

Longitudinal study on antibiotic susceptibility in commensal *E. coli* from geese raised in free-range production systems

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ABSTRACT The transmission of antimicrobial resistance bacteria from animals to humans has become an important concern. The extended-spectrum beta-lactamase (ESBL) -AmpC- producing *Escherichia coli* (ESBL-AmpC EC) and quinolones resistant *E. coli* are of particular interest. The present study aimed to evaluate the load and prevalence of antibiotic-resistant commensal *E. coli* along the goose production cycle on 2 free-range farms in central Italy. On A farm, oxytetracycline was administered, while the B farm did not use antibiotics during the geese productive cycle. One hundred geese of 1-day-old from the same batch were divided into the two farms. At hatching, the animals showed an average of *E. coli* loads was 6.83 ± 0.48 log CFU/g, and 0.28 ± 0.28 , 0, 5.12 ± 0.54 log CFU/g for *E. coli* resistant to nalidixic acid (*E. coli*^{nal}), to cefotaxime (*E. coli*^{cef}) and to tetracyclines (*E. coli*^{tet}), respectively. The loads of *E. coli*, *E. coli*^{nal}, *E. coli*^{cef} and *E. coli*^{tet} on 224 environmental faecal pools were determined at 8 time points. Antimicrobial susceptibility and molecular characterization of *E. coli*^{cef} isolates were performed. The ANOVA was

used to assess the difference in bacterial loads between the two farms. We described more than 50% of resistances for tetracyclines in both farms, and sulphonamides and cephalosporins in the A farm. The loads of *E. coli* and *E. coli*^{nal} in faeces were estimated at approximately 6–7 log (CFU/g) and 5–6 log (CFU/g) in the two farms, respectively. The average load of extended-spectrum beta-lactamase *Escherichia coli* (ESBL EC) in goose faeces varied broadly along the production cycle: in the first weeks, a sharp increase was observed in both farms, while later on A farm, the burden of ESBL EC remained steady until the end of the production cycle and on B farm the load dramatically decreased from 6 wk of age onward. An increase in the proportion of *E. coli*^{nal} was observed on A farm shortly after the antibiotic administration. Our study shows that the dynamics of antibiotic-resistant *E. coli* in farmed geese are similar to the ones observed in broilers. However, the risk of the emergence of antibiotic-resistant commensal *E. coli*, might be mitigated by the adoption of good management practices, including prudent use of antibiotics.

Key words: goose, antibiotic susceptibility, commensal *E. coli*, free-range farm

2021 Poultry Science 100:101230

<https://doi.org/10.1016/j.psj.2021.101230>

INTRODUCTION

Antibiotic resistance (AR) has become one of the most important threats to public health (World Health Organization, 2020). The rise of AR has been accelerated by the overuse of antibiotics, which exerts selective pressure on bacteria resulting in the emergence, spread, and persistence of antibiotic-resistant bacteria and

antibiotic resistance genes (ARGs) in microbial populations (D'Costa et al., 2011; Bennani et al., 2020). There is evidence that antibiotic-resistant bacteria and/or ARGs carried on mobile genetic elements are shared among man, animals, and the environment, amplifying the magnitude of this phenomenon (Aarestrup, 2015; EFSA, 2020). The gut microbiota is considered the largest reservoir of bacteria carrying AR transmissible genes, not only within livestock but also in humans (EFSA, 2020). Among those bacteria, *Escherichia coli*, which is ubiquitous in the gastrointestinal tract, has been used as an indicator to monitor the AR in humans, animals and food (Nhung et al., 2016; EFSA, 2020). *E. coli* can act as a donor, vector or recipients of AR genes and can be transmitted from animals to humans via

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Received October 22, 2020.

Accepted April 24, 2021.

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MATERIALS AND METHODS

Geese Farms and Husbandry

direct contact or through the food chain and the environment (World Health Organization, 2017). AR to highly critically important antibiotics in *E. coli* isolates from food animals is considered a public health problem (Myrenås et al., 2018; Niero et al., 2018). Such resistance may determine a lack of effectiveness of HPCIA in human infections, which finally results in treatment failures (EFSA, 2020). In poultry, extended-spectrum beta-lactamase (ESBL)-AmpC-producing *E. coli* (ESBL-AmpC EC) and quinolones resistant *E. coli* are of particular concern, since they can disseminate from poultry to healthy humans or patients (EFSA, 2020). As an alternative, these resistances may be transferred from *E. coli* to zoonotic bacteria by mobile genetic elements (EFSA, 2020). Consequently, the dynamics of excretion of ESBL-AmpC EC and quinolones-resistant *E. coli* has been thoroughly investigated in the last 10 years in intensively raised broilers (Reich et al. 2013, Dame-Korevaar et al., 2019; Apostolakos et al., 2020). Among poultry species, broilers, and turkeys are the most common sources of poultry meat representing 87 and 6.7% of total production, respectively (FAO, 2010). However, beside broilers and turkeys, the term “poultry” encompasses a range of domesticated species, including also ducks, geese, game birds (such as quails and pheasants) representing around the 7% of total poultry meat production (FAO, 2010). In the European Union (EU), goose production is particularly popular in Eastern European countries such as Hungary, Poland and Romania (Romanov et al., 1996). In Italy, the goose husbandry represents a small niche of the poultry farming and it is used for multipurpose productions such as meat, eggs, fatty liver, down, and feathers for stuffing (Romanov, 2011). In the Umbria region, the goose farm culture covers an important source, dealing with the tradition and the goose production ranges from semi-intensive to free-range systems. Small poultry farms and free-range production systems are often characterized by lower antibiotics use than intensive production and are perceived as being less risky in terms of AR (Rayner et al., 2019). However, data from the literature suggests the presence of antibiotic-resistant bacteria in poultry raised in small-holder and free-range farms (Borzi et al., 2018; Hussain et al., 2019). Moreover, in farms where poultry often shares the same environment with people, there is an increased risk of direct transmission of pathogens, including antibiotic-resistant bacteria, from poultry to man (Bertelloni et al., 2015). Geese are usually raised in an open environment, and this could, therefore, increase the risk that they contaminate soil and water with AR bacteria shed by faeces. At the same time, geese are exposed to environmental contamination posed by AR bacteria already present in soil or groundwater. As far as we know, no data are available on the dynamics of antibiotic-resistance in *E. coli* along the goose production cycle.

The hypothesis behind this study was that the load and prevalence of antibiotic-resistant commensal *E. coli* harbored in geese faeces varies along the production cycle and it might be influenced by management practices adopted on the farm.

A longitudinal study was performed at 2 geese farms in central Italy from April to July 2018. Both farms were located in the province of Perugia (central Italy), about 20 km away from each other. On both farms, the geese of *Romagnola* breed were raised in a free-range system for part of their production cycle, but management practices differed. The A farm was a family-run company raising geese, ducks and poultry to be sold to the local market. The capacity of the farm is 18,000 animals/year, with a maximum of 700 geese/year. This farm followed the national rules for poultry production, with antibiotics administered only for therapeutic purposes upon veterinary prescription, and unrestricted GMO administration. The B farm (30 ha) was entirely dedicated to organic grape production and had featured an experimental agroforestry system with the introduction of geese in the vineyard. This farm raised only geese, with a maximum of 200 animals every year. The geese were used to reduce pest and control weeds and they were slaughtered and used for own consumption within the vineyard. Both farms purchase geese from the same local supplier at 1 day of age. In March 2018, 100 geese of 1-day-old from the same batch were divided into the two farms as follows: 50 animals on A farm and 50 geese on B farm. From April to July 2018, samples were collected on 8 time points (from T1 = 10th April to T8 = 30th July), approximately every 2 wk. On A farm, geese were confined indoors from T1 to T3 in a building shared with broilers. At T3 animals were treated with oxytetracycline and the administration lasted 5 d (60 g each 100 liters of water). From T4 to the end of the cycle geese were moved outdoor grazing in an olive orchard, with a grazing area of approximately 10,000 m². On B farm, the geese were placed in a closed building until T2. At T3, the animals were moved to the vineyard, with only overnight shelter (wood and welded mesh) until T6. In the period prior to slaughter, at T7 and T8, grazing was stopped and animals were moved to a closed paddock with a fence (Figure 1). The geese were fed additional feedstuff (40% corn, 30% wheat and/or barley and 30% faba bean) supplied each day at evening, while water was provided ad libitum. On this farm, no antibiotic was administered to geese throughout the production cycle.

Sampling

The sample size was calculated to estimate the mean of quinolones-resistant *E. coli*, with a standard deviation of 0.9 log/CFU, according to a previous study (Pesciaroli et al., 2020), 0.5 precision and 95% confidence levels (Epitools, <https://epitools.ausvet.com.au/onemean>). On each of the 8 time points, 14 fresh faecal droppings were collected from each group of geese located in the two farms. Overall, 224 samples were tested, 112 for each farm. In addition, 16 faecal pool

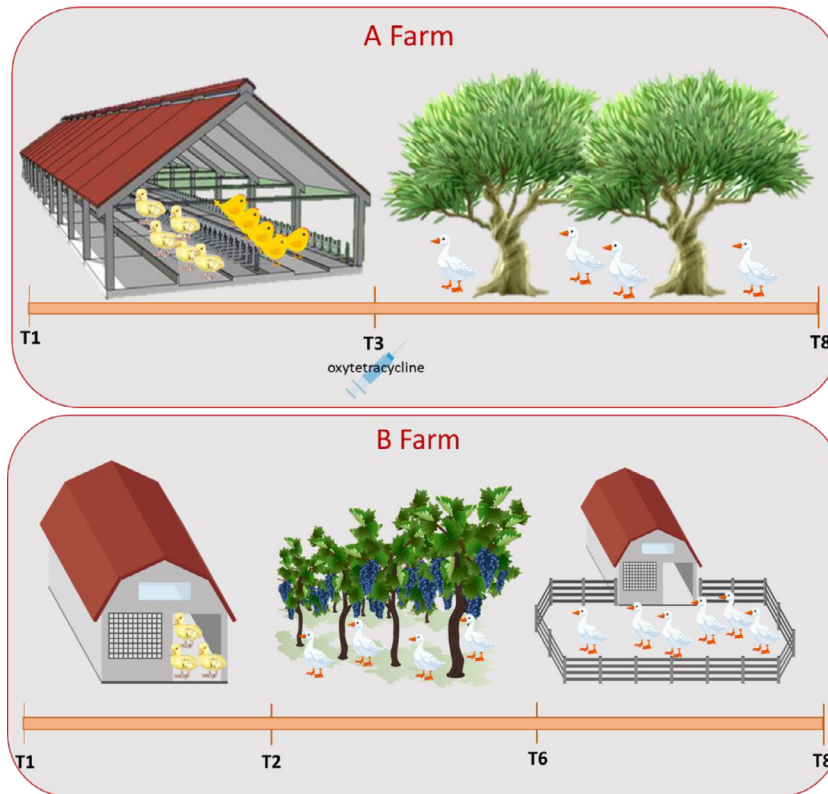


Figure 1. Graphical representation of the A farm and B farm.

samples were collected from the transport boxes, before the 1-day-old animals were allocated on the two farms (T0). All samples were immediately placed in sterile containers, kept at 4°C until processing and cultured within the following twelve hours.

Enumeration of Antibiotic-Resistant Commensal *E. coli* at Sample Level

Quantitative culture was used to determine the loads of *E. coli*, *E. coli* resistant to nalidixic acid (*E. coli*^{nal}), *E. coli* resistant to cefotaxime (*E. coli*^{cef}) and *E. coli* resistant to tetracyclines (*E. coli*^{tet}). Resistance to nalidixic acid and tetracyclines was used as an indicator of resistance to fluoroquinolones and tetracyclines, respectively, while the *E. coli*^{cef} was used as a proxy for ESBL producing *E. coli* (ESBL EC) (Duse et al., 2015; von Tippelkirsch et al., 2018; Apostolakos et al., 2020). Samples were processed as described by Duse et al. (2015). Briefly, 5 g of each sample were homogenized into a Stomacher bag in 45 mL of 0.9% saline. The suspension (10^{-1} dilution w/v) was further 10-fold diluted in 0.9% saline, from 10^{-1} to 10^{-8} . The colony-forming units (CFU) of *E. coli*, *E. coli*^{nal}, *E. coli*^{cef} and *E. coli*^{tet} were determined by plating 100 μ L of each dilution on the four selective media: 1) MacConkey agar (Sigma Aldrich- Merck KGaA, Darmstadt, Germany); 2) MacConkey agar supplemented with nalidixic acid (Sigma Aldrich- Merck KGaA, Darmstadt, Germany) (50 μ g/mL-MacConkey^{nal}); 3) MacConkey agar supplemented with cefotaxime (Sigma Aldrich- Merck KGaA, Darmstadt, Germany) (1 μ g/mL -MacConkey^{cef}) and 4) MacConkey

agar supplemented with tetracycline (Sigma Aldrich-Merck KGaA, Darmstadt, Germany) (40 μ g/mL-MacConkey^{tet}). *E. coli* ATCC 25922 was used as negative control strain for all the three selective culture media supplemented with antibiotics. A tetracycline-quinolone-resistant *Salmonella hadar* strain (Magistrali et al., 2008) was used as positive reference strain for MacConkey^{nal} and MacConkey^{tet}. For the MacConkey^{cef} medium, the quality control was carried out according to the guidelines of the European Reference Centre for antimicrobial resistance (Hasman et al., 2017), using *Salmonella* O:6,7 WHO S-17.8 as positive control strain. Plates were incubated at 37°C overnight. The number of *E. coli* colony forming units (CFU) for each sample was determined by counting pink or red colonies with a morphology resembling that of *E. coli* on MacConkey agar. For each sample, a colony was isolated from MacConkey agar plate, incubated at 37°C for 24 h and confirmed as *E. coli* using oxidase, triple sugar iron, and indole tests. Counts of *E. coli*^{nal}, *E. coli*^{cef} and *E. coli*^{tet} were determined in the same manner, but using MacConkey^{nal}, MacConkey^{cef} and MacConkey^{tet} plates. The proportion of *E. coli* isolated from each of the three antibiotic supplemented media among all *E. coli* isolates in each sample was calculated by dividing the *E. coli*^{nal}, or *E. coli*^{cef} or *E. coli*^{tet} counts by *E. coli* counts in the same sample.

Antimicrobial Susceptibility Testing at Isolate Level

For each sample, antimicrobial susceptibility of one *E. coli* isolate from a MacConkey agar without antibiotics

and 5 *E. coli* isolates from MacConkey^{cef} were tested by using the agar diffusion method on Muller Hinton agar (Oxoid Ltd., Cambridge, UK), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (The European Committee on Antimicrobial Susceptibility Testing, 2017a,b). *E. coli* ATCC 25922 was used as control strain. The following antimicrobial discs (Oxoid Ltd) were used: ampicillin (10 µg), cefotaxime (30 µg), cefazolin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), nalidixic acid (30 µg), sulfonamides (300 µg), and tetracycline (30 µg). The sizes of inhibition diameters were interpreted following the EUCAST breakpoint tables (The European Committee on Antimicrobial Susceptibility Testing, 2017b). Since the EUCAST does not provide breakpoints for cefazolin, kanamycin, nalidixic acid, sulphonamides, and tetracycline, values of the Clinical & Laboratory Standards Institute were used for these compounds (CLSI, 2018). Intermediate results were classified as “resistant”. An isolate was classified as multidrug resistant (MDR) when it exhibited resistance to at least three antibiotics representing aminopenicillins (ampicillin), first-generation cephalosporins (cefazolin), third generation cephalosporins (cefotaxime), amphenicols (chloramphenicol), quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin), sulphonamides, aminoglycosides (gentamycin), and tetracycline. Isolates resistant to up to 3 antibiotics listed above were classified as “resistant”. The *E. coli* isolated from MacConkey^{cef} were classified as ESBL *Enterobacteriaceae* using the double disk synergy test, according to EUCAST guidelines (The European Committee on Antimicrobial Susceptibility, 2013). The susceptibility to colistin was assessed using the broth microdilution method. Briefly, the sulphate salt of colistin (Sigma Aldrich SRL, Milan, Italy) was diluted in polystyrene microtiter plates (LP Italiana SpA, Milan, Italy) according to the EUCAST recommendations. *E. coli* strain ATCC 25922 and ZTA14/0097EC [kind gift of Prof. Lucas Dominguez Rodriguez, Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense, Madrid, Spain] were used respectively as negative and positive control strains. Classification of isolates as resistant was based on MIC values using criteria from EUCAST (R: MIC > 2 mg/L).

Molecular Characterization of *E. coli* From MacConkey^{cef}

All the 79 *E. coli*^{cef} isolates were tested by PCR. DNA was extracted from each sample using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction and was used as template for phyotyping-PCR and for amplification of ESBL gene. The phylogenetic group of each isolate (A, B1, B2, C, D, E and F) was determinate by using quadruplex PCR, according to Clermont et al., 2013 (Clermont et al., 2013).

The protocol described by Dallenne et al., (2010) was used for the detection of ESBL gene (*bla*_{TEM}/*bla*_{SHV}/*bla*_{OXA-1} and *bla*_{CTX-M-1,-2,-9,-15} groups).

Statistical Analysis

The bacterial counts of *E. coli* were converted to log CFU/g of faecal-pool sample for statistical analysis. A one-way analysis of variance (ANOVA) was used to assess the difference in bacterial loads from the two different farms (“farm” effect) at each time point. Moreover, a one-way ANOVA with a split plot in time arrangement was carried out to test the differences between the sampling times for each farm. The normality and homoscedasticity of the data were verified by graphical analysis of the residuals. When any ANOVA effect was significant, Tukey’s Honest Significant Differences test was used for multiple comparisons of the means. The significance level of the difference was set at $P < 0.05$. For each antibiotic molecule, the difference in the proportion of resistant isolates between samples from the two production lines was evaluated by using Pearson's χ^2 test, with a significance threshold of $P < 0.05$. The analyses were performed by using R software (v. 3.6.1) (TeamCore, 2018).

RESULTS

Loads of Antibiotic-Resistant Commensal *E. coli* at Sample Level Through the Production Cycle

At the T0, before the allocation of geese in the two farms, the average of *E. coli* loads was 6.83 ± 0.48 log CFU/g, and 0.28 ± 0.28 , 0 , 5.12 ± 0.54 log CFU/g for the *E. coli*^{nal}, *E. coli*^{cef} and *E. coli*^{tet}, respectively. All samples, irrespectively from the A farm and the sampling time, tested positive for *E. coli*, *E. coli*^{nal} and *E. coli*^{tet}, while the number of samples positive for *E. coli*^{cef} on each farm and sampling time is shown in Table S1. This latter table showed that the two farms had an opposite trend; the A farm increased the percentage of positive samples respect to the number of collected samples from T1 to T8, while in the B farm the number of positive samples for *E. coli*^{cef} decreased along the productive cycle. The one-way ANOVAs performed on the average of *E. coli* loads showed a significant “farm” effect ($P < 0.001$) for all time points except T4 and T7. Moreover taking into account the “sampling time” effect, it did result significant for both farms ($P < 0.001$). In particular the B farm showed a decreased trend from T2 (7.96 ± 0.21 log CFU/g) to T5 (6.00 ± 0.17 log CFU/g), whereas the A farm showed a slightly increased trend from T1 (6.02 ± 0.10 log CFU/g) to T4 (7.24 ± 0.21 log CFU/g) and a consequent constant trend until the end of the trial (Figure 2A). The one-way ANOVAs performed on the average *E. coli*^{cef} showed a significant “farm” effect for all the time points ($P < 0.001$) and a significant “sampling time” effect for both farms ($P <$

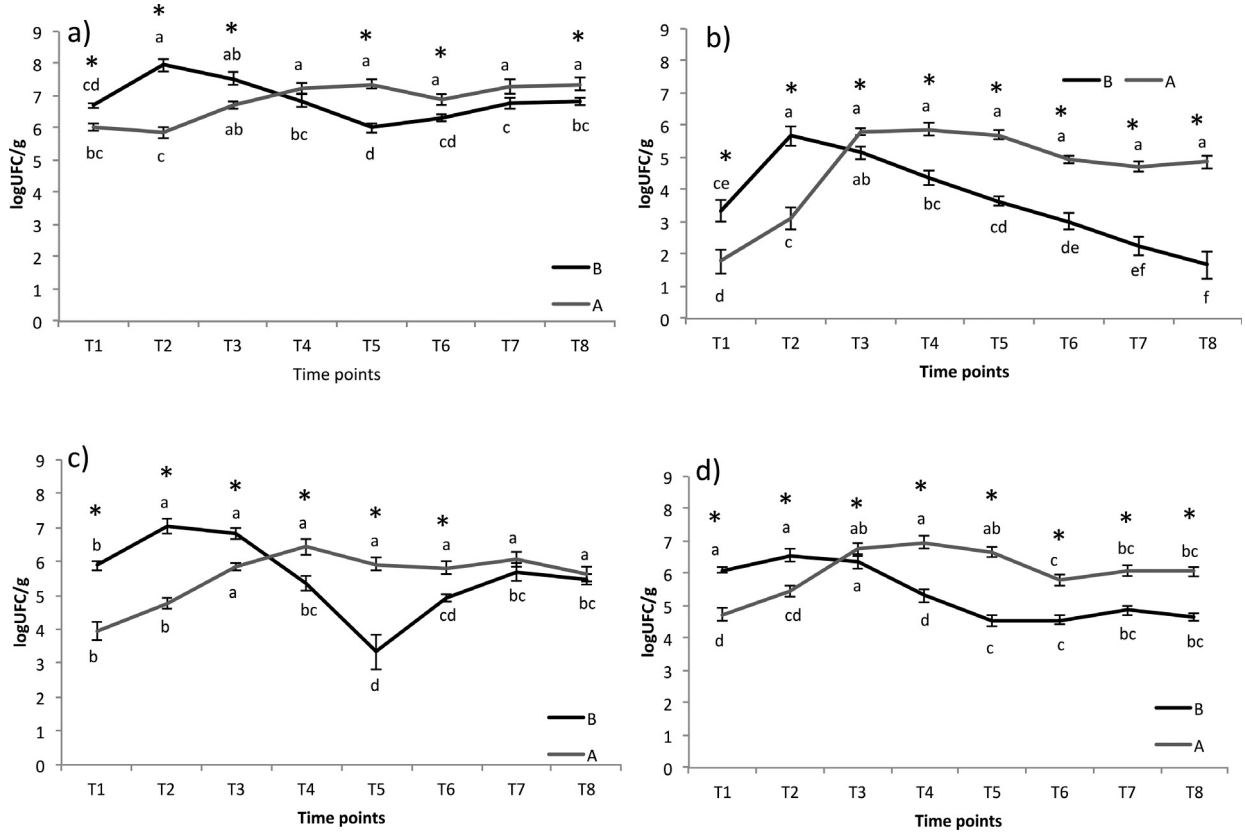


Figure 2. Results of quantitative analysis. (A) Averages of *E. coli* loads (log UFC/g); (B) averages of *E. coli*^{cef} loads (log UFC/g); (C) average of *E. coli*^{tet} (log UFC/g); (D) average of *E. coli*^{nal} (log UFC/g), in the A and B farms. Within each curve, different letters indicate significant differences between means ($P \leq 0.05$). Whereas * indicate significant differences between farms. Bars mean \pm SE (n = 14).

0.001). The main difference is that in B farm the average load increased until T2 (5.67 ± 0.30 log CFU/g) and, drastically decreased until T8 (1.67 ± 0.43 log CFU/g), while in A farm increased until T3 (5.80 ± 0.10 log CFU/g) and then remained constant over time (T8 = 4.86 ± 0.20 log CFU/g; Figure 2B). As regards to the average load of *E. coli*^{tet}, all tested effects resulted statistically significant ($P = P < 0.001$) except the “farm” effect at the T7 and T8 time points ($P = 0.078$ and $P = 0.563$, respectively). The trend curves over time started with a higher average load for the B than the A farm until T3 (6.82 ± 0.18 log CFU/g, and 5.84 ± 0.13 log CFU/g, respectively) and then a turnaround occurred until the end of the trial (Figure 2C). A similar trend was evident for the average load of *E. coli*^{nal} with a turnaround in loads between farms at T2 and T3 sampling points (T2 = 6.57 ± 0.22 log CFU/g, 5.45 ± 0.16 log CFU/g and T3 = 6.34 ± 0.18 log CFU/g, 6.78 ± 0.18 log CFU/g for B and A farms respectively; Figure 2D). If we considered the average proportion of *E. coli*^{cef} in contrast to the total *E. coli* population in the samples, the percentages were very low for both farms with the highest value reached by the A farm at T3 ($14.29\% \pm 1.92$; Figure 3A). Moreover, the average proportion of *E. coli*^{tet} in contrast to the total *E. coli* population was almost the same for both farms except for the T3, T4, and T8 time points (Figure 3B). Conversely, the average proportion of *E. coli*^{nal} in contrast to the total *E. coli* population was higher in the A

respect to the B farm for the whole trial and in particular at T3 time point ($85.30\% \pm 6.24$ and 7.04 ± 0.86 , respectively; Figure 3C).

Molecular Characterization of *E. coli* From MacConkey^{cef}

A total of 79 *E. coli*^{cef} were isolated from A farm (40) and B farm (39). The determination of the phylogenetic group of *E. coli*^{cef} revealed that all isolates belong to A group, suggesting an initial clonal relationship among the *E. coli* isolate within each farm. The same isolates were classified as ESBL EC according to the results of double disc synergy test. The analysis of genetic determinants revealed that at T1, all *E. coli*^{cef} isolated from both farms had the same β -lactamase genotype: *bla*_{SHV} positive and *bla*_{CTX-M -1, -2, -9} negative. Only 2 isolates, one per each farm, harbored the *bla*_{TEM}. From the T2 to the end of the experiment (T8), *E. coli*^{cef} isolated from B farm lost the *bla*_{SHV} determinants and acquired the *bla*_{CTX-M-1}, while the isolates from A farm maintained the same β -lactamase genotyping of the T1, except for one isolate at T7 (R8483; Figure 4). All CTX-M group 1 positive isolates showed to belong to the CTX-M-15 allelic variant. The *bla*_{TEM} was detected in another 13 of the *E. coli*^{cef} that, except for one, were isolated from A farm. At the last time point (T8) no *E. coli*^{cef} harboring *bla*_{TEM} was detected.

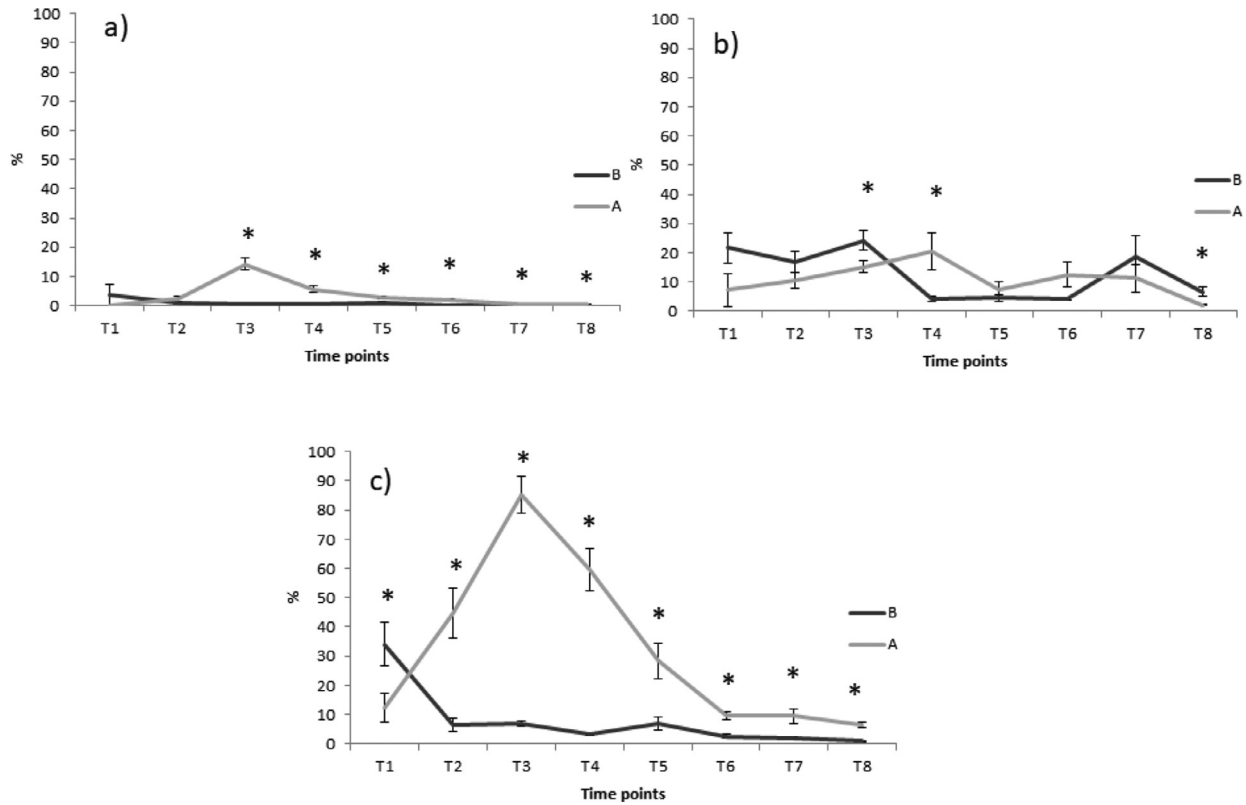


Figure 3. Averages proportion of *E. coli*^{cef} (A), *E. coli*^{tet} (B) and *E. coli*^{nal} (C) loads respect to the total *E. coli* population (%), in the A and B farms. Within each curve, different letters indicate significant differences between means ($P \leq 0.05$). Whereas * indicate significant differences between farms. Bars mean \pm SE (n = 14).

Antimicrobial Susceptibility at Isolate Level

At T0, 7 out of 16 *E. coli* isolated from MacConkey agar without antibiotics were fully susceptible to the tested antibiotics. The rest of isolates were resistant to sulfonamides (n = 8), tetracycline (n = 9), ampicillin (n = 1), and cefazolin (n = 1). No resistance was found to cefotaxime, ciprofloxacin, chloramphenicol, gentamycin and nalidixic acid. The proportion of *E. coli* isolated from T1 to T8 and resistant to each antibiotic is shown in Table 1. A prevalence of antibiotic-resistant equal or higher than 50% was found for tetracycline only, independently from the farms. The A farm also showed a

percentage ≥ 50 of isolates resistant to the first-generation cephalosporins (cephazolin) and sulphonamides. On both farms, none of the tested isolates was resistant to colistin. The MDR percentage in A and B farms is described in Table 2 and Figure 5. Only 2 isolates, one per each farm, were phenotypically classified as ESBL in all the dataset and were classified as multi-resistant. The proportion of AR was different in *E. coli* isolated from the two farms, with B farm showing the highest proportions of resistance (41.1%), while the A farm presented the highest percentage of multiresistant isolates, equal to the 65.2%. A higher percentage of fully susceptible isolates was described in the B farm (21.4%)

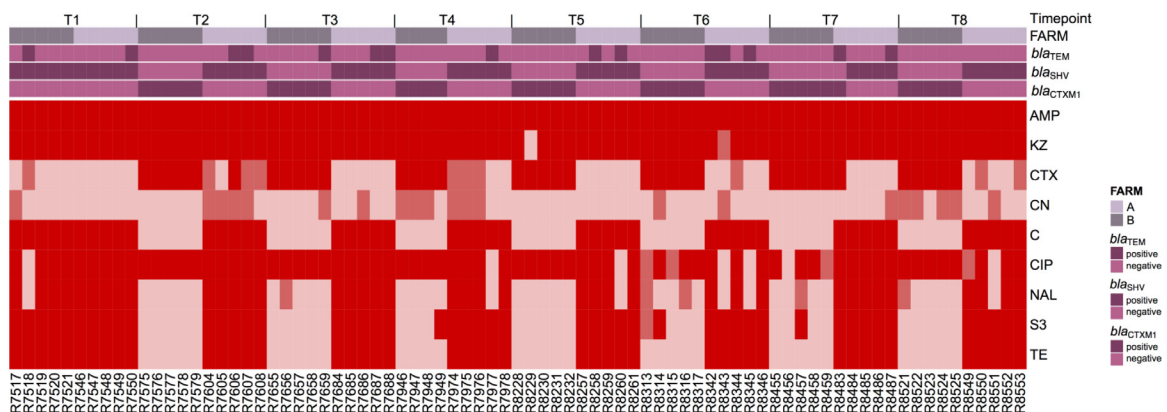


Figure 4. Heatmap showing the molecular characterization and the antibiotic susceptibility profile of each *E. coli*^{cef} isolated on the two farms. Samples are coloured according to the antibiotic molecules results: resistant (red), intermediate (light red) and susceptible (pink). (Colored version of figure is available online.)

Table 1. Antibiotic susceptibility in commensal *E. coli* of A and B farms from T1 to T8.

Antibiotic	A Farm		B Farm	
	Number resistant isolates/ Number tested isolates	Proportion of resistant isolates (95% CI)	Number resistant isolates/ Number tested isolates	Proportion of resistant isolates (95% CI)
Ampicillin	47/112	42% (32.8%–51.7%)	25/112	22.3% (15.2%–31.4%)
Cefotaxime	1/112	0.9% (0–5.6%)	2/112	1.8% (0.3%–0.7%)
Cephazolin	74/112	66% (56.4%–74.6%)	45/112	40.2% (31.2%–49.9%)
Chloramphenicol	36/112	32.1% (23.8%–41.7%)	4/112	3.6% (1.2%–9.4%)
Ciprofloxacin	54/112	48.2% (38.7%–57.8%)	23/112	20.5% (13.7%–29.4%)
Gentamicin	22/112	19.6% (13%–28.4%)	4/112	3.6% (1.1%–9.4%)
Nalidixic acid	44/112	39.3% (30.3%–49%)	19/112	17% (10.8%–25.5%)
Sulphonamides	84/112	75% (65.8%–82.5%)	32/112	28.6% (20.6%–38%)
Tetracyclines	71/112	63.4% (53.7%–72.1%)	62/112	55.4% (45.7%–64.7%)
Colistin	0	0	0	0
ESBL	1/112	0.9% (0%–5.6%)	1/112	0.9% (0%–5.6%)

Table 2. Distribution of multiresistant, resistant and fully susceptible isolates in the A and B farms from T1 to T8.

Item	A	B
Susceptible	8 (7.1)	24 (21.4)
Resistant	31 (26.7)	46 (41.1)
Multiresistant	73 (65.2)	42 (37.5)
Total	112 (100)	112 (100)

Percentages are shown in parentheses.

compared to the A farm (7.1%). Further, we compared the antibiotic susceptibility results (R vs. S) for each antibiotic molecule and we described significant differences between the A and B farms for all the tested antimicrobials (Pearson's χ^2 test: $P \leq 0.05$; Table S2 and Figure S1), except for the cefotaxime and tetracycline (Pearson's χ^2 test: $P > 0.05$; Table S2 and Figure S1). The results of the antibiotic susceptibility test of the *E. coli*^{cef} are shown in Table 3. In the A farm, more than 90% of the isolates were resistant to ampicillin, cephalosporin, chloramphenicol, sulphonamides and tetracyclines, while in the B farm for ampicillin and cephalosporin. The ESBL phenotype was confirmed in all the 79 *E. coli*^{cef} isolates belonging to the A and B farm. On both farms, all *E. coli*^{cef} isolates were considered MDR. The Figure 4 shows a global overview of the molecular results linked

with the susceptibility of antibiotic molecules of the *E. coli*^{cef} of both farms.

DISCUSSION

To the best of our knowledge, this is the first study describing the antibiotic susceptibility profile of commensal *E. coli* isolated through the complete productive cycle of geese. The antibiotic susceptibility was assessed at sample level, estimating the load of ESBL EC (*E. coli*^{cef}), quinolones-resistant-*E. coli* (*E. coli*^{nal}) and tetracycline-resistant-*E. coli* (*E. coli*^{tet}) using selective culture. Data on the antibiotic susceptibility to a panel of antibiotics was assessed at isolate level from MacConkey agar without antibiotic supplementation. While selective culture detects the presence of resistant isolates even when their prevalence in the sample is very low, the prevalence of AR in isolates from non-selective culture provides an estimate of the levels of AR in the general *E. coli* population (Apostolakis et al., 2020).

In our study, data at the isolate level show that a quite high proportion of commensal *E. coli* was resistant to antibiotics traditionally used in poultry production. We described more than 50% of resistances for tetracyclines in both farms and sulphonamides and cephalosporin in the A farm. In Europe, resistance to these antibiotic

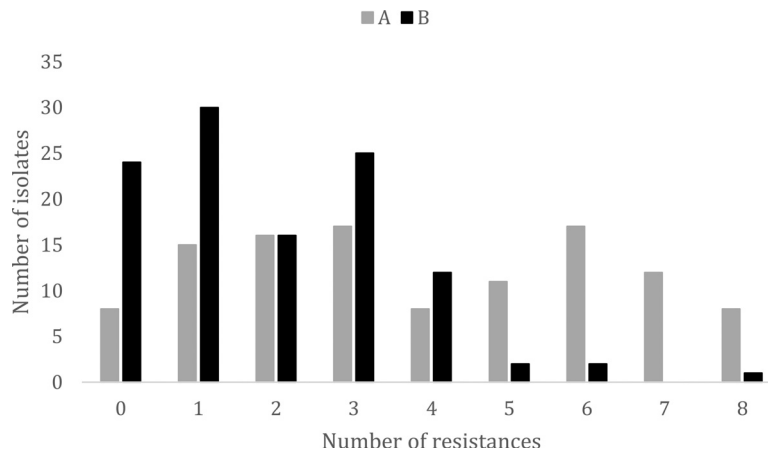
**Figure 5.** Distribution of isolates, divided according to the number of resistances, in each farm from T1 to T8. The number of isolates in the A and B farms, respectively, is shown.

Table 3. Number of resistant isolates in *E. coli*^{cef} of A and B farms from T1 to T8.

Antibiotic	A	B
Ampicillin	40 (100)	39 (100)
Cefotaxime	2 (5)	34 (87.2)
Cephazolin	39 (97.5)	38 (97.4)
Chloramphenicol	40 (100)	5 (12.8)
Ciprofloxacin	34 (85)	34 (87.2)
Gentamicin	0 (0)	0 (0)
Nalidixic acid	35 (87.5)	4 (10.3)
Sulphonamides	40 (100)	8 (20.5)
Tetracyclines	40 (100)	5 (12.8)
Colistin	0 (0)	0 (0)
ESBL	37 (92.5)	39 (100)
Total	40 (100)	39 (100)

Percentages are shown in parentheses.

molecules is reported to be present in livestock (EFSA, 2020; Pesciaroli et al., 2020) but there are no official data for the geese production systems. The described levels of resistance are probably caused by long-term usage of these antimicrobial classes in the main species of poultry production (Callens et al., 2018; Roth et al., 2018; EFSA, 2020). We did not find any isolate resistant to colistin, and this result is also consistent with what was reported by the official monitoring plan for the poultry sector, in which resistance to colistin is generally uncommon among *E. coli* isolates recovered from food-producing animals (EFSA, 2020). Furthermore, MDR isolates, exhibiting resistance to at least 3 antimicrobial classes, were frequently identified in the current study. The ampicillin-tetracyclines-sulphonamides association was the most common, as also reported for the *E. coli* strains from livestock used as indicator in Europe (EFSA, 2020).

At sample level, loads of *E. coli* and *E. coli* resistant to quinolones in goose faeces were estimated at approximately 6–7 log (CFU/g) and 5–6 log (CFU/g), respectively. Once again, these data are similar to the bacterial counts described in the cecum of broilers at slaughter (Pesciaroli et al., 2020). Therefore, despite the differences between geese and broilers, the dynamics of the *E. coli* population in these two animal species were similar. The prevalence of ESBL EC isolated from MacConkey without antibiotics was lower than 2% at the isolate level, as we already reported in broilers (Pesciaroli et al., 2020). This data underestimates the prevalence of geese shedding ESBL-AmpC EC, which are not dominant in the gut *E. coli* population (Apostolakos et al., 2020). In fact, according to selective culture on MacConkey^{cef}, geese shed ESBL EC in faeces for most of their life, as already shown in broilers (Dame-Korevaar et al., 2019). The average load of ESBL EC in goose faeces varied broadly along the production cycle. The ESBL EC were shed from the geese before the introduction in the farms even if showing low load. Such dynamic might be attributed to a vertical transmission from the parent and/or contamination at the hatchery as already suggested for broilers, where ESBL EC are already detected in 1-day-old chicken. (Bortolaia et al., 2010; Dame-Korevaar et al., 2019). In the first weeks, a sharp increase was observed: this is in line with what is

described on intensive broiler farms, where the detection frequency of ESBL-AmpC EC increases shortly after chick housing (Laube et al., 2013). This expansion of ESBL-AmpC EC is independent of the presence of antibiotic treatments and occurs even with a low colonization dosage (Laube et al., 2013; Robé et al., 2019). After the first weeks of age, the load of ESBL EC differed in the two farms: on A farm, the burden of ESBL EC remained steady until the end of the production cycle and was estimated at approximately 10⁵ CFU/g at the end of the cycle, which is in line with what observed in broilers (Laube et al., 2013). By contrast, on B farm the load of ESBL EC dramatically decreased from 6 wk of age onwards. The characterization of *E. coli*^{cef} from MacConkey^{cef} showed the presence of *bla*_{SHV}⁺, multiresistant ESBL *E. coli* isolates with a similar profile at T1 in the ducklings on the two farms. These isolates are probably derived from the farm of origin of the animals. From T2 onward, we observed a shift in the *E. coli*^{cef} population on B farm, where the multiresistant ESBL *E. coli* isolates were replaced by *bla*_{CTX-M-1}⁺ ESBL *E. coli* isolates susceptible to sulphonamides, chloramphenicol, and tetracyclines. The same shift was not observed on A farm. The management adopted by the two farms may explain this difference. In the first phases of the production cycle, the geese were kept in a confined environment on both farms, to ensure the birds remain at the right temperature. Then, the animals were left free to graze under the vineyard after about 30 d of age on B farm, while in the A farm the animals were raised indoor until the antibiotic treatment. On farm A, the geese were raised in the same building with broilers until T3, and the two groups of animal species were physically separated by double fence. The contaminated environment is a frequent source of ESBL-AmpC EC in the broiler production chain, where the residual contamination of the stable is a risk factor for the following productive cycle (Robé et al., 2019). In our experiment, since the ESBL *E. coli* isolated on farm A showed the same genetic profile along the production cycle, we could not exclude but neither confirms the hypothesis of a cross-contamination between broilers and geese. As an alternative, the presence of antibiotic residues in the barn due to previous treatments may have exerted a selective pressure for AMR bacteria (Schulz et al., 2019) on the A farm. An environmental contamination may have occurred also outdoor on both farms. Indeed some studies (i.e. Sarmah et al., 2006; Ercumen et al., 2020) reported that soil amended with animal wastes could represent a reservoir of antibiotics and enteric bacteria. If antibiotics in the environment are not efficiently degraded, they can assist in maintaining or developing antibiotic-resistant microbial populations (Witte, 1998). For instance, Hamscher et al. (2002) a field study where soil had been fertilized with liquid manure, reported that in the soil samples the concentrations of tetracycline varied from an average 86.2 µg kg⁻¹ in the topsoil (0–10 cm). We cannot exclude that the presence of residual contamination in soil may have affected the trend of ESBL-AmpC EC on the two farms.

It should be noted that geese on A farm were treated with antibiotics and this might have caused a co-selection phenomenon, favoring the expansion of multiresistant isolates (Laube et al., 2013). Consistently with this hypothesis, the proportion of the multiresistant ESBL EC increased dramatically at T3 on A farm, corresponding with the administration of tetracycline with water. A similar trend was observed for *E. coli* resistant to quinolones, where a drop of contamination along the production cycle was observed in B farm, but not on A farm. Once again, a sudden increase in the proportion of quinolones-resistant *E. coli* was observed on A farm shortly after the antibiotic administration. By contrast, the load of tetracyclines remained steady along the production cycle, with similar levels of contamination recorded at the end of the study. Quantitative data allow a better estimate of the risk for the consumer since we assume that a low load of ESBL-AmpC EC and quinolones-resistant *E. coli* in antibiotic-free geese farming before slaughter could reflect low levels of contamination and cross-contamination at the slaughterhouse and in the final product. Taken together, the analysis of quantitative data confirms a lower risk of AR on B farm than on A farm, except for tetracyclines, as already shown at the isolate level.

The Italian geese production is mainly confined in small productive realities and often products are locally sold within the region it produces. This limited geese production could also be linked to the backyard production systems, which play an important role for small farm economies in rural areas, through self-consumption or local sale of products (Hamilton-West et al., 2012; Di Pillo et al., 2019). The two farms studied applied the agroforestry system in which animals were grazing under an olive yard and a vineyard. Considering the worldwide attention on limiting the usage of antibiotics on livestock production, the agroforestry system could be considered one sustainable practice to achieve this purpose, improving the welfare and health of animals, affecting positively the environment, and increasing the soil quality (Cartoni Mancinelli et al., 2019; Massaccesi et al., 2019). However, while poultry conventional farming is highly integrated and has high biosecurity standards, the evaluation of biosecurity measures, health management, and disease control are usually very limited in those systems (Hamilton-West et al., 2012; Bravo-Vasquez et al., 2016; Di Pillo et al., 2019). Our study was carried out in 2 small farms which have different management practices, due to the lack of farms applying agroforestry system and great variability of these farms in our region. This represents a limit because we could not correlate an effect to a single variable.

Despite the limited number of farms and animals enrolled in the present study, the dynamics of antibiotic-resistant *E. coli* in farmed geese described in our study are consistent with the ones observed in broilers. In particular, we report the same loads of *E. coli* in the faeces and a sharp increase of ESBL EC in the first stages of the production cycle. Resistance to old antimicrobial classes, like tetracycline and sulphonamides, and

fluoroquinolones were common, suggesting that these antibiotic classes, widely used on broiler farms, were also administered in geese.

Notably, geese raised in extensive farming, characterized by low animal density and no antibiotic use, were characterized by a reduction in the burden of antibiotic-resistant commensal *E. coli*, as already shown in broilers (Pesciaroli et al., 2020). In conclusion, our work suggests that geese can contaminate the environment with antibiotic-resistant bacteria shed with their faeces. However, this risk of contamination can be mitigated by the adoption of good management practices, including prudent use of antibiotics and reduced bird density.

ACKNOWLEDGMENTS

The authors are grateful to the “Azienda Di Filippo” (Cannara, Pg, Italy) for hosting and encouraging this study. This work was funded by the Fondazione Cassa di Risparmio di Perugia (Grant: 2016.0151.021 “Tipicità delle produzioni locali: l’oca in vigna”).

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi: <https://doi.org/10.1016/j.psj.2021.101230>.

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