Received: 8 August 2018 | Revised: 16 March 2019 | Accepted: 18 March 2019

DOI: 10.1111/mec.15087

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Major shifts in gut microbiota during development and its relationship to growth in ostriches

Elin Videvall[1] | Se Jin Song[2] | Hanna M. Bensch[1] | Maria Strandh[1] | Anel Engelbrecht[3] | Naomi Serfontein[4] | Olof Hellgren[1] | Adriaan Olivier[5] | Schalk Cloete[3,6] | Rob Knight[2,7,8] | Charlie K. Cornwallis[1]

[1]Department of Biology, Lund University, Lund, Sweden

[2]Department of Pediatrics, University of California San Diego, San Diego, California

[3]Directorate Animal Sciences, Western Cape Department of Agriculture, Elsenburg, South Africa

[4]Western Cape Agricultural Research Trust, Elsenburg, South Africa

[5]Klein Karoo International, Research and Development, Oudtshoorn, South Africa

[6]Department of Animal Sciences, Stellenbosch University, Matieland, South Africa

[7]Department of Computer Science & Engineering, University of California San Diego, San Diego, California

[8]Center for Microbiome Innovation, University of California San Diego, San Diego, California

Correspondence
Elin Videvall, Center for Conservation Genomics, Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC.
Email: elin.videvall@gmail.com

Funding information
Stiftelsen Längmanska Kulturfonden; Stiftelsen Lunds Djurskyddsfond; Vetenskapsrådet; Knut och Alice Wallenbergs Stiftelse; Helge Ax:son Johnsons Stiftelse; Stiftelsen Lars Hiertas Minne; Kungliga Fysiografiska Sällskapet i Lund

Abstract

The development of gut microbiota during ontogeny is emerging as an important process influencing physiology, immunity and fitness in vertebrates. However, knowledge of how bacteria colonize the juvenile gut, how this is influenced by changes in the diversity of gut bacteria and to what extent this influences host fitness, particularly in nonmodel organisms, is lacking. Here we used 16S rRNA gene sequencing to describe the successional development of the faecal microbiome in ostriches (*Struthio camelus*, $n = 66$, repeatedly sampled) over the first 3 months of life and its relationship to growth. We found a gradual increase in microbial diversity with age that involved multiple colonization and extinction events and a major taxonomic shift in bacteria that coincided with the cessation of yolk absorption. Comparisons with the microbiota of adults ($n = 5$) revealed that the chicks became more similar in their microbial diversity and composition to adults as they aged. There was a five-fold difference in juvenile growth during development, and growth during the first week of age was strongly positively correlated with the abundance of the genus *Bacteroides* and negatively correlated with *Akkermansia*. After the first week, the abundances of six phylogenetically diverse families (Peptococcaceae, S24-7, Verrucomicrobiae, Anaeroplasmataceae, Streptococcaceae, Methanobacteriaceae) were associated with subsequent reductions in chick growth in an age-specific and transient manner. These results have broad implications for our understanding of the development of gut microbiota and its associations with animal growth.

KEYWORDS
colonization, microbiome, ontogeny, *Struthio camelus*, succession

1 | INTRODUCTION

The gastrointestinal tract of vertebrates is considered to be largely sterile at the time of birth (Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017; cf. Jiménez et al., 2008) and subsequently colonized by a wide array of micro-organisms, collectively termed "the gut microbiota." The microbial composition of the gut during early life has been shown to have major influences on the health and phenotype of adults through its effects on gut morphology, metabolism, immune system development and brain development (Cho et al., 2012;

Cox et al., 2014; Dominguez-Bello et al., 2010; Heijtz et al., 2011; Russell et al., 2012). For example, animals prevented from acquiring gut bacteria have smaller intestines with thinner walls, smaller lymph nodes, under-developed immune systems and reduced organ sizes (Furuse & Okumura, 1994; Gordon & Pesti, 1971; Macpherson & Harris, 2004). Similarly, animals with poorly developed gut microbiota have an altered metabolism (Cox et al., 2014) and are more susceptible to infection by pathogens (Inagaki, Suzuki, Nomoto, & Yoshikai, 1996; Round & Mazmanian, 2009; Sprinz et al., 1961). As a result, it is important to characterize how, when and which microbes are recruited and lost during ontogeny, and how this is linked to host development.

A key feature of microbiomes that has been predicted to influence host fitness is the diversity of the community (Foster, Schluter, Coyte, & Rakoff-Nahoum, 2017; Hibbing, Fuqua, Parsek, & Peterson, 2010). However, research into how gut microbial diversity changes with age has produced results that differ across species. In mice and humans, colonization of gut bacteria is initiated during birth, where the mother's vaginal and skin microbiota are important sources of bacteria, and the diversity increases gradually with age (Kundu, Blacher, Elinav, & Pettersson, 2017; Pantoja-Feliciano et al., 2013; Sommer & Bäckhed, 2013). The seeding of microbes continues through lactation, but subsequently shifts during weaning towards an adult-like bacterial community that is more stable (Koenig et al., 2011; Sekirov, Russell, Antunes, & Finlay, 2010; Yatsunenko et al., 2012). In contrast, in zebrafish (*Danio rerio*) and African turquoise killifish (*Nothobranchius furzeri*) the alpha diversity and richness of the gut microbiota is highest in neonatals and subsequently decreases during maturation (Smith et al., 2017; Stephens et al., 2016). In birds, studies examining age-related changes in gut microbial diversity are limited and show conflicting results, potentially due to differences in parental and environmental transmission of microbes across species (Dewar et al., 2017; Godoy-Vitorino et al., 2010; Grond, Lanctot, Jumpponen, & Sandercock, 2017; van Dongen et al., 2013; Yin et al., 2010). For example, older nestlings of great tits (*Parus major*) had lower cloacal microbial diversity than younger nestlings (Teyssier, Lens, Matthysen, & White, 2018), while the opposite was found in tree swallows (*Tachycineta bicolor*; Mills, Lombardo, & Thorpe, 1999), and in house sparrows (*Passer domesticus*) age did not have any effect on microbial diversity or community structure (Kohl, Brun, Caviedes-Vidal, & Karasov, 2019). In turkeys (*Meleagris gallopavo*) it has been found that gut microbial diversity initially increases and then subsequently decreases during development (Danzeisen et al., 2015; Wilkinson et al., 2017), while in chickens (*Gallus gallus*) there is often a successional increase in diversity with age (Ballou et al., 2016; Lu et al., 2003; Oakley et al., 2014; van der Wielen, Keuzenkamp, Lipman, van Knapen, & Biesterveld, 2002). Such large variation in the relationship between host age and gut microbial diversity makes it difficult to draw general conclusions about the mechanisms behind the development of gut microbiomes.

Understanding how the diversity of gut microbiota develops over ontogeny is important, as it can have large effects on host development and growth. For example, gut microbes can influence juvenile growth by altering the efficiency of the digestion process or by acting as a barrier to prevent the establishment of problematic or beneficial bacteria (Foster et al., 2017; Ley, Peterson, & Gordon, 2006; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). Studies evaluating juvenile growth in relation to gut microbiota have demonstrated that animals with low or no microbial diversity require a higher calorific intake to attain the same growth as hosts with more diverse gut communities (Bäckhed et al., 2004; Shin et al., 2011; Sommer & Bäckhed, 2013; Wostmann, Larkin, Moriarty, & Bruckner-Kardoss, 1983). However, it has also been suggested that a reduced diversity of gut microbiota may increase growth and accelerate host development (Furuse & Okumura, 1994; Gordon & Pesti, 1971). This idea is supported by numerous agricultural studies in which higher growth rates have been achieved in farm animals by using antibiotics to reduce gut bacteria, a common practice since the 1950s (Dibner & Richards, 2005; Engberg, Hedemann, Leser, & Jensen, 2000; Gaskins, Collier, & Anderson, 2002; Lin, Hunkapiller, Layton, Chang, & Robbins, 2013). Supplementing wild animals with antibiotics has also been associated with positive effects on growth (Kohl, Brun, Bordenstein, Caviedes-Vidal, & Karasov, 2017; Potti et al., 2002), although interpreting the results can be challenging as antibiotics can both increase and decrease gut microbial diversity (Crisol-Martínez, Stanley, Geier, Hughes, & Moore, 2017; Kohl et al., 2017). Likewise, some probiotic supplements have led to an increase in the growth of juvenile animals while others are associated with impaired growth (Angelakis, Merhej, & Raoult, 2013; Angelakis & Raoult, 2010; Million et al., 2012; Yin et al., 2010). Such conflicting reports on the role of gut microbial diversity in determining animal growth highlights the need for further research into the relationships between gut bacterial diversity, the relative abundance of specific bacteria and variation in host growth patterns across development. In addition, the majority of research has focused on humans, domesticated animals and model laboratory species, which emphasizes the need to broaden our understanding of the development of the host microbiome to nonmodel organisms.

In this study, we evaluated the development of gut microbiota through ontogeny in ostrich (*Struthio camelus*) chicks and its relationship to variation in juvenile growth. Repeated faecal sampling of chicks was performed under controlled conditions from 1 week after hatching until 12 weeks of age, which corresponds to the critical developmental phase in this species (Cloete, Lambrechts, Punt, & Brand, 2001; Verwoerd, Deeming, Angel, & Perelman, 1999). We also sampled adult ostriches to compare their gut microbiota with the juveniles'. Ostriches are the largest living bird species and, together with other palaeognaths, branched off from other birds at the base of the avian phylogeny, making them an evolutionary distinct group. They are a valuable economic resource, being farmed for feathers, meat, eggs and leather, yet have only been kept in captivity for a short period of time relative to other agricultural animals (~150 years; Cloete et al., 2012). The chicks are highly precocial, allowing them to be raised independently from their parents, and they reach sexual maturity from 2 years of age. Ostriches also exhibit extremely large variation in offspring growth rate, even in controlled

environments (Bonato, Evans, Hasselquist, Cloete, & Cherry, 2009; Deeming & Ayres, 1994; Engelbrecht, Cloete, Bunter, & van Wyk, 2011; Skadhauge & Dawson, 1999), and are known to suffer from bacterial gut infections (Keokilwe et al., 2015; Verwoerd, 2000). These traits make the ostrich an excellent organism for investigating host–microbiota associations, including the effects of gut microbiota on juvenile growth and development.

2 | MATERIALS AND METHODS

2.1 | Experimental setup

Ostriches were kept under controlled conditions at the Western Cape Department of Agriculture's ostrich research facility in Oudtshoorn, South Africa. Chicks were obtained from a batch of artificially incubated eggs that hatched on 30 September 2014. A total of 234 individuals were monitored from hatching until 3 months of age (12 weeks) in four groups that contained around 58 chicks each at the start of the experiment. The groups were kept in indoor pens of approximately 4 × 8 m with access to outdoor enclosures with soil substrate during the day. To reduce potential environmental variation on the development of the gut microbiota, all individuals were reared under standardized conditions with ad libitum access to food and fresh water during daytime. The chicks received a predetermined plant-based pelleted diet consisting primarily of corn, soybean and alfalfa (ratite prestarter ration, Tables S1 and S2). The adult birds were given a pelleted adult version (ratite breeder ration, Table S3) and were kept in a different area separate from the chick-rearing facility. All procedures were approved by the Departmental Ethics Committee for Research on Animals (DECRA) of the Western Cape Department of Agriculture, reference no. R13/90.

2.2 | Sample collection

Faecal samples in this study were collected from chicks at the following ages: weeks 1, 2, 4, 6, 8, and 12 (where week 1 means 7 days old, week 2 means 14 days, etc.). In addition, we sampled fresh faeces from five adult individuals kept in large outside enclosures. The sex and age of these adults were not possible to identify, but they were sexually mature, breeding individuals. All faecal samples were collected in empty plastic 2-ml microtubes (Sarstedt, cat. no. 72.693) and stored at −20°C within 2 hr of collection. Details of sample collection have been described previously (Videvall, Strandh, Engelbrecht, Cloete, & Cornwallis, 2018). The ostrich chicks were weighed during every sampling event. At the final time point (week 12), the smallest chick weighed 6 kg while the largest weighed 30 kg, representing a five-fold difference in body mass (mean = 18 kg). During the course of the study, the chicks decreased in number because some were euthanized for related studies (Videvall, 2018; Videvall, Strandh, Engelbrecht, Cloete, & Cornwallis, 2017; Videvall et al., 2018) and several died of natural causes (Videvall, 2018). We selected faecal samples from chicks that either survived the entire 3-month period or from healthy chicks that were euthanized (Table S4).

2.3 | DNA isolation, library preparation and amplicon sequencing

We prepared sample slurries with guidance from Flores, Henley, and Fierer (2012) and subsequently extracted DNA using the PowerSoil-htp 96 well soil DNA isolation kit (Mo Bio Laboratories, cat. no. 12955-4) as recommended by the Earth Microbiome Project (www.earthmicrobiome.org). For full details see Videvall et al. (2018). Amplicon libraries for sequencing of the 16S rRNA V3 and V4 regions were prepared using Illumina fusion primers containing the target-specific primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Part # 15044223 Rev.B). The samples were sequenced as 300-bp paired-end reads over three sequencing runs on an Illumina MiSeq platform at the DNA Sequencing Facility, Department of Biology, Lund University, Sweden. A total of 272 chick faecal samples, five adult faecal samples plus four negative samples were part of this study (Table S4).

2.4 | Data processing

The 16S amplicon sequences were quality-screened using FASTQC (version 0.11.5; Andrews, 2010) together with MULTIQC (Ewels, Magnusson, Lundin, & Käller, 2016). Primers were removed from the sequences using TRIMMOMATIC (version 0.35; Bolger, Lohse, & Usadel, 2014) and the forward reads were retained for analyses. Quality filtering of the reads was executed using the script multiple_split_libraries_fastq.py in QIIME (version 1.9.1; Caporaso et al., 2010). All bases with a Phred score <25 at the 3′ end of reads were trimmed and samples were multiplexed into a single high-quality multi-fasta file.

Operational taxonomic units (OTUs) were assigned and clustered using DEBLUR (version 1.0.0; Amir et al., 2017). DEBLUR circumvents the problems of clustering OTUs at an arbitrarily threshold by obtaining single-nucleotide resolution OTUs (100% sequence identity approach) after correcting for Illumina sequencing errors. The resulting OTUs are sometimes called sOTUs or amplicon sequence variants, but we refer to them here as OTUs. The minimum reads-option was set to 0 to disable filtering by DEBLUR, and all sequences were trimmed to 220 bp. We used the resulting OTU table produced after both positive and negative filtering, which by default removes any reads containing PhiX or adapter sequences, and only retains sequences matching known 16S gene sequences. Additionally, PCR-originating chimeras were filtered inside DEBLUR (Amir et al., 2017).

Taxonomic assignment of OTUs was performed using the RDP Classifier (version 2.2; Wang, Garrity, Tiedje, & Cole, 2007). We removed from all samples the following OTUs: OTUs present in the negative (blank) samples (n = 95), OTUs classifying as mitochondria (n = 7), OTUs classifying as chloroplasts (n = 18), OTUs that only appeared in one sample, and finally OTUs with a total sequence count of <10. These filtering steps removed in total ~47,000 OTUs, with 4,338 remaining for analyses. All samples were retained because none exhibited low read coverage (the lowest coverage obtained

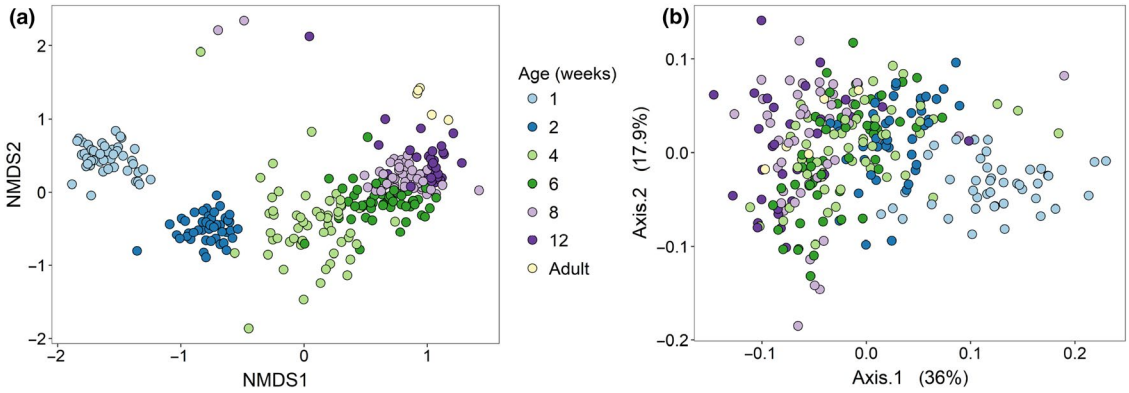

FIGURE 1 Gut microbiomes show stepwise differences with increasing age of hosts. (a) NMDS of Bray–Curtis distances and (b) PCoA of weighted UniFrac distances between samples. Colours indicate age of individuals in weeks and brackets in the PCoA display the percentage variance explained by the first two dimensions

was 1,799 reads after filtering). Furthermore, the sequence coverage per sample (mean number of filtered reads = 15,480) showed no differences across ages (ANOVA: $F = 2.01$, $p = 0.064$). The phylogenetic tree was produced in QIIME, using FASTTREE (Price, Dehal, & Arkin, 2009). Analyses were evaluated with both rarefied and nonrarefied data, which produced extremely comparable results (Figure S1). We therefore present the results from the nonrarefied data in this study.

2.5 | Data analyses

All analyses were performed in R (version 3.3.2; R Core Team, 2017). We calculated OTU richness (observed OTUs), Shannon's H index alpha diversity (hereafter referred to as alpha diversity) and Faith's weighted abundance of phylogenetic diversity (hereafter referred to as phylogenetic diversity) using absolute abundance of reads, and distance measures with Bray–Curtis and weighted UniFrac methods (Bray & Curtis, 1957; Lozupone & Knight, 2005) on relative read abundances in PHYLOSEQ (version 1.19.1; McMurdie & Holmes, 2013). The effects of age, sex, group, and chick identity on the microbiomes of chicks were examined in permutational multivariate analysis of variances (PERMANOVA) with weighted UniFrac distances, using the adonis function in the VEGAN R package (version 2.4-2; Oksanen et al., 2017) with 1,000 permutations. Age was evaluated by Z-transforming age in weeks and fitting a linear and a quadratic age term. Differences in dispersion between age groups were tested with the multivariate homogeneity of group dispersions test (betadisper) in VEGAN (Oksanen et al., 2017), followed by the Tukey's honest significant difference method. Microbiome differences across individuals within and between age groups were calculated using Bray–Curtis distance metrics on relative abundances. All comparisons between samples from the same individual were excluded prior to calculating distance metrics.

To evaluate bacterial abundances, we first modelled counts with a local dispersion model and normalized reads per sample using the geometric mean (according to the DESEQ2 manual; Love, Huber, & Anders, 2014). Differential abundances between juvenile age groups were subsequently tested in DESEQ2 with a negative binomial Wald test, while controlling for individual identity of birds, and with the beta prior set to false (Love et al., 2014). The results for specific comparisons were extracted (e.g., week 1 vs. week 2) and p-values were corrected with the Benjamini and Hochberg false discovery rate for multiple testing (Benjamini & Hochberg, 1995). OTUs were labelled as significantly differentially abundant if they had a corrected p-value (q-value) <0.01. The test between week 12 juveniles and adults was performed without individual identity in the model as this comparison did not include any repeated data measures.

We modelled juvenile growth as the weight change per week between measurements t and $t + 1$ in relation to microbial diversity at week t, including age and weight at week t as covariates, using linear mixed-effect (LME) models in the NLME R package (Pinheiro, Bates, DebRoy, & Sarkar, 2016). Separate models were run for alpha diversity, phylogenetic diversity and bacterial richness. For random effects, an unstructured (co)variance matrix was fitted to model random intercepts for individuals, random slopes for individuals across ages, and the covariance between intercepts and slopes.

To investigate the relationship between specific OTUs and chick growth, semipartial Spearman's rank correlation tests were used on the sum of the normalized abundance of all OTUs in each family during each sampling (weeks 1, 2, 4, 6 and 8) against weight change to the next sampling week (weight difference between sampling events/number of weeks between sampling events), residualized against weight. Correlations were conducted at the level of family as 76% of OTUs were missing taxonomic assignments at the level of genus or lower, whereas only 27% were missing family assignment. Spearman's rank correlation tests were used as OTU abundances were highly skewed, and not possible to correct using data transformations. Confidence intervals were calculated using bootstrapping implemented using the pcor.test function in R package RVAIDEMEMOIRE (version 0.9-69-3; Hervé, 2018). Data were filtered to only include taxa with a minimum abundance of 100 counts and where at least 10 individuals had nonzero counts. The p-values were corrected with the false discovery rate to q-values and taxa with $q < 0.05$ were

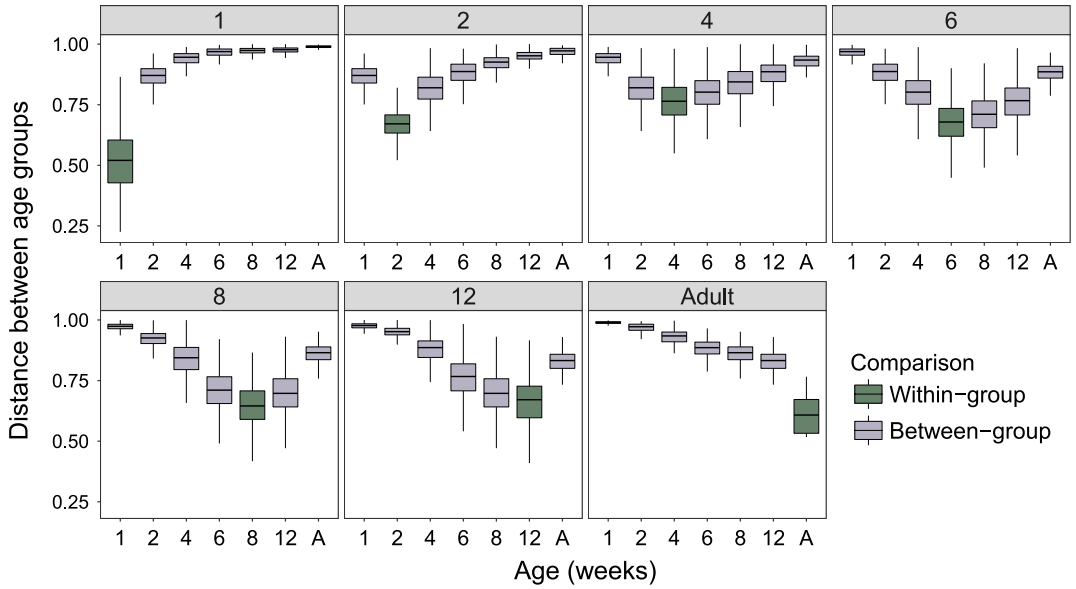

FIGURE 2 Beta diversity (Bray–Curtis distance) of gut microbiota shows most similarities within age groups. The headers display age in weeks and the x-axes show all age comparisons, with A = adults. Within-age group comparisons are highlighted in darker colour (e.g., distances between all individuals at week 1) and higher values signify more dissimilar microbiomes [Colour figure can be viewed at wileyonlinelibrary.com]

considered statistically significant. Plots were made using GGPLOT2 and GPLOTS (Warnes et al., 2018; Wickham, 2009).

3 | RESULTS

3.1 | Age has a major influence on the gut microbiota composition of juvenile ostriches

Age explained a major part of the variation observed in the ostrich chick faecal microbiome (Figures 1 and S1). There was clear chronological ordering of samples with age along the first axis of ordination in an unsupervised nonmetric multidimensional scaling (NMDS) plot of Bray–Curtis distances (Figure 1a) and in a principal coordinates analysis (PCoA) plot of weighted UniFrac distances (Figure 1b; PERMANOVA: $R^2 = 0.17$; $p < 0.001$), with the microbiota of juveniles approaching that of adult individuals as they aged. The microbiota of individuals at week 1 showed the largest differences to all other ages and clustered separately from the microbiota of individuals at week 2, which in turn clustered separately from those at week 4 and subsequent weeks (Figure 1). In addition, there was a significant quadratic effect of age ($R^2 = 0.06$; $p < 0.001$), indicating that changes in the microbial community with age slowed down as individuals got older (Figure S2). As a result, the samples from the oldest juvenile ages (weeks 6, 8 and 12) showed the least differences from each other, particularly when analysed with weighted UniFrac distances (Figure 1b).

The microbiomes within age groups were always more similar to each other than they were to other age groups (Figures 2 and S2). The degree of variation in the microbiome among individuals was similar across ages (multivariate homogeneity test of group dispersions: adjusted $p = 0.203–0.999$), indicating that PERMANOVA results were not due to differences in dispersion among the age groups. The exception was the specific comparison between weeks 2 and 4, which showed that week 4 was significantly more variable (adjusted $p = 0.009$; see Figure 1a). In line with this, our PERMANOVA showed high, but nonsignificant, variation between individuals in their microbiota ($R^2 = 0.19$, $p = 0.243$). The sex and group of juveniles did not have any effects on the variation in their microbial composition (sex: $R^2 = 0.003$; $p = 0.396$; group: $R^2 = 0.005$; $p = 0.081$).

Consistent with the analyses of microbial distances between ages, we found that the alpha diversity, phylogenetic diversity and richness of the gut microbiota increased as individuals became older (Figure 3; alpha diversity [LME], age parameter estimate (β) $SE = 0.08 \pm 0.01$, $F_{1,205} = 54.66$, $p < 0.0001$; phylogenetic diversity [LME], age $\beta \pm SE = 3.21 \pm 0.78$, $F_{1,205} = 16.95$, $p < 0.0001$; richness [LME], age $\beta \pm SE = 26.89 \pm 2.06$, $F_{1,205} = 170.40$, $p < 0.0001$). Interestingly, despite alpha diversity and richness being highest in adults, phylogenetic diversity was highest around 6 weeks of age as early-life microbiota transitioned to more adult-like microbiota (Figure 3). The age differences in diversity measures were also highly significant, even after controlling for weight (LMEs with weight as a covariate: alpha diversity, age $\beta \pm SE = 0.13 \pm 0.02$, $F_{1,204} = 55.35$, $p < 0.0001$; phylogenetic diversity, age $\beta \pm SE = 7.83 \pm 1.59$, $F_{1,204} = 18.08$, $p = 0.001$; richness, age $\beta \pm SE = 35.62 \pm 4.27$, $F_{1,204} = 182.52$, $p < 0.0001$).

3.2 | Colonizations and extinctions of bacterial groups occur throughout development

Investigating bacterial composition at different ages showed large shifts, especially during the early weeks, with differences evident even at higher levels of taxonomy (Figure 4).

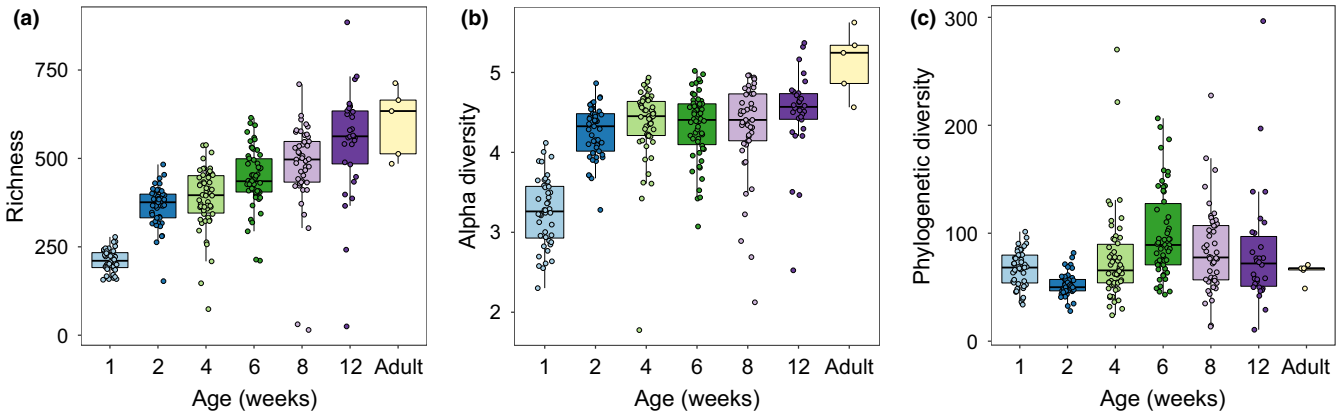

FIGURE 3 Step-wise increase of microbial diversity with host age. (a) Richness (observed OTUs), (b) alpha diversity (Shannon index) and (c) phylogenetic diversity (Faith's diversity) sampled at different ages. Adults were not included in analyses, but are shown for graphical comparison [Colour figure can be viewed at wileyonlinelibrary.com]

One-week-old juveniles had high abundances of Verrucomicrobiae and Erysipelotrichi, but by week 2 these classes were already highly reduced relative to other bacteria (Figure 4). Furthermore, Planctomycetia, Verrucomicrobiae and Gammaproteobacteria were practically absent in adults compared to juveniles. From around 8 weeks of age chicks started to establish a gut microbiome that resembled that of adults, which involved the recruitment of Bacilli and Planctomycetia and a community dominated by Clostridia (Figure 4). Other taxa had more complicated relationships with age, such as the Bacteroidia that peaked in abundance during week 2 (relative to the other classes) and subsequently decreased in the following weeks, only to increase again into adulthood (Figure 4).

Examining differences in OTU abundance between age groups produced a more detailed picture of the bacterial shifts during development. The most prevalent OTUs at different ages belonged to *Akkermansia muciniphila* during week 1 (27.2%), Bacteroidales sp. during weeks 2 and 4 (7.1%–11.9%), Clostridiaceae sp. during weeks 6, 8 and 12 (7.9%–8.6%), and Ruminococcaceae sp. in the adults (5.1%; Table S5). The relative abundance of all OTUs became more alike over time as individuals aged (Figure S3). For instance, the comparison between week 1 and week 2 showed the least similarities in overall OTU abundances, despite having the shortest interval between sampling events, while the comparison between week 8 and week 12 displayed a high similarity (Figure S3). The comparison between week 12 juveniles and adult birds also showed high correspondence, but there were several highly abundant OTUs not present in the adult gut microbiome and vice versa, indicating that further shifts of specific bacteria continued beyond 12 weeks of age (Figure S3).

We found a large number of significantly differentially abundant OTUs when comparing samples closest in time across ages (Figure 5: negative binomial Wald tests of normalized OTU abundances). Specifically, several OTUs were more abundant in 2-week-old juveniles compared to 1-week-olds (Figure 5; Table S6), with the most significant OTUs coming from the families Ruminococcaceae and Christensenellaceae. Furthermore, 16 OTUs matching Bacteroidia (families Bacteroidaceae, S24-7, Rikenellaceae and Odoribacteraceae) were more abundant at week 2 relative to week 1 (Table S6). The analysis between week 2 and week 4 yielded a large number of differentially abundant OTUs (n = 498), of which the majority (70.7%) were again more abundant in the older age group, demonstrating microbial recruitment. Notably, almost half (47.4%) of the differentially abundant OTUs were completely absent at week 2 but were present at week 4, including, for example, OTUs within Actinobacteria and Planctomycetia (Table S7). At week 6 there were again numerous colonizations (n = 166), mostly from within the classes Clostridia and Mollicutes, while some OTUs had gone locally extinct (n = 68; or to low enough levels to prevent any detection; Table S8). By week 8, extinction (n = 88) and colonization of bacteria (n = 80; Table S9) were approximately equal, and by week 12 changes in OTU abundance had slowed down with fewer differentially abundant OTUs relative to week 8 (n = 182; Table S10). The final comparison between week 12 juveniles and adults yielded 60 significant OTUs, of which all except one (*Aerococcus* sp.) were largely absent in adults (Figure 5; Table S11), although this pattern could be a direct result of the smaller number of adult samples.

3.3 | Gut microbiota is associated with the growth of hosts

Chick growth during the first week after hatching was strongly associated with alpha diversity and richness measured at 1 week of age (Figure 6; general linear model [GLM] of weight change during the first week with hatching weight as a covariate: alpha diversity $\beta \pm SE$ = 0.14 ± 0.03, $F_{1,41}$ = 17.97, p = 0.0001; richness $\beta \pm SE$ = 0.001 ± 0.0005, $F_{1,41}$ = 8.82, p = 0.005). This association appeared to be due to acquisition of bacteria during the first week as there was no relationship between hatching weight and alpha diversity or richness at week 1 (Figure 6; GLM: $\beta \pm SE$ = 0.008 ± 0.03, $F_{1,41}$ = 0.08, p = 0.78; richness $\beta \pm SE$ = 0.0002 ± 0.0004, $F_{1,41}$ = 0.21, p = 0.65). In contrast, phylogenetic diversity was not associated

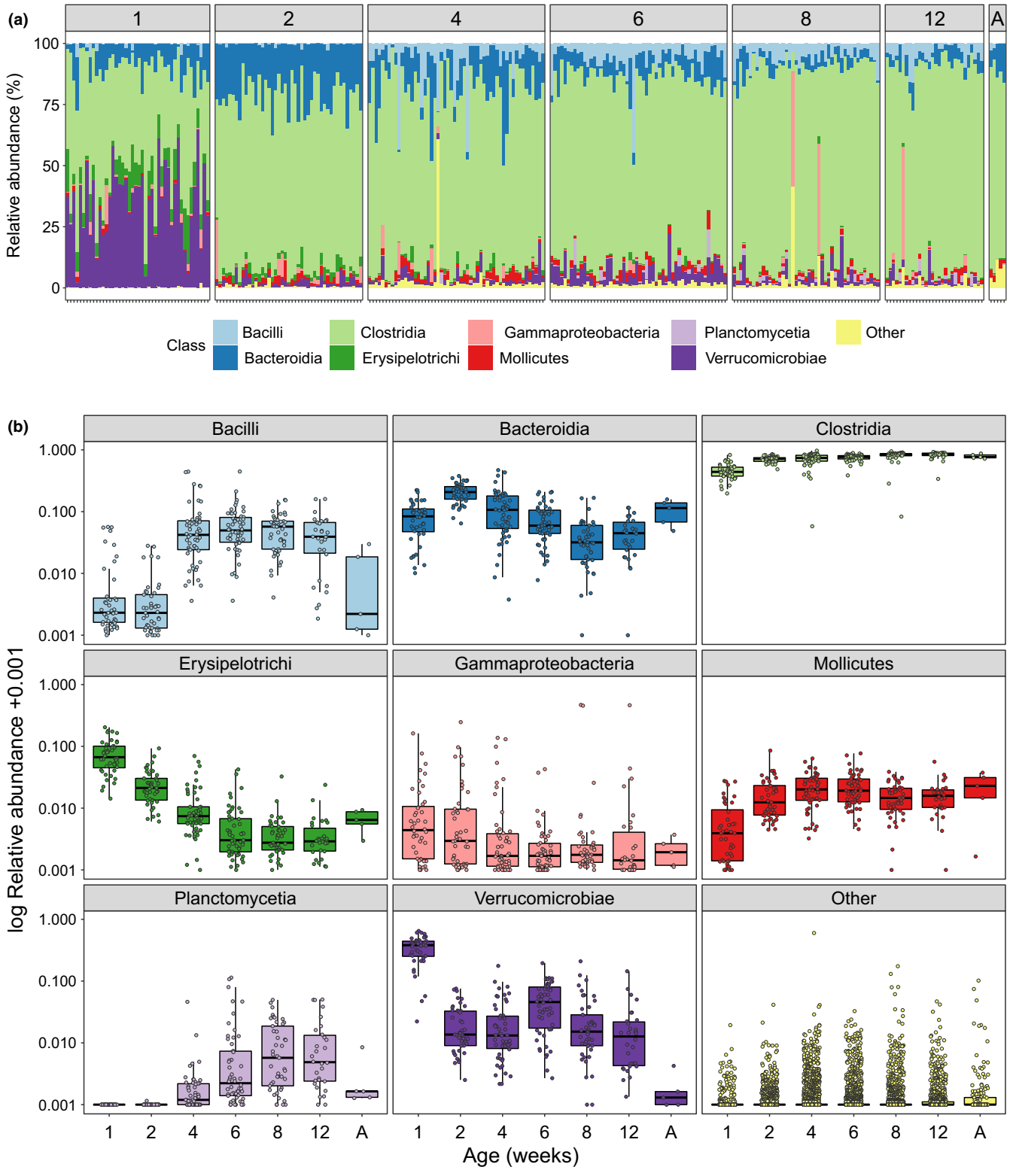

FIGURE 4 Relative abundances of bacterial groups display different trends with increasing host age. (a) Barplots showing the bacterial class composition for every host individual (bars). The headers show age in weeks, with A indicating adult individuals. (b) Boxplots illustrating the relative abundances of bacterial classes (log-transformed + 0.001). The x-axes show age in weeks, with A indicating adult individuals

with either hatching weight (GLM: $\beta \pm SE = 0.0009 \pm 0.0007$, $F_{1,42} = 1.41$, $p = 0.24$) nor growth during the first week (Figure 6; GLM: $\beta \pm SE = -0.0004 \pm 0.001$, $F_{1,41} = 0.14$, $p = 0.71$). This result suggests that there is a link between the recruitment of bacteria and juvenile growth during the first days after hatching, but it does not involve the establishment of new phylogenetic groups of

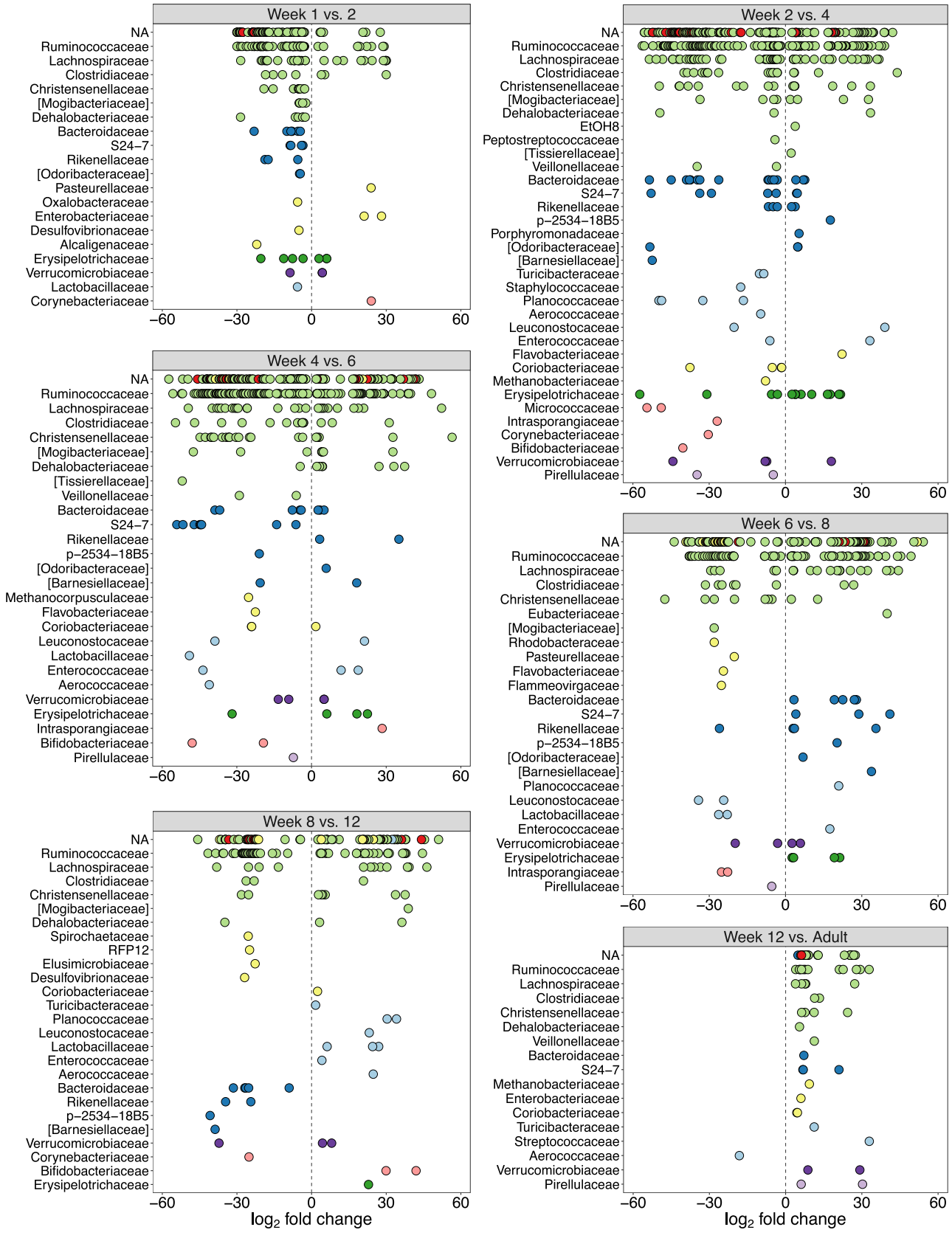
Week 1 vs. 2
Week 2 vs. 4
Week 4 vs. 6
Week 6 vs. 8
Week 8 vs. 12
Week 12 vs. Adult
NA
Ruminococcaceae
Lachnospiraceae
Clostridiaceae
Christensenellaceae
[Mogibacteriaceae]
Dehalobacteriaceae
Bacteroidaceae
S24–7
Rikenellaceae
[Odoribacteraceae]
Pasteurellaceae
Oxalobacteraceae
Enterobacteriaceae
Desulfovibrionaceae
Alcaligenaceae
Erysipelotrichaceae
Verrucomicrobiaceae
Lactobacillaceae
Corynebacteriaceae
EtOH8
Peptostreptococcaceae
[Tissierellaceae]
Veillonellaceae
p–2534–18B5
Porphyromonadaceae
[Barnesiellaceae]
Turicibacteraceae
Staphylococcaceae
Planococcaceae
Aerococcaceae
Leuconostocaceae
Enterococcaceae
Flavobacteriaceae
Coriobacteriaceae
Methanobacteriaceae
Micrococcaceae
Intrasporangiaceae
Bifidobacteriaceae
Pirellulaceae
Methanocorpusculaceae
Eubacteriaceae
Rhodobacteraceae
Flammeovirgaceae
Spirochaetaceae
RFP12
Elusimicrobiaceae
Streptococcaceae
−60
−30
0
30
60
log2 fold change
Class
Bacilli
Bacteroidia
Clostridia
Erysipelotrichi
Actinobacteria
Mollicutes
Planctomycetia
Verrucomicrobiae
Other

FIGURE 5 Significant differences in OTU abundances between host ages closest in time. Dots show differentially abundant OTUs ($q < 0.01$) between age groups, y-axes show taxonomic families, and all OTUs have been coloured at the class level. Positive $\log_2$ fold changes indicate higher relative OTU abundance in the younger age group in each comparison, and negative $\log_2$ fold changes indicate higher abundance in the older age group. NA = OTUs without family classification

bacteria. Further analyses of bacterial abundances showed that two families were significantly associated with growth in the first week: Bacteroidaceae, primarily of the genus *Bacteroides*, was positively associated with growth (semipartial Spearman's rank: $n_{individuals} = 44$, $n_{otus} = 2{,}288$, $r_s = 0.51$, confidence interval [CI] = 0.24–0.69, $q = 0.01$) and Verrucomicrobiaceae, primarily of the genus *Akkermansia*, was negatively associated with growth (semipartial Spearman's rank: $n_{individuals} = 44$, $n_{otus} = 660$, $r_s = -0.38$, CI = −0.65 to −0.08, $q = 0.01$).

After the first week, the positive association between microbial diversity and juvenile growth disappeared. Over the 12-week period of development, there was a weak negative relationship between growth and alpha diversity (LME of weight change per week [w_{t+1}–w_t], weight at time t as a covariate: alpha diversity at time t $\beta \pm SE = -0.13 \pm 0.06$, $F_{1,156} = 4.58$, $p = 0.03$), but not with bacterial richness ($\beta \pm SE = -0.0005 \pm 0.0004$, $F_{1,156} = 1.61$, $p = 0.21$) or phylogenetic diversity ($\beta \pm SE = 0.0006 \pm 0.001$, $F_{1,156} = 0.28$, $p = 0.60$). Furthermore, the relationship between juvenile growth and diversity did not differ significantly with age ($p > 0.12$ for the interaction between age and all measures of diversity). However, examining how the abundance of bacteria in different families at each age was correlated with subsequent growth in hosts showed varied relationships, both across age of chicks and different phylogenetic groups of bacteria (Figures 7 and S4). In fact, no clades of bacteria were consistently positively or negatively associated with growth at all ages, apart from Christensenellaceae, which was weakly positively related to growth throughout development (Figure 7). There were six families that were significantly correlated with juvenile growth at specific ages, all of which were associated with reduced growth (Figure 7; Table S12).

Lastly, we examined if the abundance of microbes that were correlated to growth could be explained by alpha and phylogenetic diversity. We found that the abundance of all six microbial families associated with growth varied independently of alpha diversity ($p > 0.05$; Table S13), but that the abundances of S24-7 at week 2, Verrucomicrobiaceae at week 4 and Methanobacteriaceae at week 8 were all positively correlated to phylogenetic diversity ($p < 0.05$; Table S13). Consequently, the microbes associated with reduced growth in hosts were not less prevalent in more diverse communities, and in fact they appeared to be more abundant in phylogenetically diverse gut communities.

4 | DISCUSSION

Maturation of the gut microbiota during development is a crucial process potentially affecting host fitness (Sommer & Bäckhed, 2013). We found that bacteria colonize the gut of juvenile ostriches in a successional manner and that the microbial community develops with increasing diversity and complexity as individuals age. Major compositional changes in the gut microbiota occurred during development, especially from the first to the second week of life, which coincides with a dietary switch from yolk to food. The relationships between microbiota and juvenile growth were taxon- and age-specific, potentially explaining some of the contradictions reflected in previous research. Apart from the initial period after hatching, bacterial abundance and diversity had negative and transient effects for growth, indicating that the developmental stage at which the gut microbiota is examined is crucial for understanding this relationship.

The gut microbiome of 1-week-old ostriches was highly differentiated from that of subsequent ages, with a much lower alpha diversity and a unique microbial composition dominated by Verrucomicrobiae, Clostridia, Erysipelotrichi and Bacteroidia. During the first week after hatching, ostriches are nutritionally dependent on their internal yolk sac, which is high in fat and protein

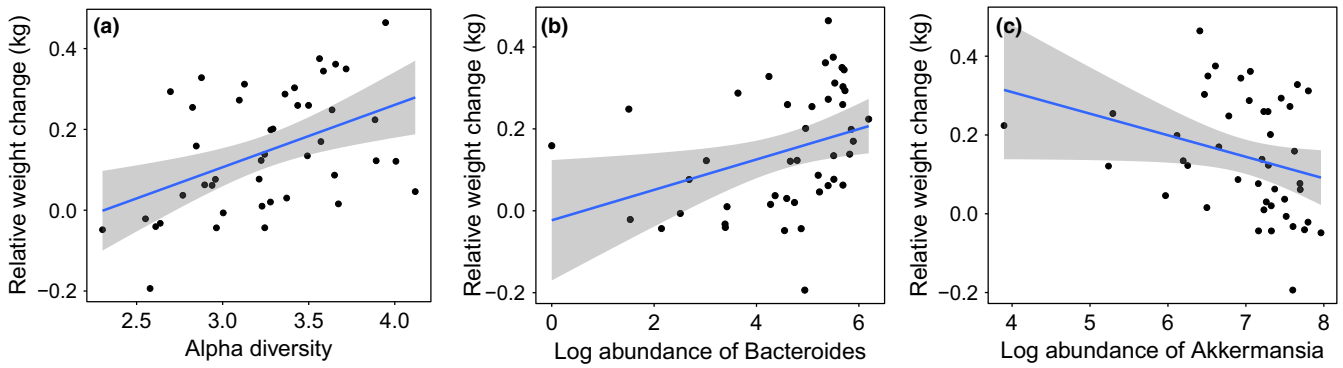

FIGURE 6 Relative juvenile growth rate during the first week after hatching in relation to (a) alpha diversity (Shannon index), (b) the abundance of *Bacteroides* and (c) the abundance of *Akkermansia*. Relative growth rate is measured as the change in weight from hatching to week 1/hatching weight. The lines represent linear regression lines and the shaded areas show the 95% confidence interval [Colour figure can be viewed at wileyonlinelibrary.com]

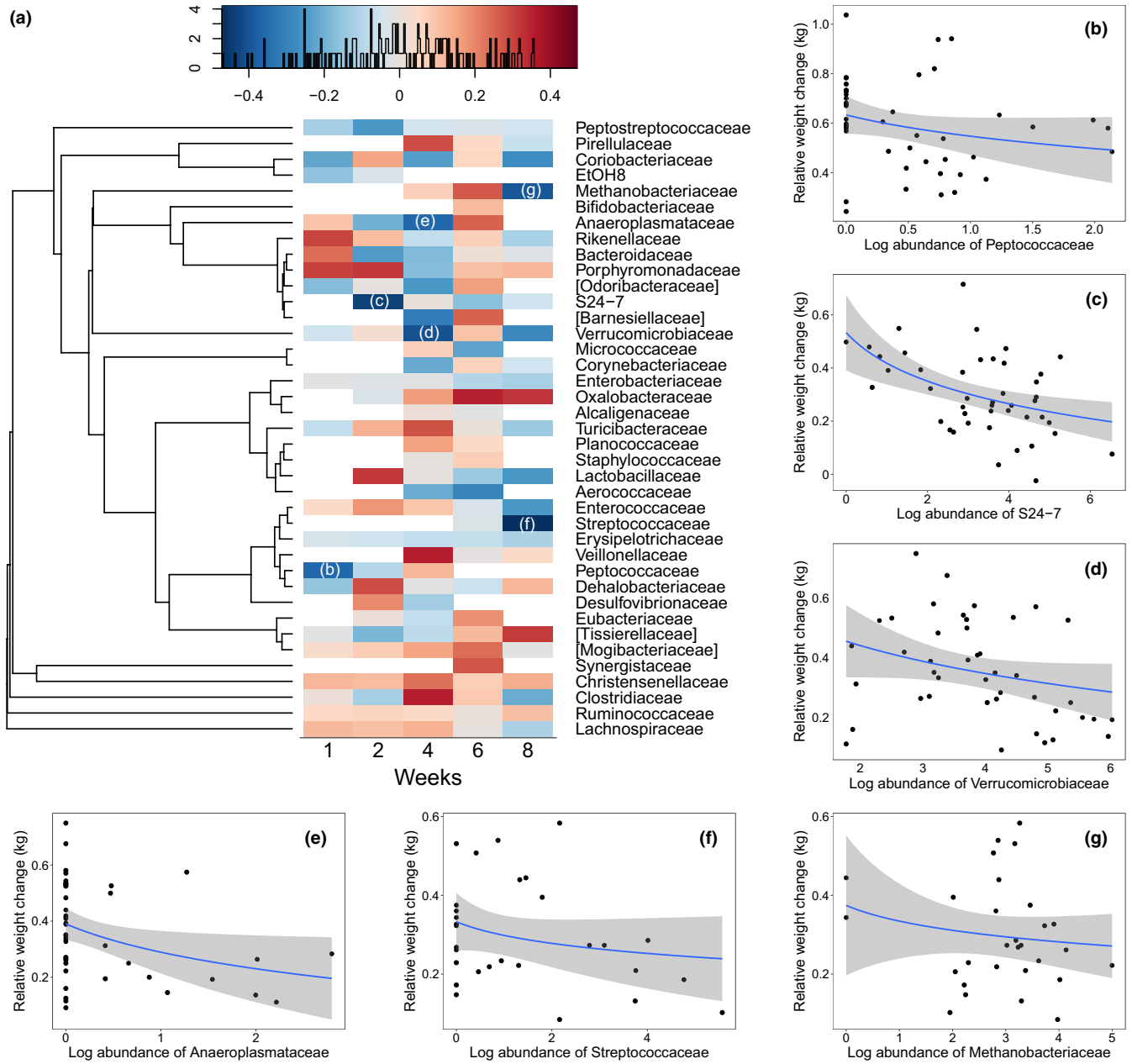

FIGURE 7 Juvenile growth in relation to microbial abundances across families. (a) Heatmap showing the strength of correlations (r_s red = positive, blue = negative, white = not present) between microbial abundances (the sum of normalized OTU abundances for each family at weeks 1–8) and juvenile growth (weight change per week after residualizing against weight). The cladogram illustrates evolutionary relationships and the key in the top shows the distribution of r_s values. Six microbial families were significantly correlated with juvenile growth to the subsequent week: (b) week 1 Peptococcaceae (r_s = −0.36, CI = −0.58 to −0.03, $n_{individuals}$ = 44, n_{otus} = 528, q = 0.04); (c) week 2 S24-7 (r_s = −0.43, CI = −0.64 to −0.12, $n_{individuals}$ = 44, n_{otus} = 2,112, q = 0.005); (d) week 4 Verrucomicrobiaceae (r_s = −0.40, CI = −0.66 to −0.12, $n_{individuals}$ = 43, n_{otus} = 645, q = 0.02); (e) week 4 Anaeroplasmataceae (r_s = −0.36, CI = −0.57 to −0.04, $n_{individuals}$ = 43, n_{otus} = 473, q = 0.04); (f) week 8 Streptococcaceae (r_s = −0.47, CI = −0.73 to −0.14, $n_{individuals}$ = 29, n_{otus} = 377, q = 0.007); (g) week 8 Methanobacteriaceae (r_s = −0.39, CI = −0.71 to −0.005, $n_{individuals}$ = 29, n_{otus} = 29, q = 0.03). White letters in the heatmap refer to the individual scatter plots. Microbial families with empty rows (no r_s values at any time point) have been excluded due to graphical size restrictions; a full-length version of this heatmap can be found at Figure S4

(Deeming, 1999). After the first week, the yolk has been largely absorbed, and they switch to external food sources (Deeming, 1999), mainly plant matter that is high in fibre. Diet has been shown to have large effects on the gut microbiome (David et al., 2014; Pan & Yu, 2014; Waite & Taylor, 2014; Xu, Hu, Xia, Zhan, & Wang, 2003), so it is likely that the dietary switch during this time has a direct impact on the differences seen in the gut community of 1- and 2-week-old chicks. One bacterial group that appears to be particularly important at this initial stage is *Bacteroides*, which was strongly positively associated with growth directly after hatching.

Bacteroides are obligate gut bacteria that are known to actively modify their environment, making it more hospitable for themselves and other microorganisms (Wexler & Goodman, 2017), for example by reducing oxygen levels (Baughn & Malamy, 2004). In chickens, this taxon seems to be more abundant in the later stages of chick development (Lu et al., 2003; Oakley et al., 2014). It is possible that the recruitment of *Bacteroides* after hatching plays a key role in the establishment of the gut microbiota and the initiation of the digestion of external food sources. By the ages 8–12 weeks, the dynamic changes of bacterial abundances had stabilized, and juveniles had largely obtained an adult-like gut community, heavily dominated by Clostridia (primarily the families Ruminococcaceae, Lachnospiraceae, and Clostridiaceae; Figure 4). This bacterial composition is similar to that of other hindgut fermenters (O' Donnell, Harris, Ross, & O'Toole, 2017), and to previous general characterizations of faecal and colon microbes in ostriches (Matsui et al., 2010; Videvall et al., 2018).

One of the most striking changes in the development of the gut microbiota was exhibited by the Verrucomicrobiae (Figure 4). This class consists of only a single species in our data, *Akkermansia muciniphila*, which dominated the gut of 1-week-old ostriches (36.1% in total), but was almost nonexistent in adults (0.09%). *A. muciniphila* is a mucin degrader found in a wide variety of animal species (Belzer & de Vos, 2012), and has been positively associated with a diet rich in polyunsaturated fat in mice (Caesar, Tremaroli, Kovatcheva-Datchary, Cani, & Bäckhed, 2015). We are not aware of any study linking *A. muciniphila* with a diet rich in yolk, but its high prevalence in 1-week-old ostriches digesting yolk and the subsequent rapid decline at older ages suggest a possible association. *A. muciniphila* has also been negatively correlated with obesity, diabetes and inflammatory gut diseases in mice and humans, as well as with body weight in chickens (Caesar et al., 2015; Derrien, Belzer, & de Vos, 2017; Everard et al., 2013; Han et al., 2016; Schneeberger et al., 2015). Our results are consistent with this evidence as we found that *A. muciniphila* was negatively associated with growth in the first weeks after hatching. This effect disappeared after 4 weeks of age (Figure 7) as the abundance of *A. muciniphila* subsequently declined. Although it is not possible to examine the factors regulating the abundance of *A. muciniphila* in our study, there are two potential mechanisms that may play a role. First, it may be that *A. muciniphila* is maintained in individuals with prolonged yolk absorption, for example due to slow or poor digestion, which is indirectly associated with reduced growth. Second, it is possible that there is a direct link between growth and *A. muciniphila* and that our results represent a failure of hosts with low growth rates to regulate their microbiota. Further research is clearly needed to establish the mechanistic basis of the effects of *A. muciniphila* in ostriches and whether it is similar to those documented in other animals.

Previous research has found contrasting effects of microbial diversity on host development and growth. We found that microbial diversity was strongly positively related to growth just after hatching when the gut microbiota was relatively simple. However, after the first week of age, alpha diversity was weakly negatively associated with growth, in line with multiple studies that have shown an increase in animal growth when the diversity of gut bacteria is reduced (Dibner & Richards, 2005; Gaskins et al., 2002). High microbial diversity has been suggested to regulate outbreaks of pathogenic bacteria by making the community more resilient (Sommer, Anderson, Bharti, Raes, & Rosenstiel, 2017). Conversely, we found that the abundance of the bacteria associated with reduced juvenile growth was either independent or greater in communities with higher phylogenetic diversity. This association suggests that during development, gut microbiomes with greater diversity are not more resilient to bacteria that reduce growth in ostriches, and highly diverse communities could even be a source of pathobionts. The specific taxa with negative effects on growth (Peptococcaceae, S24-7, Verrucomicrobiae, Anaeroplasmataceae, Streptococcaceae, Methanobacteriaceae) have all been previously associated with obesity, diabetes and metabolic disease in studies of rodents and humans (Clarke et al., 2013; Kang et al., 2014; Serino et al., 2012; Zeng, Ishaq, Zhao, & Wright, 2016). Given that birds and mammals diverged around 300 million years ago (Kumar & Hedges, 1998), our findings suggest that there are potentially conserved interactions between vertebrate gut microbiomes and their hosts. The mechanisms underlying the relationships between community diversity, abundance of specific bacteria and host traits, such as growth, clearly require further investigation. Nevertheless, our results highlight the importance of examining these factors in concert at specific developmental windows, particularly when there are major dietary shifts during ontogeny.

ACKNOWLEDGEMENTS

We are grateful to all staff at the Oudtshoorn Research Farm, Western Cape Government, for assisting with sample collection, and to three anonymous reviewers for providing valuable comments. Funding was supported by the Helge Ax:son Johnson Foundation, the Längmanska Cultural Foundation, the Lund Animal Protection Foundation, the Lars Hierta Memorial Foundation, and the Royal Physiographic Society of Lund to E.V., by a Wallenberg Academy fellowship and a Swedish Research Council grant to C.K.C., and by the Western Cape Government through the use of their facilities and animals.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

E.V. and C.K.C. planned and designed the study. S.C. provided animal facilities. A.E. supervised the experimental part of the study. N.S., A.E., C.K.C. and E.V. performed the sampling and cared for the animals. A.O. advised on sampling procedures. M.S. supervised the laboratory part of the study, and together with H.M.B. prepared the samples for sequencing. E.V. and C.K.C. performed the bioinformatic

and statistical analyses. S.J.S., R.K. and O.H. provided advice on analyses and the interpretation of results. E.V. and C.K.C. wrote the paper with input from all authors.

DATA ACCESSIBILITY

Supporting information has been made available online. Sequences have been uploaded to the European Nucleotide Archive at EBI-EMBL under accession no.: PRJEB28512.

ORCID

Elin Videvall https://orcid.org/0000-0002-9998-3689
Se Jin Song https://orcid.org/0000-0003-0750-5709
Hanna M. Bensch https://orcid.org/0000-0002-8449-9843
Anel Engelbrecht https://orcid.org/0000-0002-1372-9541
Schalk Cloete http://orcid.org/0000-0002-4548-5633

REFERENCES

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., … Knight, R. (2017). Deblur rapidly resolves single-nucleotide community sequence patterns. *mSystems*, *2*(2), e00191–e216. https://doi.org/10.1128/mSystems.00191-16

Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. Retrieved from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Angelakis, E., Merhej, V., & Raoult, D. (2013). Related actions of probiotics and antibiotics on gut microbiota and weight modification. *The Lancet Infectious Diseases*, *13*(10), 889–899. https://doi.org/10.1016/S1473-3099(13)70179-8

Angelakis, E., & Raoult, D. (2010). The increase of *Lactobacillus* species in the gut flora of newborn broiler chicks and ducks is associated with weight gain. *PLoS ONE*, *5*(5), e10463. https://doi.org/10.1371/journal.pone.0010463

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., … Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718–15723. https://doi.org/10.1073/pnas.0407076101

Ballou, A. L., Ali, R. A., Mendoza, M. A., Ellis, J. C., Hassan, H. M., Croom, W. J., & Koci, M. D. (2016). Development of the chick microbiome: How early exposure influences future microbial diversity. *Frontiers in Veterinary Science*, *3*, 1–12. https://doi.org/10.3389/fvets.2016.00002

Baughn, A. D., & Malamy, M. H. (2004). The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. *Nature*, *427*(6973), 441–444. https://doi.org/10.1038/nature02285

Belzer, C., & de Vos, W. M. (2012). Microbes inside—from diversity to function: The case of *Akkermansia*. *ISME Journal*, *6*(8), 1449–1458. https://doi.org/10.1038/ismej.2012.6

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bonato, M., Evans, M. R., Hasselquist, D., Cloete, S. W. P., & Cherry, M. I. (2009). Growth rate and hatching date in ostrich chicks reflect humoral but not cell-mediated immune function. *Behavioral Ecology and Sociobiology*, *64*(2), 183–191. https://doi.org/10.1007/s00265-009-0835-1

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349. https://doi.org/10.2307/1942268

Caesar, R., Tremaroli, V., Kovatcheva-Datchary, P., Cani, P. D., & Bäckhed, F. (2015). Crosstalk between gut microbiota and dietary lipids aggravates WAT inflammation through TLR signaling. *Cell Metabolism*, *22*(4), 658–668. https://doi.org/10.1016/j.cmet.2015.07.026

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Cho, I., Yamanishi, S., Cox, L., Methé, B. A., Zavadil, J., Li, K., … Blaser, M. J. (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature*, *488*(7413), 621–626. https://doi.org/10.1038/nature11400

Clarke, S. F., Murphy, E. F., O'Sullivan, O., Ross, R. P., O'Toole, P. W., Shanahan, F., & Cotter, P. D. (2013). Targeting the microbiota to address diet-induced obesity: A time dependent challenge. *PLoS ONE*, *8*(6), e65790. https://doi.org/10.1371/journal.pone.0065790

Cloete, S., Brand, T. S., Hoffman, L., Brand, Z., Engelbrecht, A., Bonato, M., … Malecki, I. A. (2012). The development of ratite production through continued research. *World's Poultry Science Journal*, *68*(2), 323–334. https://doi.org/10.1017/S0043933912000372

Cloete, S. W. P., Lambrechts, H., Punt, K., & Brand, Z. (2001). Factors related to high levels of ostrich chick mortality from hatching to 90 days of age in an intensive rearing system. *Journal of the South African Veterinary Association*, *72*(4), 197–202. https://doi.org/10.4102/jsava.v72i4.652

Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I., … Blaser, M. J. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell*, *158*(4), 705–721. https://doi.org/10.1016/j.cell.2014.05.052

Crisol-Martínez, E., Stanley, D., Geier, M. S., Hughes, R. J., & Moore, R. J. (2017). Understanding the mechanisms of zinc bacitracin and avilamycin on animal production: Linking gut microbiota and growth performance in chickens. *Applied Microbiology and Biotechnology*, *101*(11), 4547–4559. https://doi.org/10.1007/s00253-017-8193-9

Danzeisen, J. L., Clayton, J. B., Huang, H., Knights, D., McComb, B., Hayer, S. S., & Johnson, T. J. (2015). Temporal relationships exist between cecum, ileum, and litter bacterial microbiomes in a commercial turkey flock, and subtherapeutic penicillin treatment impacts ileum bacterial community establishment. *Frontiers in Veterinary Science*, *2*, 1–10. https://doi.org/10.3389/fvets.2015.00056

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., … Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

Deeming, D. C. (1999). *The ostrich: Biology, production and health*. Wallingford, CT: CABI International.

Deeming, D. C., & Ayres, L. (1994). Factors affecting the rate of growth of ostrich (*Struthio camelus*) chicks in captivity. *The Veterinary Record*, *135*, 617–622.

Derrien, M., Belzer, C., & de Vos, W. M. (2017). *Akkermansia muciniphila* and its role in regulating host functions. *Microbial Pathogenesis*, *106*, 171–181. https://doi.org/10.1016/j.micpath.2016.02.005

Dewar, M. L., Arnould, J. P. Y., Allnutt, T. R., Crowley, T., Krause, L., Reynolds, J., … Smith, S. C. (2017). Microbiota of little penguins and short-tailed shearwaters during development. *PLoS ONE*, *12*(8), e0183117. https://doi.org/10.1371/journal.pone.0183117

Dibner, J. J., & Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*, *84*(4), 634–643. https://doi.org/10.1093/ps/84.4.634

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(26), 11971–11975. https://doi.org/10.1073/pnas.1002601107

Engberg, R. M., Hedemann, M. S., Leser, T. D., & Jensen, B. B. (2000). Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poultry Science*, *79*(9), 1311–1319. https://doi.org/10.1093/ps/79.9.1311

Engelbrecht, A., Cloete, S. W. P., Bunter, K. L., & van Wyk, J. B. (2011). Genetic variation and heritability of ostrich weight traits. Proceedings of the Association for the Advancement of Animal Breeding and Genetics (Vol. *19*, pp. 183-186). Retrieved from http://www.aaabg.org/aaabg19/proceedings19

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., … Cani, P. D. (2013). Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(22), 9066–9071. https://doi.org/10.1073/pnas.1219451110

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Flores, G. E., Henley, J. B., & Fierer, N. (2012). A direct PCR approach to accelerate analyses of human-associated microbial communities. *PLoS ONE*, *7*(9), e44563. https://doi.org/10.1371/journal.pone.0044563

Foster, K. R., Schluter, J., Coyte, K. Z., & Rakoff-Nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature*, *548*(7665), 43–51. https://doi.org/10.1038/nature23292

Furuse, M., & Okumura, J. (1994). Nutritional and physiological characteristics in germ-free chickens. *Comparative Biochemistry and Physiology Part A, Physiology*, *109*(3), 547–556. https://doi.org/10.1016/0300-9629(94)90193-7

Gaskins, H. R., Collier, C. T., & Anderson, D. B. (2002). Antibiotics as growth promotants: Mode of action. *Animal Biotechnology*, *13*(1), 29–42. https://doi.org/10.1081/ABIO-120005768

Godoy-Vitorino, F., Goldfarb, K. C., Brodie, E. L., Garcia-Amado, M. A., Michelangeli, F., & Domínguez-Bello, M. G. (2010). Developmental microbial ecology of the crop of the folivorous hoatzin. *ISME Journal*, *4*(5), 611–620. https://doi.org/10.1038/ismej.2009.147

Gordon, H. A., & Pesti, L. (1971). The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriological Reviews*, *35*(4), 390–429. https://doi.org/10.1126/science.173.3992.171

Grond, K., Lanctot, R. B., Jumpponen, A., & Sandercock, B. K. (2017). Recruitment and establishment of the gut microbiome in arctic shorebirds. *FEMS Microbiology Ecology*, *93*(12), 1–9. https://doi.org/10.1093/femsec/fix142

Han, G. G., Kim, E. B., Lee, J., Lee, J.-Y., Jin, G., Park, J., … Kong, C. (2016). Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. *SpringerPlus*, *5*(1), 911. https://doi.org/10.1186/s40064-016-2604-8

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., … Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 3047–3052. https://doi.org/10.1073/pnas.1010529108

Herlemann, D. P. R., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME Journal*, *5*(10), 1571–1579. https://doi.org/10.1038/ismej.2011.41

Hervé, M. (2018). *RVAideMemoire: Testing and Plotting Procedures for Biostatistics*. Retrieved from https://cran.r-project.org/package=RVAideMemoire

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: Surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*, *8*(1), 15–25. https://doi.org/10.1038/nrmicro2259

Inagaki, H., Suzuki, T., Nomoto, K., & Yoshikai, Y. (1996). Increased susceptibility to primary infection with *Listeria* monocytogenes in germfree mice may be due to lack of accumulation of L-selectin+ CD44+ T cells in sites of inflammation. *Infection and Immunity*, *64*(8), 3280–3287.

Jiménez, E., Marín, M. L., Martín, R., Odriozola, J. M., Olivares, M., Xaus, J., … Rodríguez, J. M. (2008). Is meconium from healthy newborns actually sterile? *Research in Microbiology*, *159*(3), 187–193. https://doi.org/10.1016/j.resmic.2007.12.007

Kang, S. S., Jeraldo, P. R., Kurti, A., Miller, M. E., Cook, M. D., Whitlock, K., … Fryer, J. D. (2014). Diet and exercise orthogonally alter the gut microbiome and reveal independent associations with anxiety and cognition. *Molecular Neurodegeneration*, *9*(1), 36. https://doi.org/10.1186/1750-1326-9-36

Keokilwe, L., Olivier, A., Burger, W. P., Joubert, H., Venter, E. H., & Morar-Leather, D. (2015). Bacterial enteritis in ostrich (*Struthio camelus*) chicks in the Western Cape Province. *South Africa. Poultry Science*, *94*(6), 1177–1183. https://doi.org/10.3382/ps/pev084

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., … Ley, R. E. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 4578–4585. https://doi.org/10.1073/pnas.1000081107

Kohl, K. D., Brun, A., Bordenstein, S. R., Caviedes-Vidal, E., & Karasov, W. H. (2017). Gut microbes limit growth in House Sparrows nestlings (*Passer domesticus*), but not through limitations in digestive capacity. *Integrative Zoology*, *13*, 139–151. https://doi.org/10.1111/1749-4877.12289

Kohl, K. D., Brun, A., Caviedes-Vidal, E., & Karasov, W. H. (2019). Age-related changes in the gut microbiota of wild House Sparrow nestlings. *Ibis*, *161*(1), 184–191. https://doi.org/10.1111/ibi.12618

Kumar, S., & Hedges, S. B. (1998). A molecular timescale for vertebrate evolution. *Nature*, *392*(6679), 917–920. https://doi.org/10.1038/31927

Kundu, P., Blacher, E., Elinav, E., & Pettersson, S. (2017). Our gut microbiome: The evolving inner self. *Cell*, *171*(7), 1481–1493. https://doi.org/10.1016/j.cell.2017.11.024

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837–848. https://doi.org/10.1016/j.cell.2006.02.017

Lin, J., Hunkapiller, A. A., Layton, A. C., Chang, Y.-J., & Robbins, K. R. (2013). Response of intestinal microbiota to antibiotic growth promoters in chickens. *Foodborne Pathogens and Disease*, *10*(4), 331–337. https://doi.org/10.1089/fpd.2012.1348

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230. https://doi.org/10.1038/nature11550

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., & Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology*, *69*(11), 6816–6824. https://doi.org/10.1128/AEM.69.11.6816-6824.2003

Macpherson, A. J., & Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology*, *4*(6), 478–485. https://doi.org/10.1038/nri1373

Matsui, H., Kato, Y., Chikaraishi, T., Moritani, M., Ban-Tokuda, T., & Wakita, M. (2010). Microbial diversity in ostrich ceca as revealed by 16S ribosomal RNA gene clone library and detection of novel *Fibrobacter* species. *Anaerobe*, *16*(2), 83–93. https://doi.org/10.1016/j.anaerobe.2009.07.005

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Million, M., Angelakis, E., Paul, M., Armougom, F., Leibovici, L., & Raoult, D. (2012). Comparative meta-analysis of the effect of *Lactobacillus* species on weight gain in humans and animals. *Microbial Pathogenesis*, *53*(2), 100–108. https://doi.org/10.1016/j.micpath.2012.05.007

Mills, T. K., Lombardo, M. P., & Thorpe, P. A. (1999). Microbial colonization of the cloacae of nestling tree swallows. *The Auk*, *116*(4), 947–956. https://doi.org/10.2307/4089674

O' Donnell, M. M., Harris, H. M. B., Ross, R. P., & O'Toole, P. W. (2017). Core fecal microbiota of domesticated herbivorous ruminant, hindgut fermenters, and monogastric animals. *MicrobiologyOpen*, *6*(5), 1–11. https://doi.org/10.1002/mbo3.509

Oakley, B. B., Buhr, R. J., Ritz, C. W., Kiepper, B. H., Berrang, M. E., Seal, B. S., & Cox, N. A. (2014). Successional changes in the chicken cecal microbiome during 42 days of growth are independent of organic acid feed additives. *BMC Veterinary Research*, *10*(1), 282. https://doi.org/10.1186/s12917-014-0282-8

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … Wagner, H. (2017). *vegan: Community Ecology Package*. Retrieved from https://CRAN.R-project.org/package=vegan

Pan, D., & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), 108–119. https://doi.org/10.4161/gmic.26945

Pantoja-Feliciano, I. G., Clemente, J. C., Costello, E. K., Perez, M. E., Blaser, M. J., Knight, R., & Dominguez-Bello, M. G. (2013). Biphasic assembly of the murine intestinal microbiota during early development. *ISME Journal*, *7*(6), 1112–1115. https://doi.org/10.1038/ismej.2013.15

Perez-Muñoz, M. E., Arrieta, M.-C., Ramer-Tait, A. E., & Walter, J. (2017). A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: Implications for research on the pioneer infant microbiome. *Microbiome*, *5*(1), 48. https://doi.org/10.1186/s40168-017-0268-4

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team.(2016). *{nlme}: Linear and Nonlinear Mixed Effects Models*. Retrieved from https://CRAN.R-project.org/package=nlme

Potti, J., Moreno, J., Yorio, P., Briones, V., García-Borboroglu, P., Villar, S., & Ballesteros, C. (2002). Bacteria divert resources from growth for magellanic penguin chicks. *Ecology Letters*, *5*(6), 709–714. https://doi.org/10.1046/j.1461-0248.2002.00375.x

Price, M. N., Dehal, P. S., & Arkin, A. P. (2009). FastTree: Computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution*, *26*(7), 1641–1650. https://doi.org/10.1093/molbev/msp077

R Core Team. (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org/

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, *9*(5), 313–323. https://doi.org/10.1038/nri2515

Russell, S. L., Gold, M. J., Hartmann, M., Willing, B. P., Thorson, L., Wlodarska, M., … Finlay, B. B. (2012). Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Reports*, *13*(5), 440–447. https://doi.org/10.1038/embor.2012.32

Schneeberger, M., Everard, A., Gómez-Valadés, A. G., Matamoros, S., Ramírez, S., Delzenne, N. M., … Cani, P. D. (2015). *Akkermansia muciniphila* inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. *Scientific Reports*, *5*, 16643. https://doi.org/10.1038/srep16643

Sekirov, I., Russell, S. L. S., Antunes, L. C. M., & Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiological Reviews*, *90*(3), 859–904. https://doi.org/10.1152/physrev.00045.2009

Serino, M., Luche, E., Gres, S., Baylac, A., Bergé, M., Cenac, C., … Burcelin, R. (2012). Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut*, *61*(4), 543–553. https://doi.org/10.1136/gutjnl-2011-301012

Shin, S. C., Kim, S.-H., You, H., Kim, B., Kim, A. C., Lee, K.-A., … Lee, W.-J. (2011). *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*, *334*(6056), 670–674. https://doi.org/10.1126/science.1212782

Skadhauge, E., & Dawson, A. (1999). Physiology. In D. C. Deeming (Ed.), *The ostrich: Biology, production and health* (pp. 51–81). Wallingford, CT: CABI International.

Smith, P., Willemsen, D., Popkes, M., Metge, F., Gandiwa, E., Reichard, M., & Valenzano, D. R. (2017). Regulation of life span by the gut microbiota in the short-lived african turquoise killifish. *eLife*, *6*, 1–26. https://doi.org/10.7554/eLife.27014

Sommer, F., Anderson, J. M., Bharti, R., Raes, J., & Rosenstiel, P. (2017). The resilience of the intestinal microbiota influences health and disease. *Nature Reviews Microbiology*, *15*(10), 630–638. https://doi.org/10.1038/nrmicro.2017.58

Sommer, F., & Bäckhed, F. (2013). The gut microbiota — masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227–238. https://doi.org/10.1038/nrmicro2974

Sprinz, H., Kundel, D. W., Dammin, G. J., Horowitz, R. E., Schneider, H., & Formal, S. B. (1961). The response of the germ-free guinea pig to oral bacterial challenge with *Escherichia coli* and *Shigella flexneri*: With special reference to lymphatic tissue and the intestinal tract. *The American Journal of Pathology*, *39*(6), 681–695.

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. M. (2016). The composition of the zebrafish intestinal microbial community varies across development. *ISME Journal*, *10*(3), 644–654. https://doi.org/10.1038/ismej.2015.140

Teyssier, A., Lens, L., Matthysen, E., & White, J. (2018). Dynamics of gut microbiota diversity during the early development of an avian host: Evidence from a cross-foster experiment. *Frontiers in Microbiology*, *9*, 1–12. https://doi.org/10.3389/fmicb.2018.01524

van der Wielen, P. W. J. J., Keuzenkamp, D. A., Lipman, L. J. A., van Knapen, F., & Biesterveld, S. (2002). Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology*, *44*(3), 286–293. https://doi.org/10.1007/s00248-002-2015-y

van Dongen, W. F. D., White, J., Brandl, H. B., Moodley, Y., Merkling, T., Leclaire, S., … Wagner, R. H. (2013). Age-related differences in the cloacal microbiota of a wild bird species. *BMC Ecology*, *13*(1), 11. https://doi.org/10.1186/1472-6785-13-11

Verwoerd, D. J. (2000). Ostrich diseases. *Revue Scientifique Et Technique (International Office of Epizootics)*, *19*(2), 638–661. https://doi.org/10.20506/rst.19.2.1235

Verwoerd, D. J., Deeming, D. C., Angel, C. R., & Perelman, B. (1999). Rearing environments around the world. In D. C. Deeming (Ed.), *The ostrich: Biology, production and health* (pp. 163–206). Wallingford, CT: CABI International.

Videvall, E. (2018). *Evolutionary genomics of host-microbe interactions*. PhD dissertation, Lund University.

Videvall, E., Strandh, M., Engelbrecht, A., Cloete, S., & Cornwallis, C. K. (2017). Direct PCR offers a fast and reliable alternative to

conventional DNA isolation methods for gut microbiomes. *mSystems*, *2*(6), e00132–e217. https://doi.org/10.1128/mSystems.00132-17

Videvall, E., Strandh, M., Engelbrecht, A., Cloete, S., & Cornwallis, C. K. (2018). Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology Resources*, *18*(3), 424–434. https://doi.org/10.1111/1755-0998.12744

Waite, D. W., & Taylor, M. W. (2014). Characterizing the avian gut microbiota: Membership, driving influences, and potential function. *Frontiers in Microbiology*, *5*, 1–12. https://doi.org/10.3389/fmicb.2014.00223

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07.

Warnes, G., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W., Lumley, T., … Venables, B. (2018).*gplots: various R programming tools for plotting data*. Retrieved from https://CRAN.R-project.org/package=gplots

Wexler, A. G., & Goodman, A. L. (2017). An insider's perspective: *Bacteroides* as a window into the microbiome. *Nature Microbiology*, *2*(5), 17026. https://doi.org/10.1038/nmicrobiol.2017.26

Wickham, H. (2009). *ggplot2: Elegant graphics for data analysis*. New York, NY: Springer.

Wilkinson, T. J., Cowan, A. A., Vallin, H. E., Onime, L. A., Oyama, L. B., Cameron, S. J., … Huws, S. A. (2017). Characterization of the microbiome along the gastrointestinal tract of growing Turkeys. *Frontiers in Microbiology*, *8*, 1–11. https://doi.org/10.3389/fmicb.2017.01089

Wostmann, B., Larkin, C., Moriarty, A., & Bruckner-Kardoss, E. (1983). Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. *Laboratory Animal Science*, *33*(1), 46–50.

Xu, Z., Hu, C., Xia, M., Zhan, X., & Wang, M. (2003). Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poultry Science*, *82*(6), 1030–1036. https://doi.org/10.1093/ps/82.6.1030

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053

Yin, Y., Lei, F., Zhu, L., Li, S., Wu, Z., Zhang, R., … Wang, X. (2010). Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression. *ISME Journal*, *4*(3), 367–376. https://doi.org/10.1038/ismej.2009.128

Zeng, H., Ishaq, S. L., Zhao, F.-Q., & Wright, A.-D.-G. (2016). Colonic inflammation accompanies an increase of β-catenin signaling and Lachnospiraceae/Streptococcaceae bacteria in the hind gut of high-fat diet-fed mice. *The Journal of Nutritional Biochemistry*, *35*, 30–36. https://doi.org/10.1016/j.jnutbio.2016.05.015

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Videvall E, Song SJ, Bensch HM, et al. Major shifts in gut microbiota during development and its relationship to growth in ostriches. *Mol Ecol*. 2019;28:2653–2667. https://doi.org/10.1111/mec.15087