Research Square

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Fast and slow-growing breeds: characterisation of broiler caecal microbiota development throughout the growing period

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Research Article

Keywords: Broiler, growing period, microbiota, biomarker, 16S rRNA analysis

Posted Date: April 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-22748/v1

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Abstract

Background: The caecal microbiota and its modulation play an important role in animal health, productivity and disease control in poultry production. In this sense, it could be considered as a biomarker of poultry health. Furthermore, due to the emergence of resistant bacteria and the increasing social pressure to establish animal-friendly management on farms, producers are motivated to select more extensive and antibiotic-free breeds. It is therefore necessary to gain better knowledge on the development of major bacteria in healthy broilers, both in commercial fast-growing and in new slow-growing breeds. Hence, the aim of this study was to characterise caecal microbiota in two genetic poultry breeds throughout the growing period using 16S rRNA sequencing analysis.

Results: A total of 50 caecal pools (25 per breed) were sequenced by the 16S rRNA method. The complexity of caecal microbiota composition increased significantly as animals grew. Furthermore, there were statistical differences between breeds at the end of the growing period. The dominant phyla throughout the production cycle were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. The predominantly identified genera were *Ruminococcus* spp., *Lactobacillus* spp. and *Bacteroides* spp.

Conclusion: The results showed that the main caecal bacteria for both breeds were similar. Thus, these phyla or genera should be considered as biomarkers of poultry health in the evaluation of different treatments applied to animals.

Background

Microbiota are defined as the microbial community, including commensal, symbiotic and pathogenic microorganisms, which colonise different areas of animals and have an important influence on health, productivity and disease control [1–9]. Hence, the selection of beneficial microbiota plays an important role in production, protection from pathogens, control of epithelial cell proliferation and differentiation, detoxification (controlling the behavioural and neurological functions of the host) and modulation of the immune system. In fact, knowing the main microbiota composition during the growing period and how management practices could influence its modulation could help to take quick decisions at farm level [6, 10–15]. In this sense, it might be interesting to consider microbiota composition as a biomarker of poultry health [7, 9, 16]. Biomarkers are defined as characteristic measurable alterations associated with normal or pathogenic processes or responses to a treatment [17, 18]. Therefore, it is necessary to have precise information on the development of the main bacteria in healthy broilers [6].

Principal factors affecting the microbiota are age, breed, maternal elements, sex, diet, housing, hygiene, temperature, litter, antibiotic administration and gastrointestinal location [6, 19]. Referring to the last factor mentioned, the caecum is described as the organ with the greatest taxonomic diversity and abundance, which retains food for the longest period, with the greatest water absorption, and it is responsible for urea regulation and carbohydrates fermentation [6]. However, our current knowledge on the composition of the caecal microbiota of chickens is still rather underdeveloped, despite the fact that

these animals represent an important food resource for humans, while also being a potential reservoir of food-borne pathogens [1, 20].

Moreover, due to the emergence of antimicrobial-resistant bacteria, society is pressing for a reduction in antibiotic administration by finding effective alternatives to control infectious diseases at farm level [21–24]. Some of these alternatives are feed additives (prebiotics, probiotics, symbiotics, organic acids, enzymes, phytogenics and metals), alternative medical treatments (antibacterial vaccines, immunomodulatory agents, antimicrobial peptides and bacteriophages) and, finally, different broiler growth systems [25–31]. Although the beneficial effects of many of these alternatives have been demonstrated *in vitro*, the general consensus is that the effect of these products depends on the farm, management and animal characteristics, such as the breed selected [19, 23, 32].

Historically, producers have looked for breeds adapted to intensive conditions and with the best productive performance. However, in response to social pressure to establish welfare-friendly management systems in poultry production, producers are motivated to choose breeds selected for their ability to cope with the natural environment and with a lower performance [27]. Therefore, in order to make management decisions, it is necessary to have better knowledge of the effect of these alternatives under production conditions, both in commercial fast-growing and slow-growing breeds [6, 20, 33, 34].

In this context, the aim of this study was to characterise the caecal microbiota in two genetic poultry breeds, fast-growing and slow-growing, during the growing period, using 16S rRNA sequencing analysis.

Results

During this study, a total of 50 caecal pools (25 per breed) were collected, processed and sequenced. No clinical signs were observed, and the productive parameters obtained were in accordance with the breed standards. There were no statistical differences between replicates (*P*-value > 0.05).

16 rRNA profiling of fast and slow-growing breeds

The MiSeq sequencing of the 50 samples produced a total of 14 143 246 sequencing reads with an average of 282 864.9 reads per sample. Quality and chimera filtering produced a total of 12 661 675 filtered reads with an average of 253 233.5 reads per sample and ranging from 109 447 to 356 331 reads.

Assessment of rarefaction curves based on the Chao1 biodiversity index calculated for the six sequence read groups (day-old chicks, mid-period and slaughter day results for fast and slow-growing breeds) indicated that four of the curves tended to reach a plateau. However, samples from groups 1 and 2 (day-old chicks from both breeds) are at the limit of the rarefaction, leaving a rarefaction number of 72 060 reads (Fig. 1). The Chao1 alpha diversity index reveals a notable difference between the caecal microbiota depending on the age of the animals (Table 1). Statistically significant differences (*P*-value < 0.05) were found between these groups; samples from day-old chicks of both breeds (88.3 and 111.9 for the fast and slow-growing breed, respectively) displayed a lower level of complexity of the microbiota

compared to that found at mid-period (384.4 and 373.8), and samples from mid-period animals displayed a lower level of complexity than the samples from the end of the growing period (420.3 and 447.2 for the fast and slow-growing breed, respectively). Finally, there were statistically significant differences in gut microbiota diversity between both breeds at the end of the growing period (*P*-value < 0.05).

Table 1
Alpha diversity according to time of the growing period in fast (FG) and slow-growing (SG) breeds.

SAMPLING TIME	Fast-growing	Slow-growing
Arrival day	88.3[a]	111.9[a]
Mid-period	384.4[b]	373.8[b]
End	420.3[c]	447.2[d]
[a, b, c, d]: Different superscripts means significant differences with a *P*-value < 0.05.		

Differential gut microbiota composition

Inspection of predicted taxonomic profiles at phylum level for all samples is summarised in Table 2 and represented in Fig. 2. This analysis exhibited that *Firmicutes* represented the dominant phylum of the caecal community in both breeds at all sampling times in the production cycle (*P*-value < 0.05). At the onset of the growing period, *Proteobacteria* was the second prevalent phylum for fast and slow-growing breeds, outnumbering the *Bacteroidetes* phylum. However, during the rest of the production cycle, *Bacteroidetes* phylum was more abundant than *Proteobacteria* in both breeds. The longitudinal study showed that there were no statistically differences between breeds throughout the growing period (*P*-value > 0.05).

For the fast-growing breed, there were statistically significant differences depending on the time of sampling. *Proteobacteria* and *Bacteroidetes* phyla were more abundant at the arrival day (36.4% and 5%, respectively) and at the slaughter day (1.5% and 5.7%, respectively), though the high *Firmicutes* percentage was observed at mid-period (95.1%).

For the slow-growing breed, *Bacteroidetes* (5.7% and 9.3% at arrival and slaughter days, respectively) and *Firmicutes* (95.2% at mid-period) showed the same pattern as in the fast-growing breed. However, statistically significant differences were shown between day-old chicks and mid-period percentage of *Proteobacteria* (32.8% and 1.2%, respectively), which subsequently remained stable until the end of the cycle (1.7%).

Table 2

Taxonomic profiles at phylum level according to time of growing period in fast (FG) and slow-growing (SG) breeds.

Sampling time	**Arrival day**		**Mid-period**		**End**	
Breed	**FG**	**SG**	**FG**	**SG**	**FG**	**SG**
Actinobacteria	0%	0.2%	0.3%	0.3%	0.5%	0.4%
Bacteroidetes	5.0%	5.7%	1.9%	1.9%	5.7%[b]	9.3%[a]
Cyanobacteria	0%	0%	0.5%	0.4%	0.7%[b]	1.1%[a]
Firmicutes	58.6%	61.1%	95.1%	95.2%	90.3%[a]	85.6%[b]
Proteobacteria	36.4%	32.8%	1.3%	1.2%	1.5%[b]	1.7%[a]
Tenericutes	0%	0.2%	0.3%[b]	0.4%[a]	0.6%	1.1%
Unassigned	0%	0%	0.6%	0.6%	0.8%	0.8%

[a, b]: Different superscripts between breeds in the different sampling times means significant differences with a *P*-value < 0.05.

Furthermore, 46 taxa were identified at genus level (Fig. 3). Whereas 20 appeared to be present in all samples, 3 appeared to only be present in caecum samples of slow-growing breed and 23 only appeared at some times of the growing period. The most predominant genera identified were *Oscillospira* spp. (7.5%), *Ruminococcus* spp. (3.6%), *Coprococcus* spp. (2.9%), *Lactobacillus* spp. (2.5%) and *Bacteroides* spp. (2.0%). In the longitudinal study, the only statistical differences were between breeds in *Lactobacillus* spp. at the end of the growing period (2.9% and 2.8% in fast and slow-growing breed, respectively). In order to further identify differences in microbiota composition between breeds, we focused on 33 genera, which were shown to be present at an average relative abundance of more than 0.5% in at least one sample group [35].

For the fast-growing breed, the results for the genera analysis are shown in Table 3. At the arrival day, predominant bacteria of microbiota were Unclassified members (U. m.) of the *Enterobacteriaceae* family (36.4%), U. m. of *Clostridiaceae* family (6.2%), U. m. of the *Ruminococcaceae* family (5.7%), U. m. of *Lachnospiraceae* family (4.9%), *Clostridium* spp. (4.1%), U. m. of *Enterococcaceae* family (3.7%), *Oscillospira* spp. (3.5%) and *Enterococcus* spp. (3.0%).

Table 3

Taxonomic profiles at genus level according to sampling time in fast-growing breed.

Phylum	Family	Genus	Arrival day	Mid-period	End
Unassigned			0.0%	0.6%	0.8%
Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	1.5%	0.5%	3.1%
	Porphyromonadaceae	*Parabacteroides*	1.2%	0.4%	0.7%
	Rikenellaceae	-	2.0%	1.1%	1.2%
	Odoribacteraceae	*Butyricimonas*	0.3%	0.0%	0.7%
Cyanobacteria	-	-	0.0%	0.5%	0.7%
Firmicutes	*Plaococcaceae*	-	0.0%	0.5%	0.4%
	Enterococcaceae	-	3.7%	0.0%	0.0%
		Enterococcus	3.0%	0.2%	0.1%
	Lactobacillaceae	*Lactobacillus*	0.9%	3.9%	2.8%
	-	-	0.2%	0.5%	0.6%
	-	-	13.7%	29.4%	28.9%
	Christensenellaceae	-	0.0%	0.2%	0.6%
	Clostridiaceae	-	0.6%	0.0%	0.3%
		-	5.6%	0.2%	0.2%
		Clostridium	4.1%	0.5%	0.5%
	Lachnospiraceae	-	4.9%	10.4%	10.2%
		Blauria	0.7%	2.0%	2.1%
		Coprococcus	1.6%	4.0%	3.5%
		Dorea	0.2%	1.4%	1.1%
		Epulopscium	2.6%	0.0%	0.0%
		[Ruminococcus]	2.5%	3.3%	2.9%
	Ruminococcaceae	-	5.7%	18.1%	17.7%
		Anaerotruncus	0.0%	0.5%	0.4%
		Faecalibacterium	0.9%	1.5%	2.0%
		Oscillospira	3.5%	9.6%	8.8%

Phylum	Family	Genus	Arrival day	Mid-period	End
		Ruminococcus	2.1%	5.0%	4.4%
	Erysipelotrichaceae	-	0.9%	0.9%	0.4%
		Coprobacillus	0.4%	0.9%	0.5%
		cc_115	0.0%	0.9%	0.6%
Proteobacteria	*Enterobacteriaceae*	-	36.4%	1.3%	1.5%

At mid-period, the predominant genera in caecal samples were U. m. of the *Ruminococcaceae* family (18.1%), U. m. of *Lachnospiraceae* family (10.4%), *Oscillospira* spp. (9.6%), *Coprococcus* spp. (4.0%), *Lactobacillus* spp. (3.9%) and *[Ruminococcus]* spp. (3.3%). Finally, at the end of the growing period, the most prevalent bacteria were U. m. of the *Ruminococcaceae* family (17.7%), U. m. of *Lachnospiraceae* family (10.2%), *Oscillospira* spp. (8.8%), *Coprococcus* spp. (3.5%) and *Bacteroides* spp. (3.1%).

For the slow-growing breed, the results for the genera analysis are shown in Table 4. The pattern for day-old chicks was similar to that observed at this sampling time for the fast-growing breed. The most abundant bacteria were U. m. of the *Enterobacteriaceae* family (32.6%), U. m. of the *Ruminococcaceae* family (7.5%), U. m. of *Lachnospiraceae* family (6.5%), *Oscillospira* spp. (5.8%), U. m. of *Clostridiaceae* family (4.8%) and U. m. of *Enterococcaceae* family (3.6%). At mid-period, predominant genera were U. m. of the *Ruminococcaceae* family (18.4%), U. m. of *Lachnospiraceae* family (10.3%), *Oscillospira* spp. (9.6%), *Coprococcus* spp. (3.8%), *Lactobacillus* spp. (3.4%) and *[Ruminococcus]* spp. (3.3%). Lastly, at slaughter day, U. m. of the *Ruminococcaceae* family (17.0%) were the most abundant bacteria, followed by U. m. of *Lachnospiraceae* family (8.6%), *Oscillospira* spp. (7.7%), *Coprococcus* spp. (3.2%), *Bacteroides* spp. (4.1%) and *Parabacteroides* spp. (3.1%).

Table 4

Taxonomic profiles at genus level according to sampling time in slow-growing breed.

Phylum	Family	Genus	Arrival day	Mid-period	End
Unassigned			0.0%	0.6%	0.8%
Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	2.6%	0.4%	4.1%
	Porphyromonadaceae	*Parabacteroides*	1.0%	0.5%	1.1%
	Rikenellaceae	-	2.0%	1.1%	3.1%
	Odoribacteraceae	*Butyricimonas*	0.0%	0.0%	1.1%
Cyanobacteria			0.0%	0.4%	1.1%
Firmicutes	*Plaococcaceae*	-	0.2%	0.5%	0.4%
	Enterococcaceae	-	3.6%	0.0%	0.0%
		Enterococcus	1.0%	0.2%	0.4%
	Lactobacillaceae	*Lactobacillus*	1.2%	3.4%	2.9%
	-	-	0.4%	0.6%	0.3%
	-	-	14.6%	29.9%	30.0%
	Clostridiaceae	-	4.8%	0.2%	0.3%
		Clostridium	2.7%	0.4%	0.4%
	Lachnospiraceae	-	6.5%	10.3%	8.6%
		Blauria	0.8%	1.8%	1.5%
		Coprococcus	1.6%	3.8%	3.2%
		Dorea	0.8%	1.3%	0.7%
		Epulopscium	2.4%	0.0%	0.0%
		Ruminococcus	2.1%	3.3%	2.3%
	Ruminococcaceae	-	7.5%	18.4%	17.0%
		Anaerotruncus	0.0%	0.5%	0.3%
		Faecalibacterium	1.5%	1.8%	1.5%
		Oscillospira	5.8%	9.6%	7.7%
		Ruminococcus	1.7%	5.1%	3.6%
	Erysipelotrichaceae	-	1.0%	0.9%	0.6%

Phylum	Family	Genus	Arrival day	Mid-period	End
		Coprobacillus	0.4%	0.9%	0.5%
		cc_115	0.0%	0.8%	0.6%
Proteobacteria	*Enterobacteriaceae*	-	32.6%	1.2%	0.9%
Tenericutes	-	-	0.2%	0.4%	0.7%

Finally, in order to assess differences in microbiota between breeds, we analysed the beta diversity based on unweighted UniFrac for these groups, after which the UniFrac distance matrix was represented through Principal Coordinate Analysis (PCA) (Additional file 1). Statistically significant differences only appeared between breeds at the end of the growing period (P-value < 0.05). Moreover, the comparisons of beta diversity and genera presence between both breeds in the different sampling times are represented in Fig. 4, and genera data details are summarised in Additional file 2.

Discussion

The present study assessed the development of gut microbiota composition in fast and slow-growing breeds throughout the growing period under commercial farm conditions. To our best knowledge, this is the first study in the scientific literature to evaluate the relationship between both breeds on commensal bacteria evolution in caeca under the same production conditions.

As described previously, microbiota plays an important role in animal health. Moreover, its alteration (dysbacteriosis) is associated with reduced physiological functions, which represents its importance as biomarker [36–38]. It is well demonstrated that a greater complexity of the gut microbiota is observed as animals grow. However, in accordance with other studies, our findings showed that the bacterial richness became relatively stable at mid-period for both breeds under the same production conditions [19, 39–45]. This fact evidences the importance of flock management during the production cycle in terms of the microbiota status, as the transmission between environmental and intestinal bacteria is proven [2, 7, 9, 19, 42, 46–48]. It is important to highlight that bacterial diversity in the intestinal tract is higher in birds with high feed efficiency [2, 9, 49–51]. Nevertheless, in this study there were only statistically significant differences between breeds at slaughter day, probably due to the age difference of the experimental groups, but not to the breed, intestinal health or feed conversion index. Furthermore, a low diversity and evenness of the microbiota could be also a health status indicator, as it is constantly associated with poor intestinal health and, therefore, with lower performance parameters [18, 52, 53].

Regarding gut microbiota composition, the predominant phyla obtained in this study were *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria* [7, 9, 42, 45, 47, 54]. Thus, any alteration in the microbiota balance could lead to an alteration on the health or productivity of the breed. *Firmicutes* constitutes a heterogeneous phylum containing bacterial groups with different metabolic activities, and several studies have shown that a high level of this phylum is correlated with good intestinal health [18, 55]. Conversely, an increment of *Proteobacteria* is associated with dysbiosis and, consequently, with an increase in the

presence of zoonotic bacteria belonging to this phylum, such as *Salmonella* or *Campylobacter*. For this reason, it is important to ensure strict biosecurity and management control at the beginning of the growing period, when *Proteobacteria* presents its higher levels, as any stress that could produce an increment in this phylum may result in higher shedding of pathogenic bacteria and environmental contamination throughout rearing [2, 9, 18, 56, 57]. Finally, the *Bacteroidetes* phylum plays an important role in converting fermentable starch to simple sugars and these, in turn, to volatile fatty acids to meet the energy demand of the host, so their presence could be especially affected by diet components [40, 54]. It is important that any antibiotic alternative introduced in farms, such as feed additives or management practices, should not disturb microbiota balance, particularly for phyla related to pathogenic bacteria.

At genus level, the most predominant genera were *Ruminococcus* spp., *Lactobacillus* spp. and *Bacteroides* spp., in line with data reported by other authors [14, 45, 47]. In this experiment, statistically significant differences between breeds were found only for *Lactobacillus* spp. at the end of the growing period, being higher in fast-growing animals. This is an important probiotic in promoting healthy gut, as these bacteria are believed to be responsible for starch decomposition and lactate fermentation [6, 14, 42]. *Ruminococcus* spp. is known for its ability to degrade complex carbohydrates and thus may have contributed to an improved degradation of dietary fibre [58, 59]. In turn, *Bacteroidetes* spp. plays an important role in breaking down complex molecules to simpler compounds which are also essential for growth of the host and gut microbiota development. These functions are associated with higher production rates, so it might be said that high levels of these genera are indicators of adequate intestinal health in poultry [5, 6, 14, 42]. Thus, it is important to maintain these genera at higher levels throughout the growing period.

Conclusions

In conclusion, despite the demands from society for slow-growing breeds in a welfare-friendly production management and the fact that there are many possibilities of microbiota composition due to the variations between different farms and flocks, the results revealed that the main caecal bacteria in both breeds are the same, so the benefits derived thereof are also similar. For this reason, it is important to consider some important phylum or genera levels as biomarkers of gut health, controlling their development throughout the growing period to be able to evaluate the different treatments applied to animals.

Methods

In this experiment, all animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 [60].

Experiment design

The study was performed in an experimental poultry house in the Centre for Animal Research and Technology (CITA, in its Spanish acronym (Valencian Institute for Agrarian Research, IVIA, Segorbe, Spain)). To this end, 576 broilers (males and females) provided from the same hatchery were randomly housed in two identical poultry rooms (replicates A and B) and 288 animals were housed in each room (144 fast-growing and 144 slow-growing breed). In addition, animals were distributed in 24 pens (12 pens for each breed) of 1.3 m^2 in a final stocking density of 35 kg/m^2, with wood shavings as bedding material. The two commercial breeds used were one fast-growing (Ross®) and one slow-growing (Hubbard®). The fast-growing breed is characterised by efficient feed conversion and a good meat yield [61]. In contrast, the slow-growing breed is focused on the criteria of animal welfare and absence of antibiotics [62].

To simulate the real conditions of poultry production, the houses were supplied with programmable electric lighting, automated electric heating and forced ventilation. The environmental temperature was gradually decreased from 32ºC (arrival day) to 19ºC (slaughter day).

The birds received drinking water and were fed *ad libitum*. Nutritional and product analysis were assessed before the arrival of animals. Two different age commercial diets were offered to the animals: starter (1 day to 21 days) and grower (21 days to 42/63 days). Only one batch of feed per age (starter and grower) was manufactured. The starter diet was the same for both breeds, while the grower feed was the standard diet specific for each one. Mortality rates and diarrhoea presence were recorded daily. Finally, animals were weighed at weekly intervals and feed consumption per pen was recorded.

Sample collection

To assess the development of microbiota composition of broilers throughout the growing period, 30 animals from each experimental group were randomly selected and caecal samples were collected at different times of the growing period: on arrival (day-old chicks), at the mid-period (21 days old) and before slaughter (42 days of age in fast-growing, and 63 days in slow-growing). Caecal samples were taken individually and placed in sterile jars. The samples were processed immediately after collection.

DNA extraction

Caecal content was removed and homogenised. Afterwards, pools of six animals of each breed from each room were prepared (5 pools/experimental group) and the DNA of pools content was extracted according to the manufacturer's instructions (QIAamp Power Fecal DNA kit, Qiagen, Hilden, Germany) and frozen at -80ºC for shipment to the Centre for Biomedical Research of La Rioja (CIBIR, in its Spanish acronym, Logroño, Spain).

16S rRNA sequencing analysis

First, all samples received were analysed in a Fragment Analyzer (Genomic DNA 50Kb kit, AATI) to ensure their integrity. Additionally, the initial DNA concentration was measured by means of a Qubit fluorometer (dsDNA HS Assay kit, Invitrogen). From 12.5 ng of DNA (evaluated in Qubit) of each sample, the library was prepared following the instructions of the 16S rRNA Metagenomic Sequencing Library Preparation

(Illumina) protocol. The sequencing run was performed in a MiSeq (Illumina) system in 2 × 300 bp format.

The quality of the raw unprocessed reads was evaluated using the FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After removal of adapters by Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), the quality of clean reads was re-evaluated with FastQC. Then, because the fragments sequenced for each of the samples are overlapped in their central region, the V3-V4 region of the 16S rRNA gene was partially reconstructed into fragments of approximately 550–580 bp. The OTU picking and analysis was performed with Qiime (v1.9.1) pipeline [63], following the methodology "pick open reference OTUs" against the taxonomy reference base Greengenes 13.8 at 97% nucleotide identity. Finally, InteractiVenn software was used for Venn diagram construction [64].

Calculation of the alpha diversity indexes was done by Qiime (v1.9.1), which generates multiple rarefactions on the OTU table at different sequencing depths, calculates the alpha diversity indexes at each depth, and finally coheres the data, generating rarefaction graphs for each index. To Identify OTUs with differential abundance in this study, the analysis was performed using two tests: a non-parametric analysis (Kruskal-Wallis) and a parametric test (MetagenomeSeq). In both cases, starting from the standardised and filtered table of OTUs to eliminate those OTUs that may be spurious. Analysis was carried out at three taxonomic levels: Phylum, Genera and OTU.

Beta diversity was evaluated based on indices or coefficients of similarity, dissimilarity or distance between the samples from qualitative (presence/absence of OTUs) or quantitative (proportional abundance of each OTU) data. In Qiime's metagenomics protocol, beta diversity was measured through a distance or dissimilarity matrix between each pair of samples. This matrix was visualised with Principal Coordinate Analysis (PCoA) graphs in 2D and 3D, which allow analysis of the distance between each pair of samples.

Longitudinal study, focused on Phylum and Genera level was performed with the METAgenomic LONgitudinal Differential Abundance method (MetaLonDA) R-package [65, 66].

Data availability

Bioproject: PRJNA612272.

BioSample: SAMN14365530: Fast and slow-growing broiler breeds. Caecal microbiota characterisation.

Declarations

Ethics approval and consent to participate

This study was carried out using welfare principles of animal care published by Spanish Royal Decree 53/2013. All protocols were approved by the Ethical Review Panel of the Directorate-General for

Agriculture, Fisheries and Livestock from the Valencian Community by the code 2018/VSC/PEA/0067.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analysed during the current study are available in the NCBI repository, [https://www.ncbi.nlm.nih.gov/biosample/SAMN14365530/].

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by the National Institute for Agricultural Research and Experimentation and the Ministry of Economy, Industry and Competitiveness (RTA 2017-00013, Programme: MINECO, Ministerio de Economía y Competitividad) and University CEU-UCH (INDI 19/20).

Authors' contributions

LMD contributed to the acquisition of data, data analysis and manuscript writing. AV and CM contributed to the conception and design of the study. MT contributed to the data analysis. MTPG and SV contributed to the acquisition of data. All authors revised the paper critically and approved the final version of the manuscript.

Acknowledgements

The authors wish to thank the “Improvement of Production System-related Food Safety and End Products” research group (Veterinary Faculty, University CEU-Cardenal Herrera) for their technical support. English text version was revised by N. Macowan English Language Service.

References

1. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, Lee MD, Collett SR, Johnson TJ, Cox NA. The chicken gastrointestinal microbiome. FEMS Microbiol Lett. 2014;360:100–12.
2. Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl Microbiol Biotechnol. 2014;98:4301–10.
3. Pourabedin M, Zhao X. Prebiotics and gut microbiota in chickens. FEMS Microbiol Lett. 2015;362:fnv122.
4. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016;14:e1002533.

5. Banerjee S, Sar A, Misra A, Pal S, Chakraborty A, Dam B. Increased productivity in poultry birds by sub-lethal dose of antibiotics is arbitrated by selective enrichment of gut microbiota, particularly short-chain fatty acid producers. Microbiology. 2018;164:142–53.
6. Clavijo V, Flórez MJV. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult Sci. 2018;97:1006–21.
7. 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. Microbial diversity and community composition of caecal microbiota in commercial and indigenous Indian chickens determined using 16 s rDNA amplicon sequencing. Microbiome. 2018;6:115.
8. Shang Y, Kumar S, Oakley B, Kim WK. Chicken Gut Microbiota: Importance and Detection Technology. Front Vet Sci. 2018;5:254.
9. Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota. Gut Health and Chicken Productivity: What Is the Connection? Microorganisms. 2019; 7.
10. Brisbin JT, Gong J, Sharif S. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Anim Health Res Rev. 2008;9:101–10.
11. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiol Rev. 2010;90:859–904.
12. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. Cell. 2016;165:1762–75.
13. Wahlström A, Sayin SI, Marschall HU, Bäckhed F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. Cell Metab. 2016;24:41–50.
14. Hasan N, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ. 2019;7:e7502.
15. Zheng P, Zeng B, Liu M, Chen J, Pan J, Han Y, Liu Y, Cheng K, Zhou C, Wang H, Zhou X, Gui S, Perry SW, Wong ML, Licinio J, Wei H, Xie P. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. Sci Adv. 2019;5:eaau8317.
16. Wang J, Nesengani LT, Gong Y, Yang Y, Lu W. 16S rRNA gene sequencing reveals effects of photoperiod on cecal microbiota of broiler roosters. PeerJ. 2018;6:e4390.
17. Strimbu K, Tavel JA. What are Biomarkers? Curr Opin HIV AIDS November. 2010;5:463–6.
18. Ducatelle R, Goossens E, De Meyer F, Eeckhaut V, Antonissen G, Haesebrouck F, Van Immerseel F. Biomarkers for monitoring intestinal health in poultry: Present status and future perspectives. Vet Res. 2018;49:43.
19. Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA, Smidt H. Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. Front Microbiol. 2018;9:235.

20. 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. Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses. Environ Microbiol. 2017;19:4771–83.
21. WHO (World Health Organization). 2014. Antimicrobial resistance: global report on surveillance 2014. (2014). Accessed 25 Jan 2020.
22. Alós J. Resistencia bacteriana a los antibióticos: una crisis global. Enferm. Infecc. Microbiol. Clin. 2015; 33;692–699.
23. Gadde U, Kim WH, Oh ST, Lillehoj HS. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Anim Health Res Rev. 2017;18:26–45.
24. Montoro-Dasi L, Villagra A, Sevilla-Navarro S, Pérez-Gracia MT, Vega S, Marin C. In Press. The dynamic of antibiotic resistance in commensal *Escherichia coli* throughout the growing period in broiler chickens: fast-growing *vs* slow-growing breeds. Poult. Sci. 2020; 99:1591–1597.
25. Hancock RE, Nijnik A, Philpott DL. Modulating immunity as a therapy for bacterial infections. Nat Rev Microbiol. 2012;10:243–54.
26. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. Antibiotic alternatives: The substitution of antibiotics in animal husbandry? Front Microbiol. 2014;5:217.
27. Castellini C, Dal Bosco A. Poultry Quality Evaluation: Quality Attributes and Consumer Values. In: Animal Welfare and Poultry Meat in Alternative Production Systems (and Ethics of Poultry Meat Production). Technology, Elservier Ltd.: Woodhead Publishing Series in Food Science; 2017.
28. Polycarpo GV, Andretta I, Kipper M, Cruz-Polycarpo VC, Dadalt JC, Rodrigues PHM, Albuquerque R. Meta-analytic study of organic acids as an alternative performance-enhancing feed additive to antibiotics for broiler chickens. Poult Sci. 2017;96:3645–53.
29. Suresh G, Das RK, Kaur Brar S, Rouissi T, Avalos Ramirez A, Chorfi Y, Godbout S. Critical Reviews in Microbiology Alternatives to antibiotics in poultry feed: molecular perspectives. Crit Rev Microbiol. 2018;44:318–35.
30. Alagawany M, Abd El-Hack ME, Farag MR, Sachan S, Karthik K, Dhama K. The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. Environ Sci Pollut Res Int. 2018;25:10611–8.
31. Sevilla-Navarro S, Marín C, Cortés V, García C, Vega S, Catalá-Gregori P. Autophage as a control measure for *Salmonella* in laying hens. Poult Sci. 2018;97:4367–73.
32. Kogut MH, Yin X, Yuan J, Bloom L. Gut health in poultry. CAB Reviews. 2017;12:031.
33. Sirri F, Castellini C, Bianchi M, Petracci M, Merluzzi A, Franchini A. Effect of fast, medium- and slow-growing strains on meat quality of chickens reared under the organic farming method. Animal. 2011;5:312–9.
34. Lusk JL. Consumer preferences for and beliefs about slow growth chicken. Poult Sci. 2018;97:4159–66.

35. Mancabelli L, Ferrario C, Milani C, Mangifesta M, Turroni F, Duranti S, Lugli GA, Viappiani A, Ossiprandi MC, van Sinderen D, Ventura M. Insights into the biodiversity of the gut microbiota of broiler chickens. Environ Microbiol. 2016;18:4727–38.
36. Teirlynck E, Gussem MD, Dewulf J, Haesebrouck F, Ducatelle R, Van Immerseel F. Morphometric evaluation of "dysbacteriosis" in broilers. Avian Pathol. 2011;40:139–44.
37. Chen J, Tellez G, Richards JD, Escobar J. Identification of Potential Biomarkers for Gut Barrier Failure in Broiler Chickens. Front Vet Sci. 2015;2:14.
38. Díaz-Sánchez S, Perrotta AR, Rockafellow I, Alm EJ, Okimoto R, Hawken R, Hanning I. Using fecal microbiota as biomarkers for predictions of performance in the selective breeding process of pedigree broiler breeders. PLoS One. 2019;14:e0216080.
39. Van der Wielen PW, Keuzenkamp DA, Lipman LJ, Van Knapen F, Biesterveld S. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. Microb Ecol. 2002;44:286–93.
40. Lu J, Idris U, Harmon B, Hofacre C, Maurer JJ, Lee MD. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl Environ Microbiol. 2003;69:6816–24.
41. Amit-Romach E, Sklan D, Uni Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poult Sci. 2004;83:1093–8.
42. Mohd Shaufi MA, Sieo CC, Chong CW, Gan HM, Ho YW. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut Pathog. 2015;7:4.
43. Shang Y, Kumar S, Oakley B, Kim WK. Chicken Gut Microbiota: Importance and Detection Technology. Front Vet Sci. 2018;5:254.
44. Kollarcikova M, Kubasova T, Karasova D, Crhanova M, Cejkova D, Sisak F, Rychlik I. Use of 16S rRNA gene sequencing for prediction of new opportunistic pathogens in chicken ileal and cecal microbiota. Poult Sci. 2019;98:2347–53.
45. Xi Y, Shuling N, Kunyuan T, Qiuyang Z, Hewen D, ChenCheng G, Tianhe Y, Liancheng L, Xin F. Characteristics of the intestinal flora of specific pathogen free chickens with age. Microb Pathog. 2019;132:325–34.
46. Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, Joens LA, Konkel ME, Angly F, Dinsdale EA, Edwards RA, Nelson KE, White BA. Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. PLoS One. 2008;3:e2945.
47. Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. Poult Sci. 2013;92:671–83.
48. Mohammadi Ghasem Abadi MH, Moravej H, Shivazad M, Karimi Torshizi MA, Kim WK. Effects of feed form and particle size, and pellet binder on performance, digestive tract parameters, intestinal morphology, and cecal microflora populations in broilers. Poult Sci. 2019;98:1432–40.
49. Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R, Percy NJ, Ophel-Keller K. Identification and Characterization of Potential Performance-Related Gut Microbiotas in Broiler Chickens across Various Feeding Trials. Appl Environ Microbiol. 2011;77:5868–78.

50. Singh KM, Shah T, Deshpande S, Jakhesara SJ, Koringa PG, Rank DN, Joshi CG. High through put 16S rRNA gene-based pyrosequencing analysis of the fecal microbiota of high FCR and low FCR broiler growers. Mol Biol Rep. 2012;39:10595–602.
51. Stanley D, Denman SE, Hughes RJ, Geier MS, Crowley TM, Chen H, Haring VR, Moore RJ. Intestinal microbiota associated with differential feed conversion efficiency in chickens. Appl Microbiol Biotechnol. 2012;96:1361–9.
52. Maki JJ, Klima CL, Sylte MJ, Looft T. The Microbial Pecking Order: Utilization of Intestinal Microbiota for Poultry Health. Microorganisms. 2019;7(10).
53. Ocejo M, Oporto B, Hurtado A. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. Sci Rep. 2019;9:2506.
54. Kumar S, Chen C, Indugu N, Werlang GO, Singh M, Kim WK, Thippareddi H. Effect of antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance and prevalence of foodborne pathogens. PLoS One. 2018;13:e0192450.
55. Yacoubi N, Saulnier L, Bonnin E, Devillard E, Eeckhaut V, Rhayat L, Ducatelle R, Van Immerseel F. Short-chain arabinoxylans prepared from enzymatically treated wheat grain exert prebiotic effects during the broiler starter period. Poult Sci. 2018;97:412–24.
56. Neal-McKinney JM, Lu X, Duong T, Larson CL, Call DR, Shah DH, Konkel ME. Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. PLoS One. 2012;7:e43928.
57. Shin NR, Whon TW, Bae JW. *Proteobacteria*: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 2015;33:496–503.
58. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes. 2012;3:289–306.
59. Siegerstetter SC, Schmitz-Esser S, Magowan E, Wetzels SU, Zebeli Q, Lawlor PG, O'Connell NE, Metzler-Zebeli BU. Intestinal microbiota profiles associated with low and high residual feed intake in chickens across two geographical locations. PLoS One. 2017;12:e0187766.
60. Spain. Royal Degree 53/2013, 1st of February, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. Boletín Oficial del Estado, 8th of Febrary of 2013, num. 34, pp. 11370 to 11421.
61. Ross. Ross 308/Ross 308 FF broiler: Performance objectives. (2019). Accessed 22 Jan 2020.
62. Valls M, Reproductoras. & pollos crecimiento lento. / (2017). Accessed 22 Jan 2020.
63. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Gonzalez Pena A, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.

64. Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinformatics. 2015;16:169.
65. Metwally AA, Finn PW, Dai Y, Perkins DL. Detection of Differential Abundance Intervals in Longitudinal Metagenomic Data Using Negative Binomial Smoothing Spline ANOVA. BCB '17: 8th ACM International Conference on Bioinformatics, Computational Biology, and Health Informatics. Boston, Massachusetts, USA. August 2017.
66. Metwally AA, Yang J, Ascoli C, Dai Y, Finn PW, Perkins DL. MetaLonDA: a flexible R package for identifying time intervals of differentially abundant features in metagenomic longitudinal studies. Microbiome. 2018;6:32.

Additional Files

Additional file 1. A: Beta diversity represented by PCA graphic for both breeds at all sampling times. B: Beta diversity represented by Heatmap for both breeds at all sampling times.

Additional file 2. Different taxonomic profiles at genus level according to time of growing period in fast (FG) and slow-growing (SG) breeds.

Figures

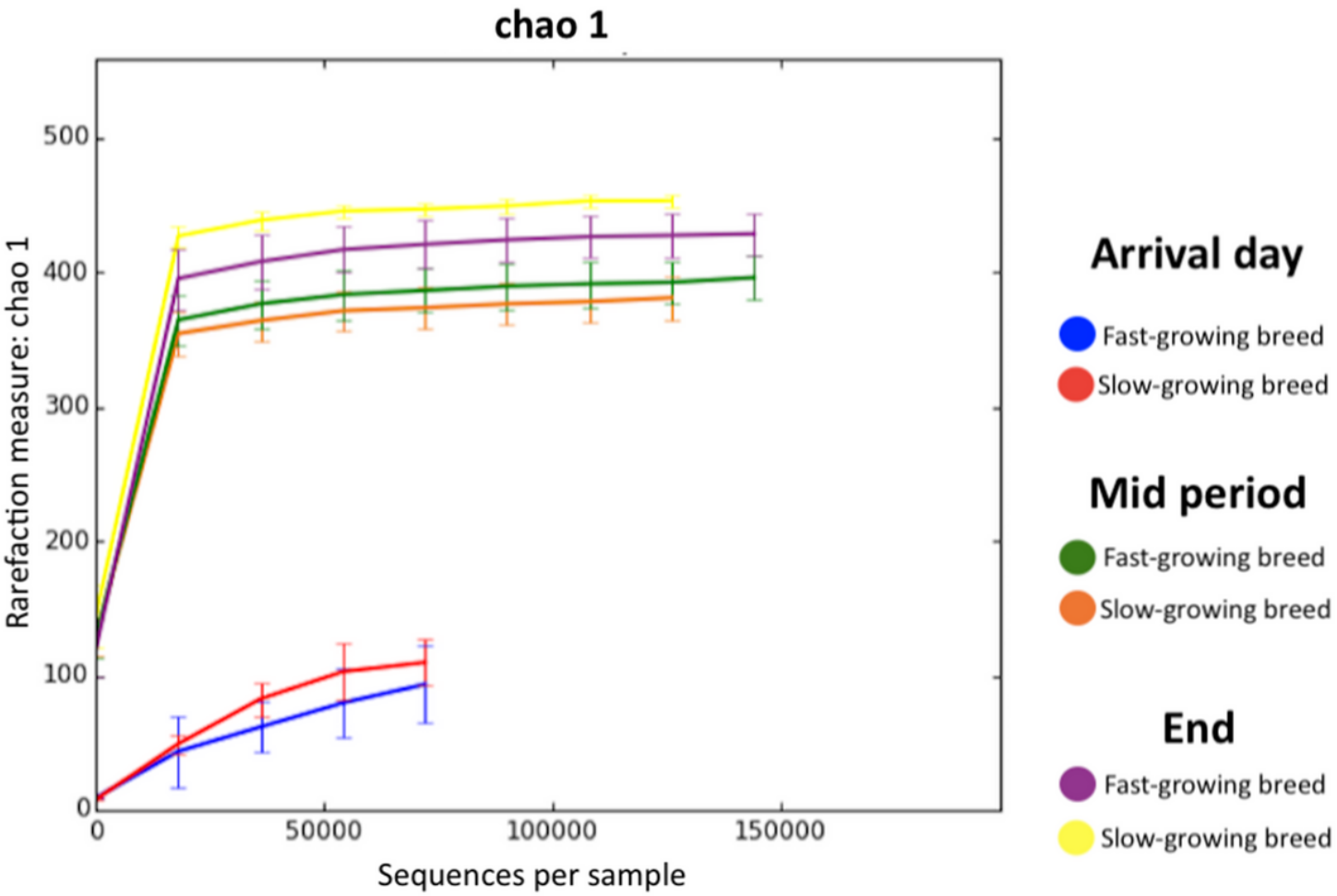


Figure 1

Evaluation of alpha diversity in fast and slow-growing breeds.

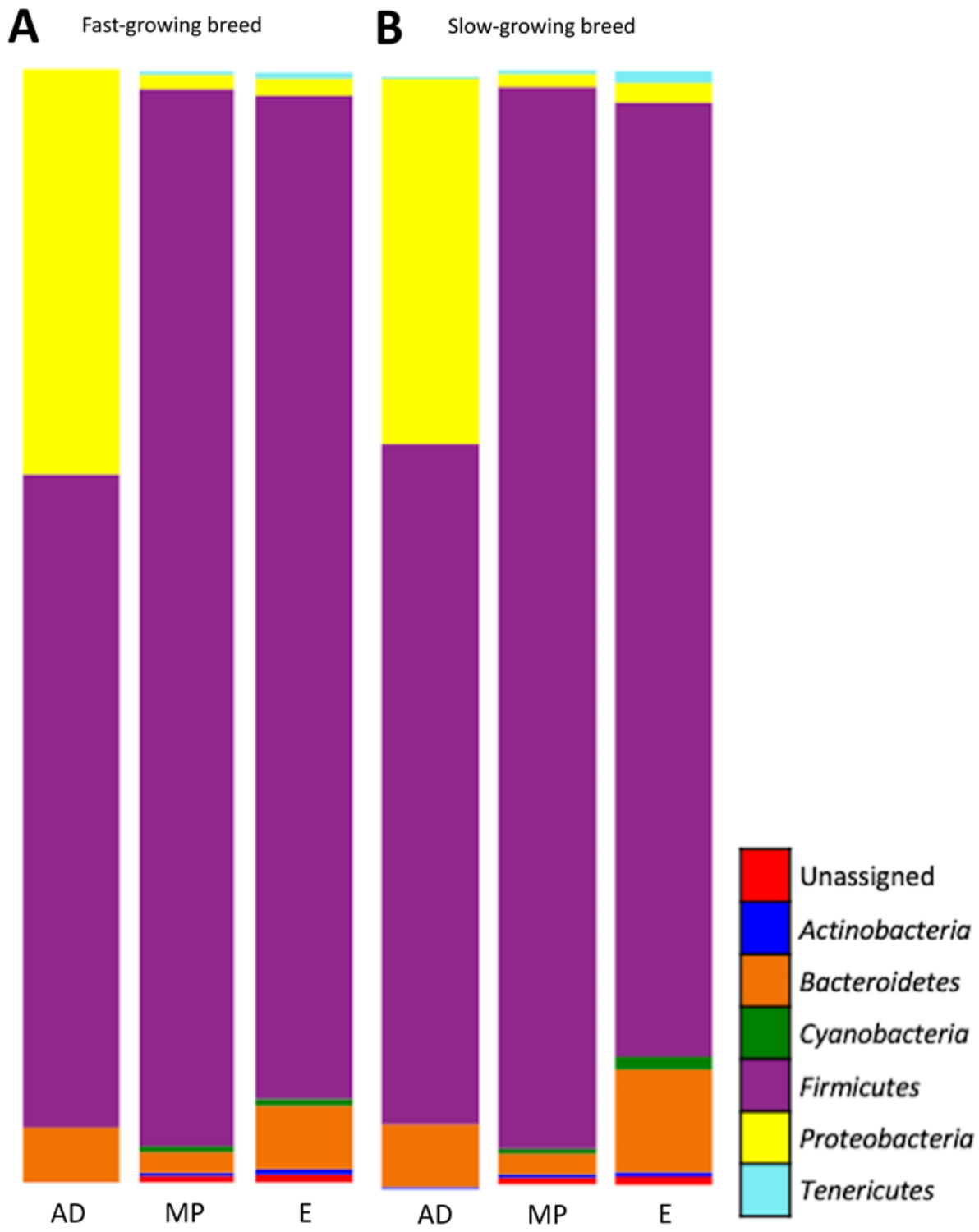


Figure 2

Taxonomic analysis at phylum level throughout the growing period. A: Phyla evolution throughout the growing period for the fast-growing breed (AD: arrival day, MP: mid-period, E: end). B: Phyla evolution throughout the growing period for the slow-growing breed (AD: arrival day, MP: mid-period, E: end).

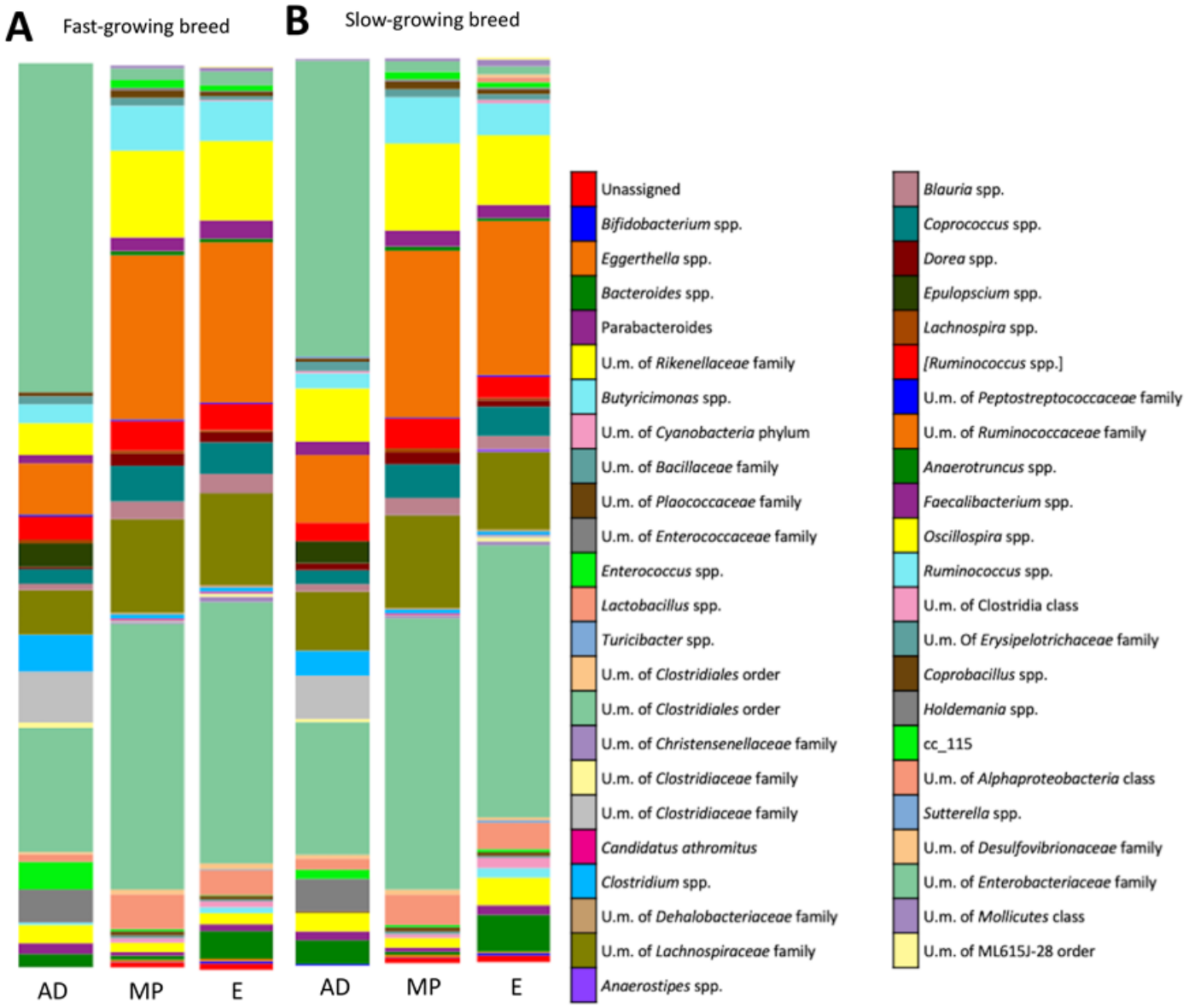


Figure 3

Taxonomic analysis at genus level throughout the growing period. A: Evolution of genera throughout the growing period for fast-growing breed (AD: arrival day, MP: mid-period, E: end). B: Evolution of genera throughout the growing period for slow-growing breed (AD: arrival day, MP: mid-period, E: end).

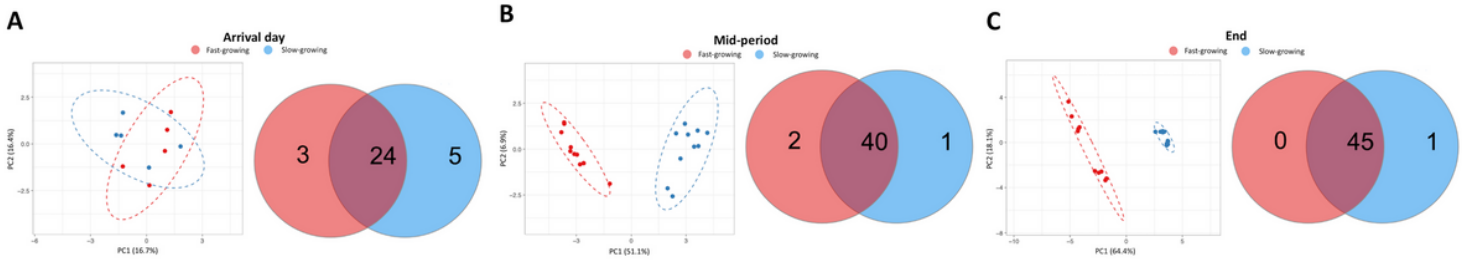


Figure 4

Evaluation of the beta diversity and comparison of genera presence in fast and slow-growing breeds. A: Beta diversity represented by PCA graphic and similar vs. different genera for both breeds at arrival day. B: Beta diversity represented by PCA graphic and similar vs. different genera for both breeds at mid-period. C: Beta diversity represented by PCA graphic and similar vs. different genera for both breeds at the end of the growing period.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile2.docx
- Additionalfile1A.tiff
- Additionalfile1B.tiff
- Additionalfile1keys.tiff