Microbial community composition and antimicrobial resistance in agricultural soils fertilized with livestock manure from conventional farming in Northern Italy

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Highlights

- Manure application does not strongly influence soil microbiome
- Fertilization enriches the abundance of some ARGs (i.e. *ermA*, *ermB*, bla_{oxa-1} and *oqxA*) harboured in agricultural soil
- Manure-derived ARGs in soil display different dissipation patterns
- Flumequine residues correlate with *oqxA* and *qnrS* abundances
- Higher ARG abundances are present in swine *vs.* dairy and poultry manure

Abstract

Antimicrobials are commonly used in conventional livestock production and manure is widely applied to agricultural lands as fertilizer. This practice raises questions regarding the effects of fertilization on (i) soil microbiota composition and (ii) spread of antimicrobials and antimicrobial resistance (AMR) in the environment. This study was conducted in a high-density farming area of Northern Italy and aimed at assessing the impact of (dairy cattle, chickens and swine) manure application on soil microbiome, antimicrobial concentrations and antimicrobial resistance gene (ARG) abundance. We found the microbial community composition in manure to be different and less diverse than in soil, with manure application altering only marginally the soil microbiome. Exceptions were the phyla Firmicutes, Tenericutes and Cloacimonetes, which significantly enriched in fertilized soil. Of the antimicrobials investigated, only flumequine concentrations increased after manure application, albeit non-significantly. ARGs were more abundant in manure, with *ermA*, *ermB,* $bla_{OXA\text{-}1}$ and *oqxA* being significantly enriched in fertilized soil. Positive correlations between *oqxA* and *qnrS* abundances and flumequine concentrations were observed, together with the co-occurrence of some ARGs and microbial taxa (e.g. *oqxA* correlated with Acidobacteria and Gemmatimonadetes). This study showed that manure application has little effect on soil microbiome but may contribute to the dissemination of specific ARGs into the environment. Moreover, flumequine residues seem to enhance the emergence of *oqxA* and *qnrS* in soil.

1. Introduction

Manure derived from conventional livestock production systems is commonly used in agriculture to improve soil quality and, as organic fertilizer, to provide nitrogen enrichment and increase organic matter (Das et al., 2017). However, microbial communities in manure may influence soil microbiome, either directly through competition or indirectly by spreading antimicrobial resistance (AMR). The extent to which the manure microbiome influences the soil microbial community remains unclear. Although some studies have found that organic manure application significantly alters the soil microbiome (Stocker et al., 2015; Zhang et al., 2020), other studies have reported changes limited to a few taxa, whereas the main microbial composition of the soil remains unmodified (Lopatto et al., 2019). Antimicrobial drugs have been widely used for several decades in conventional livestock production. While some of these antimicrobials are scarcely metabolized in the animal body and eliminated as such, others are metabolized and excreted as active or inactive metabolites in urine and faeces (Wei et al., 2011; Xia et al., 2019a). After soil fertilization, antimicrobial residues may spread into the surrounding environment, potentially inducing the emergence of resistant bacteria and antimicrobial resistance genes (ARGs) (Hou et al., 2015; Munk et al., 2018; Qiao et al., 2018; Rovira et al., 2019; Xia et al., 2019a). The dissemination of ARGs in the environment represents a great concern for public health, since they can be integrated into mobilizable genetic elements, such as plasmids or transposons, and propagated via horizontal gene transfer (HGT) among bacteria, including pathogenic and non-

pathogenic ones (Qiang et al., 2006). Indeed, ARGs disseminated into the environment have the potential to be transferred to humans, via dispersion into waterways, through runoff and drainage from the soil, or by entering the food chain (Berendonk et al., 2015; Hruby et al., 2016; Marti et al., 2013; Pruden et al., 2012). In the present study, we investigated for the first time the impact of the application of manure from three different livestock sectors, namely dairy cattle, chickens and swine, located in a high-density farming area of Northern Italy on the microbial community composition, antimicrobial concentrations and ARG abundances in agricultural soil. The main purpose of the study was then to improve our understanding of the impact of fertilization with manure from conventional livestock farms on soil microbiome and AMR spread into the environment, as well as the correlation between antimicrobial concentrations and ARG abundance. Furthermore, we aimed to assess whether the different livestock sectors differed in their microbial communities, in the concentration of antimicrobial residues and in ARG abundance.

2. Materials and Methods

2.1 Sampling procedure

Samples were collected from 31 conventional farms (10 chicken farms, 10 swine farms, and 11 dairy cattle farms) located in two regions in Northern Italy from October 2017 to March 2019. Farms were chosen because of their location in a high-density farming area and because of the application of an integrated agricultural system, meaning that the manure produced by each farm was used to fertilize the

surrounding farmland. In each farm, three samples were collected from: 1) manure or slurry (one sample), 2) soil (one sample before fertilization with manure/slurry and one sample one month after fertilization with manure/slurry), accounting for a total of 93 samples (i.e. 3 samples x 31 farms). Details of the sampled farms are summarized in supplementary material 1.

Manure/slurry samples were collected according to Kumari et al. (2015). Briefly, manure samples were collected by taking 1 kg of 10 manure cores (1.5 cm in diameter and 12 cm deep) at randomly selected locations and then pooled; slurry samples were collected by taking 1 L of slurry from five different points at 1 m depth from the surface of the storage tank. Soil samples were collected according to Dong et al. (2014) by sampling in the low layer (0-20 cm) and using an auger with a 5 cm internal diameter at five randomly selected locations and then pooled.

2.2 Quantification of antimicrobials

The analytical method was set up to detect and quantify 14 different antimicrobials: amoxicillin, ampicillin, cefquinome, ceftiofur, ciprofloxacin, danofloxacin, enrofloxacin, flumequine, marbofloxacin, erythromycin, spiramycin, tilmicosin, tylosin and colistin. The 14 antimicrobials were chosen as representing some of the most commonly used antimicrobials in animal farming and/or because listed as critically important antimicrobials by the World Health Organization (2019). Soil and manure (dairy cattle and chickens) or slurry (swine) sample purification was performed as previously described (Chiesa et al., 2018), with some modifications. An

aliquot (1 g weight) of mixed soil or manure/slurry from dairy cattle, chickens or swine, spiked with IS (enrofloxacin d5) at a final 50 ng/g, 100 µl of 20% TCA for protein precipitation and 10 ml McIlvaine buffer (pH 4.0), were combined. Samples were vortexed and sonicated for 10 minutes (min). After centrifugation at 2,500g, 4°C for 5 min, the supernatant was transferred to a clean falcon tube and defatted with 10 ml n-hexane, then vortexed and centrifuged (at the previously reported conditions) to discard the n-hexane layer. Solid phase extraction Oasis HLB cartridges was used to purify the obtained extracts; then the eluate was dried and reconstituted as reported by Chiesa et al. (2018). Chromatographic separation was obtained with the same gradient elution for all compounds except for colistin, as in Chiesa et al. (2018). For colistin, the elution started with 90% A (aqueous formic acid 0.1%; B methanol), maintained for 5 min, followed by a decrease to 10% A at 6 min, and maintained till 11 min. Subsequently, the mobile phase was gradually increased back to 90% A at 12 min and then held constantly until 17 min.

Mass spectrometric (MS) analysis was performed on a triple-quadrupole TSQ Quantum MS (Thermo Fisher Scientific, Massachusetts, U.S.A.) equipped with an electrospray interface (ESI) set in the positive (ESI+) electrospray ionization mode for all analytes (Chiesa et al, 2018). The selected diagnostic ions, one of which was chosen for the quantitation, and the collision energies are reported in supplementary material 2. Acquisition data were recorded and elaborated using Xcalibur software from Thermo Fisher Scientific.

After the identification of samples in which the absence of antimicrobials was detected, through a preliminary screening of soil or manure and slurry samples, the method was validated according to the Commission Decision 2002/657/EC criteria (European commission, 2002). For the validation procedure on manure, samples of manure/slurry from dairy cattle, swine and chickens were mixed to obtain pooled manure samples.

For each analyte, the method performance was evaluated by the determination of retention time (RT), transition ion ratios, recovery, accuracy (trueness), precision (expressed as the intra- and inter-day repeatability), linearity, as well as the decision limit (CCα) and detection capability (CCβ), which were calculated as described in SANCO/2004/2726 revision 4 (European Union, 2008). The detailed procedure is reported in Chiesa et al. (2018). All the results of the method validation for all the compounds are reported in supplementary material 3.

2.3 DNA extraction

Twenty-five grams (manure or soil) or 25 ml (slurry) of sample were placed in a sterile Filtra-bag (280 μm pore size), added with 25 ml of Phosphate Buffered Saline (PBS) and mixed by hands for 1 min. The filtered liquid was centrifuged at 4,000 rpm for 10 min at 4°C; DNA was extracted from 250 mg of the resulting pellet using DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA quality and quantity were assessed using a UV-Vis

spectrophotometer NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, United States).

2.4 16S rRNA gene amplification, sequencing, and data analysis

To evaluate differences in bacterial communities among manure/slurry, fertilized and unfertilized soils, the V3-V4 regions of the 16SrRNA gene were amplified with primers 341F/R806 (Takahashi et al., 2014) modified with overhangs to add index adapters using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). PCRs were carried out in a 2720 thermal cycler (Applied Biosystems, Waltham, MA) with 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds, followed by a final extension for 7 minutes at 72°C. PCR products were purified using the SPRIselect purification kit (Beckman Coulter, Brea, CA), and barcodes introduced via a second PCR using platform-specific barcode-bearing primers (Milan et al., 2018). Following a second purification, libraries were quantified using the Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Monza, Italy) and pooled. Pooled DNA concentration and integrity were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), and then sequenced using the Illumina MiSeq sequencing platform (San Diego, California, USA) with a 2×300 bp paired-end approach.

DADA2 package within the Quantitative Insights into Microbial Ecology 2 (QIIME2 version 2019.4) software was used for 16S rRNA data analysis (Bolyen et al., 2019; Callahan et al., 2016). To assign taxonomy categories, a Naive Bayes classifier and

the q2-feature-classifier plugin were used. Taxa assignment was carried out using SILVA- Naive Bayes sklearn trained database (Yilmaz et al., 2014). The raw sequence reads have been deposited in the NCBI Short Read Archive under the accession number PRJNA600160. The on-line based software Calypso (http://cgenome.net/wiki/index.php/Calypso) was used for microbial community characterization and α- and β-diversity statistics (Zakrzewski et al., 2017). Default parameters were employed for data filtering. Total sum normalization (TSS) and SquareRoot data transformation were used. The microbial community composition was visualized using heatmap and network analyses. To quantify the microbiome diversity within each sample group, Shannon index and Chao1 methods were employed. To assess the overall differences in microbial community composition among type of sample and livestock sectors, principal coordinate analysis (PCoA) plots and non-metric multidimensional scaling (NMDS) were used for visualization, and permutational multivariable analysis of variance (PERMANOVA) based on the Bray-Curtis dissimilar measure for significance testing using the Adonis function.

2.5 Quantitative PCR (qPCR) analysis of antimicrobial resistance genes (ARGs)

The detection of ARGs to antimicrobials commonly used in conventional farming was carried out by quantitative polymerase chain reaction (qPCR). Gene-specific qPCRs paired with melting curve analysis were employed for detecting the following ARGs: *ermA, ermB, oqxA, oqxB, qnrS, qnrA, qnrB, mcr-1, mcr-2, mcr-3, mcr-4, mcr-5,* bla_{TEM-1}, bla_{SHV}, $bla_{CTX-M-1like}$, bla_{CMY-2}, bla_{OXA-1}, bla_{OXA-48}, bla_{VIM-2} *and* bla_{NDM}. All

qPCRs were performed in a final volume of 10 µL using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) with optimal concentration of each primer (600/600nM) in a LightCycler®480 Roche (Roche, Basel, Switzerland) real-time platform. ARG-specific primers and reference strains used as positive control are summarized in supplementary material 4. Cycling conditions were as follows: initial incubation at 50 °C for 2 min, followed by 2 min at 95 °C, and 45 cycles at 95 °C for 10 sec and 50-60 °C for 40 sec. Melting curves between 40 and 95 °C were determined by adding a dissociation step after the last amplification cycle at a temperature transition rate of 4.4 °C/sec. The absolute abundance of ARGs in samples was calculated based on standard curves obtained using serial dilutions of previous end-point PCR amplicons of target DNA isolated from the reference strains. However, the absolute abundance of ARGs in a given sample is not a significant value, as it is proportional to the total DNA present in the sample; hence, ARG relative abundance was calculated, by normalizing the ARG copy number to 16S rRNA gene copy, and used in the statistical analysis.

2.6 Statistical analysis

Differences in ARG occurrence (presence/absence) over types of samples (manure/slurry, soil before fertilization, or soil 1 month after fertilization) and livestock farms (i.e. dairy cattle, swine or chickens) were tested using generalized linear models (GLMs) with a logit link function, binomial error distribution and a cluster-robust sandwich variance estimator to account for clustering of samples

collected from the same farms, while differences in ARG abundance were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons. Differences in antimicrobial residues over livestock farms and types of samples were tested using Tobit regression on log-transformed residue concentrations with the left-censoring limit set at the minimum detection threshold. To assess the association between antimicrobial residues concentration and ARG relative abundance over sample type, Spearman rank correlation analysis was performed. To assess the association between the relative abundance of microbial taxa at phylum and family levels with the ARGs, multivariate regression analysis with several dependent variables (i.e. log-transformed relative abundances of the microbial taxa) was used to jointly regress on the same independent variables (i.e. presence/absence of the different ARGs), while adjusting for livestock farm and type of sample, and accounting for clustering of observations at the farm level (cluster variable) using bias-corrected and accelerated cluster-bootstrapped standard errors (1000 replications). Since manure/slurry were considered as a unique statistical unit, hereafter we will use the term manure to refer to manure/slurry samples. Statistical analysis and data visualization were carried out in R (version 3.6.3) (https://www.r-project.org/).

3. Results

3.1 Prevalence and quantification of antimicrobials

Out of 93 samples, only 39 were found positive to at least one antimicrobial. Flumequine was found in 38 samples, although in eight it was detected as traces

250 (<CCα). Of the 14 antimicrobials investigated, six were detected in at least one
251 sample; flumequine was the most detected (38/93; 40.86% of the total samples),
252 followed by enrofloxacin (7/93; 7.53%), tylosin (3/93; 3.22%), and marbofloxacin,
253 ampicillin and ciprofloxacin (2/93; 2.15%) at a much lower prevalence. Flumequine
254 and enrofloxacin were the only compounds identified also in soil, while the
255 remainders were detected only in manure. The prevalence of antimicrobials in swine
256 and chicken farms was similar (22 *vs*. 16 samples, respectively). However,
257 flumequine was more prevalent in swine samples (73.33%) than in chicken samples
258 (53.33%), although the difference was not significant ($p > 0.05$). Marbofloxacin was
259 detected only in swine, ciprofloxacin only in chickens and swine, and ampicillin in
260 one sample of chicken and dairy cattle farms, respectively. Antimicrobial
261 concentrations ranged from below the decision limit (CCα) to 437.38 ng/g
262 (flumequine). The average concentration of flumequine in manure was 24.79 ng/g,
263 while in soil before and after fertilization was 77.77 ng/g and 109.36 ng/g,
264 respectively (Fig. 1A). However, the difference between soil and manure samples and
265 between fertilized and unfertilized soil was not significant ($p > 0.05$). Considering the
266 livestock sector, flumequine concentrations were not different in swine and chicken
267 samples, but these were significantly higher ($p < 0.001$) in comparison to samples
268 from dairy cattle, in which it was never detected (Fig. 1B).
269
270 **3.2 General description of DNA sequences**

After the quality-filter step, removal of chimeric fragments and reads merging, a total of 2,851,500 reads were obtained with 35,921 different features, with an average of 30,661 sequences per individual sample. Filtering by quality, three samples were excluded and 90 were considered in the downstream analyses. The rarefaction curves for samples plateaued, indicating that the obtained sequencing depth was good (supplementary material 5).

3.3 Composition of bacterial communities

Using 16S rRNA gene sequencing, the microbial community structure of samples was characterized. At phylum level, members of the Firmicutes and Bacteroidetes dominated the community composition of manure samples. While Bacteroidetes were dominant also in soil samples, Firmicutes were significantly ($p < 0.05$) less abundant. On the contrary, Acidobacteria and Proteobacteria were significantly more abundant ($p < 0.05$) in soil than in manure and, as a result, the heatmap at phylum level shows two main clusters, one grouping manure samples and one grouping soil samples (Fig. 2). Network analysis at operational taxonomy unit (OTU) level based on the 3,000 more abundant OTUs corroborated this finding, showing a marked separation between manure and soil samples (Fig. 3). Despite this clear separation when considering the whole bacterial community, significant ($p < 0.05$) changes in specific phyla (i.e. Cloacimonetes, Firmicutes and Tenericutes) after fertilization were observed. Within manure samples, while Firmicutes were highly abundant in all farm types, Proteobacteria and Actinobacteria phyla were more abundant in chicken

manure samples, while a higher percentage of Bacteroidetes was identified in dairy cattle and swine. As a result, two distinct clusters within manure samples were observed, one containing chicken manure samples and one containing dairy cattle and swine samples (Fig. 2).

3.4 Bacterial community α-diversity

The α-diversity, i.e. the diversity within each sample type, was evaluated at OTU level using two methods, the Shannon index and Chao1 (Fig. 4). Both measurements showed a significantly ($p < 0.05$) lower α-diversity in manure samples in comparison to soil samples, both pre- and post-fertilization. On the contrary, the α-diversity was comparable between soil samples before and after manure application. When considering the diversity within samples belonging to the same livestock sector, dairy cattle farms were characterized by the lowest α-diversity, while swine showed the highest α-diversity. The difference in α-diversity between farm type samples was highly significant ($p < 0.001$).

3.5 Comparison among bacterial communities (β-diversity)

To compare the bacterial communities between sample types (β-diversity) at OTU level, PCoA and NMDS ordination together with PERMANOVA were used. This analysis showed that the microbial community in manure was significantly different ($p < 0.001$) from that of soil before and after fertilization, whereas there was no significant difference between fertilized and unfertilized soil. This finding was

supported by PCoA and NMDS graphs (Fig. 5A, B), showing a marked difference in bacterial communities composition between manure and soil and the clustering of soil samples before and after fertilization. Pairwise PERMANOVA also showed that differences in microbial community composition between farm types were highly significant ($p < 0.001$), and PCoA and NMDS graphs confirmed these observations (Fig. 5C, D).

3.6 Prevalence and relative abundance of ARGs

Besides microbial community characterization, the prevalence and abundance of ARGs in manure and soil samples was investigated to assess whether manure application enriched ARG abundance and diversity in the soil. Of the 20 ARGs investigated, all but *qnrA* and bla_{NDM} genes, were detected in at least one sample. Bla_{TEM-1} (89.25%) and *ermB* (81.72%) were the most prevalent ARGs, followed by *ermA* (65.59%), bla_{CMY-2} (58.06%) and bla_{OXA-1} (45.16%), while the prevalence of the other genes ranged from 30.11% ($bla_{CTX-M-1LIKE}$) to 3.23% (*mcr-2*) (supplementary material 6). *Mcr-2* was detected only in manure samples, while bla_{VIM-2} was present only in soil. No target genes emerged in soil after manure application (supplementary material 6). A significant increase in the number of ARG positive soil samples after fertilization was observed for *ermA*, *ermB*, *qnrS*, bla_{CMY-2} and bla_{OXA-1} (Fig. 6A). When considering the different livestock sectors, only swine samples were positive for *mcr-2*, whereas no bla_{OXA-48} and bla_{SHV} were detected in samples from this sector.

336 The total abundance of ARGs normalized to 16S rRNA for each sample ranged from
337 1.24×10^{-14} to 0.06 ARG copy per million copy of 16S rRNA. Soil samples before
338 manure application showed lower total ARG abundance in comparison to soil after
339 fertilization, possibly reflecting the legacy of manure application, as manure samples
340 had the highest total abundance of the target genes (Fig. 6B). Target genes did not
341 show the same abundance patterns, and the relative abundance of some genes varied
342 between sample categories; *ermA*, *ermB,* bla_{OXA-1}, and *oqxA* had similar dynamics,
343 being significantly enriched ($p < 0.05$) in soil after manure application (Fig. 6C, D, E
344 and F), while $bla_{CTX-M-1LIKE}$, bla_{SHV}, and bla_{TEM-1} were significantly more abundant in
345 manure than soil ($p < 0.05$), but their relative abundance decreased after fertilization
346 (Fig. 6G, H, and J). *QnrS* was significantly more abundant in manure than in
347 untreated soil ($p < 0.001$), with trends towards enrichment after fertilization ($p =$
348 0.048) (Fig. 6I). The remaining target genes showed a low relative abundance and no
349 differences between sample categories were observed. Comparisons between
350 livestock sectors showed higher numbers of target gene copies in swine samples,
351 whereas dairy cattle samples had the lowest abundance (Fig. 6B). Specifically,
352 bla_{OXA-1}, *ermB*, *mcr-1* and *qnrS* were significantly ($p < 0.05$) more abundant in swine
353 samples than the other two sectors (Fig. 7).
354
355 **3.7 Co-occurrence between ARGs and bacterial taxa**
356 Co-occurrence patterns between the ARGs and the main microbial taxa were
357 explored at the phylum level by using multivariate regression analysis. A positive

correlation was observed between *ermA* and two phyla, Bacteroidetes (β-coefficient = 0.173, $p < 0.01$) and Hydrogenedentes (β-coefficient = 0.059, $p = 0.04$), suggesting that these phyla may harbour *ermA*. Acidobacteria (β-coefficient = 0.144, $p = 0.046$) and Gemmatimonadetes (β-coefficient = 0.138, $p = 0.044$) co-occurred with *oqxA*, while Spirochaetes may be potential hosts for bla_{OXA-1} (β-coefficient = 0.169, $p = 0.05$) and *mcr-1* (β-coefficient = 0.347, $p < 0.001$) genes.

3.8 Correlation analysis between antimicrobials and ARGs

The correlation analysis between antimicrobial concentration and the relative abundance of the 18 ARGs detected using Spearman's bivariate correlation analysis was performed considering only flumequine, since the low number of observations for the remaining antimicrobials made the analysis not possible. The analysis showed positive correlations between flumequine concentration and the abundance of *oqxA* (Spearman $r = 0.2836$, $p = 0.006$) and *qnrS* (Spearman $r = 0.2715$, $p = 0.008$), while no significant correlation was observed for the other genes conferring resistance to (fluoro)quinolones, nor any other ARGs.

4. Discussion

4.1 Antimicrobials prevalence in soil and manure

A low percentage of positive samples (42%) was found in the study, and only six out of the 14 antimicrobials screened were detected. Although flumequine is an old-generation (fluoro)quinolone relatively used in chickens and swine due to its low

costs and good tolerability mainly, it was the most detected compound (97.4%) in these livestock and the only antimicrobial detected in soil. This is in agreement with its known high persistence in manure, where it can remain after one year, thus its environmental presence was quite expected (Berendsen et al., 2018).

4.1 Impact of manure application on soil microbiome

The microbial composition for both manure and soil samples is in agreement with previous studies; the most abundant phyla identified are those typical of manure and soil microbiomes and the manure microbiome is confirmed to be less diverse than that of soil (Chen et al., 2015; Fierer, 2017; Hamm et al., 2016; Looft et al., 2012). In accordance with previous observations (Riber et al., 2014; Xie et al., 2018), manure application showed limited influence on soil microbial community and did not increase soil diversity, but rather caused significant changes only in a few phyla. Firmicutes, representing the most abundant phylum in manure, was enriched in soil after fertilization as previously reported (Rieke et al., 2018). However, several studies have shown an increase of soil microbial diversity after manure application and significant changes in the whole microbial community composition (Chen et al., 2015, 2019; Zhen et al., 2014), indicating that in some cases fertilization may have a strong impact on soil. These contrasting results might be due to the inability of most manure-associated bacteria to survive for long periods in soil, making the time of sampling a key factor and suggesting that manure microbiome might influence only temporarily the soil microbial community (Leclercq et al., 2016; Rieke et al., 2018).

Furthermore, factors other than manure application (i.e. temperature, moisture, pH, seasonality) are known to influence soil microbial composition over time, and might be accountable for such contrasting results (Classen et al., 2015; Fierer, 2017; Lopatto et al., 2019). Here, the sampling was carried out over 18 months (but mostly during winter); however, pH, humidity and temperature were comparable among most of the farms (supplementary material 1). Overall, data gathered in this study indicate that manure application has only a limited effect on soil microbiome at day 30 post-fertilization.

4.2 Abundance of ARGs and their fate in soil after fertilization

Aiming to understand whether ARGs disseminate from manure to soil, the prevalence and relative abundance of selected genes were assessed in manure and soil before and after fertilization. The highest number of total ARG copies was detected in manure and a clear increase in ARG abundance was observed in soil after fertilization. According to previous studies, this finding indicates that manure increased ARG abundance in soil (Marti et al., 2014; Xia et al., 2019a; Xiong et al., 2015; Zhao et al., 2017). None of the selected ARGs emerged in soil after manure application, suggesting that such practice might effectively enrich but not introduce any of the screened genes. The ARGs investigated in our study did not show the same abundance pattern, and only four genes (i.e. *ermA*, *ermB*, $bla_{oxa\text{-}1}$ and *oqxA*) were significantly enriched in manure-amended soil. Both *ermA* and *ermB* have been demonstrated to be able to spread and persist in soil after fertilization with swine

manure (Lopatto et al., 2019; Marti et al., 2013; Zhang et al., 2017), while an increase in bla_{oxa-1} abundance in farmland soil has been previously associated with irrigation with wastewater from swine farms showing high abundance of this gene (Yang et al., 2019). Xiong et al. (2015) reported higher *oqxA* abundance in fertilized soil in comparison with the untreated control at one month after manure application; however, at day 60, the relative abundance of the gene was lower than in the control group, potentially indicating dissipation of *oqxA* in the soil. A similar dynamic was also observed for other plasmid-mediated quinolone resistance (PMQR) genes, which disappeared within two months after manure application (Xiong et al., 2015). Lopatto et al. (2019) traced *ermB* and *ermC* abundance in treated soil over a period of six months, while the former was more abundant in the fertilized soil up to the last time point, the latter decreased after three months, suggesting that each ARG possessed a different dissipation dynamic. Bla_{TEM-1} and the extended-spectrum β-lactamase (ESBL)-encoding genes, $bla_{CTX-M-1LIKE}$ and $bla_{SHV,}$ known to be widespread in the environment (Graham et al., 2016), were significantly more abundant in manure than in soil and showed a decrease after manure application, pointing at a dilution effect in the soil after fertilization (Yang et al., 2019). Here, ARG abundance was investigated only at day 30 post-fertilization, hampering to assess the impact of manure-derived ARGs on the soil at multiple time points and to establish the dissipation dynamic of the genes in agricultural soil, and future studies should aim at this. Together with the dissemination of ARGs from manure to the environment, we investigated the differences in ARG abundance in dairy cattle, chicken and swine farms. Not

surprisingly, swine farms showed the highest total ARG abundance, and bla_{oxa-1}, *ermB*, *mcr-1* and *qnrS* were significantly more abundant in this sector in comparison to the others (Chen et al., 2019; Marti et al., 2014; Xia et al., 2019a). Differences in ARG levels between livestock sectors might rely on differences in physiologies of animals, but also on differences in manure treatment and storage (Chen et al., 2007; Sandberg and LaPara, 2016). Most of the sampled farms employed the same manure storage strategy (i.e. open-air pit), while the duration of the storage before manure application ranged from no longer than 120 days for dairy cattle farms, to 360 days for swine farms, pointing out that a longer storage might contribute to a high ARG abundance in manure. Despite the low prevalence and not being enriched by fertilization, the detection of the carbapenemase-gene bla_{oxa-48} both in manure and soil raises public health concerns due to the clinical importance of these drugs as last resort treatment in human medicine (Nordmann et al., 2011). Interestingly, the carbapenemase-gene bla_{VIM-2} was detected merely in soil, suggesting that this gene of human/animal origin can be maintained in the environment (Scotta et al., 2011). Manure application has been historically recognized as the main source of dissemination in the environment of *mcr* genes, conferring resistance to colistin, another critically important antimicrobial and last resort drug against human infections caused by multidrug resistant Gram-negative bacteria (Kempf et al., 2016; Liu et al., 2016; Xia et al., 2019b). *Mcr-1* to *mcr-5* genes were detected with a prevalence as high as 25% in manure; however, since none of the genes was enriched in soil after fertilization, and *mcr-2* and *mcr-4* were not detected in fertilized soil,

manure application may not be a driver of the spread of these genes in the environment. As a whole, the ARGs investigated did not display uniform dynamics in soil after manure application.

4.3 Correlation analysis between flumequine concentration, taxa and ARGs abundances

As previously mentioned, due to the low positivity for most of the antimicrobials investigated in this study, the possible correlation between antimicrobial concentration and ARG abundance was explored only for flumequine. The increase in flumequine concentration in soil after manure application was correlated to an increase in relative abundance of two genes conferring resistance to (fluoro)quinolones (i.e. *oqxA* and *qnrS*), suggesting that flumequine might enhance the accumulation of these PMQR genes in manure-amended soil or slow down their dissipation, as previously reported for other (fluoro)quinolones (Xiong et al., 2015). *OqxA* and *qnrS* accumulation in soil under the selective pressure of flumequine might be the result of different processes; indeed, the increase of ARGs in soil after fertilization might be due to the direct addition of bacteria originating from the manure, from proliferation of bacteria present in the soil or indirectly via gene spreading mediated by HGT (Ahmed et al., 2018; Heuer et al., 2011; Marti et al., 2014; Rieke et al., 2018). *OqxA* co-occurrence with Acidobacteria and Gemmatimonadetes, which were more abundant in soil than in manure, suggests that the application of manure carrying flumequine may promote the proliferation of

bacteria harbouring *oqxA* already present in the environment. On the contrary, *qnrS* did not correlate to any specific phyla and consequently its spread in soil might be due to HGT. Independently from the process by which the accumulation of these two genes occurred, this finding represents a concern for public health and should lead to reconsider the use of flumequine in the veterinary field. Indeed, both *oqxA* and *qnrS* confer resistance not only to flumequine, which is a first generation (fluoro)quinolone with a limited spectrum of activity (Daly and Silverstein, 2009), but also to other (fluoro)quinolones (e.g. ciprofloxacin, levofloxacin, norfloxacin, and nalidixic acid) widely used to treat human bacterial infections (Jacoby et al., 2014; Kim et al., 2009). *ErmA* co-occurred with two phyla (i.e. Bacteroidetes and Hydrogenedentes) showing similar abundance in manure and soil; hence the increase in *ermA* abundance in soil after fertilization might be due to HGT (Murphy, 1985). Spirochaetes, a phylum more abundant in manure, showed a positive correlation with bla_{oxa-1} and *mcr-1*, two genes characterized by a different dynamic in amended soil; while bla_{oxa-1} abundance significantly increased after manure application, *mcr-1* seems to follow the fate of Spirochaetes, not being enriched in the soil (Gao et al., 2019). Bla_{oxa-1} enrichment in soil might be due to HGT to other bacteria present before manure application, since this gene is commonly found in plasmids and integrons in several Gram-negative bacteria (Poirel et al., 2010).

5. Conclusion

In the present study we analysed manure and soil samples from integrated farms located in Northern Italy; by combining LC-MS/MS, qPCR and 16S rRNA gene sequencing, we demonstrated that fertilization may affect the abundance of specific ARGs in soil. The main conclusions of our study are:

- Manure-derived bacteria does not survive in soil, and manure application do not drastically affect the soil microbiome, since at 30 days after fertilization only three phyla were significantly enriched.
- ARGs showed different dynamic patterns in soil; while *ermA*, *ermB,* bla_{OXA-1}, *oqxA* and *qnrS* enriched, $bla_{CTX-M-1LIKE}$, bla_{SHV}, and bla_{TEM-1} disappeared after manure application, suggesting that different manure-derived genes experience different fates in soil.
- Flumequine may exert a selective pressure for the accumulation of *oqxA* and *qnrS* in fertilized soil; hence, the use of flumequine in the veterinary field should be reconsidered.
- The different dairy cattle, chicken and swine farms displayed different microbial communities, and the latter was characterized by the highest abundance of ARGs, of which bla_{OXA-1}, *ermB*, *mcr-1* and *qnrS* were significantly more abundant than in the other two livestock sectors.

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Declaration of competing interest

The authors declare no conflict of interest.

Author contributions

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814 **Figures**

815 **Fig. 1.** Concentration of flumequine in A) manure and soil samples and in B) dairy
816 cattle, chicken and swine samples. $p < 0.001$ as *** and $p < 0.0001$ as ****. Non-
817 significant differences are indicated by ns.

818 **Fig. 2.** Heatmap representing the microbial community composition of manure and
819 soil samples at phylum level.

820 **Fig. 3.** Network analysis representing the microbial composition of manure and soil
821 samples at OTU level. The 3,000 most abundant OTUs were considered to generate
822 the network.

823 **Fig. 4.** α-diversity within manure and soil samples (A and B) and within dairy cattle,
824 chicken and swine sectors (C and D). Boxplots represent 25th to 75th percentiles and
825 whiskers showing a maximum of 1.5x the interquartile range (IQR), and different
826 letters indicate significant differences within the α-diversity indexes ($P < 0.05$)..

827 **Fig. 5.** β-diversity between manure and soil samples (A and B) and between dairy
828 cattle, chicken and swine samples (C and D). In both NDMS and PCoA analysis,
829 samples are clustered according to Bray-Curtis distances.

830 **Fig. 6.** Prevalence and relative abundance of target genes to 16S rRNA copy number
831 in manure and soil samples. $p < 0.05$ shown as *, $p < 0.01$ as **, $p < 0.001$ as ***
832 and $p < 0.0001$ as ****. Non-significant differences are indicated by ns. For easiness
833 of representation, only ARGs showing significant differences among sample types
834 are reported.

Fig. 7. Relative abundance of target genes to 16S rRNA copy number in dairy cattle, chicken and swine samples. For easiness of representation, only ARGs showing significant differences among farm types are reported. $p < 0.05$ shown as *, $p < 0.01$ as ** and $p < 0.001$ as ***. Non-significant differences are indicated by ns.