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Letter

Coprophagy rapidly matures juvenile gut microbiota in a precocial bird

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Abstract

Coprophagy is a behavior where animals consume feces, and has been observed across a wide range of species, including birds and mammals. The phenomenon is particularly prevalent in juveniles, but the reasons for this remain unclear. One hypothesis is that coprophagy enables offspring to acquire beneficial gut microbes that aid development. However, despite the potential importance of this behavior, studies investigating the effects in juveniles are rare. Here we experimentally test this idea by examining how ingestion of adult feces by ostrich chicks affects their gut microbiota development, growth, feeding behavior, pathogen abundance, and mortality. We conducted extensive longitudinal experiments for 8 weeks, repeated over 2 years. It involved 240 chicks, of which 128 were provided daily access to fresh fecal material from adults and 112 were simultaneously given a control treatment. Repeated measures, behavioral observations, and DNA metabarcoding of the microbial gut community, both prior to and over the course of the experiment, allowed us to evaluate multiple aspects of the behavior. The results show that coprophagy causes (a) marked shifts to the juvenile gut microbiota, including a major increase in diversity and rapid maturation of the microbial composition, (b) higher growth rates (fecal-supplemented chicks became 9.4% heavier at 8 weeks old), (c) changes to overall feeding behavior but no differences in feed intake, (d) lower abundance of a common gut pathogen (*Clostridium colinum*), and (e) lower mortality associated with gut disease. Together, our results suggest that the behavior of coprophagy in juveniles is highly beneficial and may have evolved to accelerate the development of gut microbiota.

Keywords: coprophagia, fecal microbiota transplant, bacteriotherapy, microbiome development, ostrich, offspring

Lay Summary

The behavior of fecal feeding (coprophagy) is widespread among animals, yet our understanding of its causes and consequences is extremely limited. Here we experimentally tested the effects of coprophagy on the development of ostrich chicks by providing treatment groups with adult feces collected daily. We examined how coprophagy influenced the gut microbiome, juvenile growth, feeding behavior, pathogen abundance, and survival over the first 8 weeks of development. Fecal-supplemented individuals readily engaged in coprophagia, which resulted in a rapid maturation of their gut microbiome. Compared to control groups, which had no access to adult feces, treatment groups experienced a drastic increase in microbial diversity and their microbial composition rapidly shifted towards that of adults. Coprophagy also resulted in a higher juvenile growth rate, without increasing feed intake, and reduced mortality associated with gut bacterial infection. Our results suggest that the behavior of coprophagy in juveniles may have evolved to accelerate the development of gut microbiota. These findings have broad implications for animal husbandry and captive breeding programs of wildlife, and contribute to an improved general understanding of how host-associated microbes influence the evolution of animal behavior.

Introduction

Coprophagia is a behavioral trait of animals where individuals feed on fecal matter. It can be divided into the consumption of an individual's own feces (autocoprophagy), the feces of conspecifics (allocoprophagy), or the feces from other species (heterospecific coprophagy). In vertebrates, coprophagy is frequently observed in dogs, pigs, horses, and mammalian orders such as lagomorphs, rodents, and primates (Hirakawa, 2001; Sakamaki, 2010; Soave & Brand, 1991). Allocoprophagy in wild animals commonly involves juveniles feeding on the feces of adults, which has been proposed to possibly aid nutrient extraction through food redigestion and promote the acquisition of beneficial intestinal symbionts (Barnes, 1962; Ebino, 1993; Hörnicke & Björnhag, 1980; Troyer, 1982). A commonly known example is the koala, where

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offspring ingest their mother's fecal pellet in order to gain the bacteria needed for the digestion of eucalypt leaves (Blyton et al., 2022; Osawa et al., 1993). In birds, coprophagy is most often observed in herbivorous precocial species, such as ptarmigans, quails, and turkeys (Kobayashi et al., 2019; McWhorter et al., 2009; Scupham et al., 2008; Wilkinson et al., 2016), though exceptions involving heterospecific coprophagy exist among vultures (Negro et al., 2002).

While coprophagia in vertebrates has been opportunistically documented by observing animals (e.g., Leggett, 2004), studies investigating the consequences of engaging in such behavior on the gut microbial community are scarce and limited to a few species. Preventing coprophagy in rodents, rabbits, and pigs has been shown to reduce body mass, both increase and decrease food intake, and diminish the diversity and abundance of specific microbes (Aviles-rosa et al., 2019; Bo et al., 2020; Bogatyrev et al., 2020; Cree et al., 1986; Fitzgerald et al., 1964; Klaasen et al., 1990; Sukemori et al., 2006; Ushakova et al., 2008). Fecal transplantation, either through coprophagy or orally administrating selected bacteria (Barnes et al., 1980), has also been discussed as a potential method for improving the health of animals in veterinary and wildlife conservation contexts (Bornbusch et al., 2021; Guo et al., 2020; Niederwerder, 2018). For example, hand-reared kākāpō chicks are sometimes fed frozen fecal material from adults, but whether this practice alters the juvenile gut microbial community is still unknown (Waite et al., 2013).

Despite the potential importance of coprophagy for animal developmental across diverse species, experimental evidence of how it influences juvenile microbiota, growth, and survival is limited. In 1982 it was shown that juvenile iguanas hatched in captivity grew faster when they were fed adult feces (Troyer, 1982), but the cause at the time was unknown, with "microflora" being suggested as a possible explanation. With modern sequencing techniques enabling the characterization of microbial communities, there have been a handful of studies investigating the microbiota of offspring in relation to coprophagy. Combes et al. (2014) observed a reduced abundance of Ruminococcaceae and a greater abundance of Bacteroidaceae in rabbit juveniles that did not have access to maternal feces, and Bo et al. (2020) similarly found that preventing coprophagy caused changes in the gut microbiota of voles, although adults were the focus of this study. Kobayashi et al. (2019) studied the development of gut microbiota in wild ptarmigans known to engage in fecal consumption, however, without control groups preventing access to maternal feces, it was not possible to discern the specific role of coprophagia. Recently, Xiang et al. (2020) orally administered a suspension of probiotics and fecal material from sows to piglets and detected increased microbial diversity and decreased levels of inflammation in treated individuals. While their study did not evaluate coprophagic behavior per se, it provided a rare glimpse into the potential benefits of adult feces in animal development. These studies provide preliminary evidence of the potential influence of coprophagy on juvenile gut microbiota in vertebrates. However, our current understanding of why this behavior has evolved and what consequences it has for juvenile growth and survival, is very limited.

Here, we experimentally test whether coprophagy of adult feces by juveniles affects (a) gut microbiota maturation, (b) growth, (c) feeding behavior, (d) pathogen abundance, and (e) mortality. Ostriches (*Struthio camelus*) provide a great opportunity for investigating these questions as chicks regularly feed on adult fecal matter in captivity and the wild (Amado et al., 2011; Deeming, 1999), and the development of gut microbiota is associated with both juvenile growth and survival (Videvall et al., 2019, 2020). Additionally, ostrich chicks are precocial and amenable to being raised independently from parents under standardized conditions, which facilitates experimental manipulation and systematic sampling.

We monitored a total of 240 individuals for 8 weeks after hatching. Chicks were separated into 30 groups, each consisting of 8 individuals to simulate natural brood sizes (Bertram, 1992). Fecal treatment groups (n = 16) were provided with trays of freshly collected adult feces daily and control treatment groups (n = 14) were simultaneously provided with empty trays. Shortly after hatching (week 0) before experimental treatments started, we measured body weight and collected fecal samples from all individuals. After introducing adult feces, chicks were weighed during weeks 1, 2, 3, 4, 6, and 8, and fecal samples from weeks 1, 2, 4, and 8 were sequenced (16S rRNA gene) to characterize gut microbiota. We also sequenced the microbiota of 23 adult fecal samples. Behavioral observations were conducted throughout the experiment to monitor feeding rates, and feed intake per group was measured once per week by feed weigh-back. The experiment took place during two consecutive years which allowed us to increase sample sizes and account for potential annual variation (n = 120 in 2016, n = 120 in 2017). Importantly, our balanced experimental design allowed us to control for environmental, dietary, and genetic effects (see Methods, section Experimental setup for details).

Results and discussion

Coprophagy rapidly increases the diversity of gut microbiota

Coprophagy had a dramatic effect on gut microbiota diversity. Prior to the experiment (age week 0), the microbial diversity was low and did not differ between control and treatment groups (Figure 1; general linear model (GLM): treatment, richness p = .94, Shannon diversity p = .82, phylogenetic diversity p = .62; Supplementary Table S13). However, within just one week after access to adult feces were provided, fecal-supplemented chicks had acquired a much higher gut microbial diversity than control chicks, and showed a significantly faster maturation of the microbiota (Figure 1; linear mixed model (LMM): treatment × age^2, richness: $F_{1, 439}$ = 20.05, p < .0001; Shannon diversity: $F_{1, 439}$ = 6.76, p = .01; Supplementary Tables S14–S15). This pattern was particularly prominent for phylogenetic diversity (Figure 1; LMM: treatment × age^2, $F_{1, 439}$ = 47.22, p < .0001; Supplementary Table S16), indicating that the microbial community of chicks engaging in coprophagy contained substantially more phylogenetically distinct taxa than control chicks.

Coprophagy shifts the community composition of gut microbiota

The composition of the microbiota of fecal-supplemented chicks was significantly different from control chicks and remained differentiated throughout the entire experimental period (Figure 2; permutational multivariate analysis of variance (PERMANOVA): treatment, R^2 = 0.16, p = .001; Supplementary Table S2). These large structural differences were evident across all distance metrics analyzed (Jaccard, Bray-Curtis, UniFrac, weighted UniFrac; see Supplementary Figures S4–S7). Changes in the gut microbial community associated with coprophagy were apparent even at higher taxonomic ranks, such as class and order (Supplementary Figures S8–S10). Access to adult feces also had a significant interaction with age, showing that the effects of coprophagy

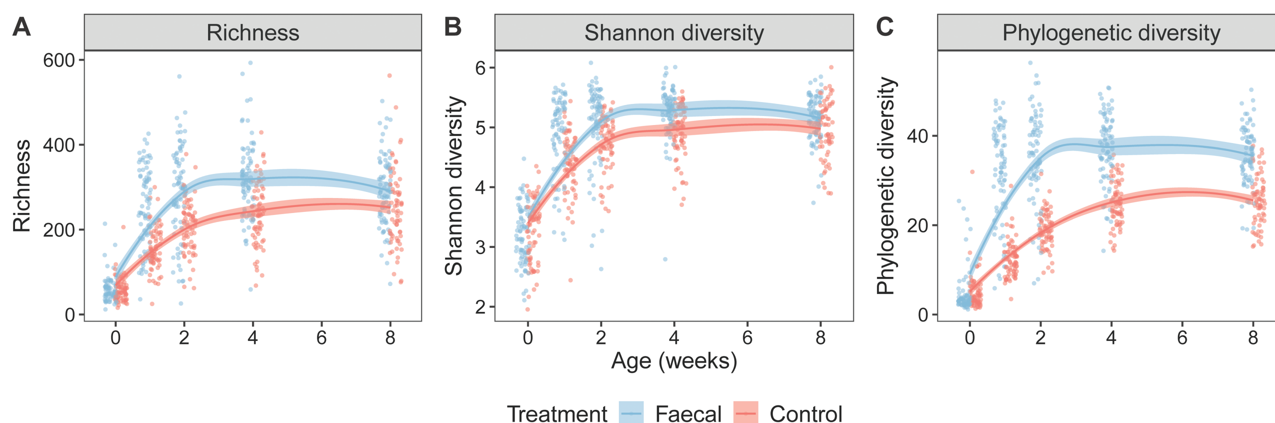

Figure 1. Coprophagy results in a rapid increase of gut microbial diversity in juvenile ostriches. (A) Richness (number of unique ASVs), (B) Shannon's diversity index, (C) Phylogenetic diversity. Lines display loess curves, shaded areas the 95% confidence interval, and each point represents a unique sample. Blue color = fecal treatment, red = control treatment. The experiment started after week 0 sampling. For associated plots showing adults and individual chicks, see Supplementary Figures S2–S3.

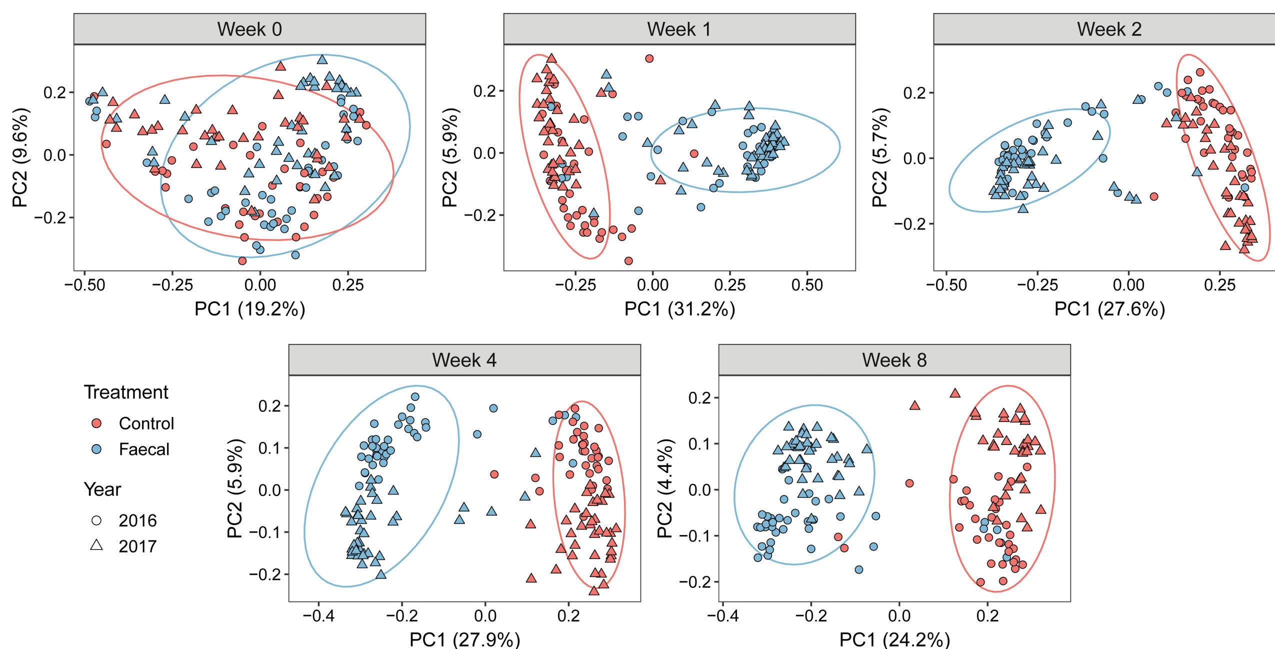

Figure 2. Coprophagy rapidly shifts community composition of the gut microbiome during development. Principal Coordinates Analysis (PCoA) plots of unweighted UniFrac distances between samples show no differences in gut microbiota prior to the experiment (week 0) and large differences between fecal treatment (blue) and control treatment (red) during the experiment (weeks 1 to 8). Each symbol portrays a unique sample, ellipses show 90% confidence intervals, and headers give ages in weeks. For PCoA graphs of other beta diversity metrics, please see Supplementary Figures S4–S7.

intensified through development (Figure 2; PERMANOVA: age × treatment, $R^2 = 0.02$, $p = .001$; age^2 × treatment, $R^2 = 0.01$, $p = .001$; Supplementary Tables S2–S4). These results demonstrate that the microbiomes of fecal-supplemented chicks were distinct, not only in community membership, but also in their composition, phylogenetic structure, and taxonomic abundance.

Coprophagy rapidly matures gut microbiota during development

To determine if the gut microbiota of fecal-supplemented individuals matured faster than control individuals, we evaluated them in relation to the microbiota of adults. Differences in microbial phylogenetic diversity (PD) between adults and chicks rapidly decreased with age in fecal-supplemented individuals, approaching adult-like diversity (Figure 3A). Beta diversity distances between chicks and adults also showed that the microbial composition of fecal-supplemented chicks approached that of adults more rapidly than control chicks (Figure 3B; generalized linear mixed model (GLMM) of UniFrac distances: treatment × age, $\chi^2 = 13.29$, $p = .0003$; Supplementary Figures S11–S14; Supplementary Table S17). Amplicon Sequence Variants (ASVs) shared between adults and chicks showed that pretreatment individuals (week 0) were highly dissimilar to adults, sharing only 7–8% of all ASVs that week (Supplementary Figure S15). However, following the start of the treatment, fecal-supplemented chicks rapidly increased in the number of ASVs they shared with adults (33% of all ASVs at week 1). In contrast, the increase in ASVs that control chicks shared with adults occurred much more slowly (still 7% at week 1; Figure 3C; Supplementary Figure S15).

Coprophagy depletes microbes associated with early life while increasing the abundance of adult-associated microbes

One week after the start of the experiment, fecal-supplemented chicks had 318 differentially abundant ASVs ($q < .01$) compared to control chicks (Supplementary Table S5). The number of ASVs with differential abundances rose to 455 during week 2 and to 427 during week 4, but decreased to 202 during week 8 (Supplementary Tables S6–S8). Two-thirds of all these differentially abundant ASVs (65.8%) were more abundant in fecal treatment groups compared to control groups, corresponding with their pattern of higher alpha diversity. However, taxa typically associated with young age in ostriches (e.g., *Akkermansia*, *Blautia*, and *Dorea*; Supplementary Table S11) were substantially underrepresented in fecal-supplemented chicks (Figure 4). For example, several ASVs of *Akkermansia muciniphila* were highly prevalent in control chicks at 1 week of age, whilst completely absent in chicks of the same age in the fecal treatment groups (mean abundance = 0; Supplementary Table S5). Conversely, genera highly associated with adults, such as

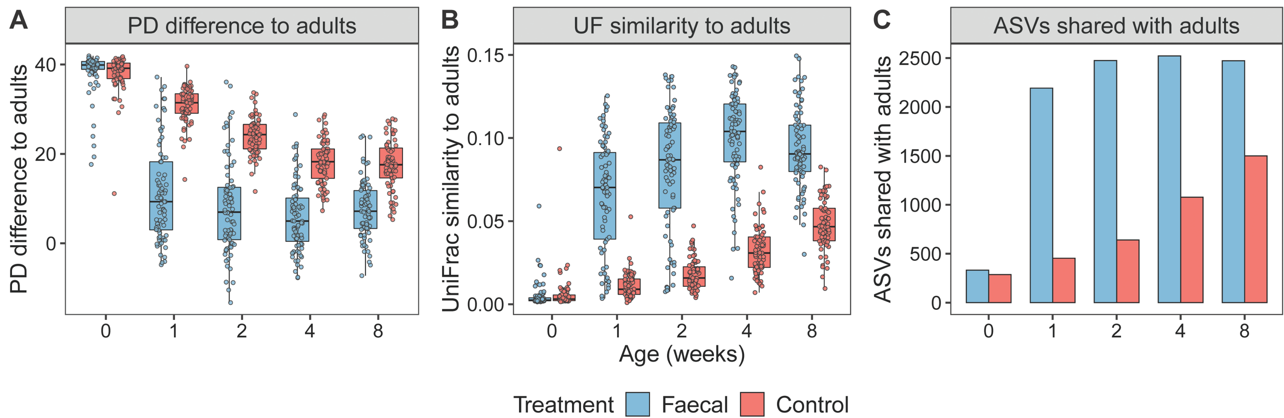

Figure 3. Coprophagy during development results in a gut microbiota more similar to that of adults. (A) Differences in phylogenetic diversity (PD) and (B) UniFrac (UF) similarity (1-distance) between chicks and the average of all adult samples. (C) Number of ASVs shared between chicks and adults. Week 0 = pretreatment. See Supplementary Figures S9–S12 for additional visualizations.

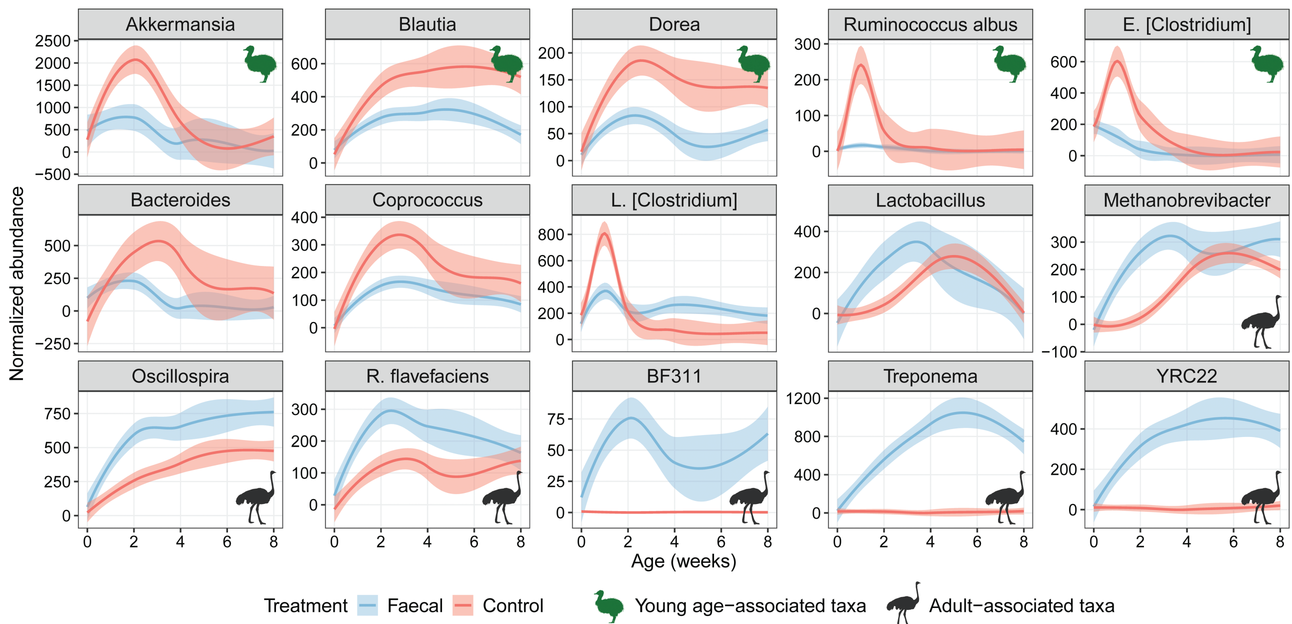

Figure 4. Coprophagy increases the abundance of adult-associated microbes and depletes microbial taxa associated with young age. Panels display genera with the most significantly differentiated ASV abundances across ages. Abundances are normalized against library size and lines show loess curves with 95% confidence intervals. The first 5 taxa are associated with young age (early life) and marked with an ostrich chick in green color. The last 6 panels show adult-associated taxa and are highlighted with a grey adult ostrich. Chicks in the control treatment (red color) have higher abundances of young age-associated taxa whilst fecal-supplemented chicks (blue color) have higher abundances of adult-associated microbes. Fecal treatment started after week 0. Ruminococcus albus and *Ruminococcus flavefaciens* are visualized separately because they showed highly distinct abundance patterns in adults and young chicks, respectively. The genus [*Clostridium*] within the Erysipelotrichaceae family is denoted with the letter "E." and [*Clostridium*] within Lachnospiraceae is labeled with "L.". The taxa shown belong to the phyla Verrucomicrobia (*Akkermansia*), Bacteroidetes (*Bacteroides*, *BF311*, YRC22), Euryarchaeota (*Methanobrevibacter*), Spirochaetes (*Treponema*), and Firmicutes (*Blautia*, [*Clostridium*], *Coprococcus*, *Dorea*, *Lactobacillus*, *Oscillospira*, *Ruminococcus*). Note that some genera, such as L. [*Clostridium*], represent multiple species and ASVs, of which only some were differentially abundant. For data on all differentially abundant ASVs, see Supplementary Tables S5–S8.

Oscillospira, *Treponema*, *Methanobrevibacter*, *BF311*, and *YRC22* (Supplementary Table S11), were more abundant in fecal-supplemented chicks compared to control chicks throughout the experiment (Figure 4).

Coprophagy-induced microbial change promotes growth without increasing feed intake

It is known that gut microbiota can affect offspring growth rates, for example, by modulating nutrient absorption and utilization, influencing immune system development, and producing hormones that regulate appetite and energy metabolism (Gensollen et al., 2016). In chickens, differences in gut microbiota have been associated with variation in feed conversion ratios and body weight (Stanley et al., 2013). Consistent with this research, we found that ostrich chicks provided with adult feces had higher growth rates than control chicks (Figure 5A; LMM: treatment × age, $F_{1,223} = 9.9$, $p = .002$; Supplementary Table S18). Consequently, by 8 weeks of age, fecal-supplemented chicks were on average 9.4% heavier (mean ± *SD*: fecal treatment, 10.90 ± 2.32 kg; control, 9.96 ± 2.21 kg; Supplementary Figure S16). Shortly after hatching, the fecal-supplemented chicks were by random chance 0.03 kg heavier on average (GLM: fecal, 0.87 kg, control, 0.84 kg; $p = .02$; Figure 5A). However, this minor difference in starting weight does not affect the growth rate (treatment × age interaction), which estimates whether the rate of weight increase over time differs between the treatments.

Not all nutrients are absorbed by the gastrointestinal tract, resulting in feces containing a small fraction of energy (~3% of total ingested energy in chickens (Masood et al., 2011)). As a consequence, the increased chick growth rate could theoretically be caused by fecal nutrients rather than the altered microbiota. We therefore evaluated whether attributes of the microbiota could predict the future growth rate of chicks and found that the rate of weight increase at early ages was strongly associated with the phylogenetic diversity of the gut microbiome (LMM: PD × age, $F_{1,545} = 53.01$, $p < .0001$; Supplementary Figure S18; Supplementary Table S19). This effect was much stronger in control chicks, particularly at 2 weeks of age, whereas microbial diversity had already plateaued by week 2 among fecal-supplemented chicks, masking any relationship with weight (LMM: treatment × PD × age, $F_{1,542} = 3.83$, $p = .05$; Supplementary Figure S18). As a result, low phylogenetic diversity of gut microbes was associated with slower growth rates and coprophagy appears to ameliorate this by elevating the diversity of chicks' gut microbiota.

Despite having higher growth rates, fecal-supplemented chicks did not consume more feed than control chicks (Figure 5B; LMM: treatment, $F_{1,31} = 0.52$, $p = .48$; Supplementary Table S20). They also spent a similar amount of time feeding on feed during development (Figure 5C; LMM: treatment × age, $F_{1,231} = 0.96$, $p = .33$; Supplementary Table S21). However, when including time spent feeding on supplemented feces, chicks in the treatment groups spent significantly more time feeding in total (on feed + feces) compared to control chicks (Figure 5D; LMM: treatment, $F_{1,27} = 10.48$, $p = .003$; Supplementary Table S23). As with growth rates, changes in feeding behavior were related to shifts in microbial diversity as individuals aged (LMM: feeding rate on feed: age × PD, $F_{1,454} = 5.99$, $p = .01$; total feeding rate: age × PD, $F_{1,445} = 6.11$, $p = .01$; Supplementary Tables S22 and S24). In control groups, young chicks with low microbial diversity

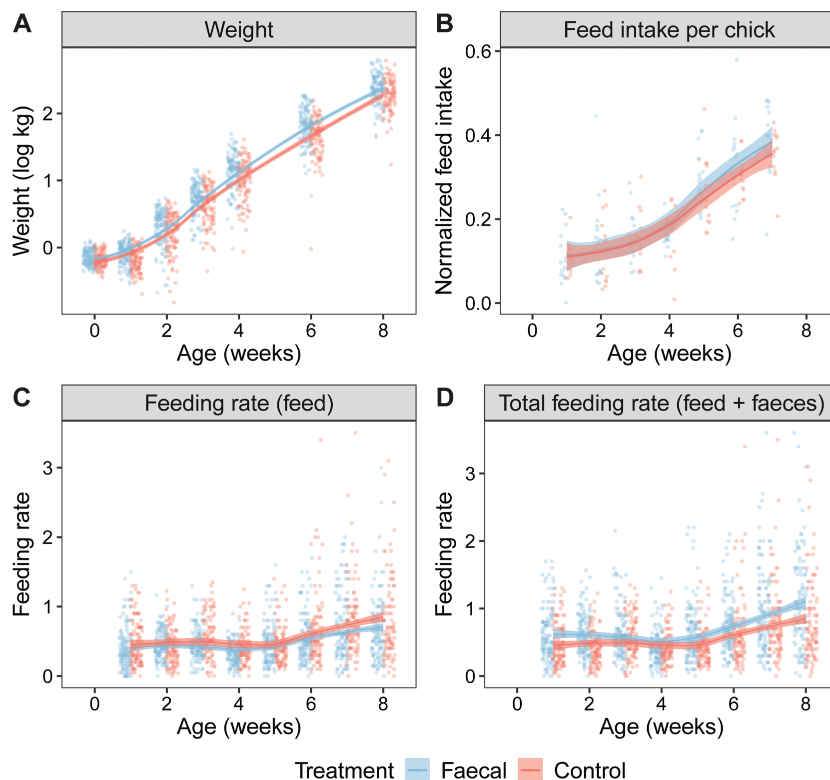

Figure 5. Coprophagic ostrich chicks grow faster without increasing feed intake. (A) Weight (log kg) during development. (B) Normalized feed intake per chick (kg), measured once per week and group, and divided by the number of individuals in the group. (C) Feeding rate (number of times individuals were observed feeding on feed divided by total minutes observed). (D) Total feeding rate, which includes feeding rate on feed + feeding rate on adult feces for the fecal treatment groups. Lines display loess curves and shaded areas denote the 95% confidence interval. Week 0 = pretreatment.

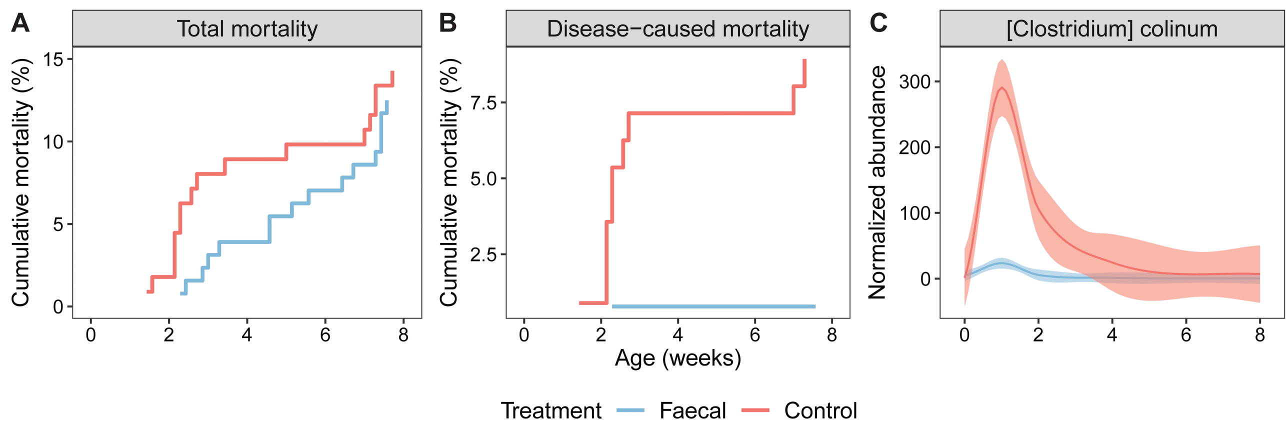

Figure 6. Coprophagy reduces mortality caused by gut disease. Cumulative step-wise lines show (A) all mortality events and (B) mortality caused by gastrointestinal disease per day during development. (C) Abundance (normalized against library size) of [*Clostridium*] *colinum* (Lachnospiraceae), a common bacterial pathogen known to cause enteritis in poultry. Lines display loess curves and shaded areas denote the 95% confidence interval. Week 0 = pretreatment. For group- and individual-level plots of *C. colinum* abundances, see Supplementary Figure S21.

spent less time feeding than chicks with more diverse gut microbiomes, whereas the feeding rates of individuals in treatment groups were largely independent of gut microbial diversity (Supplementary Figures S17 and S19–S20; Supplementary Tables S22 and S24). Together, these results suggest that the increased diversity of gut microbiota caused by coprophagy may alter the efficiency with which feed is converted to body mass and in turn, feeding behavior.

Coprophagy lowers the abundance of a common pathogen and reduces mortality caused by gut disease

Chick mortality rates were relatively low during the 2 years when the experiment was performed (13%; Figure 6A), and there were no signs of large-scale disease outbreaks that are frequently observed in ostriches (Keokilwe et al., 2015; Videvall et al., 2020). In total, 32 chicks died due to injury, disease, and leg deformities, and fecal-supplemented and control chicks had similar overall mortality rates ($n_{fecal} = 16/128$, $n_{control} = 16/112$; Figure 6A). However, when data were restricted to individuals that died from gastrointestinal diseases ($n = 11$), such as enteritis and omphalitis, control chicks had significantly higher mortality rates than fecal-supplemented chicks (Figure 6B; $\chi^2 = 7.30$, $p = .007$). Only a single death was associated with gut disease in the fecal treatment groups, whereas there were 10 deaths in the control groups attributed to gut diseases (Figure 6B). This difference in mortality corresponds with the study by Combes et al. (2014), who found that mortality was highest in rabbit juveniles without access to maternal feces. Gut microbiomes with higher diversity are generally associated with health and pathogen resilience (Lozupone et al., 2012), and our previous work has shown that low gut microbial diversity is strongly associated with both gut dysbiosis and mortality in ostrich chicks (Videvall et al., 2020).

To investigate whether there were any differences in the abundance of pathogens between treatments, we searched for bacterial species specifically known to cause gut disease in poultry and ratites. We identified one pathogen in our data with sufficient numbers of sequences: [*Clostridium*] *colinum* (family Lachnospiraceae; normalized read count, mean: 44.3, range: 0–1713.8). This species comprised several ASVs that were all significantly more abundant ($q = 3.44 \times 10^{-6}$) during 1 and 2 weeks of age in the control chicks compared to chicks in the fecal treatment (Figure 6C; Supplementary Tables S5–S6). We also investigated this pathogen on a group level during its peak at week 1 and found that it was widespread among control chicks, with 11 out of 14 control groups harboring higher average abundances of *C. colinum* than all 16 treatment groups (Supplementary Figure S21).

Clostridium colinum is a gram-positive bacterium commonly known to cause ulcerative enteritis in poultry (Prescott, 2016). It is a possible culprit behind the higher disease-related mortality rate in control chicks, but it is difficult to ascertain whether *C. colinum* was the sole cause of death, especially as we do not have sequence data from the deceased chicks at the time of death. Our previous investigation of ostrich chicks dying from enterocolitis showed that there was not just a single pathogen to blame, but instead an overall state of microbiome dysbiosis associated with higher mortality (Videvall et al., 2020). Consequently, it is possible that the consumption of adult feces provides health-related benefits that increase chick survival, such as diversified training of the immune system and increased resilience against pathogens and pathobionts.

Sex was included in all our models, but we did not observe any differences between males and females in growth, feeding rates, mortality, microbiome diversity, and microbiome composition (Supplementary Tables S14–S16, S18–S19, S21–S24). This result corroborates other evidence from ostriches showing that there is no sexual dimorphism in body mass during early development (Cilliers et al., 1995) and aligns with our previous findings related to juvenile microbiota and growth (Videvall et al., 2019).

Conclusion

Experimental evidence of the effects of coprophagy on gut microbiota and juvenile health has been severely lacking. Our study shows that the consumption of adult feces causes rapid maturation of gut microbiota in ostrich chicks. Multiple features of the microbial community were altered by coprophagia, including a major increase in diversity, shifts in taxonomic composition, phylogenetic structure, and reduced pathogen (*C. colinum*) abundance. Altogether, coprophagy by juveniles resulted in a rapid approximation toward adult gut microbiota and these microbial changes were associated with elevated growth and reduced disease-related mortality, without increasing feed intake. These differences were maintained throughout the

study period, and potentially longer, as 8-week-old fecal-supplemented chicks still maintained differentiated microbiomes compared to controls.

Some of the ingested microbes may be transient adult taxa passing through the gut, while others may establish persistent populations. The gradual shift in microbial communities towards that of adults, however, implies that consuming adult fecal matter causes persistent directional changes in microbial communities as an effect of accelerated microbiota maturation during development. If the gut microbiome of chicks had consisted primarily of transient taxa from the adults, we would have seen an instantaneous adult-like microbiota in the chicks already at week 1, and not the gradual maturation and development over time that the results demonstrate. Furthermore, the phenotypic differences measured in the fecal-supplemented chicks, including increased growth and higher survival, suggest a shift in the established chick gut microbiota. These different lines of evidence point to coprophagy altering the processes of colonization and maturation of gut bacteria during juvenile development.

The offspring of precocial animals often forage on similar food items as their parents in the wild (Starck & Ricklefs, 1998). It is therefore possible that coprophagy represents a form of vertical microbial transmission that enables juveniles to more quickly and accurately develop a microbial community tailored to digesting locally available food (Marinier & Alexander, 1995). In addition, it has been suggested that juveniles experience a critical window early in development when the immune system is being trained to tolerate important members of the microbiota (Metcalf et al., 2022). The fact that coprophagy in offspring is often seen during a limited time during ontogeny (Amado et al., 2011; Kobayashi et al., 2019; Osawa et al., 1993) adds credence to the hypothesis that the behavior evolved to acquire important microbes early in life. Consequently, consuming the feces of adults appears, for some species, to be a critical part of healthy juvenile development. These findings contribute to a better understanding of how host-associated microbes can influence the evolution of animal foraging behavior, with important implications for the health of both captive and wild species.

Methods

Experimental setup

A total of 240 ostrich chicks hatched from eggs that were artificially incubated at the Western Cape Department of Agriculture's ostrich research facility in Oudtshoorn, South Africa. Procedures were approved by the Departmental Ethics Committee for Research on Animals of the Western Cape Department of Agriculture, reference no. R13/90. We replicated the experiment during 2 years, 2016 and 2017. Each year, 120 individuals were distributed into 8 treatment groups and 7 control groups shortly after hatching, and monitored until 8 weeks of age. As part of another study, the chicks were assigned to groups according to their relatedness to each other. Relatedness was calculated from a pedigree of parents and the within-group average relatedness coefficients ranged from 0 to 0.31 (Supplementary Table S1). For groups with similar levels of genetic relatedness, we then randomly assigned them to either a fecal-supplemented treatment or a control treatment in a fully balanced factorial design. In the wild, genetic relatedness within broods can be highly variable (Kimwele & Graves, 2003) and it is unclear whether this influences gut microbiota similarity amongst chicks. Since the fecal treatments were applied in a balanced design, we could assess the effect of coprophagy whilst controlling for potential variation associated with relatedness.

Groups were randomly assigned to 15 enclosures situated next to each other. Each enclosure consisted of an indoor shelter (~4.8 × 4.8 m), where chicks were kept on cold days and at night, and an outdoor area (~4.8 × 7 m up to 4.8 × 15 m) with soil/pebble substrate where chicks were kept during the day. All individuals were reared under the same standardized conditions with ad libitum access to feed and fresh water during the daytime. The chicks received a standardized plant-based pelleted ostrich feed and adult birds were given a pelleted adult diet (details in (Videvall et al., 2019)). Adults were kept in a different area, separate from the chick facility.

Each morning, groups were provided with feed and water and released into their separate outdoor enclosures, weather permitting. Fresh feces were simultaneously collected from 4 adult ostriches (2 males and 2 females), which were not parents to the chicks in this study. Adult feces was homogenized and weighed, then placed in feed trays and distributed to all fecal treatment groups (Supplementary Figure S1). The amount of feces varied across days depending on how much fresh material was collected from the adults, although supplemented groups always received an equal amount (mean ± *SD*: 262 ± 101 g per day per group). The control groups were simultaneously provided with trays that were empty. In the afternoon, the chicks were brought inside, the trays collected, and any remaining fecal material was discarded.

Sample collection

Sample collection took place shortly after hatching before the experimental treatment started (week 0) and during the experiment at 1, 2, 3, 4, 6, and 8 weeks of age. This time window was selected based on previous data from ostrich chicks showing the greatest changes in gut microbiome composition happening in early life and up to about 8 weeks of age (Videvall et al., 2019). The weight of all individuals was recorded during each sampling event. Fecal samples were collected in empty tubes as described in (Videvall et al., 2018) and stored at −20 °C. Samples selected for sequencing comprised 5 individuals from all 30 groups at weeks 0, 1, 2, 4, and 8 during both years. While individuals for sequencing were selected randomly, those that survived the whole time period and contributed a sample every week were prioritized in order to achieve a dataset as complete as possible.

Mortality

We performed post-mortem dissections (necropsy) of all chicks that died during the course of the experiment and recorded the day and cause of death. Chicks that died due to gut disease in the form of enteritis or omphalitis were diagnosed during necropsy [see (Videvall et al., 2020) for details]. Because the chick groups were spatially randomized and situated next to each other, any potential pathogen outbreak originating in one of the groups would be equally likely to spread to both treatment and control groups. As is common in ostrich rearing systems (Gandini et al., 1986; Miao et al., 2003), a number of chicks developed deformed legs and had to be humanely euthanized. Causes of death included: developmental issues (blindness; n = 1), injuries (n = 3), gut disease (n = 11), and deformed legs (n = 17; Supplementary Table S1). Only mortalities from gut disease were statistically different between treatments (see Results).

Feeding behavior

To measure how much time individual chicks spent feeding, we conducted behavioral observations throughout the experiment.

Each group was observed at least three times a week from 1 to 8 weeks of age for a total of 270 min per group. Chicks were identified by color-coded neck tags (example in Supplementary Figure S1). Each observation period occurred for 10 min per group between the hours of 7:30 and 11:00 a.m. as chicks are usually most active during the morning (Amado et al., 2011). Observers were randomized across groups each observation day. Feeding rate was measured as the number of feeding bouts (continuous pecking at feed) performed by each chick, divided by the total minutes they were observed. Total feeding rate was measured as the feeding rate plus fecal feeding rates for fecal treatment groups. For control groups, total feeding rate was therefore identical to feeding rate. Once per week, all feed were weighed in the morning and late afternoon to estimate feed intake (including feed spill) for each group. This measure was then divided by the number of chicks present in the group to account for mortality events. Initially, we intended to measure fecal intake over time in the treatment groups via weigh-back once per week of the supplied adult feces. However, due to large variation across days in the amount of water evaporation that occurred in the fecal trays (resulting in lighter fecal weigh-backs during hot, dry days), combined with some fecal trays being tipped over, these measurements were not possible to accurately estimate and therefore discontinued.

DNA sequencing

DNA extraction was conducted using the PowerSoil DNA isolation kit and amplicon libraries prepared targeting the 16S rRNA gene V3 and V4 regions, as previously described (Videvall et al., 2018). Samples were sequenced on an Illumina MiSeq platform (paired-end, 300bp) and libraries were randomly distributed on the sequencing plates to ensure control and blank samples were not spatially separated from treatment samples. Because initial funding only allowed for one sequencing run, we first sequenced the samples collected in 2016, and when additional funds made a second sequencing run possible, we replicated the experiment in 2017 and sequenced this new set of samples. In practice, this meant we were not able to separate potential variation between years from variation due to sequencing runs, however, the effect of year/sequencing run on the microbiota turned out to be very small (R^2 = 0.009; Supplementary Table S4) and was not of major importance to the study design. Because our experimental treatments were balanced across years, including samples from 2 replications of the experiment doubled the statistical power of our analyses. In total, we sequenced 768 microbiota samples, of which 735 were chick fecal samples, 10 were blank negatives, and 23 were samples from the adult fecal material that were provided to the treatment groups.

Data processing

The sequences were quality-screened using FastQC (v. 0.11.5) and MultiQC. Filtering and trimming of reads were performed in QIIME2 (v. 2020.2; Bolyen et al., 2019), using DADA2 (v. 2020.2.0; Callahan et al., 2016). Amplicon Sequence Variants (ASVs) were trimmed to 260 bp (forward reads) and 220 bp (reverse reads) based on quality assessment. We ran the data from each sequencing run separately in DADA2, as recommended by the developers, and afterwards merged the 2 denoised ASV tables. Taxonomic assignment was trained using a naive Bayes classifier on our own data against both Greengenes (v. 13_8; McDonald et al., 2012) and Silva (v. 138; Quast et al., 2013). The taxonomic assignment against GreenGenes was substantially better for our data (Supplementary Table S12) and was used to infer taxonomy. We built a phylogenetic tree using MAFFT (Katoh & Standley, 2013) and FastTree2 (Price et al., 2010) in QIIME2. Decontam (v.1.6.0; Davis et al., 2018) was used to identify potential contaminants found in the blank samples that were prepared and sequenced simultaneously. ASVs identified with both the prevalence and frequency functions in decontam were removed. We further removed all ASVs classifying as mitochondria or chloroplast, all ASVs that appeared in fewer than 5 samples (out of 758), and all ASVs with an overall sequence count of less than 10. These filtering steps removed rare and contaminating sequences, with 8,386 unique ASVs remaining for analyses. Four samples had low coverage (library size < 500) after filtering and were removed. Number of reads per sample (mean = 18,116) showed no differences between years/sequencing runs or between treatment groups (two-way ANOVA: year, F = 0.07, p = .80; treatment, F = 0.45, p = .50).

Data analyses

We analyzed the microbial sequence data using three different alpha diversity metrics (ASV observed richness, Shannon's *H* index, Faith's phylogenetic diversity) and four different beta diversity metrics (Jaccard, Bray-Curtis, weighted and unweighted UniFrac; Bray & Curtis, 1957; Lozupone & Knight, 2005) in phyloseq (v. 1.32.0; McMurdie & Holmes, 2013). Alpha diversity was calculated using both rarefied and nonrarefied reads, and the results were identical in both approaches (Pearson's correlation: r > 0.98, p < 2.2e–16). We therefore present the nonrarefied data as recommended by McMurdie and Holmes (2014). Differences in beta diversity between treatment groups were tested in a PERMANOVA on UniFrac distances using the "adonis" function in vegan (v. 2.5-6; Oksanen et al., 2019) with 1,000 permutations. Age effects were evaluated by *Z*-transforming age in weeks and fitting both a linear and a quadratic age term. We tested for effects of dispersion using "betadisper" in vegan (Oksanen et al., 2019) and found no differences between control and treatment groups (p > .09). The only exception was at week 8 when control chicks had slightly higher variation (F = 12.9, p = .001), which does not affect the permanova results due to our balanced design (Anderson & Walsh, 2013).

Microbial abundances were normalized against library size in DESeq2 (v. 1.26.0) and tested for differential abundance using the standard negative binomial Wald test and with the "beta prior" set to false (Love et al., 2014) using ASVs with a minimum total abundance of 100 reads. The results between treatment and control groups were run separately per age group (week), while controlling for potential variation due to year. *p*-Values were corrected with the Benjamini and Hochberg false discovery rate for multiple testing and ASVs were considered significantly differentially abundant if they had a corrected *p*-value (*q*-value) < .01. The paraphyletic genera *Clostridium* within the families Lachnospiraceae, Ruminococcaceae, and Erysipelotrichaceae were renamed [*Clostridium*] due to ongoing revisions in the bacterial taxonomy and to keep all analyses of *Clostridium* strictly within the Clostridiaceae family. To differentiate them in text and figures, they are prepended with the first letter of respective family (e.g., L. [*Clostridium*]).

We considered microbial genera to be adult-associated if they (a) qualified as the top most abundant genera in adults, and were significantly more abundant in adults compared to (b) all chicks at age week 0, or (c) control chicks at age week 1 (Supplementary Table S11). Genera associated with young age were those that fulfilled the following criteria: qualified as the top most abundant genera in (a) all chicks at age week 0 or (b) control chicks at age week 1, plus were significantly more abundant in (c) all chicks at

age week 0, or (d) control chicks at age week 1, compared to adults (Supplementary Table S11). The first two chick ages were included because the microbial community at week 0 is still very simple and a large number of microbes colonize the gut during this first week after hatching (Figure 1; Supplementary Figures S2, S8–S10). Some genera, such as L. [*Clostridium*] and *Coprococcus*, were highly prevalent in both chicks and adults and therefore not associated with either (Supplementary Tables S9–S10). *Ruminococcus* was split into *Ruminococcus albus* and *Ruminococcus flavefaciens* because these 2 species showed highly distinct abundance patterns in adults and young-aged chicks, respectively (Supplementary Table S10).

A list of potential pathogens was created based on common gut diseases in poultry and ostriches documented in (Dinev, 2007; Porter, 1998; Verwoerd, 2000), and used for searches in the full taxonomic table of the chicks' microbiota. We did not find any assigned sequences from the following genera: *Salmonella*, *Escherichia*, *Pseduomonas*, *Pasteurella*, *Riemerella*, *Mycobacterium*, *Mycoplasma*, *Erysipelothrix*, *Borrelia*, and *Chlamydia*. We found trace amounts of DNA sequences matching the poultry pathogen *Clostridium perfringens* and the potential pathogenic genera *Campylobacter*, *Shigella*, and *Enterobacter*, however, these were extremely rare and present only in a few individuals from both treatments. The only known pathogen we could find with a sufficient number of sequences for analysis was [*Clostridium*] *colinum* of the Lachnospiraceae family (normalized read count, mean: 44.3, range: 0–1713.8), a well-known cause of enteritis in poultry (Porter, 1998; Prescott, 2016).

To test if fecal-supplemented and control chicks differed in their beta diversity distances to adults over time, we used a GLMM fitted with "glmer" in the lme4 R package (v. 1.1-29; Bates et al., 2015). The Bray-Curtis distances (which ranged from 0.79 to 0.999) and UniFrac distances (0.85 to 0.999) from each chick sample to all adult samples were averaged and analyzed as proportions using a binomial error distribution. Year (two-level factor), age (Z-transformed weeks), and treatment (two-level factor) were fitted as fixed effects. The interaction between age and treatment was used to test if distances between chicks and adults changed with age. The nonindependence of data arising from chicks being in the same groups and repeatedly measured was modeled by fitting random intercepts for groups, chicks nested within groups, and random slopes of chicks over age. Overdispersion was accounted for by fitting an observation level random effect.

Differences between treatment and control groups over time in phylogenetic microbial diversity, body weight (kg, log-transformed), and feeding rates were analyzed using separate LMMs fitted with the "lmer" function in the R package lmerTest (v. 3.1-3; Kuznetsova et al., 2017) on the full dataset (weeks 0, 1, 2, 3, 4, 6, 8). All response variables were modeled using a Gaussian error distribution and checked for homogeneity of variance and normally distributed residuals. To model the nonindependence of data arising due to chicks being in the same groups and repeatedly measured, random intercepts for groups and individuals within groups were fitted. To model variation in the way individuals changed with age, we fitted random slopes for chicks (random regression models). For fixed effects, treatment, age, year, and sex were fitted in all models. Interactions between age and treatment were included to test for age-dependent treatment effects. We also investigated models that included quadratic random slopes, however, this led to convergence problems in half of the models. In the remaining models, variance estimates of quadratic slopes were extremely small (~0) and did not change the interpretation of results. To avoid overcomplicating the analyses, we therefore did not include quadratic random slopes in our final analyses. In subsequent models of weight and feeding rates, phylogenetic diversity measures for chicks were included to test for the direct effects of the gut microbiome on growth and feeding behavior. For these analyses, we used a restricted version of the data that included only measurements at time points that had corresponding microbiome sequencing data (weeks 0, 1, 2, 4, 8). Interactions between diversity, treatment, and age were included to examine age- and treatment-specific effects of microbial diversity. We focused on phylogenetic diversity in these analyses as it showed the largest differences between treatment and control chicks and all three measures of microbial diversity were highly correlated. Plots were made using ggplot2 (v. 3.3.6; Wickham, 2009).

Supplementary material

Supplementary material is available online at *Evolution Letters* (https://academic.oup.com/evlett/qrad021).

Data availability

DNA sequences can be found at EMBL-EBI ENA under accession number PRJEB28516. Code used for analysis and figures can be found at the Open Science Framework repository (osf.io/ft52v).

Author contributions

E.V. and C.K.C. conceived and planned the study. S.C. provided funding and facilitated the research. E.V., C.K.C., H.M.B., and A.E. carried out the experiment. H.M.B. performed the laboratory work. E.V. and C.K.C. analysed the data, interpreted the results, and wrote the paper. All authors contributed to the manuscript.

Conflict of interest: The authors declare no competing interests.

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