Natural immunity in conventionally and organically reared turkeys and its relation with antimicrobial resistance

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ABSTRACT Suboptimal animal welfare may affect natural immunity, rendering animals more susceptible to environmentally conditioned diseases, including those requiring antimicrobial treatment, which may promote antimicrobial resistance (**AMR**) in bacterial populations. Herewith, we tested the hypothesis that conventionally raised turkeys have higher levels of AMR in indicator *Escherichia coli* bacteria, but lower levels of natural immunity, as compared to turkeys reared under organic conditions. Litter and serum samples were collected from 28 conventional and 4 organic turkey farms: *E. coli* isolates from litter were tested for resistance to 14 antimicrobials, while 3 parameters of natural immunity (i.e., lysozyme, hemolytic complement levels, and serum bactericidal activity) were assessed in the sera. Resistant *E. coli* isolates were identified in both conventional and organic farms but generally more frequently in conventional farms. High rates of resistance to ampicillin (96%), tetracycline (95%), streptomycin (82%), sulfamethoxazole (80%), ciprofloxacin (73%), and trimethoprim (71%), as well as high rates of multiresistance, were observed in conventional farms. Organically raised turkeys had significantly higher levels of lysozyme and serum bactericidal activity than conventional turkeys, and these levels were also higher in turkeys housed in farms where AMR frequency was lower. Findings support the hypothesis that conventional farming conditions may affect turkeys' natural immunity, rendering the animals more susceptible to environmentally conditioned diseases requiring antimicrobial treatment, which would in turn promote AMR. Reducing AMR in turkey farming is therefore more likely to be successful when considering animal welfare as an option to reduce the need of antimicrobial use.

Key words: antimicrobial resistance, natural immunity, Turkey, organic farming, intensive farming

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INTRODUCTION

Poultry is often raised under intensive farming conditions in which the (metaphylactic) use of large amounts of antimicrobials is considered necessary to control diseases. Intensification and specialization of husbandry practices as a consequence of profit and resource optimization are sometimes difficult to reconcile with animal welfare. For instance, a high stocking density and the use of highly concentrated feed have been reported to affect poultry health through, for example, poor litter quality and high ammonia levels (Thomas et al., 2004; Villagrá et al., 2009). Chronic stress may indeed influence the natural immune system, predisposing the animals to environmentally conditioned pathologies, as natural immunity represents the forefront of immune response against microorganisms (Kimbrell and Beutler, 2001).

Antimicrobial resistance (AMR) poses a public health threat globally, with antimicrobial (mis)use in livestock being one of its main contributors (Kaesbohrer et al., 2012; Chuppava et al., 2018). Antimicrobial resistance monitoring in gram-negative bacteria, particularly *Escherichia coli*, is of primary concern, as commensal *E. coli* is used as indicator bacteria for AMR detection (ECDC et al., 2017) and pathogenic strains are often implicated in invasive infections in humans (Tacconelli et al., 2018). Moreover, *E. coli* can live in various intestinal and extraintestinal environments, thereby favoring AMR spread among humans, animals, and the environment (Dorado-Garcia et al., 2018; Mughini-Gras et al., 2019).

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Farmed turkeys are particularly susceptible to disease (Hafez and Hauck, 2005) and therefore particularly prone to antimicrobial treatments during their relatively long commercial lifespan. This is also reflected in the high levels of antimicrobial use and AMR reported in different European countries (EFSA and ECDC, 2018). It has been shown that the rearing system influences turkey's natural immunity, with hybrid turkeys selected for high production having difficulties in coping with situational stress caused by adaptation to a backyard environment (Franciosini et al., 2011). Although the relationships among poor animal welfare, low natural immunity, and negative health outcomes (including those requiring antimicrobial treatment) are compelling (Broom, 2006), little is known about AMR as the further consequence of this cascade. In this study, we tested the hypothesis as to whether turkeys raised under conventional (intensive) conditions have higher levels of AMR in indicator *E. coli* bacteria but lower levels of natural immunity as compared to turkeys raised under organic conditions.

MATERIALS AND METHODS

Sample Collection

Samples of litter from 28 conventional turkey farms and from 4 organic turkey farms were collected for AMR testing of *E. coli* as indicator bacteria. Conventional farms were randomly selected within 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), whereas the 4 organic farms were located in central Italy (Umbria region). In conventional farms, animals were bred in littered indoor sheds at a maximum stocking density of 62 kg/m^2 and with no limitation of antimicrobial treatments in case of need. In organic farms, the stocking density was 21 kg/m^2 in the indoor area and 1 kg/m^2 in the outdoor area. Animals can be treated with antimicrobials in case of need, but no more than 3 treatments can be provided. More details on this rearing system are available in Commission Regulation (EC) No 889/2008.

Sampling of conventional farms took place twice, in late winter (February-March) and in mid-summer (July-August) of 2012–2013 as part of another study (Di Martino et al., 2018), whereas sampling of organic farms took place only once in summer. Litter samples were taken from one shed per farm using 2 pairs of boot swabs (overshoes) following the sampling protocol of Commission Regulation (EU) No 1190/2012. Briefly, the boot swabs were put on the boots and the samples were taken by walking around in the shed: swabs were then pooled into one sample and kept refrigerated until examination.

Microbiological Analyses and Antimicrobial Resistance Testing

Each sample (2 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 on a Petri dish containing the selective MacConkey Agar medium; thereafter, 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). Confirmed *E. coli* isolates were tested for antimicrobial sensitivity: minimum inhibitory concentrations (**MICs**) were determined by broth microdilution method using the semiautomatic Sensititre System (Sensititre, Trek Diagnostic Systems, UK). Briefly, 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. Results reading took place 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 antimicrobials tested was based on the indications of the European Food Safety Authority (EFSA) on monitoring of AMR in commensal *E. coli*: ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim (Decision 2013/652/EU).

The MIC results allowed to classify each tested strain as resistant or susceptible, based on the epidemiological cutoff values (**ECOFF**) established by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/), as suggested by EFSA.

Serological Analyses

Based on the AMR testing results, the 5 commercial farms with the highest and those with the lowest frequencies of resistant *E. coli* isolates, as well as the 4 organic farms, were selected for serological testing. All these 14 farms reared the same commercial hybrid turkey; 10 farms reared females, 3 reared males; and one reared both sexes. In each farm, 20 turkeys were bled at the end of their rearing cycle (average 100 days, min 98 max 105, for females; average 130 days, min 125 max 137, for males) for quantification of 3 indicator parameters of natural immunity, that is, lysozyme, hemolytic complement levels (alternative pathway), and serum bactericidal activity. Blood samples were taken from the ulnar vein using vacuum tubes without anticoagulants (Vacuette, Greiner Bio-One, Frickenhausen, Germany), which were incubated at room temperature for 2 h and centrifuged at 3,520 × g for 16 min. Serum samples were then stored under sterile conditions in aliquots at −80°C pending analysis. The natural immunity parameters were determined using the same analytical methods described in detail by Franciosini et al. (2011):

- The serum lysozyme concentrations (µg/mL) were assessed by the lysoplate assay. Serum samples were

reacted with a suspension of *Micrococcus lysodeikticus* inside an agar gel in 10-cm Petri dishes in a humidified incubator for 18 h at 37°C and then distributed in duplicate in 3-mm holes, 2 cm apart, at a regular distance of 1.5 cm from the dish edge. The diameter of the lysed areas around serum samples and lysozyme standards of known concentration in a phosphate buffer (0.066 mol, pH 6.3) was assessed by calipers or rules so that lysozyme concentration is proportional to the diameter of lysed areas, as determined by a standard curve based on reference preparations of egg white lysozyme (Sigma-Aldrich, St. Louis, MO).

- The hemolytic complement level was assessed using rabbit erythrocytes in microtiter plates at a final reagent volume of 125 μL/well (100 μL of serum dilutions + 25 μL of 3% rabbit erythrocytes). The 0 and 100% hemolysis controls were set up in each plate at the same volume in veronal buffer (pH 7.3) and distilled water, respectively. Titres were expressed as 50% hemolytic units per 100 μL (the test volume of sera). Reference standard sera were used as control toward different batches of rabbit erythrocytes.
- Serum bactericidal activity (SBA) was assessed using a turbidimetric assay in microtiter format. Nonpathogenic *E. coli* was grown until log-phase in 20 mL of brain heart infusion (BHI) broth (Biolife Italiana, Milan, Italy) and frozen at −80°C in sterile skim milk. For each test, one aliquot was thawed, resuspended in 15 mL of BHI medium, and incubated at 37°C until optical density of 590 nm was doubled. Then, bacteria were diluted 1:100 in sterile saline solution. Test reagents were distributed into wells of sterile, U-bottomed microtiter plates according to the following scheme: 50 μL of test serum (in duplicate) added to 50 μL of veronal buffer, 100 μL of BHI broth, and 10 μL of 1:100-diluted bacterial suspension. Controls of sterility were set up without bacteria (negative control). Controls of bacterial growth (positive control) were set up without serum. The missing components were replaced by veronal buffer at the same volumes. Plates were incubated in a humidified box at 37°C for 18 h. They were then read spectrophotometrically in an ELISA reader at 690 nm, with blank set on the sterility control. The percentage of SBA was then derived.

Data Analysis

Differences in prevalence of resistance to each antimicrobial between the 2 sampling seasons (summer vs. winter) in commercial farms, and between commercial and organic farms (in summer only, as organic farms were only sampled in that occasion), were tested using chi-square or Fisher's exact test, as appropriate.

Resistance to different antimicrobials in the same isolates was explored using multiple correspondence analysis (MCA) and φ coefficients. Differences in the number of multiresistances in the same isolates were assessed between seasons using Wilcoxon matched-pairs signed-rank test (as the samples were to be considered as paired samples because they originated from the same farms in 2 different sampling seasons), whereas differences in multiresistance between conventional and organic farms were assessed using the Mann–Whitney *U* test.

Differences in natural immunity parameters between conventionally and organically farmed turkeys were assessed using linear regression analysis, adjusting for age at sampling and sex (included as covariates in the models) and accounting for clustering of measurements from turkeys housed in the same farms using cluster-robust standard errors. The values of the natural immunity parameters were log-transformed before regression analysis to achieve approximately normally distributed residuals. A regression model for each natural immunity parameter was built, in which the immunity parameter was set as the outcome variable and the farm type (conventional vs. organic) was set as dichotomous response variable of interest. Linear regression was also used to test associations between each natural immunity parameter (outcome variable) and resistance to each antimicrobial by including the farm type as an instrumental variable for the resistance status to each antimicrobial (endogenous variable), and the same was done for the overall coresistance in the farms. Instrumental (linear) regression was used here because the association between natural immunity and AMR might be described by the farm type itself, which can be directly associated with natural immunity. The rearing system can also be associated with AMR, but only indirectly through the effect of the rearing system on natural immunity, that is, AMR can be expected to be the consequence of increased antimicrobial use to control diseases that occur because of stressful rearing conditions affecting the natural immune system. Statistical analysis was performed using STATA 15.1 (StataCorp, College Station, USA).

Ethical Approval

This study was approved by the Ethical Committee of the 'Istituto Zooprofilattico Sperimentale delle Venezie' (IZSVe) (CE.IZSVE.08/2014).

RESULTS

Antimicrobial Treatments and Resistance

In conventional farms, 6 ± 1 antimicrobial treatments were administered, corresponding to 30 ± 7 treatment days. The active principles administered were as follows: amoxicillin, colistin, enrofloxacin, sulfamethoxazole-trimethoprim, tylosin, and doxycyclin. All organic farms provided less than 3 treatments (details of the antimicrobials used were not available).

In all 28 conventional and 4 organic turkey farms included in this study, all the *E. coli* isolates (n = 60) were resistant to at least one of the 14 antimicrobials tested for. Table 1 shows the between-farm resistance frequency to each antimicrobial in (the 2 sampling

Table 1. Between-farm resistance levels to each antimicrobial in the 2 sampling seasons of the conventional turkey farms, and in the organic turkey farms sampled in summer.

	Conventional farms (n = 28)			Organic farms (n = 4)	
Antimicrobial	Resistance in summer (95% CI)[1]	Resistance in winter (95% CI)[1]	*P*-value Summer vs. winter	Resistance in summer (95% CI)	*P*-value Conventional (summer) vs. organic farms
Ampicillin	92.9% (73.7-98.4%)	100% (87.7–100%)	0.237	75% (21.7–97.0%)	0.340
Cefotaxime	0.0% (0.0–12.3%)[1]	0.0% (0.0–12.3%)[1]	NC	0.0% (0.0–60.2%)[1]	NC
Ceftazidime	0.0% (0.0–12.3%)[1]	0.0% (0.0–12.3%)[1]	NC	0.0% (0.0–60.2%)[1]	NC
Chloramphenicol	50.0% (31.2–68.8%)	41.4% (24.3–60.8%)	0.514	0.0% (0.0–60.2%)[1]	0.113
Ciprofloxacin	64.3% (44.1–80.4%)	79.3 (59.6–90.9%)	0.207	50.0% (11.3–88.7%)	0.620
Colistin	14.3% (5.1–33.9%)	34.5% (19.0–54.1%)	0.077	0.0% (0.0–60.2%)[1]	1.000
Florfenicol	0.0% (0.0–12.3%)[1]	6.9% (1.6–25.4%)	0.491	0.0% (0.0–60.2%)[1]	NC
Gentamicin	50.0% (31.2–68.8%)	37.9% (21.6–57.6%)	0.359	0.0% (0.0–60.2%)[1]	0.113
Kanamycin	25.0% (11.8–45.3%)	20.7% (9.1–40.4%)	0.698	0.0% (0.0–60.2%)[1]	0.552
Nalidixic acid	60.7% (41.0–77.6%)	55.2% (36.1–72.8%)	0.672	50.0% (11.3–88.7%)	1.000
Streptomycin	75.0% (54.7–88.2%)	89.7% (70.9 –96.9%)	0.146	50.0% (11.3–88.7%)	0.557
Sulfamethoxazole	67.9% (47.5–83.1%)	93.1% (74.6–98.34%)	0.016	50.0% (11.3–88.7%)	0.593
Tetracycline	89.3% (69.9–96.8%)	100% (87.7–100%)	0.112	75.0% (21.7–97.0%)	0.431
Trimethoprim	57.1% (37.5–74.8%)	86.2% (67.0–95.1%)	0.015	50.0% (11.3–88.7%)	1.000

	Average multiresistance in summer (95% CI)	Average multiresistance in winter (95% CI)	*P*-value Summer vs. winter	Average multiresistance in summer (95% CI)	*P*-value Conventional (summer) vs. organic farms
All	6.5 (5.5–7.5)	7.5 (6.9–8.1)	0.067	4.0 (0.0–9.5)	0.108

Abbreviation: NC, noncalculable.
[1]One sided, 97.5% confidence interval.

seasons of) the conventional farms, and in the organic farms (only sampled in the summer). No isolate from either conventional and organic farms was resistant to cefotaxime or ceftazidime, so no further analysis for these 2 antimicrobials were performed. In the conventional farms, overall AMR frequencies for the 2 sampling seasons combined were as follows: ampicillin 96.4% (95% CI 86.0–99.2%), chloramphenicol 46.4% (95% CI 33.1–60.2%), ciprofloxacin 73.2% (95% CI 59.3–83.7%), colistin 23.2% (95% CI 14.8–34.4%), florfenicol 3.6% (95% CI 0.8–13.9%), gentamicin 42.9% (95% CI 28.4–58.6%), kanamycin 23.2% (95% CI 14.8–34.4%), nalidixic acid 58.9% (95% CI 44.6–71.9%), streptomycin 82.1% (95% CI 66.9–91.3%), sulfamethoxazole 80.4% (95% CI 67.1–89.1%), tetracycline 94.6% (95% CI 84.1–98.3%), and trimethoprim 71.4% (95% CI 57.8–82.0). Between the 2 sampling seasons, there were significant differences in the levels of resistance to sulfamethoxazole (67.9% in summer vs. 93.1% in winter, $P = 0.016$) and trimethoprim (57.1% in summer vs. 86.2% in winter, $P = 0.015$) (Table 1). In organic farms, no isolate was found to be resistant to chloramphenicol, colistin, florfenicol, gentamicin, or kanamycin, and the resistance frequencies to the other antimicrobials were generally lower than those in conventional farms, although the small sample size of organic farms limited the achievement of statistical significance.

The average number of multiresistances in same isolates was slightly higher in winter (7.5) than in summer (6.5) in the commercial farms and was lower in organic farms (4.0) (Table 1). Multiresistance was observed significantly more often between chloramphenicol and sulfamethoxazole ($\varphi = 0.46$, $P = 0.014$), chloramphenicol and trimethoprim ($\varphi = 0.57$, $P = 0.0001$), nalidixic acid and ciprofloxacin ($\varphi = 0.74$, $P < 0.0001$), trimethoprim and sulfamethoxazole ($\varphi = 0.72$, $P < 0.0001$). These multiresistances were also reflected in the MCA (Figure 1).

Natural Immune Parameters

Summary statistics of the 3 parameters of natural immunity (lysozyme, total hemolytic complement levels, and serum bactericidal activity) are reported in Table 2. All 3 parameters showed generally higher values in organically vs. conventionally farmed turkeys, with statistically significant differences ($P < 0.0001$) being observed for lysozyme concentration and serum bacterial activity in particular (Table 2).

Association Between Antimicrobial Resistance and Natural Immunity

Natural immunity parameters were generally higher in turkeys housed in farms where the frequency of resistant *E. coli* isolates was lower (Table 3). For lysozyme concentration, significantly higher values were found in turkeys housed in farms with no detectable resistance to chloramphenicol, colistin, gentamicin, and kanamycin. For hemolytic complement levels, no significant associations with AMR were found, whereas for serum bactericidal activity, significantly higher values were found in turkeys originating from farms where resistance to chloramphenicol was not detected (Table 3).

Parameters of natural immunity in turkeys were also found to decrease according to the number of multiresistances detected (Figure 2): lysozyme concentration (linear slope –0.08, $P = 0.010$), total hemolytic complement levels (linear slope –0.02, $P = 0.650$), and serum bactericidal activity (linear slope –0.11, $P = 0.210$).

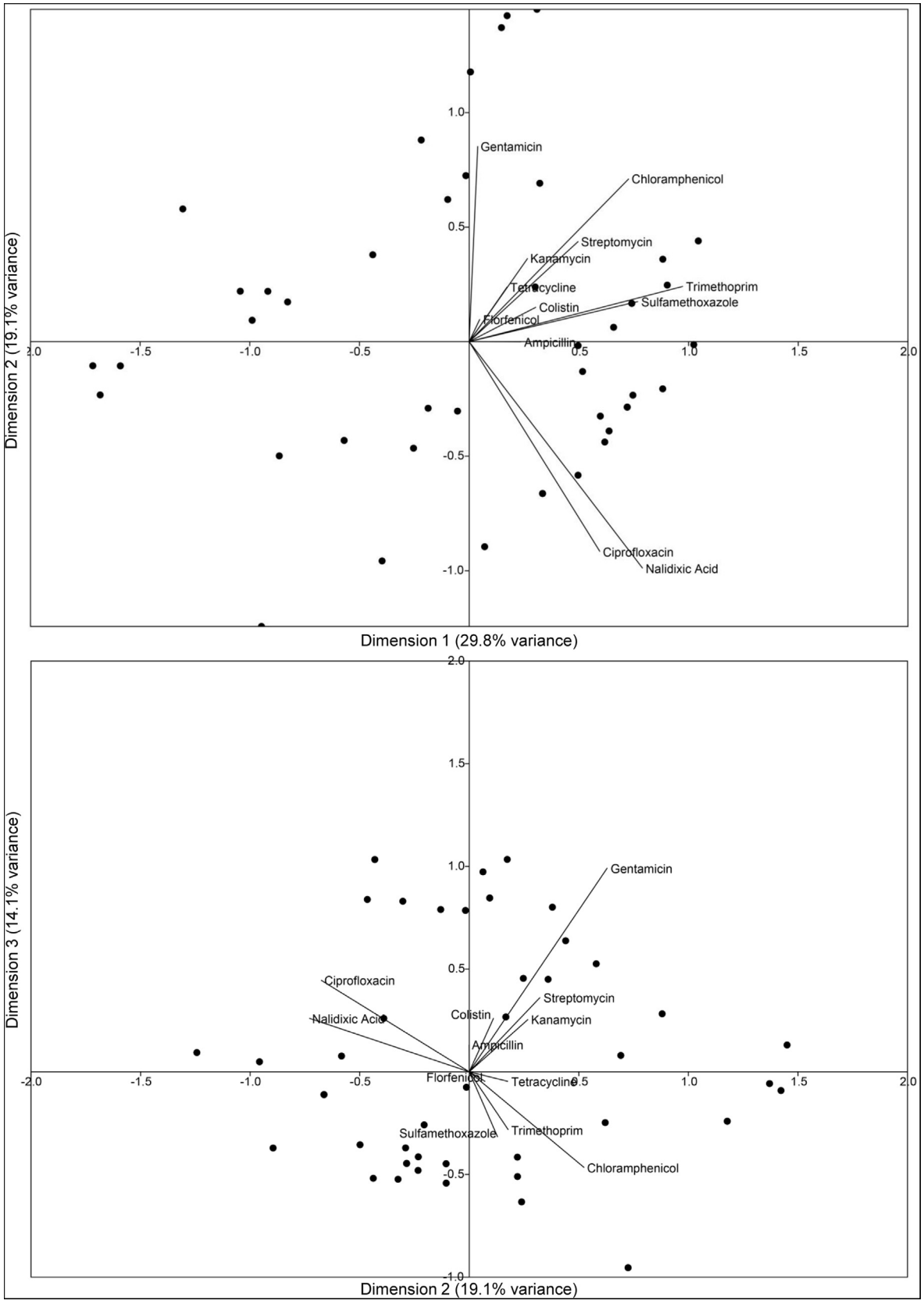

Figure 1. Scatter plots of the first vs. second (upper plot) and second vs. third (lower plot) dimensions of the multiple correspondence analysis for the antimicrobial multiresistances.

Table 2. Parameters of natural immunity in the conventionally and organically farmed turkeys.

Immune parameter	Conventionally farmed turkeys (n = 193) *Average (95% CI)*[1]	Organically farmed turkeys (n = 80) *Average (95% CI)*[1]	*P*-value
Lysozyme (μg/mL)	2.9 (2.5–3.3)	6.7 (5.5–8.3)	0.000
Hemolytic complement levels ($CH50^3$ μg/100 μL)	57.1 (41.9–77.9)	68.6 (52.7–89.2)	0.415
Serum bactericidal activity (%)	10.4 (7.0–15.4)	64.4 (44.2–93.8)	0.000

[1]Adjusted for turkeys' age at sampling, sex, and clustering at the farm level.

DISCUSSION

This study shows that the frequency of *E. coli* displaying AMR is generally high in both conventional and organic turkey farms, with AMR levels varying, to some extent, over seasons and rearing systems. In general, conventional farms had higher frequencies of AMR than organic farms, and within the conventional farms, AMR frequencies were generally higher in winter than in summer, particularly for sulfamethoxazole and trimethoprim. High rates of resistance to ampicillin (96%), tetracycline (95%), streptomycin (82%), sulfamethoxazole (80%), ciprofloxacin (73%), and trimethoprim (71%), as well as high rates of multiresistance, were observed in conventional farms. The genes conferring resistance to these antimicrobials are also frequently linked together on mobile genetic elements, resulting in coselection (EFSA and ECDC, 2018). Similar results were observed elsewhere for pathogens like *Campylobacter* (Luangtongkum et al., 2006). Although the sample size was small, these findings are of concern, as poultry has long been identified as a potential source of AMR (Furtula et al., 2010), contributing to dissemination of antimicrobial-resistant bacteria to humans via the food production chain (i.e., meat) and the environment (e.g., manure).

In conventional farms, antimicrobials can be used for treating diseases, but not as growth promotors, as this latter use has been banned in the European Union since 2006. In organic production, instead, antimicrobial use is restricted to a maximum of 3 treatments within a production cycle. In addition, organically raised turkeys must be fed only with organically produced feed and supplements, be provided with uncrowded living spaces (usually twice the space as in conventional farms), and have access to the outside environment. These characteristics of organic turkey farming might be expected to have generally positive effects on animal welfare and health, but they might also pose risks for disease introduction from outside sources (e.g., wildlife) due to the availability of outdoor access. Our study showed that the rearing system may have an effect on the natural immune system parameters, with the organically raised turkeys having significantly higher levels of lysozyme and serum bactericidal activity. Lysozyme, an important enzyme able to attack bacterial cell walls, and serum bactericidal activity linked to some complement factors and natural antibodies have higher levels in organic farms probably because the immune system of these animals is more stimulated. Natural immune system parameters were also higher in farms where the frequency of resistant *E. coli* isolates was lower, particularly for antimicrobials to which resistance was detected in conventional but not organic farms, a possible reflection of the infections in these farms. Our findings therefore support the hypothesis that turkeys raised under conventional conditions have higher levels of AMR in indicator *E. coli* bacteria, but lower levels of natural immunity, as compared to turkeys raised under organic conditions. This may entail that certain chronic stressors in

Table 3. Parameters of natural immunity in turkeys according to resistance to each tested antimicrobial.

	Lysozyme (μg/mL) n = 273			Hemolytic complement levels ($CH50^3$ μg/100 μL) n = 273			Serum bactericidal activity (%) n = 273		
	Average (95% CI)[1]			Average (95% CI)[1]			Average (95%CI)[1]		
Antimicrobial	R	S	*P*-value	R	S	*P*-value	R	S	*P*-value
Ampicillin	3.5 (2.9–4.3)	6.4 (3.2–13.2)	0.107	59.5 (49.4–71.7)	73.0 (36.9–100)	0.572	15.9 (10.2–24.8)	47.9 (9.4–100)	0.202
Chloramphenicol	**2.4 (2.1–2.9)**	**5.5 (4.6–6.5)**	**0.000**	61.7 (46.5–82.1)	59.1 (44.5–78.5)	0.840	**9.3 (5.2–16.6)**	**31.7 (17.9–56.4)**	**0.007**
Ciprofloxacin	3.4 (2.8–4.2)	5.6 (3.4–9.2)	0.081	58.2 (48.1–70.3)	76.0 (47.1–100)	0.312	14.8 (9.5–23.1)	43.4 (14.1–100)	0.083
Colistin	**2.4 (1.8–3.4)**	**4.6 (3.7–5.7)**	**0.004**	48.5 (34.7–67.6)	68.3 (54.1–86.1)	0.132	10.9 (4.7–25.4)	22.2 (12.32–40.2)	0.291
Gentamicin	**2.3 (1.9–2.9)**	**4.7 (3.9–5.5)**	**0.000**	61.3 (44.4–84.7)	59.9 (47.4–75.7)	0.915	9.6 (4.7–19.7)	23.7 (14.1–39.8)	0.054
Kanamycin	**2.5 (1.7–3.6)**	**4.5 (3.5–5.9)**	**0.025**	66.9 (45.4–98.4)	57.0 (43.8–74.3)	0.561	12.7 (4.9–33.0)	20.5 (10.6–39.4)	0.479
Nalidixic acid	3.5 (2.7–4.4)	4.2 (2.8–6.3)	0.437	58.4 (46.9–72.7)	65.8 (45.8–94.4)	0.595	18.3 (10.6–31.6)	14.7 (5.9–36.4)	0.698
Streptomycin	3.4 (2.7–4.2)	4.8 (3.1–7.4)	0.191	61.0 (49.6–75.1)	58.2 (38.5–88.2)	0.848	16.7 (9.9–28.1)	19.1 (6.8–53.7)	0.827
Sulfamethoxazole	3.4 (2.8–4.2)	5.6 (3.4–9.2)	0.081	58.2 (48.1–70.3)	76.0 (47.1–100)	0.312	14.8 (9.5–23.1)	43.4 (14.1–100)	0.083
Tetracycline	3.6 (2.9–4.4)	4.6 (2.2–9.9)	0.528	59.3 (49.3–71.3)	77.7 (39.5–100)	0.450	16.3 (10.3–25.6)	36.5 (6.9–100)	0.361
Trimethoprim	3.4 (2.7–4.2)	4.8 (3.1–7.4)	0.191	61.0 (49.6–75.1)	58.2 (38.5–88.2)	0.848	16.7 (9.9–28.1)	19.1 (6.8–53.7)	0.827

Abbreviations: NC, noncalculable; R, resistant; S, susceptible.
Bold values indicate $P < 0.05$.
[1]Adjusted for turkeys' age at sampling, sex, clustering at the farm level, and rearing system (i.e., commercial vs. organic farming, set as instrumental variable for the endogenous variable indicating resistance to the antimicrobial in question). Cefotaxime, ceftazidime, and florfenicol were excluded from this analysis because there were no *E. coli* isolates resistant to these antibiotics in the selection of 14 farms in which natural immune parameters were assessed (see also Table 1).

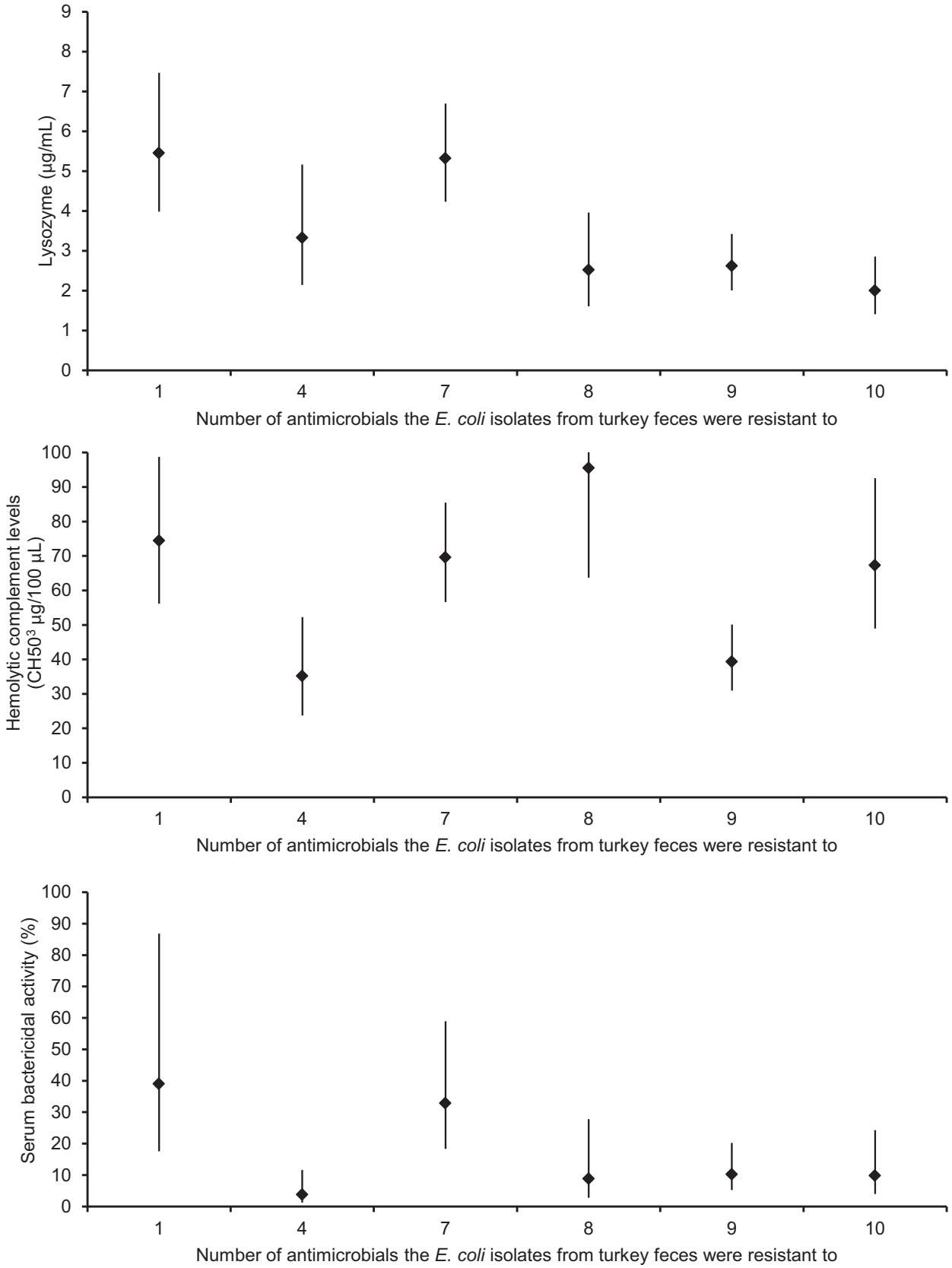

Figure 2. Parameters of natural immunity in turkeys according to the number of multiresistances detected in the *E. coli* isolates of their farms of origin. The error bars of diamond points represent 95% confidence intervals. Values are adjusted for turkeys' age at sampling, sex, clustering at the farm level, and rearing system.

conventional farms (e.g., stocking density, microclimate of shed, etc.) would affect turkeys' natural immune system, rendering the animals more susceptible to environmentally conditioned diseases requiring antimicrobial treatment, which would in turn promote AMR. The effect of the rearing system on the 3 parameters considered here has been reported previously for commercial and experimental turkeys (Franciosini et al., 2011), as well as in broilers raised in batteries and on hard floor (Stoyanchev et al., 1997) and in different hybrid turkeys raised on a slat floor and on litter (Yotova et al., 2004). Besides the rearing system, other factors might play a role in influencing the natural immune system parameters. For instance, different turkey breeds might display phenotypic variation in lysozyme and hemolytic complement levels (Bayyari et al., 1997; Sotirov et al., 1998), and serum bactericidal activity and hemolytic complement levels tend to increase with age (Franciosini et al., 2011). All these factors were controlled for in this study, as all farms reared the same commercial hybrid, and age (and sex) of the animals was always adjusted for in the analyses. Yet, we found no significant associations between the natural immunity parameters and age or sex (data not shown).

Although it is difficult to identify exactly which factors in the farm environment might influence a turkey's natural immune system, it can be hypothesized that the differences in AMR levels between conventional and organic farms, and so the third-variable relationship between AMR and natural immunity, are a reflection of the higher antimicrobial use in conventionally raised turkeys and the putatively higher rates of persistence of resistant bacteria. However, antimicrobial use alone might not be solely responsible for this because even in the absence of antimicrobial exposure, a high level of tetracycline resistance was observed in organic farms, suggesting that antimicrobial-resistant *E. coli* had been transmitted and persisted in the farm even in the absence of selection pressure (Ozaki et al., 2011), as observed for *Campylobacter* (Luangtongkum et al., 2006).

In conclusion, conventionally reared turkeys showed higher levels of AMR in indicator *E. coli* bacteria and lower levels of natural immunity as compared to turkeys reared under organic conditions. Several chronic stressors may play a role in modulating turkeys' natural immunity in a way that would render them more susceptible to environmentally conditioned diseases requiring antimicrobial treatment, which would thus promote AMR. Strategies to reduce AMR in turkey production may therefore benefit from improving animal welfare to minimize the need of antimicrobial use.

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REFERENCES

Bayyari, G. R., W. E. Huff, N. C. Rath, J. M. Balog, L. A. Newberry, J. D. Villines, J. K. Skeeles, N. B. Anthony, and K. E. Nestor. 1997. Effect of the genetic selection of turkeys for increased body weight and egg production on immune and physiological responses. Poult. Sci. 76:289–296.

Broom, D. 2006. Behaviour and welfare in relation to pathology *Appl.* Anim. Behav. Sci. 97:71–83.

Chuppava, B., B. Keller, A. A. El-Wahab, J. Meissner, M. Kietzmann, and C. Visscher. 2018. Resistance of Escherichia coli in turkeys after therapeutic or environmental exposition with Enrofloxacin depending on flooring. Int. J. Environ. Res. Public Health 15.

Di Martino, G., A. Piccirillo, M. Giacomelli, D. Comin, A. Gallina, K. Capello, F. Buniolo, C. Montesissa, and L. Bonfanti. 2018. Microbiological, chemical and physical quality of drinking water for commercial turkeys: a cross-sectional study. Poult. Sci. 97:2880–2886.

Dorado-Garcia, A., J. H. Smid, W. van Pelt, M. J. M. Bonten, A. C. Fluit, G. van den Bunt, J. A. Wagenaar, J. Hordijk, C. M. Dierikx, K. T. Veldman, A. de Koeijer, W. Dohmen, H. Schmitt, A. Liakopoulos, E. Pacholewicz, T. Lam, A. G. Velthuis, A. Heuvelink, M. A. Gonggrijp, E. van Duijkeren, A. van Hoek, A. M. de Roda Husman, H. Blaak, A. H. Havelaar, D. J. Mevius, and D. J. J. Heederik. 2018. Molecular relatedness of ESBL/AmpC-producing Escherichia coli from humans, animals, food and the environment: a pooled analysis. J. Antimicrob. Chemother. 73:339–347.

ECDC, EFSA BIOHAZ Panel and CVMP 2017. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and anti microbial consumption in humans and food-producing animals. EFSA J. 15:70.

EFSA and ECDC 2018. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. EFSA J. 16:5182.

Franciosini, M. P., A. Bietta, L. Moscati, L. Battistacci, M. Pela, G. Tacconi, I. Davidson, and P. Casagrande Proietti. 2011. Influence of different rearing systems on natural immune parameters in broiler turkeys. Poult. Sci. 90:1462–1466.

Furtula, V., E. G. Farrell, F. Diarrassouba, H. Rempel, J. Pritchard, and M. S. Diarra. 2010. Veterinary pharmaceuticals and antibiotic resistance of Escherichia coli isolates in poultry litter from commercial farms and controlled feeding trials. Poult. Sci. 89:180–188.

Hafez, H., and R. Hauck. 2005. Genetic Selection in Turkeys and Broilers and Their Impact on Health Conditions.

Kaesbohrer, A., A. Schroeter, B. A. Tenhagen, K. Alt, B. Guerra, and B. Appel. 2012. Emerging antimicrobial resistance in commensal Escherichia coli with public health relevance. Zoonoses Public Health 59(Suppl 2):158–165.

Kimbrell, D. A., and B. Beutler. 2001. The evolution and genetics of innate immunity. Nat. Rev. Genet. 2:256–267.

Luangtongkum, T., T. Y. Morishita, A. J. Ison, S. Huang, P. F. McDermott, and Q. Zhang. 2006. Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry. Appl. Environ. Microbiol. 72:3600–3607.

Mughini-Gras, L., A. Dorado-Garcia, E. van Duijkeren, G. van den Bunt, C. M. Dierikx, M. J. M. Bonten, M. C. J. Bootsma, H. Schmitt, T. Hald, E. G. Evers, A. de Koeijer, W. van Pelt, E. Franz, D. J. Mevius, D. J. J. Heederik, and E. A. Consortium. 2019. Attributable sources of community-acquired carriage of Escherichia coli containing beta-lactam antibiotic resistance genes: a population-based modelling study. Lancet Planet. Health 3:e357–e369.

Mulatti, P., M. E. Bos, L. Busani, M. Nielen, and S. Marangon. 2010. Evaluation of interventions and vaccination strategies for low pathogenicity avian influenza: spatial and space-time analyses and quantification of the spread of infection. Epidemiol. Infect. 138:813–824.

Ozaki, H., H. Esaki, K. Takemoto, A. Ikeda, Y. Nakatani, A. Someya, N. Hirayama, and T. Murase. 2011. Antimicrobial resistance in

fecal Escherichia coli isolated from growing chickens on commercial broiler farms. Vet. Microbiol. 150:132–139.

Sotirov, L. K., M. T. Lalev, M. G. Oblakova, Z. Porfirova, S. Tanchev, and N. G. 1998. Lysozyme and complement activity in different Turkey breeds. Rév Méd Vét 149:309–312.

Stoyanchev, T. K., L. K. Sotirov, I. T. Yotova, and K. Uzunova. 1997. Natural resistance peculiarities in broiler chickens of different blood-group genotypes raised in batteries and on hard floor. Bull J. Agric. Sci. 647–649.

Tacconelli, E., F. Sifakis, S. Harbarth, R. Schrijver, M. van Mourik, A. Voss, M. Sharland, N. B. Rajendran, and J. Rodriguez-Bano. 2018. Surveillance for control of antimicrobial resistance. Lancet Infect. Dis. 18:e99–e106.

Thomas, D. G., V. Ravindran, D. V. Thomas, B. J. Camden, Y. H. Cottam, P. C. Morel, and C. J. Cook. 2004. Influence of stocking density on the performance, carcass characteristics and selected welfare indicators of broiler chickens. N. Z. Vet. J. 52:76–81.

Villagrá, A., J. L. Ruiz de la Torre, G. Chacón, M. Lainez, A. Torres, and X. Manteca. 2009. Stocking density and stress induction affect production and stress parameters in broiler chickens. Anim. Welfare 18:189–197.

Yotova, I. T., L. K. Sotirov, T. K. Stoyanchev, N. A. Bozakova, D. J. Yarkov, K. T. Stoyanchev, M. G. Oblakova, and M. T. Lalev. 2004. Study on the level of natural humoral immunity in Turkey broilers bred on two floor types. Bulg J. Vet. Med. 7:51–56.