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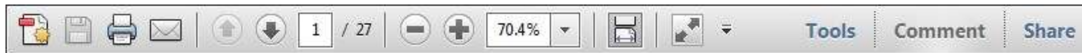

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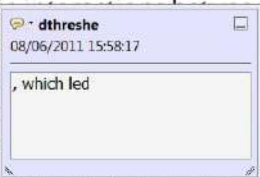

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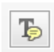

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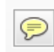

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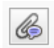

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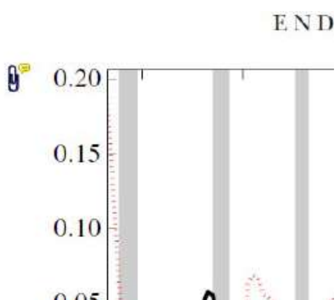

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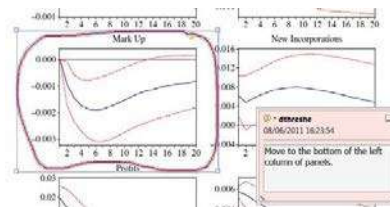

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Insights into the biodiversity of the gut microbiota of broiler chickens

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Summary

The gastrointestinal tract of poultry is densely populated with microorganisms, which are presumed to interact with the host and ingested feed. Comparison of the gut microbiota of chickens used for large-scale commercial production (Broiler Chicken, BC) and those grown in semi-wild conditions (Free-Range Chicken, FRC) revealed that at phylum level *Firmicutes* was the dominant phylum of the gut community in BC, while the gut microbiota of FRC contained higher levels of *Bacteroidetes* and *Proteobacteria*. Such differences may be due to the diet and/or the intensive use of antibiotics in BC. Indeed, analysis of the resistome of the cecal microbiomes showed a marked richness in BC datasets, with a modulation of the cecal microbiota toward antibiotic resistant bacteria. Functional characterization of the microbiome of FRC samples revealed an increase in gene pathways involved in degradation of complex carbohydrates. Furthermore, *in silico* analyses of the microbiomes of FRC and BC revealed a higher presence in genes involved in formate production in BC samples. Notably, compared to the BC microbiomes the FRC microbiomes were shown to contain a higher abundance of genes involved in the pathway for acetate production.

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Introduction

The bacterial taxa that constitute the large and complex microbial population resident in the gastrointestinal (GI) tract of chickens are believed to benefit the host by adding metabolic potential (Gerritsen et al., 2011), influencing host nutrition, gut development and physiology (Kau et al., 2011).

Chickens are considered to represent an efficient agricultural species in converting feed to lean meat, although their feed is often of low digestibility and their intestines are smaller, with shorter transit digestion times compared to those of mammals (Choct, 2009; McWhorter et al., 2009). The gut microbiota of a (healthy) chicken is presumed to play an important role in nutrient assimilation, vitamin and amino acid production and prevention of pathogen colonization (Apajalahti, 2005). The chicken gut microbiota may also act as a source of bacterial pathogens which can spread to human beings, or act as a reservoir of antibiotic-resistance determinants, which can be transferred to other microorganisms including opportunistic pathogens (Zhou et al., 2012). In poultry breeds, antibiotics are widely used to improve growth performance, while they are also employed as a prophylactic therapy so as to prevent disease development and transmission (Allen and Stanton, 2014). However, antibiotic therapy is known to cause substantial compositional alterations in microbial consortia, thus catalysing dysbiosis with a consequent detrimental impact on physiology and metabolic performance of the host that may ultimately result in the development of gut disorders (Allen and Stanton, 2014).

The most complex microbial community within the chicken gut is the one resident in the cecum, which has been shown to be dominated by the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (Sergeant et al., 2014). In this context, the cecal microbiota of a chicken is implicated in nitrogen recycling from uric acid, producing essential amino acids and digestion of non-starch polysaccharides (NSPs) (Jozefiak et al., 2004).

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Fermentation of NSPs leads to the production of short chain fatty acids (SCFAs) that are adsorbed and catabolized by the host, contributing substantially to animal nutrition and to inhibition of acid-sensitive pathogens (Turnbaugh et al., 2006). Previously, Stanley and colleagues (2013) reported the correlation between poultry cecal microbiota and the efficiency of energy extraction from feed. A number of bacterial phylotypes were identified that significantly differed in abundance between birds with high and low apparent metabolizable energy extraction ability.

Here, we performed an observational study directed to investigate the microbiota differences of 84 cecal samples collected from Broiler Chicken (BC) and Free-Range Chicken (FRC) animals. The aim of this study was to investigate compositional differences of the cecal microbiota of poultry kept under different housing regimes (either reared in a space-limiting, high-throughput production environment, or kept under semi-natural, roaming conditions, with or without antibiotic supplementation) and nutritional circumstances.

Results and discussion

16S rRNA profiling of FRC and BC

Cecal samples from 84 chickens were obtained in order to assess the microbiota composition of FRC and BC (respectively 35 and 49 samples) based on 16S rRNA-sequencing analysis as described previously (Milani et al., 2013). MiSeq-mediated sequencing of 84 samples produced a total of 6 335 983 sequencing reads with an average of 75 428 reads per sample (Table S1). Quality and chimera filtering produced a total of 5 508 392 filtered reads with an average of 65 576 filtered reads per sample, and ranging from 8230 to 189 725 reads (Table S1).

Assessment of rarefaction curves based on the Shannon and Chao1 biodiversity indexes calculated for 10 subsampling of sequenced read pools indicated that both curves tend to reach a plateau. Therefore, in all cases the obtained sequencing data was deemed adequate to cover the vast majority of biodiversity contained within the samples (Fig. 1A and B). Moreover, average rarefaction curves

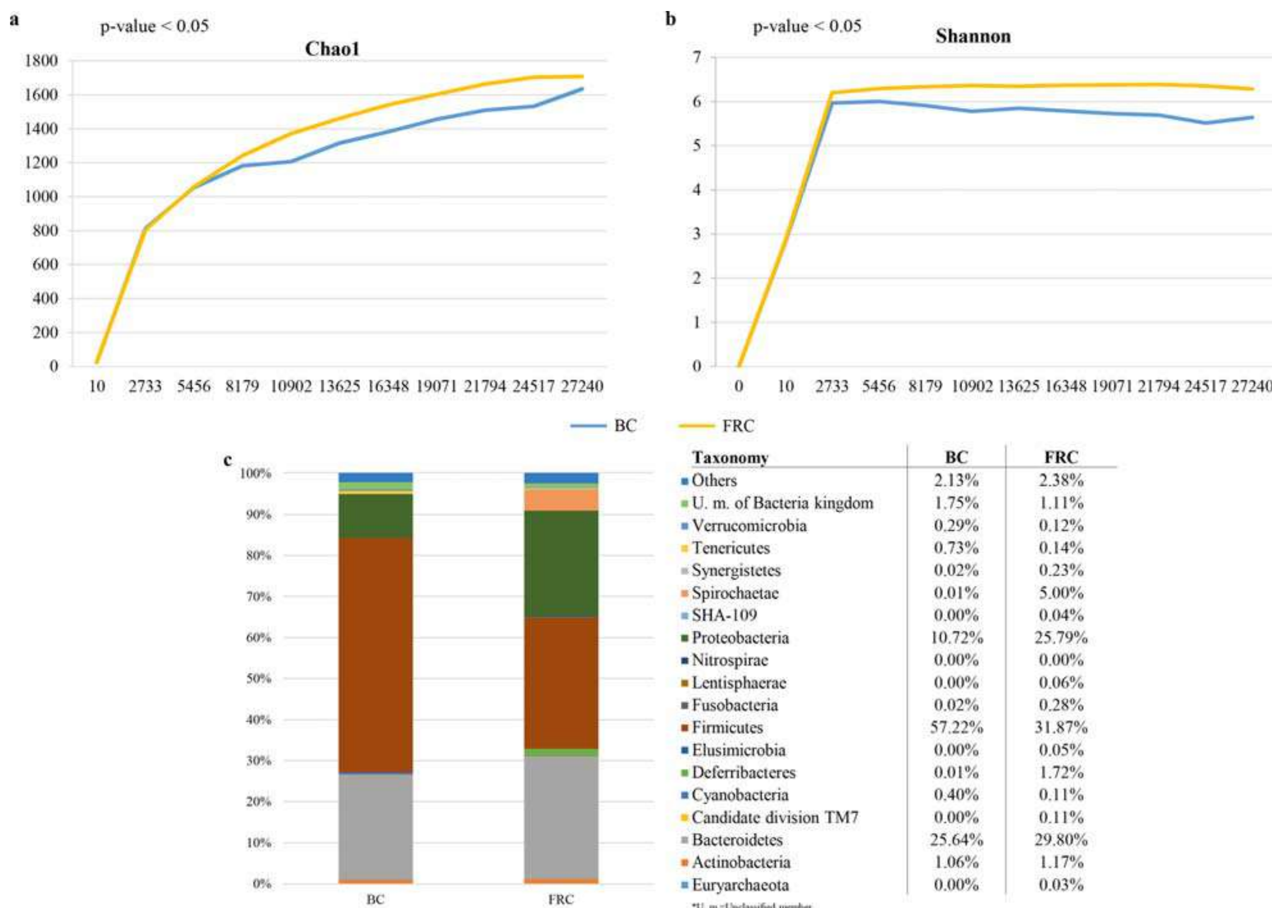

Fig. 1. Evaluation of alpha-diversity in BC and FRC samples. Panel A shows the average rarefaction curve representing variation of the Chao1 diversity index at increasing sequencing depth of BC and FRC samples. Panel B displays the average rarefaction curve representing variation of the Shannon diversity index at increasing sequencing depth of BC and FRC samples. Panel C represents a bar plot of the identified bacterial phyla in the 84 analysed samples. The legend reports the average of relative abundance of each phyla in both animal groups.

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reveal a notable difference between the gut microbiota of FRC and BC poultry (Fig. 1). Specifically, cecal samples from FRC displayed a higher level of complexity of the gut microbiota compared to that found in BC samples (Fig. 1). The two curves are significantly different based on Student's t-test statistical analysis (t-value = 0.031, dfs = 68) calculated at the highest rarefaction depths reached by all the samples (Fig. 1).

Differential Gut microbiota composition

Inspection of predicted taxonomic profiles at phylum level for all samples exhibited that *Firmicutes* (58.90%) represented the dominant phylum of the cecal community in BC chickens, outnumbering the *Bacteroidetes* (25.70%) and *Proteobacteria* (10.73%) phyla. Differences in the gut microbiota composition were detected in FRC compared to BC with a comparative reduction of *Firmicutes* (31.86%) and a concomitant increase of *Bacteroidetes* and *Proteobacteria*, represented by 30.08% and 25.82% respectively (Fig. 1C). Studies in mice, pigs and humans have suggested that the *Firmicutes* to *Bacteroidetes* (F/B) ratio based on 16S rRNA gene sequence is often correlated with weight (Singh et al., 2013). The identified F/B ratio is 3.87 (P-value < 0.01) and 1.25 (P-value < 0.01) for BC and FRC groups (Fig. 1D) respectively. However, it is not possible to correlate performances and F/B ratio since no data about animal weight are available for the samples used in this study. Interestingly, antibiotic treatment has been shown to alter gut and fecal bacterial species composition in chicken (Gong et al., 2008) towards an increased abundance of *Lactobacillus* spp., Clostridiales and Enterobacteriaceae. Similar results were reported by Singh and colleagues (2013), who showed that an increase in the F/B ratio correlated with antibiotic treatment and increase of body weight, which may in turn be related to modulation of nutrient absorption by the host.

Furthermore, at a genus level, we identified 252 taxa of which 217 appear to be present in all samples, while six and 29 appeared to be uniquely present in cecal samples of BC and FRC respectively (Fig. 2A). Notably, the presence or absence of these genera may be the

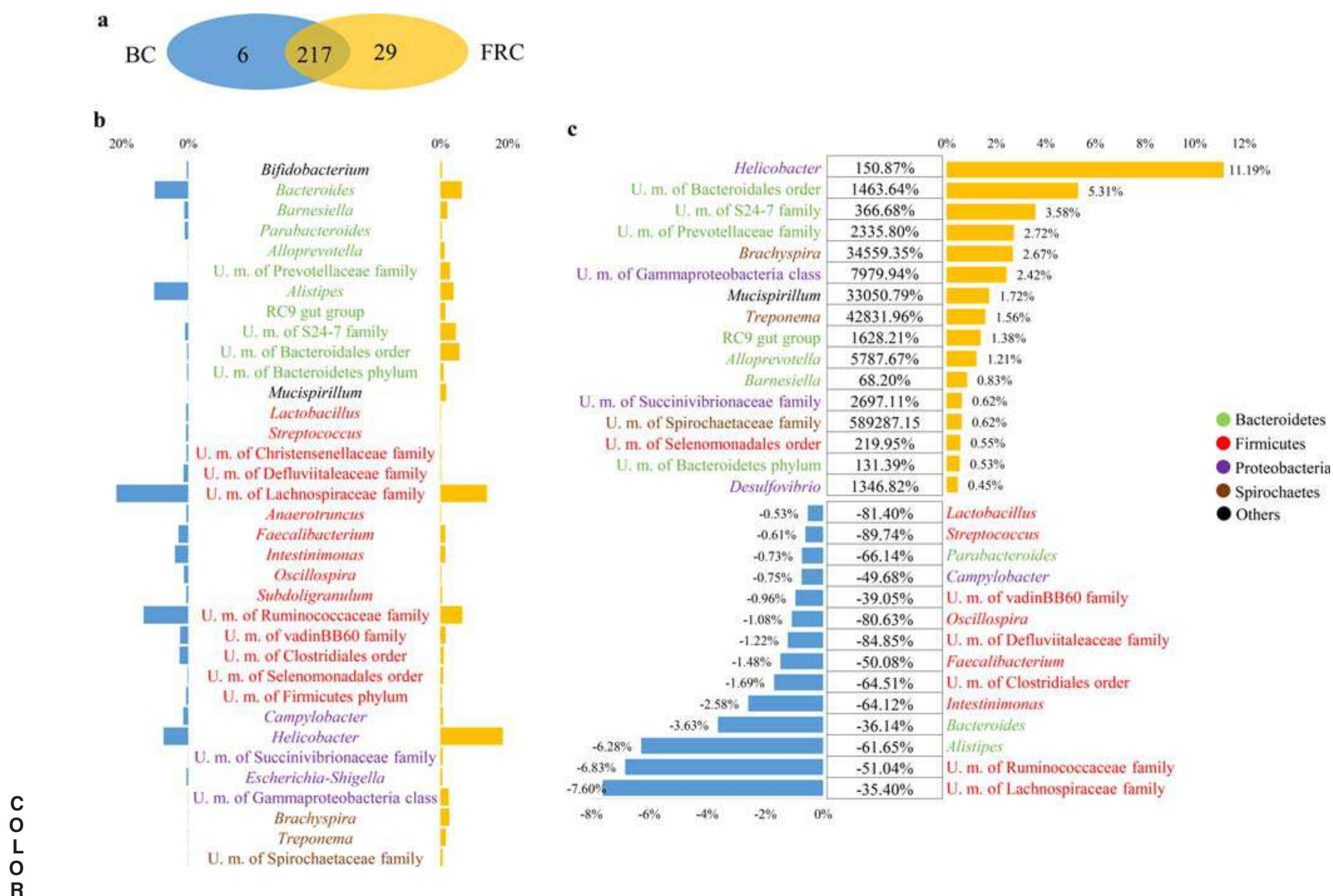

Fig. 2. Exploration of the diversity in BC and FRC groups. Panel A depicts a Venn diagram illustrating the total, unique and shared number of OTUs predicted for BC and FRC datasets. Panel B reports the core set of genera present at an average relative abundance of > 0.5% in at least one sample group. Panel c shows the variation in terms of relative abundance > 0.4% and showing increase > 50% or decrease < −30% in FRC data sets as compared to those obtained from BC samples.

COLOR

consequence of how the chickens are reared, i.e. being allowed to freely roam or being kept in a strictly confined environment, which is likely to impact on the physiology of the animals. In order to further identify differences in microbiota composition between BC and FRC groups, we focused on 97 genera, which were shown to be present at an average relative abundance of $> 0.5\%$ in at least one sample group (Fig. S1). The core set of genera obtained (Fig. 2B) and the comparison of the relative abundance average of BC and FRC (Fig. 2C) revealed a predominant presence of members of the *Firmicutes* phylum in BC samples, such as unclassified members (U. m.) of the Lachnospiraceae family, U. m. of the Ruminococcaceae family and *Intestinimonas* spp. In contrast, FRC-derived samples were shown to contain a higher diversity (compared to data obtained from BC samples) at the genus level among the *Bacteroidetes*, *Proteobacteria* and *Spirochaetes* phyla.

Notably, bacterial taxa belonging to both *Firmicutes* and *Bacteroidetes* phyla are known to be involved in the breakdown of otherwise indigestible (by the host) polysaccharides such as resistant starch and cellulose (Allen and Stanton, 2014; Stanley et al., 2012). Therefore, the observed differences in microbial composition between BC- or FRC-derived cecal samples may influence the food to energy conversion capacity in chickens housed under different conditions.

In order to evaluate microbiota differences between BC and FRC samples, we analysed the beta diversity based on unweighted UniFrac for these groups, after which the UniFrac distance matrix was represented through Principal Coordinate Analysis (PCoA) (Fig. 3). Interestingly, the samples were shown to group in two different clusters based on how the chickens had been kept, indicating that a different diet and/or prophylactic therapy impact on gut microbiota composition (Fig. 3A). Moreover, environmental samples included in this analysis, collected from BC and FRC litters, clustered with the corresponding cecal samples. Thus, the environment reflected the microbiota composition of chickens, perhaps due to contamination from the stool of the animals (Fig. 3A).

Evaluation of the beta diversity of cecal samples isolated from the BC and FRC groups, each following a particular feeding type (Fig. 3B), clearly shows a clear separation between BC and FRC animals. However, the microbiota composition of animals fed with different cereals, such as wheat and corn, clustered separately (Fig. 3B).

Analysis of the influence of antibiotic treatments on BC animals, showed a heterogeneous distribution of the samples (Fig. 3C), and it seems that different antibiotic mixes have a divergent impact on microbiota composition.

Such findings about PCoA clustering were confirmed by the obtained P-value of PERMANOVA statistical analysis (being < 0.05, dfs $= 1$), when the BC- and FRC-derived food supplementation and antibiotic treatment data sets are compared.

Prediction of the cecal microbiomes of FRC and BC

In order to evaluate the overall genetic content of the cecal microbiota of chickens, we determined the metagenome of 13 animals, representing five FRC and eight BC samples. Selection of these animals was based on the 16S rRNA microbial profiling data so as to include those birds that possess cecal microbial profiles that were closest to the average of their respective groups (Table 3).

NGS sequencing of these selected 13 samples (Table 3) produced a total of 3 379 001 raw reads that were filtered for human DNA and by quality, resulting in 331 216 filtered reads that were used for further analyses. When the taxonomic distribution predicted from the 16S rRNA profiling analysis was compared with that from the metagenomics data, there was a discrepancy in the ratio of *Firmicutes* and *Bacteroidetes*, probably due to differential amplification efficiency in the 16S rRNA profiling PCR, as previously reported (Sergeant et al., 2014). Nonetheless, the F/B ratio was shown to be higher in BC as compared to that obtained for FRC samples.

Moreover, functional classification of open reading frames based on the Cluster of Orthologous Genes (COG) obtained from assembled metagenomic datasets allowed detection of significant differences in relative abundance of COG functional categories between the two datasets. COG categories including replication, recombination and repair, energy production and conversion, carbohydrate transport and metabolism, as well as amino acid transport and metabolism were shown to be the most over-represented in both datasets (Fig. 4A). Interestingly, significant differences were detected for COG categories that are known to be involved in energy harvesting from food, such as energy production and conversion, carbohydrate transport and metabolism, amino acid transport and metabolism and lipid transport and metabolism categories (P-value < 0.05) (Fig. 4A and Table S2).

Identification of the resistome of the chicken gut microbiome

As above described, antibiotic therapy is widely used in breeding chickens (Pourabedin and Zhao, 2015). This practice impacts on the composition of the gut microbiota and is expected to influence their functionality (Allen and Stanton, 2014). However, very little is known about the occurrence of genes responsible for resistance against antibiotics, i.e. the resistome, in the chicken gut microbiome. In order to map and characterize the resistome of microbial consortia residing in the ceca of FRC and BC, the collective microbiomes of each of these two groups

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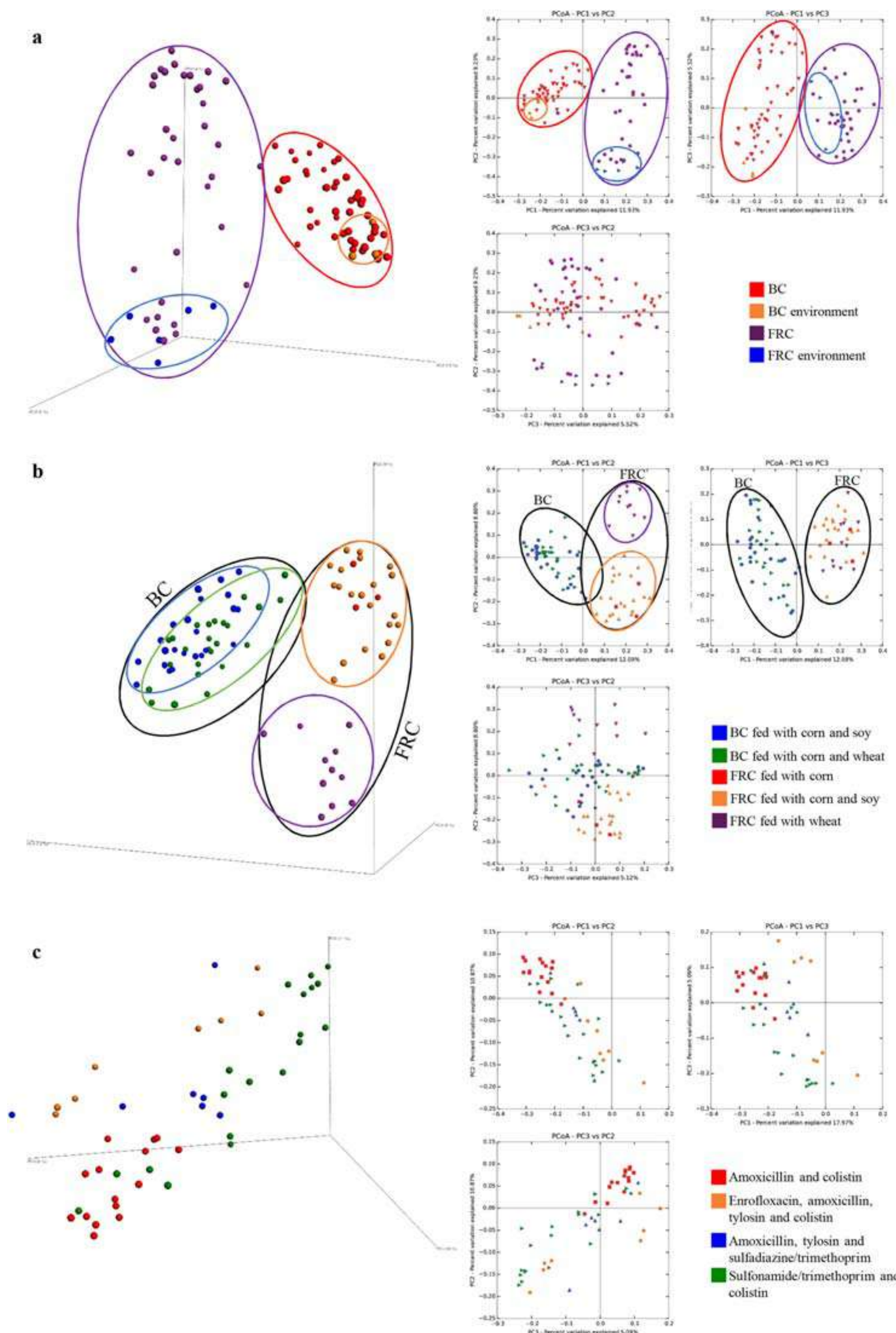

Fig. 3. Evaluation of the beta-diversity in BC and FRC samples. The predicted PCoA encompassing all 84 BC and FRC datasets is reported through two three-dimensional images as well as two-dimensional sections. Panel A shows BC, FRC and environmental datasets, and corresponding clusters are coloured in purple, red and blue/orange respectively. Panel B depicts the beta diversity of cecal samples from animals reared to different feeding types. Black circles represent BC and FRC groups, while coloured ones indicate the different cereal mix supplied. Panel c illustrates antibiotic treatments on BC animals. Antibiotic mix are reported in different colors.

were screened for known bacterial antibiotic-resistance genes (ARGs).

In silico analyses of shotgun metagenomic datasets revealed a higher abundance (16.64%) of ARGs in BC samples as compared to FRC animals (Fig. 4B). Interestingly, β-lactamase-encoding genes were shown to be the most abundant in all analyzed samples with a slight increase of 5.40% in BC samples. This might be the consequence of the fact that β-lactam-based antibiotics are the most commonly used drugs in BCs (Table 1), while β-lactamase-encoding genes are also the most commonly detected antibiotic resistance sequences found in soil and water bacteria (Allen and Stanton, 2014; Cho et al., 2014). Moreover, differences were noted in the heat map (Fig. 4B) regarding glycopeptide-based ARGs. In this context, we observed a 117.62% increase in the abundance of

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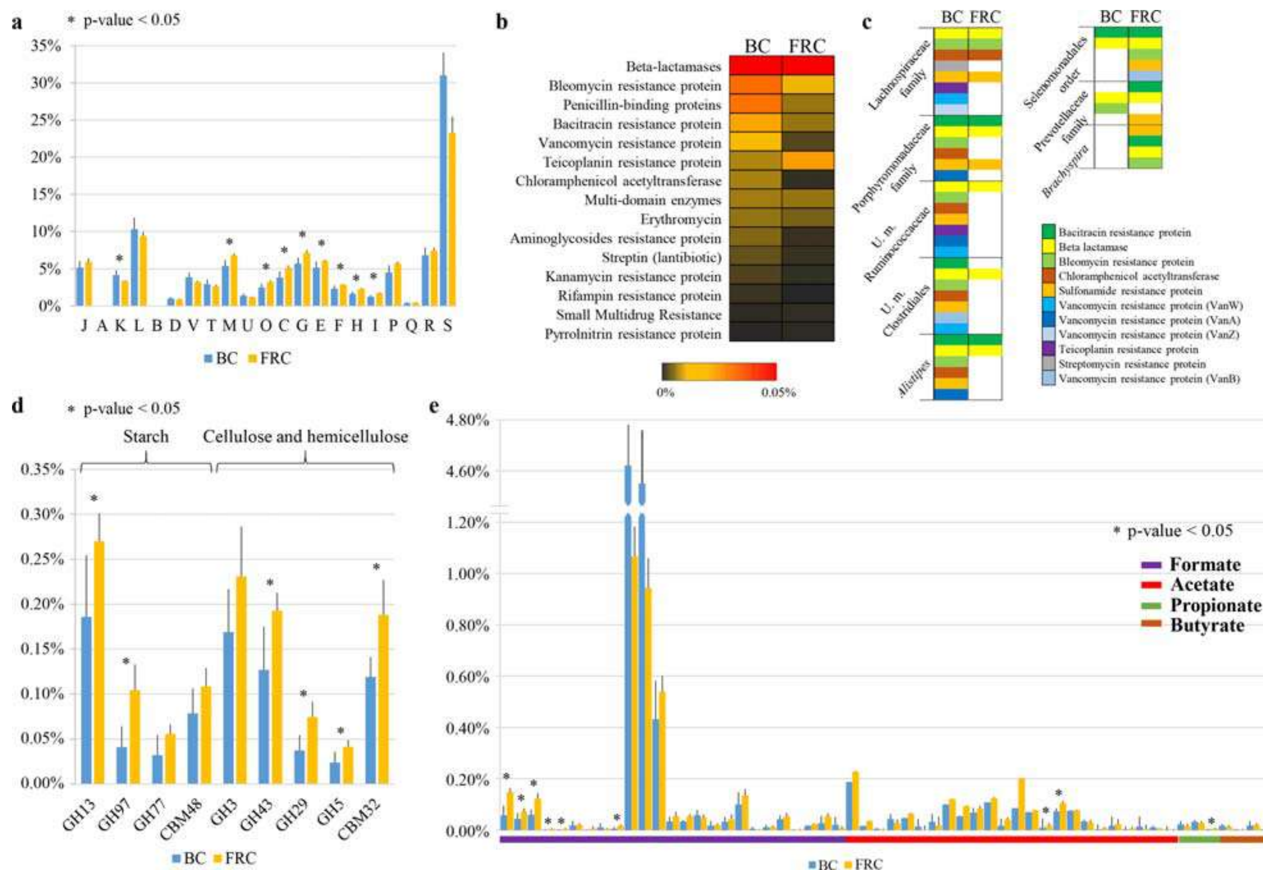

Fig. 4. Functional changes in the gut microbiome of BC and FRC. Asterisks indicate statistically significant differences between the two datasets (P-value < 0.05). Panel A depicts the functional annotation of BC and FRC metagenomic datasets according to COG categories. Each COG family is identified by a one-letter abbreviation (National Center for Biotechnology Information database). Panel B shows relative abundance of predicted enzymes involved in conveying antibiotic resistance as present in BC and FRC shotgun metagenomic datasets. Names of protein-coding genes are listed on the left, while names of sample groups used are listed at the top. Panel C exhibits ARGs in the bacterial taxa with higher variation in relative abundance in both datasets. Different colors represent various ARGs. Panel D shows changes in GH families involved in starch and plant cellulose/hemicellulose degradation in BC and FRC datasets. Panel E displays variation of pathways involved in formate, acetate, propionate and butyrate production in BC and FRC samples.

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genes encoding for vancomycin resistance in BC samples. Conversely, we observed an increase (347.24%) of genes for resistance to teicoplanin in FRC poultry with respect to BC animals. Furthermore, an increase (9.15%) of chloramphenicol acetyltransferase-encoding genes were observed in BC samples.

Taxonomic classification of shotgun metagenomic reads corresponding to ARGs allowed the identification of bacterial taxa contributing to at least 1% of the total resistome (Fig. 4C). In the BC group, we observed a prevalent presence of ARGs belonging to the *Firmicutes* phylum (40.40%). In contrast, the FRC group contains a high abundance of *Bacteroidetes* (27.32%) and *Spirochaetes* (5.03%) phyla. Focusing on bacterial taxa that exhibit a higher variation in relative ARG abundance between BC and FRC samples, we detected an increased abundance of ARGs in BC taxa compared to FRC, as well as ARGs unique to the BC group encoding chloramphenicol acetyltransferase, teicoplanin resistance protein and vancomycin resistance protein (Fig. 4C).

Thus, our findings revealed a clear selection of the microbiota members resistant to these antibiotics. Furthermore, such data confirmed the positive correlation between supplementation of antibiotics and the F/B ratio increase as reported above (Fig. 1D).

Functional characterization of the chicken cecal microbiome

As mentioned above the reconstruction of the cecal microbiomes of chickens allowed their functional classification by means of the EggNog database (Powell et al., 2014). This analysis showed that a significantly higher number of functions associated with carbohydrate transport and metabolism are present in FRC datasets (7.1%) as compared to those obtained from BC (5.7%) (P-value < 0.05).

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Table 1. Chicken samples collected in this study.

Sample name	Rearing methods	Origin	Feed	Antibiotics
P1	FRC	Parma 1 (Emilia Romagna, Italy)	Barley, wheat and wet waste	/
P2	FRC			
P3	FRC			
P4	FRC			
P5	FRC			
P6	FRC			
P7	FRC	Aulla (Toscana, Italy)	Wheat and wet waste	/
P8	FRC			
P9	FRC			
P10	BC	Cesena 1 (Emilia Romagna, Italy)	Cereals (wheat, corn), protein flour (soy, sunflower), vegetables oils (soy), mineral	Amoxicillin (20 mg Kg^{-1}) and colistin (50 mg Kg^{-1}).
P11	BC			
P12	BC			
P13	BC			
P14	BC			
P15	BC			
P16	BC			
P17	BC			
P18	BC			
P19	BC			
P20	BC	Cremona 1 (Lombardia, Italy)	Corn and soy	Amoxicillin (50 mg Kg^{-1}) and colistin (100 mg Kg^{-1}).
P21	BC			
P22	BC			
P23	BC			
P24	BC			
P25	FRC	Aulla (Toscana, Italy)	Wheat and wet waste	/
P26	FRC			
P27	BC	Cremona 2 (Lombardia, Italy)	Corn and soy	Sulfonamide/trimethoprim and colistin (100 mg Kg^{-1}).
P28	BC			
P29	BC			
P30	BC			
P31	BC			
P32	BC			
P34	BC			
P35	BC			
P36	BC			
P37	BC	Cremona 3 (Lombardia, Italy)	Corn and soy	Sulfonamide/trimethoprim and colistin (100 mg Kg^{-1}).
P38	BC			
P39	BC			
P40	BC			
P41	BC			
P42	BC			
P43	BC			
P44	BC			
P45	BC			
P46	BC			
P47	BC	Cesena 2 (Emilia Romagna, Italy)	Cereals (wheat, corn), protein flour (soy, sunflower), vegetables oils (soy), mineral	Enrofloxacin (10 mg Kg^{-1}), amoxicillin (20 mg Kg^{-1}), tylosin (50 mg Kg^{-1}) and colistin (50 mg Kg^{-1}).
P48	BC			
P49	BC			
P51	BC			
P53	BC			
P54	BC			
P55	BC			
P56	BC			
P57	BC	Cesena 3 (Emilia Romagna, Italy)	Cereals (wheat, corn), protein flour (soy, sunflower), animal fat (pig, cow, chicken oil) and minerals	Amoxicillin (20 mg Kg^{-1}), tylosin (50 mg Kg^{-1}) and sulfadiazine/trimethoprim (0.3 ml Kg^{-1}).
P59	BC			
P60	BC			
P61	BC			
P62	BC			
P63	BC			

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Table 1. *cont.*

Sample name	Rearing methods	Origin	Feed	Antibiotics
P64	BC			
P68	FRC	Varese (Lombardia, Italy)	Wheat, soy and wet waste	/
P69	FRC			
P70	FRC	Reggio Emilia (Emilia Romagna, Italy)	Corn, soy, pea, bram and minerals	/
P71	FRC			
P72	FRC			
P73	FRC			
P74	FRC			
P75	FRC			
P76	FRC			
P77	FRC			
P78	FRC			
P79	FRC			
P80	FRC			
P81	FRC			
P82	FRC			
P83	FRC			
P84	FRC			
P85	FRC			
P86	FRC			
P87	FRC			
P88	FRC			
P89	FRC	Parma 2 (Emilia Romagna, Italy)	Corn, cereal mix and wet waste	/
P90	FRC			
P91	FRC			

Rearing methods, origin of samples, feed composition and antibiotic therapies are reported.

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Moreover, we observed a difference in the COG family corresponding to energy production and conversion in FRC of 1.35% (P-value < 0.05) compared to BC datasets (Fig. 4A and Table S4). Complex polysaccharides are degraded by the gut microbiota into monosaccharides and then fermented to (mainly) produce the metabolic end products H_2, CO_2, CH_4 and SCFAs, which may then be adsorbed by the host together with any remaining digestible monosaccharides (Tremaroli and Backhed, 2012). The absorbed compounds promote hepatic triglyceride synthesis, the accumulation of triglycerides in adipocytes and subsequently, an increase in body fat (Janssen and Kersten, 2015).

In silico characterization of putative glycosyl hydrolases (GHs) responsible for degradation of complex carbohydrates, revealed that the microbiomes of FRC chickens possess a wider arsenal of GH families involved in starch, cellulose and hemicellulose degradation compared to BC samples (Fig. 4D). In particular, genes encoding predicted members of GH13, GH97 and GH77, as well as genes that specify proteins containing a CBM48 domain, are present at higher abundance (from 1.5- to 2-fold, P-value < 0.05) in the data sets from FRC animals compared to those of BC data (Fig. 4D and Table S5). GH genes encoded for α-amylases and α-glucosidases, while CBM48 is a carbohydrate-binding module known to bind various linear and cyclic α-glucans derived from starch and glycogen. Moreover, GH families GH3, GH43, GH29 and GH5, which represent α-L-arabinofuranosidase, β-D-xylopyranosidase and α-fucosidase activities (Matsuzawa et al., 2015), are more abundant in FRC datasets as compared to BC datasets (Fig. 4D). These differences can be explained by the higher abundancy of microorganisms belonging to the *Bacteroidetes* phylum in FRC chickens as compared to BC animals, where members of the former taxonomic groups are known to hydrolyze starch and plant cell wall compounds (e.g. cellulose, pectin and xylan) (Thomas et al., 2011).

Moreover, analysis of predicted bacterial metabolic pathways for SCFA production, showed a higher number of genes that are predicted to be responsible for formate production in BC compared to that found in FRC microbiomes (P-value < 0.05) (Fig. 4E, Tables S3 and S6). Formate produced in the animal gut can be degraded to CO_2 and H_2 (Sergeant et al., 2014). A high amount of hydrogen leads to reduction in fermentation and/or less energy-efficient fermentation to butyrate and propionate (Macfarlane and Macfarlane, 2003). The presence of bacteria that can metabolically act as a hydrogen sink, such as *Desulfovibrio*, *Helicobacter*, *Megamonas* and *Campylobacter* is expected to result in an increased efficiency of fermentation and SCFA production, which would ultimately exert a beneficial

effect to the host (Sergeant et al., 2014). These genera were present in the microbiota of both FRC and BC (Fig. S1).

In contrast, genes involved in acetate production were shown to be present at a significantly higher number (P-value < 0.05) in FRC microbiomes compared to BD datasets (Fig. 4E). This finding suggests a higher acetate production by the FRC microbiome, a notion that is supported by genes involved in acetate production, such as N-acetylglucosamine degradation or mycothiol biosynthesis pathways, which were shown to be significantly more abundant in FRC compared to BC (P-value < 0.05). Higher abundance of genes involved in acetate production in the microbiome of FRC animals may indirectly result in higher levels of butyrate through acetate-butyrate conversion, therefore confirming the results obtained by EggNog analysis.

In contrast, no difference in the abundance of genes predicted to be part of the metabolic pathways for butyrate and propionate synthesis was found between FRC and BC microbiomes with the exception of phosphatidylcholine resynthesis via the glycerophosphocholine pathway.

Conclusions

Improving growth performance in chickens has been one of the most important goals in poultry breeding. Recently, several studies have investigated the bacterial population that is resident in the chicken GI tract, using animals that had been reared under controlled conditions (Schokker et al., 2015; Stanley et al., 2013). The generated results suggest that the microbiota conveys benefits to the host by adding substantial metabolic potential to enhance nutrient utilization and energy conversion (Stanley et al., 2013; Waite and Taylor, 2015). Consistent with our results, these studies also found that the chicken cecal microbiota is dominated by *Firmicutes* and *Bacteroidetes*.

In this observational study, cecal samples from different slaughterhouses were collected and rearing conditions were listed to understand the microbiota composition of FRC and BC animals that had been kept at uncontrolled conditions.

The 16S rRNA gene microbial profiling data showed that the composition of the cecal microbiota of FRC is different from that of BC. In this context, *Firmicutes* dominate the BC animals, while FRC microbiota showed the predominance of bacteria belonging to the *Bacteroidetes* phylum. Furthermore, a distinct microbiota separation was detected between BC and FRC animals, apparently influenced by food supplementation and antibiotic treatment.

Analysis of the resistome of cecal microbiomes revealed that BC datasets contain higher levels of predicted ARGs compared to those of FRC, and a weak correlation was found with antibiotics used in the six different farms involved in this study (Table 1), particularly with amoxicillin. This observed increase in BC animals indicates that the use of antibiotics modulates the composition of the cecal microbiota toward antibiotic-resistant bacteria.

Functional characterization of the microbiome of FRC samples allowed the identification of key genetic features of the FRC microbiomes with respect to those of BC animals, such as an increase in the abundance of gene pathways involved in degradation of complex carbohydrates also encompassing those involved in most of the commercial chicken diets, grain. While for BC animals a higher abundance of genes involved in formate production was detected, in FRC data sets a higher number of genes was detected that are associated with acetate production, which in turn can be microbially converted to butyrate, a SCFA that can be adsorbed by the host as an energy source.

Experimental procedures

Ethic statement

In accordance with the REG CE No. 1099 of 2009 regarding the protection of animals at slaughter, this study did not require project license because no regulated procedures were carried out. Chickens were humanely killed at a designated establishment by cervical dislocation, which is an appropriate method recognized by REG CE No. 1099.

Animals and sampling

For the purpose of this study a total of 84 animals from two different poultry groups, i.e. BC (49 animals) and FRC (35 animals), were investigated (Table 1). BC were reared under commercial production conditions at six different farming centres from two geographical areas in Italy (Cesena and Cremona, Italy). These chickens were restricted in their movements due to high chicken density conditions and also they did not have access to foods other than that provided to them. All BC animals came from *Salmonella*-free breading. FRC were kept under semi-natural, free roaming conditions. This group is composed of animals from different geographical areas in the north of Italy (see Table 1). Feed composition and antibiotic treatments, i.e. name and dosage, are reported in Table 1.

All poultry GI tracts were recovered from different slaughterhouses where both BC and FRC animals were killed. Cecal samples were obtained, kept on ice and processed immediately after dissection. Briefly, each cecum was opened longitudinally. After removal of the digesta, 0.2 g of sample, composed of parts of both cecal pouches, were removed and briefly washed with RNA-later (Qiagen, Germany) to remove unattached or loosely attached bacteria from the walls. Samples were subjected to DNA extraction using the QIAamp DNA Stool Mini kit following the manufacturer's instructions (Qiagen).

Moreover, 10 environmental samples recovered from litters were included in this study. Samples were selected to represent the different rearing conditions associated with either large-scale, commercial production or the practice free-

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Table 2. Environmental samples collected in this study.

Sample name	Origin	Sample type
E4	FRC – Parma 1 (Emilia Romagna, Italy)	FRC litter
E5	FRC – Parma 1 (Emilia Romagna, Italy)	FRC litter
E7	FRC – Aulla (Toscana, Italy)	FRC litter
E11	BC – Cesena 1 (Emilia Romagna, Italy)	BC litter
E14	BC – Cesena 1 (Emilia Romagna, Italy)	BC litter
E22	BC – Cremona 1 (Lombardia, Italy)	BC litter
E23	BC – Cremona 1 (Lombardia, Italy)	BC litter
E25	FRC – Aulla (Toscana, Italy)	FRC litter
E77	FRC – Reggio Emilia (Emilia Romagna, Italy)	FRC litter
E79	FRC – Reggio Emilia (Emilia Romagna, Italy)	FRC litter

Origin of samples are reported.

T2 475 roaming (Table 2). DNA was extracted using the Power Viral 476 environmental RNA/DNA kit (Mobio, USA) following the manu- 477 facturer's instructions.

478 *16S rRNA gene amplification*

479 Partial 16S rRNA gene sequences were amplified from 480 extracted DNA using primer pair Probio_Uni and/Probio_Rev, 481 which targets the V3 region of the 16S rRNA gene sequence 482 (Milani et al., 2013). Illumina adapter overhang nucleotide 483 sequences were added to the partial 16S rRNA gene-specific 484 amplicons, which were further processed employing the 16S 485 Metagenomic Sequencing Library Preparation Protocol (Part 486 #15044223 Rev. B – Illumina; see also below). Amplifications 487 were carried out using a Verity Thermocycler (Applied Biosys- 488 tems). The integrity of the PCR amplicons were analyzed by 489 electrophoresis on a 2200 TapeStation Instrument (Agilent 490 Technologies, USA).

491 *MiSeq sequencing of 16S rRNA gene-based amplicons*

492 PCR products obtained following amplification of the 16S 493 rRNA gene sequences were purified by magnetic purification 494 step involving the Agencourt AMPure XP DNA purification 495 beads (Beckman Coulter Genomics GmbH, Bernried, Ger- 496 many) in order to remove primer dimers. DNA concentration of the amplified sequence library was determined by a fluori- 497 metric Qubit quantification system (Life Technologies). 498 AQ1 Amplicons were diluted to 4 nM and 5 μl of each diluted DNA 499 amplicons were mixed to prepare the pooled final library. 500 Sequencing was performed using an Illumina MiSeq 501 sequencer with MiSeq Reagent Kit v3 chemicals. 502

16S rRNA-microbial profiling analysis 503

The fastq files were processed using QIIME (Caporaso et al., 504 2010) as previously described (Milani et al., 2013). Paired-end 505 reads were merged and quality control retained sequences 506 with a length between 140 and 400 bp, mean sequence qual- 507 ity score >25 and with truncation of a sequence at the first 508 base if a low quality rolling 10 bp window was found. Sequen- 509 ces with mismatched forward and/or reverse primers were 510 omitted. 511

In order to calculate downstream diversity measures (alpha 512 and beta diversity indices, Unifrac analysis), 16S rRNA Opera- 513 tional Taxonomic Units (OTUs) were defined at $\geq$97% 514 sequence homology using uclust (Edgar, 2010) and OTUs 515 with less than 10 sequences were filtered. All reads were clas- 516 sified to the lowest possible taxonomic rank using QIIME 517 (Caporaso et al., 2010) and a reference dataset from the 518 SILVA database (Quast et al., 2013). Biodiversity of the sam- 519 ples (alpha-diversity) were calculated with Chao1 and 520 Shannon indexes. Similarities between samples (beta- 521

Table 3. Shotgun metagenomic data.

Sample name	Origin	Number of reads	Final read number
Poultry 1	FRC – Parma 1 (Emilia Romagna, Italy)	260147	29283
Poultry 4	FRC – Parma 1 (Emilia Romagna, Italy)	263699	22556
Poultry 5	FRC – Parma 1 (Emilia Romagna, Italy)	229129	14840
Poultry 7	FRC – Aulla (Toscana, Italy)	233002	14355
Poultry 25	FRC – Aulla (Toscana, Italy)	216209	27550
Poultry 11	BC – Cesena 1 (Emilia Romagna, Italy)	377079	74856
Poultry 12	BC – Cesena 1 (Emilia Romagna, Italy)	295779	14669
Poultry 13	BC – Cesena 1 (Emilia Romagna, Italy)	387503	75081
Poultry 14	BC – Cesena 1 (Emilia Romagna, Italy)	324878	32319
Poultry 22	BC – Cremona 1 (Lombardia, Italy)	185450	10757
Poultry 23	BC – Cremona 1 (Lombardia, Italy)	157180	2301
Poultry 28	BC – Cremona 2 (Lombardia, Italy)	269347	10527
Poultry 36	BC – Cremona 2 (Lombardia, Italy)	179599	2122

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diversity) were calculated by unweighted uniFrac (Lozupone and Knight, 2005). The range of similarities was calculated between the values 0 and 1. PCoA representations of beta-diversity were performed using QIIME (Caporaso et al., 2010).

Shotgun metagenomics

DNA was fragmented to 550–650 bp using a BioRuptor machine (Diagenode, Belgium). Samples were prepared following the TruSeq Nano DNA Sample Preparation Guide (Part#15041110Rev.D). Sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals.

Analysis of metagenomic datasets

The generated fastq files were filtered for reads with a quality score of < 25, for sequences of chicken DNA, as well as for reads < 80 bp. Bases were also removed from the end of the reads unless the average quality score in a window of 5 bp was > 25. Only paired data were further analysed. The revised gene/protein set was searched using evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG; http://eggnog.embl.de/version_4.0.beta/) databases. Interrogation of sequence reads for significant identity to known ARGs was performed using a custom script based on RapSearch2 software (Zhao et al., 2012), htseq-count (Anders et al., 2015) and the database CARD (McArthur et al., 2013), which encompasses amino acidic sequences of enzymes involved in antibiotic resistance. Reconstruction of GH profiles as well as bacterial metabolic pathways and evaluation of their abundance in the shotgun metagenomics datasets was performed using custom scripts based on RapSearch2 software (Zhao et al., 2012), htseq-count (Anders et al., 2015) and the CAZy database or the MetaCyc database (Caspi et al., 2012) respectively.

Statistical analyses

ANOVA and PERMANOVA analyses were performed with SPSS software (www.ibm.com/software/it/analytics/spss/).

Data deposition

Raw sequences of 16S rRNA gene profiling are accessible through SRA study accession number SRP064851. Shotgun metagenomics data are accessible through SRA study accession number SRP064850.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. 16S rRNA-microbial profiling of the 84 chicken samples. Only taxa with a relative abundance of >0.5% are shown. Sample names, origin and rearing methods are explained in Table 1 and in the figure.

Table S1. 16S rRNA microbial 10 profiling data.

Table S2. Average and standard deviation (SD) values of 13 the COG functional categories between the two datasets.

Table S3. Average and standard deviation (SD) values of the 17 predicted bacterial metabolic pathways for SCFA production between the two datasets.

Table S4. Degrees of Freedom (DFs), F distribution and significance 21 values obtained through Univariate ANOVA of the COG functional categories between the two datasets. Only statistically significant values were reported.

Table S5. Degrees of Freedom (DFs), F distribution and significance 26 values obtained through Univariate ANOVA of the Glycosyl Hydrolases (GH) and Carbohydrate Binging Modules (CBM) between the two datasets. Only statistically significant values were reported.

Table S6. Degrees of Freedom (DFs), F distribution and significance 31 values obtained through Univariate ANOVA of the predicted bacterial metabolic pathways for SCFA production between the two datasets. Only statistically significant values were reported.

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