Investigating the dynamics of *Salmonella* contamination in integrated poultry companies using a whole genome sequencing approach

José L. Medina-Santana ,* David Ortega-Paredes,* Sofia de Janon ,* Elton Burnett,† Maria Ishida,‡ Brian Sauders,‡ Mieke Stevens,§ and Christian Vinueza-Burgos *,1

*Unidad de investigación de Enfermedades Transmitidas por Alimentos y Resistencia a los Antimicrobianos (UNIETAR), Facultad de Medicina Veterinaria y Zootecnia, Universidad Central del Ecuador, Quito 170103, Ecuador; †Institute of Parasitology, McGill University, Montreal, QC H9X3V9, Canada; ‡Division of Food Laboratory, New York State Department of Agriculture and Markets, Albany, NY 12206, USA; and §Institute of Tropical Medicine Antwerp, Belgium

ABSTRACT The study of non-typhoid *Salmonella* in broiler integrations has been limited by the resolution of typing techniques. Although serotyping of *Salmonella* isolates is used as a traditional approach, it is not of enough resolution to clearly understand the dynamics of this pathogen within poultry companies. The aim of this research was to investigate the epidemiology and population dynamics of *Salmonella* serotypes in 2 poultry integrations using a whole genome sequencing approach. Two hundred and forty-three *Salmonella* isolates recovered from the broiler production chain of 2 integrated poultry companies were whole genome sequenced and analyzed with dedicated databases and bioinformatic software. The analyses of sequences revealed that *S.* Infantis was the most frequent serotype (82.3%). Most isolates showed a potential for resistance against medically important antibiotics and disinfectants. Furthermore, 97.5% of isolates harbored the pESI-like mega plasmid, that plays an important role in the global dissemination of AMR. SNP tree analysis showed that there were clones that are niche-specific while other ones were distributed throughout the broiler production chains. In this study, we demonstrated the potential of whole genome sequencing analysis for a comprehensive understanding of *Salmonella* distribution in integrated poultry companies. Data obtained with these techniques allow determination of the presence of genetic factors that play an important role in the environmental fitness and pathogenicity of *Salmonella*.

Key words: *Salmonella* Infantis, poultry production chain, WGS, antimicrobial resistance, pESI-like

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INTRODUCTION

Non-typhoidal *Salmonella* (**NTS**) *enterica* subsp. *enterica* is a foodborne pathogen that causes ~1.2 million infections in the United States of America (**USA**) annually (Scallan et al., 2011), and was responsible for more than 95 million cases of diarrheal disease and 50,771 deaths worldwide in 2017 (Stanaway et al., 2019). Human infections are usually mild and manifest as a self-limiting gastroenteritis. However, high-risk populations such as the infants, the elderly, and immunocompromised individuals can develop systemic disease that requires antibiotic intervention (Crump et al., 2015). Moreover, the rise of multidrug resistant (**MDR**) NTS strains have resulted in infections that are unresponsive to first-line treatment, thus requiring more complicated options (Parry, 2003; Mølbak, 2005). Studies have indicated that the increased prevalence of MDR NTS results from the overuse of antibiotics in agriculture, veterinary, and human medicine (Llor and Bjerrum, 2014; Ventola, 2015). Presumably, these practices have placed selection pressure on MDR NTS strains, leading to its clonal expansion, global dissemination, and persistence. NTS have a broad host range and occupy the gastrointestinal track of multiple species including mammals, reptiles, and birds (Evangelopoulou et al., 2013; Simpson et al., 2018; Bjelland et al., 2020; Cohen et al., 2021). Consequently, carrier species present a risk of NTS-mediated zoonoses and NTS-foodborne infections (Braden, 2006). Several serotypes that are commonly isolated from human infections, are also prevalent in poultry production systems. Despite being well-linked to NTS infections in humans (Antunes et al.,

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[1]Corresponding author: cvinueza@uce.edu.ec

2016), poultry is one of the most widely consumed and affordable protein source in the world (Magdelaine et al., 2008; Windhorst, 2017). A rising demand for these poultry products has led to more intensive farming practices in order to meet consumer demands.

Intensive poultry farming involves increased stocking densities, and faster growing breeds. Good sanitation practices are difficult to maintain under these conditions, thus enabling easier *Salmonella* transmission in the environments. Poultry meat production occurs in vertically integrated operations that consist of breeder farms, hatchery operation, broiler farms, slaughter and carcass processing (Glatz and Pym, 2015). NTS contamination can enter into any of these production steps. Moreover, NTS can contaminate the broilers' production chain via contaminated feed, rodents, wild birds, or by other breaches in biosecurity (Cox et al., 1983; Davies and Wales, 2010; Totton et al., 2012). Multiple genetic factors that confer phenotypic traits such as adhesion capacity, resistance to disinfectants and heavy metals, as well as immune-evasion mechanisms allow for various serotypes of NTS to persist in the production environment and establish successful infections in birds. Effective implementation of strategies to control NTS in integrated poultry companies is therefore difficult. Serotypes but also genotypes must be considered to understand the dynamics of this pathogen in such production systems.

Salmonella enterica serovar Infantis (***S.* Infantis**) is an emergent serotype worldwide. This serotype, has been reported as one of the most prevalent NTS in humans in Europe and USA (CDCCenters for Disease Control and, 2018; EFSA, European Food Safety Authority; European Centre for Disease Prevention and Control ECDC, 2019). Besides, several studies describe *S.* Infantis as the most prevalent serotype in poultry (Valderrama et al., 2014; Vinueza-Burgos et al., 2016; EFSA, European Food Safety Authority; European Centre for Disease Prevention and Control ECDC, 2019). Moreover, multidrug resistant phenotypes of this serotype are of public health concern (Shah et al., 2017; Mejía et al., 2020; EFSA, European Food Safety Authority; European Centre for Disease Prevention and Control ECDC, 2021).

Plasmids are the main mobile elements involved in *Salmonella* adaptation. Particularly, a rise in the incidence of *S.* Infantis was registered since 2003 worldwide. Interestingly, a large conjugative plasmid named plasmid of Emerging *Salmonella* Infantis or pESI whose structure contains determinants for resistance to various antibiotics was identified (Aviv et al., 2014). Posteriorly, pESI-like plasmids have been described in *S.* Infantis worldwide, and their implication in the rise of *S.* Infantis has been suggested (Franco et al., 2015; Aviv et al., 2016; Tate et al., 2017; Gymoese et al., 2019; Alba et al., 2020; Bogomazova et al., 2020; Cohen et al., 2020; García-Soto et al., 2020; McMillan et al., 2020b; Mejía et al., 2020; Tyson et al., 2020; Kürekci et al., 2021). Despite the relevance of these genetic elements, there is scarce information about pESI-like plasmids in the Andean region (Vallejos-Sánchez et al., 2019; Burnett et al., 2021).

The aim of this study was to investigate the epidemiology and population dynamics of *Salmonella* serotypes present within 2 integrated poultry production facilities (from hereafter called integrations), and to determine the presence of resistance and virulence genetic factors that may contribute to the environmental fitness and pathogenicity of NTS, using a whole genome sequencing approach.

MATERIALS AND METHODS

Study Design

For this study, *Salmonella* isolates originating from 2 integrated poultry companies (integrations A and B) were characterized and compared using whole genome sequencing (**WGS**). *Salmonella* was isolated from the feed mills, broiler farms, and broiler processing plants comprising both integrations. Specific details on sampling design and *Salmonella* isolation have been published in previous papers (Villagómez et al., 2017; Vinueza-Burgos et al., 2019). The distribution of *Salmonella* isolates within each integrated poultry company is presented in Table 1.

Table 1. Distribution of *Salmonella* isolates used in this study within each integrated poultry company.

Sample origin	Sampling location	Integration A	Integration B	Total
Raw feed materials	Feed mill plant	1	30	31
Compound feed	Feed mill plant	NA	2	2
Transport paper*	Broiler farms	1	1	2
Overshoes**	Broiler farms	28	44	72
Caeca content***	Slaughterhouse	2	3	5
Skin after final washing	Slaughterhouse	22	50	72
Carcasses (Skin after chilling)	Slaughterhouse	14	44	58
Turkey house	Turkey farm	NA	1	1
Total		68	175	243

*One day old chicks.
**Broiler-litter sweep swab of 30-d-old chicken.
***Samples were taken on the day of slaughter.
NA, no isolate was recovered from these locations for WGS.

DNA Extraction and Whole-Genome Sequencing

All *Salmonella* isolates were confirmed by PCR as previously described (Vinueza-Burgos et al., 2019). Genomic DNA was then extracted and purified using Wizard Genomic DNA Purification Kit (Promega, MD), and the concentrations measured using a Qubit fluorometer using the Qubit dsDNA High Sensitivity (**HS**) Assay kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA). Additionally, quality assessments were made using the NanoDrop 2000 UV-Vis (Thermo Fisher Scientific) for determination of A260/280 values. DNA extracts containing a minimum concentration of 10 ng/μL and an A260/280 ratio of 1.75 to 2.05 were used for posterior analysis. DNA extracts outside this range that were sequenced and found to provide acceptable data quality based on coverage (>40X) and other quality assessments inherent in the analysis pipelines below were included in the analysis.

WGS was performed using the MiSeq platform (Illumina, San Diego, CA) according to the harmonized FDA GenomeTrakr/CDC PulseNet protocol (CDC PulseNet, 2018). Trimming, assembly and quality control of raw reads was performed using the EnteroBase pipeline (Zhou et al., 2020). Sequences read data were uploaded to NCBI Sequence Read Archive (**SRA**). Accession numbers of all genomes, studied genes, and their metadata are listed in the Supplementary File 2.

Bioinformatics Analyses

Raw reads were submitted to EnteroBase (Zhou et al., 2020) in order to perform the primary analysis and confirm serotype designation using the SISTR algorithm. EnteroBase tools were also used to determine the Multilocus sequence typing (**MLST**) Sequence Type (**ST**) profile of each isolate (Acthman scheme) and perform Single Nucleotide Polymorphism (**SNP**) tree analysis of the isolates in each collection. Visualization of the SNP tree analysis and related metadata was performed using IToll (Letunic and Bork, 2019).

The identification of genes that determine antimicrobial resistance was performed using the ResFinder database ((Zankari et al., 2012; Bortolaia et al., 2020). Virulence genes were identified using the Virulence Factor database (Liu et al., 2019) and genes that confer resistance to disinfectants and heavy metals were accessed using the MEGARes database (Doster et al., 2019). All databases used were updated to September 12, 2020, and all analyses were performed using a mass screening of contigs in ABRicate software (Seemann, 2020).

To identify functional nitrofurantoin resistance-associated mutations, the wild type sequences of *nfs*A and *nfs*B genes (oxygen insensitive nitro reductase enzymes) were copied from the chromosome of *S. enterica* accession number NC_003197. All genomes were then analyzed using the map to reference tool in the Geneious prime (V. 2021.0.3) software. Sequences that possessed mutations were in silico translated and aligned with the wild type proteins sequences to visualize amino acid residue mutations.

Plasmid finder database (Camacho et al., 2009; Carattoli et al., 2014) with ABRicate software were used to identify the plasmid incompatibility groups. In order to characterize the pESI-like megaplasmids, sequences were mapped to the p-F219 plasmid (GeneBank accession number CP038508) described in *S.* Infantis that were isolated from broiler production systems in Peru (Vallejos-Sánchez et al., 2019) using the map to reference tool in the Geneious Prime software. Consensus sequences identified in each genome were depicted using Blast Atlas (Buckingham and Hogan, 2010).

RESULTS

Epidemiology and Population Dynamics of Salmonella Serotypes

Twelve different serotypes were identified among the studied isolates, 3 serotypes from integration A, and 11 from integration B. *Salmonella* Infantis was the most

Table 2. Frequency and origin of *Salmonella* serotypes in each poultry integration.

	Integration A		Integration B	
Serovar	%	Sample origin (n)	%	Sample origin (n)
Infantis	95.6	CC (2), O (26), SC (14), SW (22), TP (1)	77.1	CF (1), RM (4), O (37), CC (3), SC (40), SW (50)
Amsterdam	2.9	O (2)	10.3	RM (18)
Liverpool	-	-	5.1	RM (8) O (1),
Havana	-	-	2.3	O (1), SC (3)
Javiana	-	-	1.1	O (1), TP (1)
Saintpaul	-	-	1.1	O (1), SC (1)
Mbandaka	1.5	RM (1)	-	-
Soerenga	-	-	0.6	CF (1)
Albany	-	-	0.6	O (1)
Muenchen	-	-	0.6	O (1)
Uganda	-	-	0.6	O (1)
I 4,[5],12:i:-	-	-	0.6	T (1)
Total of isolates		68		175

Number of isolates (n), Raw feed materials (RM), Compound feed (CF), Transport paper (TP), Overshoes (O), Ceca content (CC), Skin before chilling (SW), Skin after chilling (SC); Turkey house (T).

Table 3. Genome-derived antimicrobial resistant patterns in *S.* Infantis isolates.

N°	AMR Pattern	No. of antimicrobial classes	No. of isolates (%)		
			Integration A	Integration B	Total
1	ABPRFQSTN	9	46 (70.8)	51 (37.8)	97 (48.5)
2	ABPRQSTN	8	4 (6.2)	33 (24.4)	37 (18.5)
3	ABQSTN	6	-	31 (23)	31 (15.5)
4	ABRFQSTN	8	6 (9.2)	2 (1.5)	8 (4)
5	ABPQSTN	7	-	7 (5.2)	7 (3.5)
6	APRQSTN	7	-	4 (3)	4 (2)
7	AQ	2	-	4 (3)	4 (2)
8	ABPFQSTN	8	2 (3.1)	1 (0.7)	3 (1.5)
9	ABPRFQN	7	3 (4.6)	-	3 (1.5)
10	APRFQSTN	8	1 (1.5)	1 (0.7)	2 (1)
11	ABFQSTN	7	1 (1.5)	-	1 (0.5)
12	ABPRQN	6	1 (1.5)	-	1 (0.5)
13	ABRQSTN	7	1 (1.5)	-	1 (0.5)
14	AQN	3	-	1 (0.7)	1 (0.5)
Total of isolates			(65)	(135)	(200)

Aminoglycoside (A), Beta-lactam (B), Phenicol (P), Trimethoprim (R), Fosfomycin (F), Quinolone (Q), Sulfonamide (S), Tetracycline (T), Nitrofurans (N).

Table 4. Genome-derived antimicrobial resistant patterns and sequence types of non *S.* Infantis serovars isolated at Integrated poultry companies.

Serovar	ST	AMR pattern	No. of antimicrobial classes	No. of isolates (%)	
				Integration A	Integration B
Albany	292	AQ	2	-	1 (2.5)
Amsterdam	2,090	AQ	2	2 (66.7)	18 (45)
Havana	588	ARQS	4	-	2 (5)
		ABPRMQST	8	-	1 (2.5)
		ARQST	5	-	1 (2.5)
I 4,[5],12:i:-	19	ABS	3	-	1 (2.5)
Javiana	1,674	AQ	2	-	1 (2.5)
		ABQ	3	-	1 (2.5)
Liverpool	1,959	AQ	2	-	9 (22.5)
Mbandaka	413	AQ	2	1 (33.3)	-
Muenchen	83	AQT	3	-	1 (2.5)
Saintpaul	50	A	1	-	1 (2.5)
		ABPRMST	7	-	1 (2.5)
Soerenga	1,659	AQ	2	-	1 (2.5)
Uganda	684	AQ	2	-	1 (2.5)
Total of isolates				(3)	(40)

Sequence type (ST), Aminoglycoside (A), Beta-lactam (B), Phenicol (P), Trimethoprim (R), Macrolide (M), Quinolone (Q), Sulfonamide (S), Tetracycline (T).

prevalent serotype in both integrations, accounting for 82.3% (n = 200) of all isolates. Interestingly, *S.* Infantis and *S.* Amsterdam were present in both integrations while the remaining serotypes were isolated in only one of the two companies. Furthermore, *S.* Infantis was observed throughout the production chain (Table 2).

Antimicrobial Resistance

a) *Salmonella* Infantis

Most *S.* Infantis isolates possessed genetic determinants of antimicrobial resistance (**GDAMR**) against 2 or more antimicrobial classes (Tables 3 and 5). In fact, 82.5% of these isolates were grouped into patterns 1, 2, and 3, which possessed genetic determinants of resistance against 9, 8, and 6 antimicrobial classes, respectively. On the other hand, patterns 7 and 14 (integration B-specific) contained genetic determinants for 2 and 3 antimicrobial classes, respectively. Patterns 1, 2, 4, 8, and 10 were common to isolates originating from both integrations.

a) Other *Salmonella* serotypes

Isolates belonging to serotypes Havana, I 4,[5],12:i:-, Javiana, Muenchen, and Saintpaul also possessed GDAMR against >2 antimicrobials classes (Table 1 in Supplementary File 1). The most common genomic resistance pattern observed among these isolates included both aminoglycosides and quinolones (Table 4).

All isolates contained the aminoglycoside resistance gene *aac(6′)-Iaa*. No genetic determinant was observed among the *S.* Infantis isolates that conferred macrolide resistance. It was also noteworthy that all isolates possessed genetic determinants for resistance against quinolones, except for those belonging to *S.* Saintpul and *S.* I 4,[5],12:i:- serotypes. Furthermore, the *qnrB19* quinolone resistance gene (Cloeckaert and Chaslus-

Table 5. Genetic determinants of antimicrobial resistance (GDAMR) of *S.* Infantis for each antimicrobial class at different locations of integrated poultry companies.

	GDAMR	Ceca content	Compound feed	Overshoes	Raw feed materials	Skin after chilling	Skin after final washing	Transport paper	Total
Antibiotic Group		No. of isolates (%).							
Aminoglycoside	*aac*(6′)-Iaa	5 (100)	1 (100)	63 (100)	4 (100)	54 (100)	72 (100)	1 (100)	200 (100)
	ant(3″)-Ia	5 (100)	-	63 (100)	-	53 (98.1)	69 (95.8)	1 (100)	191 (95.5)
	aph(3′)-Ia	3 (60)	-	32 (50.8)	-	34 (63)	37 (51.4)	-	106 (53)
	aac(3)-Iva	5 (100)	-	51 (81)	-	49 (90.7)	58 (80.6)	1 (100)	164 (82)
	aph(4)-Ia	5 (100)	-	51 (81)	-	49 (90.7)	58 (80.6)	1 (100)	164 (82)
Quinolone	*par*C (P.T57S)	5 (100)	1 (100)	63 (100)	4 (100)	54 (100)	72 (100)	1 (100)	200 (100)
	*gyr*A (p.D87Y)	5 (100)	-	63 (100)	-	54 (100)	72 (100)	1 (100)	195 (97.5)
	*qnr*B19	-	-	1 (1.6)	-	-	-	-	1 (0.5)
Nitrofurans	*nfs*A (W159*)	5 (100)	-	63 (100)	1 (25)	54 (100)	72 (100)	1 (100)	196 (98)
	*nfs*B (Q137*)	5 (100)	-	63 (100)	1 (25)	54 (100)	72 (100)	1 (100)	196 (98)
Tetracycline	*tet*(A)	5 (100)	-	63 (100)	-	53 (98.1)	69 (95.8)	1 (100)	191 (95.5)
Sulfonamide	*sul*1	5 (100)	-	63 (100)	-	53 (98.1)	69 (95.8)	1 (100)	191 (95.5)
Beta-lactamase	$bla_{\text{CTX-M-65}}$	5 (100)	-	62 (98.4)	-	51 (94.4)	70 (97.2)	1 (100)	189 (94.5)
Phenicol	*flo*R	3 (60)	-	48 (76.2)	-	49 (90.7)	54 (75)	-	154 (77)
Trimethoprim	*dfr*A14	5 (100)	-	46 (73)	-	47 (87)	54 (75)	1 (100)	153 (76.5)
Fosfomycin	*fos*A3	4 (80)	-	35 (55.6)	-	32 (59.3)	42 (58.3)	1 (100)	114 (57)
	*fos*A6	-	-	1 (1.6)	-	2 (3.7)	-	-	3 (1.5)

Dancla, 2001) was detected in only 6 isolates, while mutations were observed in either the *parC* (P.T57S) and/or *gyrA* (p.D87Y) genes of the remaining isolates. The extended-spectrum beta-lactamase (ESBL) gene $bla_{\text{CTX-M-65}}$ was present in all *S.* Infantis isolates. Overall, only 2 mutations were observed in the *nfs*A (W159*) and *nsf*B (Q137*) genes, potentially conferring resistance against nitrofurans (Table 5). All isolates possessed the genes *mdsA, mdsB, mdsC, mdtA, mdtB*, and m*dtC* that confer resistance to biocides, heavy metals and some β-lactams through efflux pups (Nagakubo et al., 2002; Blair et al., 2015). The *mdtK* and *AcrD* genes related with multidrug efflux pumps for norfloxacin, doxorubicin, acriflavine and aminoglycosides were also found in all isolates (Rosenberg et al., 2000; Nishino et al., 2006). Besides, the *pmrG* that confers AMR and host immune evasion capabilities (Negi et al., 2007) was observed in all but one *S.* Amsterdam isolate. On the other hand, the *qacl* gene that encodes for a quaternary ammonium compound efflux pump (Slipski et al., 2019) was detected in a single *S.* Saintpaul and one *S.* Havana isolate (Table 1 in Supplementary File 1).

Virulence Genes

A total of 116 virulence genes were detected among the *Salmonella* collection (Supplementary File 2). Integration B presented a greater diversity of serotypes and virulence genes between both integrations, but the number of isolates sequenced was also greater than integration A (Table 2 and Graphic 2 in Supplementary File 1). Interestingly, in both integrations the most frequent virulence groups were group 37 (73.5%; 103 virulence genes) and group 32 (14%; 102 virulence genes) (Supplementary File 2).

All serotypes possessed virulence genes encoding for fimbrial and nonfimbrial adherence, magnesium uptake, and secretion systems (Table 2 in Supplementary File 1). Serotype *S.* I4,[5],12:i:- possessed genes encoding for serum resistance and the virulence-associated spv locus. These genes have been related with the bacteremia and evasion of the complement system in invasive nontyphoid *Salmonella* (Guiney and Fierer, 2011; Mambu et al., 2017). Most *S.* Infantis isolates also contained genes encoding for iron uptake, while *S.* Javiana isolates possessed the *cdtB* typhoid toxin coding gene. Genetic determinants encoding for stress adaptation and virulence were also observed in *S.* I4,[5],12:i:- and *S.* Saintpaul isolates (Table 2 in Supplementary File 1).

Genotypic Profiles of S. Infantis Isolates

All *S.* Infantis isolates from this study belonged to the MLST sequence type (ST) 32. Sequence type designation for the remaining serotypes is presented in Table 4. The SNP tree analysis showed that isolates that clustered together generally originated from the same farm, and/or the same site within a specific farm. However, one genotype was isolated from different farms and production stages within integration B, suggesting that this clone can occupy multiple niches within the operation (orange cluster in Figure 2). Additionally, some clones (yellow clusters) were observed on the carcass surfaces post-final rinsing, demonstrating a trend to survive the sanitation process in the slaughter facilities of both integrations (Figures 1 and 2). Interestingly, clones belonging to serovar Infantis were observed persisting throughout all levels of the production chain of integration A (blue cluster, Figure 1). The SNP tree analysis also revealed that some clones were distributed across multiple farms and were able to persist, ultimately contaminating carcasses in the slaughter facilities of both integrations (yellow cluster in Figures 1 and 2). Some farms produced broilers whose carcasses were observed

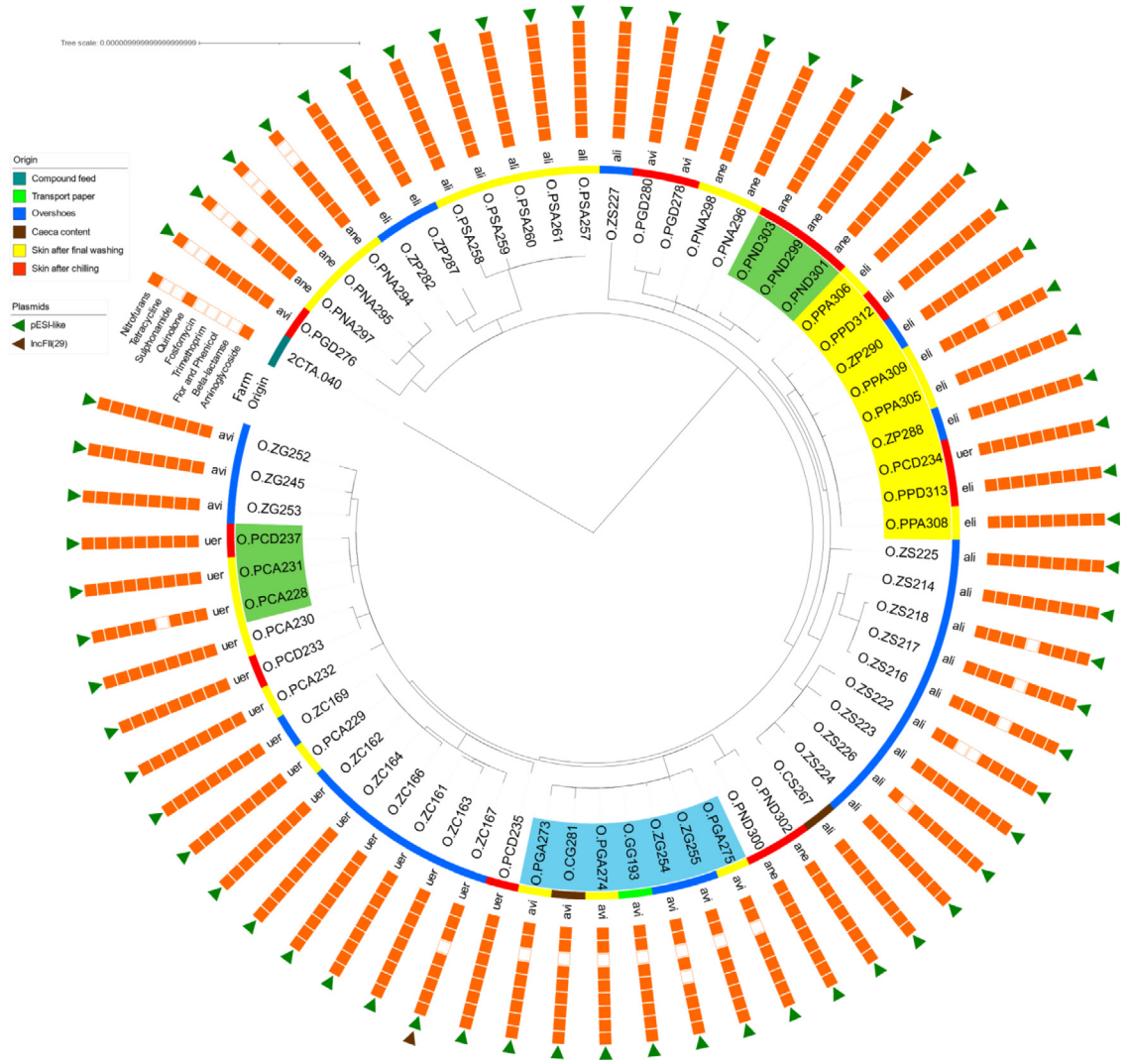


Figure 1. SNP tree analysis of *S.* Infantis isolates of integration A.

to be contaminated with clones only recovered from slaughter facilities within both integrations (green clusters in Figures 1 and 2). Remarkably, one *S.* Infantis genotype only isolated at slaughterhouse level, was present in carcasses originated from different farms (red cluster in Figure 2). Finally, isolates of *S.* Infantis that were detected in feed and its raw material formed a unique cluster that possessed minimal GDAMR and the lack of pESI-like plasmids (Figure 2).

Plasmid Identification

Four incompatibility groups were detected among the isolates in this study using Plasmid finder, including Col, IncFIB, IncFII, and IncI1 (Table 3 in Supplementary File 1). Furthermore, the map to reference analysis identified pESI-like plasmids in most (195/200) of the *S.* Infantis isolates in this study, showing homology to the reference p-F219 plasmid. In the isolates that originated in the feed mill, plasmids were not identified. All pESI-like plasmids presented genes associated to the success of *S.* Infantis in the poultry production environment and related to human infections. These genetic elements include: antimicrobial resistance genes $bla_{\text{CTX-M-65}}$, *fos*A3, *aph*(4)-Ia, *aph*(6), *aac*(3)-VI, *tet*A, *flor*R, *dfr*A, *sul*1; antiseptic resistance genes *mer* and *qac*E delta 1; and, adherence fimbria type 1 and F17 genes (Figure 3 and Supplementary File 3). The IncFIB(pN55391) plasmid was also identified in all *S.* Infantis isolates. However, after a map to reference verification, it was concluded that IncFIB(pN55391) plasmids were indeed pESI-like plasmids camouflaged in its chimeric structure.

DISCUSSION

This study examined the population structure, dynamics, and genetic signatures of *Salmonella* serotypes throughout the production chain of two poultry integrators. *S.* Infantis was the most prevalent serotype in this study, thus adding additional evidence to multiple reports that have described this serovar as a global emerging pathogen in poultry production in the Americas (Valderrama et al., 2014; Cunha-Neto et al., 2018;

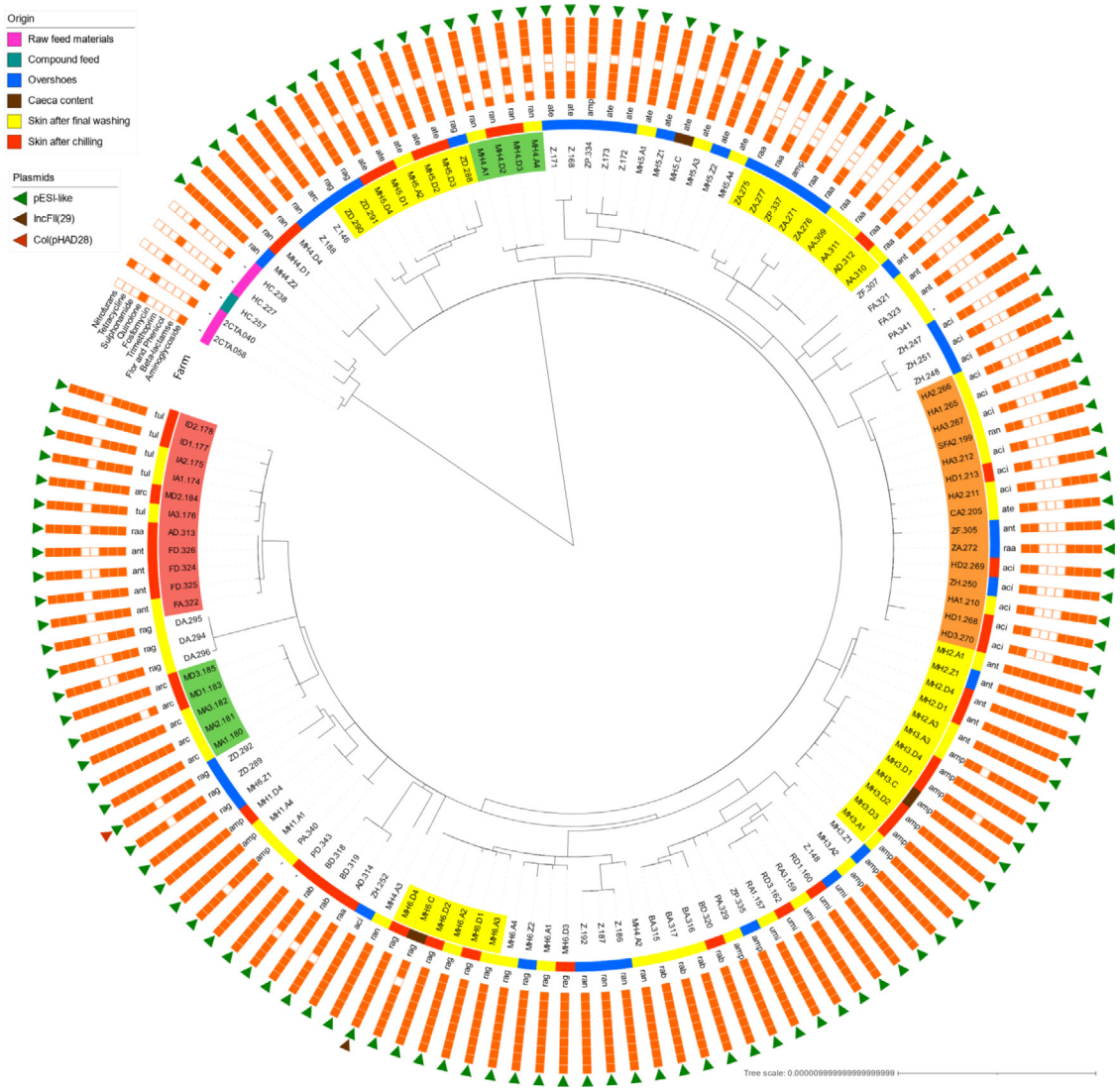
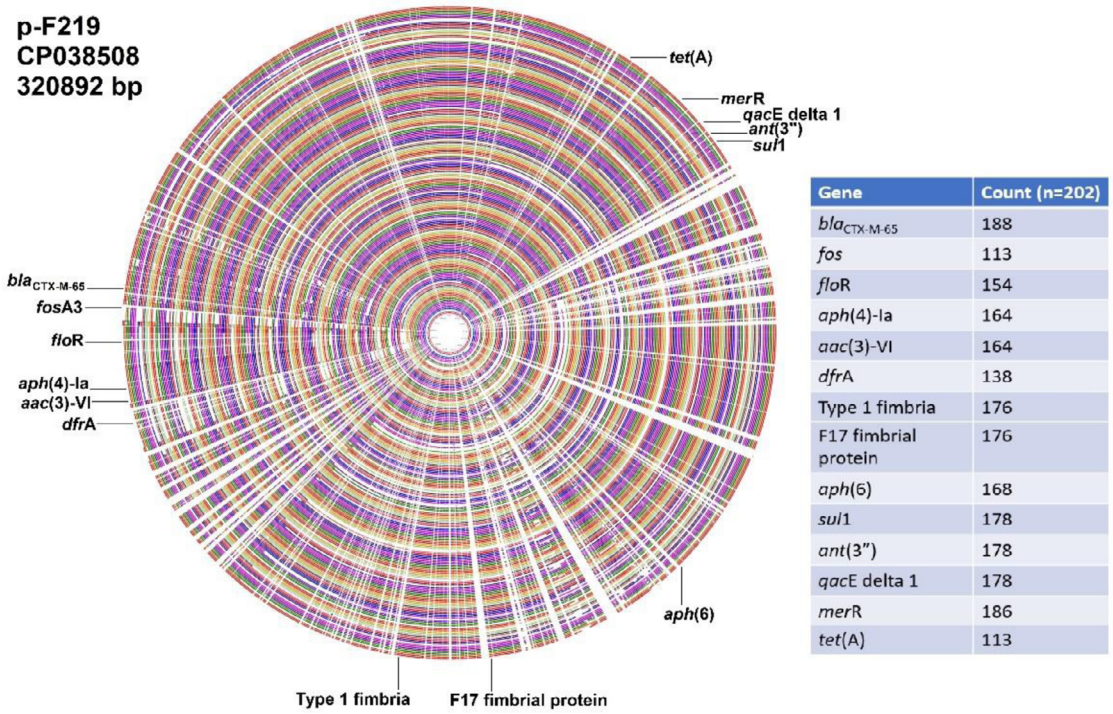


Figure 2. SNP tree analysis of *S.* Infantis isolates of integration B.

Gymoese et al., 2019; Lapierre et al., 2020; Mejía et al., 2020), Europe (EFSA, European Food Safety Authority; European Centre for Disease Prevention and Control ECDC, 2019), and Asia (Ishihara et al., 2020; Li et al., 2020).

In fact, the strong association between *S.* Infantis and poultry in Ecuador, and its governed territory of the Galapagos Islands, is well-described (Vinueza-Burgos et al., 2016, 2019; Burnett et al., 2021). Moreover, its wide distribution in both integrations highlights the trend of *S.* Infantis to thrive in various environments. *Salmonella* Infantis has been reported to be the most common serotype in live chickens and their byproducts (Salazar et al., 2019; Mejía et al., 2021). Although human isolates were not included in this study, *S.* Infantis is frequently isolated from NTS diarrheal disease (Cartelle Gestal et al., 2016; Mejía et al., 2020; EFSA, European Food Safety Authority; European Centre for Disease Prevention and Control ECDC, 2021). Furthermore, NTS outbreaks in Europe and USA have been linked to travelers returning from Ecuador and South America (Brown et al., 2018; Alba et al., 2020; Bokhary et al., 2021). This highlights the need to implement strong monitoring and control measures to mitigate the presence of this pathogen.

The population dynamics of *Salmonella* in the production chain of broiler chickens has been investigated using various methods including serotyping (Foley et al., 2011), ribotyping and pulsed field gel electrophoresis (**PFGE**) (Liebana et al., 2001; CDC, 2016; Vinueza-Burgos et al., 2019). Despite the discriminatory power of PFGE, this method has clear limitations when used to analyze highly similar bacterial genomes as is the case of *S.* Infantis. Moreover, the typification by MLST shows to be of insufficient resolution when looking for the transmission of specific genotypes throughout the broiler production chain. In fact, all *S.* Infantis isolates in this study belonged to ST32 which is the most prevalent ST of this serotype (Achtman et al., 2020). It is here where a WGS approach takes place for the analysis of the transmission of *Salmonella* genotypes in integrated poultry companies, since WGS gives a level of resolution that cannot be reached with other techniques.



Gene	Count (n=202)
$bla_{CTX-M-65}$	188
fos	113
*flo*R	154
aph(4)-Ia	164
aac(3)-VI	164
*dfr*A	138
Type 1 fimbria	176
F17 fimbrial protein	176
aph(6)	168
*sul*1	178
ant(3")	178
*qac*E delta 1	178
*mer*R	186
tet(A)	113

Figure 3. General plasmid alignment of pESI-like plasmid PSI-like plasmids showed high similarity with the Peruvian reference. However, entire blocks are reorganized or absent in some strains. A dynamic view of the alignment can be seen in Supplementary File 3.

For example, the red cluster in Figure 2 shows that a single genotype, only found at slaughterhouse level, contaminates carcasses from different farms. The fact that this genotype was not isolated in previous stages of the production chain, could suggest that this clone is well adapted to the slaughterhouse environment (e.g., forming biofilms) and could provoke cross-contamination events of carcasses at this level. Furthermore, our analysis shows that *Salmonella* genotypes originated in specific farms can enter into the slaughter line and reach carcasses of different farms (orange and yellow clusters in Figure 2). These facts highlight the role of the slaughter process as source of *Salmonella* contamination of broiler carcasses (Rasschaert et al., 2006; Rouger et al., 2017). However, the role of transportation of broilers (which was not considered in this study) should be taking in account in further studies.

Interestingly, isolates originating from the feed mill were observed to be the most genetically unique, when compared to the remaining collection. Moreover, these isolates presented fewer GDAMR. Besides, this fact could demonstrate that *Salmonella* strains originated in raw materials and feed compound are not important players in the epidemiology of this bacteria in farther stages of broiler production, at least in this dataset. However, these strains are closely related (<16 SNPs) to one strain (PNUSAS084421) originated in a human clinical case from USA (www.ncbi.nlm.nih.gov/pathogens/tree/#Salmonella/PDG000000002.2314/PDS000038582.4?accessions=PDT000526141.1). It must be considered that a significant part of the raw materials used for poultry feed production in Ecuador comes from USA. Therefore, the presence on these strains in Ecuador could be explained by the trade of raw materials between these countries.

On the other hand, the close relation between strains from chicken carcasses and human clinical cases of salmonellosis (linked to travelers) has been reported before (Tate et al., 2017; Brown et al., 2018; Alba et al., 2020; Burnett et al., 2021).

The isolate originated in meconium sampled in transport paper of 1-day-old chicken showed to be highly related to the ones originated in farms and slaughterhouse of integration A (blue cluster). This observation reveals that the production system of 1-day-old chickens could play an important role in the epidemiology of *S.* Infantis in the poultry production. Therefore, earlier stages of production (e.g., breeders, hatchery, transport, etc.) should be included when planning surveillance programs for *Salmonella*. Moreover, the data presented here show an evident clustering of isolates in each integration, evidencing the suitability of this technique to track pathogens up to their origin in integrated poultry companies (Graphic 1 in Supplementary File 1). Besides, WGS allows to identify hotspots of *Salmonella* contamination in the broiler production chain.

Our observations suggest that *Salmonella* isolates are able to persist, despite sanitation steps at various points of the production chain. Unsurprisingly, genetic determinants capable of conferring resistance against disinfectants, including quaternary ammonium compounds, as well as heavy metal tolerance, were observed in multiple isolates.

The ability of *S.* Infantis to persist in food and food processing environments and to establish successful infections in humans has resulted in its rise as an

emerging pathogen. Some genetic studies suggested that *S.* Infantis isolates possess important GDAMR and virulence-associated genes that contribute to its ability to adapt and cause successful infections (Acar et al., 2019; Bogomazova et al., 2020; Proietti et al., 2020). Most *S.* Infantis isolates detected in this study possessed GDAMR that could potentially confer resistance to first-line antibiotics used to treat humans NTS infections (beta lactams, quinolones, and sulfonamides) (Onwuezobe et al., 2012).

The selective pressure caused by the usage of antibiotics at farm level could explain the presence of several of these GDAMR, as it has been reported before (Vinueza-Burgos et al., 2016; Villagómez et al., 2017).

A similar observation was made for the other serovars; however, phenotypic studies are needed to confirm the genome predicted resistance. Nonetheless, studies have been conducted in Ecuador that correlate the presence of GDAMR with phenotypic resistance in *S.* Infantis (Villagómez et al., 2017; Vinueza-Burgos et al., 2019; Mejía et al., 2020). In fact, it has been shown a high correlation of the presence of GDAMR and resistant phenotypes (Bortolaia et al., 2020).

Virulence genes including the plasmid-encoded *spv* have been associated with increased virulence in *Salmonella* isolated from clinically ill patients (Guiney et al., 1995; Guiney and Fierer, 2011) and their presence in isolates from this study represents a potential risk to final consumers (Tate et al., 2017; EFSA, European Food Safety Authority; European Centre for Disease Prevention and Control ECDC, 2019; Tyson et al., 2020).

In this study the pESI-like megaplasmid was identified in almost all *S.* Infantis isolates throughout the production chain of the two integrations. In fact, 95% of them harbored the $bla_{\text{CTX-M-65}}$ gene that confers resistance to Third Generation Cephalosporins (**3GC**). Although 3GC are not used in poultry production, the presence of the $bla_{\text{CTX-M-65}}$ gene could be explained by a co-selection of resistance genes to antibiotics that are commonly utilized in broiler production (Franco et al., 2015; Pal et al., 2015; McMillan et al., 2020a). This is the case of the genes *fos*A3 (fosfomycin), *tet*A (tetracycline), *dfr*A (trimethoprim), *sul*1 (sulfonamide); and the antiseptic resistance gene *qac*E delta 1 found in the analyzed pESI-like plasmids. However, more research is needed to demonstrate this hypothesis in pESI-like plasmid of *S.* Infantis.

These genes have also been described in *S.* Infantis isolated from broiler production in Peru (Vallejos-Sánchez et al., 2019). The presence of $bla_{\text{CTX-M-65}}$ in these plasmids could be implicated with their permanence in environments with β-lactam antibiotics pressure (e.g., contaminated water sources or soil). The high rates of pESI-like plasmids in 3GC-resistant *S.* Infantis originated in poultry environments have been reported in several studies worldwide (Franco et al., 2015; Alba et al., 2020; Bogomazova et al., 2020; García-Soto et al., 2020; McMillan et al., 2020b; Kürekci et al., 2021). Although the dynamics of pESI-like plasmids remains largely unknown, it has been claimed that the specificity of pESI-like plasmids in *S.* Infantis could be associated with the inhibition of conjugation with other *Salmonella* serogroups in the chicken gut, mainly mediated by temperature and the presence of bile salts (Thomas and Nielsen, 2005; García-Soto et al., 2020). Additionally, the inhibition of self-transmission of these plasmids to *S.* Typhimurium and *Escherichia coli* has been demonstrated in laboratory (Aviv et al., 2014, 2016). However, more studies are needed to characterize the ecological barriers for intra- and interspecific transmission of pESI-like plasmids to other bacteria in the poultry industry.

Another important feature of pESI-like plasmids found in this study is the presence of several fimbriae genes (type 1 and F17 fimbria), that could increase the capacity of attachment to epithelial cells of these bacteria (Aviv et al., 2014, 2016). Altogether, these factors could represent major drivers for the increase of human infections caused by ESBL-producing *S.* Infantis (McMillan et al., 2020b). Therefore, the study of the molecular epidemiology of pESI-like plasmids should be included as a part of the surveillance programs to reduce the dissemination of this microorganism in the food chain.

It has been reported that pESI-like plasmids display chimeric characteristics that can cause its description as IncFIB plasmid or its variants in other studies (Aviv et al., 2014; García-Soto et al., 2020; Burnett et al., 2021). For this reason, this characteristic should be considered when reporting findings of the molecular epidemiology of this plasmid. It is also worth to mention that pESI-like plasmids can enhance the fitness of specific *S.* Infantis strains, displacing other genotypes in niches within the poultry industry (Bogomazova et al., 2020).

It is important to mention that the in-silico analysis of genomes is in continuous development. Therefore, the utilization of updated tools and dedicated databases must be considered for the detection of new genetic components. In this study we demonstrate the usefulness of a WGS approach to have an in-depth understanding of the epidemiology of *Salmonella* in integrated poultry companies. This kind of analysis can help to implement and evaluate interventions aiming to control *Salmonella* in the broiler production chain. Moreover, we report that *S.* Infantis is the main *Salmonella* serotype in studied integrations and that the pESI-like plasmids found in these isolates harbor important resistance and virulence genes.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101611.

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