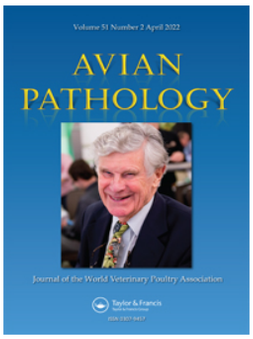

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ORIGINAL ARTICLE

Short-term and long-term effects of antimicrobial use on antimicrobial resistance in broiler and turkey farms

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ABSTRACT

Antimicrobials have been widely used in poultry, promoting antimicrobial resistance (AMR) emergence and spread. Resistant bacteria selected by antimicrobial use (AMU) can contaminate the farm environment and transfer resistance genes to other bacteria, providing opportunities for persistence and (re-)colonization of subsequent flocks and potentially jeopardizing antimicrobial treatments. We investigated the effects of AMU on AMR in poultry in the long-term (due to historical AMU in the farm) and in the short-term (due to current AMU in a flock). Litter samples from 35 broiler and 35 turkey farms in North-East Italy were sampled longitudinally for AMR testing of *E. coli* indicator bacteria in 2019/2020. Differences in AMR as a function of historical AMU (Defined Daily Doses in 2016–2018), current AMU in the sampled flock, farm size and season were tested using Generalized Estimating Equation regression analysis. In both broilers and turkeys, the highest resistance levels were observed for sulfamethoxazole (>70%), followed by ampicillin (54–60%). Only a few positive associations between historical levels of penicillin use and the specific resistance levels to penicillin in broiler farms, and the overall historical AMU and resistance to trimethoprim in turkey flocks, were significant. Current AMU showed significant effects on resistance to sulfamethoxazole, trimethoprim, ciprofloxacin and tetracycline in turkey flocks. Significant effects of farm size on some AMR levels were also identified. We found a stronger association between current AMU and AMR compared to historical AMU and AMR. AMR persistence in the farm environment in the absence of direct AMU pressure needs to be further investigated.

RESEARCH HIGHLIGHTS

- Sulfamethoxazole and ampicillin resistance are the highest in *E. coli* isolates of both broilers and turkeys.
- Short-term effects of antibiotic use on resistance are seen more often than long-term effects.
- Historical penicillin use is associated with penicillin resistance in broilers.
- There is an association between historical use and resistance to trimethoprim in turkeys.

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Antibiotic resistance; antibiotic use; epidemiology; genomics; broilers; turkeys

Introduction

Antimicrobial resistance (AMR) is one of the main challenges facing public health today, with antimicrobial use (AMU), or else its mis/overuse, being one of the main drivers of AMR emergence and spread in both human and animal populations, including poultry (Chantziaras *et al.*, 2013; Chuppava *et al.*, 2018). The use of antimicrobials as growth promoters in animal feed has been banned in the European Union (EU) since 2006 and the concept of prudent use of antimicrobials has been largely promoted and implemented in the EU. However, poultry are often reared under intensive farming conditions in which the metaphylactic use of antimicrobials is often necessary to treat (environmentally conditioned) infections, among others, which in turn would promote AMR (Mughini-Gras *et al.*, 2020). Resistant bacteria emerging by selection pressure of veterinary AMU can be transferred to humans through exposure to animals, food and the environment (Mughini-Gras *et al.*, 2019), contributing to increased mortality, morbidity and healthcare costs (Cassini *et al.*, 2019).

Poultry have long been characterized by high AMR levels in *E. coli* (EFSA & ECDC, 2018), which may be explained, at least in part, by widespread mass administration of antimicrobials (Persoons *et al.*, 2012). Although *E. coli* are often used as indicator bacteria

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for AMR in humans and different animal populations (EFSA & ECDC, 20180), in the context of avian pathology, not all *E. coli* are "harmless" indicators. Indeed, avian pathogenic *E. coli* (APEC), the causative agent of colibacillosis, is the pathogen causing the highest economic impact on the broiler industry, and a recent Italian study found an increasing incidence of colibacillosis from chicks to end-cycle broilers that may be related to a shift in APEC genotypes indicating a trend from commensalism to pathogenicity across the different broiler production stages (Apostolakos *et al.*, 2021). Therefore, AMR levels in *E. coli* can have direct clinical consequences for poultry. Italy is one of the main poultry producers in the EU, with more than 1,000,000 tonnes of broiler meat and more than 300,000 tonnes of turkey meat produced, on average, every year (Caucci *et al.*, 2019). Although overall AMU in Italy's poultry farms has decreased considerably in recent years, with about 71% and 56% AMU reductions in broilers and turkeys, respectively, between 2015 and 2017 (Caucci *et al.*, 2019), it remains considerably higher than in other EU countries, at least until 2018 (EMA, 2020).

Generally, when antimicrobials are used, susceptible bacteria are eliminated, leaving behind those bacteria able to resist the antimicrobials in question. These resistant bacteria can then multiply and become predominant, and, as such, are able to contaminate the (farm) environment and transfer, both horizontally and vertically, the genes responsible for their resistance to other bacteria (Munita & Arias, 2016). Therefore, resistant bacteria that persist in the farm environment provide opportunities for (re-)colonization of new flocks and/or transfer of resistance genes to endogenous microbiota, potentially jeopardizing future antimicrobial treatments as well. It is still unclear to what extent the effects of AMU on AMR in poultry are observable in the long-term due to the aforementioned mechanisms of AMR transfer and/or environmental persistence linked to historical AMU or whether these effects are mainly short-term. If the latter, AMR in a farm would mainly occur as a direct result of antimicrobial treatment in a flock and would then be expected to fade over time, thereby being independent of the historical AMU levels.

In this study, we focused on fattening broiler and turkey farms to test the hypothesis as to whether their (historical) AMU levels in previous years show significant (long-term) effects on the current AMR levels in *E. coli* as indicator bacteria for AMR in the farm environment (regardless of AMU and AMR in the previous production phases), while accounting for the potential short-term effects of current AMU and other variables of interest, such as farm size and season.

Materials and methods

Sampling

Litter samples from 35 broiler farms and 35 turkey farms were collected for antimicrobial susceptibility testing of *E. coli*. The broiler farms were sampled longitudinally four times, once every season, starting in June 2019 and ending in August 2020 (spring: March-May; summer: June-August; autumn: September-November; winter: December-February). Sampling of broilers occurred at ~30 days of age. At each subsequent sampling on the same farm, therefore, a different broiler flock was sampled, as the previously sampled flock had already been slaughtered when the following sampling took place. The turkey farms were sampled twice, at the beginning (~15 days) and at the end (~100 days) of a flock's production cycle. Sampling occurred between June 2019 and March 2020. Both broiler and turkey farms were located in the densely populated poultry area of North-East Italy, which is characterized by the highest density of poultry in Italy and one of the highest in Europe (Mulatti *et al.*, 2010). At each sampling, two samples were taken from one shed in the farm using two pairs of boot swabs (overshoes) following the sampling protocol of Commission Regulation (EU) No. 1190/2012, as also described in a previous study (Mughini-Gras *et al.*, 2020). The swabs were then pooled and kept refrigerated until examination.

The sampled farms were randomly selected among four groups of farms, depending on their size (i.e. total capacity in terms of number of birds reared) and average AMU in the previous 3 years (2016–2018). These groups were defined based on the data distribution (first and last tertiles). For broilers, farms of small and large size had a capacity of 12,000–30,000 and 56,000–136,000 birds, respectively. For turkeys, the size ranges were 5500–13,700 (small) and 22,000–74,000 (large). AMU was expressed as Italian Defined Daily Doses (DDDita/kg) as reported in Caucci *et al.* (2019); high users were farms with DDDita values of 1.2–4.0 for broilers and 15.9–24.1 for turkeys, whereas low users were farms with DDDita values of 0.0–0.8 for broilers and 2.6–15.4 for turkeys. The sampled farms were distributed over these four groups of eight or nine farms, each depending on their combination of size and AMU. Besides information on previous AMU (average DDDita for the years 2016–2018), information on current AMU (i.e. whether or not antimicrobial treatment was administered to the sampled flock) was also available. All recruited farms belonged to the same integrated poultry company, which declared specific management actions to counteract AMR, as reported in (Caucci *et al.*, 2019):

> relocation of parent farms to low-density areas to reduce *Mycoplasma* prevalence (2010); training

farmers regarding health management and animal welfare; improvements in ventilation and biosecurity. In 2014, tetracyclines were banned in broilers and used in turkeys only for *Mycoplasma*. Moreover, turkey eggs are disinfected in the hatchery with nebulized peroxides. Since 2016, infrastructural and managerial interventions have also been implemented at the hatcheries (e.g. strict egg quality checks, ban of formalin, all-in/all-out incubators). In 2017, colistin was banned in broilers and fluoroquinolones have been used only in exceptional cases. Strict all-in/all-out procedures were applied with cleaning and disinfection and an empty period of 21 days for turkeys and 7 days for broilers.

Sample processing and antimicrobial susceptibility testing

Sample processing and antimicrobial susceptibility testing were performed as described before (Mughini-Gras *et al.*, 2020). In brief, each sample (two pairs of boot swabs) was pre-enriched by incubation at 37 ± 1°C for 18 ± 2 h in 250 ml of buffer peptone water. Subsequently, 1 µl of pre-enrichment medium was inoculated onto a Petri dish containing the selective MacConkey Agar medium. Afterwards, inoculated plates were incubated at 37 ± 1°C for 24 ± 3 h. One well-isolated colony with typical morphology per plate was confirmed to be *E. coli* using a commercial biochemical test (API20 E Biomeriux) and tested for antimicrobial susceptibility. Minimum inhibitory concentrations (MICs) were determined by broth microdilution method using the semiautomatic Sensititre System (Sensititre, Trek Diagnostic Systems, UK): a volume of 50 µl of bacterial suspension (containing approximately 1×10^5 CFU/ml) was added to each well of a 96-well commercial microdilution tray containing geometrically increasing concentrations of antimicrobials. Outcomes were assessed after 18–24 h of incubation at 37 ± 1°C by detecting, for each antimicrobial, the first well with no turbidity or deposit and identifying the corresponding antimicrobial concentration as the MIC value. The panel of tested antimicrobials included ampicillin (AMP), azithromycin (AZI), cefotaxime (FOT), ceftazidime (TAZ), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), gentamicin (GEN), meropenem (MER), nalidixic acid (NAL), sulfamethoxazole (SMX), tetracycline (TET), tigecycline (TGC), and trimethoprim (TMP). This panel was based on the indications of the European Food Safety Authority (EFSA) on monitoring of AMR in commensal *E. coli* (Decision 2013/652/EU). The MIC results were then used to classify each isolate as resistant or susceptible based on the epidemiological cut-off values (ECOFF) of the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/).

Statistical analysis

All descriptive statistics accounted for clustering of measurements from broilers and turkeys reared in the same farms using cluster-robust standard errors. Differences in prevalence of resistance to each antimicrobial between broilers and turkeys were tested using a two-sample test of equality in proportions. Differences in the occurrence of resistance to each antimicrobial as a function of farm size, historical AMU of the farm (2016–2019, i.e. long-term effect), current AMU (i.e. short-term effect due to the antimicrobial treatments performed in the sampled flocks), and season, were tested for statistical significance using generalized estimating equations (GEE) regression models with a log link function and binomial error distribution. The GEE approach was used to account for the panel-longitudinal nature of the data, which were collected over multiple samplings and clustered at the farm level. In these models, the presence/absence of resistance to each antimicrobial was included as a binary dependent variable, with historical AMU, current AMU, farm size and season included as predictors. Besides including historical AMU in total, sub-analyses were also performed using historical AMU for each antimicrobial class. Separate analyses for broilers and turkeys were performed and the associations were expressed as odds ratios (ORs) and corresponding 95% confidence intervals (95% CI). For multi-resistance, i.e. multiple contemporaneous resistances to different antimicrobials in the same isolates, the GEE model was also used, with a log link function and negative binomial error distribution. Statistical analysis was performed using STATA 16.0 (StataCorp, College Station, USA).

Results

AMR prevalence

In 129 out of 140 samples from broiler farms and in 44 out of 70 samples from turkey farms, resistance to at least one of the antimicrobials tested for in the retrieved *E. coli* was observed. Table 1 shows the overall prevalence of resistance to each antimicrobial in (all the samplings of) the broiler and turkey farms. No isolate from either broiler or turkey farms was resistant to MER, and AZI resistance was not assessable, so no further analyses for these antimicrobials were performed. In broiler farms, the highest AMR prevalence was observed for SMX (70.22%), followed by AMP (60.3%), CIP (50.38%), NAL (45.8%), TMP (38.17%), TET (37.4%), and CHL (15.27%), whereas resistance to the other antimicrobials was considerably lower (0.76–6.87%). Over the four samplings in broiler farms, only the prevalence of SMX resistance showed a significantly decreasing trend (linear slope -0.126, 95% CI −0.223/−0.029, P value = 0.011) (Figure 1).

Table 1. Prevalence of antimicrobial resistance in the 35 sampled broiler and turkey flocks.

	Broilers			Turkeys		
Resistance	Prevalence (%)*	95% CI*		Prevalence (%)*	95% CI*	
SMX	70.22	60.50	78.41	72.86	61.99	81.54
TMP	38.17	28.92	48.36	39.29	28.74	50.93
CIP	50.38	39.76	60.97	30.00	21.42	40.25
TET	37.40	28.95	46.70	45.00	34.61	55.85
MER	0.00	–	–	0.00	–	–
AZI[a]	–	–	–	–	–	–
NAL	45.80	36.07	55.86	22.14	15.59	30.46
FOT	0.76	0.09	5.61	0.00	–	–
CHL	15.27	10.14	22.32	13.57	7.42	23.53
TGC	0.76	0.09	5.61	0.00	–	–
TAZ	0.76	0.09	5.61	0.00	–	–
AMP	60.30	51.18	68.77	53.57	40.92	65.79
COL	6.87	3.78	12.16	4.29	1.97	9.07
GEN	6.87	3.76	12.16	2.14	0.07	6.55
	Average multi-resistance*	95% CI*		Average multi-resistance*	95% CI*	
All	3.34	2.90	3.77	2.82	2.30	3.35

Note: *Estimates are adjusted for clustering of repeated observations at the farm level.
[a]Epidemiological cut-off value (ECOFF) is unavailable for AZI, so prevalence could not be calculated.

In turkey farms, no isolate was found to be resistant to FOT, TGC or TAZ, and the resistance prevalence to the other antimicrobials was generally similar to those in broiler farms. The highest AMR prevalence in turkey farms was found for SMX (72.86%), followed by AMP (53.57%), TET (45.00%), TMP (39.29%), CIP (30.00%), NAL (22.14%), and CHL (13.57%), whereas resistance to COL (4.29%) and GEN (2.14%) was the lowest detected. The prevalence of SMX resistance increased significantly between the two samplings (OR 1.22, 95% CI 1.01–1.46, P value = 0.036), whereas the prevalence of NAL decreased significantly (OR 0.41, 95% CI 0.20–0.82, P value = 0.012) (Figure 2). Comparing AMR prevalence between broilers and turkeys, significant differences were observed only for CIP and NAL ($P < 0.001$), both being higher in broilers than turkeys (Table 1).

Resistance to up to eight different antimicrobials was observed in one isolate from broilers, with a median multi-resistance of three antimicrobials (Interquartile Range [IQR] two to five). In turkeys, the maximum number of antimicrobials an isolate was contemporaneously resistant to was seven, which was observed in nine occasions, with median multi-resistance of three antimicrobials (IQR one to four). The average number of multi-resistances did not differ significantly between broilers and turkeys.

Factors associated with AMR in broilers

In total, among all samplings, 11 antimicrobial treatments were administered to the sampled broiler flocks. The antimicrobials administered were amoxicillin (AMX), enrofloxacin (ENRO), SMX, TMP, and thiamphenicol (THA). Overall, no significant effects of these treatments (i.e. current AMU) on the AMR prevalence rates were found. Yet, a significant effect of farm size was found for CHL resistance, with

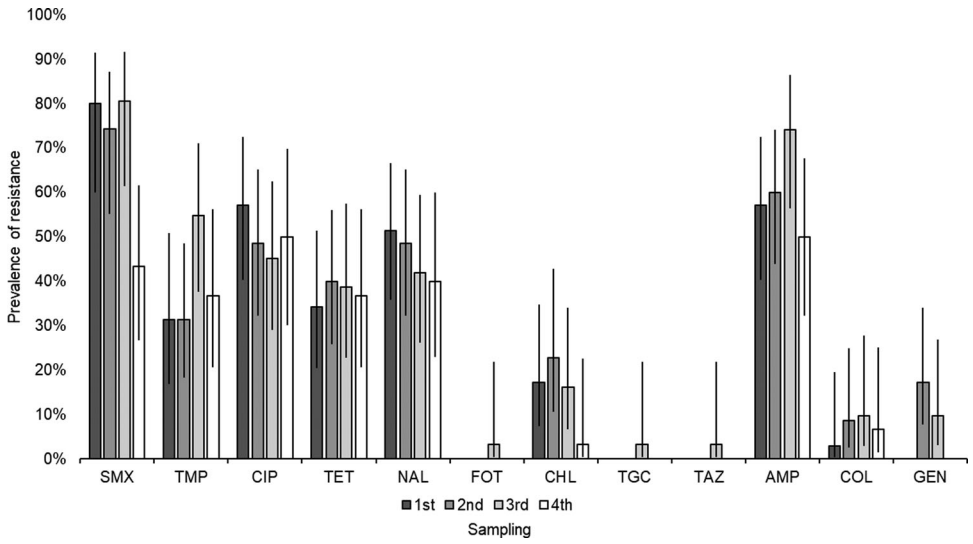

Figure 1. Prevalence of antimicrobial resistance at each sampling of the recruited 35 broiler farms. Notes: error bars indicate 95% confidence intervals. AMP = ampicillin; FOT = cefotaxime; TAZ = ceftazidime; CHL = chloramphenicol; CIP = ciprofloxacin; COL = colistin; GEN = gentamicin; NAL = nalidixic acid; SMX = sulfamethoxazole; TET = tetracycline; TGC = tigecycline; TMP = trimethoprim.

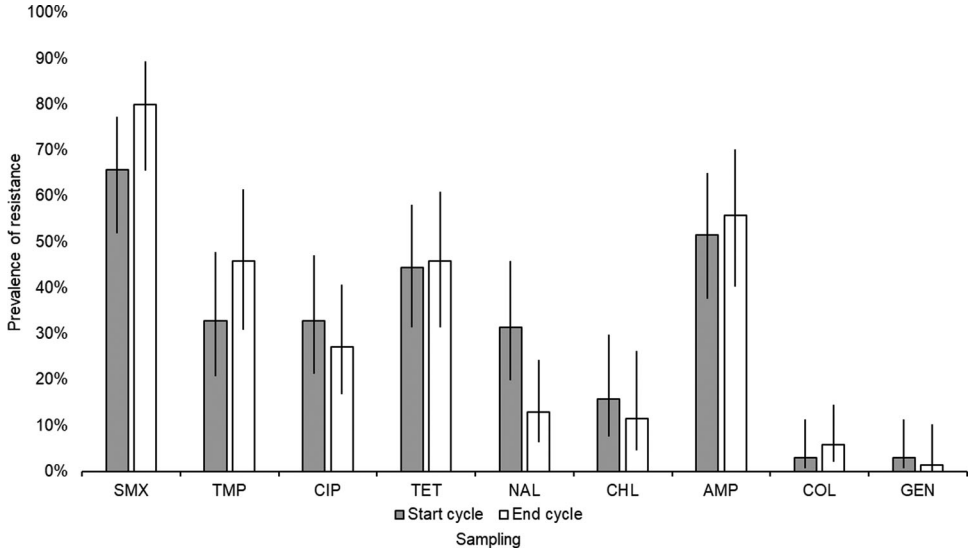

Figure 2. Prevalence of antimicrobial resistance at the start and at the end of the fattening cycles of the recruited 35 turkey farms. Notes: error bars indicate 95% confidence intervals. AMP = ampicillin; CHL = chloramphenicol; CIP = ciprofloxacin; COL = colistin (COL); GEN = gentamicin; NAL = nalidixic acid; SMX = sulfamethoxazole; TET = tetracycline; TMP = trimethoprim.

Table 2. Significant differences in AMR prevalence as a function of farm size, historical AMU in the farm, current AMU in the sampled flock, and sampling season, in broilers.

AMR	Variable	*n*	Adj. prevalence (%)*	Adj. OR*	*P* value
CHL	Farm size				
	Small	69	20.89 (12.20-29.60)	Ref.	
	Large	68	9.25 (3.01-15.49)	0.44 (0.20-0.98)	0.046
TET	Farm size*Past AMU				
	Small size & Low AMU	105	49.49 (32.18-66.81)	Ref.	
	Small size & High AMU			Ref.	
	Large size & Low AMU			Ref.	
	Large size & High AMU	32	20.13 (7.64-32.63)	0.41 (0.17-0.96)	0.040

Notes: *Estimates are adjusted for farm size, the level of past AMU, current AMU, sampling season, and clustering of observations at the farm level. Ref. = reference group. AMU = antimicrobial use. AMR = antimicrobial resistance. OR = Odds Ratio. Adj. = adjusted. TET = tetracycline. CHL = chloramphenicol.

large broiler farms having a significantly lower prevalence of CHL resistance (Table 2). For TET, a significant interaction between farm size and historical AMU was found, with farms of large size and with high historical AMU levels having a significantly lower prevalence of TET resistance. No other significant effects were found for any of the antimicrobials tested for, nor for multi-resistance. However, when looking at antimicrobial class-specific historical AMU and the corresponding resistance, only for penicillin (PEN), was its previous AMU significantly associated with increased resistance to PEN (OR 1.40, 95%CI 1.07–1.81, *P* value = 0.013).

Factors associated with AMR in turkeys

In total, among all samplings, 21 antimicrobial treatments were administered to the sampled turkey flocks (i.e. current AMU). The antimicrobials administered were AMX, SMX, TMP, COL, doxycycline (DOX), aminosidin (AMI) and TET. Overall, current AMU was significantly associated with increased resistance to SMX, TMP, CIP and TET (Table 3). Historical AMU was significantly associated with increased TMP resistance. A significant effect of farm size was observed for TMP and CIP, with large farms having a significantly higher prevalence of TMP and CIP resistance. No other significant effects were found for any of the antimicrobials tested for, nor for multi-resistance. Also when looking at antimicrobial class-specific AMU and the corresponding resistance, no significant associations were found.

Discussion

Overall, this study shows that the short-term effects of AMU on AMR in *E. coli* isolates from broiler and turkey farms are more likely to be significant than the long-term effects. Indeed, of the several associations tested, only the historical levels of PEN use and the specific resistance levels to PEN in broiler farms, and the overall historical AMU and resistance to TMP in turkey flocks were significant. Moreover, no significant effects were found for multi-resistance. On the contrary, the antimicrobial treatments in the sampled flocks showed significant effects on AMR, i.e. short-

Table 3. Significant differences in AMR prevalence as a function of farm size, historical AMU in the farm, current AMU in the sampled flock, and sampling season, in turkeys.

AMR	Variable	*n*	Adj. prevalence (%)*	Adj. OR*	*P* value
SMX	Current treatment				
	No	98	67.14 (57.21–77.07)	Ref.	
	Yes	42	85.50 (75.05–95.95)	1.27 (1.06–1.53)	0.009
TMP	Farm size				
	Small	72	29.01 (17.34–40.68)	Ref.	
	Large	68	49.40 (38.26–60.54)	1.70 (1.10–2.64)	0.017
	Past antibiotic use				
	Low	67	26.71 (14.06–39.36)	Ref.	
	High	73	48.86 (37.15–60.56)	1.83 (1.08–3.09)	0.024
	Current treatment				
	No	98	29.73 (19.64–39.82)	Ref.	
	Yes	42	58.02 (45.13–70.90)	1.95 (1.35–2.81)	0.000
CIP	Farm size				
	Small	72	22.62 (13.09–32.14)	Ref.	
	Large	68	38.97 (28.02–49.93)	1.72 (1.04–2.84)	0.033
	Current treatment				
	No	98	22.23 (14.19–30.27)	Ref.	
	Yes	42	59.77 (33.47–86.07)	2.69 (1.43–5.06)	0.002
TET	Current treatment				
	No	98	38.96 (28.27–49.64)	Ref.	
	Yes	42	58.48 (43.73–73.24)	1.50 (1.07–2.11)	0.020

Notes: *Estimates are adjusted for farm size, the level of past AMU, current AMU, sampling season, and clustering of observations at the farm level. Ref. = reference group. AMU = antimicrobial use. AMR = antimicrobial resistance. OR = Odds Ratio. Adj. = adjusted. SMX = sulfamethoxazole. TMP = trimethoprim. TET = tetracycline. CIP = ciprofloxacin.

term effects. This was the case for SMX, TMP, CIP and TET in turkey flocks. Furthermore, relatively high rates of resistance to these antimicrobials in addition to AMP were observed in both broiler and turkey farms, as also observed previously (Mughini-Gras *et al.*, 2020). Indeed, the genes conferring resistance to these antimicrobials are often associated with mobile genetic elements (MGEs), resulting in co-selection (EFSA & ECDC, 2018). These findings are of concern, as *E. coli* thrives in various intestinal and extra-intestinal niches, thereby favouring AMR spread among humans, animals and the environment (Dorado-Garcia *et al.*, 2018; Mughini-Gras *et al.*, 2019). Studies have also reported the long-term viability of *E. coli* in dust samples collected from poultry houses (Schulz *et al.*, 2016), with *E. coli* being able to survive in stored sediment samples for more than 20 years. Yet, *E. coli* survival in secondary habitats is affected by several factors, and survival times of nearly 1 year were estimated (van Elsas *et al.*, 2011), suggesting that the farm environment may function as a carrier of (resistant) *E. coli*.

Antimicrobials have been widely used for decades in intensive poultry farming. While some of these antimicrobials are scarcely metabolized and eliminated as such, others are excreted as metabolites (Xia *et al.*, 2019), contributing to the environmental pollution of antimicrobial residues (Kemper, 2008) and further promoting the occurrence of AMR in bacterial populations (Munk *et al.*, 2018). Poultry farms are environments with generally high bacterial loads combined with high levels of selective pressure due to AMU, a good recipe for AMR emergence and spread. In particular, dust-bound resistant bacteria, which are mostly of faecal origin (Cambra-López *et al.*, 2011; Luiken *et al.*, 2020), can become airborne and spread across the poultry house, as well as being emitted outdoor via the ventilation system, exposing other animals (including subsequent poultry production cycles) and people via dispersion into the environment or by entering the food chain (Berendonk *et al.*, 2015; Luiken *et al.*, 2020). The dissemination of AMR in the (farm) environment is a matter of concern, as the AMR genes can be integrated into MGEs like plasmids or transposons and be propagated via horizontal gene transfer among bacteria. In other studies on broilers, significant associations have been found between AMU (expressed as the incidence of antimicrobial treatments based on purchased products by the whole farm in the year before sampling) and gene-level AMR (Luiken *et al.*, 2019). This type of AMU data resembles ours in the sense that these are also a proxy for the overall historical AMU in the farm. While these historical AMU levels and the presence of antimicrobial residues have been suggested to affect the development and spread of resistant bacteria not only in the treated flocks, but also in the farm environment (Larsson *et al.*, 2018; Luiken *et al.*, 2020), studies have also found that the associations between gene-level AMR in poultry farm dust and historical AMU are not maintained after correction for faecal AMR. Thus, the association between faeces and dust might be so strong that it is difficult to conclude whether AMU has an additional effect on environmental contamination in addition to the effect of faeces (Luiken *et al.*, 2020). As AMU in breeding flocks could also lead to the selection of resistant bacteria that might then be transmitted to the progeny regardless of the specific AMU in the fattening flocks, AMR could then theoretically also be

acquired at the hatchery if disinfection practices are not applied properly. It is worth stressing that this study does not explicitly address AMU and vertical transmission of AMR from parent flocks to fattening broilers and turkeys, which is a major source of AMR as shown by, for example, studies on extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase (pAmpC)-producing *E. coli* (ESBL/pAmpC) showing that ESBL/pAmpC in the broiler production pyramid is prevalent and that substantial transfer between subsequent production levels does occur (Apostolakos *et al.*, 2019). Therefore, our results have to be interpreted as pertaining specifically to the (fattening) farm environment, regardless of what happened in the previous phases of the poultry production pyramid. Yet, future studies on the topic would benefit from including these phases as well.

The turkey flocks in which antimicrobials were used in the sampled animals had a higher prevalence of SMX, TMP, CIP and TET resistance. Moreover, while the prevalence of SMX resistance increased significantly from the beginning to the end of the fattening cycle, the prevalence of NAL resistance decreased. Moreover, positive associations between (current) AMU and AMR in poultry faeces have already been reported, particularly for TET, MLS (macrolides-lincosamides-streptogramins), TMP and aminoglycoside resistance (Luiken *et al.*, 2019; Xiong *et al.*, 2018). This is largely consistent with our findings, which also showed that AMU in a flock is associated with a higher prevalence of AMR in that same flock, although these antimicrobials have been used in poultry for a long time already. Indeed, current AMU alone might not be solely responsible for the observed AMR because, even in the absence of direct antimicrobial exposure in the sampled flocks, a relatively high level of AMR was observed, suggesting that antimicrobial-resistant *E. coli* can be transmitted and persist in the farm even in the absence of direct selection pressure (Ozaki *et al.*, 2011).

In general, our results showed stronger effects of current AMU (i.e. treatments in the sampled flock) than historical AMU (i.e. AMU in the whole farm in the previous 3 years). As reported by others (Luiken *et al.*, 2019), AMU data at the flock-level are "superior" to the farm-level AMU data if the association with AMR is hypothesized to occur by selection pressure in the actual flock. Yet, historical AMU data provide another perspective, namely on the long-term effects of AMU on AMR, which might occur through, for example, re-circulation of resistant bacteria within a farm, from one flock to another and/or to the next flock, with environmental pollution from antimicrobial residues also supporting the maintenance of resistant bacteria (Luiken *et al.*, 2019). These effects, however, are difficult to disentangle. Moreover, some of the measured AMR levels could not be explained by either historical or current AMU, and, as mentioned before, we cannot exclude a contribution from the parent flocks. Indeed, relatively high AMR levels in farms that do not use or use very little antimicrobials are not uncommon (Luiken *et al.*, 2019; Mughini-Gras *et al.*, 2020). Several reasons for AMR being present at high levels without substantial AMU-induced selection pressure have been suggested. These could be the large amounts and different types of antimicrobials that have been used indiscriminately in livestock well before this study period, basically since the 1950s (Davies & Davies, 2010), as well as AMU in other (higher) strata of the poultry production pyramid, which might influence AMR in lower sections through (pseudo)vertical transmission (Apostolakos *et al.*, 2019; Börjesson *et al.*, 2016; Dierikx *et al.*, 2013).

Conventionally reared turkeys are particularly susceptible to infections (Hafez & Hauck, 2005) and therefore particularly prone to antimicrobial treatments during their relatively long commercial lifespan. This is reflected in the high AMU and AMR levels reported in turkeys in the EU (EFSA & ECDC, 2018), as also shown in our study where we observed higher historical AMU levels and more frequent treatments being performed in the sampled flocks. Moreover, it has been shown that the rearing system influences the natural immunity of turkeys (Franciosini *et al.*, 2011), with conventional *vs*. organic farming conditions adversely affecting natural immunity, rendering the turkeys more susceptible to environmentally conditioned diseases requiring antimicrobial treatment, which would, in turn, promote AMR (Mughini-Gras *et al.*, 2020). Although it is difficult to identify exactly which factors might influence the different effects observed between broilers and turkeys in this study, it can be hypothesized that these differences are also the result, at least in part, of a third-variable relationship between natural immunity, AMU and AMR, i.e. a reflection of the higher need for AMU in conventionally raised turkeys leading to higher rates of selection pressure and persistence of resistant bacteria in those farms, as suggested elsewhere (Mughini-Gras *et al.*, 2020). This hypothesis would also be compatible with the positive associations we observed with farm size, as large turkey farms (which usually rely on intensive farming systems) were significantly more likely to have increased AMR prevalence. However, it is also true that all farms in our sample were intensive (conventional) farms, so all were of the same type, with the number of animals reared being essentially determined by the number of poultry houses and their size in the farm. Therefore, the effects of farm size on AMR observed here might not be associated with the farm management *per se*, especially since larger farms tend to have higher-profile, standardized approaches to farm management,

including higher biosecurity levels as observed for pig farms (Laanen *et al.*, 2013).

Conclusions

All in all, this study showed that, especially in turkeys, increased AMR levels were more often associated with AMU in the sampled flocks than with the historical (farm-level) AMU. This means that the short-term effects of AMU are generally stronger than its potential long-term effects. Generally speaking, this can be interpreted as a reassuring finding, as the poultry production sector does not seem to be inevitably doomed to bear the consequences of decades of indiscriminate AMU. However, it is also clear that the reasons that AMR appears in poultry farms are complex and diverse, which complicate the interpretation of the associations between AMU and AMR. Indeed, vertical transmission from parent flocks was not explicitly addressed here, and clearly once AMR is present on a farm, re-circulation, transfer and persistence of resistant bacteria and AMR genes in the (farm) environment is possible (Huijbers *et al.*, 2016). Furthermore, resistance gene carriage does not necessarily compromise microbial fitness, which makes the presence of AMR genes in the absence of AMU pressure likely (Fischer *et al.*, 2014; Holmes *et al.*, 2016). From these findings, it can therefore also be questioned to what extent AMR can be reduced only through decreasing AMU in specific flocks. The resulting (veterinary) public health implications are therefore difficult to conclude upon.

Ethical statement

This study was based on analyses of environmental samples and registry-based AMU data, so no experimental work with animals was included in this study. Poultry in the sampled farms were reared according to EU conventional husbandry practices in compliance with Council Directive 98/58/EC and Council Directive 2007/43/EC.

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