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Investigating the cecal microbiota in broiler poultry farms and its potential relationships with animal welfare

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ABSTRACT

The present study assessed the modulation of cecal microbiota and correlations with *Campylobacter* colonization and animal welfare status. For these purposes, we conducted a cross sectional study of the cecal microbiota from 187 broilers reared in 13 batches from 10 poultry farms by performing 16S rRNA sequencing (regions V3–4). The welfare of each batch was assessed using a simplified Welfare Quality® protocol, scoring higher in organic batches, compared to both antibiotic-free and conventional batches. The bioinformatics analyses were conducted in QIIME 2 and a linear discriminant analysis determined the association between microbiota and animals with different *Campylobacter* carriage status and welfare levels. In the microbiota from the subjects negative for *Campylobacter* or with high welfare scores, *Bacteroidetes* was the predominant phylum with the genus *Megamonas* significantly increased in abundance. A greater abundance of *Parabacteroides*, *Phascolarctobacterium*, *Helicobacter* in poultry negative for *Campylobacter* was also found at the genus level. Animals with the lowest welfare scores showed an increased abundance of *Proteobacteria*. The results suggested a different microbial composition and diversity in the analyzed groups.

1. Introduction

Campylobacter jejuni is the most important cause of human campylobacteriosis (Dearlove et al., 2016; Hazards, 2011; Ono and Yamamoto, 1999; Skarp et al., 2016), being the leading foodborne infection in Europe. The disease is characterized by self-limiting gastroenteritis with abdominal pain and diarrhea, and sometimes rare neurological complications including Guillain-Barré and Miller-Fisher syndromes (Ang et al., 2001).

Chickens are the main reservoir of *C. jejuni* that can colonize the chicken gut asymptomatically but very rarely do chickens develop pathological lesions (Bronzwaer et al., 2009; Frost, 2001; Hermans et al., 2012; Pielsticker et al., 2012). In many countries, the prevalence of drug-resistant *Campylobacter* has increased in recent years, becoming a significant public health concern (Blaser and Engberg, 2008). Biosafety measures are crucial to reduce *Campylobacter* on farms, and prevent contamination of poultry products in order to obtain a significant reduction of campylobacteriosis in humans (Russa et al., 2005). The *Campylobacter* colonization in broiler chickens happens at the farms during rearing and is age-dependent. Broiler chicken less than two to three weeks old are rarely colonized naturally by *Campylobacter* (Stern et al., 2001). The prevalence within the farms reaches almost 100% but the time and the individual colonization may vary widely (Federighi, 2017; Newell, 2002). The sources of infection include contaminated drinking water or fomites, personnel, infected livestock or free-living animals (Blaut and Clavel, 2007). Nevertheless, colonization of broiler chickens by *Campylobacter* could be influenced by competitive exclusion culture containing effective microbial strains, which could be an efficient approach to reduce the abundance of undesired or pathogenic bacterial strains (Conlan et al., 2007). Moreover, the microbiota has a profound impact on the gut immune system and on the host reaction to bacterial colonization (Broom and Kogut, 2018).

Recent studies that demonstrated the existence of a connection between microbial communities and host social behavior and animal health have proven that microbiota plays a crucial role in the lives of animals (Archie and Theis, 2011; Davis et al., 2013; Ezenwa et al., 2012;

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Ezenwa and Williams, 2014; Lize et al., 2013; Lombardo, 2008; Stilling et al., 2014). Several of these roles are exerted by microbial metabolism in releasing volatile substances detectable to animals, and because the host behavior can influence the composition of microbiota, microbial communities have the potential to communicate significant information about animals (Archie and Theis, 2011; Ezenwa et al., 2012; Ezenwa and Williams, 2014). Furthermore, microbes can directly influence the host nervous system to increase microbial transmission, manipulating host behavior for the benefit of their own survival (Alcock et al., 2014).

Animal welfare is a factor considered more and more crucial in livestock productions, concerning the quality of the meat but also regarding the safety of the consumers. According to the last Animal Welfare Global Strategy of the World Organization for Animal Health (OIE), more attention to animal welfare in food production systems can improve productivity, quality, food safety, and economic returns (OIE, 2017). In fact, ethical, economic and zoonotic aspects related to farm should be seen as complementary, and all tightly linked to the welfare of these animals. Several studies on animal welfare have discussed functional implications of the gut microbiota, not only on immunity, growth and metabolism of the host, but also on brain development and behavior. It would be interesting to explore the poultry microbiota compared in different levels of farms welfare in meat production (Kraimi et al., 2019). Very recently, a study has observed the presence of statistically significant relationship between the animal welfare level, measured with the Welfare Quality® protocol, and the contamination of poultry meat with *Salmonella* and *Campylobacter* (Iannetti et al., 2020). The effects of the animal welfare on the composition of broiler chicken gut microbiota and the consequent competition between different bacterial species could be one reason for this condition. However, more studies focusing on the gut microbiota composition in relation to the animal welfare scores of different batches were still needed to shed some light on the biological mechanisms that influence bacterial shedding and microbiological contamination of poultry meat.

The aims of this study were to explore broiler chicken microbiota and the relationship with the animal welfare and *Campylobacter* colonization in broiler poultry farms. Classical microbiological analyses were used to determine the *Campylobacter* colonization status of individual chickens sampled at the slaughterhouse. Moreover, the welfare of the animals was analyzed in different farms based on level of welfare considering feeding, housing, health and appropriate behavior. Then the results were correlated with the compositional differences of the cecal microbiota. We identified specific microorganisms that potentially negatively correlated the presence of *Campylobacter* in poultry farms, or are linked to animal welfare.

2. Materials and methods

2.1. Sample selection

One-hundred-eighty-seven Ross 308 broiler chickens, aged 6 (n = 165) and 12 weeks (n = 22), were randomly chosen from batches with commercial destination, all made available by the same private poultry company and included in the study. The animals belonged to 10 poultry farms (farm: premise where groups of animals – batches - are reared at different times, in different poultry houses) and 13 different batches (batch: group of animals reared in the same conditions, in the same poultry house, during the same period of time) located in 4 regions of Northern and Central Italy (Veneto - 45°17′N 11°38′E; Emilia-Romagna - 44°08′N 12°04′E; Marche 43°22′N 13°12′E; and Abruzzi - 42°21′N 14°24′E), including 7 conventional batches, 3 organic batches and 3 antibiotic-free batches. The total number of animals in each batch ranged from 5973 to 28,976.

The area of the poultry houses where batches were kept ranged from 675 to 2160 square meters. Birds from the same farms were always from the same hatchery.

As concerns the rearing system, organic batches were certified by an independent certification body to be compliant to regulations EC 834/2007 (Anonymous, 2007) and 889/2008 (Anonymous, 2008), that provide specific housing condition and husbandry practices for organic broilers including, among other requirements: access to open air area for at least one/third of life, poultry houses with a maximum of 21 kg liveweight/m2, no more than 4800 animals per poultry house or more only if separated in groups, age at slaughter of at least 81 days. Poultry houses were mechanically ventilated with controlled temperature, however they were structured so that, when needed, it was possible to disable the mechanical ventilation system and open exit/entry pop-holes on one side of the building in order to give to the animals the possibility to access to the open air areas. These open air areas were mainly covered with vegetation; when in open air, the animals had always the possibility to find shelter and food in the nearby poultry house. According to regulation EC 834/2007, only organic and GMO-free feed (GMO: Genetic Modified Organism) was given to the organic batches; moreover, the organic poultry included in this study was fed only with feed of vegetal origin.

On the other hand, no mandatory legislation was in force relating the production of antibiotic-free broiler batches, all provisions were voluntarily taken by the producer, and precisely: no use of antibiotics, coccidiostats and chemical antibacterial during the whole life of the animals; moreover, only feed of vegetable origin was used. For the rest, no clear differences were highlighted in the management of antibiotic-free batches compared to conventional ones, as they were kept in mechanically ventilated poultry houses with controlled temperature and without access to open air areas.

Each batch was named with an anonymous code including a letter indicating the farm of origin (A, B, C, D, E, F, G, H, I, J), a progressive number indicating the batch sampled in that farm (1 or 2), and the abbreviation of the type of management system (AF for “antibiotic-free”, Conv for “conventional” and Org for “organic”). Batches belonging to different management systems were always reared in different poultry houses and at different times, but were, in certain cases, reared in the same farms. In particular, Farms A, D, and G reared both conventional and antibiotic-free batches. All the other farms reared only organic (B, E, H) or conventional (C, F, I, J) batches. For example, the first batch, antibiotic-free, sampled in farm A was named A_1_AF, while the second batch, conventional, sampled in farm A was named A_2_Conv.

Samples were taken from the slaughtering processing chain, at the moment of evisceration. Slaughtering was carried out at the age of 44 days (6 weeks) in the antibiotic-free and conventional batches and at the age of 84 days (12 weeks) in the organic batches. All the sampled animals were males. The females, only present in the conventional batches, were removed at the age of about 30–32 days to be destined to the production of rotisserie chicken.

During the slaughtering process, ceca were removed from the carcasses and transported to the laboratory in a portable cooler at 2–4 °C for immediate processing, and however no later than 6 h after sampling.

2.2. Animal welfare evaluation

The Welfare Quality® protocol (1), modified according to De Jong et al. (de Jong et al., 2016), was applied for the evaluation of each single batch (13 batches) enrolled in the study. The data was collected on farm the day before the slaughtering, then integrated at the slaughterhouse with the data related to diseases and lesions reported from carcass inspection. The protocol is based on the internationally recognized “five freedoms” according to the World Organization for Animal Health (OIE). Animal welfare measures used in the protocol are based on 4 animal welfare principles (good feeding, good housing, good health, appropriate behavior), and further divided into 12 criteria, 9 of which applied in the conditions of this study and were therefore evaluated. In Table 1 are detailed all criteria, and relative measures, that were considered for the animal welfare assessment. These criteria are animal-based (observations of the animal response to the environment) and resource-based

Table 1
List of the principles, criteria and relative measures that were assessed for animal welfare evaluation in each batch, according to the Welfare Quality® protocol for broilers.

Principles	Criteria	Measures
Good feeding	Absence of prolonged thirst	Drinker space
	Absence of prolonged hunger	Percentage of emaciated carcasses at slaughterhouse
Good housing	Comfort around resting	Plumage cleanliness, litter quality, dust sheet test
	Thermal comfort	Panting, huddling
	Ease of movements	Stocking density
Good health	Absence of injuries	Lameness (gait score), hock burns, foot pad dermatitis
	Absence of diseases	On farm mortality, culls on farm, percentage of carcasses at slaughterhouse with signs of disease
Appropriate behavior	Good human-animal relationship	Avoidance distance test
	Positive emotional state	QBA (qualitative behavior assessment)

(evaluation of the structures and the environment where the animals are kept and evaluation of their management), and include measures such as number of drinkers available per animal, stocking density, cleanliness of the plumage, intensity of foot pad dermatitis and hock burns, quality of litter and mortality. The evaluation of each batch required about two hours; further evaluation at the slaughterhouse included the percentage of carcasses showing signs of emaciation (included in the "good feeding" principle) and the measurement of the percentage of carcasses with signs of diseases such as ascites, dehydration, septicaemia, hepatitis, pericarditis, abscess (included in the "good health" principle). One score for each of the four animal welfare principles was therefore produced for each batch, ranging from 0 to 100. A general animal welfare score was also calculated according to Tuyttens et al. (Tuyttens et al., 2015) and assigned to each batch. This score is composed by the sum of the scores assigned to each of the four animal welfare principles (maximum score 100) and can range from 0 to 400. Data were analyzed with the statistical software XLstat (Addinsoft, Belmont, USA), to verify the presence of statistically significant differences between welfare scores of each group of batches (conventional vs antibiotic-free vs organic). The analysis was carried out considering both the total welfare score and the four principles that compose it (good feeding, good housing, good health, appropriate behavior), individually considered. A *t*-test for independent means was used, with significance set at $p < 0.05$.

2.3. Campylobacter detection

The samples were processed immediately at arrival in the laboratory. The cecal content was harvested and *Campylobacter* detection was carried out according to part 1 of the ISO 10272-1:2017 method (ISO, I.F.-I.O.F.S, 2017). Briefly, the cecal content was mixed with a loop and plated directly on mCCD agar and on Karmali agar. The selective solid media were incubated at 41.5 °C in a microaerobic atmosphere and examined after 44 h to detect the presence of colonies. The suspect *Campylobacter* colonies were examined for morphology and motility using a microscope and sub-cultured on a non-selective blood agar, and then confirmed by detection of oxidase activity. Finally species confirmation for the isolates was performed by multiplex PCR as described by Wang (Wang et al., 2002), using 50 µl volumes containing 25 µl PCR Master Mix 2× (Promega Corporation, Madison, WI, USA), 25 mM $MgCl_2$, 0.5 µM *C. jejuni* and *Campylobacter lari* primers; 1 µM *Campylobacter coli* and *Campylobacter fetus* primers, 2 µM *Campylobacter upsaliensis* primers 1 ng of genomic DNA/µl. DNA amplification was carried out in a DNA thermal cycler 9700 Applied Biosystems (Applied Biosystems, Foster City, CA, USA). As as positive controls *C. coli* NCTC 11353; *C. fetus* ATCC 19438; *C. jejuni* ATCC 33291; *C. upsaliensis* NCTC 11541 and *C. lari* NCTC 11552 were used, while as negative controls Nuclease-free water was used. PCR results were analyzed on 1.5% agarose gels and gel stain Sybr Safe DNA gel (Invitrogen, Carlsbad, CA, USA).

2.4. DNA extraction from cecal samples

The samples were weighed (about 0.2 g of cecal content was used). Two-hundred µl of water as negative controls was included with every batch of extraction. The material was placed in a sterile, round-bottom 2 ml tube, 1 ml InhibitEX Buffer was added to each cecal content sample. The tubes were vortexed continuously for 1 min or until the cecal content sample was thoroughly homogenized then centrifuged at the maximum speed for 1 min. Then, 25 µl of Proteinase K (> 600 mAU/ml, solution) was added to 600 µl of supernatant, vortexed and incubated for 10 min at 70 °C. DNA extraction was then completed using the Qiagen™ QIAamp DNA Stool Mini Kit (Hilden, Germany) following the manufacturer's instructions. Extracted DNA was quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Celbio Srl., Milan, Italy). For each sample the amount of DNA ranged between 0.150 and 1 µg with a concentration higher than 5 ng / µl. DNA was stored at −20 °C until further use.

2.5. 16S rRNA gene amplification, and Illumina MISeq sequencing

Hypervariable regions V3-V4 of the 16S rRNA gene were amplified for cecal samples and negative controls consisting of nuclease-free water using the universal primers with Illumina adapters suggested by Klindworth et al., 2013 (Klindworth et al., 2013). PCR products were visualized on an agarose gel. The negative controls showed no visible bands thus were not submitted to sequencing. Sequencing was carried out using an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA) with 600 cycles (300 cycles for each paired read and 12 cycles for the barcode sequence) according to the manufacturer's instructions. Binary Base Call (BCL) raw data files generated by the Illumina sequencers were converted and demultiplexed in a single step by bcl2fastq software generating a new directory in the Run folder which contains all of the demultiplexed compressed FASTQfiles. The bcl2fastq software combined these per-cycle BCL files from a run and translated them into FASTQ files. Standard Illumina barcodes were used.

2.6. Bioinformatics and statistics

The 16 s rRNA gene amplicon data were analyzed with the QIIME2 software suite v2019.1 (Caporaso et al., 2010). The raw FASTQ data were imported using the "Fastq manifest" command creating a text file called a "manifest file". Sequences were denoised with DADA2 pipeline removing also chimeric sequences (Callahan et al., 2016).

The obtained sequences were BLAST searched against the Greengenes database (greengenes.lbl.gov) to determine the phylogeny of the OTUs. Taxonomy was assigned using the Naïve Bayes classifier trained on Greengenes 13_8 99% database and sequences were clustered into operational taxonomic units (OTUs) (available at https://data.qiime2.org/2018.4/common/gg-13-8-99-nb-classifier.qza).

A rarefaction analysis was performed using Faith pd., Shannon and observed OTUs indexes to determine the completeness of the microbial communities. Then we computed the alpha indexes observed OTUs and Chao1; and beta diversity metrics with unweighted UniFrac distance, weighted UniFrac distance. To explore the principal coordinates (PCoA) plots emperor tool was used in the context of sample metadata, generating Emperor plots for Unweighted UniFrac distance (Vazquez-Baeza et al., 2013).

Group significance between alpha and beta diversity indexes was calculated with the Kruskal–Wallis (pairwise) test, the beta-group-significance command testing the distances between samples within a group, and the statistical analysis was carried out using PERMANOVA

(Anderson, 2001) with 999 permutations (beta-group-significance command in *diversity* plugin), respectively.

The linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/lefse/) was used to characterize the microorganism features, the statistical significance and biological relevance. LEfSe method uses the Kruskal-Wallis rank sum test to detect features with significantly different abundances between assigned taxa, and the effect size of each feature was estimated by LDA. LEfSe with default parameters was applied (alpha value was set at 0.05, the logarithmic LDA score threshold set at 3.0) (Segata et al., 2011).

3. Results

3.1. Broiler chicken microbiota

Two MiSeq runs were made to generate the data then the raw sequences were merged for analysis. The Illumina MiSeq paired-end sequencing produced 42,633,792 raw reads. The quality filtering and denoise step with DADA left 31,623,475 reads with a mean of 169,109 reads per sample. A total of 18,970,850 sequences were clustered into a total of 15,079 OTUs at 99% sequence similarity grouped into phylum, classes, orders, families, and genera (Supplementary material 1). For the uniform comparison of the samples, the dataset was rarefied at 14,453 corresponding to the minimum number of reads obtained in the experiment, retaining 2,702,711 (14.25%) sequences in 187 (100.00%) samples at the specified sampling depth (Supplementary material 1). The rarefaction based on the Faith pd. index indicated that a sampling depth of 14,453 sequences was sufficient to fully observe the richness of the samples, which was further confirmed using the Shannon index and number of observed OTUs (Fig. S1, S2, S3 Supplementary material 2). The chosen sampling depth did not exclude any sample from the analyses.

According to the sequence data, the broiler cecal microbiota consisted mainly of phyla: *Firmicutes* (48.22%), *Bacteroides* (31.95), *Proteobacteria* (11.94%), *Cyanobacteria* (3.28%) and *Tenericute* (1.15%) (Fig. S4 in Supplementary material 2)(Supplementary material 1). Four families—*Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae* and *Bacteroidaceae*—predominated in the bacterial populations with different relative abundance depending on the experimental conditions.

The richness of the bacterial population was found significantly different comparing most of the farms as shown by the Chao and OTUs' indexes. Moreover, the segregation according to the farms was clear based on the beta diversity and the unweighted UniFrac distance and the Principal Coordinate Analysis (PCoA)(data not shown). Interestingly, batches belonging to the same farm revealed microbiota with similar alpha and beta indexes. The differences in the microbiota with alpha diversity indexes was also observed between conventional and antibiotic-free raising systems. No significant differences were observed in bacterial community in the chickens at 44 days (6 weeks) and 84 days (12 weeks) (Fig. 1c) (Supplementary material 1) while in the farm type analyses Antibiotic-free animals versus Conventional and Organic were statistically significantly different with 0.002 and 0.003 *p*-values respectively (Fig. 1d) (Supplementary material 1). Beta diversity was statistically significantly different comparing animal by Age and Farm Type (Pairwise PERMANOVA p-value =0.001) (Fig. 2c, d) (Supplementary material 1).

3.2. Microbiota and Campylobacter colonization

The results of *Campylobacter* detection, including the number of animals sampled per each batch, are detailed in Table 2. *Campylobacter* was isolated in all the farms visited but 35 broiler chickens tested negative for the bacterium. The individual prevalence of *Campylobacter* spp. was 81.2%. According to the microbiological isolation results, the tested population was split in the following two groups: *Campylobacter* positive animals (CPs) and *Campylobacter* negative animals (CNs).

Chao1, and the observed OTUs' indexes indicated that there were significant differences among the microbiota in CNs vs CPs, with CNs showing higher microbiota richness, which was found significant according to the Kruskal-Wallis test (*p*-value = 0.000144)(Fig. 1a)

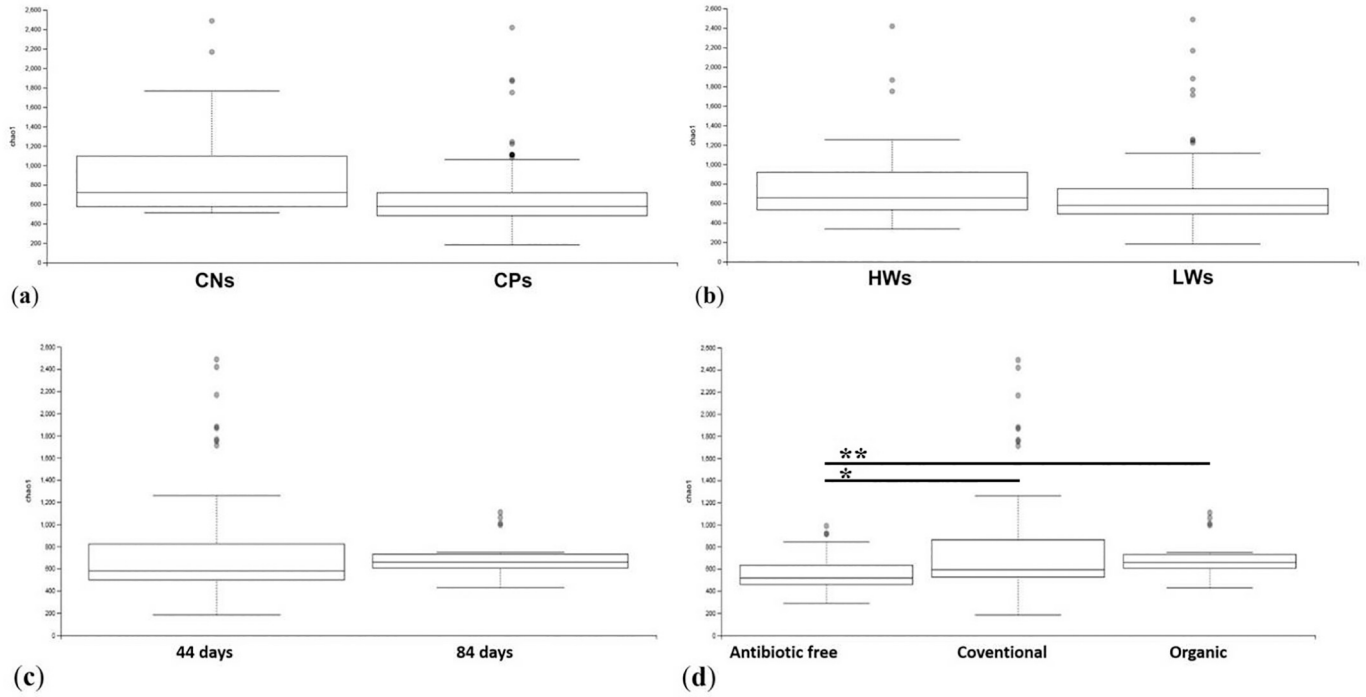

Fig. 1. Alpha diversity estimation, Chao1 diversity index (a) CPs (*Campylobacter* Positive Samples) Vs CNs (*Campylobacter* Negative Samples). (b) HWs (high welfare animals) and LWs (low welfare animals) group. (c) 44 Vs 84 days group. (d) Antibiotic-free Vs Conventional and Organic, statistically significant represented by "*" and "**", not indicated when not significant.

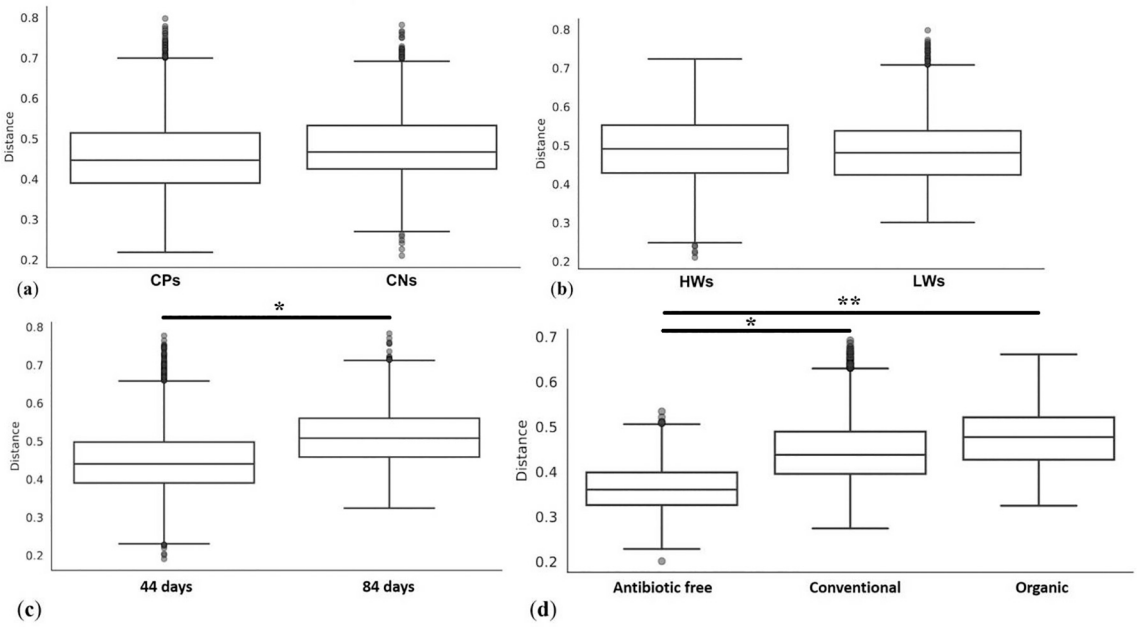

Fig. 2. Beta diversity results. (a) CPs (*Campylobacter* Positive Samples) and CNs (*Campylobacter* Negative Samples) groups. (b) HWs (high welfare animals) and LWs (low welfare animals) groups. (c) Beta diversity index of 44 and 84 days groups, statistically significant represented by "*". (d) Antibiotic-free, Conventional and Organic groups, statistically significant represented by "*" and "**", not indicated when not significant.

Table 2
CPs raw prevalence of considered animals.

Batch	Sampled animals	CP	CN	Prevalence (%)	95% LCL	95% UCL
A_1_AF	15	15	0	100,0%	79,4%	100,0%
A_2_Conv	15	15	0	100,0%	79,4%	100,0%
B_1_Org	8	5	3	62.5%	29,9%	86,3%
C_1_Conv	12	12	0	100,0%	75,3%	100,0%
D_1_Conv	15	12	3	80,0%	54,4%	92,7%
D_2_AF	15	15	0	100,0%	79,4%	100,0%
E_1_Org	6	6	0	100,0%	59,0%	100,0%
F_1_Conv	16	11	5	68.75%	44,0%	85,8%
G_1_Conv	15	15	0	100,0%	79,4%	100,0%
G_2_AF	15	15	0	100,0%	79,4%	100,0%
H_1_Org	8	8	0	100,0%	66,4%	100,0%
I_1_Conv	25	8	17	32,0%	17,2%	51,8%
J_1_Conv	22	15	7	68,2%	47,1%	83,6%
Total	**187**	**152**	**35**	**81,3%**	**75,1%**	**86,2%**

CPs: Campylobacter positive. CNs: Campylobacter negative. LCL: Lower confidence limit. UCL: Upper confidence limit.

(Supplementary material 1).

To evaluate microbiota differences between CNs vs CPs samples, we analyzed the beta diversity based on the weighted and unweighted UniFrac distances. The distances showed that the microbiota from the CNs was significantly different and could be distinguished from the CPs, thus demonstrating that the inter-group variation was significantly greater than intra-group variation (Pairwise PERMANOVA p-value =0.001) (Fig. 2a)(Supplementary material 1).

Additionally, the unweighted UniFrac distance matrix was represented through Principal Coordinate Analysis (PCoA) (Fig. 3). Interestingly, the samples grouped into two different clusters based on the segregation made in the experiment (CNs vs CPs), which seemed to be associated with the gut microbiota composition (Fig. 3a).

The relative abundance of the most prevalent phyla in the CPs and CNs groups were respectively: *Firmicutes* (49.74% vs 41.63%), *Bacteroidetes* (30.40% vs 38.68%) *Proteobacteria* (11.82% vs 12.44%), *Tenericutes* (1.21% vs 0.92%) and *Cyanobacteria* (3.29% vs 3.27%) (Fig. S5 in Supplementary material 2) (Supplementary material 1).

The relative abundance of the most prevalent families in CPs and CNs groups were respectively: *Rikinellaceae* (10.15% vs 17.46%), *Ruminococcaceae* (19.16% vs 15.17%), *Bacterirodacae* (8.64% vs 8.23%), *Lachnospiraceae* (8.70% vs 7.36%), *Barnesiellaceae* (5.86% vs 3.45%), *Helicobacteraceae* (5.27% vs 9.19%) and *Campylobacteraceae* (3.98% vs 0.80%) (Fig. S7 in Supplementary material 2) (Supplementary material 1).

Linear discriminant analysis (LDA) effect size (LEfSe) was employed to identify specific microbes in the CPs and CNs groups. LEfSe detected at Phylum level *Firmicutes* and at the family level, *Campylobacteriaceae* and *Christensenellaceae* were more abundant in the CPs while *Bacteroitedes*, *Bacillaceae, Helicobacteraceae* and *Rikenellaceae* were found more abundant in the CNs groups (p-value <0.05) (Fig. 4a,b). At the genus level, *Campylobacter, Faecalibacterium, Ruminococcus, Barnesiella,* and *Alistipes* were overrepresented in the CPs, while *Parabacteroides, Phascolorctobacterium, Megamonas* and *Helicobacter,* were more common in the CNs group (p-value <0.05) (Fig. 4b).

3.3. Microbiota and animal welfare

The results of animal welfare evaluations are reported in Fig. 5, detailing, for each batch, the results for each welfare principle (good feeding, good housing, good health and appropriate behavior) and the total welfare score. The organic batches scored averagely higher than the others (mean welfare score 225), with statistically significant difference compared to conventional ($t = -2.5995$, $p = 0.0158$) and antibiotic-free ($t = 2.7949$, $p = 0.0245$) batches. No statistically significant difference was highlighted between the welfare scores of conventional and antibiotic-free batches (mean welfare scores counting 155 and 165, respectively). The "good feeding" principle scored at its maximum (100) in all organic batches, with statistically significant difference compared to conventional ($t = -2.3509$, $p = 0.0233$) and antibiotic-free ($t = 5.4183$, $p = 0.0028$), while it did not clearly differ between antibiotic-free and conventional batches, with averages of 60 and 66, respectively. Also the "appropriate behavior" principle scored averagely higher in the organic batches, with statistically significant difference only if compared to conventional ($t = -2.5985$, $p = 0.0158$). The other two principles scored quite similarly in the three management

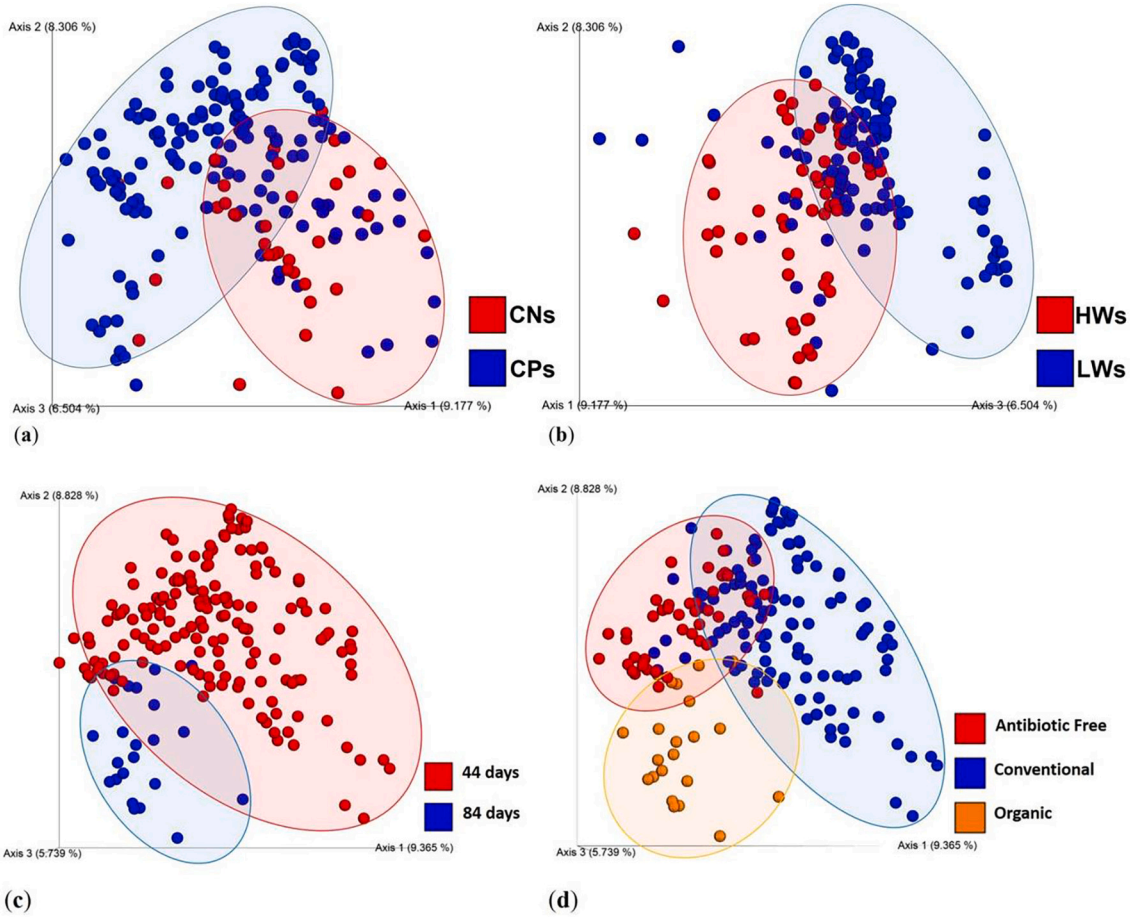

Fig. 3. Principal coordinates analysis (PCoA) of unweighted UniFrac distances. Percent of dataset variability explained by each principal coordinate is shown in brackets in axis titles (a) Emperor PCoA plot based on Unweighted UniFrac distance matrix. CPs (*Campylobacter* Positive Samples) (blue sphere) Vs CNs (*Campylobacter* Negative Samples) (red sphere). (b) HWs (high welfare animals) (red sphere) Vs LWs (low welfare animals) (blue spheres). (c) 44 Vs 84 days (blue spheres). (d) By farm type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

categories including the "good health" principle, that in the antibiotic-free batches scored in average very close to the organic (39 vs 41) and higher than the conventional (39 vs 30), however without statistically significant differences.

The microbial composition was observed in relationship with the welfare scores to explore the relationship with the microbiota. Total animal welfare scores calculated for each batch including feeding, housing, health, and behavior of the animals, ranged from 97.9 to 250.1, with a 75th percentile of 193 points. The animals were divided according to the following two categories: the high welfare animals (HWs), including 59 birds from 5 batches (38.5%, three organic and two conventional), that scored equal or greater than 193 points, and the low welfare animals (LWs), including 128 birds from 8 batches (61.5%, five conventional and three antibiotic-free) that scored less than 193. It should be highlighted that part of the HWs (22 out of 59, 37%) had a different age at the time of animal welfare and microbiota evaluations compared to all the others birds (12 weeks vs 6 weeks old).

The Chao1, and the observed OTUs indexes indicated that there were significant differences among the microbiota in animals from HWs vs LWs, with HWs group with higher microbiota richness found significant according to the Kruskal-Wallis test (p-value = 0.0161) (Fig. 1b) (Supplementary material 1).

To evaluate microbiota differences between HWs vs LWs samples, we analyzed the beta diversity distances showing that the microbiota from the HWs was significantly different and could be distinguished from the LWs demonstrating that the inter-group variation was significantly greater than intra-group variation (Pairwise PERMANOVA p-value =0.001) (Fig. 2b) (Supplementary material 1). Moreover, the distance matrix based on unweighted UniFrac distance was represented through PCoA. Notably, the samples were shown to group into two different clusters based on high and low welfare level of chickens (Fig. 3b).

The relative abundance of the most prevalent phylum in the LWs and HWs groups were respectively: *Firmicutes* (50.16% vs 44.01%), *Bacteroidetes* (29.31% vs 37.68%) *Proteobacteria* (12.61% vs 10.49%), *Cyanobacteria* (3.78% vs 2.21%) and *Tenericutes* (1.96% vs 0.93%) (Fig. S4, Supplementary material 2)(Supplementary material 1).

The relative abundance of the most prevalent families in the LWs and HWs groups were respectively: *Ruminococcaceae* (19.02% vs 17.11%), *Rikinellaceae* (9.19% vs 16.57%), *Bacterirodacae* (7.27% vs 11.35%), *Lachnospiraceae* (8.35% vs 8.68%), *Barnesiellaceae* (7.16% vs 1.61%), *Helicobacteraceae* (6.30% vs 5.36%) and *Campylobacteraceae* (3.70% vs 2.69%) (Fig. S8, Supplementary material 2)(Supplementary material 1).

LEfSe detected at family level, HWs group (Fig. 4d), *Rikinellaceae, Deferribacteraceae, Victivallaceae, Lentisphaerae, Synergistaceae* were overrepresented in the HWs group while *Lactobacillacae, Christensenellaceae, Barnesiellacae* were most abundant in the LWs group (p-value <0.05) (Fig. 4d). At the genus level, *Synergisyeses, Mucispirillum*, and *Megamonas* were overrepresented in the HWs, while *Lactobacillus*, *RF39 (unclassified Enterococcaceae), Alistipes*, *RF32(unclassified Streptococcaceae)*, *Faecalibacterium*, *YS2(rumen bacterium)*, *4C0d_2(uncultured rumen bacterium)* were overrepresented in the LWs group (p-value <0.05) (Fig. 4d).

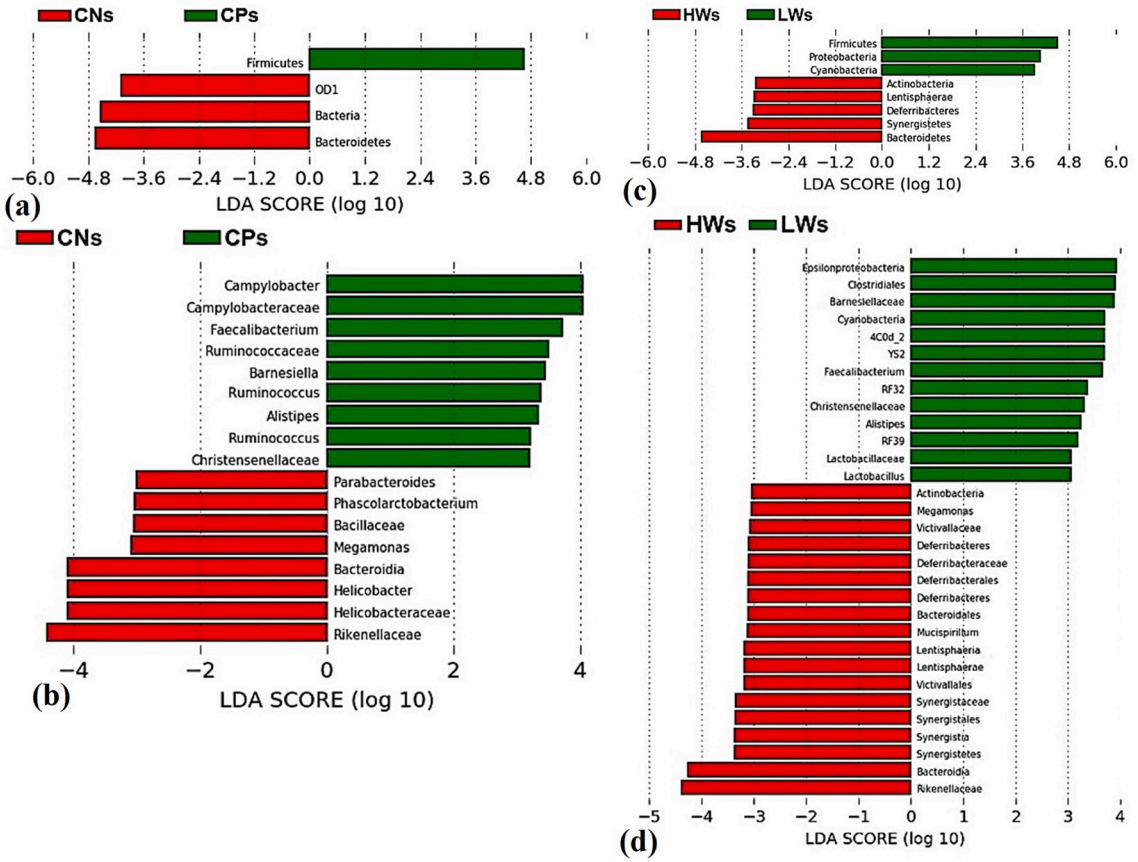

Fig. 4. LEfSe identifies the taxa with the greatest differences in abundance between CNs (*Campylobacter* Negative Samples) (Red) and CPs (*Campylobacter* Positive Samples) (green) groups at the phylum level (a) and genus level (b). The LWs (low welfare animals) taxa are indicated (green) and HWs (high welfare animals) (red) groups at the phylum level (c) and genus level (d). Only the taxa meeting a significant LDA threshold value of >3 are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

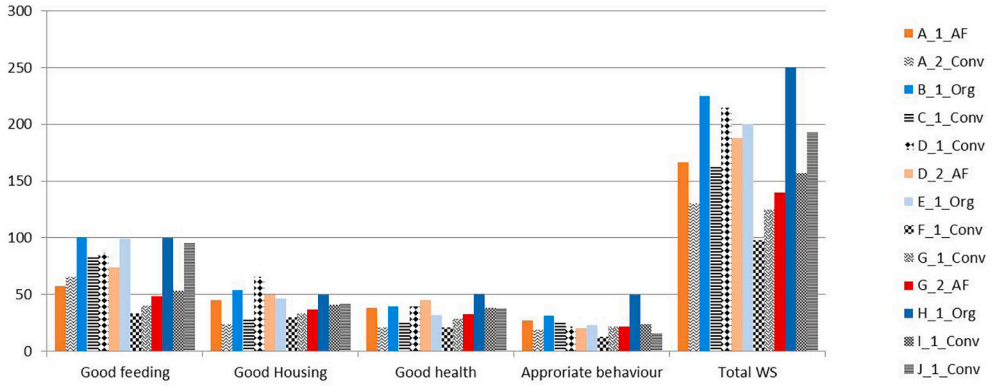

Fig. 5. Results of the animal welfare assessment with the Welfare Quality® protocol. The results of the four principles (good feeding, good housing, good health, appropriate behavior), and the total Welfare Score per each of the 13 batches are displayed. Antibiotic-free batches are in reddish colors, Organic batches are in bluish colors, Conventional batches are in black and white.

4. Discussion

An efficient strategy to control campylobacteriosis should involve monitoring of the entire farm-to-fork chain, with particular attention given to the elimination of *Campylobacter* in farmed animals. Controlling the presence of *Campylobacter* in primary production would greatly impact the burden of the disease, which is currently estimated to exceed 9 million cases per year in the EU with 0.35 million disability-adjusted life years (DALYs) per year and total annual costs of 2.4 billion Euro (Hazards, 2011). Previous studies demonstrated that a 2-log reduction would result in a significant decrease of the public health risk (Rosenquist et al., 2003).

The present study focused on the intestinal flora of broiler chickens aged from 6 to 12 weeks, belonging to different poultry batches and farms in Italy, also in relation to the animal welfare level. The cecum tract was used as the most representative gut segment for the colonization of *Campylobacter*, because it holds the highest microbial diversity and the content remains there for longer.

As previously reported in healthy chickens (Oakley et al., 2014; Wei et al., 2013), the present study confirmed that the cecal microbiota of

the broiler chickens studied was mostly represented by the phyla *Firmicutes, Bacteroidetes*, and *Proteobacteria*, with a ratio *Firmicutes/Bacteroides* of 1.5.

The alpha diversity recorded comparing animal by age and farm type suggests that poultry raised in the organic batches possess a higher microbiota diversity. Considering that the animals in the organic farms were older than in the other analyzed farm types, it is possible that the difference in chicks' age contributed to the differences in microbiota diversity we observed. Intestinal microflora in chicks at the age of 1–2 days is simple and contains a small number of bacteria belonging to a few species (Cui et al., 2017). After being placed in different housing systems, including organic and conventional, the chicks are exposed to different sources of bacteria that can enrich their immature intestine. Since there is little resistance to colonization in the young gastrointestinal tract, many bacteria can readily colonize within it (van der Wielen et al., 2002). As they grow, their intestine becomes increasingly diversified and complex (Cui et al., 2017). Therefore, as we observed, the age and farm management system could have a significant effect on the process of development and composition of the gastrointestinal microflora.

In relationship to *Campylobacter* colonization, we observed that the CNs group showed a higher alpha diversity. High diversity levels have been reported to maintain the intestinal microbiota stability after environmental stress (Konopka, 2009; Naeem and Li, 1997; Xue et al., 2015) and determine the colonization resistance against invading pathogens (Hentges et al., 1985; Shah et al., 2021; Wilson and Freter, 1986). These findings together suggest a possible link between low alpha diversity and an increased *Campylobacter* presence. The beta diversity indicated a clear separation of bacterial communities originating from CPs and CNs groups.

Statistical analyses showed in CNs group, an increased presence at the phylum level of *Bacteroidetes* was recorded, while in the CPs group we noted an increased presence of *Firmicutes*. A higher abundance of *Bacteroitedes* has been shown to be involved in the interactions with *Campylobacter,* in fact increasing proportion of *Firmicutes* and *Proteobacteria* and a decreased presence of *Bacteroitedes* result in an increased levels of *Campylobacter* and by limiting the colonization of the intestine by potential pathogenic (Sakaridis et al., 2018). Moreover Microorganisms belonging to *Bacteroides* are able to produce acetic acid, propionic acid, formic acid, and butyric acid, concentrations of particular volatile fatty acids demonstrated a negative correlation to absolute abundance of *Campylobacteraceae* (Hankel et al., 2019; Swiatkiewicz et al., 2021). Another study suggested that the reaction of the host immune system is probably more determinant than the microbiota in the decrease of *Campylobacter* presence (Chintoan-Uta et al., 2020).

Recent studies have shown that *Campylobacter* closely interacts with other microorganisms, with intestinal microbiota being important for *Campylobacter* colonization in broiler chickens (Patuzzi et al., 2021). In another study, the cecal microbiota in *Campylobacter*-free and *Campylobacter*-colonized broiler chickens differed considerably (Sofka et al., 2015), while yet another research group has shown the opposite result (Han et al., 2017). Taken together, these results suggest that multiple factors are likely involved in the successful colonization of poultry with *Campylobacter* species.

LefSe analyses was performed in order to find a specific abundance shift in the microbiota population. First, we found that the CPs contained a larger quantity of *Campylobacter* cells thus confirming the microbiological tests. The CNs group had an overall minor quantity of *Campylobacter* suggesting that the negativity was likely related to the sensitivity of the direct microbiological test or to the presence of either viable not-cultivable *Campylobacter* or dead cells (Ugarte-Ruiz et al., 2015). It is important to note that the CNs group had a higher quantity of *Helicobacter* cells but also a higher quantity of *Parabacteroides*, *Phascolorctobacterium* and *Megamonas*. Interestingly *Megamonas*, which is a genus within *Firmicutes,* was previously considered a "biomarker" of diet and lifestyle in humans (Wei et al., 2013). A previous study indicated that *Megamonas* acts as a hydrogen sink in the cecum of broiler chickens by increasing the production of short-chain fatty acids (Chen et al., 2019; Sergeant et al., 2014). Some *Parabacteroides* produce bacteriocins that are considered antagonistic substances produced by microorganisms, important for the maintenance of the resident microbiota in different ecological niches and are able to prevent exogenous bacterial colonization and invasion, and consequently the development of infectious diseases (Nakano et al., 2013).

The present study was designed also to collect more information on the potential impact that the animal welfare level could have on the *Campylobacter* colonization of broiler guts. In fact, recent studies have found evidence of direct relationships between the stress that broilers experience at farm and the shedding of foodborne pathogens, including *Campylobacter* (Alpigiani et al., 2017; Iannetti et al., 2020). This should be tightly connected with the microbiota composition, as environmental factors such as litter, feed access, and climate, can affect the composition of intestinal microbiota in chickens, both layer- and meat-type (Kers et al., 2018). Even if the present study was of descriptive type and not sufficient to demonstrate a casual link between microbiota composition and animal welfare level, a number of observations were collected that could be a reference for further research in this field, and the presence of statistically significant differences between different welfare groups was highlighted. In this regard, we found out that the animals in high welfare batches (HWs group) showed higher microbiota richness and a clear separation compared to the LWs group. Welfare was measured through the evaluation of different parameters and good welfare seems to be associated with a more diverse microbiota. The LefSe analyses showed several different species that are overrepresented in the two groups demonstrating that different environmental variables could really impact the microbial composition. In evaluating these results, it should be highlighted that a minor part (37%) of the birds in the HWs group were of different age compared to all the others (12 weeks old vs 6 weeks old). A number of studies have investigated the effect of the age on the evolution of the microbial population of broiler's gut (Shang et al., 2018, Oakley et al., 2014, Oakley and Kogut, 2016). Most of them agree on a changing microbial composition with age, even if changes are most evident during the first part of broiler's life (from 1 to 6 weeks) (Lu et al., 2003). Therefore, the statistically significant differences between HWs and LWs microbiota composition, presumably animal welfare –related, could also be partially due to the different age of part of the birds included in the HWs group. Also, for this reason, more studies are needed to confirm our results.

In HWs samples, an increased presence at the phylum level of *Bacteroidetes* was recorded, while in LWs samples we saw an increased presence of *Fimicutes, Proteobacteria*, *Cyanobacteria* and *Verrucomicrobia*. Abundance of *Bacteroidetes* is correlated with propionate production in the gut. Propionate, a short-chain fatty acid (SCFA), is a microbial metabolite that exerts multiple positive effects on the poultry gut (Liu et al., 2021), on the growth of normal epithelial cells also in other species (Clausen and Mortensen, 1995; Józefiak et al., 2004; Ritzhaupt et al., 1998), and carries multiple benefits for gut integrity and health by stimulating intestinal homeostasis, epithelial renewal and repair (Bilotta et al., 2021). Moreover, biological functions of this molecule, including its ability to inhibit pathogenic bacteria, modify immune and inflammatory responses (Langfeld et al., 2021), seem to be correlated with the global well-being of the host (Chamba et al., 2014; Chang et al., 2014; Martin-Gallausiaux et al., 2018; Sauer et al., 2007). Recently propionate has been considered a safe alternative to antibiotics in feed (Mehdi et al., 2018). The increased presence of *Bacteroidetes* might be due to access to an outdoor range, while a lower *Firmicutes/Bacteroidetes* ratio proves the fundamental impact of housing in poultry production (Xu et al., 2016). On the contrary it is interesting to observe that *Proteobacteria* show higher levels in LWs, as in literature the direct connection of this phylum with dysbiosis and risk to develop the disease has been reported (Shin et al., 2015).

To a certain extent our data suggest similarity in the bacterial

community of animals raised in good conditions (HWs) and animals negative to *Campylobacter* isolation (CNs). In those cases, we observed that the phylum *Bacteroidetes* was predominant with a relatively larger abundance and the genus *Rikinellaceae* and the species *Megamonas.* It was also evident that *Proteobacteria* were dominant in animals raised poorly (LWs) and positive to microbiological isolation (CPs). Although we cannot equate the negative to *Campylobacter* isolation to a complete absence of the microbe in the chickens, it is likely that those individuals were colonized with low concentration and consequentially less prone to be efficient shedders. The presence of those individual birds among the majority of positive animals is probably proof of resistance to colonization driven by the specific bacterial communities.

Finally, the results of this study also provided an overview of the welfare levels measured in different kinds of broiler poultry systems, that are more and more appreciated by the consumers, including organic and antibiotic-free. As previously reported (Iannetti et al., 2020), organic batches scored averagely higher than all the others, while antibiotic-free batches showed welfare scores similar to conventional. Interestingly, and differently from what has been reported in other studies (Karavolias et al., 2018), the health of antibiotic-free broiler chickens did not seem to be affected by this kind of management; more studies are required on this issue, also considering the small number of antibiotic-free and organic batches included in our research, due to the poor availability of farms of these types compared to the conventional ones.

5. Conclusions

The richness of the gut microbiota helps the chickens to maintain the microbial homeostasis thus avoiding the colonization by pathogens and might help to contain *Campylobacter* colonization. In the present study it was observed a significant dissimilarity of microbiota composition between *Campylobacter* positive animals (CPs) and *Campylobacter* negative animals (CNs) groups, suggesting its possible influence on *Campylobacter* presence. This could be explained considering that a microbiota with specific composition, containing high levels of certain microbial species, could negatively regulate the presence of *Campylobacter* through a competition mechanism, bacteriocin production or direct stimulation of the host's immune system. Moreover, on the contrary a specific microbiota composition can also facilitate the *Campylobacter* permanence in the broiler's intestine. However, this was basically an observational study, as just the presence of statistically significant relationships between the composition of broiler gut microbiota, the level of animal welfare that these animals show, and the presence or absence of *Campylobacter* were described. More research focusing on the functional characteristics of both *Campylobacter* and possibly counteracting bacterial species that we identified would be highly recommended to deepen the knowledge on the mechanisms that are at the basis of our observations and confirm the potential link between the broiler chicken microbiota composition and different animal welfare standards.

Author contributions

Conceptualization, E.D.G. and G.G.; methodology, E.D.G., G.G., L.D.M and G.D.S.; software, L.D.M.; validation, E.D.G., and G.G.; formal analysis, G.G. and L.D.M.; investigation, L.D.M., G.D.S. L.I., M.P.V., Q.S.; resources, E.D.G. and G.G.; data curation, G.G. and L.D.M.; writing—original draft preparation, G.G. and L.D.M. and F.M.; writing—review and editing, E.D.G., G.G., A.J., L.D.M, Q.S., L.I., M.P.V.; visualization, L.D.M. and G.G.; supervision, E.D.G. and G.G.; project administration, E.D.G. and G.G.; funding acquisition, E.D.G. and G.G. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A. Supplementary data

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