Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses

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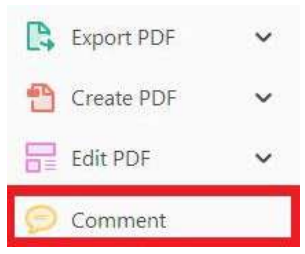

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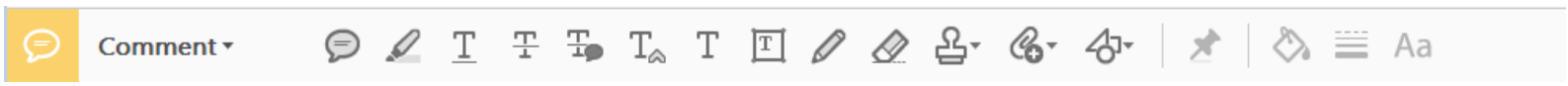

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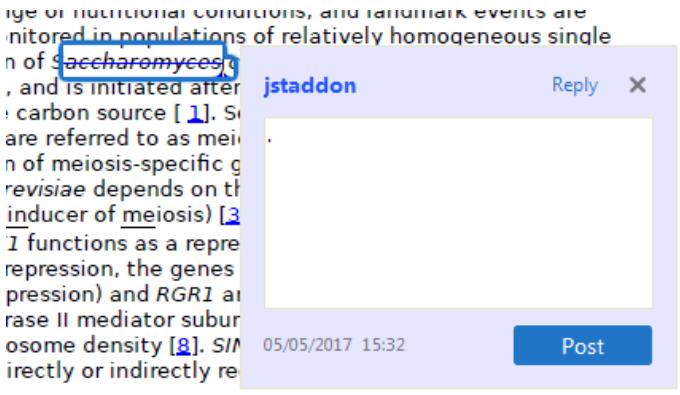

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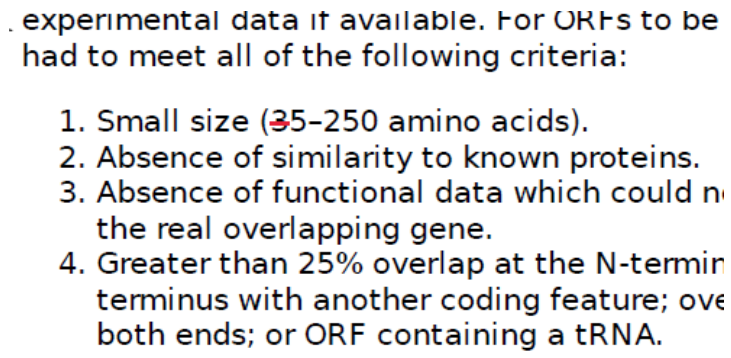
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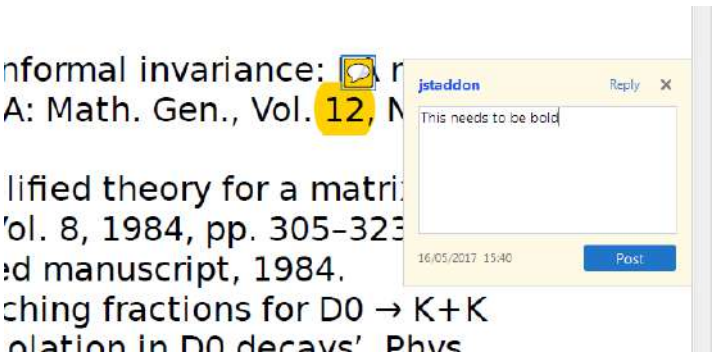

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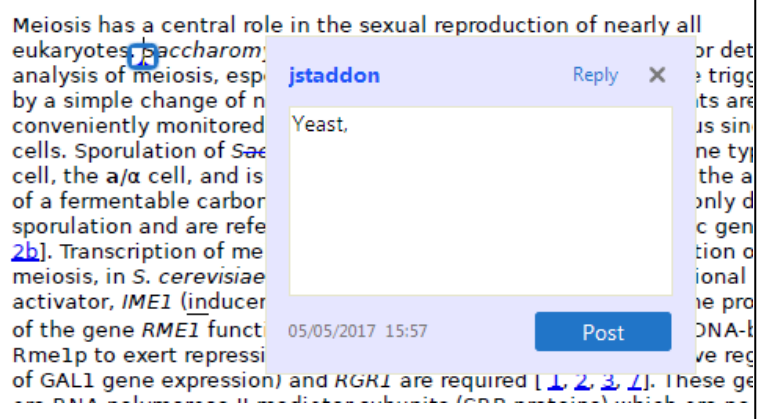

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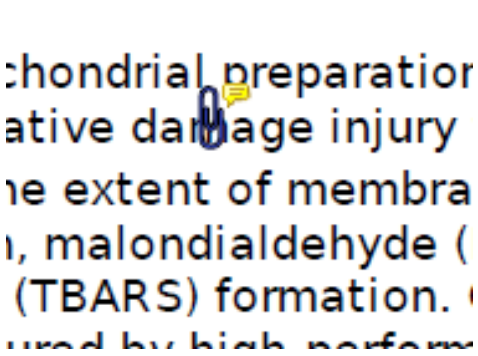

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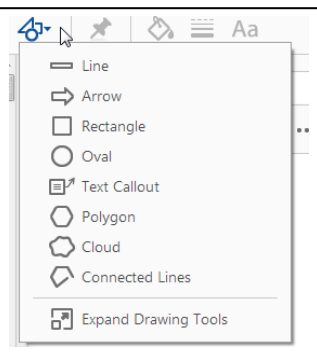

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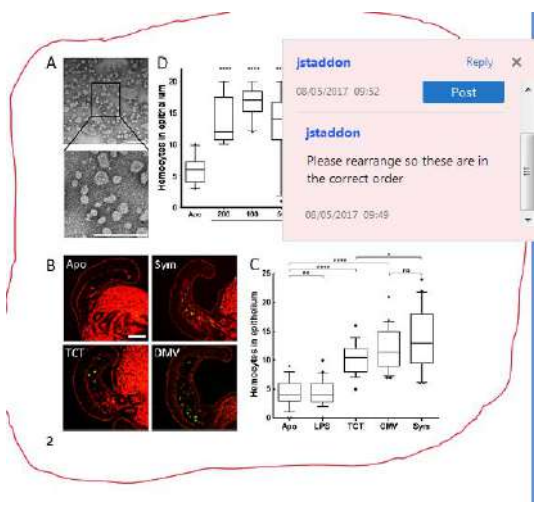

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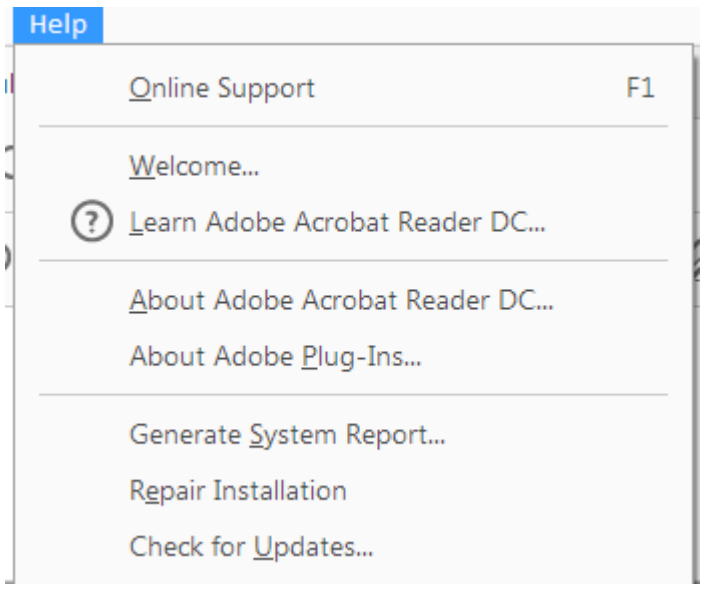

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environmental microbiology

Environmental Microbiology (2017) **00**(00), 00–00 doi:10.1111/1462-2920.13943

Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses

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Summary

Different factors may modulate the gut microbiota of animals. In any particular environment, diet, genetic factors and human influences can shape the bacterial communities residing in the gastrointestinal tract. Metagenomic approaches have significantly expanded our knowledge on microbiota dynamics inside hosts, yet cultivation and isolation of bacterial members of these complex ecosystems may still be necessary to fully understand interactions between bacterial communities and their host. A dual approach, involving culture-independent and -dependent techniques, was used here to decipher the microbiota communities that inhabit the gastro intestinal tract of free-range, broiler and feral chickens. *In silico* analysis revealed the presence of a core microbiota that is typical of those animals that live in different geographical areas and that have limited contact with humans. Anthropic influences guide the metabolic potential and the presence of antibiotic resistance genes of these different bacterial communities. Culturomics attempts, based on different cultivation conditions, were applied to reconstruct *in vitro* the microbiota of feral chickens. A unique strain collection representing members of the four major phyla of the poultry microbiota was assembled, including bacterial strains that are not typically retrieved from the chicken gut.

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Introduction

Analysing the microbiota composition of breeding animals has gained growing interest because this allows a prediction of the compositional structure and associated metabolites of such communities, which are believed to fundamentally impact on all aspects of host physiology (Hiergeist *et al.*, 2015). The compositional analysis of the gut microbiota has enjoyed rapid advances in recent years thanks to metagenomic approaches based on high throughput sequencing methods (Fraher *et al.*, 2012; Weinstock, 2012; Milani *et al.*, 2013). However, in order to obtain detailed mechanistic insights into the roles of gut microorganisms with regards to interactions with their host and/or other bacteria in their natural ecosystem, isolation and cultivation of individual members of the gut microbiota is required (Browne *et al.*, 2016; Lagier *et al.*, 2016).

A large proportion of the gut microbiota of animals, including that of humans, is represented by not yet cultured microorganisms, including bacteria that are highly sensitive to oxygen and/or highly nutritionally exigent, thus preventing cultivation by classical laboratory approaches (Lagier *et al.*, 2016). Most of the current knowledge concerning the gut microbiota is still restricted to humans (Clemente *et al.*, 2012; Lozupone *et al.*, 2012; Marchesi *et al.*, 2016), although information on the gut microbiota of breeding-

animals, including poultry, is increasing (Oakley *et al.*, 2014; Mao *et al.*, 2015; McCormack *et al.*, 2017). In this context, both culture-independent and cultivation-based approaches have revealed that the majority of the human gut microbiota consists of members of the phyla *Bacteroidetes* and *Firmicutes*, yet many additional players (i.e., other bacterial phyla present in the human gut microbiota, principally *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia*), are part of this complex ecosystem (Human Microbiome Project Consortium, 2012; Lozupone *et al.*, 2012; Mirzaei and Maurice, 2017). Similar results were reported for farm-based animals, such as broiler chickens (Mohd Shaufi *et al.*, 2015; Mancabelli *et al.*, 2016). Nevertheless, and in contrast to human beings, our current knowledge on the composition of the gut microbiota of chickens is still rather underdeveloped, despite that fact that these animals represent an important food resource for humans, while also being a potential reservoir of food-borne pathogens (Oakley *et al.*, 2014).

Feralization is a process by which domesticated species have escaped their controlled environment and colonized new habitats. It is also called 'domestication in reverse' (Gering *et al.*, 2015; Johnsson *et al.*, 2016), as it involves the removal of direct anthropogenic control over natural and sexual selection regimes. Animals, such as poultry, subjected to a feralization process could be useful to elucidate the impact of human care on the development of microbiota. With this aim, we report here an in-depth cataloguing of the microbiota composition of free-range, broiler and feral (i.e., formerly domesticated, wild-living) chickens (abbreviated here as FRC, BC and FC respectively) by means of omics approaches that used metagenomic and culturomics techniques, and also involved bacterial cultivation approaches based on multiple culture conditions (Lagier *et al.*, 2015; Lagier *et al.*, 2016). The results allowed us to understand what happens to the cecal microbiota of domesticated chickens during the process of feralization, and to obtain a unique chicken cecal microbiota strain collection, which includes representatives of the four major phyla characteristic of this complex bacterial community.

Results and discussion

Microbial diversity of feral chicken

The microbiota composition of 15 cecal samples of chickens originating from Bermuda (Table 1) was assessed based on 16S rRNA amplicon sequencing as described previously (Milani *et al.*, 2013). These animals comprised wild animals or chickens reared under distinct circumstances, with variable degrees of human control or interference, called Bermuda Feral, Free-Range Bermuda Broilers and Free-Range Bermuda Layers, as described in the Experimental Procedures section. Illumina-based 16S rRNA microbial profiling produced a total of 855 136 sequencing reads with an average of filtered 53 177 reads per sample (Supporting Information Table 1). The microbiota analysis of Bermuda poultry (encompassing Bermuda Feral, Free-Range Bermuda Broilers and Free-Range Bermuda Layers) obtained here, was compared with that of 84 previously characterized BC (broiler chicken) and FRC (free-range chicken) samples (Table 1) (Mancabelli *et al.*, 2016), where collection of biological materials and protocols for DNA isolation and 16S rRNA gene microbial profiling had been performed in an identical manner to those used here (Mancabelli *et al.*, 2016).

Assessment of rarefaction curves based on the Shannon and Simpson biodiversity indexes calculated for ten subsamplings 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 reveal a difference between the cecal microbiota of BC, FRC and Bermuda poultry (Fig. 1). Specifically, cecal samples from Bermuda displayed a higher level of complexity of the cecal microbiota compared to that found in BC samples (Fig. 1) (Student's *t*-test statistical analysis p-value < 0.05). In contrast, the curves are not significantly different between Bermuda poultry and FRC (p-value > 0.05) calculated at the highest rarefaction depths reached by all samples (Fig. 1).

The microbiota of Bermuda Feral was shown to be dominated by the *Bacteroidetes* phylum (average value 40%), followed by *Firmicutes* and *Proteobaceria* (at average values of, respectively, 30% and 18%). In particular, the most abundant bacterial taxa are Unclassified Member (U. m.) of the *Prevotellaceae* family (11.74%), *Bacteroides* spp. (9.13%) and U. m. of *Bacteroidales* order (5.46%) (Supporting Information Figures S1, S2 and S3). Non-significant differences in microbiota composition were observed in Free-Range Bermuda Broilers, and Free-Range Bermuda Layers compared to Bermuda Ferals, at phylum level, despite the finding that the *Proteobacteria* phylum was shown to be present at a lower relative abundance (about 10% in both groups) (Supporting Information Figure S1). Both Free-Range Bermuda Broilers, and Free-Range Bermuda Layers were dominated by the *Bacteroides* genus (respectively 18% and 30%) followed by U. m. of *Ruminococcaceae* family (9% and 7% respectively) (Supporting Information Figure S3).

Differences in cecal microbiota composition between BC, FRC and FC chickens

In order to evaluate microbiota differences between Bermuda poultry, BC and FRC samples, we analysed the β-diversity based on unweighted UniFrac for these groups, after which the UniFrac distance matrix was represented

Table 1. Chicken samples collected in this study.

Samples name	Rearing methods	Origin	Feed	Antibiotics	References
BER50, BER52, BER53, BER55, BER56, BER57A, BER57B, BER58, BERXX, BER	FC	St. George (Bermuda)	Wet waste[a]	/	This study
BER100, BER101, BER102, BER103, BER104	FRC	St. George (Bermuda)	Wheat and wet waste, soy-free, corn-free	/	
P1, P2, P3, P4, P5, P6	FRC	Parma 1 (Emilia Romagna, Italy)	Barley, wheat and wet waste	/	(Mancabelli *et al.*, 2016)
P7, P8, P9	FRC	Aulla (Toscana, Italy)	Wheat and wet waste	/	
P10, P11, P12, P13, P14, P15, P16, P17, P18, P19	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}).	
P20, P21, P22, P23, P24	BC	Cremona 1 (Lombardia, Italy)	Corn and soy	Amoxicillin (50 mg kg^{-1}) and colistin (100 mg kg^{-1}).	
P25, P26	FRC	Aulla (Toscana, Italy)	Wheat and wet waste	/	
P27, P28, P29, P30, P31, P32, P33, P34, P35, P36	BC	Cremona 2 (Lombardia, Italy)	Corn and soy	Sulfonamide/trimethoprim and colistin (100 mg kg^{-1}).	
P37, P38, P39, P40, P41, P42, P43, P44, P45, P46	BC	Cremona 3 (Lombardia, Italy)	Corn and soy	Sulfonamide/trimethoprim and colistin (100 mg kg^{-1}).	
P47, P48, P49, P51, P53, P54, P55, P56	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}).	
P57, P59, P60, P61, P62, P63, P64	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}).	
P68, P59	FRC	Varese (Lombardia, Italy)	Wheat, soy and wet waste	/	
P70, P71, P72, P73, P74, P75, P76, P77, P78, P79, P80, P81, P82, P83, P84, P85, P86, P87, P88	FRC	Reggio Emilia (Emilia Romagna, Italy)	Corn, soy, pea, bram and minerals	/	
P89, P90, P91	FRC	Parma 2 (Emilia Romagna, Italy)	Corn, cereal mix and wet waste	/	

a. Wet waste: invertebrates, seeds, shoots, household and business garbage.
Origin of samples, antibiotic treatments and feed composition are reported.

through Principal Coordinate Analysis (PCoA) (Fig. 1C). Such analyses showed that the Bermuda samples grouped in the FRC group, being clearly separated from BC. Interestingly, a detailed analysis of the FRC group revealed a split in two different clusters that do not correlate with the geographical origin of the animals (Fig. 1D). In detail, the first cluster, called the FRC dominant group (FRC-group) was composed of FRC samples derived from different geographical areas and or livestock in Italy (Parma 2, Varese and Reggio Emilia), while the second one, indicated as the Feral Chicken-dominant group (FC-group) encompassed samples collected in Italy (Parma 1 and Aulla) and all those obtained from Bermuda (Bermuda Ferals, Free-range Bermuda Broilers and Free-Range Bermuda Layers), despite differences in diet and rearing methods (Table 1). Such findings were shown to be statistically validated by a p-value of < 0.001, as obtained by PERMANOVA analysis, when the data sets of the two clusters were compared. Thus, chickens from different lifestyles (FC vs. FRC), ancestries and rearing localities can

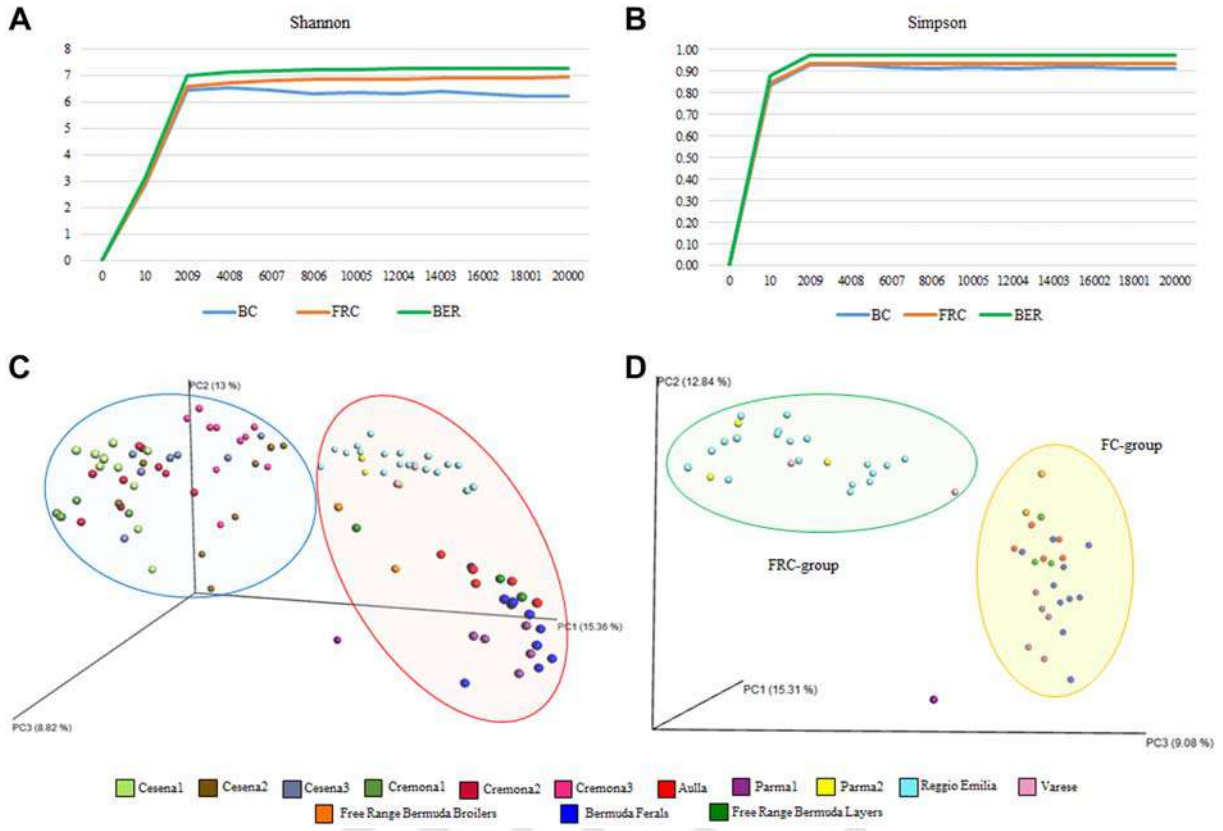

Fig. 1. Evaluation of α- and β- diversity in various poultry groups.
A. The average rarefaction curve representing variation of the Shannon diversity index at increasing sequencing depth of Bermuda, BC and FRC samples.
B. The average rarefaction curve representing variation of the Simpson diversity index at increasing sequencing depth of Bermuda, BC and FRC samples.
C. The predicted PCoA encompassing all Bermuda, BC and FRC data sets through three-dimensional images.
D. the predicted PCoA encompassing all Bermuda and FRC data sets through three-dimensional images.

exhibit striking similarities in core cecal microbiota. Moreover, the existence of a core microbiota, being typical of chickens that live in different geographical areas with little if any human contact and feeding on a natural diet, was observed (Fig. 1C).

Inspection of the predicted microbiota in FRC, BC and FC groups

Exploring the predicted taxonomic profiles at phylum level for the analysed samples clearly shows that the BC, FRC and FC groups possess a distinct microbiota composition (Fig. 2A–C and Supporting Information Figs. S1, S2 and S3). Sample categorization in FRC, BC or FC was performed subsequent to obtaining the results from the PCoAs as reported in Fig. 1C and D. The bacterial community of the BC and FRC cecal samples was shown to be dominated by the *Firmicutes* phylum, although at a significantly different level (p-value < 0.05, with an average relative abundance of 59.8% and 33.4% respectively), outnumbering the *Bacteroidetes* (26.2% and 29.2%) and *Proteobacteria* (10.9% and 28.3%) phyla (Fig. 2A). Conversely, the FC-group cecal microbiota was dominated by members of the *Bacteroidetes* phylum (with an average relative abundance of 38.6%). *Firmicutes* in the FC-derived samples were present at a lower level in comparison with those from BC samples (32%, p-value < 0.05), while the *Proteobacteria* phylum was shown to be present at a significantly lower abundance (18.5%, p-value < 0.05) in comparison to the FRC cecal samples.

In order to further identify differences in microbiota composition between BC, FRC and FC, the microbiota composition was explored at genus level, evaluating differences of bacterial genera present at an average relative abundance of $> 0.5\%$ in at least one sample group (Fig. 2C and Supporting Information Fig. S3). The definition of the core microbiota, that is, bacterial species that are present in all samples of a given sample set (Salonen *et al.*, 2012), also revealed particular differences between FC, FRC and BC animals (Fig. 2D). In detail, unclassified

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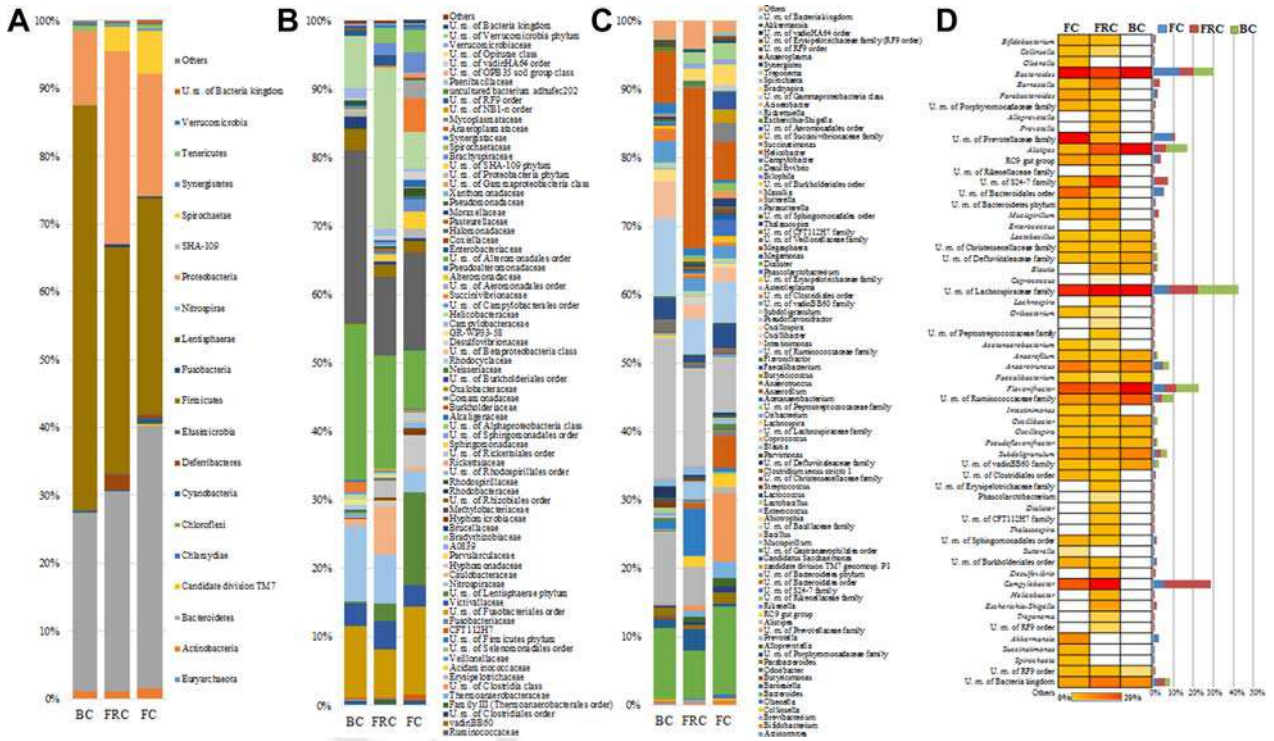

Fig. 2. Evaluation of the predicted microbiota in FRC, BC and FC groups. Sample categorization in FRC, BC or FC was performed subsequent to obtaining the PCoA results as reported in Fig. 1C and D.
A–C. The 16S rRNA-microbial profiling of aggregate FC, BC and FRC groups at phylum, family and genus level (only taxa with a relative abundance of $>0.5\%$ are shown) respectively.
D. A heat map reporting the presence/absence of core species detected in the microbiota of the three data sets. Moreover, the relevance of each genus in the FC, FRC and BC data sets is reported on the right. U. m.: unclassified member.

members (U. m.) of the *Prevotellaceae* family, U. m. of the *Bacteroidales* order and *Succinimonas* spp. were preponderant in FC samples as compared to FRC and BC sample sets (p-value < 0.05) (Fig. 2C). In contrast, the cecal samples from the FRC-group showed a higher presence, at genus level, of U. m. of the *S24-7* family, *Barnesiella* spp., *Mucirospirillum* spp. and *Helicobacter* spp. The BC group elicited a preponderance in *Alistipes* spp., U.m. of *Ruminococcaceae* family and *Intestinimonas* spp. The microbial genera that were shared among the three chicken groups, representing a putative 'core' cecal microbiota, that is, defined as persistent members of a microbial community (Astudillo-Garcia *et al.*, 2017), which co-evolved with chickens in these different environments, were predicted to consist of 18 taxa (Fig. 2D). These core bacterial species belonged to the *Firmicutes* phylum, indicating the maintenance of a common core phylogeny under different ecological circumstances.

Prediction of the cecal microbiomes of FC

In order to evaluate the overall genetic content of the cecal microbiota of chickens, we decoded the microbiomes of three FC animals (BER, BER50 and BERXX, characterized as Bermuda Feral) by a metagenomics approach. Selection of these animals was based on the 16S rRNA microbial profiling data so as to include those birds showing cecal microbial profiles that were closest to the average of their corresponding group (Table 2). The obtained data were then compared to microbiome reconstruction information from a similar shotgun sequencing effort of three BC animals (Mancabelli *et al.*, 2016), selected to understand how anthropometric influences could drive the microbiota composition and its capability. Next Generation Sequencing of these selected six FC- and BC-samples (Table 2) produced a total of 53 219 426 raw reads that were filtered for human and poultry DNA and by quality, resulting in 13 876 919 filtered reads that were used for further analyses.

Notably, the taxonomic prediction achieved by the metagenomic analyses was consistent with that obtained by 16S rRNA profiling analysis, highlighting a preponderance in the relative abundance of bacteria belonging to the *Bacteroidetes* and to *Firmicutes* phyla in FC and BC animals respectively (Supporting Information Table S2 and Supporting Information Figure S4).

Table 2. Shotgun metagenomics raw data of FC and BC samples.

Sample name	Rearing category	Pe reads pre-filtering	Pe reads post-filtering
BER	FC	13819331	3260366
BER50	FC	19697499	5233692
BERXX	FC	18613136	4853492
P11	BC	377079	178602
P13	BC	387503	221710
P14	BC	324878	129057

Functional characterization of the feral chicken cecal microbiome

The reconstruction of the cecal microbiome of feral chickens allowed functional classification through the EggNog database (Huerta-Cepas *et al*., 2016). Data were compared with BC data sets (Mancabelli *et al*., 2016) and significant differences (p-value < 0.05) were identified for the EggNog families encompassing Transcription, Cell wall/membrane/envelope biogenesis, Carbohydrate transport and metabolism, Nucleotide transport and metabolism and Inorganic ion transport and metabolism (Fig. 3A and Supporting Information Fig. S3).

Metabolic capability to convert complex carbohydrates from diet into simpler glycans and to subsequently transform them into short chain fatty acids (SCFAs) is driven by carbohydrate degrading enzymes, such as glycosyl hydrolases (GHs), and by several enzymes belonging to the SCFA biosynthetic pathways (den Besten *et al*., 2013). *In silico* characterization of putative GHs responsible for the degradation of oligo/polysaccharides highlighted that the microbiomes of domesticated chickens (BC) possess a larger GH repertoire, in particular associated with the degradation of complex glycans derived from plant and cereals, such as mannan and (arabino)xylan (p-value <0.05) (Fig. 3B and Supporting Information Table S4). As reported previously, captive birds fed with grain were predicted to share a microbiota with increased capability for carbohydrate metabolism (Waite and Taylor, 2014). In particular, genes encoding β-galactosidases, α-amylases and β-glucosidases, encompassing members of the GH2, GH13 and GH3 families, were the most abundant in cecal microbiomes of BC (Fig 3B). These BC microbiomes were shown to be enriched in bacteria belonging to the *Firmicutes* phylum, which are able to degrade otherwise indigestible carbohydrates including cellulose and starch (Stanley *et al*., 2013).

The level and types of SCFAs that are generated in the ceca are largely influenced by the amount of starch introduced in the ceca (Roto *et al*., 2015). It is worth mentioning that the pronounced presence of genes for SCFA synthesis is related to acetate and formate production for both BC and FC animals (Fig. 3C). In this context, the FC microbiomes showed a reduced occurrence of genes predicted to encode enzymes involved in the production of formate, acetate, yet also propionate and butyrate (respectively, 11, 2.5, 4.3 and 3.6 time less) (Fig. 3C) in comparison to BC microbiomes. Indeed, only genes involved in a small number of pathways related to acetate (e.g., chitin degradation to ethanol, vanillin biosynthesis and rhamnogalacturonan type I degradation pathways) and formate production (e.g., tetrahydrobiopterin biosynthesis I and II, estradiol biosynthesis I and II, tryptophan degradation to 2-amino-3-carboxymuconate semi-aldehyde and formaldehyde oxidation) were enriched (p-value < 0.05) in the microbiomes of FC, compared to those of BC (Fig. 3D and Supporting Information Table S5).

The prevalence of genes related to carbohydrate metabolism and energy recovery from food is higher in the microbiomes of BC animals than those from Bermuda. This may be explained by the fact that farm animals have undergone a genetic selection toward maximum increase in body weight in a short time and a concurrent maximal conversion of diet into body weight. Thus, one may argue that the cecal microbiota of BC has been selected toward an enrichment of those microorganisms that perform a very efficient energy recovery from the diet (Ley *et al*., 2008). In contrast, the FC animals have reduced dimensions, are more agile and fast (Gering *et al*., 2015). Thus, microbiomes with a lower prevalence of genes related to carbohydrate metabolism and energy recovery from food, may convey a selective advantage for balancing mobility and body size.

Prediction of the resistome of feral chicken

In recent years increasing interest has emerged on how the gut microbiota may act as a reservoir for antibiotic resistance genes (ARGs) (Salyers *et al*., 2004; Hu *et al*., 2013; Yassour *et al*., 2016). In this context, the investigation of ARGs in the poultry microbiome may represent a serious global health safety issue since such ARGs may be subject to horizontal transmission to pathogenic bacteria in the gut environment (von Wintersdorff *et al*., 2016). For this reason, the FC resistome, that is, all genes predicted to be involved in antibiotic resistance (Wright, 2007), were mapped and compared with data previously obtained

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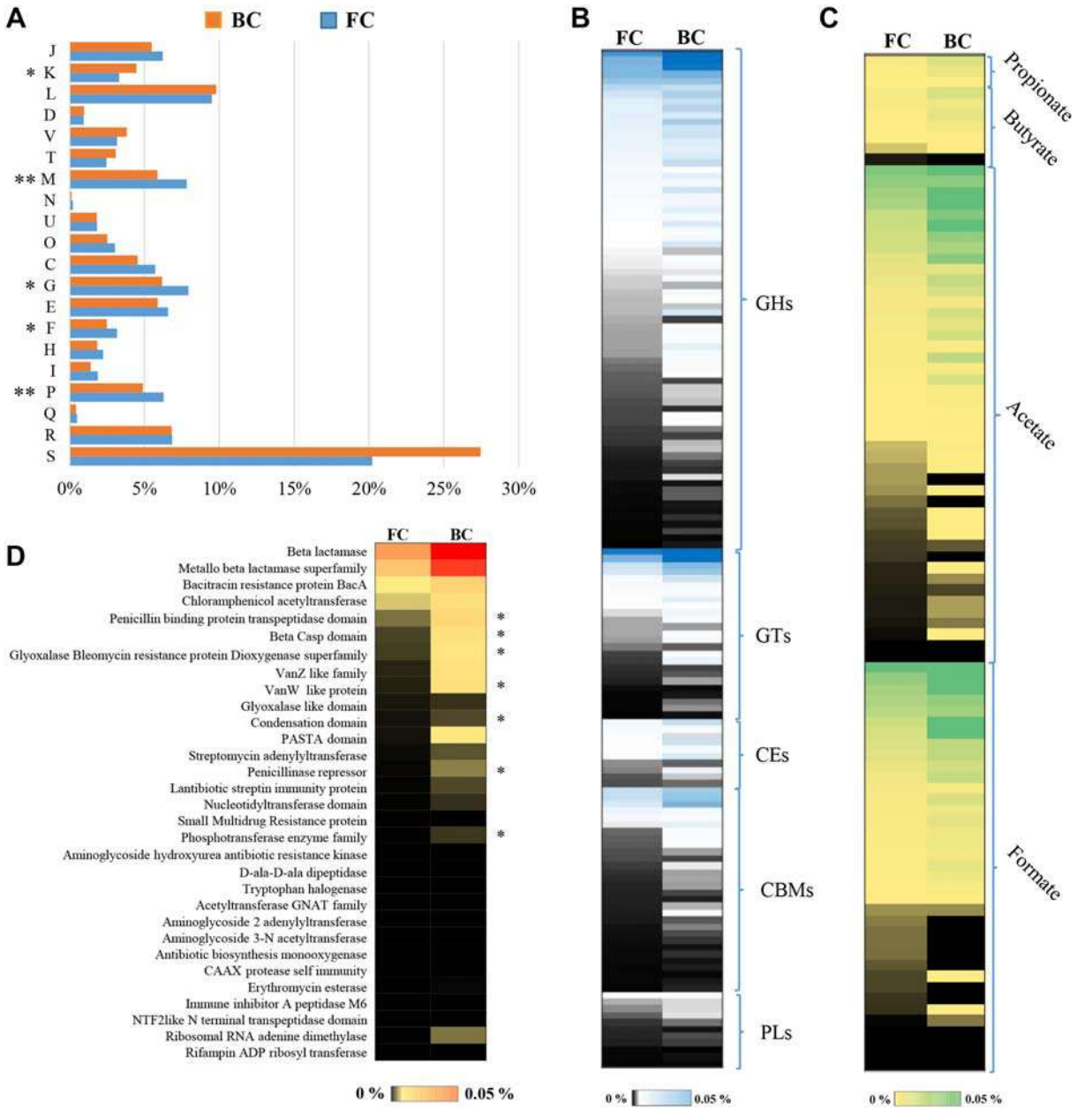

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Fig. 3. Functional changes in the cecal microbiome of BC and FC. Asterisks indicate statistically significant differences between the two data sets (p-value < 0.05).
A. The functional annotation of BC and FRC metagenomic data sets according to COG categories. Each COG family is identified by a one-letter abbreviation (National Center for Biotechnology Information database).
B. Changes in carbohydrate degradation related genes in BC and FC data sets. GH, GT, CE, CBM and PL indicate, respectively, glycosyl hydrolase, glycosyl transferase, carbohydrate esterase, carbohydrate-binding module and polysaccharide lyase.
C. A heat map reporting variation of pathways involved in formate, acetate, propionate and butyrate production in BC and FRC samples.
D. Relative abundance of predicted enzymes involved in conveying antibiotic resistance as present in BC and FC shotgun metagenomic data sets. Names of protein-encoding genes are listed on the left, while names of sample groups used are listed at the top.

from BC animals (Mancabelli *et al.*, 2016), in which an antibiotic therapy was routinely administered (Table 1). *In silico* analysis of shotgun data revealed a higher abundance (70%) of ARGs in BC compared to FC animals. Interestingly, and as reported previously (Mancabelli *et al.*, 2016), β-lactamase-encoding genes (as well as β-lactamase and metallo-β-lactamase superfamily) were the most abundant ARGs in all analysed samples (Fig. 3D), in particular in BC animals, which all had received amoxicillin, a β-lactamic antibiotic (Table 1).

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Notably, the resistome of FC animals are enriched in ARGs encoding predicted β-lactamase activity and resistance to bacitracin or chloramphenicol (Fig. 3D and Supporting Information Table S6). The presence of ARGs genes in wild animals was previously demonstrated to be related to human contact (Osterblad *et al.*, 2001; Thaller *et al.*, 2010). In this context, the feralization process with accompanying detachment from human care is correlated with the reduction of ARGs in the FC microbiome (Fig. 3D). Notably, a statistically significant difference (p-value < 0.05) was detected for genes related to β-lactamase, and resistance to bleomycin or vancomycin, with an increase of about 66%, 81% and 91% in BC samples respectively (Supporting Information Table S6).

In vitro *reconstruction of the core microbiota of FC poultry*

Culturomics approaches were applied to those cecal samples of FC and FRC animals that had been included in the FC-group (Fig. 1C and D) from the 16S rRNA gene microbial profiling. These attempts were developed in order to reconstruct the microbial community encompassing the FC cecum. A total of 26 samples were analysed, involving samples that exhibited a typical FC-type microbiota based on 16S microbial profiling results and PCoA analysis (Fig. 1D). Selective and un-selective growth conditions based on the use of different modified media as well as various growth parameters such as 0_2 availability, antibiotics, micro- and macro- nutrient availability, fresh-sample and frozen sample inoculation, were applied to assess the microbial composition of these 26 cecal samples. About 1000 colonies were picked from plates prepared for all tested cultivation conditions. Furthermore, each of these colonies was subjected to additional purification processes and taxonomic identification based on 16S ribosomal RNA gene sequencing. Among the different cultivation conditions tested, the most effective, which resulted in the isolation of about 40% of the total number of identified bacterial species-based on the microbiota analysis, was cultivation on PYG in an anaerobic atmosphere followed by incubation for five days.

These analyses allowed the reconstruction of part of the FC core microbiota (FC-microbiota) consisting of 417 strains representing 43 different species, representative of 15 genera and belonging to the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Fig. 4). The FC-microbiota isolates were dominated by *Firmicutes*,

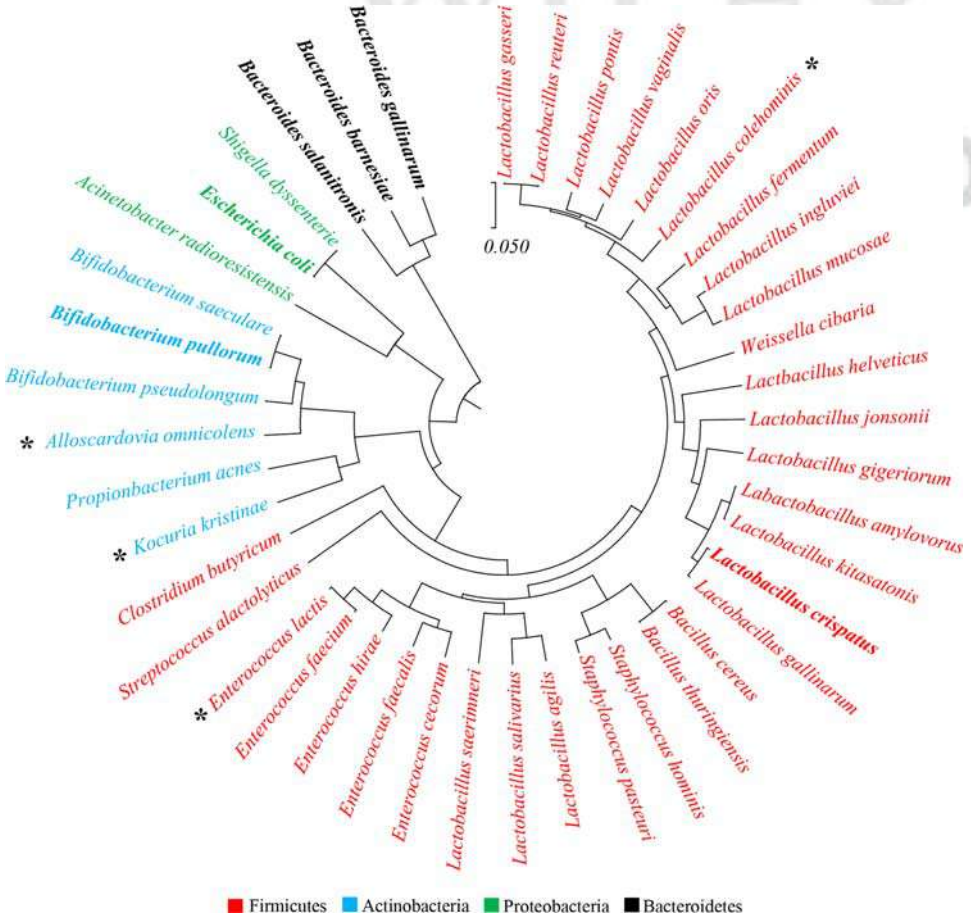

Fig. 4. Diversity of the FC poultry microbiota strain collection.
The picture shows the UPGMA phylogenetic tree based on the analysis of the sequenced 16S rRNA gene illustrating the classification of the isolated FC poultry microbiota members at species level. Only unique sequences were used. The tree is colour coded according to the phyla. Non-typical poultry isolates were marked with an asterisk. Bacterial taxa identified by culturomic and shotgun metagenomic approaches were reported in bold. The tree was generated using MEGA v. 7.0.2.

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reflecting the abundant presence of this phylum in the poultry intestine. In detail, *Lactobacillus* spp., *Enterococcus* spp. and *Escherichia* spp. were the most frequently isolated bacterial genera of FC-microbiota, representing 53%, 10% and 2.5% of the isolates respectively. However, only a small number of isolates were classified as belonging to the *Bacteroidetes* phylum (Fig. 4). Arguably, conservation, manipulation or cultivation procedures of samples have impacted on the persistence of members of this bacterial taxon, apparently being particularly sensitive to the applied laboratory growth conditions, thus reducing the variety of isolated bacterial species.

The overall biodiversity of the FC-microbiota based on these culturomic analyses allowed us to reconstruct the phylogenetic tree shown in Fig. 4.

Interestingly, of the 420 strains forming the FC-microbiota, four taxa, that is, *Alloscardovia omnicolens*, *Enterococcus lactis*, *Kokuria kristinae* and *Lactobacillus colehominis*, representing approximately 4% of the number of taxa of FC-microbiota, have never been isolated from poultry previously, while the other strains are typical inhabitants of the chicken intestine (Collado and Sanz, 2007; Stanley *et al.*, 2014).

To assess the relevance of the culturomic approach reported here, a comparison between the cultivation-based method and high-throughput shotgun sequencing data was performed. Only six of the 43 species identified with culturomic-based methods were detected in shotgun results (about 15% of the total species detected by culturomics). Interestingly, these six bacterial species were spread across the four phyla identified using culture-based methods (Fig. 4).

Notably, comparison of 16S rRNA microbial profiling data with those achieved with culture-based approaches, highlighted that 60% of the bacterial genera detected with the culturomics approach were also identified with molecular methods. In contrast, just 3% of the bacterial genera identified by the 16S rRNA microbial profiling approach were isolated from our cultures of poultry cecal samples.

These findings highlight a substantial discordance between the sets of bacteria identified by these two approaches, at both genus and species level (Lagier *et al.*, 2016). Indeed, the ability of high throughput cultivation-based approaches to isolate bacteria from environmental samples that are not detected in genomic and metagenomic studies is far from effective. At the same time, large-scale molecular studies have a detection limit that prevents the detection of microorganisms that are present below a particular threshold (Lagier *et al.*, 2012).

Conclusions

Farm animals that undergo feralization follow a reverse domestication process, where they may gain certain traits that reflect their ancestors, while maintaining others that had been selected by humans such as brain dimension and organization, plumage and animal size (Gering *et al.*, 2015; Callaway, 2016; Johnsson *et al.*, 2016). Such animals also represent an intriguing example of microbiota evolution subsequent to human influences, which include changes in environment, diet and host genetics. To investigate how feralization modulates the microbiome, *in silico* and *in vitro* approaches were used to decipher the cecal microbiota of FC animals originated from Bermuda (and compare them to domesticated BC).

Notably, despite the fact that these feral animals live on an island, are not exposed to human husbandry, and follow a 'natural' diet, the Bermuda Poultry, that is, Feral, Free-range broilers and layers, microbiota displayed a composition that still resembles that of FRC cecal samples collected in very different and distant geographical regions (Mancabelli *et al.*, 2016). Interestingly, the reconstructed microbiota of FRC and Bermuda animals were shown to be characterized by a higher abundance of strains belonging to the *Bacteroidetes* phylum, being clearly different from the cecal microbiota of domesticated animals (i.e., BC). Furthermore, analysing the cecal microbiota of FC, BC and FRC, 18 taxa belonging to the *Firmicutes* phylum were present in all samples, thus forming a proposed core microbiota. Although the low number of analysed FC cecal samples may represent a limitation of this study, the interesting results obtained prompted us to sequence total DNA of Bermuda Feral cecal samples through a shotgun metagenomics approach. The functional microbiota characterization highlighted a selection in FC microbiota of those bacterial genera that are less efficient for energy recovery (or host weight gain), when compared to BC, yet carry a lower number of genes related to antibiotic resistance.

In addition, we performed a first attempt to reconstruct the cecal microbiota of FC. Culturomics approaches were applied to *in vitro* reconstruct the microbiota of FC animals, providing comprehensive culture conditions simulating or mimicking the environmental conditions present in the cecum of chickens. This approach enabled a large-scale cultivation of bacteria and the generation of a unique strain collection of about 420 isolates, including bacterial strains that are not typically retrieved from the chicken gut, and representing the four major bacterial phyla present in the cecal microbiota of feral poultry. Many efforts will still be needed to obtain a complete bacterial collection representing the complexity of the poultry microbiota, but these 15 samples are a starting point to understand the evolution of this bacterial community. Additional feral samples collected from other geographical locations as well as further growth media/cultivation conditions may result in a more complete culture collection of the feral chicken cecal microbiome.

Experimental procedures

Ethical statement

Collection and export of samples derived from feral chickens was approved by the Bermuda Department of Environment and Natural Resources in support of the Bermuda Biodiversity Project. The samples manipulation and DNA extraction protocols were approved by the 'Comitato di Etica Università degli Studi di Parma', Italy. All procedures were performed in strict compliance with national guidelines (Decreto legislativo 26/2014) on the protection of animals used for scientific purposes.

Animals, sampling and DNA extraction

A total of 15 chickens from St. George's island, Bermuda, were investigated (Table 1). Ten animals were reared under natural conditions, without human control or interference, and therefore categorized as Bermuda Feral. Bermuda supports a large, self-sustaining population of feral chickens that have been living in the wild since at least the mid-1980s. Based on preliminary genetic and morphological analyses, these birds appear to be an admixed flock originating from several breeds that are popular sources of meat and eggs in the Western hemisphere. Our ad hoc observations of gut content suggest a highly variable diet including locally-occurring invertebrates (e.g., snails and insects), local ornamental and/or natural vegetation (e.g., seeds and shoots) and garbage from Bermuda households and businesses. We also sampled five birds from a Bermuda farm that were categorized as FRC since some human impact (e.g., shelter, ad libitum formulated diet and vaccination) was present. Two of these (BER 100–101, Free-Range Bermuda Broilers) were broiler breed males raised in a common, large outdoor enclosure and fed an ad libitum diet of growth-formulated feed. Three female brown pullets (BER 102–104, Free-Range Bermuda Layers) were also included that were housed in coops with an outdoor run, immediately adjacent to the broilers. All of the individuals from the Bermuda farm originated from a hatchery on the mainland US, where they were vaccinated for Marek's Disease Virus.

Cecal samples were obtained, kept under anaerobic conditions, transferred to the laboratory and maintained at −80°C until processing. We selected the ceca as organs of particular interest as they harbour the highest microbial cell densities (up to 10^{11} cells g^{-1}), have the longest residence time (12–20 h) of digesta in the gastrointestinal tract, and are important sites for carbohydrate fermentations, water regulation and recycling of urea (Oakley *et al.*, 2014; Sergeant *et al.*, 2014; Waite and Taylor, 2014). For DNA extraction, after removal of the digesta, 0.2 g of sample, composed of parts of both cecal pouches, were removed and briefly washed with Ringer's solution (Sigma, Italy) 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, USA).

16S rRNA gene amplification and MiSeq sequencing

Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni and/Probio_Rev, which target the V3 region of the 16S rRNA gene sequence (Milani *et al.*, 2013). Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons, which were further processed using the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B – Illumina; see also below) as previously reported (Mancabelli *et al.*, 2016). Purified amplicons were diluted to 4 nM and 5 μl aliquots of each diluted DNA amplicon were mixed to prepare the pooled final library. Sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals.

16S rRNA-microbial profiling analysis

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

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 NextSeq 500 sequencer with NextSeq Mid Output v2 Kit chemicals.

Analysis of metagenomic data sets

The generated fastq files were filtered for reads with a quality score of < 25, for sequences of chicken genomic 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 AQ2 sequence reads for significant identity to known antibiotic resistance genes (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 acid sequences of enzymes involved in antibiotic resistance. Reconstruction of glycosyl hydrolase profiles and bacterial metabolic pathways, and evaluation of their abundance in the shotgun metagenomic data sets 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.

In vitro *reconstruction of poultry microbiota*

For microbiota cultivation, different media were assessed, including Proteose Yeast Glucose (PYG) agar modified media (Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und

Zellkulturen GmbH) with modifications (0.5% resistant starch, 0.05% pectin from citrus peel and 0.25% inulin), YCFA agar (Duncan *et al*., 2002) supplemented with 0.2% of glucose, maltose and cellobiose, brain heart infusion (BHI) agar modified (10 mg L^{-1} hemin, and 1 mg L^{-1} vitamin K, 0.05% L-cysteine hydrochloride), de Man, Rogosa and Sharpe (MRS) agar supplemented with 0.05% L-cysteine hydrochloride and Chicken cecal medium (CCM), based on VL broth prepared as reported by Nisbet and colleagues (1993) and added with a chicken cecal content solution (autoclaved at 121°C for 15 min, 5% w/v) at a final concentration of 1% w/v. Finally, PYG medium was also supplemented with streptomycin (16 μg ml^{-1}) or vancomycin (2 μg ml^{-1}) after autoclaving.

All reagents were placed in the anaerobic cabinet (Ruskin, in which the atmosphere consisted of 17% CO_2, 80% N_2 and 2.99% H_2) for 24 h prior to use in order to create anaerobic conditions.

Cecal samples were obtained from animals categorized as FC-group members after 16S rRNA microbial profiling. All samples were processed immediately after their arrival at the laboratory.

About 0.2 g of cecum samples were washed with phosphate buffer solution (pH = 7.0), 10-fold diluted and then inoculated in all media previously described. Plates were incubated at 37°C for 5 days in anaerobic conditions, in 5% CO_2 and in aerobic conditions. Colonies were randomly picked, pure-cultured and DNA was extracted from each isolate through rapid mechanic cell lysis as described previously (Turroni *et al*., 2009). Isolates from each sample were stored at −80°C in the presence of glycerol (30%, v/v).

Taxonomic identification of FC isolates

&&Identification of each isolate was performed by PCR amplification of a portion of the 16S rRNA gene using primers P0 (5′-GAAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′). Each 25 μl PCR reaction contained approximately 30 ng of genomic DNA, Platinum PCR SuperMix 1X (Invitrogen, USA) and 100 pM of each oligo. PCR reactions were performed on a Verity Thermocycler (Applied Biosystems, USA). Electrophoretic profiles were visualized by SYBR Safe DNA gel stain (Invitrogen). PCR product purification was performed using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following manufacturer instructions.

Each 16S rRNA gene thus generated from individual colonies originating from fecal samples was sequenced and it was then subjected to a BLAST search against the GenBank database.

Statistical analyses

ANOVA and PERMANOVA analyses were performed using Tuckey HSD post hoc test. All statistical analyses were performed with SPSS software (www.ibm.com/software/it/analytics/spss/).

Data deposition

16S rRNA-based microbial profiling data sets obtained in this study were deposited in SRA under accession numbers SRP114489. Shotgun metagenomic data sets are accessible through SRA study accession number SRP114492.

Acknowledgements

This work was funded by the EU Joint Programming Initiative – A Healthy Diet for a Healthy Life (JPI HDHL, http://www.healthydietforhealthylife.eu/) to D.v.S. (in conjunction with Science Foundation Ireland (SFI), Grant number 15/JP-HDHL/3280) and to M.V. (in conjunction with MIUR, Italy). We thank GenProbio srl for financial support of the Laboratory of Probiogenomics. L.M. is supported by Fondazione Cariparma, Parma, Italy. D.v.S. is a member of The APC Microbiome Institute funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan (Grant number SFI/12/RC/2273). The authors declare that they have no competing interests. Part of this research is conducted using the High Performance Computing (HPC) facility of the University of Parma.

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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 chicken samples at phylum level, encompassing BC, FRC and FC animals. Sample categorization in FRC, BC or FC was performed subsequent to obtaining the results from the PCoAs as reported in Figure 1c and d. U. m.: unclassified member.
Fig. S2. 16S rRNA-microbial profiling of the chicken samples at family level, encompassing BC, FRC and FC animals. Sample categorization in FRC, BC or FC was performed subsequent to obtaining the results from the PCoAs as reported in Figure 1c and d. U. m.: unclassified member.
Fig. S3. 16S rRNA-microbial profiling of the chicken samples at genus level, encompassing BC, FRC and FC animals. Sample categorization in FRC, BC or FC was performed subsequent to obtaining the results from the PCoAs as reported in Figure 1c and d. Only taxa with a relative abundance of $>0.5\%$ are shown. U. m.: unclassified member.
Fig. S4. Taxonomic data at phylum level obtained from shotgun metagenomics of FC and BC cecal samples.
Table S1. 16S rRNA microbial profiling data.
Table S2. Bacterial taxonomy from shotgun metagenomics sequencing of FC and BC.
Table S3. Functional annotation of FC metagenomic data sets according to COG categories. Each COG family is identified by a one-letter abbreviation
Table S4. Carbohydrate degradation related enzymes of FC microbiomes.
Table S5. Presence of pathways involved in formate, acetate, propionate and butyrate production in FC microbiomes.
Table S6. Presence of predicted enzymes involved in antibiotic resistance in FC shotgun metagenomics data set.