German Journal of Veterinary Research
eISSN:2703-1322

Research article

Isolation of *Salmonella* from tissue and environmental samples and assessment of risk factors in commercial layers in Argentina

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Article History:
Received: 7-Dec-2024
Accepted: 28-Jan-2025
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Abstract

This study aimed to evaluate the isolation methods of *Salmonella* from tissues and environmental samples collected during natural *Salmonella* outbreaks in commercial layer farms in Argentina. We also assessed key risk factors for contamination with *Salmonella* serovar Gallinarum biovar Gallinarum (SG) in poultry houses. To achieve this, we collected tissue samples from 20 houses (n=398; including spleen, liver, ovary, cecal contents, and bone marrow) and environmental samples (n=510; including feed, eggs, feces, and water) from deceased laying hens and their surroundings. Various isolation procedures were employed using different media. *Salmonella* was identified based on biochemical tests and serotyping using agglutination tests. Results showed that out of 398 tissue samples, 247 (62%) were positive for SG: bone marrow (62/80), spleen (59/79), liver (57/80), ovary (51/80), and cecum 18/79). *Salmonella* spp. was detected in 25% (1/4), 17% (2/12), 10.6% (10/94), and 3.6% (4/110) in boot swabs, egg nests, feces, and feed, respectively. Additionally, samples of yolk egg pool (1/74) and eggshell pool (1/74) were positive for SG. Among isolated serovars, *S.* ser. Cerro was the most frequently isolated serovar, followed by SG, *S.* ser. Livingstone, *S.* ser. Enteritidis, *S.* ser. Derby, *S.* ser. Corvallis, *S.* ser. Infantis, *S.* ser. Mbandaka, *S.* ser. Montevideo, *S.* ser. Schwarzengrund, and *S.* ser. Heidelberg, which were all isolated from environmental samples. Notably, four poultry houses exhibited contamination with multiple serotypes, with three or four serotypes present. Multiple logistic regression analyses revealed that vaccination against SG and effective insect control notably decreased the spread of *Salmonella* on poultry farms. Our results emphasize the importance of using diverse sample types and detection techniques for isolating and identifying *Salmonella*. This data could also contribute to developing improved control programs and intervention strategies at the farm level to minimize *Salmonella* contamination in poultry.

Keywords: *Salmonella*, Detection, Environmental samples, Laying-hen farm

Citation: Soria, M. A., Godano, E. 1., Leiva, L. E., Shehata, A. A., and Bueno, D. J. 2025. Isolation of *Salmonella* from tissue and environmental samples and assessment of risk factors in commercial layers in Argentina. Ger. J. Vet. Res. 5 (2): 1-14. https://doi.org/10.51585/gjvr.2025.2.0129

Introduction

Salmonella enterica subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) is responsible for fowl typhoid (FT), a serious systemic disease primarily affecting adult birds. FT significantly impacts the poultry sector in various countries, leading to economic losses from decreased egg production and high mortality rates, reaching up to 80% in affected flocks (WOAH, 2018; Zhou et al., 2022; Wales and Lawes, 2023). On the other hand, paratyphoid *Salmonellae* are a diverse group of motile *Salmonella* serotypes that are not explicitly adapted to hosts and are commonly found in poultry. Infections caused by

paratyphoid *Salmonella* in chickens are typically asymptomatic, although they can occasionally result in persistent colonization of the intestinal tract and internal organs, potentially leading to carcass contamination (Gast and Porter, 2020). *Salmonella* ser. Enteritidis (SE) can be isolated from various organs of commercial layer hens infected with SG, indicating that it is crucial to investigate the dynamics of *Salmonella* spp. infection and to determine the serotypes circulating within the same ecological niche (Pulido-Landınez et al., 2014; Sreekantapuram et al., 2021).

Like many other bacterial diseases, FT can be transmitted in several ways. Infected birds, whether they are reactors or carriers, play a crucial role in spreading *Salmonella* through horizontal and egg transmission. Additionally, droppings from infected birds and contaminated feed, water, and litter can serve as sources of *Salmonella* (Shivaprasad and Barrow, 2013; Cocciolo et al., 2020; Yeakel, 2022). However, the difficulty in detecting this bacteria at low concentrations represents a crucial problem, leading to false-negative results, particularly when poultry feces (Soria et al., 2012b), feed (Soria et al., 2011), or water (Soria et al., 2013a) samples are used.

Clinical signs and lesions of FT are similar to *Salmonella* biovar Pullorum infection. Therefore, the diagnosis can be confirmed by isolating and identifying the isolated pathogen (Yeakel, 2022). It is well-established that the methods for isolating SG depend on the origin of the samples. While recovery from cloacal samples and feces may be less successful, examining tissues obtained during post-mortem is generally more effective (WOAH, 2018).

Indeed, numerous agar media are utilized to isolate *Salmonellae*. At least two different media, preferably with dissimilar indicator systems, should be used to differentiate *Salmonella* from other pathogens (WOAH, 2018; Gast and Porter, 2020). Our study employed statistical analysis to determine the most effective samples and selective-differential media for isolating *Salmonella* from tissue and environmental samples. Additionally, we identified the primary risk factors for SG contamination, which might significantly impact decision-making policy with targeted interventions.

Material and methods

History of chicken farms

Table 1 provides brief data on the history of tested farms. Between June 2013 and September 2016, twenty-layer houses across two states in Argentina were investigated: three located in Santa Fe and 17 in Entre Rios. The chickens had a history of high mortality rates and a significant drop in egg production, previously testing positive for the isolation of SG from bone marrow samples.

Among the sampled poultry houses, four were backyard laying hens, while 16 were industrial laying hens. Some layer hens exhibited acute illness at sampling, characterized by ruffled feathers, difficulty breathing, reduced feed intake, and watery diarrhea. Seventeen layer hen flocks were in the egg-laying phase, with 13 housed in battery cage systems and four in backyard systems. Three hen flocks were not yet in the egg-laying phase, with ages ranging from 2 to 4 months. The age of hens kept in other flocks was 13 months (ranging from 6 to 21 months). Flock sizes varied significantly, from 70 to 30,000 hens. Seven flocks had a vaccination history against SG with the SG-9R vaccine Table 1.

Sampling and isolation of *Salmonella* from tissues

Based on the availability of deceased birds, one to ten dead laying hens were gathered from each house. The samples were transported in iceboxes to the Poultry Health Laboratory (Agriculture Experimental Station of the National Institute of Agricultural Technology in Concepción del Uruguay, Entre Ríos, Argentina), where necropsies were performed to collect the liver, spleen, ovarian follicles, ceca, and legs.

All organs were processed individually and/or together. Differential-selective agar plating method was used for *Salmonella* isolation from the liver and spleen samples on MacConkey agar (MCA; Acumedia-Neogen, Lansing, MI, USA) and *Salmonella-Shigella* agar (SSA; Merck (Darmstadt, Germany). Furthermore, parts of the content of the ceca and ovarian follicles were taken and put on the same differential-selective agar media plates as described above. Besides, a sterile cotton swab, premoistened with sterile 0.85% sodium chloride solution, was inserted into the bone marrow from the shank, rotated gently, and then streaked in a part of the same plates described before. After that, all plates were streaked with a sterile loop and incubated at 37°C for 18–24 hrs. If there was no bacterial

growth, the plates were reincubated for 48 hrs. Pooled portions of liver, spleen, ceca, and ovaries from 3 to 10 laying hens per house were enriched in tetrathionate broth (TTB) containing TTB-base (Acumedia-Neogen, Lansing, MI, USA), 20 mL/L iodine potassium iodide solution (containing 6 g of iodine, 5 g of potassium iodide, and 20 mL of demineralized water), 0.1% brilliant green (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and 40 mg/mL novobiocin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After incubating for 18 hours at 37°C, a loopful of the broth was streaked onto MCA and SSA agar, followed by further incubation at 37°C for 18-24 hours.

Table 1: History of the examined farms

House number	Age/ weeks	Breed	Flock size	Location	Housing type	SG vaccination	Drop in egg production %
1	54	Hy-Line	4,000	Entre Rios	Manual	-	ND
2	42	Hy-Line	6,000	Santa Fe	Manual	+	ND
3	18	ND	70	Entre Rios	Floor backyard	-	ND
4	120	Bataraza	70	Entre Rios	Floor backyard	-	ND
5	52	Hy-Line	24,600	Entre Rios	Automatic	-	25.0
6	30	Pro-Huerta	450	Entre Rios	Floor backyard	-	33.3
7	55	Hy-Line	5,400	Santa Fe	Manual	+	47.0
8	24	ND	35,000	Santa Fe	Automatic	-	10.0
9	16	Hy-Line	7,000	Entre Rios	Manual	-	ND
10	9	Hy-Line	22,000	Entre Rios	Manual	+	ND
11	72	Hy-Line	30,000	Entre Rios	Automatic	ND	30.0
12	80	Hy-Line	4,200	Entre Rios	Manual	-	35.0
13	65	Hy-Line	23,000	Entre Rios	Automatic	+	25.0
14	45	Ross	600	Entre Rios	Floor backyard	-	60.0
15	87	Hy-Line	25,000	Entre Rios	Automatic	-	15.0
16	84	Hy-Line	8,500	Entre Rios	Manual	-	8.0
17	37	Hy-Line	18,000	Entre Rios	Manual	+	20.0
18	15	Hy-Line	18,000	Entre Rios	Manual	+	ND
19	76	ND	520	Entre Rios	Manual	-	50.0
20	35	Hy-Line	23,500	Entre Rios	Automatic	-	40.0

ND: no data

Sampling and isolation of *Salmonella* from feed

Approximately 2–3 kg of feed was collected from various sources, including the feed chute of manual battery cage houses, layer feeders, and external silos. In floor housing systems, samples were collected from feeders and/or feed bags in sterile plastic bags. Isolation was conducted using the Salmosyst method (Soria et al., 2013b). Briefly, two samples of 25 g each) were pre-enriched in 225 mL of Salmosyst broth (Merck, Darmstadt, Germany). Then, 10 mL from this culture was transferred to sterile tubes and supplemented with a Salmosyst selective supplement tablet (Merck, Darmstadt, Germany). After incubation at 37°C for 24 hrs, a loopful of the broth was streaked onto xylose lysine deoxycholate (XLD) agar (Oxoid, Basingstoke, Hampshire, UK) supplemented with 4.6 mL/L tergitol-4 (XLDT, Sigma, St. Louis, MO, USA) and EF-18 agar (Acumedia-Neogen, Michigan, USA), followed by another incubation at 37°C for 24 hrs.

Sampling and isolation of *Salmonella* from eggs

Thirty eggs per house, particularly dirty eggs, were collected and grouped into five pools, each containing six eggs. The yolk, albumen, and eggshells were processed separately in sterile plastic bags (five pools of six samples each). Samples were collected and processed as described by Soria et al. (2012a). Samples were

pre-enriched in 225 mL of tryptic soy broth (TSB; Merck, Darmstadt, Germany), supplemented with ferrous sulfate (35 mg of ferrous sulfate/Liter). The mixture was incubated at 37°C for 18–24 hrs. Then, 1 mL of the broth was transferred to 10 mL TTB. This mixture was then incubated at 37°C for another 18–24 hrs. Finally, a loopful of the incubated selective enrichment broth was streaked on Hektoen (Acumedia-Neogen, Michigan, USA) and XLD (Oxoid, Basingstoke, Hampshire, UK) agar plates, which were then incubated at 37°C for an additional 18–24 hrs.

Sampling and isolation of *Salmonella* from feces

Fecal samples (~250 g) were collected from all belts in each house line after the manure removal system (automatic) was activated. In the case of the manual battery cage system, approximately 250 g of mixed fresh feces were collected from 60 locations beneath the cages in the dropping pits. In all cases, sterilized spoons were used for sample collection. Two pairs of boot swabs were used to sample the poultry house in the case floor system. The swabs were worn over boots, disinfected, and moistened with a 0.85% sterile saline solution. Samples were obtained by walking around the poultry house floor, ensuring that about 50% of the floor area was covered with each pair of boot swabs. Swabs from each poultry house were pooled into one sample and placed in a sterile plastic bag.

Additionally, two pools of at least 25 g of egg nest material were gathered into sterile plastic bags. Furthermore, two 25 g subsamples were taken from each feces and egg nest material for *Salmonella* isolation. *Salmonella* isolation from feces was done according to Soria et al. (2012b).

Briefly, following pre-enrichment in 225 mL of buffered peptone water (BPW, Merck, Darmstadt, Germany) for 18 to 24 hours at 37°C, 30 µL of the incubated BPW culture was inoculated semisolid Rappaport-Vassiliadis (MSRV) medium (Acumedia-Neogen, Michigan, USA), supplemented with 1 mL/L of a 2% novobiocin solution, and incubated at 40°C for 18–24 hours. The cultures were then streaked onto XLDT and EF-18 (Acumedia-Neogen, Michigan, USA) agar.

Sampling and isolation of *Salmonella* from water

Water samples, each 1 liter, were collected from the external water tanks and taps inside the hen houses on each farm. Before collection, the water was allowed to run for approximately 3 to 5 minutes. The faucet was sterilized using a flame, and the water was then collected in sterile bottles. Two 50 mL subsamples from each 1-liter sample were pre-enriched in 450 mL of Buffered BPW at a double concentration. After an incubation period of 18 to 24 hours at 37°C, 30 µL of the incubated BPW culture was streaked onto MSRV medium and subsequently subcultured onto XLDT and EF-18 agar plates (Acumedia-Neogen, Michigan, USA) (Soria et al., 2013a).

Identification of *Salmonella* spp.

To confirm the presence of *Salmonella*, two suspected colonies from each selective differential agar plate were biochemically tested using a variety of media, including triple-sugar iron agar (Acumedia-Neogen, Michigan, USA), lysine iron agar, Simmons citrate agar, sulfide indole motility medium (Merck, Darmstadt, Germany), Jordan's tartrate agar, and phenylalanine agar (Hi-Media, Thane, India). After confirmation, all *Salmonella* isolates were stored on nutritive slant agar (Merck, Darmstadt, Germany) until serotyping was carried out.

Serotyping

Serotyping was conducted following the White-Kauffmann-Le Minor scheme, utilizing somatic (O) and flagellar (H) antisera (INPB-A.N.L.I.S., Dr. Carlos G. Malbran, Buenos Aires, Argentina), as described by Grimont and Weill (2007).

Acriflavine agglutination test

A 1:1000 dilution of acriflavine (Sigma-Aldrich, Co., St Louis, USA) was prepared in distilled water to distinguish between smooth and rough strains during SG isolation. A loop of culture was then mixed with a drop of the acriflavine solution on a slide. The slide was gently rotated, and after 30 seconds, the degree of agglutination was observed. In positive cases, slow formation of granules indicated that the *Salmonella* strain was rough, while smooth-phase colonies did not show agglutination (WOAH, 2018).

Analysis of risk factors associated with SG outbreaks

A questionnaire was carried out in hen/poultry houses with fowl typhoid outbreaks, and it had different sections related to farm identification data, animal vaccination plan, biosecurity

measures applied on the farm, feed characteristics, water supply, egg destination, type disposal of animal dead. The survey was conducted on the same day as the sample collection. The farm owner or the person responsible for the farm was interviewed by the primary author or the veterinarian who coordinated with the farm. The survey consisted of closed-ended questions (dichotomous or multiple-choice) related to biosecurity practices for poultry farms, following the guidelines set by the Argentinean National Agrifood Health and Quality Service regulation 542/2010 (Senasa, 2010). Data were coded and recorded in an Excel database (Microsoft Corporation) for Infostat Software analysis (Herrero et al., 2020).

To examine the relationship between various risk factors and the presence or absence of SG, we utilized survey data from 20 hen houses previously reported by Soria (2013) as negative for both SG isolation and SG antibodies. Each house was treated as a separate unit due to the diverse types of commercial hen houses on the farms (backyard laying hens, manual and automatic battery cages). A two-step statistical approach was applied to assess the association between the survey variables and SG status in the sampled hen houses. Quantitative and qualitative variables with multiple levels were categorized into two groups. Hen age was divided based on the average age at first molting in laying hens: those ≥72 weeks old and those <72 weeks old. Similarly, based on the average number of hens per house, the two categories for laying hens were <10,000 and ≥10,000. Univariate analysis was used to test all potential risk factors, and only variables with a p-value <0.15 were selected (Fisher's or x^2 test). In the first step, significant variables were checked for collinearity using the Phi test to remove any correlated independent variables. Variables with a Phi value greater than 0.5 were considered collinear, and in cases of collinearity, only the more biologically relevant variable was retained for further analysis. A logistic multiple-regression model was used to assess the remaining variables, with the Wald chi-square test applied for the p-value criterion. A significance level of p<0.05 was considered, and results were presented as odds ratios (OR) with 95% confidence intervals (CI) for all significant variables.

Statistical analysis

The effectiveness of the differential-selective agars and samples was evaluated by calculating relative sensitivity (RSe) and agreement metrics, which included the Kappa coefficient and McNemar's test. A sample was classified as a relative true positive if it tested positive for *Salmonella* spp. in at least one of the differential-selective agars. Conversely, a relative true negative was defined as a sample where *Salmonella* was not detected in any of the agars.

Kappa coefficients were evaluated based on the criteria described by Dawson and Trap (2004): excellent agreement (0.93 to 1.00), very-good agreement (0.81 to 0.92), good agreement (0.61 to 0.80), fair agreement (0.41 to 0.60), slight agreement (0.21 to 0.40), poor agreement (0.01 to 0.20), and no agreement (<0.01). McNemar's test was performed using a chi-square approximation with a significance level of p≤0.05 (GraphPad Software, 2024). Additionally, the agreement between different samples for isolating *Salmonella* from dead laying hens on farms experiencing fowl typhoid (FT) outbreaks was examined using the Kappa coefficient and McNemar's test.

Results

Isolation of *Salmonella* from tissue samples collected from dead birds

A total of 398 tissue samples were collected from deceased laying hens, of which 247 (62.0%) tested positive for *Salmonella* spp. SG was isolated from the bone marrow, spleen, liver, ovaries, and cecum (see Table 2). The results of biochemical tests and serotyping can be found in Supplementary Table 1. The rate of SG isolation varied between 22.8% and 77.5% across different types of samples. No statistically significant differences were observed in SG isolation rates among bone marrow, spleen, liver, and ovarian follicle contents. Specifically, 53.8% (7 out of 13) of pooled samples from the liver, spleen, and ovaries were positive for SG isolation. In contrast, only 7.7% (1 out of 13) of cecal pools tested positive.

The results regarding the agreement of selective-differential media used for SG isolation from various tissue samples during the FT outbreak are presented in Table 3. The level of agreement ranged from fair (in cecum content) to excellent (in bone marrow and liver) for the comparison between MCA and SSA in organ samples, with no significant difference (p>0.05).

Table 4 illustrates the agreement levels for SG isolation within tissue samples. Kappa coefficients indicated that the agreement ranged from poor to good, depending on the samples being compared. A good agreement was found for SG isolation between the liver and both the spleen and ovarian follicle. In contrast, there was a fair agreement between liver and bone marrow as well as between spleen and bone marrow, with no significant difference (p>0.05). However, a fair agreement was noted between the spleen and ovarian follicles, which showed a significant difference (p<0.05). The agreement between ovarian follicle and both bone marrow and cecum content was slight, with a significant difference (p<0.05). Additionally, the agreement between cecum content and liver, spleen, or bone marrow was poor, with a significant difference (p<0.05).

Table 2: Isolation rate of *Salmonella* ser. Gallinarum biovar Gallinarum (SG) from tissues collected from dead laying hens in fowl typhoid outbreaks.

SG isolation from organs	Isolation rate (%, CI[a])
Bone marrow	62/80[A] (77.5, 0.671-0.854)
Spleen	59/79[A] (74.7, 0.640-0.830)
Liver	57/80[A] (71.2, 0.605-0.800)
Ovarian follicle content	51/80[A] (63.7, 0.528-0.734)
Cecum content	18/79[B] (22.8, 0.148-0.332)

[A–B]Different uppercase letters in the same column are significantly different (p<0.05). 95% confidence interval (CI).

Table 3: Agreement (Kappa coefficient and McNemar's test) of *Salmonella* ser. Gallinarum biovar Gallinarum (SG) isolation between different selective-differential plating media used to isolate *Salmonella* from tissues.

SG isolation from different samples	Plating media	Kappa Coefficient[a]	*p*-value[b]
Liver	MCA/SSA	0.94	0.480
Spleen		0.82	0.220
Ovarian follicle content		0.84	0.683
Cecum content		0.51	1.000
Bone marrow		0.96	1.000

[a] Kappa is significantly non-zero (p< 0.05). [b] Determined with McNemar's chi-square test for paired samples. MCA: MacConkey agar, SSA: *Salmonella Shigella* agar

Table 4: Agreement (Kappa coefficient and McNemar's test) between different tissue samples for isolation of *Salmonella* Gallinarum.

Comparison between SG isolation from different organ samples	Kappa Coefficients[a]	*p*-value[b]
Liver/spleen	0.78	0.450
Liver/ovarian follicle content	0.66	0.150
Liver/cecum content	0.18	0.000
Liver/bone marrow	0.51	0.302
Spleen/ovarian follicle content	0.56	0.040
Spleen/cecum content	0.19	0.000
Spleen/bone marrow	0.51	0.790
Ovarian follicle content/cecum content	0.27	0.000
Ovarian follicle content /bone marrow	0.38	0.029
Cecum content /bone marrow	0.13	0.000

[a] Kappa is significantly non-zero (p<0.05) [b] Determined with McNemar's chi-square test for paired samples

Isolation of *Salmonella* from environmental samples

Table 5 presents the results of *Salmonella* isolation from various sources, including feed, feces, boot swabs, egg nests, and egg content samples collected from 20 laying-hen houses. A total of 510 samples were analyzed using different isolation methods, and 19 samples tested positive for *Salmonella* spp., resulting in a positivity rate of 3.7%. Notably, all water samples (n=68) and albumen pools (n=74) were negative for *Salmonella*. The positivity rates for *Salmonella* spp. among the different sample types were as follows: boot swabs at 25% (1 out of 4), egg nests at 17% (2 out of 12), feces at 10.6% (10 out of 94), and feed at 3.6% (4 out of 110). Additionally, only one sample of each of the yolk egg pool (1 out of 74) and eggshell pool (1 out of 74) tested positive for SG from 222 egg samples.

The distribution of multiple *Salmonella*

serotypes in environmental samples from the commercial layer hen houses is summarized in Table 6. Eight out of the sampled hen houses (40%) tested positive for *Salmonella* spp., with a total of 33 *Salmonella* strains isolated. These isolates were categorized into 11 serotypes: *S.* ser. Cerro (6 isolates), SG (5), *S.* ser. Livingstone (4), SE (4), *S.* ser. Schwarzengrund (3), S. ser. Derby (3), *S.* ser. Corvallis (3), *S.* ser. Infantis (2), *S.* ser. Mbandaka (1), *S.* ser. Montevideo (1), and *S.* ser. Heidelberg (1). Four hen houses were contaminated with only one *Salmonella* serotype, while four others showed contamination with multiple serotypes (ranging from 3 to 4). *S.* ser. Cerro was isolated from manual battery cage systems and floor backyard chickens. In contrast, *S.* ser. Livingstone and *S.* ser. Corvallis were found in both manual and automatic battery cage systems. *Salmonella* ser. Enteritidis (SE) was exclusively detected in feces from the automatic battery cage system, whereas SG was isolated from eggshells, egg yolks, feces, and feed across different houses.

Table 7 shows the results of the recovery sensitivity (RSe) and agreement (Kappa coefficient) calculations for the selective differential media used. The RSe for XLDT agar ranged from 0.50 to 0.92, while for EF-18 agar, it ranged from 0.50 to 0.83, with no significant difference between the two. However, there was no consensus on the suitability of XLDT and EF-18 agar for feed samples, although both were deemed suitable for feces samples. The morphology of motile and non-motile *Salmonella* observed on MSRV is illustrated in Figure 1. Moreover, all isolated SG strains were classified as smooth-type (Figure 1).

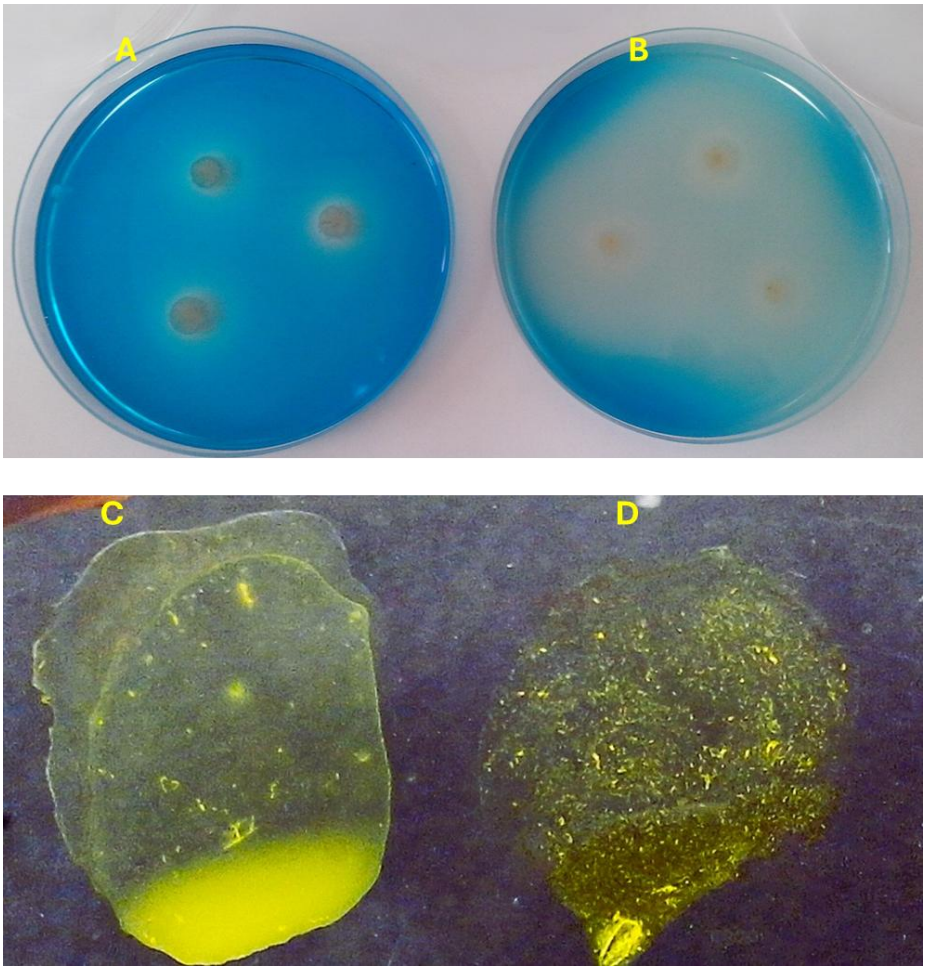

Figure 1: Colony morphology of non-motile *S. Gallinarum* (A) and motile *Salmonella* (B) on the modified semisolid Rappaport-Vassiliadis (MSRV) media. Acriflavine agglutination test of SG: C) Smooth-phase colonies of *S. Gallinarum* do not produce agglutination. D) Rough-phase colonies of SG 9R produce agglutination.

Table 5: Detail overview of *Salmonella* isolation in feed, feces, boot swabs, egg nests, and egg samples from 20 hen houses in fowl typhoid outbreaks of Entre Rios, Argentina.

House number	Number of positive samples of *Salmonella* spp./total number of samples processed (%)[a]							
	Feces	Boot swab	Egg nest	Feed	Water	Egg samples		
						Eggshell	Albumen	Egg yolk
1	0/8	-	-	1/8[b]	0/4	0/5	0/5	0/5
2	1/12[b]	-	-	0/4	0/4	0/5	0/5	0/5
3	-	0/1	0/2	0/4	0/4	0/1	0/1	0/1
4	-	1/1[c]	2/4[c]	0/2	0/2	0/1	0/1	1/1[b]
5	2/4[c]	-	-	0/4	0/4	0/5	0/5	0/5
6	-	0/1	0/2	0/4	0/6	0/2	0/2	0/2
7	2/10[c]	-	-	0/6	0/4	0/5	0/5	0/5
8	0/4	-	-	0/6	0/2	0/5	0/5	0/5
9	0/12	-	-	0/12	0/4	-	-	-
10	2/6[c]	-	-	3/12[b,c]	0/4	-	-	-
11	3/4[c]	-	-	0/4	0/4	0/5	0/5	0/5
12	0/4	-	-	0/8	0/4	0/5	0/5	0/5
13	0/4	-	-	0/4	0/4	0/5	0/5	0/5
14	-	0/1	0/4	0/2	0/2	0/5	0/5	0/5
15	0/4	-	-	0/4	0/2	0/5	0/5	0/5
16	0/4	-	-	0/8	0/2	0/5	0/5	0/5
17	0/4	-	-	0/6	0/4	0/5	0/5	0/5
18	0/6	-	-	0/6	0/2	-	-	-
19	0/4	-	-	0/2	0/4	1/5[b]	0/5	0/5
20	0/4	-	-	0/4	0/2	0/5	0/5	0/5
Total	10/94 (10.6)	¼ (25.0)	2/12 (17.0)	4/110 (3.6)	0/68 (0)	1/74 (1.4)	0/74 (0)	1/74 (1.4)

[a] Total number of samples processed includes the duplicate samples. [b] Isolation of *S.* Gallinarum. [c] Isolation of motile *Salmonella* spp.

Table 6: Multi-serotype contamination in eight *Salmonella*-positive environmental hen houses with fowl typhoid outbreaks.

Number of houses (County, type of poultry house)	Number of serotypes isolated	Serotypes isolate					
		Feed	Feces	Egg nest	Boot swab	Eggshell pool	Egg yolk pool
1 (Paraná, manual)	1	SG[a]	-	-	-	-	-
2 (Santa Fe, manual)	1	-	SG	-	-	-	-
4 (Gualeguaychu, floor backyard chickens)	3	-	-	Cerro	Schwarzengrund	-	SG
5 (Paraná, automatic)	1	-	Enteritidis	-	-	-	-
7 (Santa Fe, manual)	4	-	Corvallis, Mbandaka, Livingstone, Cerro	-	-	-	-
10 (Diamante, manual)	4	Montevideo SG	Livingstone, Infantis	-	-	-	-
11 (Diamante, automatic)	3	-	Heidelberg, Corvallis, Derby	-	-	-	-
19 (Gualeguaychu, manual)	1	-	-	-	-	SG	-

[a] SG: *S.* ser. Gallinarum biovar Gallinarum.

Table 7: Relative sensitivity (RSe) and agreement (kappa coefficient) for selective-differential plating media used in feed and feces for *Salmonella* isolation from hen houses of Entre Ríos and Santa Fe, Argentina, with fowl typhoid outbreaks.

Type of sample	Media	Relative sensitivity	Kappa coefficient [a]	*p*-value [b]
Feed	XLDT	0.50[A] (0.15-0.85)	0.00	0.617
	EF-18	0.50[A] (0.15-0.85)		
Feces	XLDT	0.92[A] (0.62-1.00)	0.79	0.625
	EF-18	0.83[A] (0.54-0.96)		

[A,B] Values with different superscripts in the same column are significantly different ($p<0.05$), [A] Kappa is significantly non-zero (P<0.05) [B] Determined with McNemar's chi-square test for paired samples. XLDT = xylose lysine desoxicholate agar plus 4.6 mL/L of tergitol 4.

Analysis of risk factors associated with *Salmonella* Gallinarum outbreaks

All hen houses with fowl typhoid outbreak that had received the SG 9R vaccine (30%, 6/20) were positive for isolating SG in tissue samples. On the other hand, from the other 14 poultry houses with fowl typhoid outbreak, SG was isolated from all houses, and only SE was isolated in one of them (from feces). Furthermore, 19 poultry houses, free from *Salmonella* spp., were vaccinated with SG 9R.

Table 8 shows the results of univariate analysis for risk factors identification of SG isolation in a hen house as the sampling unit. Ten variables were significant for this step of analysis ($p<0.15$): number of hen/poultry houses, equipment by ingress of people, disposal of dead poultry, insect control, bacteriological test of drinking water, number of hens, number of hens per cage, hen ages, antibiotics use, and SG vaccination.

Moreover, the multiple logistic regression analysis included these 10 variables (Table 9). Fly control ($p=0.010$) and SG vaccination ($p=0.002$) were significant and turned out to be a risk factor for SG presence. The OR for these variables were 25 and 93, respectively. Therefore, the laying-hen houses that did not apply insect control and SG vaccination had 25 and 93-fold greater odds for SG presence than the hen houses that used these control strategies. Also, the Hosmer-Lemeshow test was insignificant ($p=0.73$), indicating that the model fits the data.

Discussion

This study reports on the occurrence of *Salmonella* spp. in 19 laying hen farms with FT outbreaks in Entre Rios, Argentina, using animal and environmental samples. Of them, four poultry houses belonged to backyard laying hens, and 16 poultry houses were industrial laying hens. To our knowledge, there is a lack of research on SG and other *Salmonella* serovars in Argentina. Examining samples originating from chickens showed significant variations in the incidence of SG according to gender, breed, style of raising, economic use, and growth stage (Zhou et al., 2022). For SG isolation, the WOAH (2018) recommends tissue samples from infected birds, such as their caecal tonsils, liver, and spleen, rather than environmental and fecal samples. Because of this bacteria's irregular shedding and the low sensitivity of bacteriological detection techniques, fecal and environmental samples are not the ideal samples for identifying the existence of *Salmonella* (Soria et al., 2011; 2012b; 2013a; WOAH, 2018). This aligns with our results, where we only isolated SG from feed, feces, eggshell pool, and egg yolk pool from 2, 1, 1, and 1 poultry houses, respectively. We used the Salmosyst broth method for feed samples, which Soria et al. (2013b) could recover SG from 3×10^1 colony forming unit (CFU)/25 g onward in artificially contaminated poultry feed. The presence of SG in this matrix indicated the possible route of entry of this bacterium in hens. In this study, we detected the occurrence of SG in 23–78% of the tissue samples. Indeed, bone marrow, spleen, liver, and ovarian follicle content samples showed the highest SG rate isolation (64–78%). Soria et al. (2023), who used the same technique for *Salmonella* isolation, found that 31–37% of the same organ samples were positive for SG isolation from sacrificed layers in a naturally occurring FT outbreak. On the other hand, Haque et al. (2021) detected SG in 42% of dead hens. However, this bacterium was isolated based on selective enrichment of the samples in Rappaport Vassiliadis Soya Broth following streaking on XLD agar and in farms with low-biosecurity measures. So, from the same hen samples, the rate of SG isolation depends on whether the hen is alive or not, the technique used for isolation, and whether there is an outbreak.

Table 8: Results from univariate analysis for risk factors identification of *Salmonella* biovar Gallinarum (SG)*.

Variable		Number of houses	% of positives hen houses for SG	p-value
County	Parana and La Capital	19	52.6	0.752
	Others counties	21	47.6	
Number of hen/poultry house	< 5 poultry houses	19	63.2	0.113
	≥ 5 poultry house and backyard hen	21	38.1	
Knows about regulation N° 542/2010	No	30	43.3	0.273
	Yes	10	70.0	
Fenced	No	6	66.7	0.661
	Yes	34	47.1	
Tracking compliance on distance with neighboring farms	No	16	62.5	0.242
	Yes	23	43.5	
Disinfection equipment	No	25	48.0	0.744
	Yes	15	53.3	
Equipment by ingress of people	No	33	60.6	0.008
	Yes	7	0.0	
Other species in the farm	No	5	20.0	0.342
	Yes	35	54.3	
Disposal of dead poultry	Open systems	20	65.0	0.058
	Close systems	20	35.0	
Fly control	No	16	81.3	0.003
	Yes	24	29.2	
Rodent control	No	6	83.3	0.182
	Yes	34	44.1	
Bound registration book	No	38	52.6	0.487
	Yes	2	0.0	
Feed sources	Own production	25	56.0	0.327
	Purchases to third parties	15	40.0	
Feed presentation	Floor	34	55.9	0.182
	Pellet or both	6	16.7	
Bacteriological test of drinking water	No	24	70.8	0.003
	Yes	16	18.8	
Egg fiber carton reuse	No	5	80.0	0.342
	Yes	35	45.7	
Number of hens	< a 10,000	18	33.3	0.057
	≥ a 10,000	22	63.6	
Number of hens per cage	2 to 4 hens	26	36.0	0.048
	5 and backyard	14	71.4	
Hen age	< a 72 weeks	25	60.0	0.146
	≥ a 72 weeks	15	35.7	
Antibiotics use	No	27	33.3	0.006
	Yes	13	84.6	
SG vaccination	No	15	92.9	0.000
	Yes	25	26.1	

*40 farms were used for data analysis; 20 had fowl typhoid outbreaks (Table 1), and 20 houses were negative for SG isolation and SG antibodies, previously reported by Soria (2013).

Table 9: Multiple logistic-regression model of risk factors for *Salmonella* ser. Gallinarum biovar Gallinarum (SG) isolation.

Variable	OR[a]	*p*-value
Number of hen/poultry house	5.63	0.263
< 5 poultry house		
≥ 5 poultry house and backyard hen (ref.)[b]		
Equipment by ingress of people	< 0.00	1.000
Yes		
No (ref.)		
Disposal of dead poultry	1.50	0.850
Close systems		
Open systems (ref.)		
Fly control	25.06	0.010
Yes		
No (ref.)		
Drinking water bacteriological test	1.70	1.000
Yes		
No (ref.)		
Numbers of hens	0.18	0.140
≥ a 10,000		
< a 10,000 (ref.)		
Number of hens per cage	< 0.00	1.000
5 and backyard		
2 a 4 hens (ref.)		
Hens ages	1.40	0.818
≥ a 72 weeks		
< a 72 weeks (ref.)		
Antibiotics uses	0.41	0.463
Yes		
No (ref.)		
SG vaccination		
Yes	92.85	0.002
No (ref.)		

*40 farms were used for data analysis; 20 had fowl typhoid outbreaks (Table 1), and 20 houses were negative for SG isolation and SG antibodies, previously reported by Soria (2013). [a] OR: odds ratio. [b] Ref.: Indicate the reference variable to OR calculation

Our results also showed that at least 2 dead hens/poultry houses in a suspected natural FT outbreak could be used to have a chance of isolating this bacterium from spleen, liver, ovarian follicle content, or bone marrow. These implied fewer animals were needed to detect SG than was proposed to sacrifice hens in the natural FT outbreak (Soria et al., 2023).

In a pure culture, SG grows well in non-selective media. However, chemicals in enrichment and selective media might prevent the growth of extraneous organisms (WOAH, 2018). Different authors reported an agreement of very good and excellent for SG isolation from liver, spleen, and bone marrow from dead hens using MCA and SSA in monitoring study (Soria, 2013) and the sacrificed layers in a naturally occurring FT outbreak (Soria et al., 2023). Using MCA/SSA, we found the same outcomes in our investigation.

Because paratyphoid *Salmonellae* are persistent in the environment, there are always opportunities for infection to spread horizontally within and between flocks (Gast and Porter, 2020). The environmental matrices most reported in the literature for detecting *Salmonella* in laying hens were dust and feces (Pacholewicz et al., 2023). The last was the sample in which we found the highest rate of *Salmonella* isolation among environmental samples. Furthermore, we isolated 8 paratyphoid *Salmonellae* serotypes (in addition to SG), namely: *S.* ser. Cerro, *S.* ser. Livingstone, SE, *S.* ser. Derby, *S.* ser. Corvallis, *S.* ser. Infantis, *S.* ser. Mbandaka, and *S.* ser. Heidelberg. One of the most prevalent serovars of *Salmonella* in humans in Argentina and in several countries is SE (Chaname-Pinedo et al., 2023). Although we only isolated SE from feces in the automatic hen house, fecal shedding and intestinal colonization by this serovar in laying hens can be impacted by stocking density. SE could be detected for up to 10 weeks post inoculation by hens in different housing treatment groups (Gast et al., 2017), which is challenging to implement control measures.

Salmonella may originally contaminate the

eggs during their development within the ovary or during their passage through the oviduct, and horizontal transmission may occur through trans-shell contamination (Roy Rodriguez et al., 2015; Shaji et al., 2023). On the other hand, the cloaca of birds is a crucial colonization site and plays a role in subsequent egg infection (El-Tras et al., 2010). Collecting eggs during the sampling is a direct epidemiological measure of the farm's health status (Holt et al., 2011). Furthermore, usually, the contents of 10 to 30 eggs are pooled for culturing because of the low incidence and level of *Salmonella* contamination (Soria et al., 2017). This criterion was used in our study. Soria et al. (2012a) could recover SG from the egg content in the lowest dilutions tested (5 to 13 CFU/25 mL).

On the other hand, Betancor et al. (2010) studied the prevalence of *Salmonella enterica* in eggs in Uruguay from 620 pools of eggs belonging to 21 poultry farms, only four of which were positive for SG. Furthermore, *Salmonella* spp. was not detected in 240 eggs; each sample consisted of six eggs from conventional battery-cage systems (Solis et al., 2023). Although the previous works were not carried out on farms with FT outbreaks, the negative results of the egg samples in this work align with the low prevalence found by these authors.

Salmonella may originally contaminate the eggs during their development within the ovary or during their passage through the oviduct, and horizontal transmission may occur through transshell contamination (Roy Rodriguez et al., 2015; Shaji et al., 2023). On the other hand, the cloaca of birds is a crucial colonization site and plays a role in subsequent egg infection (El-Tras et al., 2010). Collecting eggs during the sampling is a direct epidemiological measure of the farms. Soria et al. (2011; 2012b) compared the RSe of XLDT agar and EF-18 agar in artificially contaminated feed and fecal samples for motile and non-motile *Salmonella* strains, utilizing the same culture method presented in the current study. They could not find any differences between these media. However, Soria et al. (2017) found that EF-18 agar had better relative sensitivity than XLDT agar for *Salmonella* spp isolation from naturally contaminated feed samples. In the current study, it was observed that both EF-18 and XLDT agar had the same relative sensitivity for the feed samples, although there was a low percentage of *Salmonella* spp. isolation. This also agrees with Poppe et al. (2004), who indicated that non-motile *Salmonella* strains represent less than 1% of the isolates from feed balanced for animal consumption.

Implementing effective biosecurity measures is essential to preventing *Salmonella* spp. from spreading and enhancing food safety (Shaji et al., 2023). *Salmonella* is widely distributed in flies and less frequently in beetles and mites. Farms offer excellent and suitable niches such as manure, dust, spilled feed, and long production periods without cleaning (Wales et al., 2010; Zamora-Sanabria and Molina Alvarado, 2017). We detected that laying hen houses that did not implement insect control were more likely to have SG in their facilities. In contrast to these findings, Soria (2013) did not find a potential risk of flies in *Salmonella* infection based on a yes or no analysis.

One crucial strategy for successfully reducing the infection of virulent strains, such as *Salmonella* serotypes, in chicken flocks is the implementation of systematic vaccination programs (Haque et al., 2021). Vaccines are used to increase resistance to infection and may improve the short-term responsiveness of control programs, but the problems are not entirely eliminated (Zamora-Sanabria and Molina Alvarado, 2017). The live vaccine SG 9R contains a rough SG strain that can also offer partial protection against *Salmonella* Enteritidis.

Although vaccination with SG 9R may lower flock losses, field strain infection cannot be avoided. The SG 9R immunization demonstrated that although it guarantees good protection, fecal shedding has been observed for long to 24 hours after vaccination, and it may revert to the virulent smooth type that caused several outbreaks (WOAH, 2018; Farhat et al., 2024). We determined that laying-hen houses that did not apply SG vaccination had 93-fold greater odds for SG presence than in vaccinated flocks. However, hen houses whose birds received this vaccine (6/20) were positive for SG isolation in organ samples from dead birds, even in flocks that have received vaccinations. This can be explained by the inability of these vaccines to provide sterile immunity (Toka and Geinor, 2023). On the other hand, from 14 poultry houses that were not vaccinated with SG 9R, only SE was isolated from the fecal samples of one house. This finding could explain why this last serovar was not isolated in the vaccinated farm.

Conclusion

This study shows the importance of considering different types of samples and detection methods during a fowl typhoid outbreak study. Bone marrow, spleen, liver, and ovarian follicle content are the best samples for SG isolation from dead hens. Furthermore, there are no statistical differences for *Salmonella* species isolation between MacConkey agar and *Salmonella-Shigella* agar in these samples, and EF-18 agar and xylose lysine deoxycholate agar supplemented with tergitol 4 in feed and feces samples. Our study also highlights the importance of feed as a source for SG in layers and the implementation of sound biosecurity practices such as fly control and SG vaccine to control this bacterium. This data could be valuable in establishing better control programs and intervention strategies at the farm level to reduce *Salmonella* spp. contamination of animals.

Article Information

Funding. This work was supported by PhD fellowship and grant from Instituto Nacional de Tecnología Agropecuaria (PDI 114).

Conflict of interest. The author declared no conflict of interest.

Acknowledgment. We thank Marcelo L. Signorini (Instituto Nacional de Tecnología Agropecuaria EEA Rafaela, Argentina) for statistical assistance.

Authors contribution. Conceptualization, M.A.S. and D.J.B., funding acquisition, D.J.B., investigation, M.A.S., E.I.G., L.E.L. and D.J.B., methodology, M.A.S., and D.J.B., project administration, D.J.B., supervision, visualization, and writing—original draft preparation, M.A.S, and D.J.B., writing—review and editing, M.A.S., A.A.S and D.J.B. All authors have read and agreed to the published version of the manuscript.

Data availability. All data are shown in the manuscript and tables.

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