Research Note: Persistent *Salmonella* problems in slaughterhouses related to clones linked to poultry companies

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ABSTRACT Salmonellosis remains one of the main foodborne zoonoses in Europe, with poultry products as the main source of human infections. The slaughterhouse has been identified as a potential source for *Salmonella* contamination of poultry meat. Despite the mandatory programme of the EU, there are companies with persistent *Salmonella* that are unable to remove the bacteria from their processing environment, compromising the entire production line. In this context, an intensive sampling study was conducted to investigate a slaughterhouse with persistent *Salmonella* problems, establishing the genetic relationship among *Salmonella* strains isolated during the slaughter process. A total of 36 broiler flocks were sampled during processing at the slaughterhouse. *Salmonella* was identified based on ISO 6579-1:2017 (Annex D), serotyped by Kauffman-White-Le-Minor technique, and the genetic relationship was assessed with ERIC-PCR followed by PFGE. The outcomes showed that 69.4% of the batches sampled carried *Salmonella* upon arrival at the slaughterhouse and that 46.3% of the different samples from carcasses were contaminated with *Salmonella*. The two serovars isolated at the different steps in the slaughterhouse were Enteritidis (98.2%) and Kentucky (1.8%). Pulsed-field gel electrophoresis analysis revealed a low genetic diversity, with all *S.* Enteritidis isolates showing a nearly identical pulsotype (similarity >85%) and *S.* Kentucky strains showed the same XbaI PFGE profile (95.0% genetic similarity). The results of this study showed a high genetic relationship among isolates recovered from carcasses and environmental samples in the slaughterhouse from both *Salmonella*-positive and *Salmonella*-free flocks. *Salmonella* strains re-circulated across to poultry flocks and re-entered the slaughterhouse to survive on the processing line. Thus, it is necessary to implement molecular diagnosis methods in time at the field level to determine the *Salmonella* epidemiology of the flock, to make rapid decisions for the control of *Salmonella* and prevent entry into the slaughterhouse environment.

Key words: *Salmonella*, slaughterhouse, poultry, PFGE, zoonosis

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INTRODUCTION

Salmonellosis remains one of the main foodborne zoonoses in Europe, with 87,923 cases in 2019 confirmed by a laboratory test or based on the clinical symptoms and epidemiological link (EFSA and ECDC, 2021), and poultry products are the main source of human infection. Poultry and poultry meat can become contaminated with *Salmonella* throughout the entire poultry production chain (breeder farms, fattening farms, transportation, slaughterhouse, and retail; Ramirez-Hernandez et al., 2021). Thus, to ensure effective control, insights into the occurrence of *Salmonella* and factors affecting their prevalence are essential.

Previous studies have identified the slaughterhouse as a potential source for *Salmonella* contamination of poultry meat, representing a critical stage for controlling its dissemination in the food chain (Zeng et al., 2021). During slaughter, carcasses may become contaminated by bacteria found in the intestinal content of the animals, either from the same flock or in previously slaughtered

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flocks (Zeng et al., 2021). In recent years, strict biosecurity and management protocols have been established at farm level, which have made it possible to reduce and control the prevalence of the bacteria to a target established by National *Salmonella* Control Programmes (**NSCP**) set up by the European Commission. However, despite the important economic investment made by the governments and the poultry sector, there are companies with persistent *Salmonella* strains that cannot be removed from the processing environment, compromising the entire production line. Therefore, when *Salmonella* enters into the slaughterhouse from the farm, the bacterium is able to persist in the slaughterhouse environment despite cleaning and disinfection procedures available in the agri-food chain, remaining viable for up to 15 mo (Barron and Forsythe, 2007). Due to this situation, the rapid detection of the bacteria, characterization of the strain and identification of the contamination routes at the slaughterhouse are essential for field level control and to safeguard the food supply effectively (Bell et al., 2016). In this perspective, the use of techniques with discriminatory power to explore the possible relationships among the circulating strains is of particular interest for source attribution studies. To this end, an intensive sampling study was conducted to investigate a slaughterhouse with persistent *Salmonella* problems, establishing the genetic relationship among *Salmonella* strains isolated during the slaughter process.

MATERIALS AND METHODS

All the procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments.

Study Design

Over a year (2015), a poultry slaughterhouse with persistent *Salmonella* was intensively sampled. Overall, 36 broiler chicken flocks were sampled in 18 different visits (the first- and last-processed flocks per visit). The processing line constituted part of an integrated broiler chicken company and operated under standard commercial conditions. The flock definition used was a group of chickens reared in a broiler house during the same time period and under the same rearing conditions.

Sample Collection

From each flock, samples were collected at different times: when animals arrived at the slaughterhouse, before slaughtering the flock, and while processing carcasses (Figure 1).

From each flock studied, 2 feces samples (200−300 g each, n = 72) were collected directly from truck crates during unloading at the slaughterhouse (EC, 2005). The flock was declared infected if at least one of the samples was tested as positive (n = 36). Before processing the carcasses, environmental samples were taken. For this purpose, one 10 cm^2 sample was collected by rubbing the entire area with sterile gauze pads wet with a disinfectant neutraliser (AES Laboratories, Bruz Cedex, France) from the steel surface that came into contact with carcasses in 6 selected stages: exsanguination, scalding, defeathering, evisceration, grading, and air-chilling (Figure 1). One sample of the foodstuff contact surfaces of clean finished product transporting crates was also collected. Finally, one carcass was taken from each of the 3 selected processing line stages: after the exsanguination, defeathering, and air-chilling stages. Each carcass was collected under aseptic conditions and placed into an individual sterile bag (Seward, Worthing, UK). All the collected samples were transported in a cool box at ≤4°C to the laboratory for microbial analyses and were analysed within 2 h after sampling.

Laboratory carcass sampling was carried out by collecting neck skin and internal cavity surface samples. The skin samples (25 g) were placed into a sterile bag and diluted at 1:10 vol/vol in Buffered Peptone Water 2.5% (BPW, Scharlau, Barcelona, Spain). The mix was homogenized by stomaching at 230 rpm for 120 s (Stomacher 400 circulator, Seward Ltd., Worthing, UK). Samples from the internal cavity surface of each carcass were also collected with sterile wet gauze pads (AES laboratories, Bruz Cedex, France). To this end, each carcass was

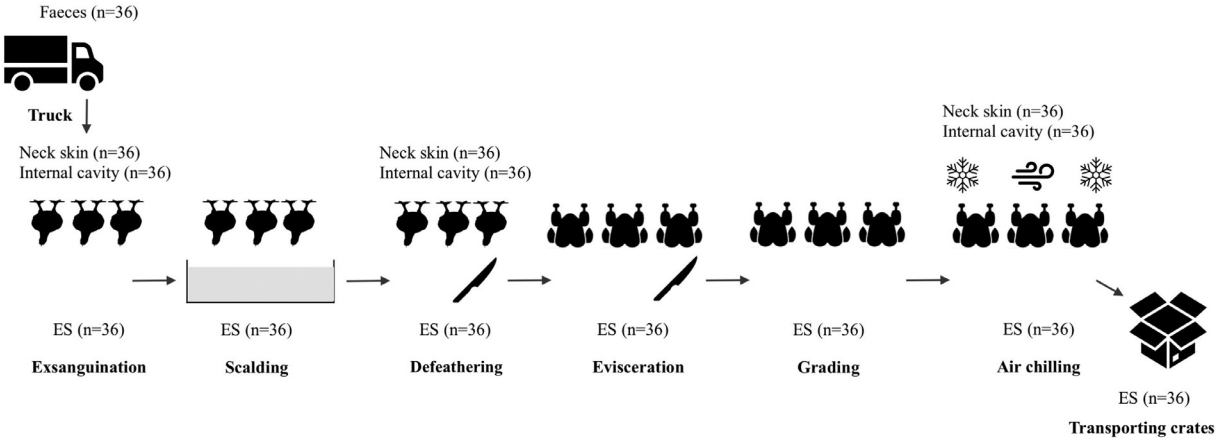

Figure 1. Samples collected at different times of the working day at the slaughterhouse. Abbreviation: ES, environmental samples.

aseptically opened and internally rubbed with sterile wet gauze.

Salmonella Isolation and Serotyping

For *Salmonella* detection, faeces (25g) and environmental wet gauze pads, neck skin homogenates, and internal surface cavity were analyzed (ISO 6579:2017). According to ISO, samples were first pre-enriched in 1:10 vol/vol BPW 2.5% and incubated at 37 ± 1°C for 18 ± 2 h. Pre-enriched samples were transferred to Semi--Solid Rappaport Vassiliadis agar plates (MSRV, Difco, Valencia, Spain) and incubated at 41.5 ± 1°C for 24 to 48 h. Suspicious plates were transferred to 2 different agar plates, ASAP (AES Laboratories, Bruz Cedex, France) and Xylose Lysine Deoxycholate agar (XLD, Liofilchem, Valencia, Spain), and were incubated at 37 ± 1°C for 24 ± 3 h. After the incubation period, 5 *Salmonella* suspect colonies were selected and transferred to a nutrient agar plate (Scharlab, Barcelona, Spain) incubated at 37 ± 1°C for 24 ± 3 h. Finally, an API-20E biochemical test (API-20, bioMerieux, Madrid, Spain) was performed to confirm *Salmonella* spp.

Salmonella isolates were serotyped by Kauffman-White-Le-Minor technique at the *Centro de Calidad Avícola y Alimentación Animal* of the Valencian Community (Castellón, Spain).

Molecular Typing of Salmonella Isolates

Two different subtyping methods were used for genotyping *Salmonella* isolates. Clonality among the *Salmonella* isolates was initially assessed by enterobacterial repetitive intergenic consensus (**ERIC**)-PCR, as previously described by Moré et al. (2017). Representative isolates from the different *Salmonella* ERIC-PCR patterns identified per sample were further analyzed by pulsed-field gel electrophoresis (**PFGE**).

PFGE was performed according to the PulseNet standardized protocol ''Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*" (www. pulsenetinternational.org). Isolates were analyzed using the Xbal restriction enzyme (Roche Applied Science, Indianapolis, IN).

ERIC-PCR and PFGE band patterns were analysed using Fingerprinting II software, v3.0 (Bio-Rad, Hercules, CA). Similarity matrices were calculated with the Dice coefficient and a cluster analysis was performed by the unweighted-pair group method with arithmetic mean (**UPGMA**). The isolates with a minimum level of similarity of 90% were considered genetically similar or identical and were assigned the same pulsotype.

Statistical Analysis

A generalized linear model, which assumed a binomial distribution for the colonising of *Salmonella,* was fitted to the data to determine the presence and diversity of the bacteria in live chickens upon arrival at the slaughterhouse, in carcasses in the different poultry slaughter process stages, and the impact of each processing stage on the microbiological contamination of chicken broiler carcasses. A flock was declared infected if at least one of the samples collected was positive. The association with the status of live broiler chicken flocks and *Salmonella*-contaminated carcass samples (neck skin and internal cavity surface) from the exsanguination, defeathering and air-chilling stages was also studied. A carcass sample was stated as being contaminated if at least one of the samples (neck skin and/or internal cavity surface) from each stage tested positive. In all the tests, an error was designated as having a binomial distribution and the probit link function was used. The binomial data for each sample were assigned 1 if they presented prevalence of *Salmonella,* or 0 otherwise. A P value of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard deviation. The analyses of *Salmonella* results were carried out by a commercially available software program (SPSS 16.0 software package; SPSS Inc., Chicago, IL, 2002).

RESULTS AND DISCUSSION

Since the National *Salmonella* Control Plans (NSCP) were set out in poultry production, a significant reduction of human cases of infection has been achieved. However, we have reached a prevalence of *Salmonella* that is difficult to eradicate from poultry production systems, with the presence of "persistent cases" in poultry facilities that allow *Salmonella* to remain the leading cause of food outbreaks in Europe (EFSA and ECDC, 2021).

During this study, a total of 504 samples were collected from eight points of the slaughterhouse. Samples were collected from feces (n = 36), environmental surfaces (n = 252), neck skin (n = 108), and internal cavity surfaces (n = 108).

From all samples collected at the slaughterhouse, 45.0% (227/504) were positive for *Salmonella*. From environmental surfaces, 40.4% (102/252) of the samples were positive for *Salmonella*, with statistically significant differences between the different stages, with defeathering and grading (69.0 ± 7.7 and 56.0 ± 8.6%, respectively) being the most contaminated stages in the slaughterhouse (P = 0.00). These stages were followed by evisceration, crate-transporting (47.0 ± 8.3%, both) and exsanguination (44.0 ± 8.3%), and finally air-chilling and scalding (14.0 ± 5.8 and 6.0 ± 3.8%, respectively; $P < 0.05$).

According to the different batches sampled, 69.4% (25/36) arrived at the slaughterhouse shedding *Salmonella* in faeces, and 46.3% (100/216) of the different samples from carcasses (neck skin and internal cavity surfaces) were also contaminated with *Salmonella*. The frequency of *Salmonella*-positive samples collected from the chicken neck skin in the exsanguination,

defeathering and air-chilling stages was 67.0 ± 7.9, 81.0 ± 6.6, and 31.0 ± 7.7%, respectively, being the percentage of the *Salmonella*-positive samples statistically significantly lower at the air-chilling stage ($P < 0.05$). For the internal cavity surface, the frequency of *Salmonella*-positive samples in the exsanguination, defeathering, and air-chilling stages was 3.0 ± 2.7, 44.0 ± 8.3, and 53.0 ± 8.3%, respectively, being the percentage of the *Salmonella*-positive samples statistically significantly lower at the exsanguination stage ($P < 0.05$). For each stages, there were statistically significant differences in both types of chicken carcass samples (neck skin and internal cavity surfaces; $P < 0.05$).

According to the status of the broiler flocks upon their arrival at the slaughterhouse: when the *Salmonella* status of live flocks were positive (n = 25), 48 ± 7.1% (12/25), 56 ± 7.0% (14/25), and 55 ± 7.3% (13/25) of the chicken carcass samples (neck skin and internal cavity surfaces) were *Salmonella*-positive in the exsanguination, defeathering and air-chilling stages, respectively. However, when the *Salmonella* status of live flocks was negative (n = 11), 5 ± 4.4% (1/11), 77 ± 8.9% (8/11), and 80 ± 6.8% (9/11) of the collected chicken carcass samples were *Salmonella*-positive in the exsanguination, defeathering and air-chilling stages, respectively. The chicken carcasses sampled from the exsanguination stage showed significant differences according to live flock status (positive or negative) and when compared to the different stages studied ($P = 0.00$). Nevertheless, no significant differences were found between the *Salmonella*-contaminated chicken carcass samples from either the defeathering or the air-chilling stage, nor among stages when considering life flock status before slaughter ($P \geq 0.05$).

During the study, 227 *Salmonella* strains from 2 different serovars were isolated: 98.2% belonged to serovar Enteritidis (223/227) and 1.8% to serovar Kentucky (4/227). *Salmonella enterica* subsp. *enterica* serovar Enteritidis was found in all different sample types collected at all stages. Meanwhile, *Salmonella enterica* subsp. *enterica* serovar Kentucky was found in faeces and neck skin samples (exsanguination and defeathering) from one flock. Moreover, *S.* Kentucky was recovered from the environment in the exsanguination stage, after processing the *S.* Kentucky-contaminated flock.

The status of the flock when it arrives at the slaughterhouse is a challenge to avoid cross-contamination during processing, and an important factor for safe finished product quality. Notably, *Salmonella* Enteritidis is the most frequent serovar isolated in this study. Since the 1980s, this serovar has been the most frequently involved in human salmonellosis in Europe, representing 72.4% of confirmed foodborne outbreaks in 2019 (EFSA and ECDC, 2021).

The persistent cases of *Salmonella* can be explained by reasons such as inaccurate cleaning and disinfection procedures (Zeng et al., 2021), the development of biofilms (Marin and Lainez, 2009), or the high resistance of the circulating strains (Bridier et al., 2019). Indeed, in this study, the same strain of *S.* Enteritidis was able to survive the cleaning and disinfection protocols, leading to cross-contamination among different slaughter stages. In this study, exsanguination, evisceration, and crate-transporting were particularly relevant stages in the bacteria epidemiology. Exsanguination is one of the first steps in the slaughter process, where birds can carry significant loads of microorganisms from the feathers, skin, and feces from the farm environment and from their transport from the farm to the slaughterhouse. Moreover, the evisceration step is particularly relevant, where a rupture of the intestinal tract could contaminate the equipment. Finally, similarly to other studies, the crate-transporting stage was considered a critical stage due to the possibility of *Salmonella* adhesion to wet surfaces and formation of biofilms. Bacteria within biofilms are up to 1,000 times more tolerant to disinfectants, mechanical removal, and other stresses (Dieltjens et al., 2020), facilitating bacterial survival.

To assess the genetic relationship among isolates recovered from the different steps at the slaughterhouse, the isolates were initially typed by ERIC-PCR. The result of this screening technique showed 89 different patterns. One strain from each of the 89 different ERIC-PCR patterns was selected for further analysis by PGFE. Strains of serovar Enteritidis were grouped in 2 PFGE clusters (≥90% similarity each) which were closely related (≥ 85.0% genetic similarity). Moreover, *S.* Kentucky strains showed two different pulsotypes among the 4 *S.* Kentucky isolates, showing the *S.* Kentucky strains from the environmental samples the same XbaI PFGE profile (95.0% genetic similarity; Figure 2).

Salmonella infection of chickens during rearing has been studied by several authors in different countries, including under Spanish conditions (Marin and Lainez, 2009). Nevertheless, in many cases, the results obtained were difficult to interpret, so it was not always possible to find a solution to the problem. Currently, the genotypic characterisation of isolates makes it possible to identify the different genotypes found within a particular serovar present in the slaughterhouse. In this study, the same *Salmonella* serovar were isolated in 98% of the samples. Considering that the same genotypes could be isolated from different farms, owned by the same integrated company, it strongly suggests a cross-contamination throughout the broiler supply chain (Ha et al., 2018).

This study demonstrated that in an integrated company with a high presence of persistent cases of *Salmonella*, there was a high genetic relationship among the strains isolated from the incoming flocks, the slaughter environment and their carcasses (Zeng et al., 2021). This fact could be explained by the survival of certain clones linked to the companies, with a high resistance to adverse conditions that allows them to recirculate continuously throughout the production chain. In addition, it has been observed that regardless of whether the flocks arrive at the slaughterhouse excreting the bacteria or not, the slaughterhouse surfaces continued to be highly contaminated (Zeng et al., 2021).

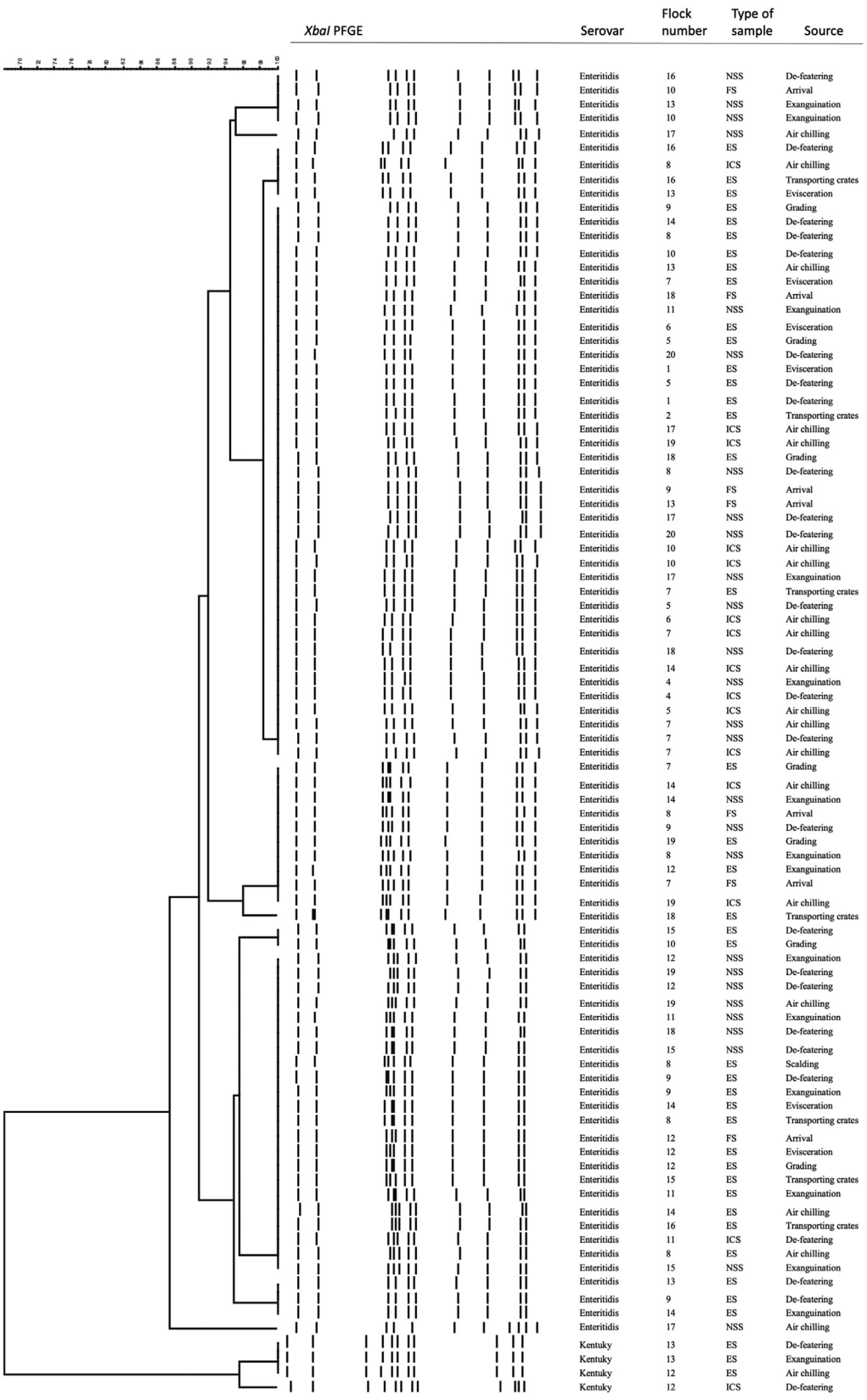

Figure 2. Dendrogram of the XbaI PGFE profiles from a subset of *Salmonella* strains isolated from environmental and chicken samples (n = 89). Abbreviations: ES, environmental sample; FS, feces sample; ICS, Internal surface cavity; NSS, neck skin sample. Arrival: Truck crates.

In Spain, 80% of the broiler production chain is controlled by integrated companies which have not only broiler farms but also feed plants, breeders, and slaughterhouses. This has been considered a risk for the transmission and maintenance of other zoonotic bacteria such as *Salmonella, Listeria* or *Campylobacter*. This highlights the need for improvement of the biosecurity measures and vaccination programmes to control

persistent *Salmonella* strains from breeder farms to broiler farms, to reduce infection pressure in the slaughterhouse. In addition, it is necessary to implement molecular diagnosis methods in time at the field level, enabling us to track the potential source of strains, to make rapid decisions to control the pathogen, limiting the entry of the bacterium into the slaughterhouse.

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DISCLOSURES

The authors declare no conflict of interest.

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