (Dibner & Richards 2005). Finally, bacteria that are enriched under antibiotic treatment could drive enhanced food conversion (Dibner & Richards 2005). In our study we observed an increased abundance in the genus *Lactococcus* in antibiotic-treated nestlings. This microbial genus has been used as

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a probiotic to enhance growth in a variety of animals (Harzevili *et al*. 1998; Timmerman *et al*. 2006; Heo *et al*. 2013), and, thus, may drive the enhanced growth observed in our study.

Antibiotic treatment also seemed to disrupt the relationship between the luminal and mucosal microbial communities. The current study and other previous research demonstrates that the lumen and mucosa harbor distinct microbial communities (Lu *et al*. 2014; Malmuthuge *et al*. 2014). It has been hypothesized that the mucosal microbiota may be more stable in the face of perturbation when compared to the luminal community (Donaldson *et al*. 2016). Indeed, antibiotic treatment explained more variation in microbial community structure in the lumen than in the mucosa. In addition, luminal communities exhibited larger increases in *Lactococcus* abundances compared to the mucosal communities. These differential responses resulted in the decoupling of mucosal and luminal microbial diversity observed in the antibiotic-treated animals. It would be interesting to study the recovery of mucosal and luminal communities from antibiotic perturbation.

In summary, treatment with antibiotics enhances growth of altricial nestlings, although this effect is likely not mediated through enhanced digestive capacity. Hosting a diverse and abundant microbiota seems to come at a cost for developing birds, limiting their growth. Given that juvenile body size is predictive of later survival (Magrath 1991; Rieger 1996), microbes could impact host fitness. However, it must be recognized that microbes may have many other functions during early development. For example, gut microbes are imperative in the training of the immune system (Round & Mazmanian 2009), which is still developing during the nestling period (Killpack *et al*. 2013). Therefore, limited microbial diversity or abundance may cause hosts to be more susceptible to pathogens later in life. For example, frogs that were reared in sterile water as tadpoles are more susceptible to infection by parasitic worms in adulthood (Knutie *et al*. 2017). Understanding the costs and tradeoffs associated with hosting microbial communities represents a frontier for the field of host–microbe interactions.

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SUPPLEMENTARY MATERIALS

Figure S1 Mass gain of nestlings subjected to different diet and antibiotic treatments. HC, high carbohydrate; HP, high protein. Mass gain over the course of the experiment was determined by subtracting mass at the beginning mass (measured at 13:30 hours at 3 days old) from the mass at a given time. Nestlings were dissected in the morning of day 7 post-hatch. Bars represent means ±SEM.

Figure S2 Faith's phylogenetic diversity of the mucosal microbial communities from nestling House Sparrows subjected to various diets and antibiotic treatments. Bars represent means ± SEM.

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