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Review Article

The temporal fluctuations and development of faecal microbiota in commercial layer flocks

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ABSTRACT

The microbiota of the gastrointestinal tract influences gut health, which in turn strongly impacts the general health and productivity of laying hens. It is essential to characterise the composition and temporal development of the gut microbiota in healthy layers raised under different management systems, to understand the variations in typical healthy microbiota structure, so that deviations from this might be recognised and correlated with production and health issues when they arise. The present investigation aimed to study the temporal development and phylogenetic composition of the gut microbiota of four commercially raised layer flocks from hatch to end of the production cycle. Non-intrusive faecal sampling was undertaken as a proxy to represent the gut microbiota. Sequencing of 16S rRNA gene amplicons was used to characterise the microbiota. Beta diversity analysis indicated that each faecal microbiota was different across the four flocks and had subtly different temporal development patterns. Despite these inter-flock differences, common patterns of microbiota development were identified. Firmicutes and Proteobacteria were dominant at an early age in all flocks. The microbiota developed gradually during the rearing phase; richness and diversity increased after 42 d of age and then underwent significant changes in composition after the shift to the production farms, with Bacteroidota becoming more dominant in older birds. By developing a more profound knowledge of normal microbiota development in layers, opportunities to harness the microbiota to aid in the management of layer gut health and productivity may be more clearly seen and realised.

1. Introduction

The gut microbiota has been defined as the collection of all microbes found in the gastrointestinal tract. The microbiota establishes a symbiotic association with the host and has been shown to influence host physiology. It plays a critical role in the development and training of the immune system, intestinal health and physiology, and nutrient release and absorption in the host (Khan et al., 2020). Food animals are an important protein source for the growing human population, and efficient production is critical to minimising the ecological impact of the industries (Henchion et al., 2017). Thus, understanding the structure of the typical healthy gut microbiota and how it can be maintained and positively manipulated is likely to be an important way to enhance bird productivity and health and therefore maximise the efficiency of animal protein production.

Egg and chicken meat industries constitute a large and growing proportion of the food-producing animal industries because of their production efficiency and competitive pricing. For poultry, most microbiota research has mainly been focused on broilers (Stanley et al., 2013a, 2013b). The meat-producing birds represent a large sector of the poultry industry, and broiler studies are less

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laborious due to their short commercial life span. Previous research has shown that birds from the same parent stock (i.e. similar genetics), from the same hatchery, raised in the same housing conditions, and on the same feed, had highly variable gut microbiota (Stanley et al., 2013b). The authors hypothesised that this variation in gut microbiota could be attributed to random colonisation with environmental bacteria in the absence of maternally derived bacteria in the very clean hatcheries that are typical within the industry (Stanley et al., 2013b). A comparative study conducted in slow-growing chickens of different breeds raised in a cage and free-range systems showed that the composition and diversity of the gut microbiota might be influenced by the different housing systems (Sun et al., 2018). Other studies have also shown that housing systems can play a key role in the establishment of gut microbiota in birds (Al-Ajeeli, 2017; Hubert et al., 2019; Ocejo et al., 2019; Adhikari et al., 2020; Seidlerova et al., 2020). However, it is important to note that these studies were conducted in the controlled environments of experimental/research animal facilities, which are different from field conditions.

Laying hens are genetically different, have different feed and housing, and have a much longer production lifespan than broilers. It was expected that the dynamics of gut microbiota establishment and development in layers are markedly different from broilers (Kers et al., 2018). In laying hens, the housing systems are substantially different (cage, free-range, barn) than broilers which are generally raised on deep litter systems. In the last few decades, multiple studies have focused on the effect of housing systems on poultry welfare (Duncan, 2001; Ferrante, 2009; Janczak and Riber, 2015; Meseret, 2016). In 1999 the European Union issued a directive to phase out battery cages and shift layer production to alternate housing systems (Appleby, 2003). Other countries, like New Zealand and Canada, have announced plans to phase out cage systems in the future (Hartcher and Jones, 2017). In Australia, the majority of eggs are produced in free-range (47%) and cage systems (40%), with a small percentage of barn production systems (11%) (Australian Eggs, Annual Report 2019). The pullet rearing practices in Australia are variable across the egg industry. Generally, the flocks are raised on the floor until birds approach the point of lay and then shifted to free-range, barn, or cage production systems. Earlier studies in layer flocks focused on single flocks, but it is difficult to determine the general relevance of results from studies based on a single flock (Videnska et al., 2014; Ngunjiri et al., 2019). Some studies recently analysed the impact of the environment on the gut microbiota of layers (Hubert et al., 2019; Adhikari et al., 2020; Seidlerova et al., 2020). However, these studies were performed on hens during their early production. In the previous studies, the effects of cage-free vs. caged housing systems (Hubert et al., 2019), conventional cages vs. enriched colony cages (Adhikari et al., 2020), and indoor housing vs. outdoor housing (Seidlerova et al., 2020) on gut microbiota were compared. These controlled pen trials investigated how gut microbiota composition was influenced by a few variables while attempting to reduce variability in other factors (e.g., the same feed). The controlled environments of research facilities do not entirely represent the field relevant practices followed by commercial farms. This study investigated faecal microbiota temporal development and structure from hatching to the end of the production cycle of four commercial layer flocks, reared in the barn, free-range, and cage housing systems. Faecal microbiota was studied because it can be collected without sacrificing birds and is the type of sample that could be taken in the future for routine monitoring and assessment of some aspects of the gut microbiota. Previous work has demonstrated that faecal samples give a good accounting of the microbial species present in the caecum but gives a less accurately estimate of their relative abundance in the caecum (Stanley et al., 2015).

2. Materials and methods

2.1. Animal ethics

All the animal work was conducted according to national and international animal welfare guidelines and complied with the ARRIVE guidelines (Percie du Sert et al., 2020). The experiment was approved by the Animal Ethics Committee, University of Adelaide (Approval No. S-2018-015). The protocol was carried out in accordance with the guidelines specified in the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition 2013.

2.2. Farms

Four commercial layer flocks were selected for the study. The selection of the farms was based on the willingness of the farmers to participate in the study and the distance from the research laboratory. The flocks A and B were reared on dirt floors from 1 d old and transferred to free-range and barn housing production systems, respectively. In flock A, a Spotty Liver Disease (SLD) outbreak (caused by *Campylobacter hepaticus* infection (Van et al., 2017)) was noted at the age of 34 weeks, which resulted in a drop in egg production (20%) and recovered in the next 2 weeks (Fig. S1). The flock was treated with chlortetracycline (60 mg/kg) through water for 1 week. Flock C was reared on a concrete floor from 1 d old and transferred to a multi-age cage production system (four flocks of different ages housed in the same shed, the birds in each flock were housed in different rows within the shed). Flock D was in cage systems from rearing to the end of production. For sampling birds in cages, each cage had a colony of 6 to 20 birds, each cage was labelled, and same cage was sampled at each time point. All four flocks were transported to production houses at the age of 16 weeks. The birds in all four flocks belonged to the same breed, Hyline, and originated from the same hatchery. All four flocks were reared at different locations but in the same season (autumn-winter). Flock A was vaccinated for infectious bronchitis virus, coccidia, infectious laryngotracheitis virus, Newcastle disease virus, fowl pox, avian encephalomyelitis virus, fowl cholera, egg drop syndrome, and Marek's disease virus. Flock B and flock C were vaccinated against all the above diseases except coccidia and fowl pox. Flock D was vaccinated for infectious bronchitis, infectious laryngotracheitis, Newcastle disease, avian encephalomyelitis, fowl cholera, egg drop syndrome, and Marek's disease.

All four flocks received wheat and soya-based diet and the ingredients used in the feeds are shown in Table S1. All flocks met the expected egg laying performance as per the Hyline breed standard (Fig. S1), and so it was concluded that all diets were of high quality and provided adequate nutrition. The details regarding the timing of feed changes are listed in Table S2. The details of each flock are listed in Table S3.

2.3. Sample collection

Meconium samples ($n = 30$ each) were collected from all three flocks by scrapping hatching trays in sterile sample containers from each flock at the hatchery (TechnoPlas, P10065SU). Faecal samples ($n = 30$) were collected over the course of the study, from hatch to 70 weeks (days 0, 2, 5, 10, 20, weeks 6, 12, 18, 20, 22, 28, 34, 40, 46, 52, 58, 64, 70; for flock D, there was an additional sampling conducted at week 75), by cloacal swabbing. The faecal material on each swab was resuspended in 500 μL of phosphate buffered saline (PBS), then transported on ice from the hatchery or farm to the laboratory and stored at −20 °C until processed for DNA extraction.

2.4. DNA extraction

DNA was extracted and purified from cloacal swabs ($n = 1999$) using the QIAamp Fast DNA Stool Mini kit (Qiagen) using a modified protocol (Knudsen et al., 2016) which was further modified for this study. Briefly, 200 mg of faecal sample (semi-solid faecal slurry suspended in PBS) was vortexed after adding 1 mL of preheated (70 °C) InhibitEx buffer (Qiagen). The samples were homogenised after the addition of 390 mg glass beads (Sigma glass beads, acid washed, 450 to 600 μm (180 mg) and 106 μm (210 mg)) using a Bullet Blender (Next Advance) for 5 min. The samples were incubated on ice for 30 s before incubating in a 95 °C heat block for 7 min. The samples were centrifuged for 2 min and the supernatant was collected and then further processed according to the modified QIAamp Fast DNA Stool Mini kit protocol (Knudsen et al., 2016). The DNA was eluted in 100 μL of ATE buffer (Qiagen) and stored at −20 °C.

2.5. Amplicon sequencing

The V3–V4 region of the 16S rRNA gene was amplified with Q5 high fidelity DNA polymerase (New England Biolabs), using dual indexing, variable spacer primers; the forward primer was 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer was

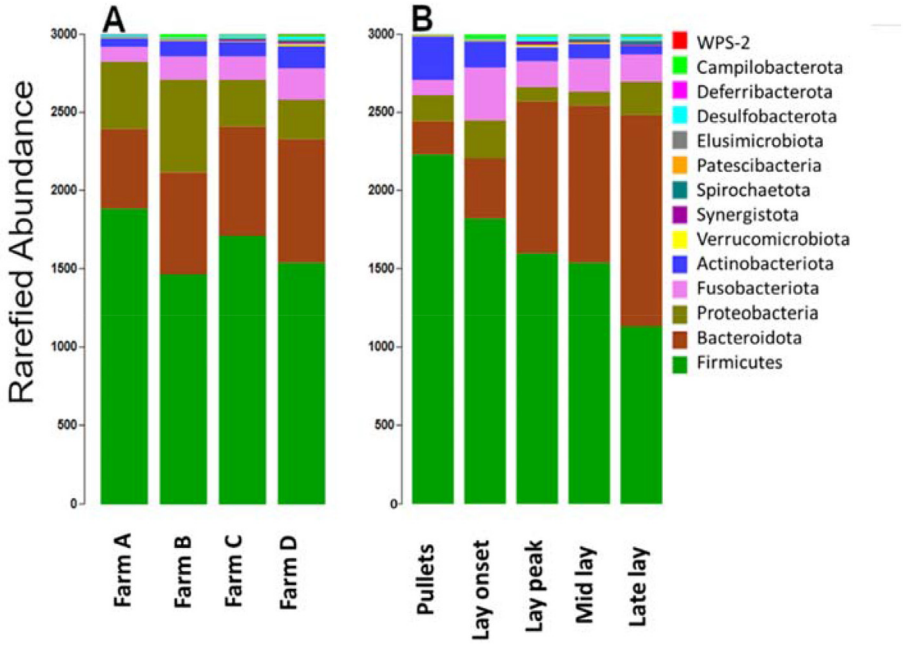

Fig. 1. Stacked bar-charts showing phylum level microbiota compositions by the farm (A) and by stage of lay (B).

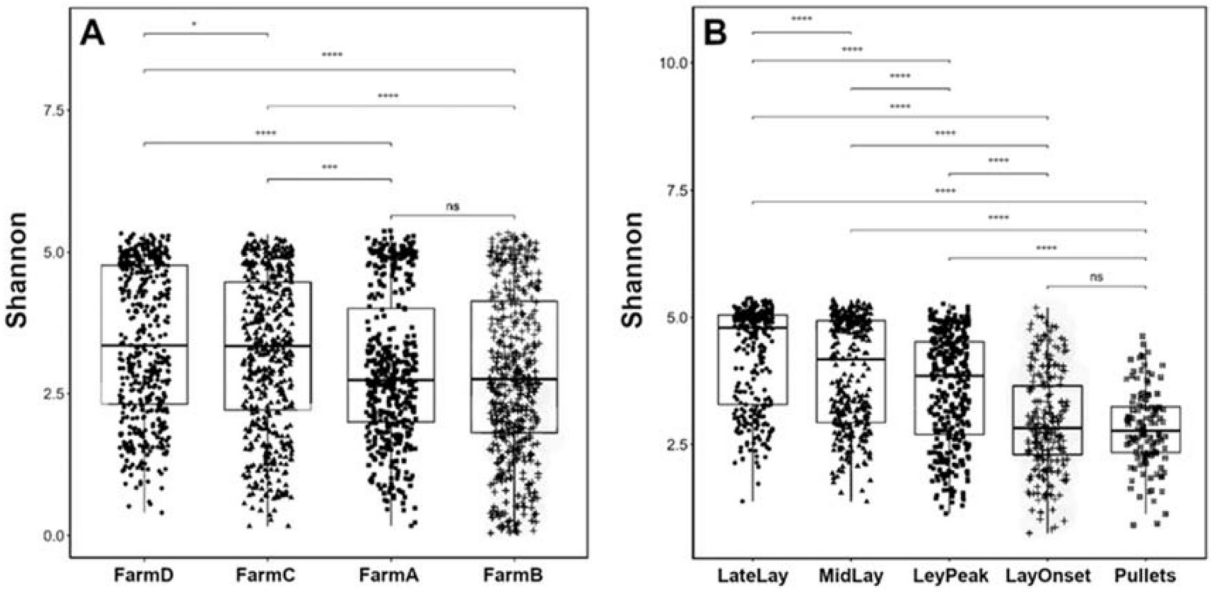

Fig. 2. Shannon diversity index by the farm (A) and the stage of lay (B). The groups are sorted from highest to lowest diversity. Significance calculated via the Kruskal–Wallis test is shown via asterisk (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$).

806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Fadrosh et al., 2014). The cycling conditions for PCR were 98 °C for 1 min, 35 cycles of 98 °C for 10 s, 49 °C for 30 s, and 72 °C for 30 s and final extension at 72 °C for 10 min. The amplicon sequencing was performed using an Illumina MiSeq system (2x300 bp). The data was demultiplexed with Cutadapt (Martin, 2011), and the microbiota analysis was performed in Quantitative Insights into Microbial Ecology 2 (QIIME2) (Bolyen et al., 2019). Quality filtering, denoising, and chimera removal were done using Dada2 (Callahan et al., 2016) as a QIIME2 plugin with all recommended parameters. Taxonomy was assigned using SILVA v138.1 database (Quast et al., 2013).

2.6. Statistical analysis

The microbiota analysis was performed at amplicon sequence variant (ASV) and higher levels using a range of R packages most notably Phyloseq (McMurdie and Holmes, 2013), Phylosmith (Smith, 2019), Vegan (Dixon, 2003), and Microeco (Liu et al., 2021). Primer 7e with the Permanova+ plugin (Anderson et al., 2008) was also used in the data analysis. The ASV level table was rarefied to 3000 sequences per sample. This study collected a total of 1999 swab samples from four flocks. The data were analysed in total and separated by the individual farm. Stage of lay was used as a variable

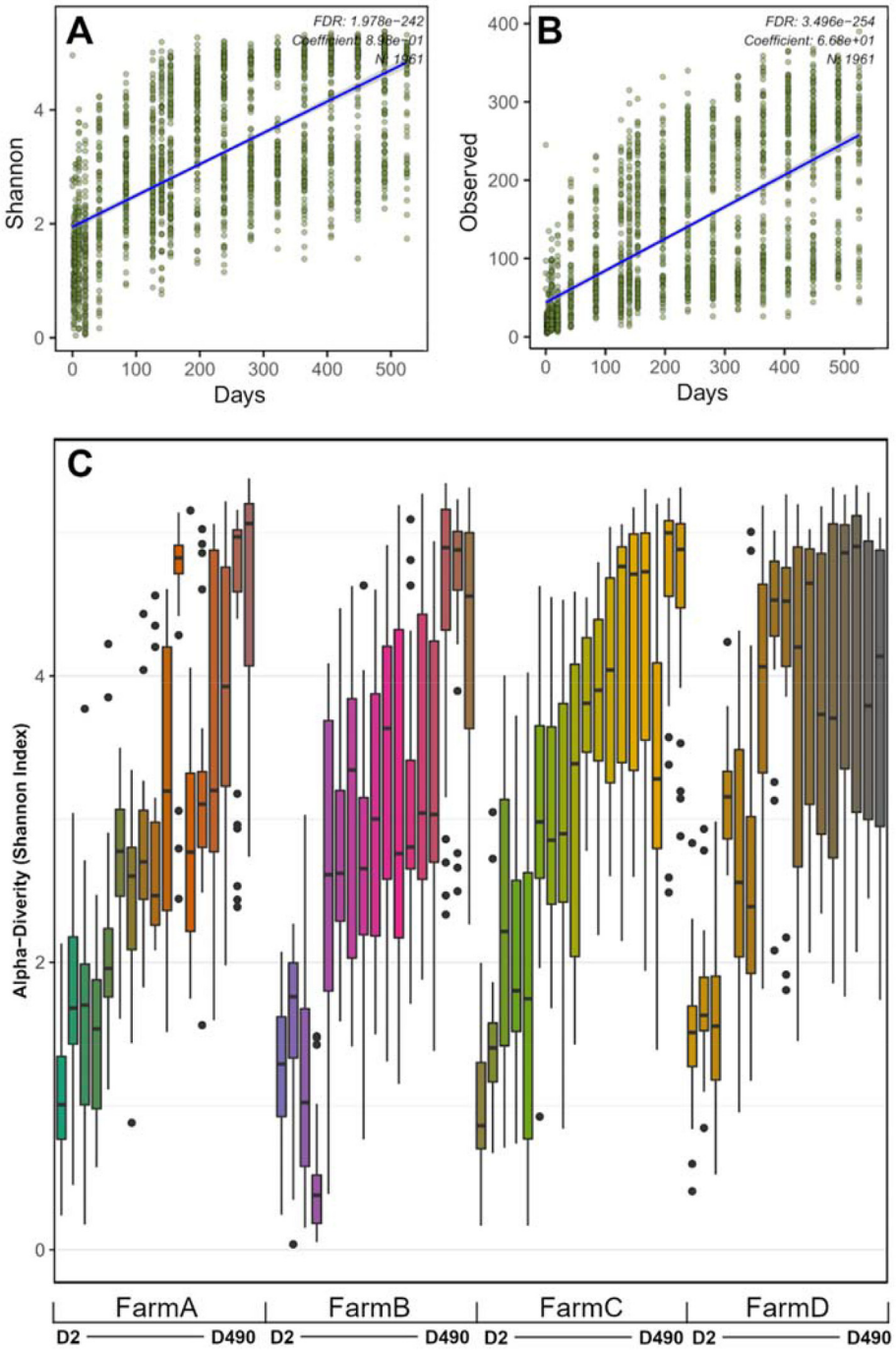

Fig. 3. Regression plots (A and B) showing Shannon and Observed amplicon sequence variant (ASV) index correlation with time, and Shannon index in each farm (C) presented per sampling time (d 2 to 490).

Fig. 4. Multiple dimension scale (MDS) plot based on unweighted UniFrac.

by separating birds into pullets (weeks 10 to 14), lay onset (weeks 18 to 20), the peak of lay (weeks 22 to 34), mid to lay (weeks 46 to 58), and late lay (weeks 64 to 75). In graphs and analysis using stage of lay, all sampling timepoints within the selected time range were included in each category. All the statistical methods used are specified in the results section.

3. Results

An extensive dataset was generated. After rarefaction, the dataset consisted of 1961 samples, each rarefied to 3000 sequences, and covered 13,318 ASVs. This dataset has provided deep insights into microbiota development and maturation in layers across different production systems, from hatch to the end of production. Because of the size of the dataset and the detailed analysis, extensive supplementary data given in Appendix file, comprising 12 Figures and 8 Tables, are included to support the findings presented in the results section.

3.1. Overall microbiota structure across the four farms

Despite the farms being at different locations, and having differences in the feed and production systems, the same major phyla were noted in each flock. The dominant bacterial phyla were Firmicutes, Bacteroidota, Proteobacteria and Fusobacteriota (Fig. 1). The relative abundance levels of these phyla changed over time between the major life stages of the production hens (Fig. 1). The change in phylum level composition across the different stages of lay for each farm is shown in Fig. S2. These major time dependent shifts were even more evident at the genus level. The genus level compositions changed across the life stages of the birds across the whole dataset (Fig. S3), and the general trends were also replicated in each of the individual farms (Fig. S4). More detailed analysis revealed that *Lactobacillus*, *Dickeya*, and uncultured *Peptostreptococcaceae* and *Bacteroides* genera were the most abundant genera across the four farms (Fig. S4), however, the dominance was stage of lay dependent and changed over time (Figs. S2 to S4).

3.2. Richness and diversity

Richness and diversity measures of the microbiota steadily increased over time on all four farms. Fig. 2 shows the alpha

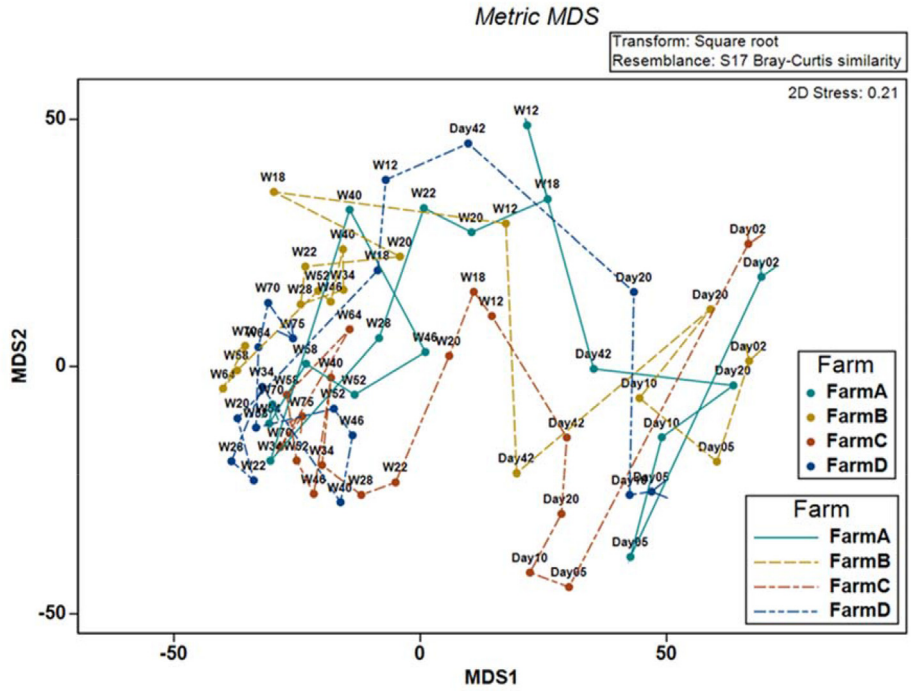

Fig. 5. Genus level timeline of the temporal development of each farm from d 2 to week 75. Each line follows the timeline of the microbiota development for one farm. MDS = multiple dimension scale; W = week.

diversity measures at an ASV level across the complete dataset, shown by the farm (Fig. 2A) and by the stage of lay (Fig. 2B). Individual significance comparisons were calculated using the Kruskal–Wallis test. The association of major diversity indices with the age was tested with linear models using MaAsLin2 R package (Mallick et al., 2021). The possible confounding effects of farms was controlled by including farm as a random factor in the models. The regression analysis showed significant positive temporal correlations with a steady increase in richness and diversity (Fig. 3A and B, Table S4). Fig. 3C shows an increase in diversity for each farm over time, confirming that this temporal enrichment trend was consistent across all four farms.

3.3. Beta diversity

All variables (farm, lay stage, and age) were significantly correlated with changes in microbiota composition (Permanova using weighted and unweighted UniFrac, all $P < 0.001$). This extended to individual within group pairwise comparisons by the farm (between farms) and lay stage (between stages) using both weighted and unweighted UniFrac distances. The only exception was a comparison of lay peak and mid lay by weighted UniFrac, where there were no significant changes. Some of the individual comparisons of sampling times are not significantly different. Fig. 4 presents a multiple dimension scale (MDS) plot based on unweighted UniFrac distances. The plot shows higher dispersion of microbiota communities at earlier stages of lay and more compact communities in older birds, most notable in the late lay stage, indicating greater similarity amongst the faecal microbiota compositions of older birds. Figs. S4 and S5 show distance-based redundancy analysis (dbRDA) plots with data coloured by the farm (Fig. S5) and by the time of sampling (Fig. S6) with genera level vectors selected by Pearson correlation.

3.4. Dynamics of microbiota development

The changes over time in microbiota compositions in each farm and comparison in the microbiota maturation process between the farms are illustrated in Fig. 5, at the genus level, and Fig. S7, at the phylum level. The distances between the average microbiota timepoints for each farm were calculated using Bray Curtis on square root transformed data and plotted using Primer 7 software. Both figures show a clear central shift towards mature microbiota from d 42 (6 weeks of age) for Farms A, B and C and d 20 in Farm D (Fig. 5). This shift at the phylum level becomes apparent between weeks 12 and 22 (Fig. S7). The clustering of samples in an MDS plot, using a complete linkage algorithm (Fig. 6) showed the separation of samples. Irrespective of farm origin, the samples from d 2 to 42 clustered together, as did the samples from older birds. The plot displays genus-level vectors showing known genera that include opportunistic pathogens, including *Clostridium*, *Escherichia-Shigella*, *Streptococcus*, *Staphylococcus*, *Campylobacter* and *Gallibacterium*. Fig. 6 indicates that the abundance and hence risk from *Clostridium*, *Escherichia-Shigella*, *Streptococcus* and *Staphylococcus* is higher at earlier ages and *Campylobacter* and *Gallibacterium* towards the middle of the production cycle. Fig. S8 shows the same graph in 3D with a superimposed complete linkage tree.

3.5. Taxa contributing to laying stage succession

The taxa involved in the temporal shifts in microbiota and farm-to-farm differences were further investigated. First, Simper analysis (Primer 7e) which calculates taxa contribution to differences between specified groups of samples, was used. Simper analysis at the genus level showed that the most distinctive shift was from pullets to point of lay (POL), while all other microbiota shifts were driven by *Lactobacillus*, uncultured *Peptostreptococcaceae*, *Fusobacterium*, and *Bacteroides*. Figs. S8 to S12 show the temporal changes in the abundance of these genera for each farm separately to confirm the high reproducibility of their temporal patterns across four very different farms. The shifts in the abundance between these taxa contributed to around 50% of dissimilarity in all other shifts (Table 1). Table S5 is an extended version of Table 1, showing an average abundance of groups, average dissimilarity, and SD. At the phylum level, the major contributors of 70% of stage shift dissimilarities were Firmicutes, Bacteroidota and Fusobacteriota (Table S6).

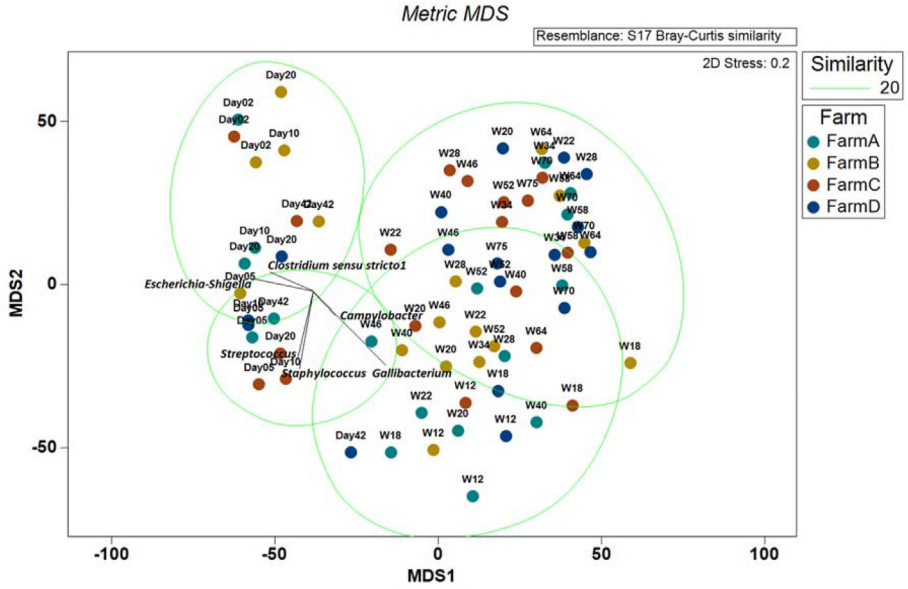

Fig. 6. Multiple dimension scale (MDS) plot, with samples coloured by the farm and labelled by the time of sampling. Samples were clustered using a complete linkage algorithm in Primer 7e software, and the green ellipses show clusters of samples with a minimum of 20% Bray–Curtis similarity. W = week.

Table 1
Genera that contribute to the significant microbiota shifts between the stages of lay.

Comparison (groups 1 & 2)	Contributing genera	Contribution, %	Cumulative contribution, %
Pullets & POL	Uncultured *Peptostreptococcaceae*	14.83	14.83
Pullets & POL	*Lactobacillus*	11.71	26.54
Pullets & POL	*Streptococcus*	9.33	35.87
Pullets & POL	*Fusobacterium*	8.32	44.19
Pullets & POL	*Turicibacter*	5.19	49.39
Pullets & POL	*Enterococcus*	4.62	54.01
Pullets & POL	*Gallibacterium*	3.49	57.50
Pullets & POL	*Bacteroides*	3.34	60.84
Pullets & POL	*Dickeya*	2.99	63.83
Pullets & POL	*Alistipes*	2.96	66.8
Pullets & POL	Unclassified *Bacteroidales*	1.98	68.78
Pullets & POL	Unclassified	1.92	70.70
POL & lay peak	*Lactobacillus*	14.09	14.09
POL & lay peak	Uncultured *Peptostreptococcaceae*	11.46	25.55
POL & lay peak	*Fusobacterium*	8.84	34.39
POL & lay peak	*Bacteroides*	8.03	42.42
POL & lay peak	*Streptococcus*	6.11	48.53
POL & lay peak	Unclassified *Bacteroidales*	4.38	52.91
POL & lay peak	*Gallibacterium*	2.77	55.69
POL & lay peak	*Turicibacter*	2.58	58.26
POL & lay peak	*Dickeya*	2.51	60.78
POL & lay peak	Unclassified *Prevotellaceae*	2.26	63.03
POL & lay peak	*Enterococcus*	2.22	65.26
POL & lay peak	*Faecalibacterium*	1.68	66.94
POL & lay peak	*Phascolarctobacterium*	1.60	68.54
POL & lay peak	*Alistipes*	1.58	70.12
Lay peak & mid lay	*Lactobacillus*	17.74	17.74
Lay peak & mid lay	Uncultured *Peptostreptococcaceae*	12.39	30.13
Lay peak & mid lay	*Bacteroides*	10.60	40.72
Lay peak & mid lay	*Fusobacterium*	7.36	48.08
Lay peak & mid lay	Unclassified *Bacteroidales*	5.90	53.98
Lay peak & mid lay	Unclassified *Prevotellaceae*	2.75	56.73
Lay peak & mid lay	*Turicibacter*	2.21	58.94
Lay peak & mid lay	*Rikenellaceae_RC9*	2.13	61.07
Lay peak & mid lay	*Gallibacterium*	2.08	63.15
Lay peak & mid lay	*Prevotella*	2.02	65.17
Lay peak & mid lay	*Phascolarctobacterium*	1.96	67.13
Lay peak & mid lay	Unclassified *Lachnospiraceae*	1.59	68.72
Lay peak & mid lay	Unclassified	1.54	70.26
Mid lay & late lay	*Lactobacillus*	15.35	15.35
Mid lay & late lay	*Bacteroides*	11.99	27.34
Mid lay & late lay	Uncultured *Peptostreptococcaceae*	10.96	38.29
Mid lay & late lay	*Fusobacterium*	7.55	45.84
Mid lay & late lay	Unclassified *Bacteroidales*	6.27	52.12
Mid lay & late lay	*Gallibacterium*	4.14	56.25
Mid lay & late lay	*Prevotella*	3.20	59.45
Mid lay & late lay	Unclassified *Prevotellaceae*	2.71	62.16
Mid lay & late lay	*Rikenellaceae RC9*	2.08	64.24
Mid lay & late lay	*Turicibacter*	2.00	66.25
Mid lay & late lay	*Phascolarctobacterium*	1.60	67.84
Mid lay & late lay	Unclassified *Lachnospiraceae*	1.49	69.33
Mid lay & late lay	Unclassified	1.48	70.81

POL = point of lay.
Simper analysis identifies genera that contribute to significant microbiota shifts between the stages of lay. Table 1 shows genus level contribution (Contribution %) to groups dissimilarity and cumulative contribution up to 70%, which was considered as a cut-off for low contributions. Comparisons are independent from one another.

3.6. Biomarker discovery

In addition to Simper analysis, to identify the taxa contributing to microbiota shifts associated with stages of lay, the linear discriminant analysis effect size (LEfSe) biomarker discovery algorithm was used to identify genera enriched in different farms (Fig. 7) and stages of lay (Fig. 8).

The onset of the lay stage was characterised by a high temporal prevalence of pathogen-rich genera like *Escherichia-Shigella*, *Campylobacter*, *Clostridium sensu stricto 1*, but also genera often regarded as beneficial, such as *Bifidobacterium*, *Akkermansia*, *Candidatus Arthromitus* and *Butyricicoccus*. Only the *Ruminococcus torques* group reached the selected threshold as characteristic for lay peak. *Lactobacillus* was characteristic of the mid lay phase, and *Bacteroides* and *Faecalibacterium* are among those representing the late lay and more mature birds (Fig. 8).

3.7. Taxa temporal regression analysis

The temporal trend of abundance of different phyla (Table S7) and genera (Table S8) was tested with linear models controlling the confounding effects of farms using the MaAsLin2 R package (Mallick et al., 2021). The significant phyla with the highest absolute coefficient included Bacteroidota, that increased in relative abundance over time, and Proteobacteria and Firmicutes that fell in abundance over time (Fig. 9).

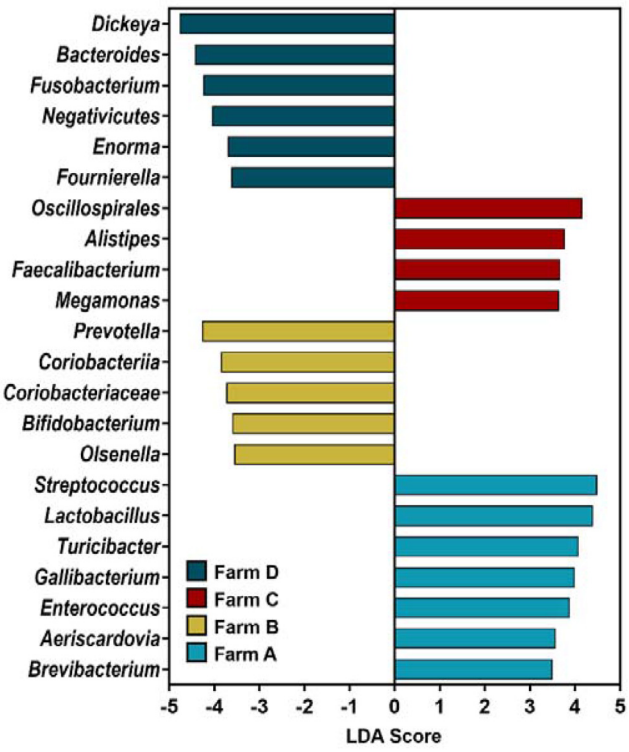

Fig. 7. LEfSe graph showing genera ($P < 1.4E\text{-}5$ and LDA > 3.5) associated with different farms. LDA = linear discriminatory analysis; LEfSe = LDA effect size.

4. Discussion

The four flocks included in this study had egg production that matched the breed standard (apart from a one-week production decline in flock A due to a SLD outbreak), indicating that they were healthy 'normal' flocks. Each flock had significantly different microbiota composition and patterns of microbiota development. These significant differences in the faecal microbiota compositions and beta diversity between different flocks could be due to multiple factors such as feed (Haberecht et al., 2020), environment, or housing systems (Kers et al., 2018). It is clear that there is no single ideal composition for the faecal microbiota; microbiota of varying composition can result in equally satisfactory outcomes for the host. This is also in agreement with previous findings in broiler chickens that catalogued the high variability in gut microbiota that still resulted in fully functional and healthy outcomes (Stanley et al., 2013b). The effect of age on gut microbiota composition has been documented in earlier longitudinal studies in layers (Videnska et al., 2014; Ngunjiri et al., 2019). Although the exact compositions of gut microbiota were significantly different in these studies, some common patterns associated with the temporal development of the gut microbiota composition were observed, which were similar to the findings reported in the current study. For example, the dominance of the Firmicutes phylum at an early age and the rise in the abundance of the Bacteroidota phylum in older hens. These findings were also supported by other published temporal studies conducted in layers (Callaway et al., 2009; Adhikari et al., 2020).

The faecal microbiota richness in flocks A, B, C and D increased with age. A high richness of gut microbiota has been associated with improved gut health and productivity (Diaz Carrasco et al., 2019). Further, the richer microbiota are generally considered an indicator of better gut health as rich populations tend to be more robust in resisting and recovering from perturbations and have a broader metabolic potential (Dethlefsen et al., 2008; Stanley et al., 2014).

The significant decrease in richness of gut microbiota at week 40 in flock A coincided with a SLD outbreak (caused by *C. hepaticus*) at week 34. *C. hepaticus* is known to colonise in the small intestine and caeca of infected birds (Van et al., 2017). This drop in gut microbiota richness could be because of the SLD outbreak. Recent analysis has shown that the gut microbiota is affected by *C. hepaticus* infection, leading to lower alpha diversity (Van et al., 2022). The temporal patterns of richness and diversity are slightly different between the flocks, with flocks A, B and C steadily increasing in diversity while farm D reaches the diversity plateau after the peak of lay. These findings suggest that the gut microbiota development was distinct in different flocks, which was supported by an earlier study (Stanley et al., 2013b).

It is well known in mammals that infants are seeded with maternal gut microbiota (Dominguez-Bello et al., 2010; Gritz and Bhandari, 2015). However, in birds, the embryonated eggs are not connected to the hen by the umbilical cord or placenta. Despite this, it has been suggested that there may be some acquisition of gut microbiota from the hen, however, this hypothesis is still being tested (Ding et al., 2017; Lee et al., 2019). Multiple studies reported that the post-hatch environment is the primary source of microbiota acquisition (Stanley et al., 2013a; Kers et al., 2018). As part of this study, not reported in the results, meconium samples from chick transport boxes were taken and analysed, but the yields of DNA, 16S rRNA gene amplification, and sequences generated were very low and inconsistent. No useful results could be confidently reported, hence, they have not been included; but we suspect there is a very low and variable bacterial load in meconium, often with nothing detectable.

It was observed that faecal microbiota was present from d 2 onwards and continued to develop over time. It is assumed that the early microbiota is acquired from the environment in a random and variable process in which the microbiota is acquired from a variety of sources, including the hatchery equipment and environment, human handlers, transport boxes, the shed atmosphere, and the very earliest water, litter and feed supplies that they have access to (Stanley et al., 2013b). Influencing the stochastic process of early microbiota acquisition by introducing standardised and structured beneficial primary microbiota exposure may provide a more consistent and predictable gut microbiota development. It may be possible to achieve this by seeding the birds with feed supplements such as probiotics or prebiotics at an early age, such as by in ovo inoculation or immediately upon placement as farm 1-d-old chicks (Roto et al., 2016; Baldwin et al., 2018).

The Firmicutes and Proteobacteria were the most dominant phyla throughout the rearing phase in all the flocks. An earlier study found that the gut microbiota was dominated by phylum Proteobacteria (family Enterobacteriaceae in phase 1 (first week of life)), phylum Firmicutes in phase 2 (2 to 4 weeks), followed by an increase in Bacteroidota in phase 3 (2 to 6 months) (Videnska et al., 2014). In the current study, all the flocks showed a high abundance

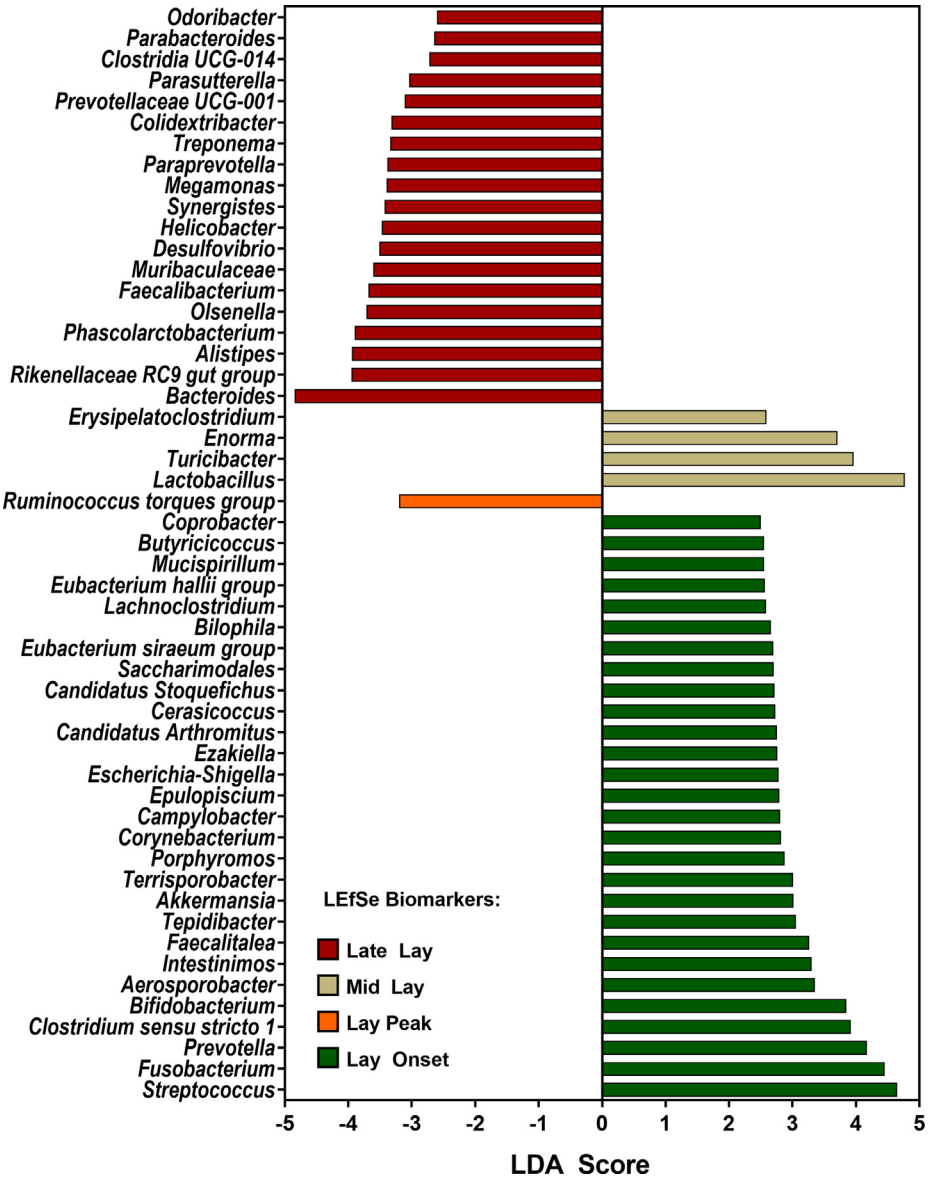

Fig. 8. LEfSe graph showing genera ($P < 0.01$ and LDA > 3) associated with different stages of lay. Genera associated with the Pullet stage were below the LDA score threshold. LDA = linear discriminatory analysis; LEfSe = LDA effect size.

of phylum Proteobacteria on d 2 (Fig. S2); however, only days later, the Firmicutes had become the dominant phylum. The high abundance of Firmicutes at an early age can be related to the high energy demand as the growth rate at an early age is rapid (Videnska et al., 2014).

Within the earliest stages of rearing, the lactobacilli represented a large proportion of the Firmicutes (Fig. 10D). Many *Lactobacillus* strains have been used as probiotics in chickens (De Cesare et al., 2017; Forte et al., 2018). *Lactobacillus* strains produce short-chain fatty acids (SCFA) and cross-feed and encourage the growth of other SCFA-producing bacteria. The SCFA, butyrate, is the preferred energy substrate of gut epithelial cells (Roediger, 1982; Scheppach, 1994; Martin-Gallausiaux et al., 2021). A previous study in broiler chickens demonstrated beneficial effects such as reduced pathogenic species and increased SCFA-producing bacteria after administration of *Lactobacillus* strains after hatch (Baldwin et al., 2018). However, it should be noted that not all *Lactobacillus* are beneficial for health, and some studies have indicated that some *Lactobacillus* spp., are correlated with poor growth performance in broiler chicken (Torok et al., 2011; Stanley et al., 2012a). Some *Lactobacillus* spp. reduce lipid intake and cause dietary energy losses in broilers (De Boever et al., 2000). Given the limited understanding of the role of *Lactobacillus* spp. in layers, further studies focused on the role of *Lactobacillus* spp. in layers reared in different housing systems are needed. Thus, the high abundance of the Firmicutes phylum may support rapid growth at an early age.

In an earlier study, it was found that Bacteroidota succeeded Firmicutes between week 4 to week 26 of flock age, and members of the phylum Bacteroidota accounted for 55% of the total gut microbiota (Videnska et al., 2014). In the current study, the shift from Firmicutes to Bacteroidota occurred somewhat later in flocks A and B, beginning from around week 40, and the two phyla were mostly co-dominant after 70 weeks of age (Fig. S2). In flocks C and D, the Bacteroidota became the second most dominant phylum at week 20 (Figs. S1 and S9). Nordentoft et al. (2011) analysed gut microbiota of 18-week-old hens and found that the most abundant

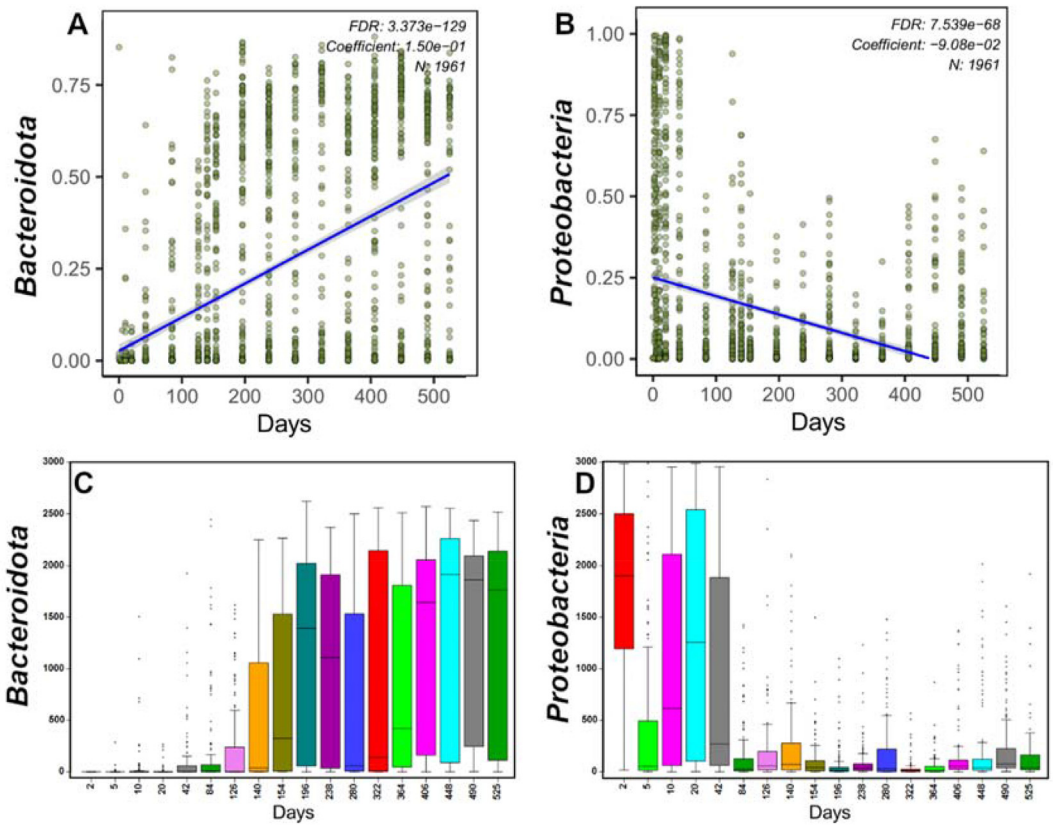

Fig. 9. Regression plots (A and B) confirmed by temporal boxplots of the same phyla. A table with all significantly temporally affected phyla is given in Table S7.

phyla were Firmicutes, and Bacteroidota, followed by a lower abundance of phyla Actinobacteria, Proteobacteria and Fusobacteria and the same phyla dominated in all four of the flocks investigated in the current study, with the difference in ratios in each farm and across the timeline. The dominance of the phylum Bacteroidota, noted in the late lay phase, was also recorded in another earlier study (Callaway et al., 2009).

Overall, the abundance of phylum Firmicutes was higher at an early age, and phylum Bacteroidota succeeded in the mid and late-lay phases. In layers, there is a high energy requirement during early growth and development and then throughout egg production (Bryden et al., 2021). Bacteria of the Bacteroidota phylum have the metabolic potential to digest complex carbohydrates, including cellulose and resistant starch (Stanley et al., 2013a). Propionate and acetate are the primary fermentation products resulting from such digestion (Wrzosek et al., 2013; Yang et al., 2013). It is hypothesised that the high abundance of Firmicutes in the rearing and early lay phase is related to the high physiological energy requirement for growth and egg production, while during mid and late lay, the host energy requirement is comparatively less and thus, to maintain the energy balance, Bacteroidota partially replace Firmicutes. Thus, the gut microbiota might be modulated in response to the changing host energy use. Although the exact mechanism of the gut microbiota host communication is unclear, previous research has indicated the role of host molecules like miRNAs (miR-199a-5p, miR-1226), hormones (insulin, estradiol, norepinephrine), cytokines (IL-1β, TNF-α), host sensing molecules (NLRP6), and metabolic signalling pathways (FXR signalling agonist (GW4064) and antagonist (Gly-MCA), fucose) in communication with the resident and pathogenic microbiota (White et al., 2020).

The comparisons in this study and with other published studies indicate that flocks do not closely follow the same trends in the development of gut microbiota, and the abundance of each phylum and the phylogenetic composition within each phylum differ depending on time and study (Videnska et al., 2014; Ngunjiri et al., 2019). Comparison of microbiota composition across different studies can be difficult because of fundamental differences such as housing, genetics, feed, and environmental influences (Kers et al., 2018). It has also been documented that comparisons of microbiota analysis between different studies can be challenging as different data acquisition and analysis methods are used, and any such differences can influence the outcomes and how they are reported (D'Amore et al., 2016; Allali et al., 2017). In the current investigation, all the data acquisition and analysis methods were standardised and consistent across the whole study, so it can be concluded that the differences between flocks have biological or stochastic origins and are not caused by technical variations.

The gut microbiota composition changed significantly between the pullet and at the point of lay stages. This change possibly occurred due to physiological changes such as the onset of sexual maturity, housing systems, and feed and transportation of birds. The transition from rearing to production is a point of particular vulnerability for layer flocks when their health can be compromised by several pathogens such as *Salmonella*, and *C. hepaticus* (SLD) (Gole et al., 2014; Phung et al., 2020). Efforts to minimise gut microbiota disruption after transport may lead to beneficial health and productivity outcomes.

The gut microbiota analysis showed variation in the abundance of beneficial genera at different time points in all four flocks. The abundance of the genus *Faecalibacterium* was in the top 1% most abundant genera in all four flocks (Fig. S4). The genus *Faecalibacterium*, a gram-positive strict anaerobe, is a common inhabitant of gut microbiota and produces butyrate and other short-chain fatty acids by fermenting dietary fibres (Martín et al.,

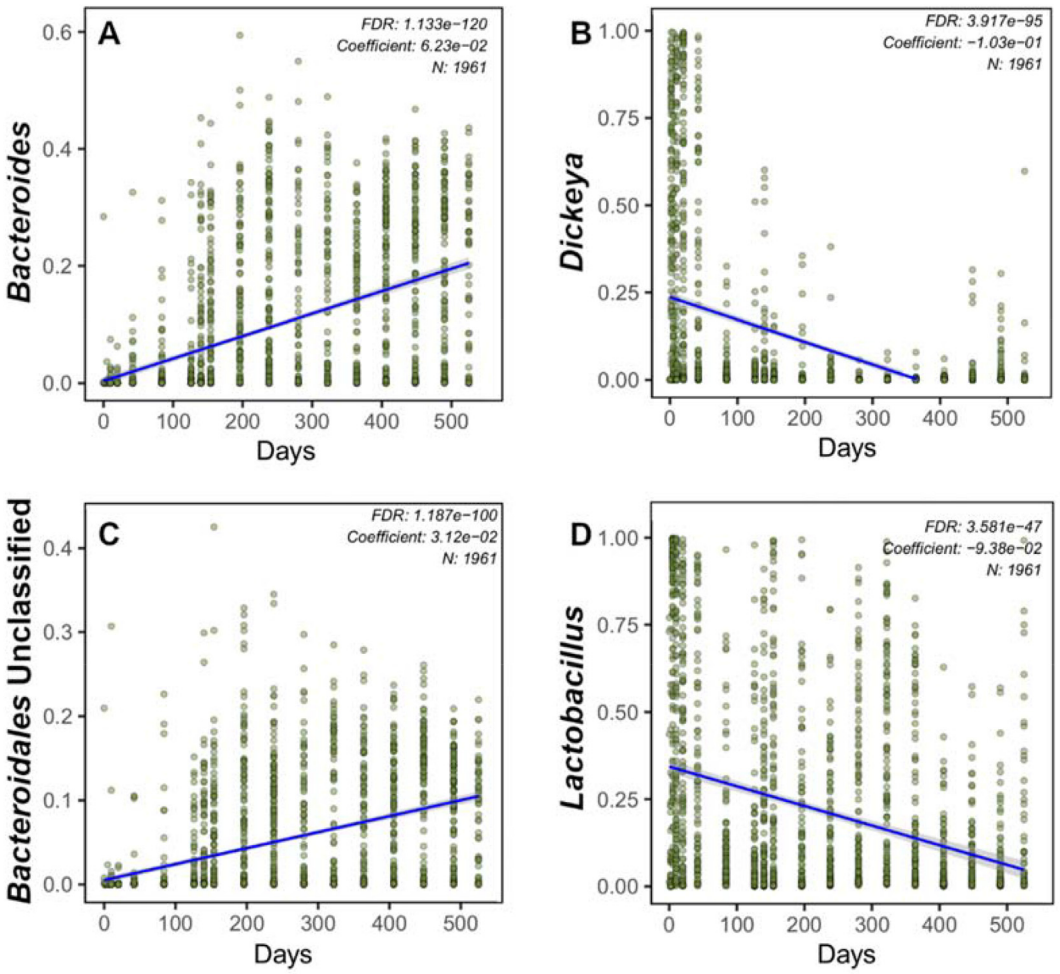

Fig. 10. Regression plots showing some of the most significantly altered genera *Bacteroides* (A), *Dickeya* (B), Unknown *Bacteroidales* (C), and *Lactobacillus* (D). The significantly temporally affected genera are given in Table S8.

2017). It is also associated with improved FCRs and body weight gain in birds and may have a role in directing the development of the immune system (Stanley et al., 2016). The abundance of *Bifidobacterium* was also in the top 1% most abundant genera in all farms and high in flocks B and C. *Bifidobacterium* is associated with the production of lactic acid as a primary product of glucose fermentation and has been reported to improve gut health (Binda et al., 2018).

The faecal microbiota analysis also identified potentially pathogenic genera in all four flocks. Genera that include opportunistic pathogens, such as *Gallibacterium* (Bojesen et al., 2007), *Streptococcus* (Saif et al., 2011), and *Enterococcus* (Saif et al., 2011) were identified at variable levels in the flocks. The abundance of genera *Enterococcus*, *Fusobacterium*, *Gallibacterium*, and *Streptococcus* were higher in pullets reared on a dirt floor (flocks A and B). This could be attributed to the difficulty of thoroughly cleaning and disinfecting dirt floors. The increased abundance of opportunistic pathogens like *Gallibacterium* and *Fusobacterium* at week 18 may be linked to the stress caused by transportation and the onset of sexual maturity. Further studies are needed to investigate the factors that influence the abundance of potentially pathogenic bacteria in the gut of layers.

5. Conclusions

This longitudinal field study has investigated microbiota development in commercial flocks that met the established breed performance standards. The study has identified the stages of flock life that had the greatest influence on the richness of gut microbiota and the carriage of potentially pathogenic taxa. This information will help the industry understand disease risks and adopt mitigation strategies. The data generated in this study, along with those from other layer microbiota studies, help define the development, structure, and natural variability in normal healthy microbiota. With a sound knowledge of healthy microbiota established, future studies could be focused on the analysis of the gut microbiota of the flocks with poor growth and production characteristics. That will allow contrast to be drawn between fully functional and poorly functioning microbiota. By understanding such differences and the temporal development of the microbiota from hatch to full maturity, guidance will be provided for the design of bacterial consortia that can be applied to ensure healthy microbiota development. This will allow the important step of moving beyond cataloguing and describing microbiota to practical ways to manipulate and harness the full potential of the gut microbiota.

Data availability

The 16S rRNA sequence data are available from the Sequence Read Archive (SRA) data database under the accession number PRJNA895927.

Author contributions

Nitish Joat: Methodology, Formal analysis, Writing – Original Draft. **Yadav S. Baiagai:** Formal analysis. **Thi Thu Hao Van:** Methodology. **Robert J. Moore:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – Review & Editing, Funding acquisition. **Dragana Stanley:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – Review & Editing, Funding acquisition. **Kapil Chousalkar:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – Review & Editing, Project Administration, Funding acquisition. All authors have read and approved the final manuscript.

Dedication to Professor Mingan Choct

We would like to acknowledge the significant role Professor Mingan Choct has had in nurturing and encouraging the sort of research presented in this manuscript. In his role as one of the main driving forces behind the establishment and management of the Australian Poultry Cooperative Research Centre, he had the foresight to support a wide range of researchers, many of whom were new to poultry research. The influx of researchers that he introduced to poultry research brought new skills and ideas to apply to industry issues. Professor Choct had the foresight to support some of the very earliest work that applied the then newly developing technologies of next generation high throughput DNA sequencing to chicken gut microbiota analysis. Through his support and mentoring, this enabled some of the authors of this current manuscript to produce some of the very earliest work to characterise the poultry gut microbiota and associate it with poultry pathogen carriage and productivity (Stanley et al., 2012a, 2012b). Professor Choct's championing of important areas of poultry research, both new and established, and his mentoring of many scientists, using his deep knowledge and connections within the poultry industry, has had a profound and ongoing positive impact on both Australian and international poultry research.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.07.006.

References

Adhikari B, Jun S-R, Kwon YM, Kiess AS, Adhikari P. Effects of housing types on cecal microbiota of two different strains of laying hens during the late production phase. Front Vet Sci 2020;7:331.
Al-Ajeeli MNH. Evaluation of performance of Hy-line Brown laying hens fed soybean and soybean-free diets using cage and free-range rearing systems. Poult Sci 2017;97:812–9.
Allali I, Arnold JW, Roach J, Cadenas MB, Butz N, Hassan HM, et al. A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. BMC Microbiol 2017;17:194.
Anderson MJ, Gorley RN, Clarke KR. PERMANOVA+ for PRIMER: guide to software and statistical methods. Plymouth: PRIMER-E Ltd; 2008. p. 218.
Appleby MC. The European Union ban on conventional cages for laying hens: history and prospects. J Appl Anim Welf Sci 2003;6:103–21.
Baldwin S, Hughes RJ, Hao Van TT, Moore RJ, Stanley D. At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. PLoS One 2018;13:e0194825.
Binda C, Lopetuso LR, Rizzatti G, Gibiino G, Cennamo V, Gasbarrini A. Actinobacteria: a relevant minority for the maintenance of gut homeostasis. Dig Liver Dis 2018;50:421–8.
Bojesen AM, Christensen JP, Bisgaard M. Infections and other avian Pasteurellaceae. Poult Dis 2007:160.
Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 2019;37:852–7.
Bryden WL, Li X, Ruhnke I, Zhang D, Shini S. Nutrition, feeding and laying hen welfare. Anim Prod Sci 2021;61:893–914.
Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581–3.
Callaway T, Dowd S, Wolcott R, Sun Y, McReynolds J, Edrington T, et al. Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. Poult Sci 2009;88:298–302.
D'Amore R, Ijaz UZ, Schirmer M, Kenny JG, Gregory R, Darby AC, et al. A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. BMC Genom 2016;17:55.
De Boever P, Wouters R, Verschaeve L, Berckmans P, Schoeters G, Verstraete W. Protective effect of the bile salt hydrolase-active *Lactobacillus reuteri* against bile salt cytotoxicity. Appl Microbiol Biotechnol 2000;53:709–14.
De Cesare A, Sirri F, Manfreda G, Moniaci P, Giardini A, Zampiga M, et al. Effect of dietary supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) on caecum microbioma and productive performance in broiler chickens. PLoS One 2017;12:e0176309.
Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 2008;6:e280.
Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota, gut health and chicken productivity: what is the connection? Microorganisms 2019;7:374.
Ding J, Dai R, Yang L, He C, Xu K, Liu S, et al. Inheritance and establishment of gut microbiota in chickens. Front Microbiol 2017;8:1967.
Dixon P. VEGAN, a package of R functions for community ecology. J Veg Sci 2003;14:927–30.
Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci USA 2010;107:11971–5.
Duncan IJ. Animal welfare issues in the poultry industry: is there a lesson to be learned? J Appl Anim Welf Sci 2001;4:207–21.
Fadrosh DW, Ma B, Gajer P, Sengamalay N, Ott S, Brotman RM, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2014;2:6.
Ferrante V. Welfare issues of modern laying hen farming. Ital J Anim Sci 2009;8:175–89.
Forte C, Manuali E, Abbate Y, Papa P, Vieceli L, Tentellini M, et al. Dietary *Lactobacillus acidophilus* positively influences growth performance, gut morphology, and gut microbiology in rurally reared chickens. Poult Sci 2018;97:930–6.
Gole VC, Caraguel CG, Sexton M, Fowler C, Chousalkar KK. Shedding of *Salmonella* in single age caged commercial layer flock at an early stage of lay. Int J Food Microbiol 2014;189:61–6.
Gritz EC, Bhandari V. The human neonatal gut microbiome: a brief review. Front Pediatr 2015;3:17.
Haberecht S, Bajagai YS, Moore RJ, Van TTH, Stanley D. Poultry feeds carry diverse microbial communities that influence chicken intestinal microbiota colonisation and maturation. AMB Express 2020;10:143.
Hartcher KM, Jones B. The welfare of layer hens in cage and cage-free housing systems. Worlds Poult Sci J 2017;73:767–82.

Henchion M, Hayes M, Mullen AM, Fenelon M, Tiwari B. Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. Foods 2017;6:53.
Hubert SM, Al-Ajeeli M, Bailey CA, Athrey G. The role of housing environment and dietary protein source on the gut microbiota of chicken. Animals 2019;9:1085.
Janczak AM, Riber AB. Review of rearing-related factors affecting the welfare of laying hens. Poult Sci 2015;94:1454–69.
Kers JG, Velkers FC, Fischer EA, Hermes GD, Stegeman JA, Smidt H. Host and environmental factors affecting the intestinal microbiota in chickens. Front Microbiol 2018;9:235.
Khan S, Moore RJ, Stanley D, Chousalkar KK. Gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety. Appl Environ Microbiol 2020;17:e00600-e00620.
Knudsen BE, Bergmark L, Munk P, Lukjancenko O, Priemé A, Aarestrup FM, et al. Impact of sample type and DNA isolation procedure on genomic inference of microbiome composition. mSystems 2016;1:e00095-e00016.
Lee S, La T-M, Lee H-J, Choi I-S, Song C-S, Park S-Y, et al. Characterization of microbial communities in the chicken oviduct and the origin of chicken embryo gut microbiota. Sci Rep 2019;9:6838.
Liu C, Cui Y, Li X, Yao M. microeco: an R package for data mining in microbial community ecology. FEMS Microbiol Ecol 2021;97.
Mallick H, Rahnavard A, McIver L, Ma S, Zhang Y, Nguyen LH, et al. Multivariable association discovery in population-scale meta-omics studies. PLoS Comput Biol 2021;17:e1009442.
Martin-Gallausiaux C, Marinelli L, Blottiere HM, Larraufie P, Lapaque N. SCFA: mechanisms and functional importance in the gut. Proc Nutr Soc 2021;80:37–49.
Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J 2011;17:10–2.
Martín R, Miquel S, Benevides L, Bridonneau C, Robert V, Hudault S, et al. Functional characterization of novel *Faecalibacterium prausnitzii* strains isolated from healthy volunteers: a step forward in the use of *F. prausnitzii* as a next-generation probiotic. Front Microbiol 2017;8:1226.
McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8:e61217.
Meseret S. A review of poultry welfare in conventional production system. Livest Res Rural Dev 2016;28:12.
Ngunjiri JM, Taylor KJ, Abundo MC, Jang H, Elaish M, Mahesh K, et al. Farm stage, bird age, and body site dominantly affect the quantity, taxonomic composition, and dynamics of respiratory and gut microbiota of commercial layer chickens. Appl Environ Microbiol 2019;85:e03137-e03118.
Nordentoft S, Mølbak L, Bjerrum L, De Vylder J, Van Immerseel F, Pedersen K. The influence of the cage system and colonisation of *Salmonella* Enteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 pyrosequencing. BMC Microbiol 2011;11:187.
Ocejo M, Oporto B, Hurtado A. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. Sci Rep 2019;9:2506.
Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol 2020;18:e3000410.
Phung C, Vezina B, Anwar A, Wilson T, Scott PC, Moore RJ, et al. *Campylobacter hepaticus*, the cause of Spotty Liver Disease in chickens: transmission and routes of infection. Front Vet Sci 2020;6:505.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–6.
Roediger WE. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology 1982;83:424–9.
Roto SM, Kwon YM, Ricke SC. Applications of in ovo technique for the optimal development of the gastrointestinal tract and the potential influence on the establishment of its microbiome in poultry. Front Vet Sci 2016;3:63.
Saif Y, Fadly AM, Glisson J, McDougald L, Nolan L, Swayne DE. Diseases of poultry. John Wiley & Sons; 2011.
Scheppach W. Effects of short chain fatty acids on gut morphology and function. Gut 1994;35:S35–8.
Seidlerova Z, Kubasova T, Faldynova M, Crhanova M, Karasova D, Babak V, et al. Environmental impact on differential composition of gut microbiota in indoor chickens in commercial production and outdoor, backyard chickens. Microorganisms 2020;8:767.
Smith SD. phylosmith: an R-package for reproducible and efficient microbiome analysis with phyloseq-objects. J Open Source Softw 2019;4:1442.
Stanley D, Denman SE, Hughes RJ, Geier MS, Crowley TM, Chen H, et al. Intestinal microbiota associated with differential feed conversion efficiency in chickens. Appl Microbiol Biotechnol 2012a;96:1361–9.
Stanley D, Geier MS, Chen H, Hughes RJ, Moore RJ. Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. BMC Microbiol 2015;15:51.
Stanley D, Geier MS, Denman SE, Haring VR, Crowley TM, Hughes RJ, et al. Identification of chicken intestinal microbiota correlated with the efficiency of energy extraction from feed. Vet Microbiol 2013a;164:85–92.
Stanley D, Geier MS, Hughes RJ, Denman SE, Moore RJ. Highly variable microbiota development in the chicken gastrointestinal tract. PLoS One 2013b;8:e84290.
Stanley D, Hughes RJ, Geier MS, Moore RJ. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: challenges presented for the identification of performance enhancing probiotic bacteria. Front Microbiol 2016;7:187.
Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl Microbiol Biotechnol 2014;98:4301–10.
Stanley D, Keyburn AL, Denman SE, Moore RJ. Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. Vet Microbiol 2012b;159:155–62.
Sun J, Wang Y, Li N, Zhong H, Xu H, Zhu Q, et al. Comparative analysis of the gut microbial composition and meat flavor of two chicken breeds in different rearing patterns. BioMed Res Int 2018:4343196.
Torok VA, Allison GE, Percy NJ, Ophel-Keller K, Hughes RJ. Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. Appl Environ Microbiol 2011;77:3380–90.
Van TTH, Gor MC, Anwar A, Scott PC, Moore RJ. *Campylobacter hepaticus*, the cause of spotty liver disease in chickens, is present throughout the small intestine and caeca of infected birds. Vet Microbiol 2017;207:226–30.
Van TTH, Lee Nen That LFM, Perera R, Anwar A, Wilson T, Scott PC, et al. Spotty liver disease adversely affect the gut microbiota of layers hen. Front Vet Sci 2022;9:1039774.
Videnska P, Sedlar K, Lukac M, Faldynova M, Gerzova L, Cejkova D, et al. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. PLoS One 2014;9:e115142.
White JR, Dauros-Singorenko P, Hong J, Vanholsbeeck F, Phillips A, Swift S. The role of host molecules in communication with the resident and pathogenic microbiota: a review. Med Microecol 2020;4:100005.
Wrzosek L, Miquel S, Noordine M-L, Bouet S, Chevalier-Curt MJ, Robert V, et al. *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. BMC Biol 2013;11:61.
Yang J, Martínez I, Walter J, Keshavarzian A, Rose DJ. In vitro characterization of the impact of selected dietary fibers on fecal microbiota composition and short chain fatty acid production. Anaerobe 2013;23:74–81.