Antibiotic resistance genes load in an antibiotic free organic broiler farm

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ABSTRACT Antibiotic resistance is a serious concern for public health. Farm environments are relevant reservoirs of antibiotic resistant bacteria and antibiotic resistance genes (**ARGs**), thus strategies to limit the spread of ARGs from farms to the environment are needed. In this study a broiler farm, where antibiotics have never been used for any purpose, was selected to evaluate if this measure is effective in reducing the ARGs load in farm environment (**FE**) and in meat processing environment (**MPE**). Faecal samples from FE and MPE were processed for DNA extraction. Detection and quantification of the 16S rRNA gene and selected ARGs (*bla*$_{\text{TEM}}$, *qnr*S, *sul*2, and *tet*A) were carried out by PCR and digital droplet PCR (**ddPCR**), respectively. Generally, the relative abundance of the quantified ARGs in FE was similar or higher than that measured in intensive farms. Furthermore, apart for *tet*A, no differences in relative abundances of the other ARGs between FE and MPE were determined. These results suggest that the choice to not use antibiotics in broiler farming is not so effective to limit the ARGs spread in MPE and that further sources of ARGs should be considered including the preceding production phase with particular reference to the breeding stage.

Key words: antibiotic resistance, antibiotic free, poultry farm, ddPCR

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INTRODUCTION

The use of antibiotics in human and veterinary medicine causes the selection and spread of antibiotic resistance (Nhung et al., 2017). The latest ESVAC report, published in November 2021, shows that the volume of sales of antimicrobials for use in food-producing animals in Europe fell by more than 43% between 2011 and 2020 with Italy accounting for 696.7 tonnes of which 181.8 were dedicated to food-producing animals. The distribution of the various food-producing animals by country, expressed by Population Correction Unit (**PCU**) revealed an Estimated PCU (in 1,000 tonnes) of the population of poultry, for Italy, in 2020, equal to 766 (European Medicine Agency, 2021) . The annual monitoring of antimicrobial resistance in animals and food within the EU related to 2018 data was specifically focused on poultry and revealed for Italy a scenario were only about 28% of Salmonella isolates from broilers were fully susceptible (EFSA, 2020)

The circulation of potentially mobile antibiotic resistance genes (**ARGs**) in farms contributes to the dissemination of antibiotic resistance in environment. Thus, farms can be considered as reservoirs of antibiotic resistant bacteria and of ARGs. Poultry farms deserve particular attention in this respect because of the high antibiotic consumption in chicken production (148 mg per kg of animal produced, (Boeckel et al., 2015), second only to the pigs production, and because "poultry is one of the most widespread food industry worldwide" (Nhung et al., 2017) with the production of eggs and poultry meat that is globally increasing. One hypothetical way to reduce the spread of antibiotic resistance in farms could be not to use antibiotics at all, thus limiting the selection of ARGs because of the selective pressure exerted by the antibiotics. Although ARGs can persist in environment in absence of the antibiotic selection pressure, the strategy of not using antibiotics in poultry farms needs investigation to understand if it really affects the presence and spread of ARGs. In this study we selected a broiler farm where antibiotics are not used along the production cycle and we evaluated the presence and abundance of selected ARGs in feces collected in different houses of the farming environment (**FE**) and

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samples collected in the meat processing environment (**MPE**). Their abundances were compared with those detected in other production systems, previously investigated and already published, where antibiotics are used, and with those measured in environment with a different degree of anthropogenic pollution. Furthermore, we analyzed if the relative abundance of ARGs was significantly different in FE and in MPE, estimating the contribution of FE to the spread of antibiotic resistance in MPE.

MATERIALS AND METHODS

Sampling Scheme

The selected farm is a family-run organic broiler production facility (certified by the organic productions organism of certification namely ICEA) which is located in the middle of Padana plain in the north part of Italy. The farm is exclusively dedicated to chicken meat production by posing great attention to the respect of nature as a whole; animals are raised in the more natural way as possible by mimicking a wild raising environment thus guarantying the balance among animals-environment-human beings. One of the characterizing farming aspects is that no antibiotics are administered to animals. The farm includes not only the facilities for the farming of broilers (small houses in the FE) but also a small slaughterhouse that is integrated with the food processing and packaging room. Day-old chickens (hubbard breed) are bought from a commercial hatchery; on each occasion around 3,400 animals are housed in 2 different premises (males and females are kept separated in 2 different houses) and moved when they are 35 to 40 days old to the final house. The farm consists in total of 31 small houses, each one hosting from 800 to 1,200 animals and includes an open-air area freely accessible. Chickens are slaughtered in small groups when they are 81 to 110 days old (the depopulation of each house may take up to 2 wk). The empty house is cleaned and disinfected and populated again after more than 15 d. The slaughter plant operates 2 d per week. In addition it is family-operated facility with 5 full-time staff.

A feedmill was located onsite, and raw ingredients are largely plant-based (or grains) sourced from local organic grain producers.

The sampling was designed in order to collect representative samples of farmed animal (FE, samples from 1 to 3), slaughtering, processing and packaging (MPE, samples from 4 to 6) in occasion of the visit (performed on January 29, 2020), although recognizing the diversity among the samples within the same environment, that is, FE and MPE. Sampling points and methodology are described in detail below.

In all cases the final samples (namely from 1 to 6) were obtained by pooling together the elementary samples and were immediately transported to the laboratory under refrigeration temperature.

Sample 1: Fecal material collected using three pairs of boot swabs in one random house hosting 2 wk old chickens;

Sample 2: Secal material collected using three pairs of boot swabs in one random house hosting 60 days old chickens;

Sample 3: Fecal material collected using three pairs of boot swabs in one random house just depopulated;

Sample 4: Slaughterhouse environmental samples collected by using three sponges immediately after slaughtering before cleaning and disinfection (samples were collected from surface were organic matter was clearly present such as floor, evisceration area, drainage channels, surface of slaughtering equipment);

Sample 5: Processing environmental samples collected by using 3 sponges during the working session (samples were collected from the surface of working tables, knives, drainage channels, floor and walls);

Sample 6: Packaging environmental samples collected by using 3 sponges during the working session (samples were collected from walls, floor, working table, packaging machine, food trays)

At the laboratory each final sample was suspended with 100 mL distilled water, stomached for 1 min at room temperature in order to obtain a homogenized sample. A volume of 1 mL was used for DNA extraction. Two replicates were processed for each sample.

Antibiotic Resistance Determinants Selection

We selected ARGs widespread in farms and in environment. In brief, we tested the presence of bla_{TEM} as representative gene coding for resistance to β-lactams; *qnr*S, as representative of the quinolones resistance genes; *sul*2 and *tet*A as representative resistance genes against 2 of the oldest discovered antibiotics, sulfonamides, and tetracycline, respectively. Furthermore *mcr*-1 was selected as ARG particularly relevant at clinical level. The five selected ARGs were firstly screened by PCR end-point and if positive they were quantified by ddPCR. Droplet PCR was used for this scope instead of quantitative real time PCR because of its higher sensitivity, higher resistance to PCR inhibitors and unnecessary standard curves construction, particularly relevant when working with DNA extracted from environmental samples.

DNA Extraction

DNA extraction was performed with Qiagen QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions. Before proceeding with PCR end-point and ddPCR, the DNA concentration was measured with the NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher).

Antibiotic Resistance and 16S rRNA Gene Detection and Quantification

DNA samples were analyzed by PCR end-point (testing all the above mentioned ARGs) and ddPCR. PCR end-point assays were carried out in 25 μL with 2.5 μL of DNA (with range between 4.75 ng and 69 ng), 0.4 μM of each primer, 2 mM of $MgCl_2$, 200 μM of dNTPs, 1X PCR Buffer II (Thermo Fisher Scientific) and 2.5 U of AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific). PCR program was 95°C for 2 min, 25 cycles of 95°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, and the final extension was set at 72°C for 5 min. PCR products were run in agarose electrophoresis gel at 2%. Only positive samples by PCR were tested with ddPCR. DNA extracts from S. Typhimurium 2011_2776 for *Bla*$_{TEM}$, *sul*2, *tet*A, *E.coli* 2019_82 for *mcr*-1 and Monophasic Variant of S. Typhimurium 2019_112 for *qnr*S were used as amplification controls.

Positive DNA samples were tenfold diluted before the analysis with ddPCR. ddPCR assays were carried out with 22 μL of reaction mix prepared assembling QX200 ddPCR EvaGreen Supermix with primers at the concentration of 3 μM, 2 μLof DNA and nuclease free water. Aliquots of 20 μL of each sample were transferred to the DG8 Cartridge together with 70 μL of QX200 Droplet Generation Oil. DG8 Cartridge was placed in QX200 Droplet Generator (Bio-Rad). Droplets were carefully transferred to a 96-well PCR plate for the amplification on a C1000 Touch Thermal Cycler (Bio-Rad). Positive controls (amplified target gene) and NTC (No template controls) were included in each run. The program, recommended by Bio-Rad, was 95°C for 5 min, 40 cycles of 95°C for 30 s and annealing/extension temperature (optimized for each tested gene) for 1 min with a ramp rate of 2°C s^{-1} and 2 final steps at 4°C for 5 min and 90° C for 5 min. The plates were transferred in QX200 Droplet Reader (Bio-Rad) to acquire data. Reactions with more than 10,000 droplets were analyzed. Thresholds to discriminate between positive and negative droplets were manually set up and only samples with ≥3 positive droplets (Di Cesare et al., 2016b) were considered as positive. Data were expressed as gene copy μL^{-1} using QuantaSoft Analysis Pro software (Bio-Rad) for the analysis.

Statistical Analysis

Gene abundances were normalized by dividing the copy number of the quantified gene by the corresponding 16S rRNA gene copy number. In order to evaluate the presence and abundance of ARGs in our system, the difference in their abundances according to sampling environment ("Farming" vs. "Meat Processing") was assessed by ANOVA. For the test, as response variables were used the relative abundances of the genes transformed, prior to be analyzed, into the arcsine of the square root of their values, because they represent proportion data. The results were considered significant at $P < 0.05$. The statistical analyses were performed in the R environment version 3.6.0.

RESULTS AND DISCUSSION

Apart the *mcr*-1 gene, codifying for resistance to colistin, which is the last resort antibiotic used to treat multidrug resistant gram-negative bacterial infections, all the other ARGs, tested by PCR, were positive. This result is not surprising taking into account that the detected ARGs, *bla*$_{TEM}$, *qnr*S, *sul*2, and *tet*A, are widely spread in the farm environments. On the contrary, although previously found in chicken farms, *mcr*-1 could not have been extensively disseminated in farm environments (Petrin et al., 2019). Moreover *bla*$_{TEM}$ has previously been detected as one of the most frequent clinically relevant ARGs in poultry farms (Wang et al., 2021), widely distributed on conjugative plasmids, *qnr*S is located at plasmid level and frequently detected in potential human pathogenic bacteria, for example, *Escherichia coli, Salmonella*, isolated from humans, poultry, and pigs (Literak et al., 2010), *sul*2 and *tet*A are widely distributed in poultry farm environments (Mazhar et al., 2021).

Thus, *bla*$_{TEM}$, *qnr*S, *sul*2, and *tet*A were further analyzed by ddPCR. Droplets were $1.11 \times 10^4 \pm 1.11 \times 10^3$ for *bla*$_{TEM}$, $1.23 \times 10^4 \pm 1.12 \times 10^3$ for *qnr*S, $1.36 \times 10^4 \pm 4.33 \times 10^2$ for *sul*2 and $1.22 \times 10^4 \pm 1.18 \times 10^3$ for *tet*A. The most abundant ARG was *sul*2, its relative abundance ranged between 8.70×10^{-4} gene copy/16Sr RNA copy in MPE and 4.90×10^{-1} gene copy/16S rRNA copy in FE. The *tet*A relative abundance was comprised between 4.00×10^{-4} gene copy/16S rRNA copy in MPE and 3.60×10^{-3} gene copy/16S rRNA copy in FE. The *qnr*S relative abundance ranged from 0.00 to 1.20×10^{-2} gene copy/16S rRNA copy in MPE. *bla*$_{TEM}$ was the lowest abundant resistance gene with a relative abundance ranging from 1.00×10^{-3} gene copy/16Sr RNA copy in MPE to 2.60×10^{-3} gene copy/16S rRNA copy in FE.

The mean value of the relative abundance of *sul*2 in FE (2.11×10^{-1} gene copy/16Sr RNA copy, Figure 1) resulted similar to what previously detected in chicken and duck farms of comparable dimensions (25,000 chicken and 38,000 ducks), where antibiotics use could not be excluded (Cheng et al., 2013). The *sul*2 gene is widely distributed in environment and also used as marker of the antibiotic resistance spread since the beginning of the antibiotic era. It was previously found in low anthropogenic impacted environments, for example, polar marine sediment environment (Tan et al., 2018) and in highly anthropogenic polluted environments, for example, wastewater treatment plant effluents (Di Cesare et al., 2016a). Comparing the relative abundance of *sul*2 in those environments with that measured in this farm, it is clear that this gene was highly concentrated in FE, being at least 2 Log more abundant than the high and low anthropogenic polluted environments above mentioned. Furthermore, a tendency of

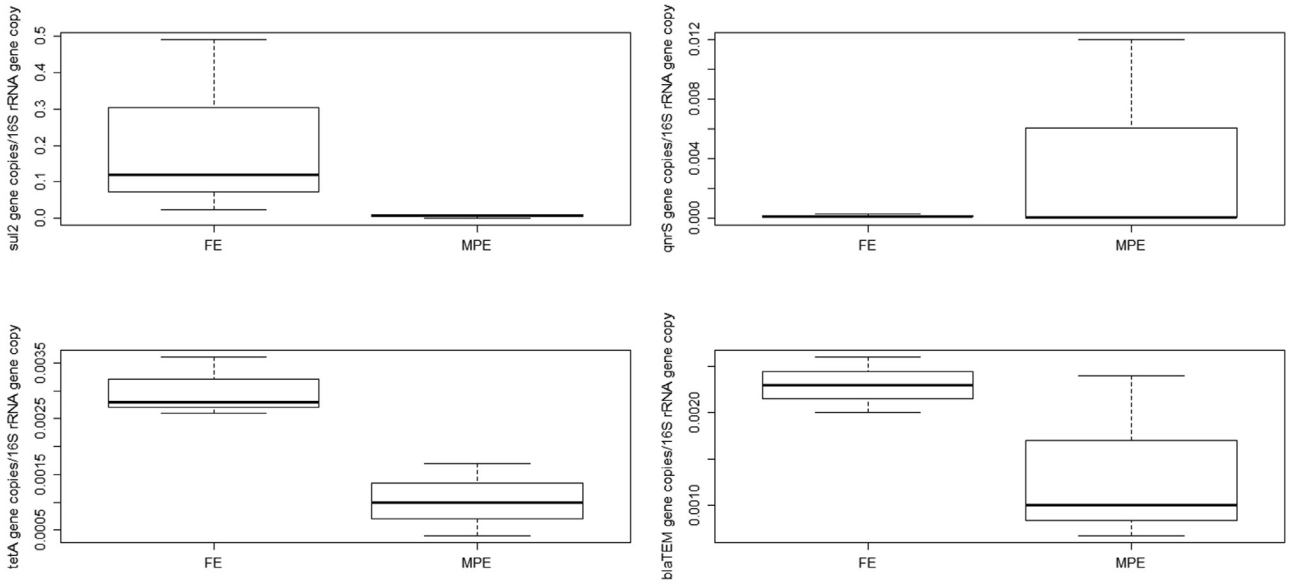

Figure 1. Relative abundances of ARGs according to sampling environment. Boxplots of the distribution of abundances of *sul*2, *qnr*S, *tet*A, and bla_{TEM}, quantified by ddPCR, in the samples from the sites where the animals were farmed, "Farming Environment" (FE), and in the samples collected in sites where the meat was processed, "Meat Processing Environment" (MPE).

*sul*2 to increase moving from the sample 1 (younger animals) to the sample 3 (older animals) was observed, possibly due to a higher chance to have contamination of the FE by pests or management practices with time in older chickens derived samples. Thus, this (organic) chicken farm also in absence of the selective pressure exerted by the use of antibiotics could significantly contribute to the spread of the sulfonamide resistance in environment. The mean value of the relative abundance of *tet*A in FE was 3.00×10^{-3} gene copy/16S rRNA copy (Figure 1), resulting higher than that measured in other farm environments where the use of antibiotics could not be excluded (Petrin et al., 2019), and similar to that measured in anthropogenic impacted environments, for example, wastewater treatment plant effluents (Di Cesare et al., 2016b), and at least 3 Log higher than that measured in low anthropogenic polluted environment, for example, polar marine sediment (Tan et al., 2018). The mean value of the relative abundances of the other 2 tested ARGs in FE (2.30×10^{-3} and 1.52×10^{-4} gene copy/16S rRNA copy for bla_{TEM} and *qnr*S respectively, Figure 1) placed in between those measured in highly (Di Cesare et al., 2016b) and low (Tan et al., 2018) anthropogenic polluted environments.

The relative abundances of the tested ARGs in MPE resulted relatively lower if compared with those in FE (Figure 1), however, because of the high intra group variability observed due to the high diversity among the samples within the same environment, in particular for *sul*2, the difference in ARG abundances between the two different groups was not statistically confirmed (Table 1). Only the relative abundance of *tet*A was significantly higher in FE than in MPE (Table 1). This result opens to two possible scenarios: a) the ARGs stabilize in bacterial communities isolated from the environmental farm irrespective of the analyzed matrices, that is, feces in FE and meat processing facility in MPE, eventually reaching the final products. Indeed it is commonly recognized that meat form animals never treated with antibiotics could harbor antibiotic resistant bacteria and b) other sources, for example, meat handlers, meat processing surfaces hosting bacteria resistant to disinfection procedures, could contribute to the ARGs stabilization in the bacterial communities in MPE.

Overall our results showed that, although in the analyzed broiler farm the antibiotic use was banned, ARGs were present in the bacterial communities isolated from the animal feces and in environmental samples of meat processing facilities. The relative abundance of the tested ARGs measured in FE was generally comparable to that measured in nonantibiotic free farms suggesting that the efficacy of the choice to not use antibiotics is limited by the fact that chicks could be colonized by antibiotic resistant bacteria at their arrival. Furthermore, the ARGs relative abundance in FE was for some genes higher or comparable to that measured in high anthropogenic polluted environments, and for others in between the high and low anthropogenic impacted environments, suggesting the possible contribution of the antibiotic free farms polluted to the spread of ARGs in environment. Finally, this study, although limited to only one antibiotic free farm, lacking of baseline

Table 1. Statistical results for the analysis of variance (ANOVA) assessing the influence of the sampling environment ("Farming" vs. "Meat Processing") on the abundance of ARGs.

Gene	Df	Sum Sq	Mean Sq	F value	*P* value
*sul*2	1	1.8648e-05	1.8648e-05	3.6619	0.1282
*qnr*S	1	1.1515e-07	1.1515e-07	0.6143	0.477
*tet*A	1	8.4291e-08	8.4291e-08	12.421	**0.02435***
bla_{TEM}	1	2.3065e-08	2.3065e-08	2.9799	0.1594

*$P < 0.05$, indicates that there is a significant difference in the abundance of *tet*A between the samples collected in the "Farming" environment and those collected in the "Meat Processing" environment.

microbial data, and to the quantification of selected ARGs instead of using a shotgun sequencing approach (thus providing a complete overview of the whole antibiotic resistome), highlights that the choice of not using antibiotics in animals, alone, is not effective to limit the spread of ARGs along the chicken meat production chain, thus claiming for additional measures to be taken.

DISCLOSURES

The authors declare no conflicts of interest.

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