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Insights into the biodiversity of the gut microbiota of broiler chickens

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standard framework for the analysis of microeconomic activity. Nevertheless, it also led to the development of a number of strategic approaches. The number of competitors is that the structure of the industry is mainly composed of firms at the national level, are extremely important. Words on City by G. H. M. henceforth we open the black b



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there is no room for extra profits as mark-ups are zero and the number of firms (set) values are not determined by Blanchard and ~~Kiyotaki~~ (1987), perfect competition in general equilibrium of aggregate demand and supply in the classical framework assuming monopoly for an exogenous number of firms

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dynamic responses of mark-ups consistent with the VAR evidence



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labor and supply shocks. Most of the variance in the number of firms is due to standard frictions. New evidence on the number of competitors and the impact is that the structure of the sector



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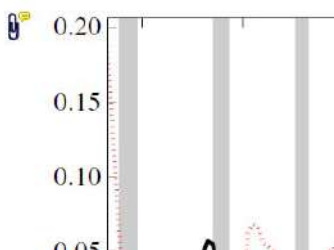


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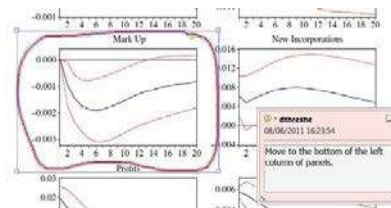


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Insights into the biodiversity of the gut microbiota of broiler chickens

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19 Summary

20 **The gastrointestinal tract of poultry is densely popu-**
 21 **lated with microorganisms, which are presumed to**
 22 **interact with the host and ingested feed. Comparison**
 23 **of the gut microbiota of chickens used for large-scale**
 24 **commercial production (Broiler Chicken, BC) and**
 25 **those grown in semi-wild conditions (Free-Range**
 26 **Chicken, FRC) revealed that at phylum level *Firmi-***
 27 ***cutes* was the dominant phylum of the gut**
 28 **community in BC, while the gut microbiota of FRC**
 29 **contained higher levels of *Bacteroidetes* and *Proteo-***
 30 ***bacteria*. Such differences may be due to the diet**
 31 **and/or the intensive use of antibiotics in BC. Indeed,**
 32 **analysis of the resistome of the cecal microbiomes**
 33 **showed a marked richness in BC datasets, with a**
 34 **modulation of the cecal microbiota toward antibiotic**
 35 **resistant bacteria. Functional characterization of the**
 36 **microbiome of FRC samples revealed an increase in**
 37 **gene pathways involved in degradation of complex**
 38 **carbohydrates. Furthermore, *in silico* analyses of the**
 39 **microbiomes of FRC and BC revealed a higher pres-**
 40 **ence in genes involved in formate production in BC**

41 **samples. Notably, compared to the BC microbiomes**
 42 **the FRC microbiomes were shown to contain a higher**
 43 **abundance of genes involved in the pathway for ace-**
 44 **tate production.**
 45

46 Introduction

47 The bacterial taxa that constitute the large and complex
 48 microbial population resident in the gastrointestinal (GI)
 49 tract of chickens are believed to benefit the host by adding
 50 metabolic potential (Gerritsen et al., 2011), influencing
 51 host nutrition, gut development and physiology (Kau et al.,
 52 2011).

53 Chickens are considered to represent an efficient agri-
 54 cultural species in converting feed to lean meat, although
 55 their feed is often of low digestibility and their intestines are
 56 smaller, with shorter transit digestion times compared to
 57 those of mammals (Choct, 2009; McWhorter et al., 2009).
 58 The gut microbiota of a (healthy) chicken is presumed to
 59 play an important role in nutrient assimilation, vitamin and
 60 amino acid production and prevention of pathogen coloni-
 61 zation (Apajalahti, 2005). The chicken gut microbiota may
 62 also act as a source of bacterial pathogens which can
 63 spread to human beings, or act as a reservoir of antibiotic-
 64 resistance determinants, which can be transferred to other
 65 microorganisms including opportunistic pathogens (Zhou
 66 et al., 2012). In poultry breeds, antibiotics are widely used
 67 to improve growth performance, while they are also
 68 employed as a prophylactic therapy so as to prevent dis-
 69 ease development and transmission (Allen and Stanton,
 70 2014). However, antibiotic therapy is known to cause sub-
 71 stantial compositional alterations in microbial consortia,
 72 thus catalysing dysbiosis with a consequent detrimental
 73 impact on physiology and metabolic performance of the
 74 host that may ultimately result in the development of gut
 75 disorders (Allen and Stanton, 2014).

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

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84 Fermentation of NSPs leads to the production of short
 85 chain fatty acids (SCFAs) that are adsorbed and catabol-
 86 ized by the host, contributing substantially to animal
 87 nutrition and to inhibition of acid-sensitive pathogens (Turn-
 88 baugh et al., 2006). Previously, Stanley and colleagues
 89 (2013) reported the correlation between poultry cecal
 90 microbiota and the efficiency of energy extraction from
 91 feed. A number of bacterial phylotypes were identified that
 92 significantly differed in abundance between birds with high
 93 and low apparent metabolizable energy extraction ability.

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

Results and discussion

16S rRNA profiling of FRC and BC

104
 105
 106 Cecal samples from 84 chickens were obtained in order to
 107 assess the microbiota composition of FRC and BC
 108 (respectively 35 and 49 samples) based on 16S rRNA-
 109 sequencing analysis as described previously (Milani et al.,
 110 2013). MiSeq-mediated sequencing of 84 samples pro-
 111 duced a total of 6 335 983 sequencing reads with an
 112 average of 75 428 reads per sample (Table S1). Quality
 113 and chimera filtering produced a total of 5 508 392 filtered
 114 reads with an average of 65 576 filtered reads per sample,
 115 and ranging from 8230 to 189 725 reads (Table S1).

116 Assessment of rarefaction curves based on the Shan-
 117 non and Chao1 biodiversity indexes calculated for 10
 118 subsampling of sequenced read pools indicated that both
 119 curves tend to reach a plateau. Therefore, in all cases the
 120 obtained sequencing data was deemed adequate to cover
 121 the vast majority of biodiversity contained within the sam-
 122 ples (Fig. 1A and B). Moreover, average rarefaction curves

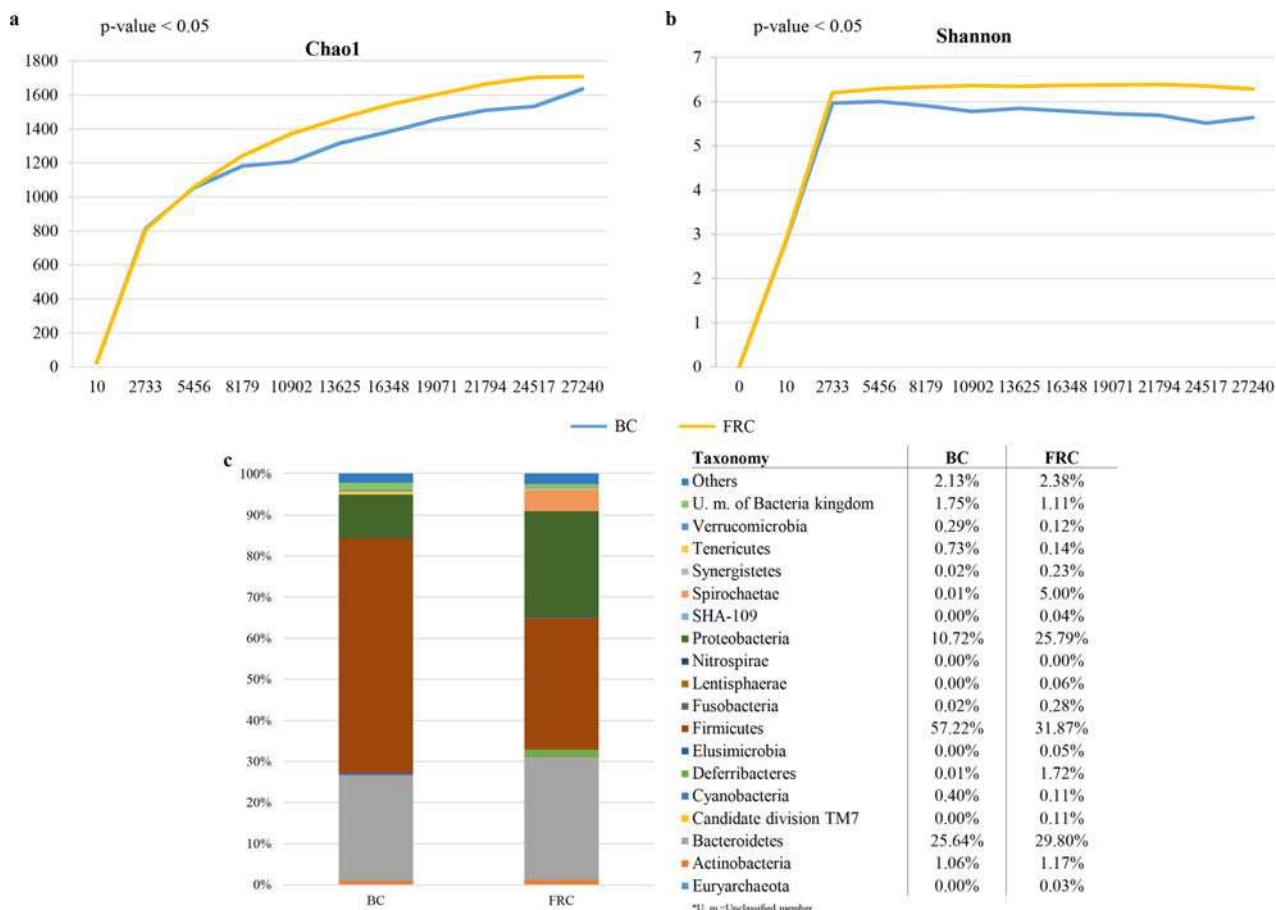


Fig. 1. Evaluation of alpha-diversity in BC and FRC samples. Panel A shows the average rarefaction curve representing variation of the Chao1 diversity index at increasing sequencing depth of BC and FRC samples. Panel B displays the average rarefaction curve representing variation of the Shannon diversity 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.

COLOR

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

131 *Differential Gut microbiota composition*

132 Inspection of predicted taxonomic profiles at phylum level
 133 for all samples exhibited that *Firmicutes* (58.90%) repre-
 134 sented the dominant phylum of the cecal community in BC
 135 chickens, outnumbering the *Bacteroidetes* (25.70%) and
 136 *Proteobacteria* (10.73%) phyla. Differences in the gut
 137 microbiota composition were detected in FRC compared
 138 to BC with a comparative reduction of *Firmicutes* (31.86%)
 139 and a concomitant increase of *Bacteroidetes* and *Proteo-*
 140 *bacteria*, represented by 30.08% and 25.82% respectively
 141 (Fig. 1C). Studies in mice, pigs and humans have sug-

gested that the *Firmicutes* to *Bacteroidetes* (F/B) ratio 142
 based on 16S rRNA gene sequence is often correlated 143
 with weight (Singh et al., 2013). The identified F/B ratio is 144
 3.87 (P-value < 0.01) and 1.25 (P-value < 0.01) for BC and 145
 FRC groups (Fig. 1D) respectively. However, it is not possi- 146
 ble to correlate performances and F/B ratio since no data 147
 about animal weight are available for the samples used in 148
 this study. Interestingly, antibiotic treatment has been 149
 shown to alter gut and fecal bacterial species composition 150
 in chicken (Gong et al., 2008) towards an increased 151
 abundance of *Lactobacillus* spp., Clostridiales and Entero- 152
 bacteriaceae. Similar results were reported by Singh and 153
 colleagues (2013), who showed that an increase in the F/B 154
 ratio correlated with antibiotic treatment and increase of 155
 body weight, which may in turn be related to modulation of 156
 nutrient absorption by the host. 157

Furthermore, at a genus level, we identified 252 taxa of 158
 which 217 appear to be present in all samples, while six 159
 and 29 appeared to be uniquely present in cecal samples 160
 of BC and FRC respectively (Fig. 2A). Notably, the 161 F2
 presence or absence of these genera may be the 162

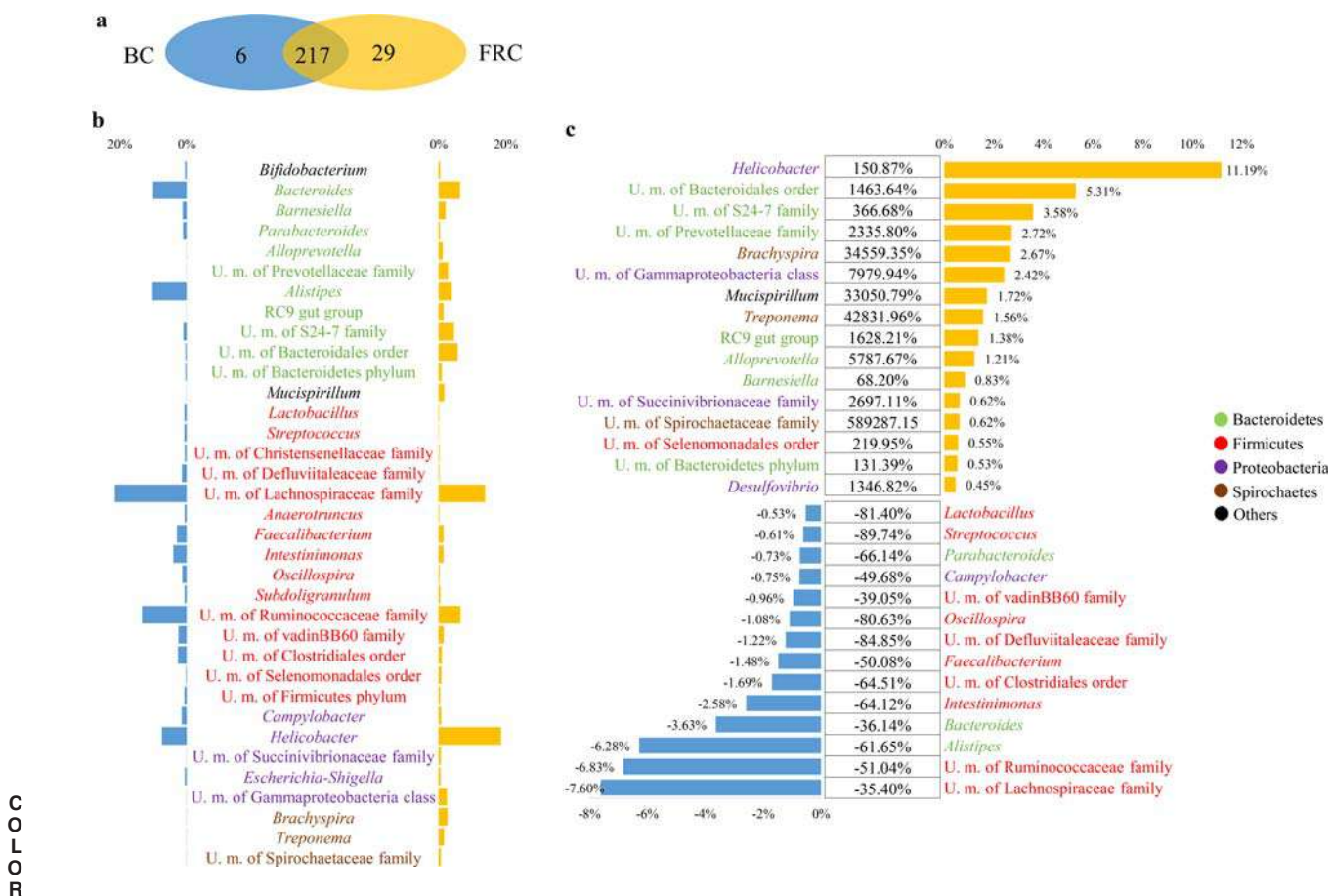


Fig. 2. Exploration of the diversity in BC and FRC groups. Panel A depicts a Venn diagram illustrating the total, unique and shared number of OTUs predicted for BC and FRC datasets. Panel B reports the core set of genera present at an average relative abundance of > 0.5% in at least one sample group. 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.

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- 163 consequence of how the chickens are reared, i.e. being
 164 allowed to freely roam or being kept in a strictly confined
 165 environment, which is likely to impact on the physiology of
 166 the animals. In order to further identify differences in micro-
 167 biota composition between BC and FRC groups, we
 168 focused on 97 genera, which were shown to be present at
 169 an average relative abundance of > 0.5% in at least one
 170 sample group (Fig. S1). The core set of genera obtained
 171 (Fig. 2B) and the comparison of the relative abundance
 172 average of BC and FRC (Fig. 2C) revealed a predominant
 173 presence of members of the *Firmicutes* phylum in BC sam-
 174 ples, such as unclassified members (*U. m.*) of the
 175 Lachnospiraceae family, *U. m.* of the Ruminococcaceae
 176 family and *Intestinimonas* spp. In contrast, FRC-derived
 177 samples were shown to contain a higher diversity (com-
 178 pared to data obtained from BC samples) at the genus
 179 level among the *Bacteroidetes*, *Proteobacteria* and *Spiro-*
 180 *chaetes* phyla.
- 181 Notably, bacterial taxa belonging to both *Firmicutes* and
 182 *Bacteroidetes* phyla are known to be involved in the break-
 183 down of otherwise indigestible (by the host)
 184 polysaccharides such as resistant starch and cellulose
 185 (Allen and Stanton, 2014; Stanley et al., 2012). Therefore,
 186 the observed differences in microbial composition between
 187 BC- or FRC-derived cecal samples may influence the food
 188 to energy conversion capacity in chickens housed under
 189 different conditions.
- 190 In order to evaluate microbiota differences between BC
 191 and FRC samples, we analysed the beta diversity based
 192 on unweighted UniFrac for these groups, after which the
 193 UniFrac distance matrix was represented through Principal
 F3 194 Coordinate Analysis (PCoA) (Fig. 3). Interestingly, the
 195 samples were shown to group in two different clusters
 196 based on how the chickens had been kept, indicating that
 197 a different diet and/or prophylactic therapy impact on gut
 198 microbiota composition (Fig. 3A). Moreover, environmental
 199 samples included in this analysis, collected from BC and
 200 FRC litters, clustered with the corresponding cecal sam-
 201 ples. Thus, the environment reflected the microbiota
 202 composition of chickens, perhaps due to contamination
 203 from the stool of the animals (Fig. 3A).
- 204 Evaluation of the beta diversity of cecal samples isolated
 205 from the BC and FRC groups, each following a particular
 206 feeding type (Fig. 3B), clearly shows a clear separation
 207 between BC and FRC animals. However, the microbiota
 208 composition of animals fed with different cereals, such as
 209 wheat and corn, clustered separately (Fig. 3B).
- 210 Analysis of the influence of antibiotic treatments on BC
 211 animals, showed a heterogeneous distribution of the sam-
 212 ples (Fig. 3C), and it seems that different antibiotic mixes
 213 have a divergent impact on microbiota composition.
- 214 Such findings about PCoA clustering were confirmed by
 215 the obtained P-value of PERMANOVA statistical analysis
 216 (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* 219
- 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 taxo-
 nomic 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 amplifica-
 tion 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, signifi-
 cant 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* 255
- 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 micro-
 biome. 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

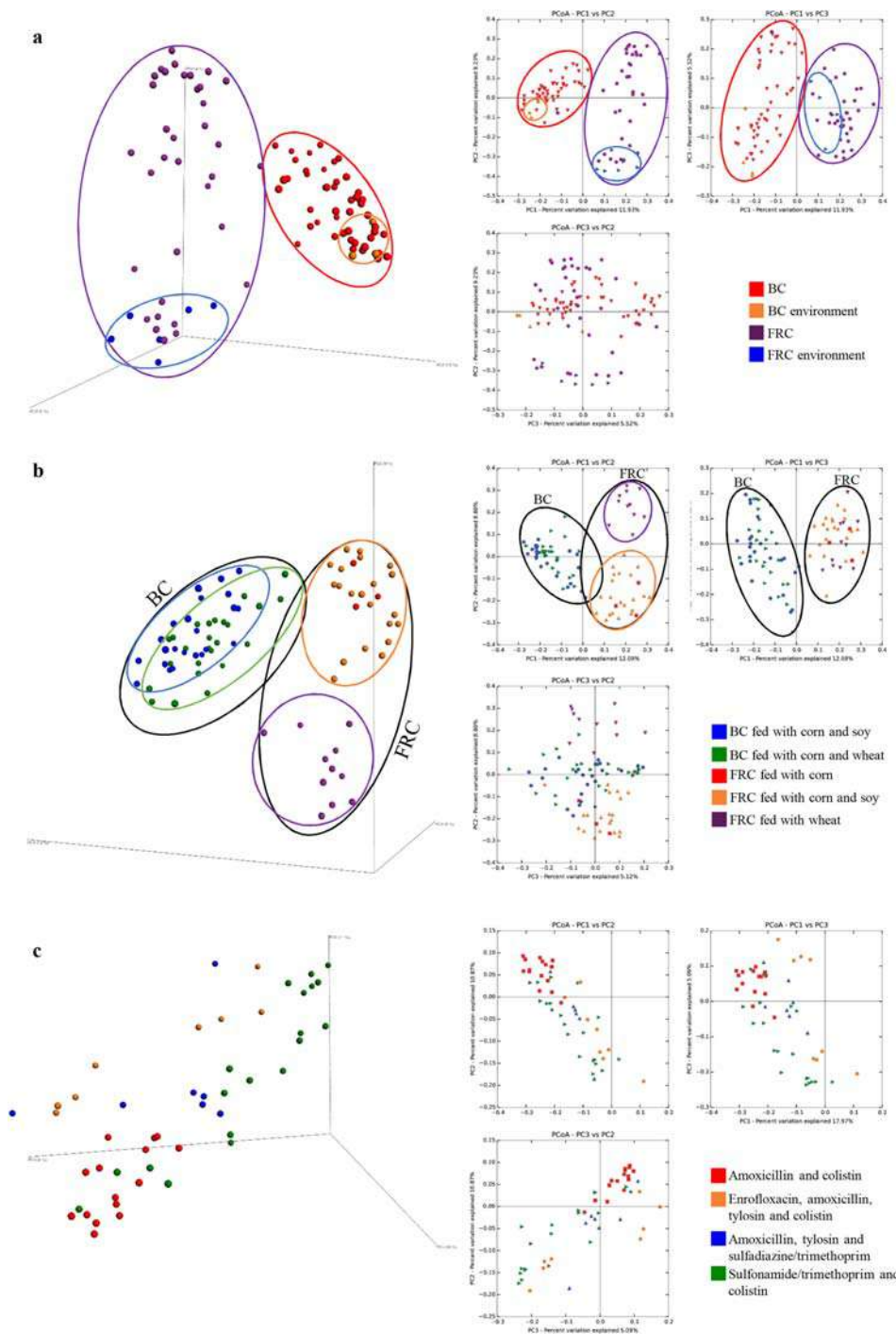


Fig. 3. Evaluation of the beta-diversity in BC and FRC samples. The predicted PCoA encompassing all 84 BC and FRC datasets is reported through two three-dimensional images as well as two-dimensional sections. Panel A shows BC, FRC and environmental datasets, and corresponding clusters are coloured in 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.

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

269 *In silico* analyses of shotgun metagenomic datasets
270 revealed a higher abundance (16.64%) of ARGs in BC
271 samples as compared to FRC animals (Fig. 4B). Interest-
272 ingly, β -lactamase-encoding genes were shown to be the
273 most abundant in all analyzed samples with a slight
274 increase of 5.40% in BC samples. This might be the con-

sequence of the fact that β -lactam-based antibiotics are 275
the most commonly used drugs in BCs (Table 1), while β - 276 T1
lactamase-encoding genes are also the most commonly 277
detected antibiotic resistance sequences found in soil and 278
water bacteria (Allen and Stanton, 2014; Cho et al., 2014). 279
Moreover, differences were noted in the heat map 280
(Fig. 4B) regarding glycopeptide-based ARGs. In this con- 281
text, we observed a 117.62% increase in the abundance of 282

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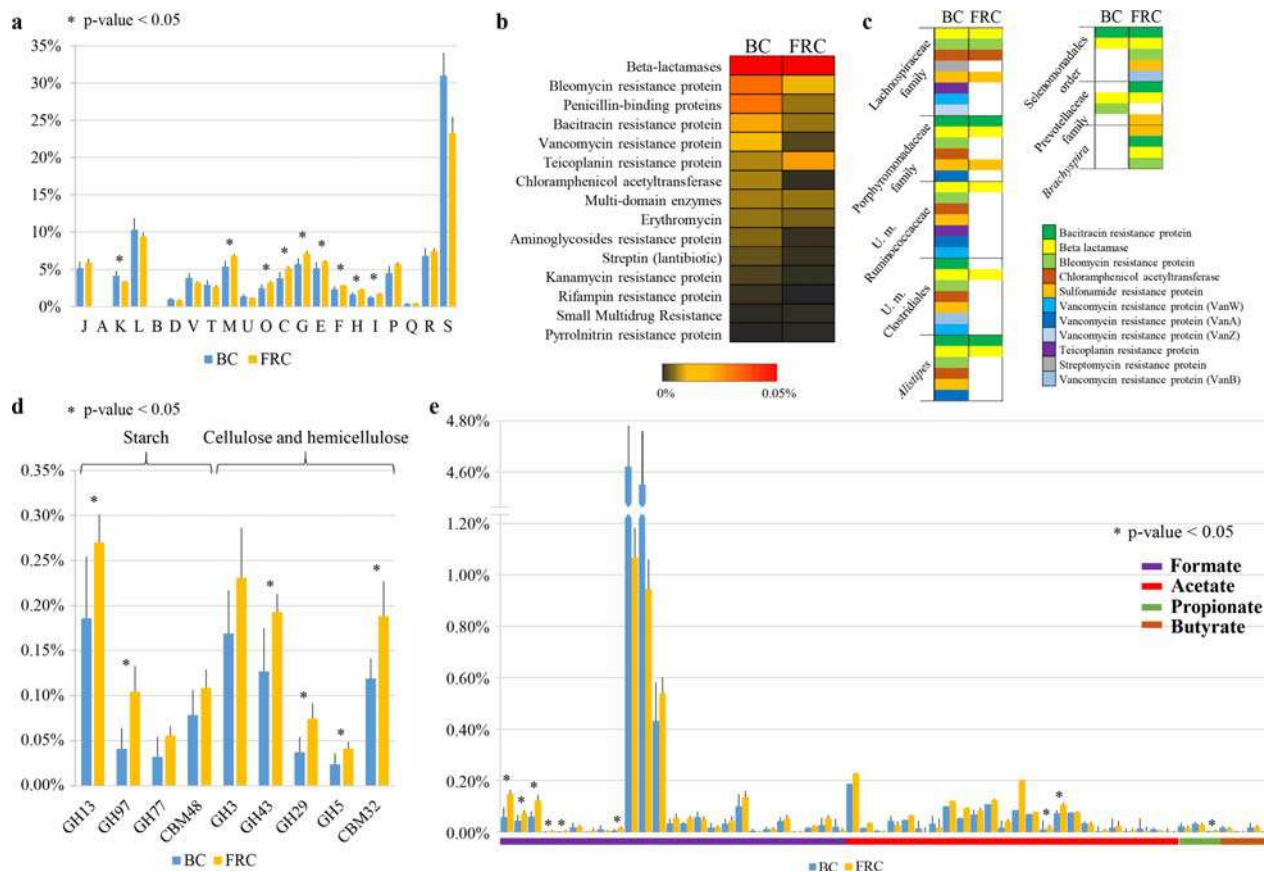


Fig. 4. Functional changes in the gut microbiome of BC and FRC. Asterisks indicate statistically significant differences between the two datasets (P-value < 0.05). Panel A depicts the functional annotation of BC and FRC metagenomic datasets according to COG categories. Each COG family is identified by 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.

283 genes encoding for vancomycin resistance in BC samples.
 284 Conversely, we observed an increase (347.24%) of genes
 285 for resistance to teicoplanin in FRC poultry with respect to
 286 BC animals. Furthermore, an increase (9.15%) of chloram-
 287 phenicol acetyltransferase-encoding genes were observed
 288 in BC samples.

289 Taxonomic classification of shotgun metagenomic reads
 290 corresponding to ARGs allowed the identification of bacteri-
 291 al taxa contributing to at least 1% of the total resistome
 292 (Fig. 4C). In the BC group, we observed a prevalent pres-
 293 ence of ARGs belonging to the *Firmicutes* phylum
 294 (40.40%). In contrast, the FRC group contains a high
 295 abundance of *Bacteroidetes* (27.32%) and *Spirochaetes*
 296 (5.03%) phyla. Focusing on bacterial taxa that exhibit a
 297 higher variation in relative ARG abundance between BC
 298 and FRC samples, we detected an increased abundance
 299 of ARGs in BC taxa compared to FRC, as well as ARGs
 300 unique to the BC group encoding chloramphenicol acetyl-

transferase, teicoplanin resistance protein and vancomycin
 resistance protein (Fig. 4C).

Thus, our findings revealed a clear selection of the
 microbiota members resistant to these antibiotics. Further-
 more, 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 micro-
 biomes 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 com-
 pared to those obtained from BC (5.7%) (P-value < 0.05).

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	Aulla (Toscana, Italy)	Wheat and wet waste	/
P7	FRC			
P8	FRC			
P9	FRC	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).
P10	BC			
P11	BC			
P12	BC			
P13	BC			
P14	BC			
P15	BC			
P16	BC			
P17	BC			
P18	BC			
P19	BC	Cremona 1 (Lombardia, Italy)	Corn and soy	Amoxicillin (50 mg Kg ⁻¹) and colistin (100 mg Kg ⁻¹).
P20	BC			
P21	BC			
P22	BC			
P23	BC	Aulla (Toscana, Italy)	Wheat and wet waste	/
P24	BC			
P25	FRC			
P26	FRC	Cremona 2 (Lombardia, Italy)	Corn and soy	Sulfonamide/trimethoprim and colistin (100 mg Kg ⁻¹).
P27	BC			
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	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).
P46	BC			
P47	BC			
P48	BC			
P49	BC			
P51	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).
P53	BC			
P54	BC			
P55	BC			
P56	BC			
P57	BC			
P59	BC			
P60	BC			
P61	BC			
P62	BC			
P63	BC			

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.

317 Moreover, we observed a difference in the COG family cor-
 318 responding to energy production and conversion in FRC of
 319 1.35% (P-value < 0.05) compared to BC datasets (Fig. 4A
 320 and Table S4). Complex polysaccharides are degraded by
 321 the gut microbiota into monosaccharides and then fer-
 322 mented to (mainly) produce the metabolic end products
 323 H₂, CO₂, CH₄ and SCFAs, which may then be adsorbed by
 324 the host together with any remaining digestible monosac-
 325 charides (Tremaroli and Backhed, 2012). The absorbed
 326 compounds promote hepatic triglyceride synthesis, the
 327 accumulation of triglycerides in adipocytes and subse-
 328 quently, an increase in body fat (Janssen and Kersten,
 329 2015).

330 *In silico* characterization of putative glycosyl hydrolases
 331 (GHs) responsible for degradation of complex carbohy-
 332 drates, revealed that the microbiomes of FRC chickens
 333 possess a wider arsenal of GH families involved in starch,
 334 cellulose and hemicellulose degradation compared to BC
 335 samples (Fig. 4D). In particular, genes encoding predicted
 336 members of GH13, GH97 and GH77, as well as genes
 337 that specify proteins containing a CBM48 domain, are
 338 present at higher abundance (from 1.5- to 2-fold, P-val-
 339 ue < 0.05) in the data sets from FRC animals compared to
 340 those of BC data (Fig. 4D and Table S5). GH genes
 341 encoded for α -amylases and α -glucosidases, while
 342 CBM48 is a carbohydrate-binding module known to bind

various linear and cyclic α -glucans derived from starch and 343
 glycogen. Moreover, GH families GH3, GH43, GH29 and 344
 GH5, which represent α -L-rabinofuranosidase, β -D-xyl- 345
 opyranosidase and α -fucosidase activities (Matsuzawa 346
 et al., 2015), are more abundant in FRC datasets as com- 347
 pared to BC datasets (Fig. 4D). These differences can be 348
 explained by the higher abundancy of microorganisms 349
 belonging to the *Bacteroidetes* phylum in FRC chickens as 350
 compared to BC animals, where members of the former 351
 taxonomic groups are known to hydrolyze starch and plant 352
 cell wall compounds (e.g. cellulose, pectin and xylan) 353
 (Thomas et al., 2011). 354

Moreover, analysis of predicted bacterial metabolic path- 355
 ways for SCFA production, showed a higher number of 356
 genes that are predicted to be responsible for formate pro- 357
 duction in BC compared to that found in FRC microbiomes 358
 (P-value < 0.05) (Fig. 4E, Tables S3 and S6). Formate pro- 359
 duced in the animal gut can be degraded to CO₂ and H₂ 360
 (Sergeant et al., 2014). A high amount of hydrogen leads 361
 to reduction in fermentation and/or less energy-efficient 362
 fermentation to butyrate and propionate (Macfarlane and 363
 Macfarlane, 2003). The presence of bacteria that can met- 364
 abolically act as a hydrogen sink, such as *Desulfovibrio*, 365
Helicobacter, *Megamonas* and *Campylobacter* is expected 366
 to result in an increased efficiency of fermentation and 367
 SCFA production, which would ultimately exert a beneficial 368

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

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

385 In contrast, no difference in the abundance of genes pre-
386 dicted to be part of the metabolic pathways for butyrate
387 and propionate synthesis was found between FRC and BC
388 microbiomes with the exception of phosphatidylcholine
389 resynthesis via the glycerophosphocholine pathway.

390 Conclusions

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

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

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

416 Analysis of the resistome of cecal microbiomes revealed
417 that BC datasets contain higher levels of predicted ARGs
418 compared to those of FRC, and a weak correlation was
419 found with antibiotics used in the six different farms
420 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 ani-
mals, such as an increase in the abundance of gene
pathways involved in degradation of complex carbohy-
drates 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 dif-
ferent 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 move-
ments 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 breeding. 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 slaughter-
houses where both BC and FRC animals were killed. Cecal
samples were obtained, kept on ice and processed immedi-
ately 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. Sam-
ples 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 repre-
sent the different rearing conditions associated with either
large-scale, commercial production or the practice free-

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 497
 476 environmental RNA/DNA kit (Mobio, USA) following the manu- 498
 477 facturer's instructions. 499
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 501
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 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).
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 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
 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
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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

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

526 *Shotgun metagenomics*

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

533 *Analysis of metagenomic datasets*

534 The generated fastq files were filtered for reads with a quality
535 score of < 25, for sequences of chicken DNA, as well as for
536 reads < 80 bp. Bases were also removed from the end of the
537 reads unless the average quality score in a window of 5 bp
538 was > 25. Only paired data were further analysed. The
539 revised gene/protein set was searched using evolutionary
540 genealogy of genes: Non-supervised Orthologous Groups
541 (eggNOG; http://eggnoG.embl.de/version_4.0.beta/) data-
542 bases. Interrogation of sequence reads for significant identity
543 to known ARGs was performed using a custom script based
544 on RapSearch2 software (Zhao et al., 2012), htseq-count
545 (Anders et al., 2015) and the database CARD (McArthur
546 et al., 2013), which encompasses amino acidic sequences of
547 enzymes involved in antibiotic resistance. Reconstruction of
548 GH profiles as well as bacterial metabolic pathways and evalu-
549 ation of their abundance in the shotgun metagenomics
550 datasets was performed using custom scripts based on Rap-
551 Search2 software (Zhao et al., 2012), htseq-count (Anders
552 et al., 2015) and the CAZy database or the MetaCyc database
553 (Caspi et al., 2012) respectively.

554 *Statistical analyses*

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

557 *Data deposition*

558 Raw sequences of 16S rRNA gene profiling are accessible
559 through SRA study accession number SRP064851. Shotgun
560 metagenomics data are accessible through SRA study acces-
561 sion 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 pro-
duction between the two datasets. Only statistically signifi-
cant values were reported.

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