Investigating the cecal microbiota of broilers raised in extensive and intensive production systems

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ABSTRACT Intensive broiler production practices are structured to prevent the introduction and spread of pathogens; however, they can potentially minimize the exposure of broilers to beneficial commensal bacteria. In this study, we used 16S rRNA amplicon sequencing to characterize the cecal microbiota of 35-day-old broilers from 22 independent commercial farms rearing broilers under intensive (IPS) or extensive production systems (EPS). We aimed to determine which bacteria are normal inhabitants of the broiler ceca and which bacteria might be missing from broilers in IPS. In addition, we generated a collection of 410 bacterial isolates, including 87 different species, to be used as a resource to further explore the effects of selected isolates on bird physiology and to elucidate the role of individual species within the cecal microbial community. Our results indicated significant differences in the microbiota of broilers between systems: the microbiota of broilers from EPS was dominated by Bacteroidetes {55.2% ± 8.9 [mean ± standard deviation (SD)]}, whereas Firmicutes dominated the microbiota of broilers from IPS (61.7% ± 14.4, mean ± SD). Bacterial taxa found to be core in the EPS microbiota, including *Olsenella*, *Alistipes*, *Bacteroides*, *Barnesiella*, *Parabacteroides*, *Megamonas*, and *Parasutterella*, were shown to be infrequent or absent from the IPS microbiota, and the EPS microbiota presented higher phylogenetic diversity and greater predicted functional potential than that of broilers in IPS. The bacteria shown to be depleted in broilers from IPS should be further investigated for their effects on bird physiology and potential application as next-generation probiotics.

IMPORTANCE Production practices in intensive farming systems significantly reduce the introduction and spread of pathogens; however, they may potentially minimize the exposure of animals to beneficial commensal microorganisms. In this study, we identified core bacteria from the cecal microbiota of broilers raised in extensive production systems that are missing or reduced in birds from intensive systems, including *Olsenella*, *Alistipes*, *Bacteroides*, *Barnesiella*, *Parabacteroides*, *Megamonas*, and *Parasutterella*. Furthermore, the cecal microbiota of broilers from extensive systems showed higher diversity and greater functional potential than that of broilers from intensive systems. In addition, a collection of bacterial isolates containing 87 different species was generated from the current study, and this important resource can be used to further explore the role of selected commensal bacteria on the microbial community and bird physiology.

KEYWORDS broiler, gut microbiota, cecal microbiota, poultry, extensive production system, intensive production system, commensal

The community of bacteria living in the gastrointestinal tract (GIT) of poultry has been shown to influence many aspects of host physiology, including nutrient digestion (1–3), immune system maturation and tuning (4–6), disease resistance and tolerance (7–9), and intestinal development (10). Throughout evolution, this association has resulted in bacteria that are highly adapted to the GIT of specific host species (11), that are

likely to colonize the GIT after a single exposure (12), and that can significantly affect host metabolism (13). Moreover, the microbiota can respond quickly to environmental conditions and promote changes in host phenotype that aid acclimation and adaptation to new situations (14, 15). Therefore, it is expected that individuals hosting beneficial bacteria are more likely to survive, reproduce, and transmit their commensal bacteria to the next generation, favoring the persistence of both the host species and beneficial commensal organisms (15, 16).

Despite growing evidence indicating the importance of proper microbial colonization (17), intensive farming practices are structured in a way that reduces opportunities for the selection and transmission of beneficial commensal bacteria across generations. In intensive production systems (IPS), broilers are hatched in artificial hatcheries and moved to enclosed barns, which limit contact with the microbiota of mature birds. In addition, broilers in IPS are fed standardized diets with a limited number of ingredients, which may contain antibiotics. In contrast, broilers reared in extensive production systems (EPS) might have access to outdoor environments and be fed antibiotic-free diets, which offer more opportunity for exposure and colonization by a wide variety of microorganisms. In face of the restrictions on the use of antibiotics due to concerns about the development of antimicrobial resistance, there is a need to find alternative strategies to promote animal growth and control disease occurrence in IPS. The restoration of the chicken's native microbiome using next-generation probiotics, which include bacterial strains that reside in the intestinal ecosystem, could be a potential alternative (18). This contrasts with "traditional" probiotic products that usually contain bacterial strains harvested from soil and fermented food, which may fail to effectively colonize and persist in the chicken GIT unless constantly provided (12, 19). The lack of host-adaptation traits coupled with individual variabilities in broiler microbiota communities could be the reason why probiotics that aim to improve performance and disease resistance have inconsistent results (20, 21).

Domesticated animals colonized with microbiota from wild counterparts have shown reduced inflammatory responses and increased survival following infection challenges (22), and their microbiota was shown to be more resilient to disturbances caused by factors such as dietary and environmental changes as well as antibiotic use (23). Bacteria from chickens from EPS were shown to have higher antagonist activity against pathogens and less resistance to antimicrobials than bacteria from chickens from IPS (24, 25). In addition, studies have shown that poultry raised in IPS may lack the core bacteria that are found in free-range and feral birds (26–28).

Previous studies comparing the microbiota of poultry raised in IPS and EPS presented some limitations, such as small sample sizes and the unavailability of samples from similar-aged birds from each system (25, 28). In the present study, we characterized the cecal microbiota of age-matched broilers from 22 independent commercial farms under IPS (n = 12 farms) or EPS (n = 10 farms), aiming to determine which bacteria are normal inhabitants of the chicken GIT and which bacteria might be missing from broilers in IPS. Further genomic characterization of selected commensal bacterial isolates has also been performed to guide the development of next-generation probiotics and further studies aiming to understand the role of individual bacteria within the gut microbiota.

RESULTS

The amplicon sequencing of 105 cecal samples generated an average of 52,457 ± 36,444 [mean ± standard deviation (SD)] reads that were assigned to 12,331 amplicon sequence variants (ASVs) and 792 taxa. Samples were rarefed at 11,360 reads, resulting in the removal of 4,172 ASVs and 100 taxa that were not present in any sample after random subsampling. Two samples that presented less than 11,360 reads were excluded from downstream analysis. Analyses of 103 rarefied samples (n = 45 from EPS and n = 58 from IPS) indicated the presence of 8,159 ASVs and 692 taxa.

Production systems significantly affect cecal microbiota composition

The cecal microbiota of broilers raised in EPS had higher phylogenetic diversity than that of birds in IPS ($P < 0.001$) (Fig. 1A), but no significant differences were observed in alpha-diversity indices including Chao1 (Fig. 1B), Shannon, and Simpson (Fig. S1) ($P = 0.06$, $P = 0.93$, and $P = 0.35$, respectively). Permutational multivariate analysis of variance (PERMANOVA) analysis indicated that the microbiota composition was significantly different between systems ($P = 0.001$, $R^2 = 0.10$) (Fig. 1C). Hierarchical clustering analysis showed a clear separation of samples according to rearing system, except for a single sample obtained from a broiler from IPS that was clustered with samples obtained from broilers from EPS (Fig. 2A). In addition, samples of birds from the same farm clustered tightly, except for three samples from three different farms that were separated from the remaining samples obtained from the same farm (Fig. S2). Bacteroidetes dominated the cecal microbial community in EPS birds [55.2% ± 8.9 (mean relative abundance ± SD)], whereas Firmicutes dominated the cecal microbial community in IPS broilers (61.7% ± 14.4) (Fig. 2B). Six phyla were exclusively detected in broilers from EPS, including Deferribacteres (0.8% ± 1.1, $P < 0.001$), Elusimicrobia (0.9% ± 3.4, $P < 0.001$), Fusobacteria (0.1% ± 1.3, $P < 0.001$), Patescibacteria (0.3% ± 0.8, $P < 0.001$), Spirochaetes (2.7% ± 4.1, $P < 0.001$), and Synergistetes (0.4% ± 0.5, $P < 0.001$). Besides the six unique phyla, the cecal microbiota of EPS broilers presented a higher relative abundance of Actinobacteria (1.2% ± 0.9 vs 0.7% ± 1.1, $P < 0.001$), Bacteroidetes (55.2% ± 8.9 vs 27.9% ± 11.9, $P < 0.001$), *Lentisphaera* (0.5% ± 0.5 vs 0.01% ± 0.04, $P < 0.001$), Proteobacteria (4.7% ± 3.0 vs 0.3% ± 1.0, $P = 0.002$), and Verrucomicrobia (0.7% ± 1.3 vs 0.3 ± 1.0, $P < 0.001$) than that of IPS broilers. On the other hand, IPS broilers had enriched Firmicutes (61.7% ± 14.4 vs 28.7% ±
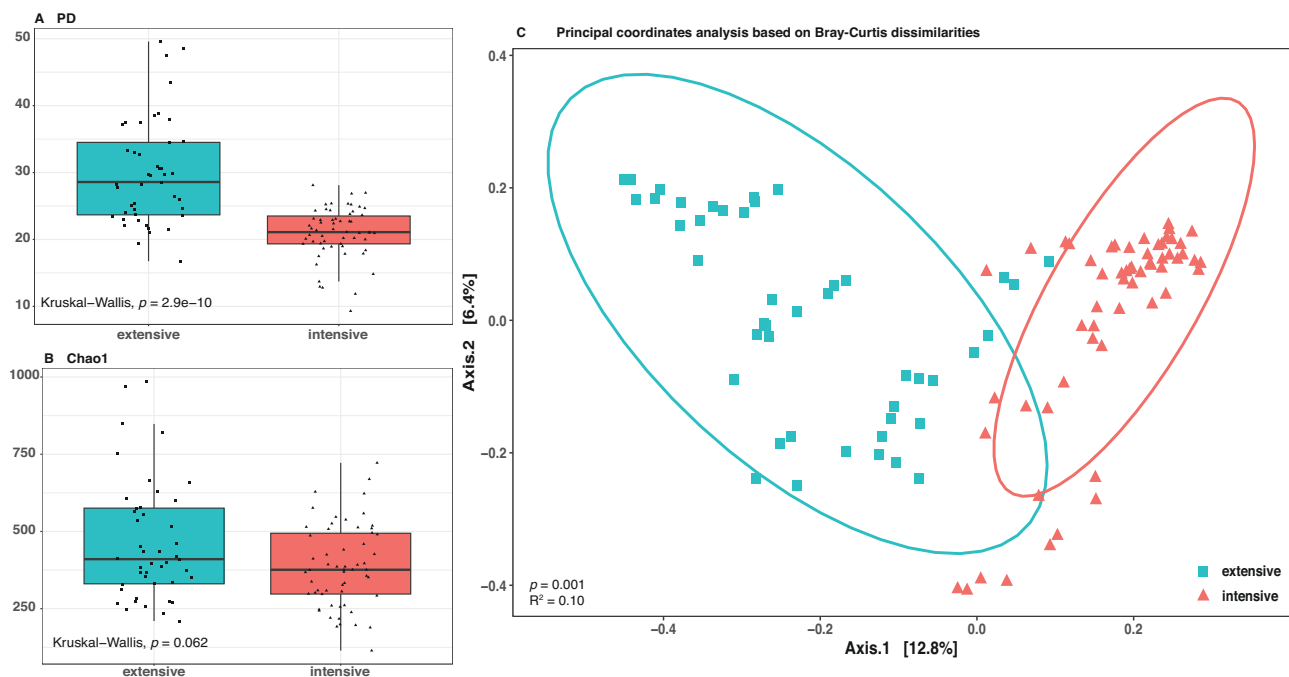
FIG 1 (A, B) Alpha-diversity indices and (C) PCoA generated based on Bray-Curtis dissimilarity of cecal samples obtained from 35-day-old broilers from extensive or intensive production systems. Samples are colored and shaped according to treatments, and data ellipses represent the 95% confidence region for group clusters assuming a multivariate *t*-distribution.
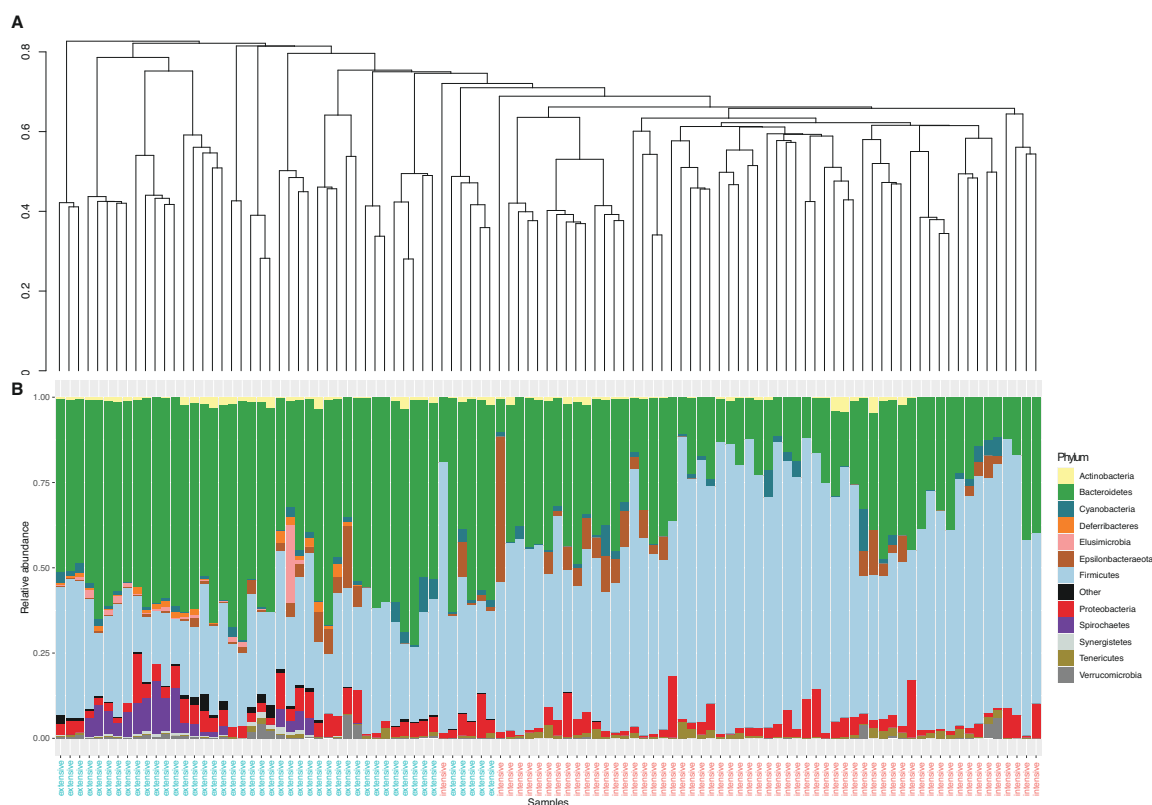
FIG 2 (A) Dendrogram showing hierarchical clustering and (B) bar plots showing the relative abundance of phyla in cecal samples obtained from 35-day-old broilers from extensive or intensive production systems. Phyla observed in less than 5% of samples and that had less than 1% relative abundance were combined as "other" (black).

7.5, $P < 0.001$) and Tenericutes (1.0% ± 1.1 vs 0.4% ± 0.6, $P = 0.003$). The most abundant phyla within the microbiota of broilers in both systems were Firmicutes, Bacteroidetes, and Proteobacteria (Fig. S3).

EPS cecal microbiotas harbor unique ASVs

Among the total number of ASVs detected, 52.5% were found to be unique to birds raised in EPS, 33.4% were unique to birds raised in IPS, and 14.3% were shared between both systems. An average of 67.8% ± 14.4 (mean relative abundance ± SD) of the cecal microbial community in EPS broilers was composed of ASVs that were unique to this system, whereas the ASVs unique to the microbiota of IPS broilers composed only 14.5% ± 6.7 of the cecal microbial community in IPS broilers (Fig. S4A). We further looked at phyla assignment for shared and unique ASVs and found that Bacteroidetes was the most abundant phyla detected within the unique microbial members of extensively and intensively raised broilers, composing 71.2% ± 10.9 of the unique community of EPS and 46.6% ± 22.9 of the unique community in IPS (Fig. S4B). Most of the ASVs shared by both systems were Firmicutes, with relative abundances of 73.7% ± 17.4 in EPS and 72.5% ± 15.0 in IPS (Fig. S4C).

Differential abundance analyses at the ASV level were performed after aggregating all ASVs not seen in more than 20 birds into a single feature named "rare." This reduced the number of ASVs from 8,153 to 452 ASVs that represented an average relative abundance of 72.6% ± 9.5 (mean ± SD) of the microbial community in IPS birds and 35.5% ± 9.5 of the community in EPS. Within these 452 ASVs, 158 and 33 ASVs were enriched in broilers from IPS and EPS, respectively. ASVs that were aggregated as rare were enriched and composed most of the microbial community (64.5% ± 9.5) in EPS birds. The microbiota

of IPS birds was mostly composed of ASVs that were not differentially abundant between the systems (39.8% ± 12.3). It was also noteworthy that ASVs that were enriched in IPS birds were consistently found in EPS birds, with an average relative abundance reaching 16.5% ± 9.0 in the latter, whereas the ASVs enriched in EPS birds rarely occurred in IPS broilers, which had an average relative abundance of only 0.6% ± 0.8 in the latter (Fig. 3; Table S1). Without performing aggregation of rare ASVs, differential abundance analyses resulted in 249 ASVs enriched in EPS broilers and 130 ASVs enriched in IPS broilers.

The family Enterobacteriaceae is enriched in IPS microbiotas

Differential abundance analyses at the family level were performed after combining families not detected in more than 20 birds into a single category called rare, which reduced the number of bacterial families from 97 to 53. Analyses of these 53 families indicated that five bacterial families were unique to the microbiota of EPS broilers, namely, Deferribacteraceae (0.8% ± 1.0, $P < 0.001$), Elusimicrobiaceae (0.9% ± 3.4, $P < 0.001$), Spirochaetaceae (2.7% ± 4.0, $P < 0.001$), Synergistaceae (0.4% ± 0.5, $P < 0.001$), and *Victivalles vadin BE97* (0.1% ± 0.2, $P < 0.001$). In addition, nine families were found to be enriched in EPS birds, whereas seven families were enriched in IPS birds, including Enterobacteriaceae ($P < 0.001$). The average relative abundance of Enterobacteriaceae in IPS and EPS broilers was 2.4% ± 3.9 and 0.2% ± 0.3, respectively (see Fig. S5).

IPS cecal microbiotas are missing microbes

At the taxonomic level, 49.4% of the total number of taxa was shared between the cecal microbiota of broilers from both systems, whereas 41.5% and 9.1% of taxa were unique to the cecal microbiota of broilers raised in EPS and IPS, respectively. Analyses of features assigned to the genus and species levels indicated 10 genera and 6 species to be missing from the microbiota of EPS broilers, whereas 55 genera and 31 species were indicated to be missing from the microbiota of IPS broilers (Table S2). All taxa found to be missing from EPS broilers presented rare occurrences in IPS broilers, and the same was true for most of the taxa found to be missing from IPS birds. However, some taxa commonly found in EPS birds were completely absent from IPS birds, namely,
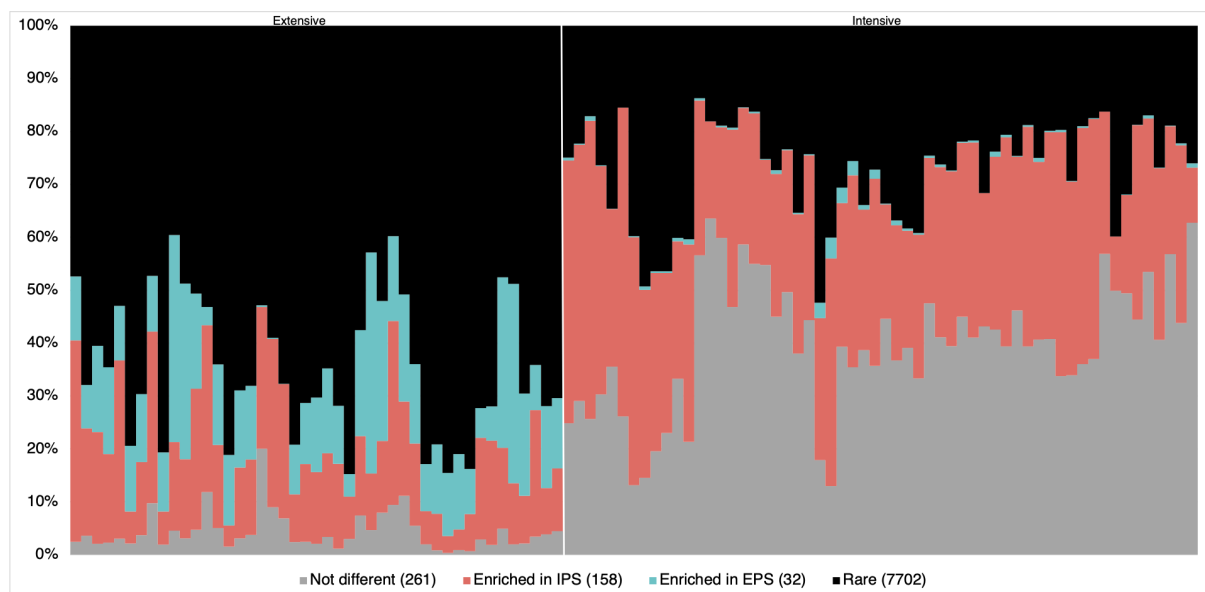
FIG 3 Simplified taxa plots showing the relative abundance of ASVs in the cecal contents of 35-day-old broilers from extensive or intensive production systems. ASVs that were found to be enriched in the microbiota of extensively or intensively raised birds are shown in green and red, respectively. ASVs that were not differently abundant between the systems are shown in gray. ASVs observed in less than 20 birds were aggregated into a single category called rare, and their relative abundance is indicated in black. The numbers between brackets indicated how many ASVs were included in each grouping category.

the species *Bacteroides plebeius* [2.3% ± 3.9 (mean relative abundance ± SD) , *P* < 0.001],
Bacteroides salanitronis (0.2% ± 0.4, *P* < 0.001), as well as the genera *Alloprevotella* (2.2% ±
2.9, *P* < 0.001), Prevotellaceae UCG-001 (1.6% ± 0.8), *Mucispirillum* (0.8% ± 1.1, *P* < 0.001),
Elusimicrobium (0.9% ± 3.4, *P* < 0.001), and Synergistetes (0.4% ± 0.5, *P* < 0.001).

Differential abundance analyses at the taxa level were performed after combining all
taxa present in less than 20 birds as a single taxon called rare, which reduced the number
of taxa from 692 to 241. We found 39 taxa enriched in EPS birds and 36 taxa enriched in
IPS birds. Most of the differentially abundant taxa showed an average relative abundance
below 0.5% (Fig. 4; Fig. S6). A total of 164 taxa were assigned as core components of the
broiler cecal microbiota, 90 of which were shared between birds in both systems (Fig.
S7). In addition, 44 taxa were assigned as core in EPS birds, and 31 taxa were assigned as
core in IPS birds (Fig. 5). The cecal microbiota of IPS broilers was shown to be depleted
of taxa that were core members of EPS birds, such as *Olsenella* (0.3% ± 0.22 vs 0.01%
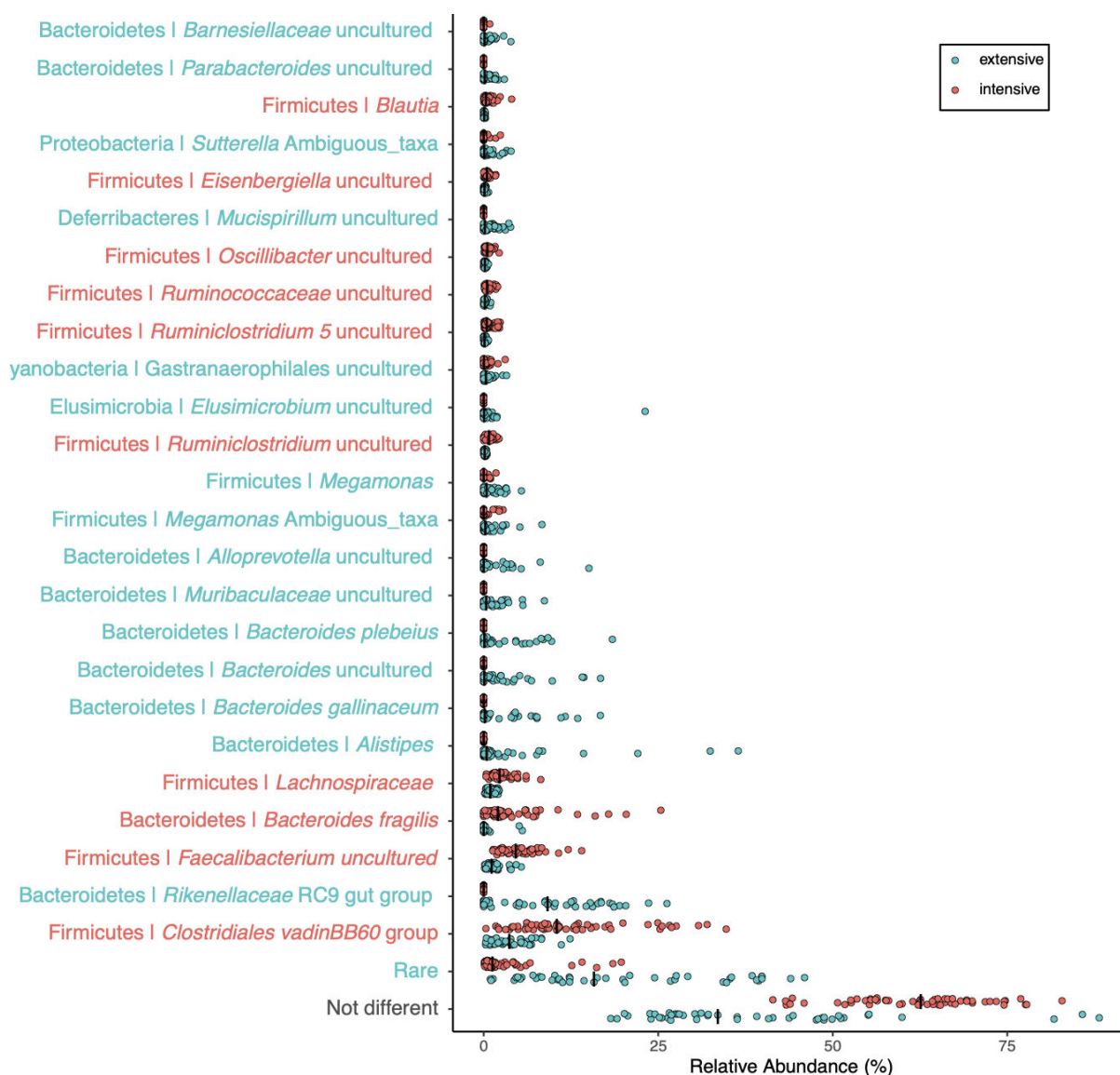
FIG 4 The relative abundance of microbial taxa that were shown to be differently abundant in the cecal microbiota of 35-day-old broilers from extensive or
intensive production systems. Taxa names are color coded according to the system in which they were found to be enriched: green for an extensive system
and red for an intensive system. Dots represent the relative abundance of taxa in individual samples. Taxa observed in less than 20 birds were combined into a
single category called rare and shown to be significantly enriched in EPS broilers. The relative abundance of taxa that were not differently abundant between the
systems is shown as "not different."
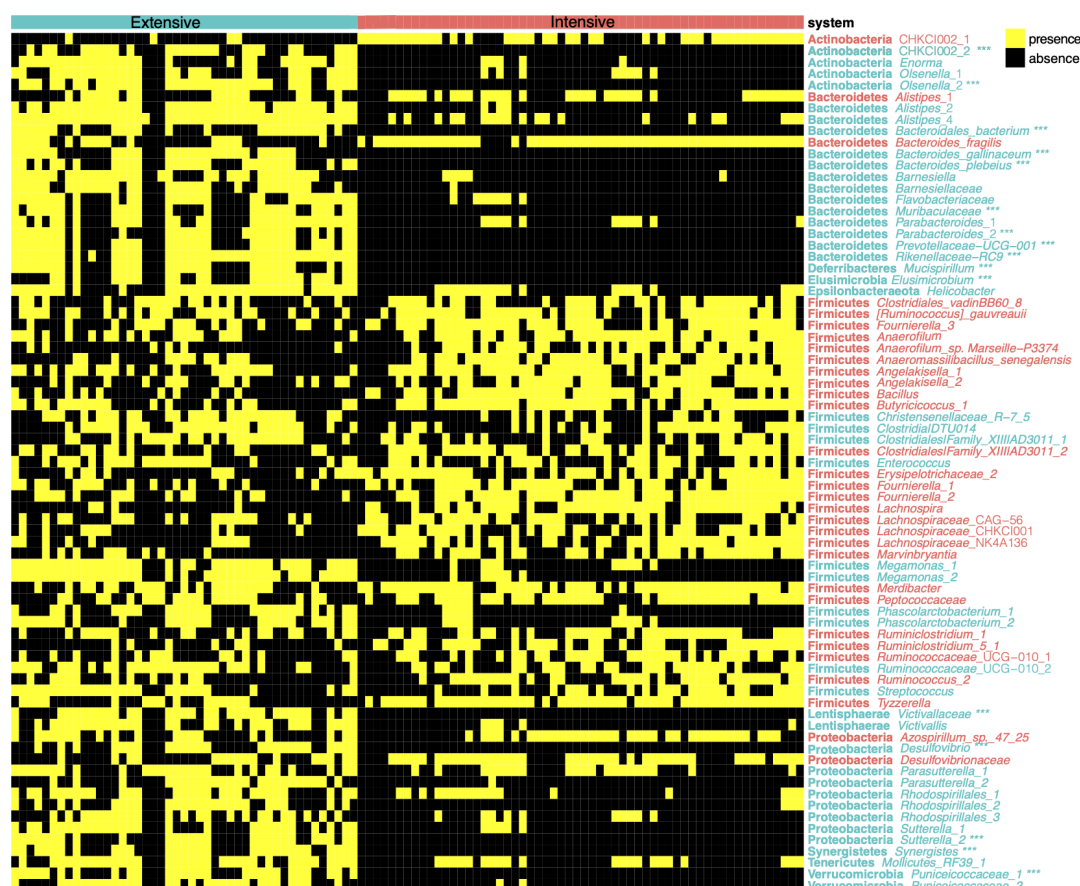
FIG 5 Heatmap indicating the presence (yellow) and absence (black) of core microbes in the microbiota of IPS and EPS broilers. Each column represents a sample and is colored green (left side) or red (right side) if obtained from EPS or IPS broilers, respectively. Taxa names are colored green or red if found to be core taxa within the microbiota of EPS or IPS broilers, respectively.

± 0.03, $P = 0.001$), Bacteroidales (38.1% ± 3.0 vs 20.7% ± 1.9, $P < 0.001$), *Bacteroides gallinaceum* (2.6% ± 4.2 vs 0%, $P < 0.001$), *Bacteroides plebeius* (2.3% ± 3.9 vs 0%, $P < 0.001$), Muribaculaceae (1.3% ± 1.9 vs 0%, $P < 0.001$), *Parabacteroides* (0.9% ± 0.7 vs 0.4% ± 0.9, $P < 0.001$), Prevotellaceae UCG-001 (1.6% ± 0.8 vs 0%, $P < 0.001$), Rikenellaceae RC9 (9.2% ± 7.6 vs 0%, $P < 0.001$), *Mucispirillum* (0.8% ± 1.1 vs 0%, $P < 0.001$), *Elusimicrobium* (0.9% ± 3.4 vs 0%, $P < 0.001$), Victivallaceae (0.1% ± 0.2 vs 0%, $P < 0.001$), *Desulfovibrio* (0.4% ± 0.5 vs 0.0 ± 0.1%, $P < 0.001$), *Sutterella* (0.9% ± 0.8 vs 0.1% ± 0.4, $P < 0.001$), Synergistetes (0.4% ± 0.5 vs 0%, $P < 0.001$), and Puniceicoccaceae (0.2% ± 0.2 vs 0%, $P < 0.001$). The microbiota of EPS birds presented a lower frequency of Firmicutes that were core members of IPS birds, although complete depletion was not observed for any taxa in EPS birds.

The EPS microbiota has greater predicted functional potential

Principal component analysis of predicted Enzyme Commission genes and MetaCyc pathways indicated a clear separation between samples obtained from EPS and IPS broilers (Fig. S8). A total of 167 pathways were shown to be differentially present, of which 95 were enriched in EPS broilers and 52 were enriched in IPS broilers. With an effect size of 0.5 as a threshold, 60 pathways were identified as biologically relevant (Fig. 6). A total of 75 ASVs were estimated to contribute to the enriched pathways, and most of the contributing ASVs were assigned to the order Clostridiales (Table S3). The microbiota of EPS broilers presented 53 enriched pathways that were mainly involved in the biosynthesis of amino acids (L-arginine, L-serine, and L-tyrosine), cofactors and
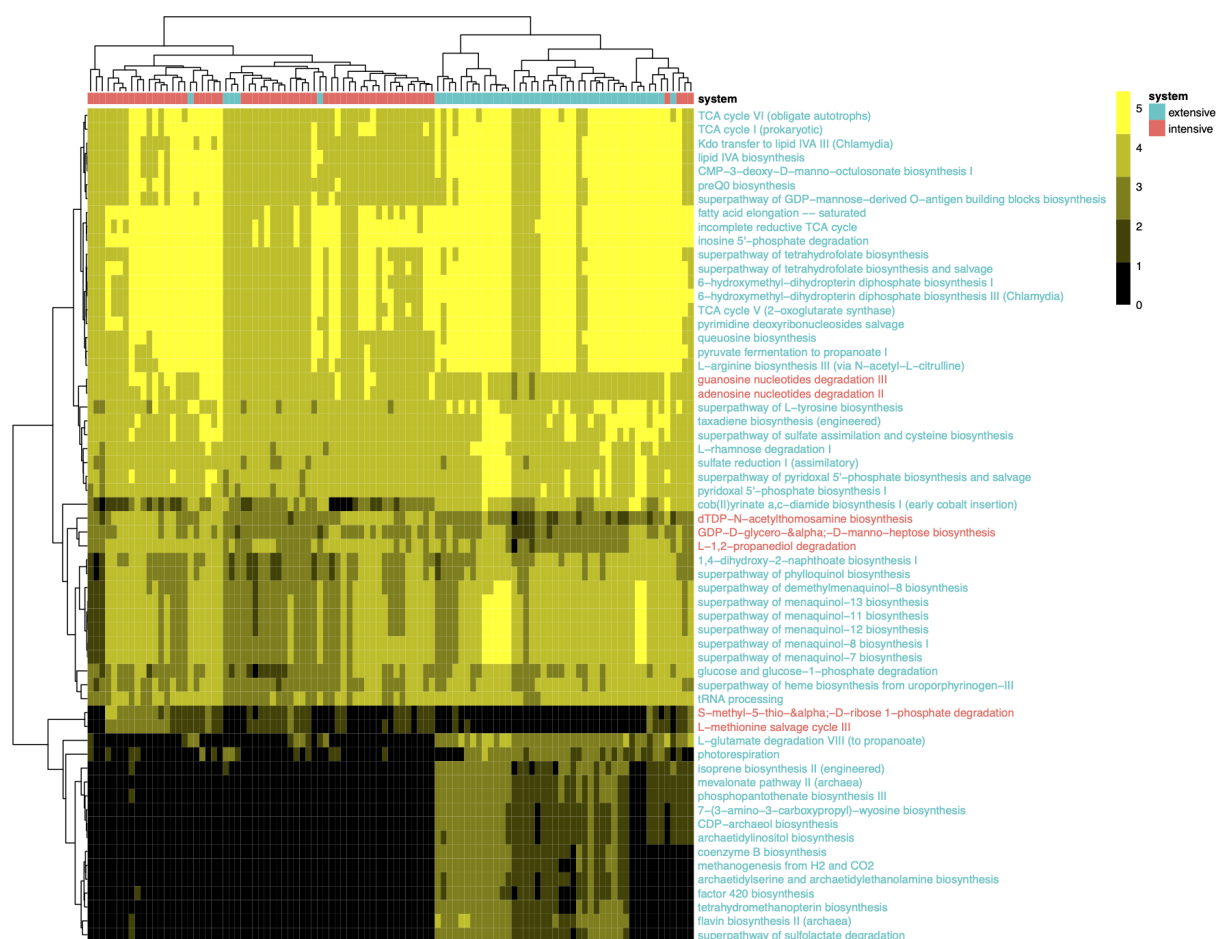
FIG 6 Heatmap indicating the log-transformed abundance of predicted pathways shown to be differently present in the microbiota of broilers from extensive (green) and intensive (red) rearing systems. Each column represents a sample and is colored green or red if obtained from EPS or IPS broilers, respectively. Pathway names are colored green or red if found to be enriched in the microbiota of EPS or IPS broilers, respectively.

vitamins (B6, B9, B12, K2, coenzyme A), fatty acids, and carbohydrates. In addition, the microbiota of EPS broilers showed several enriched pathways related to nutrient degradation and assimilation, including rhamnose degradation and sulfur assimilation, and pathways related to the generation of precursor metabolites such as methane and propionic acid. On the other hand, only seven pathways were shown to be enriched in the microbiota of IPS broilers. These pathways were shown to be involved in the biosynthesis of L-methionine, dTDP-N-acetylthomosamine (an important antigen in the outer membrane of Enterobacteriaceae), and heptose sugars (commonly found on the cell surface of many bacteria).

Isolation of chicken commensals and whole-genome sequencing

We collected and identified 410 isolates, which were assigned to 87 species from 6 phyla. Most isolates were members of Firmicutes, comprising 53 species, followed by 14 species from Bacteroidetes, 10 from Actinobacteria, 7 from Proteobacteria, 2 from Cyanobacteria, and 1 species from Fusobacteria (Fig. 7; Table S4). We selected 24 isolates from the phyla Actinobacteria, Bacteroidetes, and Firmicutes for whole-genome sequencing (WGS) analysis, of which 5 were considered new species. The isolates considered as new species had low OrthoANI values compared to reference genome sequences of *Collinsella massiliensis* (75.93%), *Bacteroides gallinaceum* (74.2%), *Bacteroides uniformis* (75.03%), *Barnesiella viscericola* (85.73%), and *Ruminococcus torques* (74.3%) (Table S5).
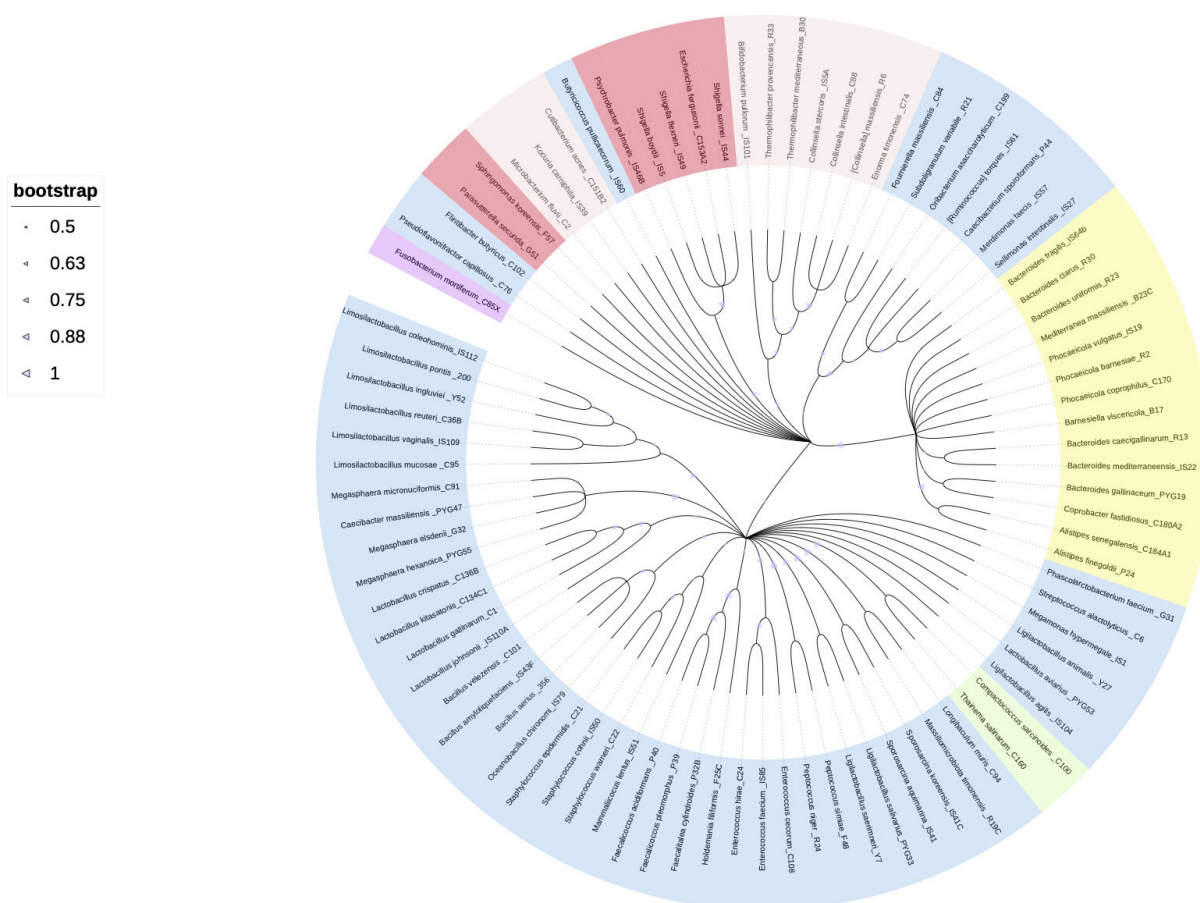
FIG 7 Phylogram showing the diversity of the 87 bacterial species isolated from the cecal microbiota of chickens. Letters and numbers after the species name indicate the identification number of a representative isolate for each species. The colors in the circle represent the phyla Actinobacteria (salmon), Bacteroidetes (yellow), Cyanobacteria (green), Firmicutes (blue), Fusobacteria (purple), and Proteobacteria (pink).

The presence of genes encoding bacteriocins and identified virulence factors is indicated in Tables S6 and S7. The presence of antibiotic resistance genes is illustrated in Fig. 8.

DISCUSSION

Identifying bacteria that are normal inhabitants of the cecal microbiota of broilers is an initial step in using microbial manipulation in favor of broiler production. As intensive production practices may hinder the transmission of commensal microbes across generations (29), exploring potential missing microbes in intensively raised broilers compared with broilers reared in extensive systems can guide the development of microbial strategies to manipulate broiler health and performance. In the current study, we characterized the cecal microbiota of market-aged broilers raised in 22 independent commercial farms under intensive and extensive production systems. We found that the microbiota of EPS broilers was significantly different and had higher phylogenetic diversity than the microbiota of IPS broilers. Numerous bacteria were completely absent from IPS broilers compared with EPS broilers. Among these missing bacteria, some were cored in broilers from EPS, including *Olsenella*, *Bacteroides gallinaceum*, *Bacteroides plebeius*, *Parabacteroides*, *Mucispirillum*, *Elusimicrobium*, Victivallaceae, *Sutterella*, *Desulfovibrio*, and Synergistetes. On the other hand, a few missing bacteria, such as Fusobacteria and Patescibacteria, were only occasionally observed in EPS broilers and therefore considered less relevant.

At the phylum level, there was a clear difference between the systems, with the cecal microbiota of EPS broilers presenting a higher relative abundance of Bacteroidetes and
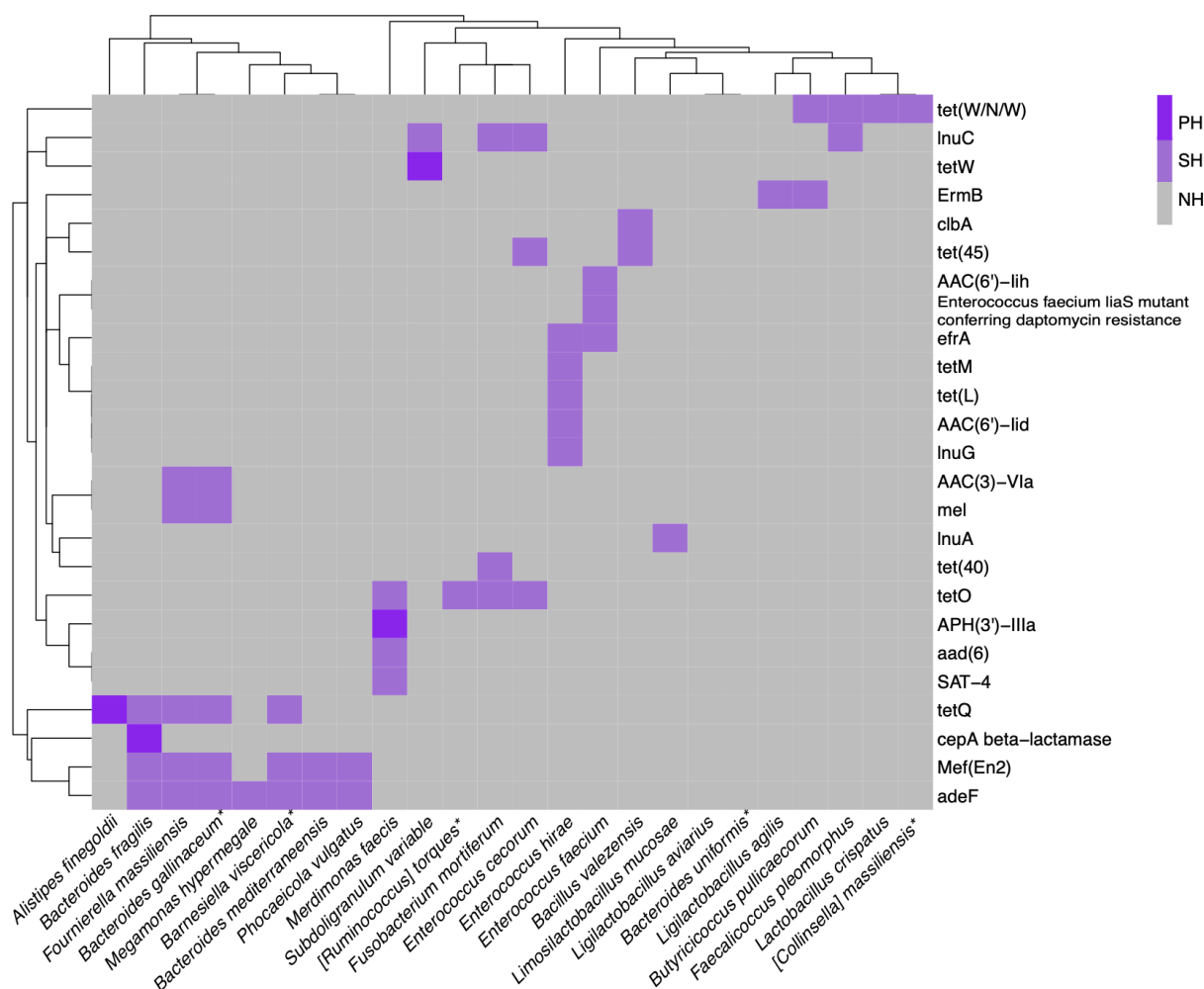
FIG 8 Heatmap indicating the presence of genes conferring antimicrobial resistance in the genomes of selected isolates. The dark purple color indicates a resistance gene identifier (RGI) match 100% identical to the reference protein sequence (perfect hit, PH); the light purple color indicates an RGI match with a bit-score greater than the curated BLASTP bit-score cut-off (strict hit, SH); and the gray color indicates no RGI match (NH). Species names followed by an * indicate isolates considered novel species.

Proteobacteria, while the cecal microbiota of IPS broilers was dominated by Firmicutes. This was consistent with previous studies comparing poultry in extensive and intensive systems (25, 27, 30–33) and has also been observed in humans living in rural and urbanized societies (34, 35), as well as in wild and captive rodents (22, 36). The similarity among results is not surprising since broilers are captive animals and current production practices resemble the changes in lifestyle that human society underwent during urbanization, which include sanitation, the use of antibiotics, and reduced microbiota transmission between mothers and offspring (37).

Previous studies have indicated that spore-forming and aerotolerant bacteria, such as most Firmicutes and Proteobacteria, are likely acquired from the environment, whereas bacteria not equipped to survive in aerobic conditions, such as Bacteroidetes (non-spore-forming strict anaerobes), need to be transmitted from mother to offspring (38). EPS broilers are more likely to present higher Bacteroidetes abundance due to their contact with a less sanitized environment and potential encounter with fecal material from mature birds, while IPS broilers are more likely to be colonized by Firmicutes present in the environment. Non-spore-forming strict anaerobes present a higher colonization capacity and a higher degree of host adaptation than that of spore-formers and aerotolerant bacteria (12). In that way, the reduction of Bacteroidetes and other

obligate anaerobes such as *Megamonas* and *Parasutterella* indicates that intensively raised broilers are missing host-adapted species that likely co-evolved with birds in nature.

Among enriched Bacteroidetes species in the cecal microbiota of EPS broilers, species *Alistipes*, *Barnesiella*, *and Parabacteroides* have been shown to be efficient colonizers of the broiler ceca (39, 40) and to be enriched in chicks raised in contact with an adult hen (41). Specifically, *Alistipes*, one of the first colonizers of the chicken GIT (42), has been positively correlated with broiler body weight (43) and has been shown to be more abundant in the ceca of healthy broilers compared to coccidiosis-infected broilers (33) and in the ceca of free-range chickens compared to commercial broilers (28). *Barnesiella* has been shown to be enriched in the cecal material of free-range chickens (28), high-performing broilers (33), and chicks colonized with cecal material (40). *Parabacteroides* has been shown to be positively correlated with broiler body weight (44) and to be more abundant in older chickens (28, 45–47), which could be an indicator of a more mature microbiota.

Within the genus *Bacteroides*, *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* were enriched in the microbiota of IPS broilers, whereas *Bacteroides salinitronis* DSM 18170, *Bacteroides plebeius*, and *Bacteroides gallinaceum* were enriched in the cecal microbiota of EPS broilers. *B. plebeius*, *B. gallinaceum*, and *B. salinitronis* have been previously shown to be chicken-adapted species, whereas *B. thetaiotaomicron* has been shown to be human adapted (48), thus indicating that IPS broilers are likely being colonized by *Bacteroides* of human origin instead of chicken commensals. The reduction of host-adapted species may affect several aspects of bird physiology (6, 9, 49). For example, it was recently shown that week-old broilers with high *Bacteroides* abundance had increased short-chain fatty acid concentration, higher cecal claudin-1 and IL-10 expression, and lower expression of interleukin-1β compared to broilers with low *Bacteroides* abundance, suggesting that *Bacteroides* can promote polysaccharide degradation, improve intestinal barrier, and modulate immune responses toward downregulation of inflammatory pathways (50).

Within the phylum Firmicutes, *Megamonas*, a core member of the broiler cecal microbiota, was shown to be less abundant in IPS broilers. *Megamonas* has been shown to be an efficient colonizer of the broiler gut and to be enriched in broilers inoculated with adult cecal content and defined communities (6, 39, 41, 51, 52). Interestingly, enriched *Megamonas* has also been observed in wild and free-range birds compared to birds in captivity (26, 28, 53, 54). In addition to being a free hydrogen utilizer and short-chain fatty acid producer, *Megamonas* has been shown to inhibit *Salmonella* growth *in vitro* (55, 56), which warrants further exploration of the function of this genus in the chicken GIT.

Within the phylum Proteobacteria, the cecal microbiota of EPS broilers showed a higher relative abundance of *Sutterella*, *Parasutterella*, and *Desulfovibrio*. *Desulfovibrio* is an effective colonizer of the chicken ceca (39, 41), which can consume free hydrogen present in the gut environment (42). *Parasutterella* has been identified as a core member of the gut microbiota of 35-day-old broilers (46), and *Sutterella* has been positively associated with broiler body weight (44). Although there has been no specific study evaluating the role of these genera in the gut microbiota of chickens, *Parasutterella* has been shown to significantly impact host physiology by modulating bile acid and cholesterol metabolism in mice (13).

In the current study, six phyla were exclusively detected in extensively raised birds. Among these six unique phyla, Fusobacteria was a minor component of the broiler microbiota, which agrees with other studies (42). Despite its relative low abundance (0.1% ± 1.3), some Fusobacteria species can degrade uric acid (1, 55), which could be of importance to broiler physiology. Spirochaetes were detected in 46% of the EPS broilers, with an average relative abundance of 2.7% ± 4.1. Members of this phylum have been reported to be enriched in free-range chickens (57) and Indian native chicken breeds (32) compared to commercial broilers. The main Spirochaetes detected in our study were

Treponema, *Sphaerochaeta*, and *Brachyspira*, which have been considered as potential pathogens (58). Some *Treponema* species can degrade cellulose and xylan (59), and the observed enrichment in EPS chickens could result from access to high-fiber substrates. The phylum Elusimicrobia was present in 50% of the EPS broilers, with an average relative abundance of 0.9% ± 3.4. *Elusimicrobium* was previously shown to be missing from commercial broilers compared to indigenous breeds (32) and to be a core microbe in the cecal microbiota of 81-day-old free-range chickens (28). Interestingly, an increase in *Elusimicrobium* was observed in the microbiota of laying hens fed with insect-based diet (60). As a common member found in the GIT of insects (61), the presence of *Elusimicrobium* in EPS broilers is likely a consequence of these birds having access to insects as food sources. The phylum Synergistetes was detected in most EPS birds, with an average relative abundance of 0.4% ± 0.5. This phylum has been reported both as a minor and major component of the chicken gut microbiota (41, 62–65). Synergistetes species can degrade toxic compounds from plants (66) and were shown to be abundant in the ceca of wild capercaillie but absent from captive birds. Capercaillie relies on conifer plants as a feed source during the winter, and it is speculated that the low survival rates observed in captive birds re-introduced to wild environments result from a lack of Synergistetes within the gut microbiota of captive birds (53). The reduction in Synergistetes seen in captive birds, coupled with the impaired host ability to detoxify toxic compounds and digest plant materials, warrants further investigation of the impact of Synergistetes on the cecal microbiota of broilers.

The microbiota of IPS broilers had a reduced frequency of *Olsenella* and *Victivallis*. In contrast with our results, a previous study observed *Olsenella* to be increased in indoor hens compared to outdoor-reared hens (25). It is possible that the contradictory results are due to differences in *Olsenella* species present across studies. In our study, the only ASV assigned to the species level was *Olsenella* sp. Marseille-P3256, which has recently been reclassified as *Thermophilibacter mediterraneus* (67). We were able to isolate *Thermophilibacter mediterraneus* and *Thermophilibacter provencensis*, which are former *Olsenella* species that could be employed in future studies to evaluate the impact of *Olsenella* on host physiology. In addition, in agreement with our results, *Victivallis* was previously shown to be enriched in extensively raised chickens (25, 28), which could be related to access to insects, as *Victivallis* was shown to be enriched in chickens fed insect larvae (68).

The microbiota of IPS broilers showed an enrichment in *Blautia*, *Faecalibacterium*, and *Oscillibacter*, which were core microbes shared by birds in both systems. *Blautia* and *Oscillibacter* were previously observed to be enriched in indoor- compared to outdoor-raised chickens (25). Interestingly, chicks inoculated with a competitive exclusion product containing *Blautia* and *Oscillibacter* were shown to have a lower relative abundance of *Blautia* than non-inoculated chicks, while no *Oscillibacter* was detected in either group (69). This suggests that *Blautia* and *Oscillibacter*, although ubiquitous in the broiler cecal microbial community, can be displaced by other bacteria if other bacteria are available. *Faecalibacterium* is a major butyrate producer (70) that has been associated with improved growth performance and gut health in broilers (21, 33, 44, 71–73); however, the characterization of the effect of *Faecalibacterium* on broiler physiology is still needed. In addition, several genera with a relative abundance lower than 0.5% were shown to be enriched in the microbiota of IPS broilers. Among these enriched genera, *Bacillus* and *Butyricicoccus* have been previously mentioned as core members of the broiler microbiota (28, 31, 74). Several *Bacillus* species have been used as probiotics and suggested to confer benefits to broilers (75); however, *Bacillus* species are usually not effective colonizers of the chicken gut and need to be constantly provided to exert effects (12, 41). *Butyricicoccus* has been considered a potential probiotic due to its production of butyrate (76) and positive association with broiler performance and disease resilience (72, 77); however, it has been reported that pure cultures of *Butyricicoccus pullicaecorum* inoculated in day-old chicks failed to colonize the ceca (39).

A greater predictive functional potential observed in the microbiota of EPS broilers may be related to different dietary patterns between systems. Although greater functional potential could be considered beneficial, the pathways enriched in the EPS microbiota may not necessarily benefit IPS broilers, which are fed relatively simplified diets. Nonetheless, identifying and harvesting bacteria capable of providing functional potential could aid the use of alternative feed ingredients if coupled with the supplementation of specific bacteria that can utilize these ingredients. Moreover, bacteria harvested from EPS chickens could potentially have a lower incidence of antimicrobial resistance genes and a higher ability to inhibit pathogen growth. A limitation of our results is that the accuracy of functional predictions based on 16S rRNA sequencing is dependent upon the availability of reference genomes, which could potentially result in limited annotation of particular ASVs. Future studies using techniques such as metagenomic sequencing will help improve the accuracy of the functional annotation of microbial communities in EPS and IPS chickens.

Conclusion

In the current study, we focused on bacteria from EPS chickens since these bacteria are more likely to be host adapted and to have evolved with chickens in nature. We identified *Olsenella*, *Alistipes*, *Bacteroides*, *Barnesiella*, *Parabacteroides*, *Megamonas*, and *Parasutterella* as core microbes within the broiler cecal microbiota to be further investigated for their effects on bird physiology and potential applications as next-generation probiotics. These genera seem to be depleted in IPS broilers but are frequently found in EPS broilers and readily colonize the ceca after a single exposure. The collection of bacterial isolates generated in this study will be used as a resource to further explore how differences in microbiota composition can influence bird physiology and to elucidate the role of individual species within the microbial community.

MATERIALS AND METHODS

Samples

Farms that participated in this research project were recruited with the assistance of poultry industry workshops, producer associations, and local veterinarians. Details about the research project were introduced to participating producers, and samples were collected with research consent. Cecal samples from IPS broilers ($n = 59$) were collected from 12 independent commercial farms. The broilers were euthanized on farm using cervical dislocation, and the cecal contents were collected using sterile techniques into an empty tube or a tube containing liquid casein yeast (LCY) media supplemented with 30% glycerol and 0.05% L-cysteine. Samples were transported on dry ice and stored at −80°C until use. Cecal samples from EPS broilers ($n = 46$) were collected from 10 independent commercial small-scale farms that supply poultry products to local farmers' markets. Among these 10 farms, one farm that raised free-range broilers in an organic system was visited, and samples were collected as described for IPS broilers. Samples from the remaining nine EPS farms were collected from a provincially inspected slaughterhouse. Specifically, broilers were electrically stunned, bled, and eviscerated, and intestinal tracts (from ileum to cloaca) were collected in sterile plastic bags and transported on ice to a laboratory within 3 h. Cecal tissues were subsequently dissected in an anaerobic chamber (Bactron 300, Sheldon Manufacturing Incorporated; gas conditions: 5% CO_2, 5% H_2, and 90% N_2) to collect the cecal contents as described above.

Cecal samples from additional chickens were collected in LCY supplemented with 30% glycerol and 0.05% L-cysteine for culturing and isolating bacteria. Samples were obtained from 2-year-old backyard bantam rosters ($n = 2$), 17-week-old roosters from heritage breeds raised without antibiotics and with access to the outdoors ($n = 5$), 1-, 5-, and 40-week-old layers raised in an organic system with access to the outdoors ($n = 5$ per age), and 40-week-old layers raised in cages in an intensive system ($n = 5$). These

birds were euthanized on farm using cervical dislocation, and the cecal contents were transported on dry ice and stored at −80°C until use.

DNA extraction

The extraction of DNA from cecal contents, reagent control, and a gut microbial community standard (ZymoBIOMICS, ZymoResearch) were performed using the QIAamp DNA Stool Mini Kit (Qiagen Inc., US) according to the manufacturer's instructions with the addition of a bead-beating step. Approximately 100 mg of cecal content was mixed with Inhibitex buffer and 2.0 mm garnet beads (BioSpec Products, Bartlesville, OK, USA) and lysed by bead-beating twice at 6.0 m/s for 30 s (FastPrep-24TM 5G, MP Biomedicals). The purity and concentration of the extracted DNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Scientific). The Illumina 16S Metagenomic Sequencing Library Preparation Protocol targets the V3-V4 region of the 16S rRNA gene (primers forward 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each 25 µL of polymerase chain reaction (PCR) reaction contained 12.5 µL of 2× KAPA HiFi HotStart ReadyMix, 5 µL of 1 µM forward primer, 5 µL of 1 µM reverse primer, and 2.5 µL of DNA template (5 ng/µL). The PCR program consisted of an initial denaturation step of 3 min at 95°C, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step of 72°C for 5 min. Amplicons were purified using AMPure XP beads prior to and after the attachment of Illumina sequencing adapters. The final library was diluted to 4 nM and sequenced using paired-end 2 × 300 cycles on an Illumina MiSeq Platform (Illumina Inc., San Diego, CA, USA). All DNA extractions and sequencing procedures were performed by the same person.

16S rRNA amplicon sequencing analysis

Raw sequencing data were processed using Quantitative Insight into Microbial Ecology 2 (QIIME 2 v2021.4) (78) and DADA2 for pairing, denoising, de-replication, and chimera filtering (79). Sequences were truncated at 270 (forward) and 220 (reverse) base pairs based on the median quality score and discarded if they presented more than six expected errors. Mafft and fastree methods (80, 81) were used to align sequences and generate phylogenetic trees. Taxonomy was assigned using the q-2-feature-classifier plugin (82). The Naïve Bayes classifier (83) was pretrained on the SILVA 138 QIIME compatible database (84). Sequences were clustered at 99% identity using majority taxonomy strings. Data were analyzed using phyloseq v.1.40.0 (85), microbiome v. 1.18.0 (86), and qiime2R v. 0.99.6 (87) packages in R v.1.4.1717 (88). ASVs assigned to the mitochondria family, chloroplast order, archaea kingdom, or unassigned were removed from the data set, and the remaining reads were rarefied at an even count for downstream analysis. Phylogenetic diversity, Chao1, and Simpson indices were used to evaluate alpha diversity. The Bray-Curtis distance matrix and principal coordinates analysis (PCoA) were used to evaluate beta diversity. Hierarchical clustering was performed based on the Bray-Curtis distance matrix and the single linkage ("friends-of-friends") method (stats package). Testing for differentially abundant features was performed at the ASV, taxa, family, and phylum levels. Analysis at the taxonomic level was performed by merging all ASVs exhibiting the same taxonomy string using the *tax_glom* function (phyloseq package). Differential abundance analyses were done using the limma-voom tool v.3.52.4 (89, 90), DESeq2 (v.1.36.0) with apeglm for logarithmic fold change shrinkage and false discovery rate (FDR) correction (91, 92), and the non-parametric factorial Kruskal-Wallis sum-rank test from the linear discriminant analysis effect size algorithm (93). To reduce the occurrence of type I error, features were considered differentially abundant if differences were consistently detected by a combination of the three methods, with an alpha level of 0.05. The core microbiota for each system was defined as taxa present in at least 50% of birds in each group (94). Taxa concomitantly assigned as core in the microbiota of IPS and EPS broilers were defined as members

of the core cecal microbiota. Figures were generated using the ggplot2 v.3.4.0 (95) and pheatmap v.1.0.12 (96) packages.

A phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2, v.2.1.4-b) (97) was used to predict the functional potential of the microbiota based on ASVs. Sequences were aligned to reference trees using EPA-ng (98), and hidden state predictions were performed by Castor package v. 1.7.8 (99). Enzyme Commission numbers and MetaCyc pathways databases were used to predict microbial gene families and pathways.

Bacterial isolation and identification

Cecal samples collected in LCY supplemented with 30% glycerol and 0.05% L-cysteine were thawed on ice, homogenized by vortexing, and serially diluted in 1× PBS inside an anaerobic chamber. Diluted samples were plated on nine types of media (Table S1) and incubated at 37°C using three gas conditions: aerobic, anaerobic (5% CO_2, 5% H_2, and 90% N_2), and high CO_2 (20% CO_2, 10% H_2, and 70% N_2). After 72 h of incubation, single colonies with distinct morphologies were selected from each plate, streaked on fresh media plates, and incubated for 72 h to obtain isolates. The purified colonies were further characterized by Sanger sequencing. Amplicon PCR amplifying 16S rRNA gene was performed using primers 8F/926R (forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 5′-CCGTCAATTCNTTTRAGT-3′). Each 50 µL of PCR reaction contained 5 µL of 10× Taq polymerase buffer (Invitrogen, Carlsbad, CA, USA), 2 µL of 10 µM forward primer, 2 µL of 10 µM reverse primer, 2 µL of 10 mM deoxynucleotide triphosphate mix (Invitrogen), 2 µL of 50 mM $MgCl_2$ (Invitrogen), 0.5 µL of 1 U/µL Taq polymerase (Invitrogen), and 1 µL of nuclease-free water containing the harvested bacterial colony. The PCR program consisted of an initial denaturation step of 10 min at 94°C, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 7 min. The amplicon products were purified using a GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific). The purity and concentration of amplicon products were determined using Nanodrop 2000 (Thermo Scientific) and sent for Sanger sequencing (Molecular Biology Service Unit, University of Alberta). The taxonomy of the resultant sequences was analyzed using the rRNA/ITS database of 16S rRNA within the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). The maximum likelihood phylogenetic tree based on the 16S rRNA gene was constructed using MEGAX software, and the phylogenetic tree was visualized using Interactive Tree Of Life (iTOL v6) software. In addition, sterile 1× PBS was added to each type of plate, and the surface was scraped using an inoculation loop to collect microbial cells, which were mixed with LCY supplemented with 0.05% L-cysteine and 50% glycerol (final concentration, 25%), and stored at −80°C.

WGS analysis

The extraction of genomic DNA from selected isolates was conducted using the Wizard Genomic DNA Purification Kit (Promega Corp., USA) following the manufacturer's instructions. Purity and concentration of extracted DNA were determined using the Nanodrop 2000 spectrophotometer and the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Scientific), respectively. The library preparation was performed using the NEBNext Ultra II DNA Library Prep kit (New England Biolabs Inc., CA, USA), followed by 150bp paired-end sequencing on an Illumina NovaSeq 6000 platform (Illumina Inc., USA). The quality of the resultant paired-end 150 bp sequences was analyzed using FastQC (v.0.11.9) (100), and sequence adapters were trimmed using Trimmomatic (v. 039) (101). Draft genome assemblies were performed using SPAdes assembler v.30.10.1 (102), and the quality of assembled genomes was evaluated using QUAST v.5.5.0 (103). For determination of taxonomy, the contig file of selected isolates was compared to reference genomes (RefSeq Genome Database) of suspected species using the OrthoANIu algorithm (104). Isolates that concomitantly presented 16S rRNA identity below 98% against the NCBI rRNA/ITS database using BLAST and OrthoANIu values lower

than 95% against NCBI reference genomes were considered new species (105). Genome annotation was performed on the Rapid Annotation using Subsystem Technology system (106) and visualized using SEED Viewer (107). Genemark (108) and BLASTp (109) were used to predict genes and align the resulting amino acid sequences of coding genes. The amino acid sequences were aligned against the Bacteriocin database (110) for identification of genes encoding bacteriocins and the Virulence Factor Database for identification of genes encoding virulence factors using identity thresholds of >60% (Blastp). Additionally, the Comprehensive Antibiotic Resistance Database (111) was used to annotate antibiotic resistance genes using default parameters.

Statistical analysis

Alpha-diversity indices were analyzed using one-way analysis of variance (ANOVA) and the Tukey honestly significant difference when data were normally distributed and the Kruskal-Wallis and Wilcoxon rank sum test with FDR adjustment if data were not normally distributed. Beta-diversity distance matrices were analyzed using PERMANOVA to test for differences in the distances to centroids and dispersion of the groups and permutation test for homogeneity of multivariate dispersions to test for homogeneity of dispersions between groups (vegan package). Differences in the gene abundance or MetaCyc pathways in extensive and intensive systems were identified using ANOVA-like differential expression (ALDEx) analysis (ALDEx2 package v.1.30.0 (112). An alpha level of 0.05 was considered for all the analyses.

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Camila S. Marcolla, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review and editing | Tingting Ju, Formal analysis, Methodology, Visualization, Writing – review and editing | Hannah L. Lantz, Methodology, Writing – review and editing | Benjamin P. Willing, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review and editing

DATA AVAILABILITY

The data sets generated and analyzed in the current study are available in the NCBI SRA repository under accession no. PRJNA955389.

ETHICS APPROVAL

Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Alberta in accordance with the Canadian Council on Animal Care guidelines (AUP00002373) and by the Human Research Ethic Board at the University of Alberta in accordance with the Tri-Council Policy Statement: Ethical Conduct of Research involving Humans (Pro00071050).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (Spectrum02352-23_S0001.docx). Fig. S1 to S8.
Supplemental tables (Spectrum02352-23_S0002.xlsx). Tables S1 to S7.

REFERENCES

1. Mead GC. 1989. Microbes of the avian cecum: types present and substrates utilized. J Exp Zool 252:48–54. https://doi.org/10.1002/jez.1402520508

2. Józefiak D, Rutkowski A, Martin SA. 2004. Carbohydrate fermentation in the avian ceca: a review. Anim Feed Sci Technol 113:1–15. https://doi.org/10.1016/j.anifeedsci.2003.09.007

3. Apajalahti J, Vienola K. 2016. Interaction between chicken intestinal microbiota and protein digestion. Anim Feed Sci Technol 221:323–330. https://doi.org/10.1016/j.anifeedsci.2016.05.004

4. Broom LJ, Kogut MH. 2018. The role of the gut microbiome in shaping the immune system of chickens. Vet Immunol Immunopathol 204:44–51. https://doi.org/10.1016/j.vetimm.2018.10.002

5. Meijerink N, Kers JG, Velkers FC, van Haarlem DA, Lamot DM, de Oliveira JE, Smidt H, Stegeman JA, Rutten V, Jansen CA. 2020. Early life inoculation with adult-derived microbiota accelerates maturation of intestinal microbiota and enhances NK cell activation in broiler chickens. Front Vet Sci 7:584561. https://doi.org/10.3389/fvets.2020.584561

6. Zenner C, Hitch TCA, Riedel T, Wortmann E, Tiede S, Buhl EM, Abt B, Neuhaus K, Velge P, Overmann J, Kaspers B, Clavel T. 2021. Early-life immune system maturation in chickens using a synthetic community of cultured gut bacteria. mSystems 6:e01300-20. https://doi.org/10.1128/mSystems.01300-20

7. Rubio LA. 2019. Possibilities of early life programming in broiler chickens via intestinal microbiota modulation. Poult Sci 98:695–706. https://doi.org/10.3382/ps/pey416

8. Chintoan-Uta C, Wisedchanwet T, Glendinning L, Bremner A, Psifidi A, Vervelde L, Watson K, Watson M, Stevens MP. 2020. Role of cecal microbiota in the differential resistance of inbred chicken lines to colonization by *Campylobacter jejuni*. Appl Environ Microbiol 86:e02607-19. https://doi.org/10.1128/AEM.02607-19

9. Zaytsoff SJM, Montina T, Boras VF, Brassard J, Moote PE, Uwiera RRE, Inglis GD. 2022. Microbiota transplantation in day-old broiler chickens ameliorates necrotic enteritis via modulation of the intestinal microbiota and host immune responses. Pathogens 11:972. https://doi.org/10.3390/pathogens11090972

10. Forder REA, Howarth GS, Tivey DR, Hughes RJ. 2007. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. Poult Sci 86:2396–2403. https://doi.org/10.3382/ps.2007-00222

11. Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, Walter J. 2010. Diversification of the gut symbiont lactobacillus reuteri as a result of host-driven evolution. ISME J 4:377–387. https://doi.org/10.1038/ismej.2009.123

12. Kubasova T, Seidlerova Z, Rychlik I. 2021. Ecological adaptations of gut microbiota members and their consequences for use as a new

generation of probiotics. Int J Mol Sci 22:5471. https://doi.org/10.3390/ijms22115471

13. Ju T, Kong JY, Stothard P, Willing BP. 2019. Defining the role of parasutterella, a previously uncharacterized member of the core gut microbiota. ISME J 13:1520–1534. https://doi.org/10.1038/s41396-019-0364-5

14. Zilber-Rosenberg I, Rosenberg E. 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol Rev 32:723–735. https://doi.org/10.1111/j.1574-6976.2008.00123.x

15. Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML, Gilbert MTP. 2016. Do vertebrate gut metagenomes confer rapid ecological adaptation? Trends Ecol Evol 31:689–699. https://doi.org/10.1016/j.tree.2016.06.008

16. Foster KR, Schluter J, Coyte KZ, Rakoff-Nahoum S. 2017. The evolution of the host microbiome as an ecosystem on a leash. Nature 548:43–51. https://doi.org/10.1038/nature23292

17. Blaser MJ, Falkow S. 2009. What are the consequences of the disappearing human microbiota? Nat Rev Microbiol 7:887–894. https://doi.org/10.1038/nrmicro2245

18. Langella P, Guarner F, Martín R. 2019. Editorial: next-generation probiotics: from commensal bacteria to novel drugs and food supplements. Front Microbiol 10:1973. https://doi.org/10.3389/fmicb.2019.01973

19. Jha R, Das R, Oak S, Mishra P. 2020. Probiotics (direct-fed microbials) in poultry nutrition and their effects on nutrient utilization, growth and laying performance, and gut health: a systematic review. Animals (Basel) 10:1863. https://doi.org/10.3390/ani10101863

20. Schneitz C. 2005. Competitive exclusion in poultry - 30 years of research. Food Control 16:657–667. https://doi.org/10.1016/j.foodcont.2004.06.002

21. Stanley D, Hughes RJ, Geier MS, Moore RJ. 2016. 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 7:187. https://doi.org/10.3389/fmicb.2016.00187

22. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, Hickman HD, McCulloch JA, Badger JH, Ajami NJ, Trinchieri G, Pardo-Manuel de Villena F, Yewdell JW, Rehermann B. 2017. Wild mouse gut microbiota promotes host fitness and improves disease resistance. Cell 171:1015–1028. https://doi.org/10.1016/j.cell.2017.09.016

23. Rosshart SP, Herz J, Vassallo BG, Hunter A, Wall MK, Badger JH, McCulloch JA, Anastasakis DG, Sarshad AA, Leonardi I, Collins N, Blatter JA, Han S-J, Tamoutounour S, Potapova S, Foster St Claire MB, Yuan W, Sen SK, Dreier MS, Hild B, Hafner M, Wang D, Iliev ID, Belkaid Y, Trinchieri G, Rehermann B. 2019. Laboratory mice born to wild mice have natural microbiota and model human immune responses. Science 365:eaaw4361. https://doi.org/10.1126/science.aaw4361

24. Souza MR, Moreira JL, Barbosa FHF, Cerqueira M, Nunes AC, Nicoli JR. 2007. Influence of intensive and extensive breeding on lactic acid bacteria isolated from gallus gallus domesticus ceca. Vet Microbiol 120:142–150. https://doi.org/10.1016/j.vetmic.2006.10.019

25. Seidlerova Z, Kubasova T, Faldynova M, Crhanova M, Karasova D, Babak V, Rychlik I. 2020. Environmental impact on differential composition of gut microbiota in indoor chickens in commercial production and outdoor, backyard chickens. Microorganisms 8:767. https://doi.org/10.3390/microorganisms8050767

26. Scupham AJ, Patton TG, Bent E, Bayles DO. 2008. Comparison of the cecal microbiota of domestic and wild turkeys. Microb Ecol 56:322–331. https://doi.org/10.1007/s00248-007-9349-4

27. Ferrario C, Alessandri G, Mancabelli L, Gering E, Mangifesta M, Milani C, Lugli GA, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, Hiyashi R, Mackie R, van Sinderen D, Ventura M. 2017. Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses. Environ Microbiol 19:4771–4783. https://doi.org/10.1111/1462-2920.13943

28. Ocejo M, Oporto B, Hurtado A. 2019. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. Sci Rep 9:2506. https://doi.org/10.1038/s41598-019-39323-x

29. Nurmi E, Rantala M. 1973. New aspects of salmonella infection in broiler production. Nature 241:210–211. https://doi.org/10.1038/241210a0

30. Xu Y, Yang H, Zhang L, Su Y, Shi D, Xiao H, Tian Y. 2016. High-throughput sequencing technology to reveal the composition and function of cecal microbiota in dagu chicken. BMC Microbiol 16:259. https://doi.org/10.1186/s12866-016-0877-2

31. Pandit RJ, Hinsu AT, Patel NV, Koringa PG, Jakhesara SJ, Thakkar JR, Shah TM, Limon G, Psifidi A, Guitian J, Hume DA, Tomley FM, Rank DN, Raman M, Tirumurugaan KG, Blake DP, Joshi CG. 2018. Microbial diversity and community composition of caecal microbiota in commercial and indigenous Indian chickens determined using 16S rDNA amplicon sequencing. Microbiome 6:115. https://doi.org/10.1186/s40168-018-0501-9

32. Paul SS, Chatterjee RN, Raju M, Prakash B, Rama Rao SV, Yadav SP, Kannan A. 2021. Gut microbial composition differs extensively among Indian native chicken breeds originated in different geographical locations and a commercial broiler line, but breed-specific, as well as across-breed core microbiomes, are found. Microorganisms 9:391. https://doi.org/10.3390/microorganisms9020391

33. Bindari YR, Gerber PF. 2022. Centennial review: factors affecting the chicken gastrointestinal microbial composition and their association with gut health and productive performance. Poult Sci 101:101612. https://doi.org/10.1016/j.psj.2021.101612

34. Das B, Ghosh TS, Kedia S, Rampal R, Saxena S, Bag S, Mitra R, Dayal M, Mehta O, Surendranath A, Travis SPL, Tripathi P, Nair GB, Ahuja V. 2018. Analysis of the gut microbiome of rural and urban healthy Indians living in sea level and high altitude areas. Sci Rep 8:10104. https://doi.org/10.1038/s41598-018-28550-3

35. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107:14691–14696. https://doi.org/10.1073/pnas.1005963107

36. Bensch HM, Tolf C, Waldenström J, Lundin D, Zöttl M. 2023. Bacteroidetes to firmicutes: captivity changes the gut microbiota composition and diversity in a social subterranean rodent. Anim Microbiome 5:9. https://doi.org/10.1186/s42523-023-00231-1

37. Marcolla CS, Alvarado CS, Willing BP, Miglior F. 2019. Early life microbial exposure shapes subsequent animal health. Can J Anim Sci 99:661–677. https://doi.org/10.1139/cjas-2019-0029

38. Karasova D, Faldynova M, Matiasovicova J, Sebkova A, Crhanova M, Kubasova T, Seidlerova Z, Prikrylova H, Volf J, Zeman M, Babak V, Juricova H, Rajova J, Vlasatikova L, Rysavka P, Rychlik I. 2022. Host species adaptation of obligate gut anaerobes is dependent on their environmental survival. Microorganisms 10:1085. https://doi.org/10.3390/microorganisms10061085

39. Kubasova T, Kollarcikova M, Crhanova M, Karasova D, Cejkova D, Sebkova A, Matiasovicova J, Faldynova M, Sisak F, Babak V, Pokorna A, Cizek A, Rychlik I. 2019. Gut anaerobes capable of chicken caecum colonisation. Microorganisms 7:597. https://doi.org/10.3390/microorganisms7120597

40. Ramírez GA, Richardson E, Clark J, Keshri J, Drechsler Y, Berrang ME, Meinersmann RJ, Cox NA, Oakley BB. 2020. Broiler chickens and early life programming: microbiome transplant-induced cecal community dynamics and phenotypic effects. PLoS One 15:e0242108. https://doi.org/10.1371/journal.pone.0242108

41. Kubasova T, Kollarcikova M, Crhanova M, Karasova D, Cejkova D, Sebkova A, Matiasovicova J, Faldynova M, Pokorna A, Cizek A, Rychlik I. 2019. Contact with adult hen affects development of caecal microbiota in newly hatched chicks. PLoS One 14:e0212446. https://doi.org/10.1371/journal.pone.0212446

42. Rychlik I. 2020. Composition and function of chicken gut microbiota. Animals (Basel) 10:103. https://doi.org/10.3390/ani10010103

43. Farkas V, Csitári G, Menyhárt L, Such N, Pál L, Husvéth F, Rawash MA, Mezőlaki Á, Dublecz K. 2022. Microbiota composition of Mucosa and interactions between the microbes of the different gut segments could be a factor to modulate the growth rate of broiler chickens. Animals (Basel) 12:1296. https://doi.org/10.3390/ani12101296

44. Johnson TJ, Youmans BP, Noll S, Cardona C, Evans NP, Karnezos TP, Ngunjiri JM, Abundo MC, Lee C-W, Elkins CA. 2018. A consistent and predictable commercial broiler chicken bacterial microbiota in antibiotic-free production displays strong correlations with

performance. Appl Environ Microbiol 84:e00362-18. https://doi.org/10.1128/AEM.00362-18

45. Videnska P, Sedlar K, Lukac M, Faldynova M, Gerzova L, Cejkova D, Sisak F, Rychlik I. 2014. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. PLoS One 9:e115142. https://doi.org/10.1371/journal.pone.0115142

46. Jurburg SD, Brouwer MSM, Ceccarelli D, van der Goot J, Jansman AJM, Bossers A. 2019. Patterns of community assembly in the developing chicken microbiome reveal rapid primary succession. Microbiologyopen 8:e00821. https://doi.org/10.1002/mbo3.821

47. Ngunjiri JM, Taylor KJM, Abundo MC, Jang H, Elaish M, Kc M, Ghorbani A, Wijeratne S, Weber BP, Johnson TJ, Lee C-W. 2019. 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 85:e03137-18. https://doi.org/10.1128/AEM.03137-18

48. Kollarcikova M, Faldynova M, Matiasovicova J, Jahodarova E, Kubasova T, Seidlerova Z, Babak V, Videnska P, Cizek A, Rychlik I. 2020. Different bacteroides species colonise human and chicken intestinal tract. Microorganisms 8:1483. https://doi.org/10.3390/microorganisms8101483

49. Yan C, Xiao J, Li Z, Liu H, Zhao X, Liu J, Chen S, Zhao X. 2021. Exogenous fecal microbial transplantation alters fearfulness, intestinal morphology, and gut microbiota in broilers. Front Vet Sci 8:706987. https://doi.org/10.3389/fvets.2021.706987

50. Fan Y, Ju T, Bhardwaj T, Korver DR, Willing BP. 2023. Week-old chicks with high bacteroides abundance have increased short-chain fatty acids and reduced markers of gut inflammation. Microbiol Spectr 11:e0361622. https://doi.org/10.1128/spectrum.03616-22

51. Poudel B, Shterzer N, Sbehat Y, Ben-Porat N, Rakover M, Tovy-Sharon R, Wolicki D, Rahamim S, Bar-Shira E, Mills E. 2022. Characterizing the chicken gut colonization ability of a diverse group of bacteria. Poult Sci 101:102136. https://doi.org/10.1016/j.psj.2022.102136

52. Wongkuna S, Kumar R, Ghimire S, Drescher SM, Maji A, Ambat A, Antony L, Chankhamhaengdecha S, Janvilisri T, Nelson E, Doerner KC, Behr M, Scaria J. 2022. Identification of a microbial sub-community from the Feral chicken gut that reduces salmonella Colonization and improves gut health in a Gnotobiotic chicken model. Microbiology. Microbiology, Microbiology. https://doi.org/10.1101/2022.07.23.501241

53. Wienemann T, Schmitt-Wagner D, Meuser K, Segelbacher G, Schink B, Brune A, Berthold P. 2011. The bacterial Microbiota in the ceca of capercaillie (Tetrao urogallus) differs between wild and captive birds. Syst Appl Microbiol 34:542–551. https://doi.org/10.1016/j.syapm.2011.06.003

54. Yadav S, Caliboso KD, Nanquil JE, Zhang J, Kae H, Neupane K, Mishra B, Jha R. 2021. Cecal microbiome profile of hawaiian feral chickens and pasture-raised broiler (commercial) chickens determined using 16S rRNA amplicon sequencing. Poult Sci 100:101181. https://doi.org/10.1016/j.psj.2021.101181

55. Barnes EM, Impey CS. 1974. The occurence and properties of uric acid decomposing anaerobic bacteria in the avian caecum. J Appl Bacteriol 37:393–409. https://doi.org/10.1111/j.1365-2672.1974.tb00455.x

56. Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ. 2014. Extensive microbial and functional diversity within the chicken cecal microbiome. PLoS One 9:e91941. https://doi.org/10.1371/journal.pone.0091941

57. Mancabelli L, Ferrario C, Milani C, Mangifesta M, Turroni F, Duranti S, Lugli GA, Viappiani A, Ossiprandi MC, van Sinderen D, Ventura M. 2016. Insights into the biodiversity of the gut microbiota of broiler chickens. Environ Microbiol 18:4727–4738. https://doi.org/10.1111/1462-2920.13363

58. Sueyoshi M, Adachi Y. 1990. Diarrhea induced by Treponema hyodysenteriae: a young chick cecal model for swine dysentery. Infect Immun 58:3348–3362. https://doi.org/10.1128/iai.58.10.3348-3362.1990

59. Tokuda G, Mikaelyan A, Fukui C, Matsuura Y, Watanabe H, Fujishima M, Brune A. 2018. Fiber-associated spirochetes are major agents of hemicellulose degradation in the hindgut of wood-feeding higher termites. Proc Natl Acad Sci U S A 115:E11996–E12004. https://doi.org/10.1073/pnas.1810550115

60. Borrelli L, Coretti L, Dipineto L, Bovera F, Menna F, Chiariotti L, Nizza A, Lembo F, Fioretti A. 2017. Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens. Sci Rep 7:16269. https://doi.org/10.1038/s41598-017-16560-6

61. Geissinger O, Herlemann DPR, Morschel E, Maier UG. 2009. The ultramicrobacterium "Elusimicrobium minutum" gen. nov., sp. nov., the first cultivated representative of the termite group 1 phylum. Appl environ Microbiol 75:2831–2840. https://doi.org/10.1128/AEM.02697-08

62. Hou Q, Kwok L-Y, Zheng Y, Wang L, Guo Z, Zhang J, Huang W, Wang Y, Leng L, Li H, Zhang H. 2016. Differential fecal microbiota are retained in broiler chicken lines divergently selected for fatness traits. Sci Rep 6:37376. https://doi.org/10.1038/srep37376

63. Nan S, Yao M, Zhang X, Wang H, Li J, Niu J, Chen C, Zhang W, Nie C. 2022. Fermented grape seed meal promotes broiler growth and reduces abdominal fat deposition through intestinal microorganisms. Front Microbiol 13:994033. https://doi.org/10.3389/fmicb.2022.994033

64. Wang J, Fan H, Han Y, Wei J, Zhao J, Zhou Z, Plaizier J. 2017. Pyrosequencing of the broiler chicken gastrointestinal tract reveals the regional similarity and dissimilarity of microbial community. Can J Anim Sci 97:302–313. https://doi.org/10.1139/cjas-2015-0120

65. Yang Q, Ren S, Niu T, Guo Y, Qi S, Han X, Liu D, Pan F. 2014. Distribution of antibiotic-resistant bacteria in chicken manure and manure-fertilized vegetables. Environ Sci Pollut Res Int 21:1231–1241. https://doi.org/10.1007/s11356-013-1994-1

66. Leong LEX, Khan S, Davis CK, Denman SE, McSweeney CS. 2017. Fluoroacetate in plants - a review of its distribution, toxicity to livestock and microbial detoxification. J Anim Sci Biotechnol 8:55. https://doi.org/10.1186/s40104-017-0180-6

67. Zgheib R, Anani H, Meng MM, Mailhe M, Ricaboni D, Morand A, Caputo A, Traore SI, Fontanini A, Armstrong N, Raoult D, Fournier PE. 2021. New human-associated species of the family atopobiaceae and proposal to reclassify members of the genus olsenella Int J Syst Evol Microbiol 71. https://doi.org/10.1099/ijsem.0.004819

68. Colombino E, Biasato I, Ferrocino I, Bellezza Oddon S, Caimi C, Gariglio M, Dabbou S, Caramori M, Battisti E, Zanet S, Ferroglio E, Cocolin L, Gasco L, Schiavone A, Capuccio MT. 2021. Effect of insect live larvae as environmental enrichment on poultry gut health: gut mucin composition, microbiota and local immune response evaluation. Animals (Basel) 11:2819. https://doi.org/10.3390/ani11102819

69. Dame-Korevaar A, Kers JG, van der Goot J, Velkers FC, Ceccarelli D, Mevius DJ, Stegeman A, Fischer EAJ. 2020. Competitive exclusion prevents colonization and compartmentalization reduces transmission of ESBL-producing Escherichia coli in broilers. Front Microbiol 11:566619. https://doi.org/10.3389/fmicb.2020.566619

70. Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F, Rychlik I, Dozois CM. 2016. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Appl Environ Microbiol 82:1569–1576. https://doi.org/10.1128/AEM.03473-15

71. Siegerstetter S-C, Schmitz-Esser S, Magowan E, Wetzels SU, Zebeli Q, Lawlor PG, O'Connell NE, Metzler-Zebeli BU. 2017. Intestinal microbiota profiles associated with low and high residual feed intake in chickens across two geographical locations. PLoS One 12:e0187766. https://doi.org/10.1371/journal.pone.0187766

72. Lundberg R, Scharch C, Sandvang D. 2021. The link between broiler flock heterogeneity and cecal microbiome composition. Anim Microbiome 3:54. https://doi.org/10.1186/s42523-021-00110-7

73. Pedroso AA, Lee MD, Maurer JJ. 2021. Strength lies in diversity: how community diversity limits salmonella abundance in the chicken intestine. Front Microbiol 12:694215. https://doi.org/10.3389/fmicb.2021.694215

74. van Kuijk SJA, Han Y, Garcia-Ruiz AI, Rodiles A. 2021. Hydroxychloride trace minerals have a positive effect on growth performance, carcass quality and impact Ileal and cecal microbiota in broiler chickens. J Anim Sci Biotechnol 12:38. https://doi.org/10.1186/s40104-021-00553-7

75. Lutful Kabir SM. 2009. The role of probiotics in the poultry industry. Int J Mol Sci 10:3531–3546. https://doi.org/10.3390/ijms10083531

76. Geirnaert A, Steyaert A, Eeckhaut V, Debruyne B, Arends JBA, Van Immerseel F, Boon N, Van de Wiele T. 2014. Butyricicoccus pullicaecorum, a butyrate producer with probiotic potential, is intrinsically tolerant to stomach and small intestine conditions. Anaerobe 30:70–74. https://doi.org/10.1016/j.anaerobe.2014.08.010

77. Eeckhaut V, Wang J, Van Parys A, Haesebrouck F, Joossens M, Falony G, Raes J, Ducatelle R, Van Immerseel F. 2016. The probiotic butyricicoccus pullicaecorum reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers. Front Microbiol 7:1416. https://doi.org/10.3389/fmicb.2016.01416

78. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS II, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857. https://doi.org/10.1038/s41587-019-0209-9

79. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869

80. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780. https://doi.org/10.1093/molbev/mst010

81. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490. https://doi.org/10.1371/journal.pone.0009490

82. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's Q2-feature-classifier plugin. Microbiome 6. https://doi.org/10.1186/s40168-018-0470-z

83. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Weiss R, Dubourg V, Vanderplas J, Passos A, Cournapeau D, Brucher M, Perrot M, Duchesnay E. 2011. Scikit-learn: Machine learning in python. J Mach Learn Res 12:2825–2830. https://www.jmlr.org/papers/volume12/pedregosa11a/pedregosa11a.pdf.

84. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–6. https://doi.org/10.1093/nar/gks1219

85. McMurdie PJ, Holmes S. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:e61217. https://doi.org/10.1371/journal.pone.0061217

86. Lahti L, Shetty S. Introduction to the microbiome R package

87. Bisanz JE. Qiime2R: importing QIIME2 artifacts and associated data into R sessions

88. RStudio Team. 2020. Rstudio: integrated development for R

89. Law CW, Chen Y, Shi W, Smyth GK. 2014. METHOD open access voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol 15:15. https://doi.org/10.1186/gb-2014-15-2-r29

90. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47. https://doi.org/10.1093/nar/gkv007

91. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DEseq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8

92. Zhu A, Ibrahim JG, Love MI. 2019. Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. Bioinformatics 35:2084–2092. https://doi.org/10.1093/bioinformatics/bty895

93. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. 2011. Metagenomic biomarker discovery and explanation. Genome Biol 12:R60. https://doi.org/10.1186/gb-2011-12-6-r60

94. Martínez I, Stegen JC, Maldonado-Gómez MX, Eren AM, Siba PM, Greenhill AR, Walter J. 2015. The gut microbiota of rural papua new guineans: composition, diversity patterns, and ecological processes. Cell Rep 11:527–538. https://doi.org/10.1016/j.celrep.2015.03.049

95. Wickham H. 2016. Ggplot2 elegant graphics for data analysis. Cham. https://doi.org/10.1007/978-3-319-24277-4

96. Kolde R. 2019. Pheatmap: pretty heatmaps

97. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille MGI. 2020. PICRUST2 for prediction of metagenome functions. Nat Biotechnol 38:685–688. https://doi.org/10.1038/s41587-020-0548-6

98. Barbera P, Kozlov AM, Czech L, Morel B, Darriba D, Flouri T, Stamatakis A. 2019. EPA-ng: massively parallel evolutionary placement of genetic sequences. Syst Biol 68:365–369. https://doi.org/10.1093/sysbio/syy054

99. Louca S, Doebeli M. 2018. Efficient comparative phylogenetics on large trees. Bioinformatics 34:1053–1055. https://doi.org/10.1093/bioinformatics/btx701

100. Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data

101. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170

102. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021

103. Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29:1072–1075. https://doi.org/10.1093/bioinformatics/btt086

104. Yoon S-H, Ha S-M, Lim J, Kwon S, Chun J. 2017. A large-scale evaluation of algorithms to calculate average nucleotide identity. Antonie Van Leeuwenhoek 110:1281–1286. https://doi.org/10.1007/s10482-017-0844-4

105. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat Commun 9:5114. https://doi.org/10.1038/s41467-018-07641-9

106. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics 9:75. https://doi.org/10.1186/1471-2164-9-75

107. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R. 2014. The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). Nucleic Acids Res 42:D206–14. https://doi.org/10.1093/nar/gkt1226

108. Besemer J, Borodovsky M. 2005. Genemark: web software for gene finding in prokaryotes, eukaryotes and viruses. Nucleic Acids Res 33:W451–4. https://doi.org/10.1093/nar/gki487

109. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

110. van Heel AJ, de Jong A, Montalbán-López M, Kok J, Kuipers OP. 2013. BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides. Nucleic Acids Res 41:W448–53. https://doi.org/10.1093/nar/gkt391

111. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen A-L, Cheng AA, Liu S, Min SY, Miroshnichenko A, Tran H-K, Werfalli RE, Nasir JA, Oloni M, Speicher DJ, Florescu A, Singh B, Faltyn M, Hernandez-Koutoucheva A, Sharma AN, Bordeleau E, Pawlowski AC, Zubyk HL, Dooley D, Griffiths E, Maguire F, Winsor GL, Beiko RG, Brinkman FSL, Hsiao WWL, Domselaar GV, McArthur AG. 2020. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 48:D517–D525. https://doi.org/10.1093/nar/gkz935

112. Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB. 2013. ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-seq. PLoS One 8:e67019. https://doi.org/10.1371/journal.pone.0067019