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ORIGINAL RESEARCH

Influence of farm management on the dynamics of *Salmonella enterica* serovar Infantis shedding and antibiotic resistance during the growing period of broiler chickens

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Abstract

Background: *Salmonella enterica* serovar Infantis is a zoonotic pathogen isolated in broilers causing great economic losses in the European poultry sector. It is demonstrated that an investment in management measures at farm level could directly affect the control of food chain microorganisms. The aim of this study was to investigate the development of *S.* Infantis antimicrobial resistance (AMR) patterns during the growing period, according to flock density and ventilation management, without antibiotic administration.
Methods: The experiment was performed in two identical poultry houses, evaluating commercial and optimal farm conditions. At 24 h of rearing, 20% of the animals were orally infected with a *S.* Infantis strain susceptible to all the antibiotics tested. To study *Salmonella* shedding, faeces samples from each experimental group were taken weekly and analysed as per ISO/TS 6579-2:2017. Antibiotic susceptibility was assessed according to Decision 2013/653.
Results: *Salmonella* shedding showed that the lowest counts were observed in the first week post-infection and highest at slaughter day for both groups. Moreover, 100% of the isolates were multi-resistant.
Conclusion: The acquisition of AMR by *S.* Infantis starts at the onset of the production cycle and is maintained until the end, demonstrating the importance of transmission of AMR in zoonotic bacteria at farm level.

KEYWORDS
antimicrobial resistance, broiler, growing period, multidrug-resistance, salmonella

INTRODUCTION

Animal welfare and food safety are increasing concerns for poultry product consumers.[1] Both issues are closely related, as it has been demonstrated that if animals are in good welfare status, their resilience is increased, and they can cope with environmental challenges or infectious diseases.[2–5] For that reason, an investment in more efficient and animal friendly management measures in the poultry sector could directly affect animal health.[3,6–9] In this sense, a good ventilation system is essential for heat stress management, a factor that undermines the productivity and immunology of livestock.[10,11] Likewise, high stocking density also has an adverse effect on the performance and immune status of broilers.[12–14]

Furthermore, despite the strict legislation against *Salmonella*, these bacteria remain the principal source of human foodborne disease in Europe, and poultry products are the main source involved in human outbreaks.[1,15,16] Moreover, *Salmonella enterica* serovar Infantis is an emerging serovar of great concern for European broiler production, as it has been demonstrated that this serovar is present in 50%

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of *Salmonella* contaminated broiler meat samples analysed.[16,17] Consequently, nowadays *S.* Infantis control at farm level is one of the main objectives for the poultry sector.

One hypothesis that explains the emergence of *S.* Infantis in the poultry sector is its ability to gain antimicrobial resistance (AMR) from the gut microbiota and/or environment.[18–20] Moreover, a novel megaplasmid has been identified that represents a recent evolutionary change in the pathogenicity and stress tolerance of local *S.* Infantis population.[21] In this context, AMR control in the field requires effective surveillance programs, proper food handling practices and prudent use of antibiotics throughout the production cycle.[22,23] However, to be able to establish adequate control measures, it is necessary to have better knowledge of the epidemiology of this serovar.

In accordance with the increasing consumer concern for animal welfare and the public health issue of AMR, the objective of this study was to investigate the development of *S.* Infantis AMR during the broiler growing period, according to density and ventilation management.

METHODS

In this experiment, all animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.[24]

Experiment design

The study was performed in two identical poultry houses of an experimental poultry farm at the Center for Research and Animal Technology (CITA-IVIA, in its Spanish acronym *Valencian Institute for Agrarian Research*, Segorbe, Spain). For this purpose, two different environmental farm conditions, commercial farm conditions (CFC) (house 1) and optimal farm conditions (OFC) (house 2), were evaluated. For CFC, chicks were housed at 35 kg/m^2 density, and non-optimal parameters of ventilation were applied (allowing a maximum concentration of ammonia of 25 ppm). While in OFC, the animals were housed in low density at 17 kg/m^2, and ventilation was provided within the optimal parameters (allowing a maximum concentration of ammonia of 10 ppm).

To this end, day-old-chicks (Ross) (males and females) were distributed in two identical poultry houses (n = 1062, 531 per house). Within each of the houses, 204of 531 animals were located in 12 pens with wood shavings as bedding material. The rest of the animals (327/531) were housed in the remaining space using also wood shavings as bedding material to simulate production conditions. The house was supplied with programmable electrical lights, automated electric heating and forced ventilation. The environmental temperature was gradually decreased from 32°C (1 day) to 19°C (42 days) following common practice in poultry production. The experimental pelleted feed was commercial feed according to standard diets for broilers. Two different diets were offered to the birds: starter (1–21 days) and grower (21–42 days). Only one batch of feed per age (starter and grower) was provided. Nutritional and product analysis were assessed before the arrival of animals. Feed was weighed, manually distributed and added *ad libitum*. Furthermore, the mortality and the presence of diarrhea were registered daily. Finally, animals were weighed at weekly intervals, and feed consumption per pen was recorded.

Salmonella infection

At 24 h after placing, 20% of birds/pen were orally infected with *S.* Infantis. The experimental infection was done with 100 µl of a *S.* Infantis diluted at an infective titter of 10^4 CFU/ml. The strain was selected from a database of *Salmonella* strains isolated from the *Salmonella* National Control Program (CECAV, in its Spanish acronym *Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana*, Castellón, Spain). To ensure that this strain was susceptible to all antibiotics studied, antimicrobial susceptibility was tested according to the European Committee on Antimicrobial Susceptibility Testing guidelines.[25] The source for zone diameters used for interpretation of the test was http://www.eucast.org/clinical_breakpoints/. The strain of *S.* Infantis was inoculated into Mueller-Hinton agar (Scharlab, S.L., Barcelona, Spain) to form a bacterial lawn, the antibiotic disks were added, and plates were incubated at 37°C ± 1°C for 24 ± 3 h.

Salmonella detection and identification

Salmonella status of the chicken houses was tested before the arrival of the animals in accordance with ISO 6579-1:2017.[26] In addition, *Salmonella* status of the flock was tested at the arrival day, collecting samples of meconium (n = 250) and delivery box liners (n = 10).[27]

Salmonella enumeration was assessed as per ISO/TS 6579-2:2017.[28] Animals were sampled at different times throughout the growing period (7, 14, 21, 28, 35 and 42 days of age). For each sampling time and house (CFC vs. OFC), faeces samples (25 g) were directly collected from each pen per duplicate (n = 24). Once in the laboratory, two pools of samples from each replicate per house (n = 2 pools/treatment/house) were homogenized and transferred into 225 ml of Buffered Peptone Water (Scharlab, S.L., Barcelona, Spain). Afterwards, 2.5 ml of the suspension was transferred into an empty tube. Serial 1:5 dilutions were made from each tube and incubated at 37°C for 18 ± 2 h. After incubation, 20 µl was transferred onto Rappaport Vasiliadis agar plates (MSRV, Difco, Valencia, Spain) and incubated at 41.5°C for 24–48 h. Suspected plates were streaked into XLD medium (Scharlab, S.L., Barcelona, Spain) and incubated at 37°C ± 1°C for 24 ± 3 h. Then, API-20E test (Biomerieux, S.L., Barcelona, Spain) was performed to confirm *Salmonella*. Finally, for the estimation of Most Probable Number, the software described by

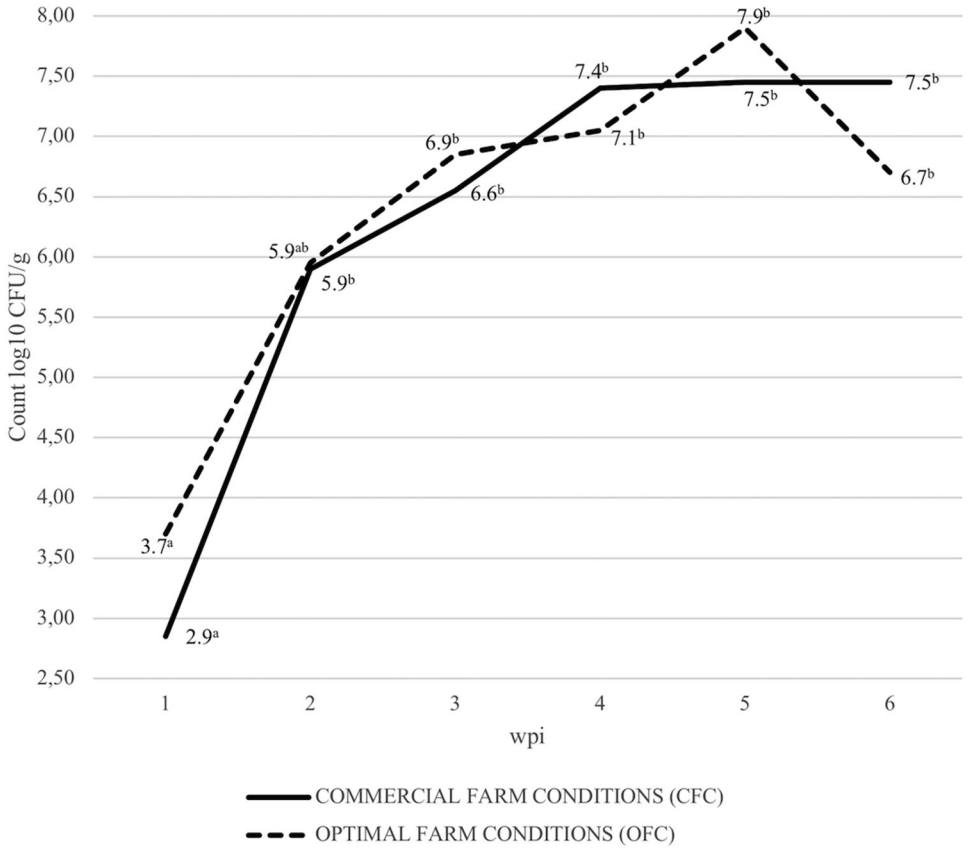

FIGURE 1 *Salmonella* excretion dynamic in CFC and OFC during growing period.
[a,b]: different superscripts means significant differences with a p-value < 0.05

Jarvis et al was used, and the results were transformed into logarithms ($\log_{10}$ CFU/g).[29] To confirm that the isolates were obtained from the original inoculum, *Salmonella* strains were serotyped at CECAV, using the Kauffman-White-Le Minor scheme.[30]

Antimicrobial susceptibility test

Antimicrobial susceptibility of the strains isolated was tested as reported above. The antibiotics selected were those set forth in Decision 2013/652[31] including two quinolones: ciprofloxacin (CIP) (5 µg) and nalidixic acid (NAL) (30 µg); three b-lactams: ampicillin (AMP) (10 µg), cefotaxime (CTX) (30 µg) and ceftazidime (CAZ) (30 µg); one phenicol: chloramphenicol (CHL) (5 µg); one potentiated sulfonamide: trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 µg); one polymyxin: colistin (CST) (10 µg); one macrolide: azithromycin (AZM) (15 µg); one glycylcycline: tigecycline (TGC) (15 µg); one aminoglycoside: gentamycin (GEN) (10 µg) and one pyrimidine: trimethoprim (TMP) (5 µg). MDR was defined as acquired resistance to at least one agent in two or more antimicrobial classes.[32]

Statistical analysis

ANOVA test was used to study the dynamics of *S.* Infantis shedding and AMR during growing period under different farm conditions (CFC and OFC). A p-value < 0.05 was considered to indicate a statistically significant difference. Analyses were carried out using a commercially available software application (SPSS 24.0 software package; SPSS Inc., Chicago, IL, 2002).

RESULTS

During this experiment, all the productive parameters were according to the breed standards, and no signs of intestinal disease were observed. Thus, no antibiotics were administered in this study.

Salmonella excretion

At the start of the trial, negative *Salmonella* status of the chicken houses and the day-old-chickens was confirmed. Moreover, all the *Salmonella* strains isolated during this study were serotyped as *S.* Infantis.

Results obtained for CFC and OFC are presented in Figure 1. For both environmental farm conditions studied, the lowest excretion of *S.* Infantis was observed in the first week post-infection (wpi). Then, for CFC, *S.* Infantis detection increased until 14 days and then became stable until the end of growing period (p-value < 0.05). However, for OFC, *S.* Infantis counts increased until 21 days of the growing period and then remained stable

TABLE 1 Antibiotic resistance isolates according to the antibiotic and the moment of the growing period in commercial (CFC) and optimal farm conditions (OFC)

Environmental conditions	wpi	N pools	CIP	NAL	CTX	CAZ	AMP	CHL	SXT	CST	AZM	TGC	GEN	TMP
CFC	**1**	**2**	2	2	2	1	0	0	2	0	0	0	0	2
	2	**2**	2	2	1	1	0	0	2	1	0	0	0	2
	3	**2**	2	2	0	0	0	0	2	0	0	0	0	2
	4	**2**	2	2	0	1	0	0	2	0	0	0	0	2
	5	**2**	2	2	0	0	0	0	1	0	0	0	0	2
	6	**2**	2	2	0	0	0	0	2	0	2	0	0	2
	Total	**12**	**12**	**12**	**3**	**3**	**0**	**0**	**11**	**1**	**2**	**0**	**0**	**12**
OFC	**1**	**2**	2	2	2	1	0	0	2	0	0	0	0	2
	2	**2**	2	2	1	0	1	1	2	0	0	0	0	1
	3	**2**	2	2	0	0	0	0	2	0	0	0	0	2
	4	**2**	2	2	1	1	1	0	2	0	0	0	0	2
	5	**2**	2	2	0	0	0	0	2	0	0	0	0	2
	6	**2**	2	2	0	0	0	0	2	0	2	0	0	2
	Total	**12**	**12**	**12**	**4**	**2**	**2**	**1**	**12**	**0**	**2**	**0**	**0**	**11**

Abbreviations: AMP, ampicillin; AZM, azithromycin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; GEN, gentamycin; NAL, nalidixic acid; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; TMP: trimethoprim.

until the end of growing (p-value < 0.05). However, no statistically significant differences were found between treatments (CFC vs. OFC) in *Salmonella* counts (p-value > 0.05).

Prevalence of antibiotic resistance and multidrug-resistance

Although the *S.* Infantis strain used to infect the animals was completely susceptible to all antibiotics tested at the time of infection, and no antibiotics were administered during the growing period, *Salmonella* isolates obtained from both groups (n = 24) were MDR after 1 wpi. No statistically differences were found between experimental conditions (CFC vs. OFC) and the time of sampling (p-value > 0.05).

For CFC, the highest percentages of AMR were found to CIP (100%, n = 12), NAL (100%, n = 12) and TMP (100%, n = 12), followed by SXT (91.7%, n = 11), CTX (25.0%, n = 3), CAZ (25.0%, n = 3), AZM (16.7%, n = 2) and finally, CST (8.3%, n = 1). No resistance was found against AMP, CHL, GEN and TGC. Regarding resistance dynamic through the entire growing period, at 1 wpi, *S.* Infantis strains showed resistance to CIP, NAL, CTX, CAZ, SXT and TMP. It is important to highlight that at 2 wpi, resistance to CST also appeared. However, from the third wpi onwards, only resistance to CIP, NAL, SXT and TMP remained until slaughter day (Table 1).

In the case of OFC, the highest AMR percentages were observed to CIP (100%, n = 12), NAL (100%, n = 12) and SXT (100%, n = 12), followed by TMP (91.7%, n = 11). The remaining antibiotics showed a lower AMR percentage: CTX (33.3%, n = 4), CAZ (16.7%, n = 2), AMP (16.7%, n = 2), AZM (16.7%, n = 2) and CHL (8.3%, n = 1). Regarding the AMR dynamics during the growing period, at 1 wpi and at the slaughter day, *S.* Infantis strains were resistant to the same antibiotics of CFC isolated strains. However, in OFC no resistance to CST appeared during the growing period (Table 1).

Abbreviations: AMP, ampicillin; AZM, azithromycin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; GEN, gentamycin; NAL, nalidixic acid; TGC, tigecycline; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole.

Antibiotic resistance patterns

A number of *Salmonella* strains isolated resistant to the different antibiotics tested according to different environmental farm conditions (CFC and OFC) are presented in Table 2.

Overall, 11 different resistance patterns were observed. The combination of CIP-NAL-SXT-TMP (37.5%, n = 9) was the pattern most frequently observed, followed by CIP-NAL-SXT-AZM-TMP (16.67%, n = 4).

DISCUSSION

The present study examined the development of *S.* Infantis AMR in broiler chickens during the growing period, comparing two different environmental conditions according to density and ventilation parameters. To our best knowledge, this is the first study in scientific literature to evaluate the effect of these management measures at farm level on *S.* Infantis epidemiology.

TABLE 2 Number of Salmonella strains isolated resistant to the different antibiotics tested according to different environmental farm conditions

Environmental conditions	Number of AMR to the indicated number of antibiotics											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total**
CFC	0	0	0	1	4	5	2	0	0	0	0	12
OFC	0	0	0	1	5	3	1	2	0	0	0	12

Abbreviations: AMR, antimicrobial resistance; CFC, commercial farm conditions; OFC, optimal farm conditions.

On the day of placement, the negative *Salmonella* status of the chickens was confirmed. After infection, for CFC experimental group *S.* Infantis counts increased until 14 days and, for OFC until 21 days, without statistically significant differences between treatments. Our results agree with those reported previously by Marin and Lainez, when *Salmonella* detection in faeces increased until second week of age, coinciding with the maturation of the animals' immune system and remaining stable until processing day.[33,34]

In the European Union, a strict poultry welfare legislation has been set out at farm level.[35] However, a large section of society calls for a continuous increase in animal welfare during the grow-out period.[1] In fact, different authors indicate that lower stress situations increase the potential of the immune system to protect the individual against pathogens.[3,4,14,17,36–39] However, the results of our study showed that the improvement in ventilation or density parameters of the flock has no effects in terms of either *Salmonella* shedding which is in line with Velasquez et al and Pulido-Landínez.[40,41]

AMR rates of *Salmonella* isolates obtained since the start of the trial showed that no statistical differences were found between treatments, despite the improvement in management conditions. In addition, it is important to underscore that a high percentage of *S.* Infantis isolated during the growing period were MDR, although no antibiotics were administrated.[16,23,42]

Different hypotheses could explain this fact. Previous studies using genomic analysis of bacteria indicated they could acquire resistance profiles by incorporating different genetic elements through horizontal gene transfer from other bacteria and/or from the environment.[34,43–48] In this sense, the commensal microbiota could acquire the AMR and, intestinal zoonotic bacteria such as *Salmonella* could acquire the AMR by conjugation, transformation or transduction mechanisms.[49,50] For that reason, different scientific studies underline the importance of developing sanitary measures at the interface between the environment and livestock farming.[51–53] However, further studies are needed to confirm the main source of AMR of the *Salmonella* strains at farm level.

Moreover, in reference to AMR percentages obtained from different antibiotics assessed, it is important to highlight the results obtained against CST and TGC, as they are considered critically important antimicrobials used as last-resort drugs to treat human infectious diseases.[50,54] On the one hand, no isolates showed AMR to TGC. The results agree with that reported by the European Food Safety Authority (EFSA),[50] and it might be explained by the restricted use to human in hospital treatments.[55] Conversely, the presence of AMR against CST could be due to its use in animal production for several years to treat infectious diseases and as a growth promotor[56] and, as indicated by previous studies, resistant genes could remain in the environment and reach the microbiota of animals, and from there transmitted to zoonotic bacteria. Furthermore, it is important to note that the highest AMR obtained is to CIP, NAL, SXT and TMP. In 2020, EFSA reported very high levels of resistance to CIP, NAL and SXT in *Salmonella* isolated from broilers, and low levels of resistance to AMP and CHL, matching our results.[50] Moreover, specifically for SXT and TMP, one hypothesis that could explain the results obtained in this study is that these antibiotics are permitted in Spain as therapeutic agents for antibacterial therapy in animal.[55] This study reveals the importance of AMR monitoring in zoonotic and commensal bacteria in food-producing animals and their food products to be able to understand the development and diffusion of resistance, providing relevant risk assessment data, and evaluating targeted interventions.[50,57]

In conclusion, the results of this study showed that when chicks are infected with the serovar *S.* Infantis at day one of the growing period, they continue shedding the bacteria in faeces until the processing day. Besides, the acquisition of AMR began at the onset of the production cycle and continued until the end, regardless of different management conditions applied. Nevertheless, it is important to highlight that no molecular studies of the microbiota interaction have been done in this study, which may restrict the interpretation of the results obtained. Thus, further deeply studies of the plasmids, pathogenicity islands or transposons are needed to achieve a better knowledge of *S.* Infantis AMR dynamics at the farm level, in order to establish better control programs and reduce its prevalence throughout the food chain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Data curation: L. Montoro-Dasi, C. Marin and S. Vega. Formal analysis: L. Montoro-Dasi and C. Marin. Funding acquisition: A. Villagra. Investigation: L. Montoro-Dasi and C. Marin. Methodology: A. Villagra and C. Marin. Writing-original draft: L. Montoro-Dasi and C. Marin. Writing-review and editing: A. Villagra, S. Vega and C. Marin. All authors have read and agreed to the published version of the manuscript.

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