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Routes of dispersion of antibiotic resistance genes from the poultry farm system

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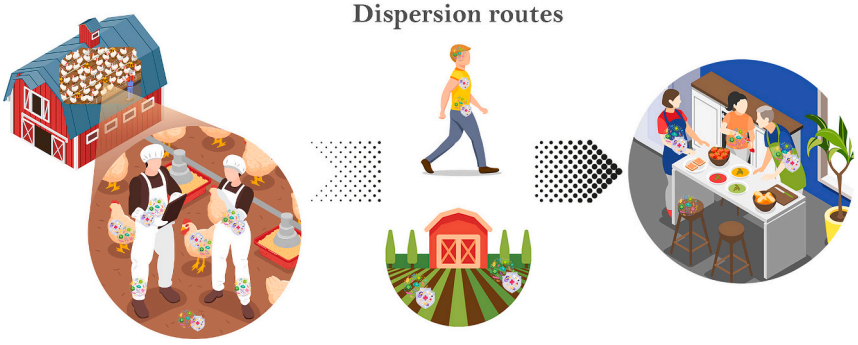
HIGHLIGHTS

- A total of 448 SGBs from the poultry farms system have been characterized.
- 180 poultry GM SGBs were spread across the farm system, reaching the farmers GM.
- These SGBs are endowed with clinically relevant ARGs, the 20 % of which on MGE.
- Workers' microbiomes are the main ARGs dispersion route from the poultry houses.
- NGS-based metacommunity surveys are strategic to monitor ARGs dispersion.

GRAPHICAL ABSTRACT



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ABSTRACT

Poultry farms are hotspots for the development and spread of antibiotic resistance genes (ARGs), due to high stocking densities and extensive use of antibiotics, posing a threat of spread and contagion to workers and the external environment. Here, we applied shotgun metagenome sequencing to characterize the gut microbiome and resistome of poultry, workers and their households - also including microbiomes from the internal and external farm environment – in three different farms in Italy during a complete rearing cycle. Our results highlighted a relevant overlap among the microbiomes of poultry, workers, and their families (gut and skin),

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with clinically relevant ARGs and associated mobile elements shared in both poultry and human samples. On a finer scale, the reconstruction of species-level genome bins (SGBs) allowed us to delineate the dynamics of microorganism and ARGs dispersion from farm systems. We found the associations with worker microbiomes representing the main route of ARGs dispersion from poultry to human populations. Collectively, our findings clearly demonstrate the urgent need to implement more effective procedures to counteract ARGs dispersion from poultry food systems and the relevance of metagenomics-based metacommunity approaches to monitor the ARGs dispersion process for the safety of the working environment on farms.

1. Introduction

Even if the use of antibiotics has transformed modern human and veterinary medicine, with benefits for human and animal health (Katz and Baltz, 2016; Hutchings et al., 2019), in the long term, it has had several downsides, including the selection of multidrug-resistant strains in humans, animals, and environmental ecosystems, posing a pressing threat in the One Health framework (Crofts et al., 2017). Antimicrobial resistance features are encoded by the so-called antibiotic resistance genes (ARGs), which allow microbes to live and grow in the presence of antibiotics. ARGs have been detected in bacteria living in both natural (Bahram et al., 2018; Zhang et al., 2020) and anthropogenic environments (Hendriksen et al., 2019; Chng et al., 2020), but their highest diffusion has been detected in environments with unrestricted antibiotic practices, due to antibiotic overuse in food systems and/or medical practices (Van Boeckel et al., 2019). This evidence implies a direct association between antibiotic use and the emergence and propagation of antibiotic resistance in microbial communities. Propagation mechanisms include the transmission of resistant microbes from host to host, directly or through an environmental passage, and/or the exchange of the genetic material coding for ARGs between different microbial strains sharing, even transiently, the same ecosystem (Kim and Cha, 2021; McCarthy et al., 2014; Liu et al., 2016; von Wintersdorff et al., 2016; Jiang et al., 2017). In any case, the spread of antibiotic resistance depends on a complex web of connections between microbes, hosts, and their environment, which are always favored in a context of high population density and sharing a confined environment, facilitating the spread of resistant bacteria and ARGs (Bruinsma et al., 2003).

Animal farms are hotspots for the development and spread of ARGs, due to limited space in relation to livestock numbers, and the extensive use of antibiotics. For years, antibiotics have been administrated for non-therapeutic purposes, such as growth promotion and disease prevention, and have consistently been detected in the livestock gastrointestinal tract at low concentrations (Woolhouse and Ward, 2013; Zhu et al., 2017; He et al., 2020). For instance, in the chicken-related food production industry, antibiotics have been utilized in breeding programs as a feed additive to improve production efficiency by limiting pathogen colonization (Khadem et al., 2014). In Europe, where antibiotic usage was banned in 2006, their administration remains still very pervasive in poultry husbandries. This because of the recent increase in infections that have required a larger number of applications of therapeutic antibiotic doses (Immerseel et al., 2004; Gaucher et al., 2017). This extensive use of antibiotics thus exerts selective pressure for bacteria in the livestock intestine for ARGs acquisition and consequent transmission to the entire food system microbiome metacommunities. However, only a few studies have provided a systematic assessment of the dynamics involved in the dispersion of antibiotic resistance from food systems (Bai et al., 2022; Mazhar et al., 2021; Zhu et al., 2021; Song et al., 2021; Duan et al., 2019). In the scenario, we hypothesize that antibiotic-resistant bacteria, once selected in the livestock gut, may be dispersed into the internal farm environment and then, may reach the external environment directly - through environmental route of dispersion - or, more importantly, in association with workers' microbiomes. In particular, the host-associated route of antibiotic resistance propagation would increase the likelihood of human exposure, particularly for farmers and those living in neighboring areas. Supporting our hypothesis, it has been recently demonstrated that the intestinal microbiome of farmers living in close contact with farmed pigs and poultry showed a higher number of resistances when compared to urban residents living in the same geographic area (Maciel-Guerra et al., 2023).

In this scenario, we assessed the presence and distribution of microorganisms and ARGs in three different poultry farms systems in Italy, in a longitudinal time setting during a commercial productive process. To this end, a food system metacommunity-based approach was implemented, including: fecal metagenomes from the farmed animals, metagenomes from the internal and the external farm environments, and human metagenomes from the workers and their households, for a total number of 281 sequenced metagenomes. By reconstructing the Metagenome Assembled Genomes (MAGs) from the generated metagenomic dataset we have been able to map ARGs dispersion routes from the farm system suggesting the importance of metagenomics-based metacommunity surveys for a more systematic evaluation of the risks associated with animal food production.

2. Materials and methods

2.1. Animals, sample collection and processing

Three commercial broiler flocks, reared between October 9th, 2019, and November 14th, 2019, in three conventional poultry houses labeled as RR, CM, ZR and located about 40 km one from the other in northern Italy, were selected for this study. All flocks were sampled up to 30 days before depopulation. The flocks reared in CM and RR were never treated with antibiotics, while the chickens reared in RR were treated with Lincospectin at day 23. Sampling time points of each tested house are summarized in Supplementary Table 1 while type and number of samples collected at each time point and from each poultry house are reported in Supplementary Table 2. Specifically, 10-cm deep soil samples were collected in 2 areas close to each tested poultry house using a sterile falcon tube (50 mL). Water samples from drinking trough were collected in 2-L sterile bottles and transported to the laboratory where water was filtered onto cellulose mixed ester 0.22-μm pore-size filters (MF-Millipore) through a vacuum filtration system. Bioaerosol samples inside the farm were collected via a pump connected to a filter with a flow rate of 2–8 L/min for a collection time of almost 6 h. One gram of fresh feces was randomly collected, using a sterile scalpel, from the litter in different areas covering the whole house, for a total of 22 samples. Boot swab samples were collected through a sterile gauze pad soaked in physiological solution and rubbed onto the operators' boots after walking inside the poultry house. Environmental swabs (wall and ventilator swabs) were collected on the left wall and on the right wall of the house, 1 each, with 1 ventilator swab at the opposite side of the entrance. Swabs were collected in individual sterile tubes and transported to the laboratory in refrigerated conditions, where they were supplemented with 1 mL of physiological solution, vortexed for 1 min, and transferred to a new sterile tube. Washing solutions of each swab type were then combined in a single sterile tube and stored at −80 °C. All other samples, except for environmental swabs and water samples, which were subjected to a pre-treatment step, were transferred to a sterile plastic tube and immediately transported to the laboratory where they were stored at −80 °C until further processing. For human samples, every worker-household couple was given an auto-sampling kit

containing sterile nylon flocked swabs for the sampling of 4 different ecosystems: stool and skin from farm workers and cohabitants, house environment, and workers' coat (approved by the Bioethics Committee of the University of Bologna on 05/20/2019, Prot. 116733). Each swab contained a preservative for room temperature storage for days until the swabs were delivered to the laboratory and stored at −20 °C until further processing.

2.2. DNA extraction and shotgun metagenome sequencing

Total DNA was extracted from all samples using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany), with the exception of water samples, which were processed using the DNeasy PowerWater kit (Qiagen), and human and animal fecal samples, for whom the QIAamp Fast DNA Stool Mini Kit (Qiagen) was used, with a modified protocol. Briefly, a bead-beating step was added at the beginning of the extraction with a TissueLyser (Qiagen) at 30 Hz for 3 × 30 s to increase DNA yield. Human skin swabs were vortexed twice and immediately sonicated for 2 min at 45 KHz. Debris was separated by centrifugation at 700 ×*g* for 1 min and the supernatant was centrifuged at 4000*g* for 1 min. Then, the pellet was treated with the QIAamp PowerFecal DNA kit (Qiagen), following the manufacturer's instructions.

A total of 281 samples (199 poultry fecal samples, 18 soil samples, 9 air samples, 9 water samples, 9 boot swab samples, 21 wall or vent farm swab samples, 6 human fecal samples, 5 human skin samples, 3 house swab samples and 2 human coat samples) were selected and processed for shotgun metagenome sequencing. DNA was quantified using a QUBIT fluorometer (Invitrogen, Waltham, MA, USA) and DNA libraries were prepared using the QIAseq FX DNA library kit (Qiagen) according to the manufacturer's instructions. For the specific details see Supplementary File 1 and a summary flow chart of the subsequent analysis is reported in Supplementary Fig. 1.

2.3. Species-level genome bins definition and antibiotic resistance genes identification

Raw reads were filtered following the standard operative procedures of the Human Microbiome Project (Turnbaugh et al., 2007), adapting the procedures to the poultry samples, when necessary, in addition MAGs were reconstructed using *megahit* and *metawrap binning* module, while the ARGs were identified using PathoFact pipeline. For all specifications in detail refer to Supplementary File 1.

2.4. Detection of strain-sharing events

To gain deeper insight into the potential sharing of microbiome components across poultry, environmental, and human metagenomes, we investigated the strain-level population structure using StrainPhlAn3 as previously illustrated (Valles-Colomer et al., 2023). For all details see Supplementary File 1.

2.5. Biostatistics

All statistical analysis was performed using R software (version 4.2.0, www.r-project.org) with packages *vegan* (version 2.6-2) (Oksanen et al., 2022), *RcppAlgo* (version 2.6.0) (Wood, 2022), *xlsx* (version 0.6.5) (Dragulescu and Arendt, 2020), *ggVennDiagram* (version 1.2.2) (Gao, 2022), *ggplot2* (version 3.4.0) (Wickham, 2016), *ComplexUpset* (version 1.3.3) (Krassowski, 2020), *RColorBrewer* (version 1.1-3) (Neuwirth, 2022), *gplots* (version 3.1.3) (Warnes et al., 2022), *viridis* (version 0.6.2) (Simon et al., 2021), *reshape2* (version 1.4.4) (Wickham, 2007), *tidyverse* (version 1.3.2) (Wickham et al., 2019), and *hrbrthemes* (version 0.80) (Rudis, 2020). Beta diversity and alpha diversity were estimated using the *vegdist* function (method = "bray") and *diversity* function, in the *vegan* package, respectively. Data separation in the Principal Coordinates Analysis (PCoA) was evaluated using a permutation test with pseudo-F ratios (function *adonis* in the vegan package). Wilcoxon rank sum test was used to assess significant differences in alpha diversity between groups. P-values were corrected using p.adjust (function in *stats* package, method = "fdr") and corrected p-values ≤ 0.05 were considered statistically significant. An antibiotic resistance gene transferability index (ARGTI) was calculated in each sample, as in the study of Cao and colleagues (Cao et al., 2022). Specifically, ARGTI represents the ratio between the sum of the abundance of ARGs that are located on phage or plasmid sequences and the sum of the abundance of the total ARGs in individual samples.

3. Results

3.1. SGBs-level characterization of microbiomes in the poultry food system

A total of 281 samples were processed for DNA extraction and shotgun metagenome sequencing. Specifically, three poultry houses were sampled longitudinally at three time points (T1 to T3), following the entire rearing cycle. At each time point and for each house we collected: (i) 22 or 23 fresh caeca drops from the litter as representative of the animal gut microbiomes; (ii) 2 or 3 swabs from the farm walls and ventilation system, 1 swabs from workers' boots and 1 farm air sample, as representative of the internal farm ecosystem; and (iii) 1 water samples from the drinkers and 2 soil samples from the surrounding area, as representative of the external farm environment. At T3 fecal samples and skin swabs from 3 workers and their cohabitants, as well as swabs from their farm coats and their house surfaces, were also collected, as representative of the worker and worker's family microbiomes. An overview of the study design together with the sampling collection through the poultry production cycles is shown in Fig. 1.

A total of 3.4 billion paired-end raw reads were generated, with an average of 12 million reads per sample. We were able to reconstruct 1741 high quality MAGs being dereplicated to 448 SGBs, considering 95 % of similarity as minimum threshold for clustering MAGs together (see Methods section for further details and Supplementary Table 3). Then, we mapped these 448 representative genomes against the previously explored MAGs (>12,000) from the available chicken gut microbiome (Feng et al., 2021) and the SGBs from >150,000 human gut microbiome MAGs, including different individuals, spanning age, geography, and lifestyle (Pasolli et al., 2019). In total, 394 SGBs (88 %) clustered together with at least one known reference genome (full list of known SGBs is reported in Supplementary Table 4). On the other hand, the remaining fraction of SGBs (53 SGBs, 22 %) showed >5 % genetic distance to any SGBs of the databases and could be considered as thus far unreported genomes.

After taxonomic profiling, 74 bacterial families were identified within the dataset of metagenomic samples (Fig. 2A). When looking at the species level (SGBs), the poultry microbiome was mostly dominated by *Lactobacillus crispatus*, *Escherichia coli*, and *Lactobacillus johnsonii*. The samples from the internal farm environment (wall, vent, boot soles, air) showed different species mainly of poultry origin, such as *Corynebacterium stationis*, *Brachybacterium intestinipullorum*, *L. johnsonii* and *L. crispatus*. As for the peculiarities of the microbiomes structure from soil samples around the farms, we found that *Pelomonas sp016790285*, *Xanthobacter autotrophicus_A* and *Sphingobium ummariense* were the most abundant SGBs. On the other hand, the human microbiome was characterized by higher relative abundances of *Bacteroides uniformis*, *Bifidobacterium adolescentis*, and *Bifidobacterium longum* (gut), and *B. uniformis*, *B. longum*, and *Phocaeicola dorei* (skin). Finally, samples from the worker houses were characterized by the presence of bacterial species from inhabitants (workers and their householders), such as *Bifidobacterium longum* and *Phocaeicola dorei*, in agreement with previous observations (Zhang et al., 2019; Cheng et al., 2022). The SGBs profile for each sample, expressed as genome copies per million reads, is reported in Supplementary Table 5.

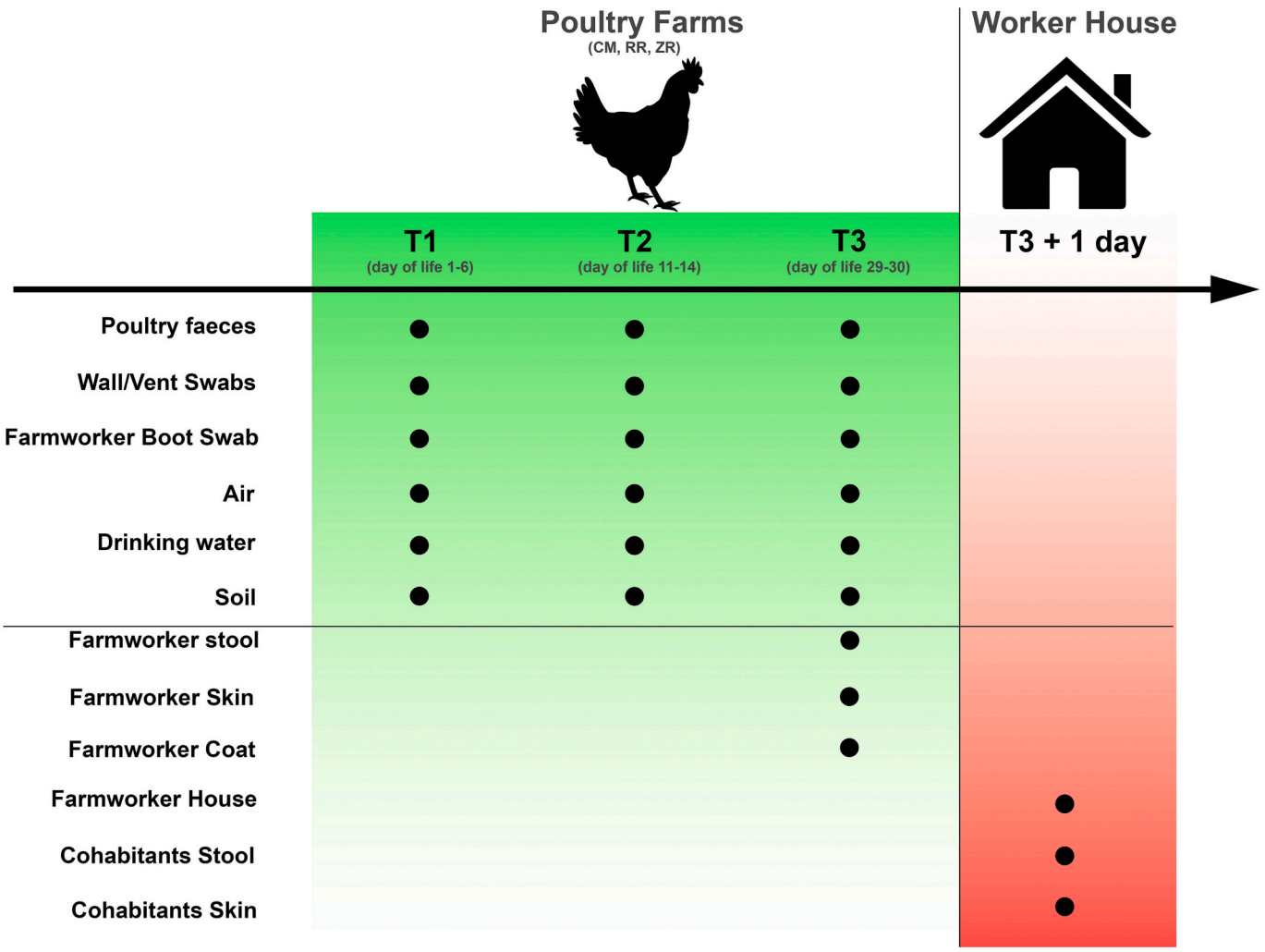

Fig. 1. Overview of the study design and sampling collection. Poultry and farm samples were collected at three different time points during the rearing cycle in three different Italian farms (CM, RR, ZR). Farm worker and cohabitant samples were taken at the last time point or the day immediately after. Black dots indicate the collection time points for each type of sample.

Based on these SGBs profiles, microbiome community structures in the three farms, including the poultry gut microbiomes, the internal and external environmental samples, and the microbiome configurations from the workers, their cohabitants and their houses, were compared by PCoA using Bray-Curtis distances. We found the SGBs-level microbiome composition from different ecosystems clustered separately, independent of farms (Fig. 2B, permutation test with pseudo-F ratio, p-value = 0.001). Alpha diversity in the external soil around the farms was lower compared to poultry and human microbiomes, when looking at the number of species (observed_features metric) but showed a more even distribution of species abundance (Shannon and Simpson indexes) (Fig. 2C).

3.2. SGBs dispersion in the farm system

We evaluated the extent to which components of the poultry microbiome were dispersed in the internal and external farm environments, to the workers' microbiome (gut and skin) and cohabitants, and to their shared living environment. To this end, we first mapped the metagenomic reads to our collection of SGBs using the metawrap tool "quant_bins", and then selected the shared species validating the presence of the same species across different samples. The SGBs abundance per sample type is provided in Fig. 3.

We found,180 poultry gut microbiome SGBs were detected in both the internal farm ecosystem and the workers' metagenomes (skin and gut) (Fig. 4A), with 175 of them also present in the corresponding microbiomes of their cohabitants (Fig. 4B). Most of these host associated SGBs (140, 78 %), representing poultry gut components able to disperse through the internal farm environment and colonize the human gut, were assigned to Bacillota phylum (Fig. 4C). At the family level, 55 out of 180 (31 %) poultry gut microbiome SGBs were assigned to *Lachnospiraceae*, 26 (14 %) to *Ruminococcaceae*, 21 (12 %) to *Oscillospiraceae* and 17 (9 %) to *Acutalibacteraceae* families (Fig. 4D). Conversely, 31 SGBs were exclusively shared between the poultry gut microbiome and the internal farm environment (wall, vent, air and boot swabs). These SGBs were mostly assigned to *Lachnospiraceae*, *Ruminococcaceae* and *Oscillospiraceae* families. One hundred twenty SGBs were shared between the poultry gut microbiome and the external soil around the farms (Supplementary Table 6). Finally, 66 cosmopolitan SGBs were detected, understood as microbial species shared among all ecosystems, including poultry gut microbiome, samples from internal (i.e., workers' boots, air, wall and vent) and external (i.e., water from the watering places and soil from the surrounding of the farm) farm environments, and workers' samples (skin, gut, house and farm coat), also including samples from their cohabitants (Fig. 4A).

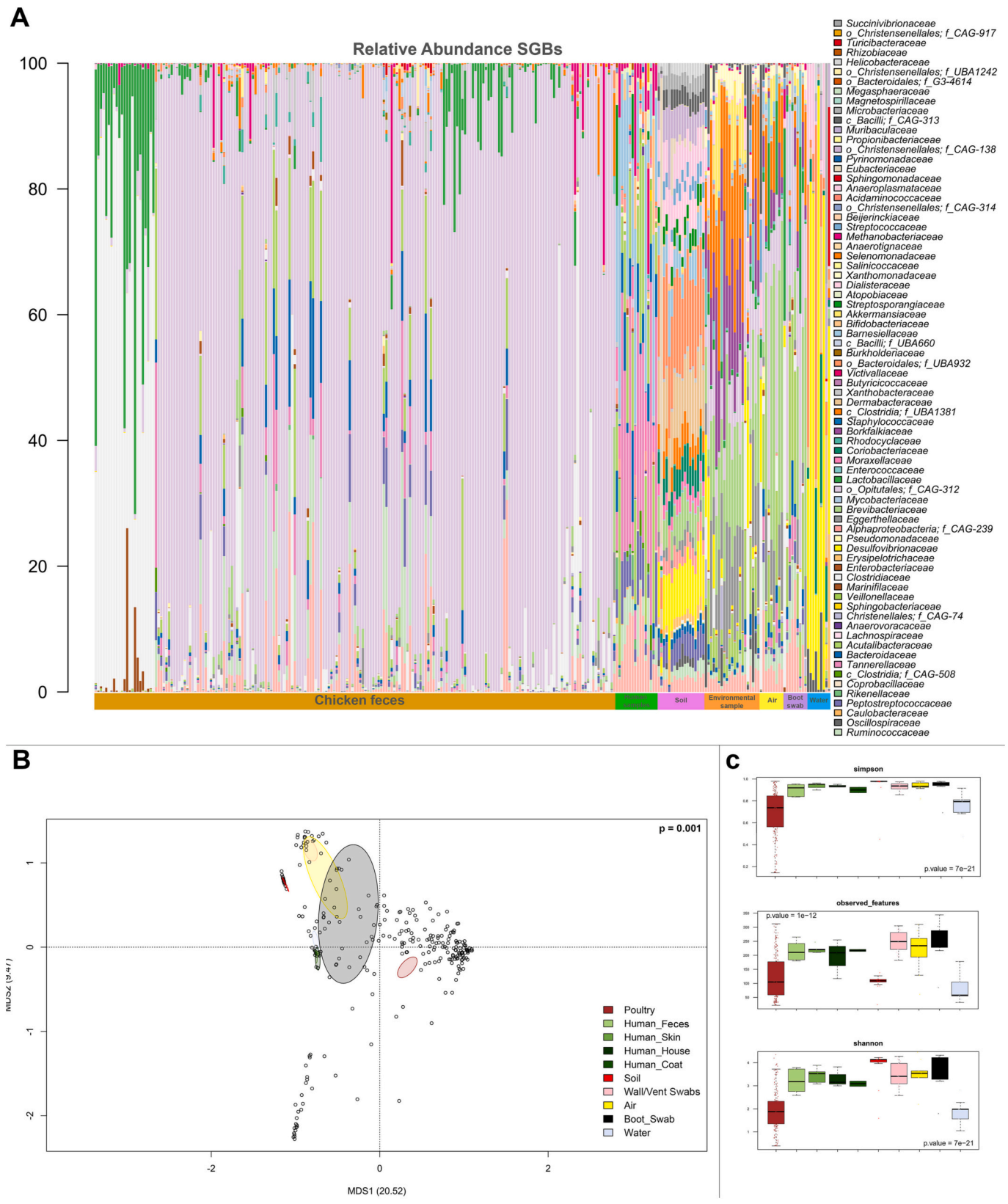

Fig. 2. Composition of humans, poultry, and farm environmental microbiomes. A) Taxonomic classification of species-level genome bins (SGBs), represented as relative abundance at the family level across samples. B) Principal Coordinate Analysis based on the Bray Curtis distances calculated on species-level genome bins (SGBs) relative abundance in each sample. The percentage of variance in the dataset explained by each axis is reported. C) Alpha diversity boxplots based on observed features (number of species-level genome bins), Shannon index and Simpson index. All metrics showed a significant variation (Kruskal-Wallis test, p-value < 0.001) of alpha diversity among microbial ecosystems.

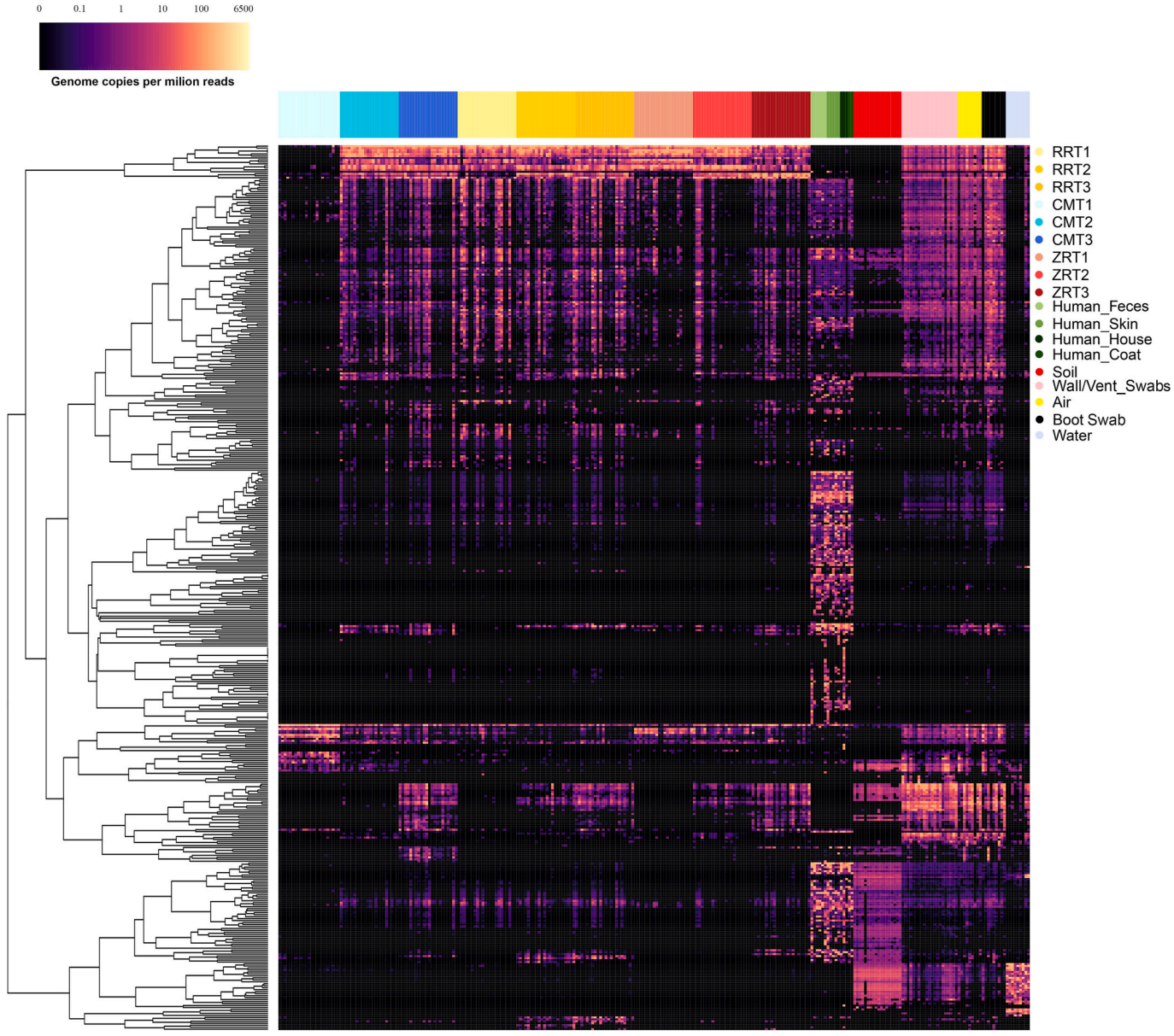

Fig. 3. Distribution of all species-level genome bins across humans, poultry, and farm environmental samples. Heatmap based on the species-level genome bins (SGBs) abundance expressed as genome copies per million reads in each sample (grouped by color legend in the top right corner).

3.3. Strain-level dispersion of poultry gut microbiome components in the farm system

In order to provide the best possible resolution for the dispersion process of poultry microbiome components outside the farm system in association with human microbiomes, we applied StrainPhlAn3 (Truong et al., 2017) on the SGB-specific marker genes, enabling the strain-level identification for the most abundant poultry SGBs being detected in the microbiomes of workers and their families. For each SGB, this approach allowed us to verify whether different microbiomes contained the same strains, thus inferring possible poultry-to-workers transmission process. Thirty-one SGBs were present in sufficient abundance in the obtained metagenomes to conduct the StrainPhlAn3 analysis. For 10 of these SGBs we were able to verify that the same strains were present in both poultry and human microbiomes, of which *Acutalibacter* ornithocaccae, *Negativibacillus faecipullorum*, *Merdibacter merdigallinarum*, *Enterocloster excrementigallinarum* and *Eisenbergiella intestinigallinarum* were of recognized poultry origin (Gilroy et al., 2021).

Generally, all these strains are found in human associated samples such as feces, coat, skin, and house environments. Conversely, we did not find evidence of strain sharing between poultry feces and the external soil around the farms. The SGB-specific normalized phylogenetic distances (nGDs) among the different metagenomes, proving that the samples containing the same bacterial strains, are reported in Supplementary Table 7.

3.4. Assessment of antimicrobial resistance determinants in the 180 host associated SGBs poultry microbiome components being detected in the microbiomes of workers and their families

In order to evaluate the impact of the SGBs dispersion from the farm system poultry house in terms of antimicrobial resistance risk, we first determined the ARGs presence in the previously detected 180 host-associated poultry gut microbiome SGBs being able to reach the microbiomes of workers and their families. To this end, we built a customized gene catalog, based on the entire set of ORF annotations

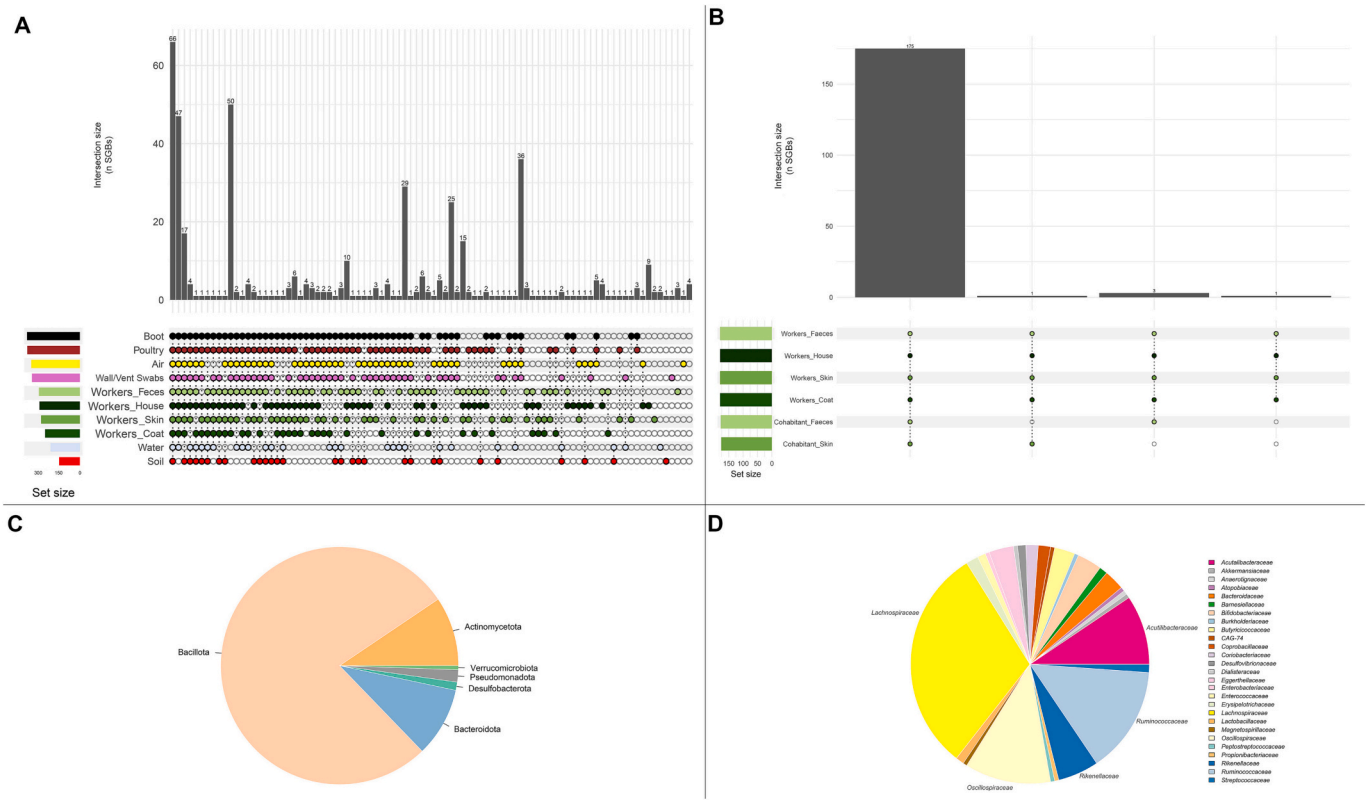

Fig. 4. Species-level genome bins sharing across humans, poultry, and farm ecosystems and their taxonomical assignment. A) Upset plots showing the species-level genome bins (SGBs) distribution across poultry, farm environment and worker ecosystems, highlighting the sharing of several species-level genome bins (SGBs) between different samples. B) Distribution of the 180 species-level genome bins (SGBs) identified as shared between poultry gut, internal farm, and worker ecosystems from the previous upset plot, in worker and cohabitant samples. C–D) Taxonomical assignment of the 180 shared species-level genome bins (SGBs) at the phylum and family level.

retrieved from the assembled genomes by applying the PathoFact pipeline (de Nies et al., 2021). Starting from 1.6 million dereplicated ORFs (at 90 % of sequence similarity), 22,162 were assigned to ARGs and included into the catalog (Supplementary File 2).

We found that the 180 SGBs shared between poultry and worker microbiomes contained 604 ARG variants, that contributed to the

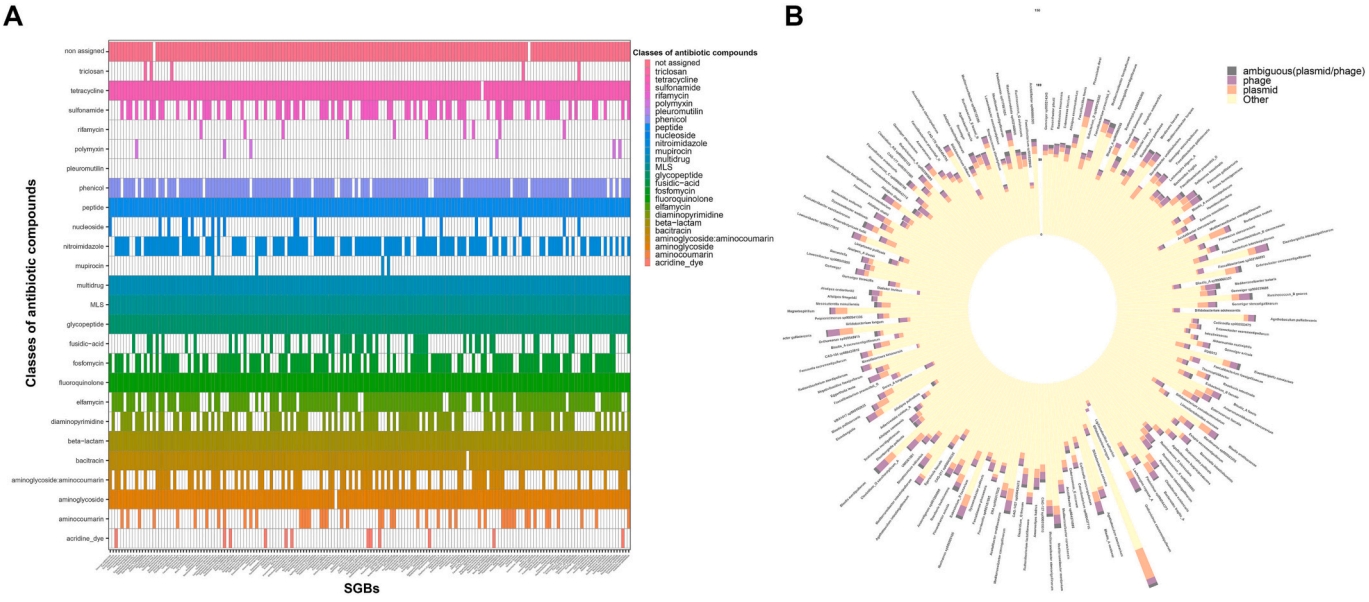

Fig. 5. Occurrence of antibiotic resistance within the shared species-level genome bins. A) Presence/absence of antibiotic classes towards which antibiotic resistance genes (ARGs), within the 180 species-level genome bins (SGBs) shared between poultry gut, internal farm, and human ecosystems, are effective. B) Circular bar plots showing the number of genes within each shared species-level genome bin (SGB) and their prediction on plasmid or phage sequences (according to the color legend on the right).

resistance against 25 different classes of antibiotic compounds (Fig. 5A; Supplementary Table 8). In particular, the resistances against tetracycline, sulfonamide, phenicol, antimicrobial peptide, nitroimidazole, multidrug, macrolide-lincosamide-streptogramin (MLS), glycopeptide antibiotics, fosfomycin, fluoroquinolone, elfamycin, diaminopyrimidine, beta-lactam, bacitracin and aminoglycoside classes were the most represented. On the other hand, the resistances for other target compounds, such as those of the classes triclosan, rifamycin, polymyxin, nucleoside, mupirocin, fusidic-acid, aminocoumarin and acridine, were extremely rare. Further examining these genes by PathoFact, we found that 20 % (121 out of 604) of these ARGs were located on mobile elements such as plasmid or phage sequences, thus being possibly more susceptible to horizontal gene transfer (HGT) events (Fig. 5B).

Interestingly, 24 ARGs within the 604 variants were present in at least 135 of the 180 shared SGBs (75 %), thus representing a putative core resistome. This core resistome was effective against several classes of antibiotic compounds such as aminoglycoside, bacitracin, beta-lactam, elfamycin, fluoroquinolone, glycopeptide antibiotics, MLS, multidrug, antimicrobial peptide, and tetracycline (Fig. 6). Four of these core ARGs were predicted to be mobile, i.e., present within plasmid or phage sequences, and to encode for a multidrug efflux pump (*mepA*), a metallo-beta-lactamase (*NDM*-8), a tetracycline efflux protein (t*etB(P)*), and point mutations in *gyrB* of *Clostridioides difficile* conferring resistance to fluoroquinolones (*Cdif_gyrB_FLO*). Interestingly, these core mobile ARGs showed the same genetic structure, including proximity to transposase genes in the different SGBs as a feature of the poultry and human microbiomes (e.g., only minoritarian in one of the two ecosystems with <0.1 genome copies per million reads) (Supplementary Table 9), possibly indicating HGT following inter-host SGBs dispersion process (Fig. 7).

In order to provide a global vision of the distribution of the 4 core mobile ARGs across different human populations, we assessed their prevalence in 927 human gut metagenomes, including samples from rural Tanzanian, Brazilian, urban Peruvian, Chinese, Indian, German, American, Swedish and Italian populations (see Supplementary Table 10). Interestingly, we found that the proportion of these core mobile ARGs were significantly higher (Wilcoxon rank sum test, p-value < 0.05) in the gut microbiome of the workers and their households from our study and in the Italian cohort, compared to all other human populations, with the highest prevalence in the Western urban population with respect to rural communities, which showed a minimal abundance of these ARGs (Fig. 8A). The highest prevalence of these 4 ARGs at the local scale - CIRCLES and Italian populations - can be the result of the combination of the observed relevant dispersion of SGBs from the poultry gut microbiome and the high ARGs transferability index (ARGTI, Cao et al., 2022) shown by poultry gut resistome (p-value < 0.01; Fig. 8B).

4. Discussion

In this study, we conducted a comprehensive and longitudinal analysis of the microbiome metacommunities from three commercial

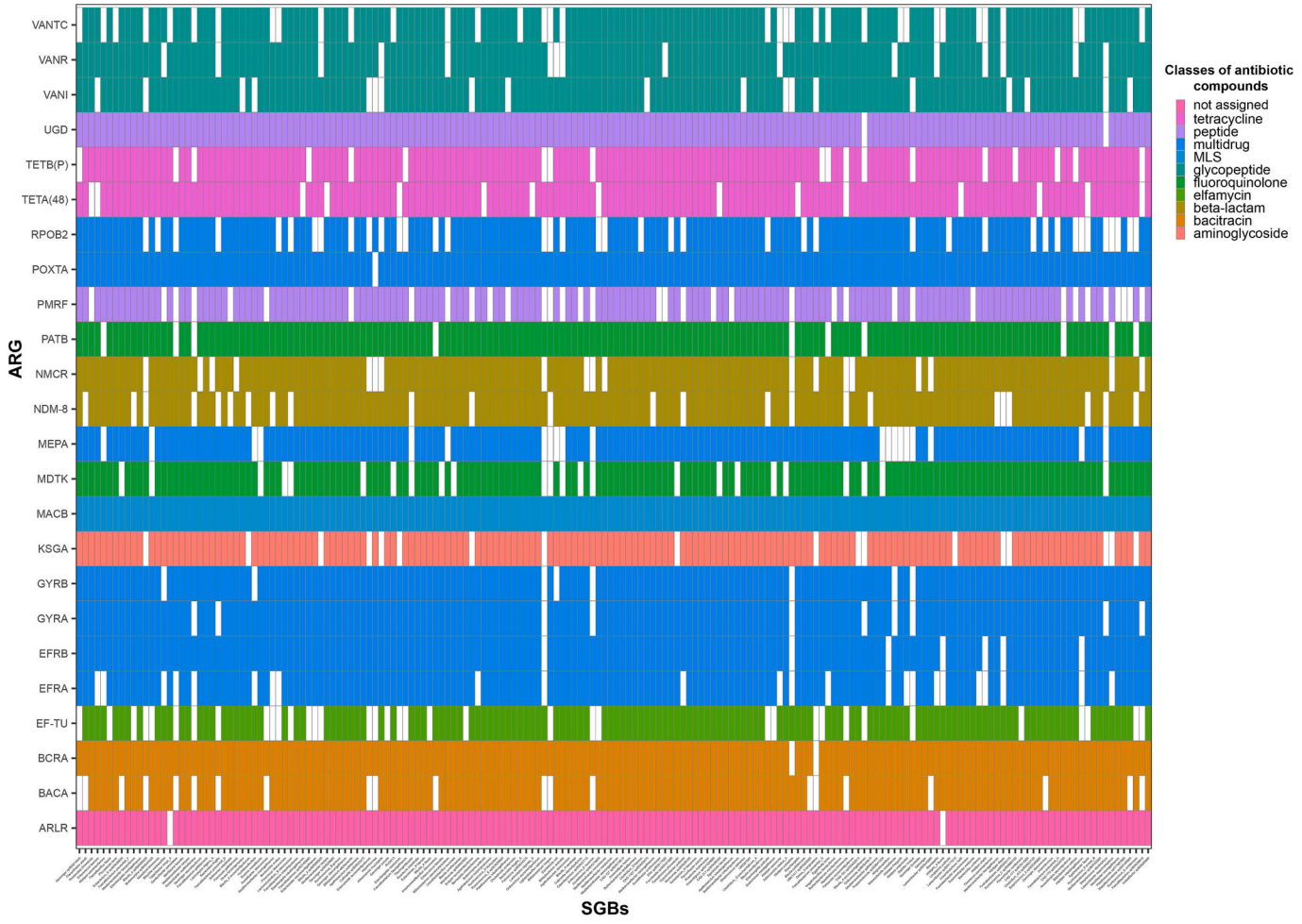

Fig. 6. Occurrence of antibiotic core resistome within the 180 shared species-level genome bins. Presence/absence plot of antibiotic resistance genes showing a prevalence higher than 75 % across the 180 species-level genome bins (SGBs) shared between poultry gut, internal farm, and human ecosystems (classes of antibiotics against which antibiotic resistance genes are effective are represented by color legend).

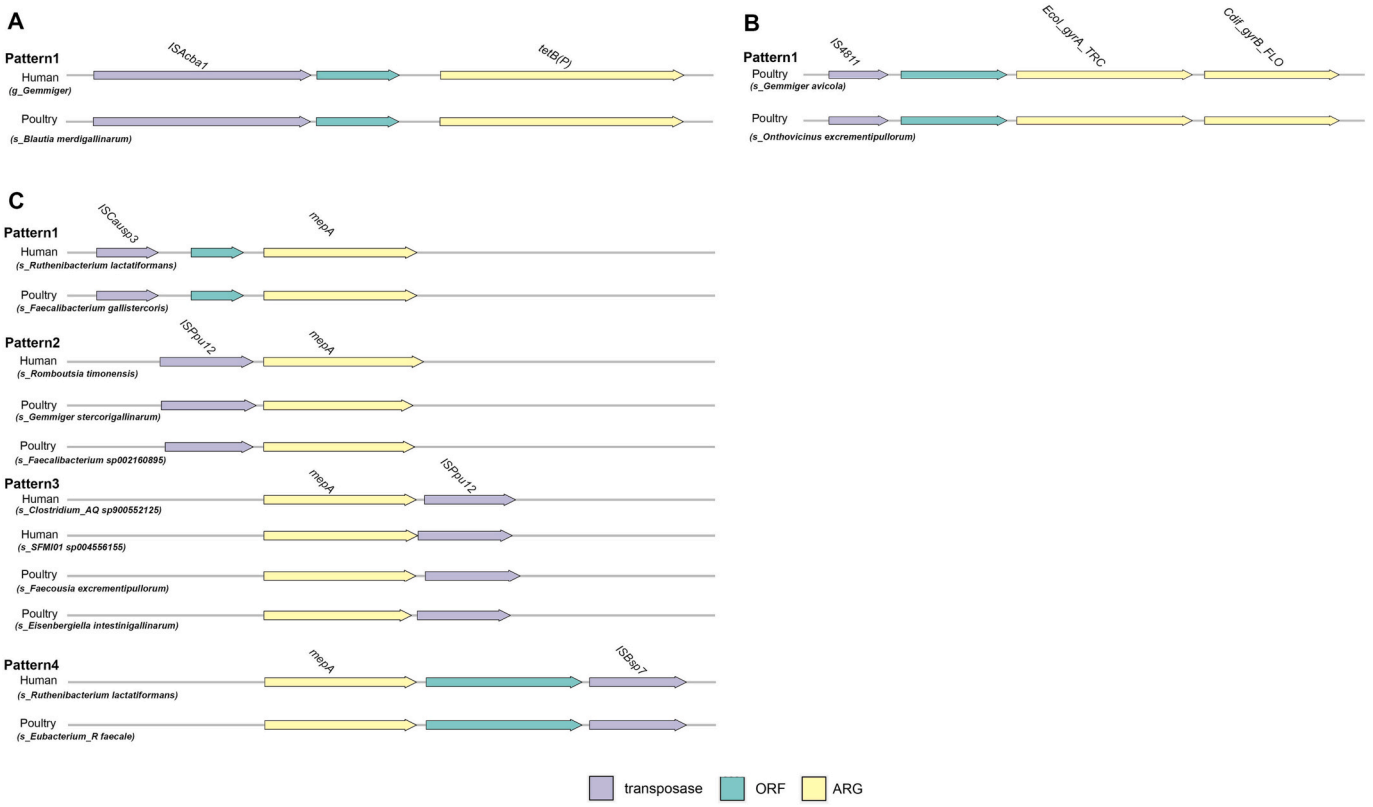

Fig. 7. Genomic structure patterns of antibiotic resistance genes across the shared species-level genome bins. Antibiotic resistance genes showing a prevalence higher than 75 % across the 180 species-level genome bins (SGBs) shared between poultry gut, internal farm, and human ecosystems with a prediction on plasmid or phage sequences highlight in some case the same genomic structure. Antibiotic resistance genes (ARGs) patterns for *tetb(P)* (A), *Cdif_gyrB_FLO* (B) and *mepA* (C) that were shared among different species-level genome bins (SGBs) with their identified lowest taxonomic level are highlighted under each genomic pattern. A color legend of the coding DNA sequences (CDSs) is located at the bottom of the figure, highlighting antibiotic resistance genes, transposases, and other open reading frames (ORFs).

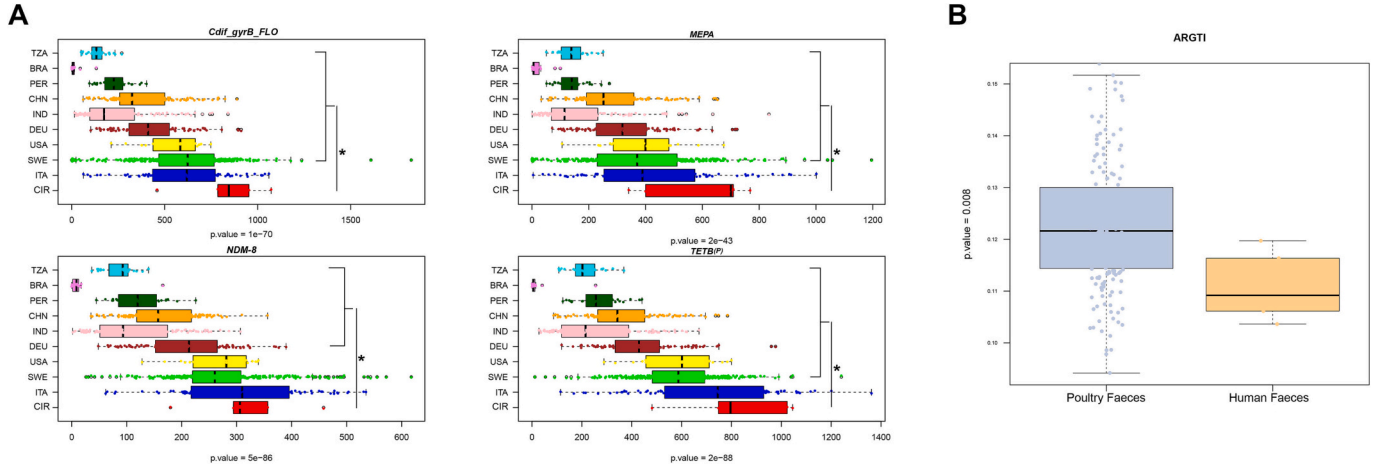

Fig. 8. Distribution of core mobile ARGs across different world populations. A) Boxplots showing reads per kilobase of genes per million reads mapped (RPKM) across 9 populations of the 4 most prevalent shared antibiotic resistance genes (ARGs) with prediction on plasmid or phage sequences, carried by the 180 shared species-level genome bins (SGBs) between poultry gut, internal farm, and human ecosystems. Human fecal samples from our study (CIR) showed a significant higher abundance of almost all genes compared to the other individuals (Wilcoxon rank sum test, p-value < 0.05). B) Boxplots showing the antibiotic resistance gene transferability index (ARGTI) values in poultry and human gut microbiomes (Kruskal-Wallis test, p-value < 0.001). TZA: Rural Tanzanian, BRA: Rural Brazilian, PER: Peruvian, CHN: Chinese, IND: Indian, DEU: German, USA: American, SWE: Swedish, ITA: Italian populations.

poultry houses, during a complete rearing cycle, in Italy. The poultry gut microbiome was assessed together with internal and external farm microbiomes, also including the microbiomes from workers and their family members (gut and skin), allowing us to provide a comprehensive description of the dispersion and circulation of poultry gut microbiome SGBs within and outside the farm system, as well as the diffusion of ARGs from the poultry house to the local and general human population.

In our study, 448 SGBs were created from 281 microbiome samples, spanning 10 different ecosystems, with 394 SGBs assigned at the species level and 53 (22 %) representing new candidate species. Interestingly,

the SGBs-level compositional structure of all the studied microbiomes clustered by ecosystem type, not by poultry house. In particular, the boot and the air microbiomes as a proxy of the internal farm environment were clustering in the between the poultry and the human associate microbiomes. This suggests a possible route of transfer of SGBs from the poultry gut to the workers' microbiomes trough their passage in the internal farm environment.

When considering in more detail the dispersion process of SGBs from the poultry gut microbiome to the internal and external farm system, including the microbiomes of workers and their families, we found different dispersion routes. For instance, 31 SGBs, mainly belonging to known anaerobic poultry gut microbiome families (e.g., *Lachnospiraceae*, *Ruminococcaceae* and *Oscillospiraceae*), were restricted to the farm environment, having been detected only in the poultry gut microbiome and samples internal to the farm (i.e., wall, vent, air and boot swabs). On the contrary, most of the poultry gut SGBs were able to reach the external environment by two different strategies, the former in association with the human host and the latter involving direct dispersion trough environmental routes. More specifically, we detected 180 poultry gut microbiome SGBs capable of pervasively dispersing in the internal farm environment (i.e., walls, ventilation systems, air, and worker's boots) and then escaping the farm system in association with the workers' microbiomes (gut and skin), finally colonizing the same human ecosystems of their family members. Within these 180 SGBs, we found 70 species of well-known poultry origin (Gilroy et al., 2021) and, among them, the most abundant in microbiomes of workers and their families were *E. coli*, *B. fragilis*, *Lawsonibacter sp900545895* and *F. avistercoris*. All these species have been assigned to 21 host-associated non-spore-forming anaerobic genera (Reimer et al., 2022), explaining the observed host-dependency for their dispersion process outside the farm system. For 10 of the 180 SGBs shared between poultry gut and workers and families' microbiomes (gut and skin), strain-level matching was confirmed, supporting inter-holobiont transmission of these microorganisms.

Differently, 120 SGB-level poultry gut microbiome components were detected in both the internal farm environment and the external soil surrounding the farms. Within these 120 SGBs, we found a total of 33 species of known poultry origin, assigned to a total of 26 different bacterial genera. Most of these genera contain species that grow under anaerobic conditions, showing a high propensity for host association, but possibly being capable of surviving transiently in the external environment. However, aerobic bacterial genera such as *Brachybacterium*, *Brevibacterium*, *Escherichia* and *Luteimonas* were also detected, suggesting their intrinsic ability to disperse externally from the farm environment via an environmental route. Finally, 66 cosmopolitan SGBs were found, able to exploit both dispersion patterns, towards the external environment or associated with human hosts.

Our data on the dispersion routes of poultry gut SGBs from the farm environment suggest an association with the workers' microbiomes as the principal route of dispersion. This dispersion, in association with human microbiomes, raises important concerns in terms of the spread of antimicrobial resistance, which can be rapidly transferred from poultry gut components to the human gut microbiome, with important implications for human health. According to our findings, the 180 SGBs shared between the poultry gut and the workers and families' microbiomes were endowed with a diverse and complex resistome, with an overall profile that well matched the most abundant antibiotics used in food-producing animal systems in Europe (EMA, 2021). This confirms the impressive impact of the antibiotic usage in the food system in shaping the gut resistome structure of farmed animals.

Interestingly, among the resistance genes detected in these 180 SGBs, the great majority provided resistance against antimicrobial classes listed as "critically important or highly important" for human health by the World Health Organization (WHO, 2023). Specifically, 15 ARGs belonging to the core resistome of the 180 SBGs conferred resistance to antimicrobial classes defined as "critically important for human health", such as *vanI*, *vanR* and *vanTC* (conferring resistance against glycopeptide antibiotics), *macB* (resistance to macrolide antibiotics), *patB* and *MdtK* (resistance to fluoroquinolone antibiotics), *ksgA* (resistance to aminoglycosides), *NmcR* and *NDM*-8 (resistance to beta-lactam antibiotics) and *rpoB2*, *poxtA, mepA*, *gyrA*, *gyrB*, *efrB*, *efrA*, conferring resistance against multiple antibiotic classes. Indeed, these resistance classes have been detected in pathogens of high clinical relevance, such as carbapenem-resistant *Acinetobacter*, *Enterobacteriaceae* and *Pseudomonas aeruginosa*, fluoroquinolone-resistant *Campylobacter* spp., *Salmonellae*, *Shigella* spp. and *Neisseria gonorrhoeae*, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Exner et al., 2017; Ma et al., 2021; ECDC, 2023).

4 of these core ARGs were classified as mobile, being present within plasmid or phage sequences. These mobile elements encode for a multidrug efflux pump (*mepA*), a metallo-beta-lactamase (*NDM*-8), a tetracycline efflux protein (*tetB(P)*), and point mutations in *gyrB* of *C. difficile*, conferring resistance to fluoroquinolone antibiotics (*Cdif_gyrB_FLO*). Suggesting the ability of these ARGs to mobilize between poultry and human gut microbiome components by HGT, for three of these 4 ARGs the same genetic cassette was detected in the SGBs characteristic of either poultry or human gut microbiome. All 4 ARGs pose a clinically important risk to human health, due to their efficacy against critically important antibiotic compounds and their identification in known human pathogens. For example, metallo-beta-lactamases, commonly observed in *E. coli* and *Klebsiella pneumoniae*, have been included into "The Big Five Carbapenemases", a group of ARGs of clinical relevance [61]. Differently, the multidrug efflux pump *mepA* is part of the resistance arsenal of MRSA, a multidrug-resistant bacterium recognized as a major mortality factor in hospital-acquired infections (Hassanzadeh et al., 2020). Indeed, MRSA is one of the most common causes of antibiotic-resistant infections worldwide and the leading cause of post-operative wound infections (ECDC, 2023). Finally, both *Cdif_gyrB_FLO* and *tetB(P)* are part of the resistance arsenal of *C. difficile* (Mac Aogain et al., 2015; Czepiel et al., 2019; Kecerova et al., 2019). *C. difficile* is one of the most common causes of nosocomial infections (Czepiel et al., 2019), which may vary from common diarrhea to fulminant colitis associated with shock, hypotension, or megacolon, which, in the worst case, can lead to death (McDonald et al., 2018).

5. Conclusions

Collectively, our results demonstrated relevant connections and exchanges between poultry, workers, and worker's family microbiomes (gut and skin), which resulted in a direct passage of clinically relevant ARGs from farmed animals to human hosts. A relevant fraction of these ARGs has been detected on mobile elements, favoring their dispersion to human-associated microorganisms at the population level. The high resolution of the applied computation tools allowed us to provide important insight into the dynamics of ARGs dispersion from the poultry houses, which involves the association of the workers' microbiomes as the main route of dispersion of resistance genes to human populations. In the current scenario, where antibiotic-resistant bacteria pose one of the greatest threats to modern medicine and are estimated to cause more than two million infections globally and 30,000 deaths in EU countries annually (Ma et al., 2021; Gržinić et al., 2023), our findings confirm the importance to minimize the antibiotic utilization in farm systems, possibly also exploiting sustainable microbiome-based solutions (e.g. probiotics and/or prebiotics) for the protection of the animal health in farm systems. Finally, the application of metagenomics-based food system metacommunity surveys for the monitoring of the ARGs dispersion route from the farm environment may become part of the routine procedures to protect the safety of the working environment on farms and, if applied on a wider scale, may become a strategic tool to control the risk of selection and dispersion of new resistance variants at the human population level.

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Ethics approval and consent to participate

Human sampling was approved by the Bioethics Committee of the University of Bologna on 05/20/2019, Prot. 116,733.

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CRediT authorship contribution statement

Daniel Scicchitano: Data curation, Formal analysis, Visualization, Writing – original draft. **Giulia Babbi:** Writing – review & editing. **Giorgia Palladino:** Writing – review & editing. **Silvia Turroni:** Writing – review & editing. **Yitagele Terefe Mekonnen:** Writing – review & editing. **Cédric Laczny:** Writing – review & editing. **Paul Wilmes:** Writing – review & editing. **Pimlapas Leekitcharoenphon:** Writing – review & editing. **Andrea Castagnetti:** Writing – review & editing. **Federica D'Amico:** Writing – review & editing. **Patrizia Brigidi:** Writing – review & editing. **Castrense Savojardo:** Writing – review & editing. **Gerardo Manfreda:** Writing – review & editing. **Pierluigi Martelli:** Writing – review & editing. **Alessandra De Cesare:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Frank Møller Aarestrup:** Writing – review & editing. **Marco Candela:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft. **Simone Rampelli:** Data curation, Formal analysis, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

High-quality reads from all samples are deposited in the European Nucleotide Archive under the project accession number PRJEB63360. SGBs are available here: https://site.unibo.it/microbiome-science-biotechnology-unit/en/microbiome-materials-and-databases.

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