Investigation into the effect of mannan-rich fraction supplementation on the metagenome of broiler chickens

Sarah Delaney[1,2], Thi Thuy Do[1], Aoife Corrigan[2], Richard Murphy[2] and Fiona Walsh[1,*]

Abstract

Antibiotic resistance is regarded as one of the most serious threats to human health worldwide. The rapid increase in resistance rates has been attributed to the extensive use of antibiotics since they became commercially available. The use of antibiotics as growth promotors has been banned in numerous regions for this reason. Mannan-rich fraction (MRF) has been reported to show similar growth-promoting effects to antibiotics. We investigated the effect of MRF on the microbial community, resistome and metabolic pathways within the caecum of commercial broilers at two different timepoints within the growth of the broiler, day 27 and day 34. The data indicated an overall increase in health and economic gain for the producer with the addition of MRF to the diet of the broilers. The only significant difference across the microbial composition of the samples was in the richness of the microbial communities across all samples. While all samples harboured resistance genes conferring resistance to the same classes of antibiotics, there was significant variation in the antimicrobial resistance gene richness across time and treatment and across combinations of time and treatment. The taxa with positive correlation comprised Bacilli and Clostridia. The negative correlation taxa were also dominated by Bacilli, specifically the Streptococcus genera. The KEGG-pathway analysis identified an age-related change in the metabolism pathway abundances of the caecal microflora. We suggest that the MRF-related increases in health and weight gain in the broilers may be associated with changes in the metabolism of the microbiomes rather than the microbial composition. The resistome variations across samples were correlated with specific genera. These data may be used to further enhance the development of feed supplements to reduce the presence of antibiotic resistance genes (ARGs) within poultry. While the ARGs of greatest concern to human or animal health were not detected in this study, it has identified the potential to reduce the presence of ARGs by the increase in specific genera.

DATA SUMMARY

The metagenome sequences have been deposited in the European Nucleotide Archive (ENA) under primary accession PRJEB29033 and secondary accession ERP111299. The corresponding analysed data is available in MGnify under the project codes MGYS00003447 and ERP111299.

INTRODUCTION

Over 1000 different species of bacteria inhabit the gastrointestinal tracts of poultry and livestock. These bacteria enter the human food chain through the consumption of meat products, which are regarded as a major source of protein for humans [1]. Poultry is the fastest growing agricultural sub-sector, with continued growth expected as the global population increases [2]. This places enormous pressure on poultry producers, with production often being large-scale and highly intensive. Within such systems, large densities of birds are housed in close proximity to each other [3], and are in constant contact with effluent and secretions from other birds [4]. This creates an ideal environment for bacteria (commensal or pathogenic) to spread throughout the flock. For example, bird-to-bird transmission of the enteric pathogen *Campylobacter* occurs rapidly within a flock, with almost the entire flock becoming colonized within a few days of when the first bird was colonized [5]. In the same manner,

antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are also disseminated throughout poultry flocks.

The threat of antibiotic resistance to global health is ever-increasing. The continued overuse and misuse of antibiotics in both humans and animals has drastically accelerated the development and spread of antibiotic resistance [6]. The link between the use of antibiotics in agriculture, whether for treatment or prevention of disease, or to promote the growth of animals, to increased resistance rates has been well documented [7–9]. Antibiotic use creates a selective pressure that allows for the proliferation of ARB [10]. The gut microbiome of food-producing animals is a known reservoir of ARGs, with bacteria having the ability to harbour these genes even in the absence of selective pressure from antibiotic use [11]. These ARGs can transfer to human and animal pathogens [12]. The caecum is the most densely populated region of the chicken gastrointestinal tract, and is known to harbour an assortment of micro-organisms, which are involved in vital processes such as the recycling of nitrogen, digestion of resistant carbohydrates, absorption of additional nutrients, prevention of colonisation with pathogens and detoxification of harmful substances [13]. However, it can also harbour pathogens such as *Salmonella enterica* and *Campylobacter jejuni*, which cause disease in humans.

Prebiotics are described as nondigestible feed additives that benefit the host by selectively stimulating the growth or metabolic activity of a small number of intestinal micro-organisms [14]. Supplementation of the diet of broilers with the prebiotic mannan oligosaccharide (MOS) has been reported to improve bird weight and feed efficiency [15]. However, how the supplementation of the broiler diet with prebiotics influences the resistome and microbiome is not fully elucidated. Our study aimed to investigate the effect of mannan-rich fraction (MRF) supplementation in the diet of commercial broiler chickens on the microbiome and resistome at two different timepoints within the growth of the broiler. A metagenomics-based approach was employed to examine any MRF-induced changes in the structure and diversity of the microbial community and the resistome within the broiler caecum. We also aimed to identify metabolic pathway changes and changes in correlation of the microbiome and resistome data across time and treatment.

METHODS

Broiler caecal sample collection

The broiler samples were collected from a commercial production site in the EU. All animals were taken from a commercial hatchery and transported to the commercial sheds on the day of hatching. Approximately 10000 birds were mirror imaged from the hatchery into the production sheds, where they received either a control standard commercial corn-soy diet or a standard diet plus MRF (Alltech Biotechnology, Dunboyne, Ireland) at the manufacturer's recommended inclusion rates (800 grams per ton (g/t-1)[starter ration], 400 g/t-1 [grower ration] and 200 g/t-1 [finisher ration]). The birds were raised

Impact Statement

Advances in molecular technologies has allowed for more sensitive detection of the components of the metagenomes from numerous environments. We examined the metagenomes of 16 broiler chickens from a commercial production facility at two different time points. Half of the broilers received a mannan-rich fraction (MRF) supplementation in their diets. We noted that variabilities were found within the ceacal microbiome communities between birds, even within the same group living in identical conditions. A large number of antibiotic resistance genes (ARGs) were identified (*n*=171) across all samples, displaying the presence of ARGs even in the absence of selective pressures from antibiotics. There was significant variation in the antimicrobial resistance gene richness across time and treatment and across combinations of time and treatment. The negative correlation between specific taxa and ARGs may be useful to develop a strategy of enhancing these bacteria within the microbiome to reduce ARGs. The presence of high numbers of ARGs in food-producing animals could adversely affect both animal and human health.

and fed under typical commercial production conditions, receiving feed and water *ad libitum*. All other conditions were kept uniform for all sheds. At days 27 and 34 post-hatch, the intact caecal pouches of four randomly caught birds per treatment group were removed immediately after euthanization. Animals were euthanized in accordance with humane killing protocols as set forth in European Union Council Regulation (EC) 1099/2009. Samples were lyophilized and stored at −80 °C before analysis. Samples 1–4=day 34 MRF treated, 5–8=day 34 control, 9–12=day 27 MRF treated, 13–16=day 27 control (Table S1, available in the online version of this article). Control samples lacked MRF in their diets.

Microbiota maturation in chickens occurs between days 15 and 22 and has been found to remain in a stable status thereafter [16]. Thus, the first timepoint of 27 days was chosen to ensure that background variability effects due to the lack of microbiota maturation would not confound the data analysis. Broilers are usually slaughtered between day 35 and 49 for meat production. The broilers at day 34 represented the last day before slaughter and as such the final day connecting the broiler feed to the broiler and then meat. This is an important day as it is the closest link between broiler and human for potential transmission of antibiotic resistance.

Analysis of broiler growth indices

Average live weight (kg) was calculated by measuring bird weights on arrival to the slaughter house and dividing by the total number of birds on arrival. Bird mortality was recorded daily on farm and reported at the end of crop as a percentage of total birds placed in the shed. Feed intake was calculated

Table 1. Comparison of the growth indices of the broilers with and without MRF in their diets

	Mortality (%)	Average live wt (kg)	Feed conversion ratio	European production efficiency factor
Control	3.71	2.09	1.65	341.54
MRF	3.44	2.44	1.57	373.54

as the amount of feed consumed during each growing phase (starter, grower and finisher). Feed conversion ratio (FCR) and European production efficiency factor (EPEF) were calculated at the end of flock based on the average liveweight, total mortality and amount of feed consumed. Feed conversion ratio (FCR) was calculated as follows: FCR (kg feed/ kg gain)=cumulative feed intake (kg)/total weight gain (kg). European production efficiency factor (EPEF) was calculated based on the following formula, where viability (%) is=100 - mortality (%)

$$EPEF = \frac{viability\ (\%) \times body\ weight\ (kg)}{Age(d) \times FCR} \times 100$$

A lower FCR indicates that the broiler needs less feed to gain the same weight. A higher EPEF indicates overall better health and more uniform weight gain.

Total DNA extraction and Illumina sequencing

Total DNA was extracted from 0.05 g of each caecal sample (*n*=16, labelled 1–16 as described for the samples) using the Qiagen DNeasy PowerSoil kit (Hilden, Germany) according to the manufacturer's guidelines. The concentration and purity of the extracted DNA was measured using an Invitrogen Qubit Fluorometer (dsDNA high-sensitivity assay kit) (Waltham, MA) and a DeNovix DS-11 spectrophotometer. The sequencing was performed at the Centre for Genomics Research, University of Liverpool. Illumina unamplified fragment libraries were prepared using the TruSeq PCR-free kit (350 bp inserts). The samples were paired end sequenced (2×150 bp) using an Illumina HiSeq 4000. Between 60 and 80 million raw reads were obtained per sample. The raw Fastq files were trimmed for the presence of Illumina adapter

sequences using Cutadapt version 1.2.1. The 3′ end of any reads, which matched the adapter sequence for 3 bp or more, were trimmed. Sickle version 1.200 was used to further trim reads, with a minimum window quality score of 20. Reads that were shorter than 20 bp after trimming were removed.

Bioinformatic analysis

The total reads per sample analysed after quality control and trimming ranged from 57465201 reads to 82809780 reads. The trimmed reads were uploaded to the European Nucleotide Archive (ENA). Metagenome assembly were performed using Megahit (v1.2.6, parameters: -continuous –kmin-1pass –k-min 27 –k-max 87 –min-contig-len 500) [17]. The metagenomic classsifer Kaiju was used for taxonomic profiling in the community (v1.7.4, -a greedy -e 5 m 11 s 75 x, NCBI RefSeq database) [18]. The genomic annotation of assembled contigs was performed using Prokka (v1.14.6) with default settings [19]. The protein sequences predicted by Prokka were subjected to the functional annotation using GHOSTKOALA [20]. Antimicrobial resistance annotation was performed using DeepARG [21]. The machine-learning solution, which utilizes CARD, ARDB and UNIPROT databases first removes low-quality reads using TRIMMOMATIC, then merges reads into one file (VSEARCH) and submits them for classification to the DeepARG algorithm[22]. The relative abundance of ARGs was normalised to the 16S rRNA content of each sample. The ARGs were assigned using the following parameters: identity: 80%, e-value: 1e-10, coverage: 50% and probability: 0.8. The resulting data were visualized and analysed using Calypso (http://cgenome.net/calypso/) [22]. Spearman's correlation analysis using the SciPy package was employed to assess the
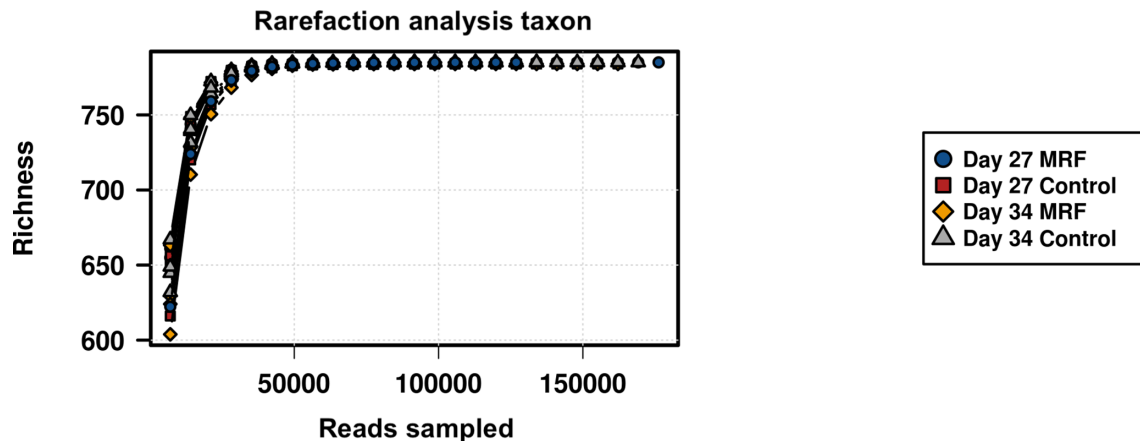
Fig. 1. Rarefaction analysis at all taxonomic ranks demonstrating a sufficient sequencing depth for each sample.
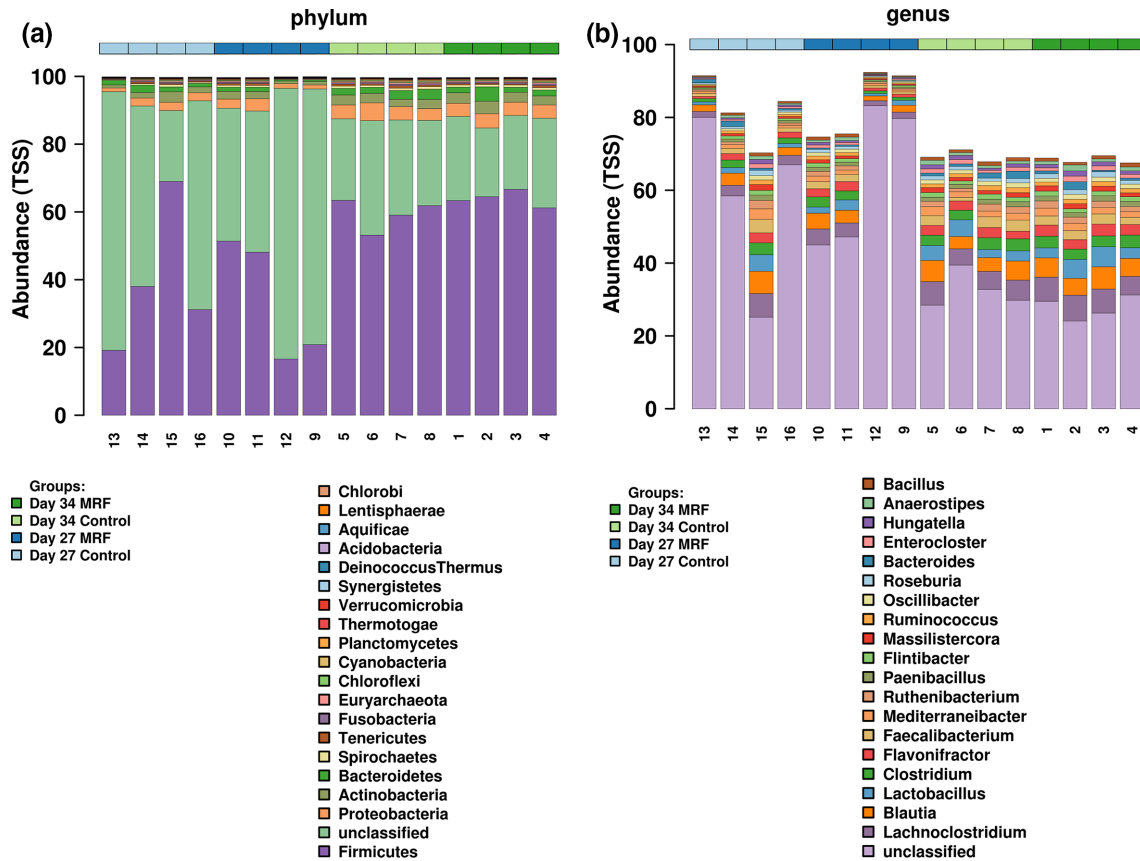
Fig. 2. Microbial community composition displaying the top 20 most abundant taxa across all samples. (a) is displayed at phylum taxonomic level. (b) is displayed at genera taxonomic level.

relationship between antibiotic resistance genes and bacterial taxa [23]. The network correlating antibiotic resistance genes with bacterial taxa was constructed using Cytoscape (v3.8.2) [24]. The KEGG Orthology (KO) tables were mapped to KEGG pathways by MinPath (v1.5) [25].
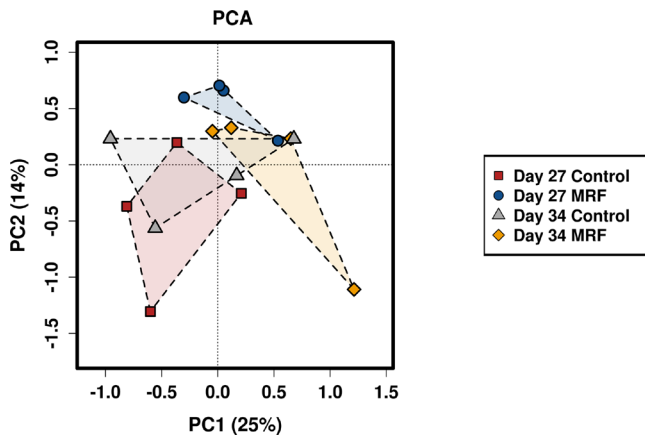

Fig. 3. PCA of the microbial taxa across each sample. Each sample is grouped according to time and treatment.

Statistical analysis

The data were normalized for statistical analysis and rare taxa, with less than 0.001% relative abundance were removed. Samples were compared based on treatment group (control vs MRF) and time-point (day 27 vs day 34). Rarefaction analyses and principal component analysis (PCA) of the microbiome were performed. The microbial community composition was quantitatively visualized by bar charts. ANOVA was used to compare the relative abundances of taxa between treatment groups. Bacterial alpha diversity was estimated using the Shannon index and richness estimated using Chao1.

Antibiotic resistance genes were assigned to the core resistome if they were present in all samples. Antibiotic resistance genes detected in at least one sample but less than the total number of samples was assigned to the accessory resistome. The statistical analysis and correlation analysis of the ARGs was performed using the PAleontological STatistics (PAST) version 3.2 [26]. Samples were compared using ANOVA Mann–Whitney pairwise tests with Bonferroni correction for multiple comparisons [27]. PCA were performed in PAST using default settings. The sequences are deposited in the European Nucleotide Archive (ENA) under primary accession PRJEB29033 and secondary accession ERP111299.
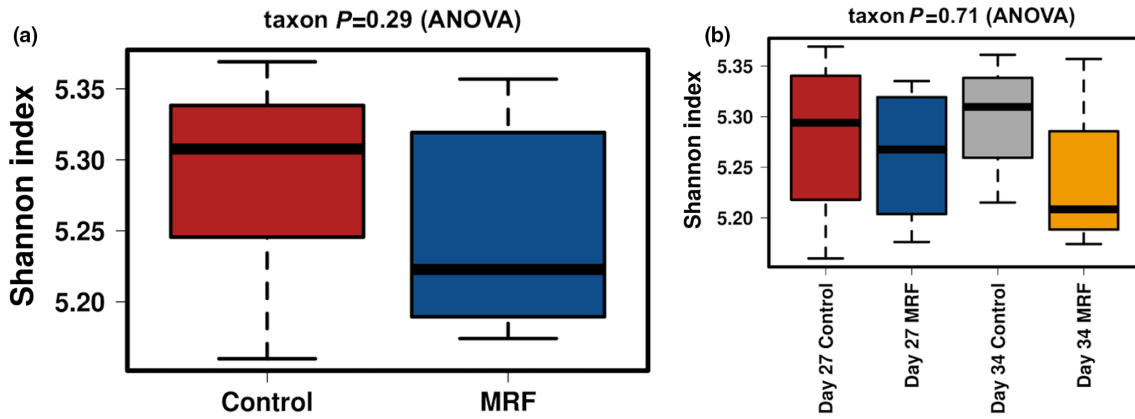
Fig. 4. Statistical analysis of taxon microbial alpha diversity using Shannon-index analysis. (a) describes the alpha diversity comparison of the samples across time. (b) describes the alpha diversity of the microbial taxa within the groups of samples based on time and treatment. The *P* value demonstrates statistically significant difference across the samples where *P*<0.05. The black line denotes the median value in each dataset.

To analyse the correlations between antibiotic resistance genes and bacterial taxa, Spearman's rank correlation coefficients were calculated. A correlation was considered strong when absolute value of Spearman's *r* >0.7 and when *P*<0.05.

RESULTS & DISCUSSION

### Broiler growth characteristics

The growth indices of the broilers fed with the addition of MRF were compared with those of the broilers lacking the MRF addition (Table 1). The lower FCR of the broilers with MRF in their diets indicated that the broilers needed less feed to gain the same weight, which is a more efficient use of feed. The higher EPEF indicated overall better health and more uniform weight gain in the broilers with MRF in their diets in comparison with the control broilers. These data indicated an overall increase in health and economic gain for the producer with the addition of MRF to the diet of the broilers.

Microbial community composition

Rarefaction analysis showed a sufficient sequencing depth was achieved (Fig. 1). The microbiome was dominated by *Firmicutes* across all samples, with an average of 39.34% of all classified reads from day 27 control, 34.24% for day 27 MRF, 59.38% from day 34 control and 63.93% from day 34 MRF (Fig. 2a, Tables S2 and S3). This was followed by *Proteobacteria*, with an average of 2.05% of all classified reads from day 27 control, 2.22% for day 27 MRF, 4.19% from day 34 control and an average of 3.96% from the day 34 MRF group. We then identified a number of unclassified reads in all samples with an average of 40.8% of all classified reads. Previous studies have also found unclassified reads within their samples[26]. The relative abundances were then followed by *Actinobacteria* and *Bacteroidete*s. Variation within sample groups was noted across *Firmicutes* and *Proteobacteria*. These findings are in keeping with other studies investigating the
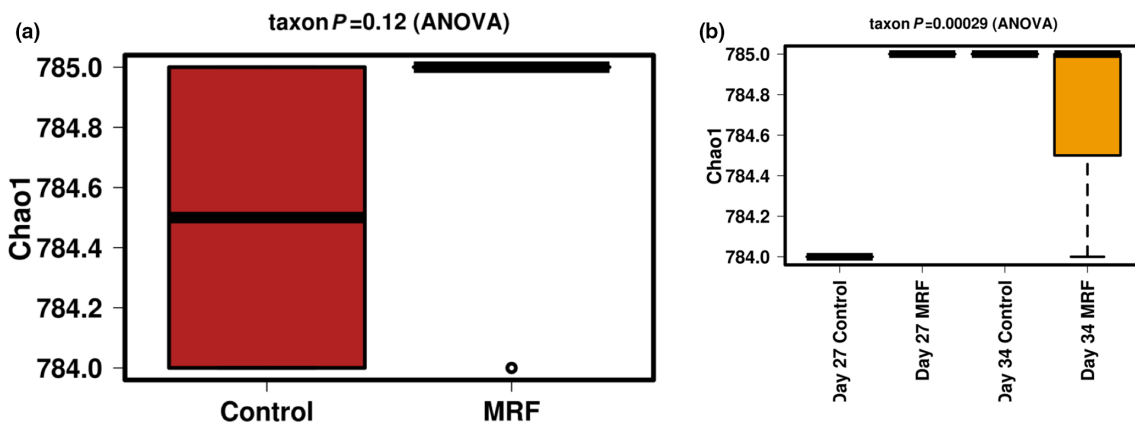

Fig. 5. The microbial community richness was assessed using Chao1. (a) describes the comparative community richness of the samples across time. (b) describes the comparative community richness of the samples based on time and treatment. The *P* value demonstrates statistically significant difference across the samples where *P*<0.05. The black line denotes the median value in each dataset.
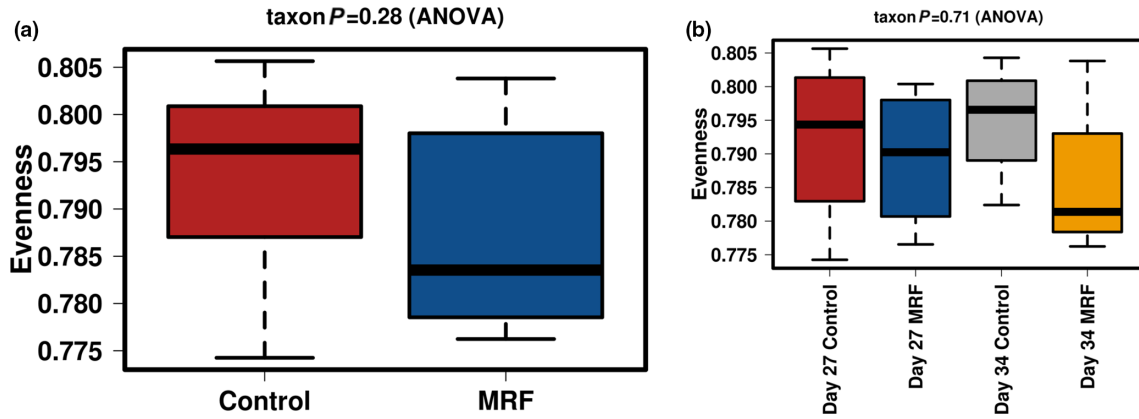
Fig. 6. The evenness of the microbial community is compared between the treated and control samples (a) or across the four sample types (b). The *P* value demonstrates statistically significant difference across the samples where *P*<0.05.

broiler microbiome [7, 28]. Variation within samples has also been observed previously, and has been attributed to factors such as farm workers, housing conditions, biosecurity level, litter and feed access [29]. An average of 45.45% of all reads in all samples were unclassified at genera level (Fig. 2b). *Lachnoclostridium* was the next most dominant, followed by *Blautia*, *Clostridium* and *Lactobacillus*. The large components of unclassified bacteria at each level of analysis of microbiome composition indicates that most of the broiler caecal microbiome is unclassified and requires targeted efforts to identify these bacteria.

A previous study into the effect of mannan oligosaccharide on the broiler microbiome found a shift from *Firmicutes* to *Bacteroides* at phylum level [30]. We did not observe this same change in microbiota, with ours remaining dominated by *Firmicutes* at phylum level. The authors also noted a change to a *Bacteroidia* dominant microbiota at class level from *Clostridia*. We saw a slightly higher relative abundance of *Bacteroidia* in the MRF group but this was not found to be significant. Mannan oligosaccharide has been described to increase the abundance of *Lactobacillus* spp. in the caecum [31]. However, *Lactobacillus* genus was already dominant
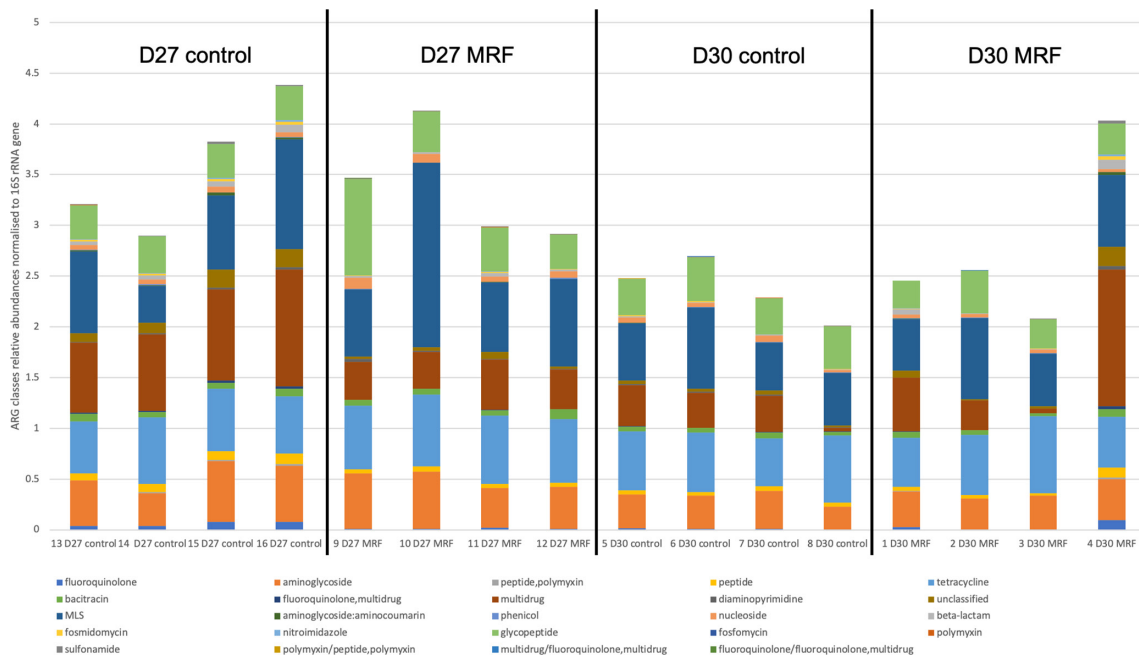
Fig. 7. Antimicrobial resistance gene relative abundances per sample as normalised using 16S rRNA gene abundances per sample grouped according to resistance to class of antimicrobial across all samples.
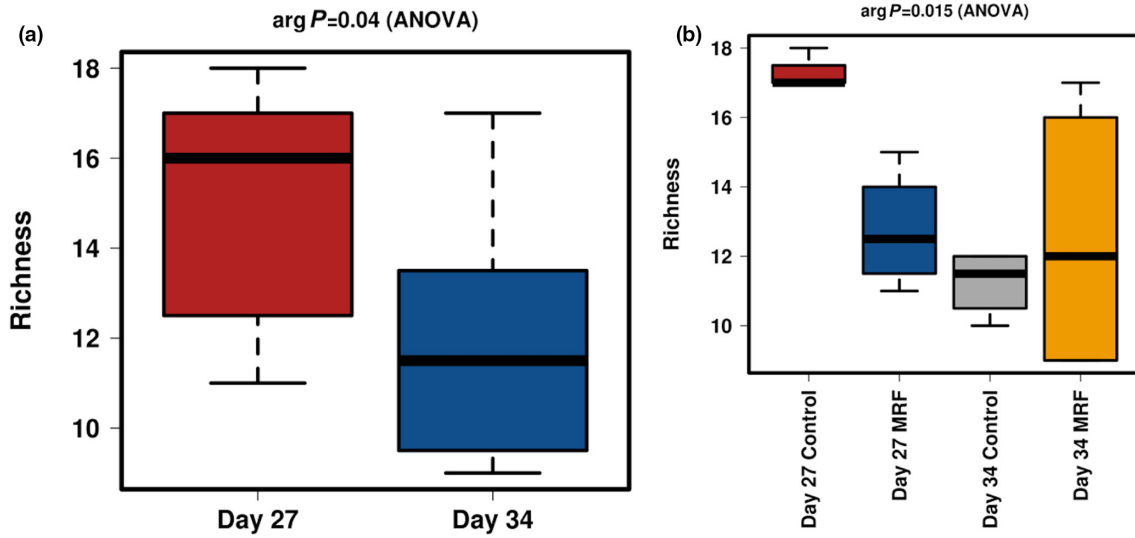
Fig. 8. Antimicrobial resistance gene richness was assessed using Chao1. (a) describes the antimicrobial resistance gene richness comparison of the samples across time. (b) describes the antimicrobial resistance gene richness within the groups of samples based on time and treatment.

within the classified taxa of all samples, and we did not observe notable changes between the control group and the group that received MRF. Studies have shown that pathogenic bacteria that possess mannose-specific fimbriae can bind to mannose, which reduces the risk of pathogens including *Salmonella* and *Escherichia coli* in the gastrointestinal tract [32].

PCA was used to plot the relative abundance of taxa (Fig. 3). The observed patterns between D27 and D34 MRF groups and one of the D34 control group samples were similar. The D27 control taxa displayed no overlap with the MRF-treated samples. The distances between individual samples of the same group, i.e. D24 control, D34 control and D34 treated were further apart than the distances between samples across

the groups. The exception was D27 MRF treated, which clustered closer together. The variation across samples within a group were as influential on the distribution as the treatments or time. Thus no significant variations were attributed to treatment or time.

Alpha diversity

The Shannon index was used to assess the microbial alpha diversity (Fig. 4). No significant differences were observed between the control and MRF-treated birds ($P=0.29$) (Fig. 4a) or across the four sample types ($P=0.71$) (Fig. 4b). The microbial community richness was assessed using Chao1. No significant differences were observed between the control and
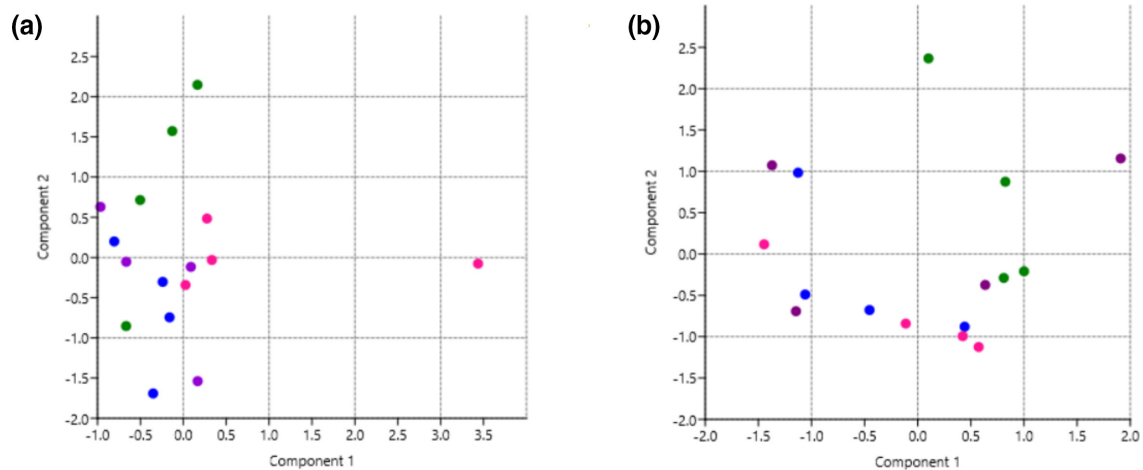
Fig. 9. PCA of samples based on the relative abundance of antimicrobial resistance genes present in the core resistome (a) and accessory resistome (b). Purple: day 34 MRF, blue: day 34 control, pink: day 27 MRF, green: day 27 control.
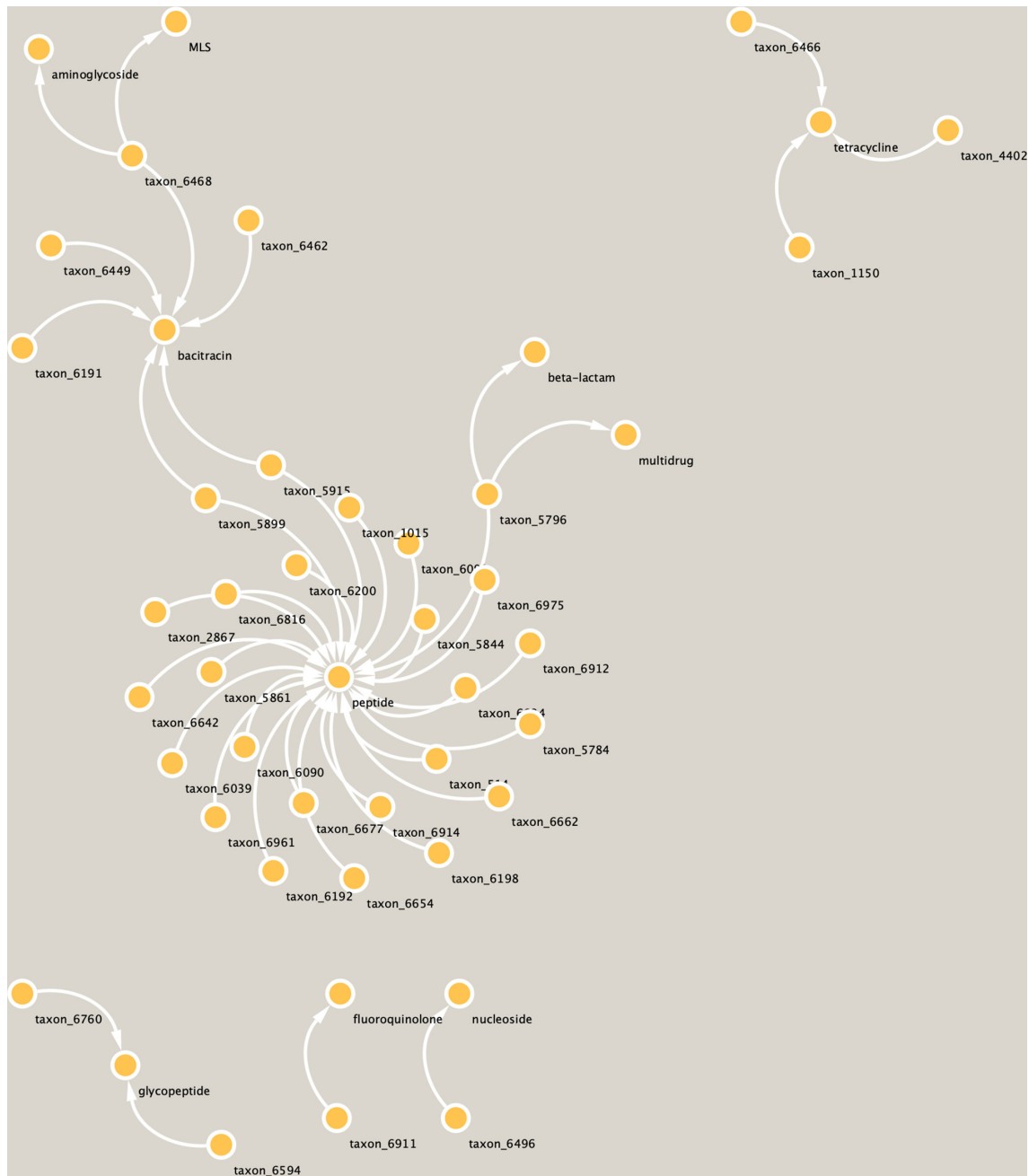
Fig. 10. Network analysis of the correlation between antimicrobial resistance genes and taxa. The figure displays the strong and significant correlation between antimicrobial resistance genes and specific bacterial taxa.

MRF-treated birds (*P*=0.12) (Fig. 5a). Significant difference (*P*=0.00029) was seen across the four sample types (Fig. 5b), indicating a lower number and therefore less rich community in the control D27 than D27 treated or D34 control and a wider distribution of community richness across the D34-treated samples. No significant differences were observed in the evenness of the microbial community between the treated and control samples or across the four sample types (Fig. 6). Thus, the only significant difference across the samples was in the richness of the microbial communities across the samples.

Resistome analysis

A total of 171 ARGs were identified across all samples. The samples were from healthy broiler chickens that had not been administered antibiotics. The trends of resistance across all samples were investigated by summing the relative abundance of ARGs per sample by antibiotic class (Fig. 7). All samples harboured resistance genes conferring resistance to the same classes of antibiotics. The greatest proportions of ARGs present in all samples included tetracycline,
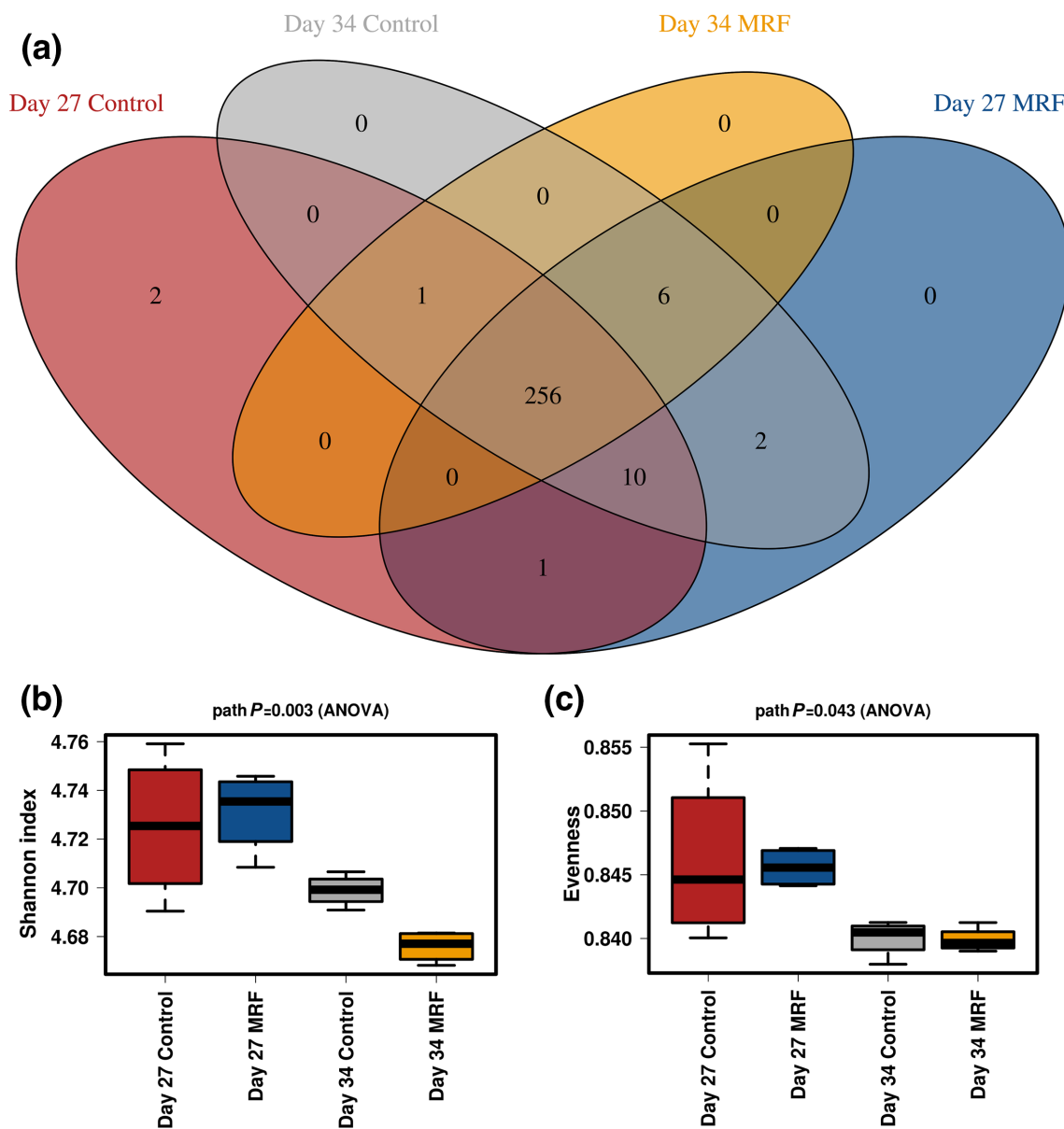
Fig. 11. KEGG-pathway analysis across all samples. (a) Distribution of the core and shared pathways across all sample types. (b) Statistical analysis of the richness of the diversity of pathways in the samples using Shannon index. (c) The evenness of the relative diversities across the KEGG pathways in the samples. The *P* value demonstrates statistically significant difference across the samples where $P<0.05$.

aminoglycoside, multidrug, glycopeptide and macrolide-lincosamide-streptogramin B (MLS_B), nucleoside and peptide resistance genes. Resistance to the remaining classes of antibiotics was relatively low. It must also be noted that the numbers of different ARGs that confer resistance to the tetracycline and aminoglycoside classes are much larger than some other classes, for example, ARGs conferring fluoroquinolone resistance. This will always increase the prevalence of those resistance genes relative to others. However, there is a wide variety of beta-lactam resistance genes detected in bacteria but in our samples they are present in very low proportions, indicating that they are not ARGs of concern in these samples.

From the total resistomes, 69 ARGs were assigned to the core resistome as they were present in all 16 samples (Table S4). The remaining ARGs that were present in at least one but not all samples were assigned to the accessory resistome, totalling 102 ARGs. The core resistome included a large number of efflux pumps (*n*=21), as well as porins (*n*=3), tetracycline (*n*=9), glycopeptide (*n*=10), beta-lactam (*n*=2), aminoglycoside (*n*=5), peptide (*n*=4), MLS_B (*n*=1), lincosamide (*n*=2), streptogramin (*n*=1), macrolide (*n*=1), unclassified (*n*=6), nucleoside (*n*=2), fluoroquinolone (*n*=1) and diaminopyrimidine (*n*=1) ARGs (Table S5). The distribution of genes was reasonably consistent across all samples within
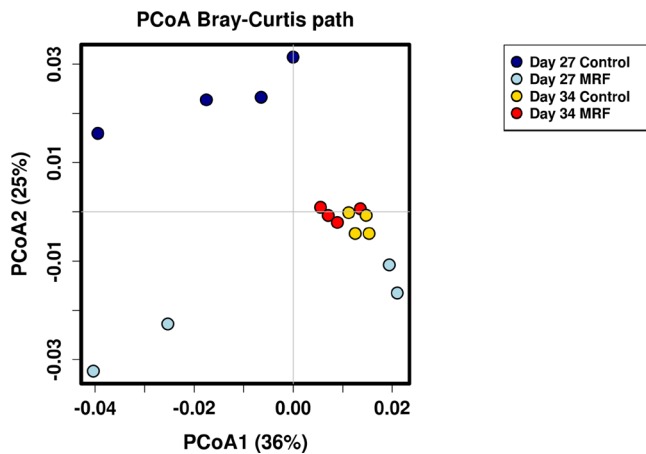
Fig. 12. PCA of samples based on the relative abundance of present in the KEGG pathways.

the core resistome. The *tetW* (tetracycline resistance), *lnuC* (lincosamide resistance) and *aadE* (aminoglycoside resistance) genes were the most abundant. A review of the faecal resistome of pigs and broilers from nine European countries also found less consistency and far more variability in the relative proportions of resistance in the broiler samples in comparison to pigs [33]. The reasons for these levels of variability is yet to be determined. However, in this study the housing conditions, feed and locations of the birds within the groups were identical and cannot explain the variability. This is an important gap in our knowledge as certain birds within a flock may pose more of a risk to the spread of ARGs than others.

The accessory resistome was comprised of 102 ARGs. The *ermF* gene and *rpoB2* were the most abundant accessory genes, which confer resistance to MLS$_B$ antibiotics and rifamycin, respectively. The following genes have been reported as co-located on the same plasmids or within the same mobile elements on plasmids isolated from food borne pathogens, such as *Salmonella* species and *Escherichia coli*: *aph(3′)-I, dfrA17, aac(3)-II, tetR, dfrA12* and *aac(3)-IV* [34, 35]. However, our study did not detect significant levels of *Salmonella* species or *E. coli* in the birds. These genes were in the accessory resistome and as such most likely to not be present on the natural microbiome chromosomes. These data suggest that there may be a non-pathogenic reservoir of the ARGs commonly identified in food-borne pathogens present in the gut microbiome.

Antimicrobial resistance gene richness (total number of detected ARGs in each sample group) were compared across time and each sample type (Fig. 8). Statistically significant differences were identified between day 27 and day 34 samples (*P*=0.04) (Fig. 8a) and between the four sample types across treatment and control (*P*=0.015). These data analysis identifies the significant variation in the resistome across time and treatment and across combinations of time and treatment.

PCA was performed on the core (Fig. 9a) and accessory (Fig. 9b) resistomes. An overlap in the core resistomes of all sampled groups was observed. The core resistomes of samples from the day 27 MRF group had a large intra-cluster distance along the PC1 axis, while the core resistomes of the 27 control, 34 control and 34 MRF had a greater intra-cluster distance along the PC2 axis. The core resistomes of samples from day 34 clustered together. Those from the day 27 groups had a defined inter-cluster distance, indicating the differences in the abundance of core ARGs between the MRF and control groups. This was also observed within the accessory resistome, where the day 27 groups also had a large inter-cluster distance, with a clear separation between the control and MRF groups. The day 34 MRF group (purple) had a larger intra-cluster distance along the PC1 axis but still clustered with the day 34 control samples (blue). It appears that MRF had an effect on ARG abundances at day 27. We conclude from the resistome analysis that MRF appears to enable a stabilization of core resistomes where they are highly variable but will not inhibit the stability of the resistomes where they are already stable.

Correlation analysis

In total 36 different taxa significantly correlated with ARGs (Tables S6 and S7). Overall 30 different taxa had significant positive correlations with ARGs and six had significant negative correlations with ARGs. Two different taxa had significant positive correlations with both bacitracin and peptide ARGs and one with beta-lactams, multidrug resistance and peptide ARGs. One taxon had significant negative correlations with aminoglycoside, bacitracin and MLS$_B$ ARGs. Among the significant correlations, 13 taxa had positive, two taxa had negative correlations with peptide resistance genes, five taxa had positive and one taxon had negative correlations to bacitracin resistance genes. Other significant positive correlations included beta-lactam, fluoroquinolone, multidrug resistance, nucleoside and tetracycline and significant negative correlations glycopeptide, MLS$_B$, aminoglycoside and tetracycline ARGs. The taxa displaying correlation were predominantly within Firmicutes. Those with positive correlation varied predominantly across *Bacilli* and *Clostridia*. The negative correlation taxa were also dominated by *Bacilli*. The genus *Streptococcus* was negatively correlated with tetracycline, aminoglycoside, bacitracin, MLS$_B$ and glycopeptide ARGs. While previous studies have analysed the correlation between ARG diversity and bacterial diversity or co-occurrence of ARGs across samples of poultry or migratory birds, they have not included a co-occurrence analysis of the microbial taxa and the ARGs [36, 37].

A network was created based on strong and significant correlations between bacterial taxa and ARGs. The network contained 46 nodes, 42 edges and formed eight clusters (Fig. 10). The arrows denote the dependency direction in the network. As presented in the network (Fig. 10), peptide resistance genes had the most edges with bacterial taxa, followed by bacitracin and tetracycline. Taxa 5796 and 6468 were significant correlated with three groups of ARGs. The network also showed that the hubs of most of the clusters

were ARGs, which indicated the important roles of ARGs in the network construction.

Functional analysis

The KEGG Orthology (KO) data were mapped to KEGG pathways by MinPath (v1.5) (Table S8). Core pathways (*n*=256) predominated across all samples types (Fig. 11a). There were few unique pathways identified in only one group of samples, e.g. the Sphingolipid signalling KEGG pathways were unique to the day 27 control samples. A statistically significant difference in Shannon's diversity index was identified within identified pathways in all samples (*P*=0.003) (Fig. 11b). The evenness of pathways was also statistically significant different across the KEGG pathways in the samples (Fig. 11c). This indicates that while the pathways were common across all samples the relative abundances of the pathway genes and in turn their availability for utilization was significantly different across the sample groups. Both richness and evenness decreased significantly from day 27 to day 34. This indicates an age-related change in the metabolism pathway abundances of the caecal microflora. This can also be seen in the PCoA (Fig. 12) with day 34 samples clustered and day 27 separated.

CONCLUSION

We suggest that the MRF-related increases in health and weight gain in the broilers may be associated with changes in the metabolism of the microbiomes rather than the microbial composition. The resistome variations across samples were correlated with specific genera. These data may be used to further enhance the development of feed supplements to reduce the presence of ARGs within poultry. While the ARGs of greatest concern to human or animal health were not detected in this study it has identified the potential to reduce the presence of ARGs by the increase in specific genera.

Author contributions
S.D. performed the experiments, analysed and interpreted the data, and wrote the manuscript. T.T.D. reanalysed the data and interpreted the data and wrote the manuscript. A.C. coordinated the data collection, collation and performed the analysis of the broiler growth indices. R.M. conceived and designed the project, organized the chicken farms and sample collection. F.W. secured the funding, conceived and designed the project, wrote the manuscript, performed data analysis.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The samples were obtained from a commercial farm that fed the poultry two different diets, both of which are used in commercial poultry farming. We therefore did not perform animal experiments but used samples from animals that were slaughtered as part of the farming and food production process. The animals were therefore not used as experimental birds and would have been killed in the farming process regardless of whether we obtained samples or not. Therefore, we did not require ethical approval for the study of the samples from

these birds. The ARRIVE and 2Rs guidelines describe animals used specifically for experiments and not those in a commercial setting, which are processed for food. The 3Rs guidelines state: 'Any procedure done for a scientific (including experimental) or educational purpose which may cause a protected animal pain, suffering, distress or lasting harm is a regulated procedure under the ASPA'. The samples from the farmed animals in our study were not treated any differently to many thousands of chicken across the EU and were killed as part of the food production process, not for our experiments.

References

1. **Allen HK**, **Stanton TB**. Altered egos: antibiotic effects on food animal microbiomes. *Annu Rev Microbiol* 2014;68:297–315.

2. **Mottet A**, **Tempio G**. Global poultry production: Current state and future outlook and challenges. *World Poul Sci J* 2017;73:245–256.

3. **Astill J**, **Fraser E**, **Dara R**, **Sharif S**. Detecting and predicting emerging disease in poultry with the implementation of new technologies and big data: A focus on avian influenza virus. *Front Vet Sci* 2018;5:263.

4. **Bolder NM**. Microbial challenges of poultry meat production. *World Poul Sci J* 2007;63:401–411.

5. **Zhang X**, **Yin T**, **Du X**, **Yang W**, **Huang J**, *et al*. Occurrence and genotypes of *Campylobacter* species in broilers during the rearing period. *Avian Pathol* 2017;46:215–223.

6. **Fair RJ**, **Tor Y**. Antibiotics and bacterial resistance in the 21st century. *Perspect Medicin Chem* 2014;6:25–64.

7. **You Y**, **Silbergeld EK**. Learning from agriculture: Understanding low-dose antimicrobials as drivers of resistome expansion. *Front Microbiol* 2014;5:284.

8. **Landers TF**, **Cohen B**, **Wittum TE**, **Larson EL**. A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep* 2012;127:4–22.

9. **Marshall BM**, **Levy SB**. Food animals and antimicrobials: Impacts on human health. *Clin Microbiol Rev* 2011;24:718–733.

10. **Fletcher S**. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. *Environ Health Prev Med* 2015;20:243–252.

11. **Allen HK**. Antibiotic resistance gene discovery in food-producing animals. *Curr Opin Microbiol* 2014;19:25–29.

12. **Thanner S**, **Drissner D**, **Walsh F**. Antimicrobial resistance in agriculture. *mBio* 2016;7:e02227-15.

13. **Chen WL**, **Tang SGH**, **Jahromi MF**, **Candyrine SCL**, **Idrus Z**, *et al*. Metagenomics analysis reveals significant modulation of cecal microbiota of broilers fed palm kernel expeller diets. *Poult Sci* 2019;98:56–68.

14. **Gibson GR**, **Roberfroid MB**. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J Nutr* 1995;125:1401–1412.

15. **Kumprecht I**, **Zobac P**, **Siske V SAE**. Effects of dietary mannanoligosaccharide level on liveweight and feed efficiency of broilers. *Poult Sci* 1997;76:132.

16. **Ranjitkar S**, **Lawley B**, **Tannock G**, **Engberg RM**. Bacterial succession in the broiler gastrointestinal tract. *Appl Environ Microbiol* 2016;82:2399–2410.

17. **Li D**, **Liu CM**, **Luo R**, **Sadakane K**, **Lam TW**. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 2015;31:1674–1676.

18. **Menzel P**, **Ng KL**, **Krogh A**. Fast and sensitive taxonomic classification for Metagenomics with Kaiju. *Nat Commun* 2016;7:11257.

19. **Seemann T**. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.

20. **Kanehisa M**, **Sato Y**, **Morishima K**. Blastkoala and Ghostkoala: KEGG tools for functional characterization of genome and metagenome sequences. *J Mol Biol* 2016;428:726–731.

21. Arango-Argoty G, Garner E, Pruden A, Heath LS, Vikesland P, *et al*. DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. *Microbiome* 2018;6:23.

22. Zakrzewski M, Proietti C, Ellis JJ, Hasan S, Brion M-J, *et al*. Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics* 2017;33:782–783.

23. Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, *et al*. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat Methods* 2020;17:261–272.

24. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;13:2498–2504.

25. Ye Y, Doak TG. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Comput Biol* 2009;5:e1000465.

26. Hammer-Muntz O, Harper D, Ryan P. PAST: paleontological statistics software package for education and data analysis version 2.09. undefined. /paper/PAST%3A-paleontological-statistics-software-package-Hammer-Muntz-Harper/a82d7e0dc7b2d3a-1b159e0902bb9f3017788a786. 2001.

27. Joyce A, McCarthy CGP, Murphy S, Walsh F. Antibiotic resistomes of healthy pig faecal metagenomes. *Microb Genom* 2019;5.

28. 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. *Scientific Reports* 2019;9:1–14.

29. Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA, *et al*. Host and environmental factors affecting the intestinal microbiota in chickens. *Front Microbiol* 2018;9:235.

30. Corrigan A, de Leeuw M, Penaud-Frézet S, Dimova D, Murphy RA. Phylogenetic and functional alterations in bacterial community compositions in broiler ceca as a result of mannan oligosaccharide supplementation. *Appl Environ Microbiol* 2015;81:3460–3470.

31. Rehman H, Vahjen W, Kohl-Parisini A, Ijaz A, Zentek J. Influence of fermentable carbohydrates on the intestinal bacteria and enteropathogens in broilers. *World Poultry Sci J* 2009;65:75–90.

32. Akhtar T, Ara G, Ali N, Ud Din Mufti F, Imran Khan M. Effects of dietary supplementation of mannan-oligosaccharide on virus shedding in avian influenza (H9N2) challenged broilers. *Iran J Vet Res* 2016;17:268–272.

33. Munk P, Knudsen BE, Lukjancenko O, Duarte ASR, Van Gompel L, *et al*. Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat Microbiol* 2018;3:898–908.

34. Glenn LM, Lindsey RL, Folster JP, Pecic G, Boerlin P, *et al*. Antimicrobial resistance genes in multidrug-resistant *Salmonella enterica* isolated from animals, retail meats, and humans in the United States and Canada. *Microb Drug Resist* 2013;19:175–184.

35. Wang J, Stephan R, Power K, Yan Q, Hächler H, *et al*. Nucleotide sequences of 16 transmissible plasmids identified in nine multidrug-resistant *Escherichia coli* isolates expressing an ESBL phenotype isolated from food-producing animals and healthy humans. *J Antimicrob Chemother* 2014;69:2658–2668.

36. Wang Y, Lyu N, Liu F, Liu WJ, Bi Y, *et al*. More diversified antibiotic resistance genes in chickens and workers of the live poultry markets. *Environ Int* 2021;153:106534.

37. Cao J, Hu Y, Liu F, Wang Y, Bi Y, *et al*. Metagenomic analysis reveals the microbiome and resistome in migratory birds. *Microbiome* 2020;8:26.