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EDITED BY

John J. Maurer,
Virginia Tech, United States

REVIEWED BY

Alejandro Piña-Iturbe,
Pontificia Universidad Católica de Chile, Chile
Chelea Matchawe,
Institute of Medical Research and Studies of
Medicinal Plants (IMPM), Cameroon

*CORRESPONDENCE

Luis Nuñez
✉ fabiann7@yahoo.es

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High contamination of *Salmonella* spp. in raw milk in Ecuador: molecular identification of *Salmonella enterica* serovars Typhi, Paratyphi, Enteritidis and Typhimurium

Anthony Loor-Giler^{1,2}, Camila Sanchez-Castro³,
Marcela Robayo-Chico³, Byron Puga-Torres⁴,
Silvana Santander-Parra⁵ and Luis Nuñez^{5,6*}

¹Laboratorios de Investigación, Dirección General de Investigación, Universidad de las Américas (UDLA), Quito, Ecuador, ²Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina, ³Facultad de Ingeniería y Ciencias Aplicadas, Carrera de Ingeniería en Biotecnología, Universidad de las Américas (UDLA), Quito, Ecuador, ⁴Facultad de Medicina Veterinaria y Zootecnia, Universidad Central del Ecuador, Quito, Ecuador, ⁵Facultad de Ciencias de la Salud, Carrera de Medicina Veterinaria, Universidad de las Américas, Quito, Ecuador, ⁶One Health Research Group, Facultad de Ciencias de la Salud, Universidad de las Américas, Quito, Ecuador

In Ecuador, the consumption of raw milk is fundamental, nonetheless it also presents risks due to the presence of *Salmonella*, a pathogen responsible for foodborne diseases (FBD). This study evaluated the prevalence of *Salmonella* spp. in raw milk from the provinces of Pichincha and Manabí, identifying the *Salmonella enterica* serovars Typhi, Paratyphi, Enteritidis and Typhimurium, in order to determine the risks associated with FBD. For this study, 600 raw milk samples were analyzed using a specific qPCR assay for *Salmonella enterica*, in parallel, bacterial isolation of the strains was performed according to ISO 6579-1:2017, and serotyped using endpoint PCR assays. Finally, the 16S rDNA of some isolates was sequenced and the identification of these isolates was confirmed. A prevalence of 37.5% ($n = 225$ positive samples) was obtained, where the climatic season had a significant influence on *Salmonella enterica* contamination, being higher in the warm season. In the analysis of serovars, *Salmonella Typhimurium* and *Salmonella Enteritidis* predominated with 62 and 60 samples respectively, followed by *Salmonella Paratyphi* with 32 samples and *Salmonella Typhi* with only 28 samples. A phylogenetic relationship was found between the 16 s samples sequences of this study and those previously reported in NCBI. The high prevalence of *Salmonella enterica* in raw milk intended for consumption in Ecuador represents a significant public health risk due to its high pathogenic potential. It is therefore essential to improve biosafety practices among livestock producers and implement sanitary control measures to reduce the risk posed by this pathogen in raw milk and similar food products.

KEYWORDS

Salmonella spp., raw milk, *Salmonella enterica* serovars, contamination, risk of infection

1 Introduction

In Ecuador, the consumption of dairy products has deep cultural roots, especially in rural areas, where production is a pillar of the economy and tradition (Vranješ et al., 2015; Puga-Torres et al., 2022). Raw milk and its derivatives are appreciated for their taste and their high nutritional value, as they contain high proportions of macronutrients (proteins, fats and carbohydrates), vitamins and minerals, which, in areas where food is limited, represent an indispensable basis of nutrition for these communities (Burgess, 2014; Thorning et al., 2016). Those same hyper-nutritious content is what makes raw milk the ideal medium for the growth of pathogenic microorganisms such as: *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus aureus*, and others (Quigley et al., 2013; Williams et al., 2023; Antoszevska et al., 2024). Among these, *Salmonella* spp. stands out as one of the main causes of foodborne diseases (FBD) worldwide, so its presence in unpasteurized dairy products represents a public health problem (Ohl and Miller, 2001; DeAngelis, 2024). According to the World Health Organization (WHO), 1.9 billion people suffer from infectious enteritis each year—resulting in 715,000 deaths—with one third of these cases linked to contaminated food, and *Salmonella* species responsible for 9% of foodborne diarrheal diseases (WHO, 2018; Pakbin et al., 2022).

Salmonella enterica, a member of the genus *Salmonella* and the *Enterobacteriaceae* family, is a gram-negative, frequently intracellular, bacillary pathogen (Cabello et al., 1993). It has a genome of approximately 5 Mb and in some serovars of *Salmonella enterica* subsp. *enterica*, a ~94 kb large virulence plasmid (pSLT) that contributes to pathogenesis through genes such as those in the *spv* operon. While pSLT is characteristic of serovars like Typhimurium, other clinically relevant serovars, such as Typhi lack this plasmid and instead rely on distinct virulence factors, including chromosomally encoded pathogenicity islands and, in the case of *S. Typhi*, the Vi capsular antigen (Johnson et al., 2018; Hiley et al., 2019; Knodler and Elfenbein, 2019). This subspecies includes most of the serotypes associated with infections in humans and animals, including the most common ones: *Salmonella Typhi*, *Salmonella Typhimurium*, *Salmonella Enteritidis*, and *Salmonella Paratyphi* (Knodler and Elfenbein, 2019). Salmonellosis is the name given to infection caused by bacteria of the genus *Salmonella*, which affects the gastrointestinal tract, although in certain cases it can lead to systemic disease (Terrier and Martinez, 2006). Based on disease presentation, symptoms and epidemiological characteristics, *Salmonella enterica* serotypes are classified as typhoidal, with *Salmonella Typhi* and *Salmonella Paratyphi* as the most distinctive, and non-typhoidal, such as *Salmonella Typhimurium* and *Salmonella Enteritidis* as the most distinguishing in regions such as North and South America, as well as Europe, according to WHO and European Food Safety Authority (EFSA) surveillance data; and may differ in other regions depending on local epidemiological patterns and food production systems (Knodler and Elfenbein, 2019; Daigle, 2021).

In Ecuador, the presence of *Salmonella* in food, especially meat, is widely documented, and its association with multidrug-resistant strains has been highlighted, which represents a growing risk to public health. In these, Typhimurium stood out as the most common serovar and the resistance genes CTX (for cephalosporins) and KPC-2 were found (Mejía et al., 2020; Mejía et al., 2021; Vinueza-Burgos et al., 2023; Villavicencio et al., 2024). Infections with non-typhoidal

serovars in humans produce an episode of enterocolitis lasting 4–7 days, causing abdominal pain, anorexia, nausea, vomiting, watery or mucoid diarrhea, rarely with blood (Ohl and Miller, 2001; Knodler and Elfenbein, 2019). Susceptibility to infection with these strains is highest in infants, elderly and immunocompromised persons (Chen et al., 2025). Most of the serovars classified as non-typhoid have a veterinary impact, especially in poultry and livestock, because they cause severe enteritis symptoms, with high mortality (Al-Adwani et al., 2013; Shaji et al., 2023; Aworh et al., 2024). In contrast, typhoid salmonellosis is severe, characterized by systemic spread of the pathogen, invading extraintestinal tissues (Cabello et al., 1993; Baqir et al., 2025). During infection for up to 30 days, in addition to the symptoms present in non-typhoid salmonellosis, weakness and pink skin patches on the extremities are present (Bhandari, 2024). Mortality due to typhoid salmonellosis is close to 10% in cases of non-resolution of infection with antibiotics, which increases in low-income countries (Pieters et al., 2018).

Controlling the occurrence of FBDs is a critical public health concern, small and sporadic outbreaks of salmonellosis have been described during the last century, mainly caused by contaminated food, with 52% of non-typhi and 37% of typhi serovars (Savage, 1929; Nelson and Snider, 1946; Ohl and Miller, 2001; Zizza et al., 2024). In Pennsylvania, USA 2007, 29 cases of salmonellosis were determined by consumption of cheese formulated from raw milk (Centers for Disease Control and Prevention (CDC), 2007). In Italy, in 2015, in relation to several cases of FBDs, high levels of contamination and non-identified bacterial counts were identified in raw milk for daily consumption (Chiarlone et al., 2025). Recently, in Poland, 50% contamination by pathogenic microorganisms was identified in organic food of animal origin, where 8% corresponded to *Salmonella enterica* (Sosnowski et al., 2024). In 2021, the Ministry of Public Health of Ecuador (MPS) identified 5,872 cases of undiagnosed food-borne infections, which could be related to the presence of bacteria, such as *Salmonella enterica*, in raw milk for daily consumption (MSP, 2021).

Taking into account the considerations outlined above and previously reported for *Salmonella* spp. in Ecuador, it is necessary to identify this bacteria with high zoonotic potential in raw milk, that contaminate high consumption and production foods, such as raw milk, which could cause FBDs. The present study aims to determine the prevalence of *Salmonella* spp. in raw milk from the two provinces with the highest production in Ecuador (Pichincha and Manabí), using isolation with selective media and molecular identification through a qPCR assay from the enriched milk; in addition, the presence of the serotypes Typhi, Paratyphi, Enteritidis and Typhimurium were determined from the bacterial isolates, thus determining the risk according to the type of salmonellosis that they would cause.

2 Methods

2.1 Sampling

In order to determine prevalence data, the sample number was calculated using the formula:
$$n = \frac{Z^2 * P_{exp} (1 - P_{exp})}{d^2}$$
 according to the

parameters previously described for an unknown population (Rodríguez

del Águila and González-Ramírez, 2014), where: n = required sample size; P_{exp} = expected prevalence; d = desired absolute precision (0.05 or 5%); z = statistic for a level of confidence (95%) = 1.96. The minimum number of total raw milk samples required would then be 384. A total of 600 raw milk samples were collected between July 2022 and June 2023 from two provinces of Ecuador (Pichincha = 300 and Manabí = 300), with approximately 50 samples collected per province per season using a simple random sampling during a climatic year. According to the National Institute of Meteorology and Hydrology (INAMHI), samples will be classified as “warm” if collected between June and September, a period characterized by drought and high temperatures of approximately 30°C; and “rainy” if collected between October and May, a period characterized by abundant rainfall and low temperatures of approximately 22°C. Additionally, the size of the producer will be taken into account, where according to the Ministerial Agreement No. 095 (MAG, 2022), those who own between 1 and 50 head of cattle will be classified as “small producers,” and those who own between 50 and 200 head of cattle will be classified as “medium producers.” Sample collection was carried out based on NTE INEN ISO 707 and Standard ISO 7218 [Instituto Ecuatoriano de Normalización (INEN), 2015]. Samples were collected in sterile containers and placed at 4°C until they were transported to the Research Laboratories of the Universidad de Las Americas (UDLA), where they were subjected to bacteriology and molecular processes necessary for the identification of *Salmonella* spp. All the processes carried out in this research were in accordance with the guidelines and approval of the Committee on the Care and Use of Laboratory and Domestic Animal resources of the Agency of Regulation and Control of Phytosanitary and Animal Health of Ecuador (AGROCALIDAD), under number #INT/DA/019.

2.2 Bacteria isolation and DNA extraction from pre-enrichment raw milk

The isolation of *Salmonella* spp. from raw milk samples was carried out in accordance with ISO 6579-1:2017 (Instituto Ecuatoriano de Normalización (INEN), 2015). Samples were placed

in Buffered Peptone Water (BPW), as a pre-enrichment method, and incubated for ~19 h at 200 rpm. After incubation, a 1 mL aliquot of the pre-enrichment was taken and subjected to DNA extraction using the GT Reagent in a phenol/chloroform based method, according to the previously described protocol (Dalla-Costa et al., 2017). In parallel, a portion of the enrichment was placed in Modified Semi-Solid Rappaport-Vassiliadis (MSRV) and incubated at 40°C for at least 24 h. Taking the greyish colored growth, it was seeded on Xylose-Lysine-Deoxycholate (XLD) agar and incubated at 37°C. After at least 19 h, red colonies with a black center or totally black colonies were picked and placed in 200uL of 1x TE Buffer, and DNA was extracted using a boiling-based method. Samples were frozen at –20°C overnight, subsequently heated to 95°C in a dry bath, centrifuged at 12,000 RCF for 10 min to pellet cellular debris, and 100uL of supernatant was in a new tube. DNA samples were placed at –20°C until use (Dashti and Dashti, 2009).

2.3 Standard curve construction

To prepare the calibration curve, the segment of the *ttr* locus corresponding to the qPCR assay to be used was amplified using the *ttr*-6 and *ttr*-4 primers (Table 1), the 2X Promega GoTaq Green Master Mix enzyme kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and applying the same thermocycling temperatures described previously (Malorny et al., 2004), and confirmed by SANGER sequencing (described in section 2.6). For this purpose, DNA extracted from *Salmonella Typhimurium* strain ATCC 14028 was used. The PCR product was purified with ExoSAP-IT™ Express PCR product Cleanup Enzyme (Applied Biosystems, Santa Clara, CA 95051, USA) and subsequently quantified on Nano Drop equipment (Thermo Fisher Scientific, California, CA, USA). Using the web-based DNA Copy Number and Dilution Calculator, the volumes needed to create a standard of 10⁹ copies of genetic material were determined. Then, 10 serial dilutions were performed to 1 copy of genetic material to determine the sensitivity of the assay.

TABLE 1 Primers and probes used in this study.

Name	Target		Assay	Sequence	Size	References
ttr-6	ttr locus	Salmonella spp	qPCR	CTCACCAGGAGATTACAACATGG	93 bp	Malorny et al. (2004)
ttr-4				AGCTCAGACCAAAAGTGACCATC		
ttr-5				TEXRED-CACCGACGCGAGACCGACTTT-BHQ2		
ST_227F	STY0307 gene	Salmonella Typhi	Duplex PCR 1	GGCAGATATACTTTCGCAGGCA	227 bp	Khokhar et al. (2022)
ST_227R				CCCAGAACCAAATTTGCTTACA		
SPAI_305F	SSPA1724 gene	Salmonella Paratyphi		CGCAGAGTGCAAGTGGAGT	305 bp	
SPAI_305R				GCATCCTCGGCCAGTCTTAC		
SEc-F	SEN0997 gene	Salmonella Enteritidis	Duplex PCR 2	GCCGAGCTTGATGACAAACCTG	171 bp	Park and Ricke (2015)
SEc-R				GCGCTTCGCTTTTCCAAGTCC		
STe-F	STM4497 gene	Salmonella Typhimurium		AACAACGGCTCCGGTAATGAGATTG	310 bp	
STe-R				ATGACAAACTCTTGATTCTGAAGATCG		
27-F	16 s rDNA	Bacteria	PCR	AGAGTTTGATCMTGGCTCAG	1.4 kb	Weisburg et al. (1991)
1,482-R				CGGTTACCTTGTTCAGACTT		

2.4 Detection of *Salmonella* spp. by qPCR

To determine the presence of *Salmonella* spp in the raw milk samples, and to confirm the veracity of the bacterial isolates, an adapted single-plex qPCR assay was run with the previously described primers and probes for *ttr* locus, changing FAM fluorophore to TexRED and quencher to BHQ2 (Table 1). The assay was run using TaqMan Universal Master Mix II, with UNG, 0.2 μ M of each primer, 0.1 μ M of each probe, 1 μ L of extracted DNA and made up to 10 μ L volume with UltraPure™ DNase/RNase-Free Distilled Water. The qPCR reactions were run on the CFX96 Touch Real-Time PCR Detection System thermal cycler (Bio Rad Laboratories, Inc., Hercules, CA 94547, USA). The reactions were run under the protocol: 1 cycle at 50°C for 2 minutes for the inactivation of UNG, 1 cycle at 95°C for 5 minutes for the initial denaturation, followed by 45 cycles at 95°C for 15 seconds for denaturation, 60°C for 45 seconds for reading and annealing, and 72°C for 30 seconds for extension. The assay was performed on DNA extracted from pre-enriched raw milk and from bacterial isolates of each sample. All reactions were run in duplicate, and each run included a positive control (*Salmonella* Typhimurium ATCC 14028) and a negative control (ddH₂O).

2.5 Identification of *Salmonella enterica* serovars Typhi, Paratyphi, Enteritidis and Typhimurium

The presence serovars of Typhi and Paratyphi using the duplex PCR 1 previously described protocol (Khokhar et al., 2022), and Enteritidis and Typhimurium using the duplex PCR 2 previously described protocol (Table 1) (Park and Rieke, 2015) were identified in DNA samples extracted from isolated strains of *Salmonella* spp. PCR reactions were run using 2X Promega GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.4 μ M of each primer, 1 μ L of DNA and made up to 10 μ L volume with UltraPure™ DNase/RNase-Free Distilled Water. Thermocycling protocols were run according to protocols previously described (Park and Rieke, 2015; Khokhar et al., 2022). The amplified samples were subjected to horizontal electrophoresis on 2% agarose gel stained with SYBR Safe DNA Gel Strain (Thermo Fisher Scientific) and compared with a 50 bp molecular weight marker (Thermo Fisher Scientific). Gels were analyzed using the ChemiDoc XRS + analysis system (Bio Rad) and identified based on molecular weight according to each target.

2.6 16S rDNA sequencing

Ten DNA samples were randomly selected from the pool of raw milk isolates Excel's RAND function, and subjected to 16S rDNA amplification to ensure representative variability (Weisburg et al., 1991). The protocol was run using 2X Promega GoTaq Green Master Mix enzyme (Promega, Madison, WI, USA), 0.3 μ M of primers 27F and 1482R (Table 1), 1 μ L of DNA extracted from the isolated bacteria and made up to 10 μ L volume with UltraPure™ DNase/RNase-Free Distilled Water. PCR products were subjected to horizontal 1.2% agarose gel electrophoresis and stained with SYBR™ Safe DNA Gel Strain (Thermo Fisher Scientific) and

compared with a 100 bp molecular weight marker (Thermo Fisher Scientific). Confirmed amplicons with a molecular weight of ~1,450 were purified with ExoSAP-IT™ Express PCR product Cleanup (Applied Biosystems, Santa Clara, CA 95051, USA) according to the manufacturer's instructions. The purified samples were sequenced forward and reverse using BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and the sequence reaction was read on the ABI 3500 Series Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed using Geneious software version 10.2.3¹ and compared with other *Salmonella* spp. sequences previously deposited in the GenBank, randomly selected to differentiate the sequences of *Salmonella bongori* and *Salmonella enterica*. Both sets of sequences were aligned using the Clustal X algorithm in ClustalX 2.1 (Larkin et al., 2007). A phylogenetic tree was then constructed using the MEGA X program (Kumar et al., 2016), using the Neighbor joining statistical method and the p-distance substitution model and phylogeny test bootstrap model with 1,000 bootstrap to differentiate the species of the *Salmonella* genus.

2.7 Statistical analysis

A descriptive statistic of the data obtained was constructed differentiating the presence of *Salmonella* spp. based on the origin of the samples, the size of the producer, and the climatic time of collection. Additionally, the differentiation of the isolated serovars was classified under these same parameters (Supplementary material). A heat map was constructed to discriminate the proportion of positives for each serovar studied in each month of collection corresponding to a climatic time using RStudio Software V2022.12.0, which was also used to determine the prevalence of *Salmonella* spp. in raw milk in Ecuador. The normality of the data was determined using a Shapiro–Wilk test. As the data were not normally distributed, Z-test tests were used to determine whether there were any statistically significant differences in the prevalence of *Salmonella* spp. between provinces (Pichincha vs. Manabí), producer sizes (small vs. medium) and climatic seasons (warm vs. rainy); and Cochran's Q test for serovars. The significance threshold for all tests was set at $p < 0.05$.

3 Results

3.1 Standard curve and sensitivity

The standard curve generated with the 9 serial dilutions in base 10 generated a curve with an efficiency of 97.6% and a correlation coefficient of 0.999 (Supplementary material). Samples with a Ct of <38 (equivalent to one copy) were considered positive. Since all points on the curve showed amplification, a limit of detection (LoD) of up to one copy of bacterial genetic material was established.

¹ <https://www.geneious.com>

3.2 Detection of *Salmonella* spp. in pre-enrichment milk by qPCR

Of the 600 raw milk samples analyzed in this study, the prevalence of *Salmonella* spp. was 37.5% (Table 2). Among the data studied, only differences between warm and rainy seasons (and not the parameters of size and locality) were found; showing that climatic season is a significant factor influencing *Salmonella* contamination rates in raw milk, regardless of province or producer size (Supplementary material). These data, in terms of climatic season, showed higher prevalence in warm weather, with 96.00% of the total positive samples in both provinces and for both producer sizes sampled ($n = 216/225$). Smallholder farms showed the highest prevalence of *Salmonella* spp. (81.78%, $n = 184$), compared to medium-sized producers. Particularly by province, Pichincha showed a slightly higher positivity for this bacterium with 55% of the total samples (Figure 1). Significant differences were identified only in the presence of *Salmonella* spp. based on the collection time (climate), where those classified as warm always showed a higher positivity ratio ($p < 0.001$). Based on the groups generated by these three parameters, the highest positivity rate was found in the group of samples collected from small producers in Pichincha during the warm season; with similar percentages in the same province and climate, but in medium-sized producers, no significant differences with the other specific groups ($p > 0.05$). Bacterial isolation of *Salmonella* spp., subsequently confirmed by qPCR, showed 94.67% agreement (213 samples) with direct detection from enriched milk and yielded a Cohen's kappa of 0.96, indicating a high level of concordance between both methods despite the greater sensitivity of the latter.

3.3 Identification of isolated serotypes Typhi, Paratyphi, Enteritidis and Typhimurium

Of the isolated samples, *Salmonella Enteritidis* and *Salmonella Typhimurium* were the most common serovars with 27.5 and 26.6% of the isolates, respectively, showing significant differences with *Salmonella typhi* ($p < 0.001$), which was the least present 12.44%

(Table 3). Of the analyzed isolates, 19.11% could not be classified in any of these serovars.

In relation to the climatic season in which the samples were collected, it was found that *Salmonella Paratyphi*, *Salmonella Typhimurium* and *Salmonella Typhi* had significant differences in terms of the positives found in months classified as warm and those classified as rainy ($p < 0.001$), being predominant in the first case. However, this behavior was visibly observed in only two of the months (Figure 2), so the high number of samples collected and subsequently found to be positive in the warm season could be a statistical bias. On the other hand, the serovar *Salmonella enteritidis* showed predominance in the months of October and February, corresponding to rainy, and in September, corresponding to warm (Figure 2); although maintaining similar values in both cases (Supplementary material). No positive samples were found in March and November.

3.4 Analysis of 16S rDNA sequences

The phylogenetic tree generated formed two general clades differentiated by containing the two species of the genus *Salmonella*. The first clade contains the *Salmonella bongori* sequences obtained from GenBank, and the other contains the *Salmonella enterica* sequences obtained in this study and those collected from NCBI distributed throughout the study (Figure 3). As we expected, the sequences obtained in this study showed a similarity of more than 98% with the sequences previously deposited in GenBank [Supplementary material (Matrix of Nucleotides)], showing their generic closeness and correspondence with this species.

4 Discussion

Microbiological contamination of raw milk is a relevant problem due to its rich nutrient composition, which makes it a favorable medium for the growth of pathogenic microorganisms (Rampling, 1996; Quigley et al., 2013; Antoszevska et al., 2024). Among these, *Salmonella* genus stands out as an agent of concern, both because of its pathogenic potential and its ability to spread through contaminated

TABLE 2 Distribution of positives values of *Salmonella* spp. classified by locality, size of producer and climate.

Real time PCR for detection of <i>Salmonella</i> spp					
Locality	Producer	Climate	(+) samples	(−) samples	Total samples
Pichincha	Small	Warm*	97 (59.87%)	65	162
		Rainy	6 (6.52%)	86	92
	Medium	Warm*	19 (52.77%)	17	36
		Rainy	2 (11.11%)	16	18
Manabí	Small	Warm*	80 (39.60%)	122	202
		Rainy	1 (2.43%)	40	41
	Medium	Warm*	20 (51.28%)	19	39
		Rainy	0 (0.0%)	10	10
Total			225 (37.50%)	375	600

*Significant differences according to statistical analysis compared with other parameters.

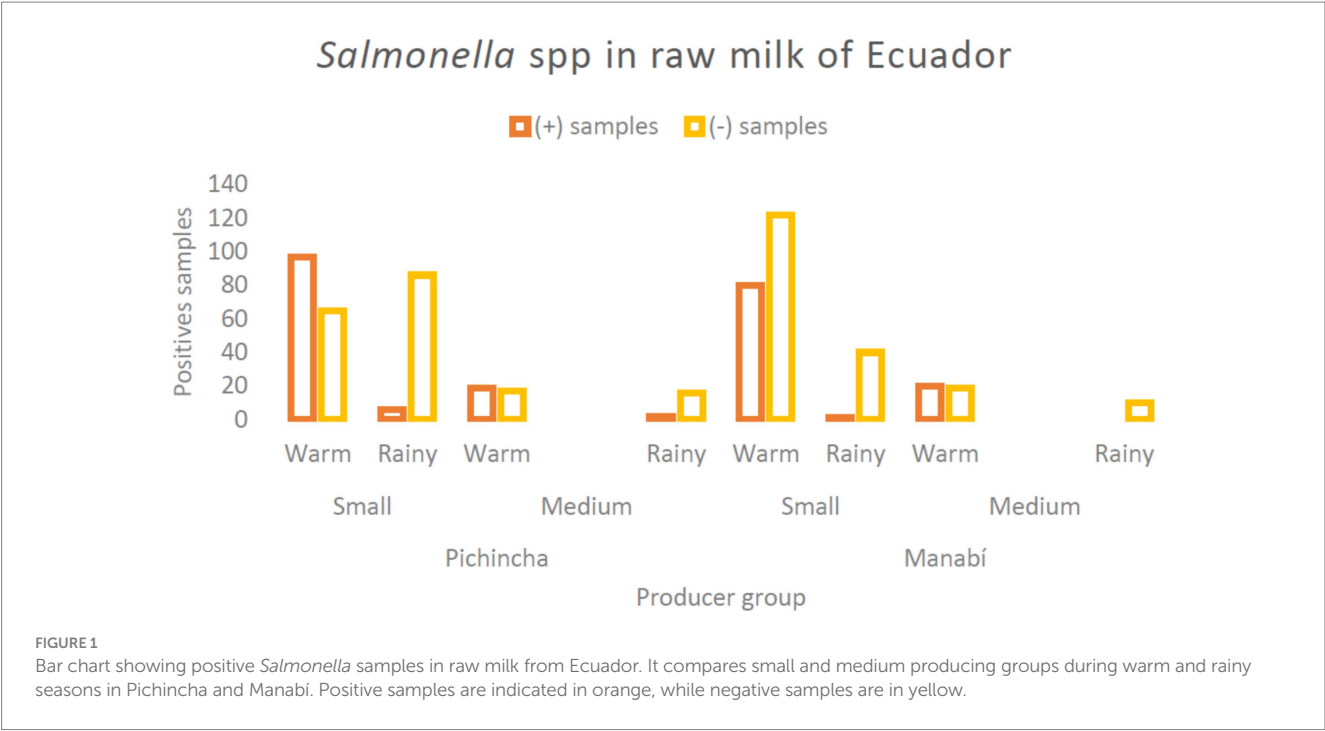
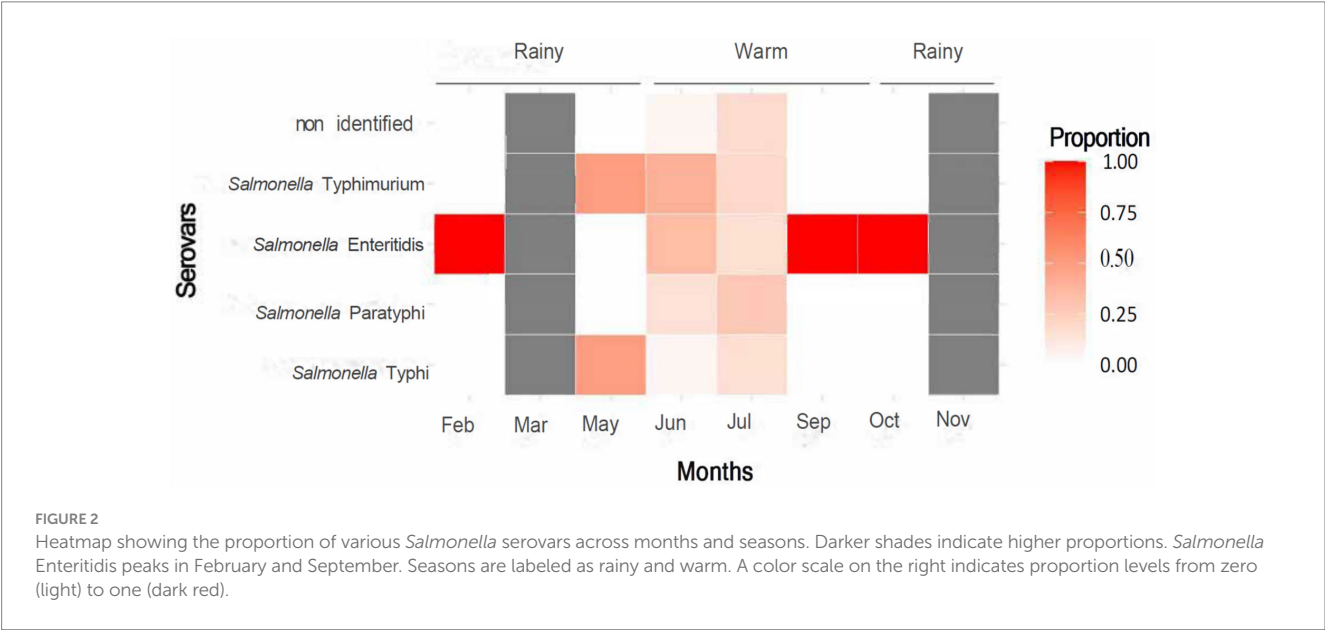


TABLE 3 Analysis of PCR results for serovars in identified *Salmonella* spp. samples.

Isolated samples	Identified serotypes				
	<i>S. Typhi</i>	<i>S. Paratyphi</i>	<i>S. Enteritidis</i>	<i>S. Typhimurium</i>	N ID
225	28	32	60	62	43
	(12.44%)	(14.22%)	(26.66%)	(27.55%)	(19.11%)

S., *Salmonella*; N ID, Non identified.



food (WHO, 2018; Bhandari, 2024). This problem has significant public health implications, especially in contexts where raw milk is consumed without adequate heat treatment (Vranješ et al., 2015). The use of PCR-based diagnostic methods for the early detection of contaminants in raw milk and other media, without the need for bacterial isolation, is presented as a highly efficient alternative with greater sensitivity and specificity (Malorny et al., 2004; Lopes et al., 2018; Demirci et al., 2019). This study shows the adaptation of a previously described qPCR protocol. The protocol was validated by constructing a standard curve and performing a sensitivity assay.

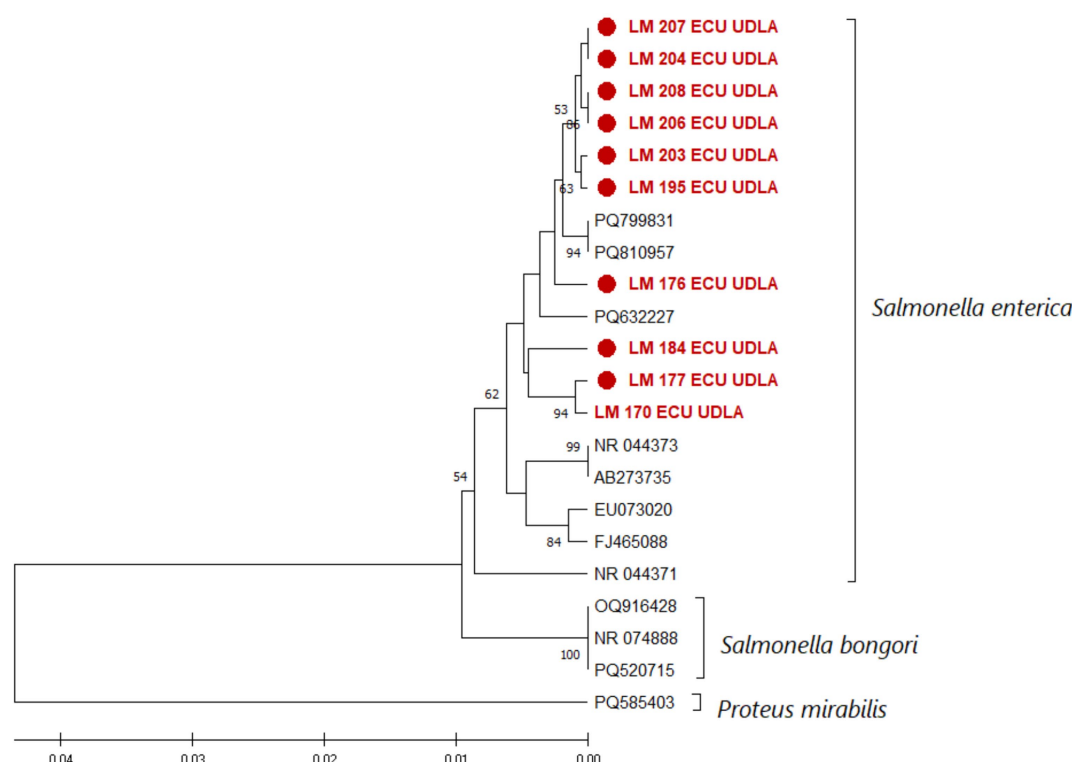


FIGURE 3

Phylogenetic relationships between the *Salmonella Enterica* sequences obtained here and other sequences collected from NCBI based on part of the 16S rDNA gene nucleotide sequence. The sequences were aligned in ClustalX 2.1. The phylogenetic tree was constructed using the MEGA X software package. Numbers along the branches refer to bootstrap values for 1,000 replicates. The scale bar represents the number of substitutions per site. A *Proteus mirabilis* 16S sequence was used as an outgroup. The sequences obtained in this study are shown in red and are marked with ●.

These tests showed that the protocol can detect as little as one copy of genetic material (Supplementary Figure 1). The protocol's efficiency meets the standards set forth in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). With this method, the presence of *Salmonella* spp. in raw milk used for consumption in Ecuador was demonstrated (Table 1).

A prevalence of 37.5% of *Salmonella* spp. was identified in the analyzed samples. The high presence of this pathogen in raw milk is presented as a risk for Ecuadorian public health, particularly in the populations that consume it. This value is consistent with previous studies in nearby regions that report the presence of this bacterium contaminating more than 30% of dairy products. This was particularly true in production systems that had inadequate hygiene (Dewey-Mattia et al., 2018; Barac et al., 2024). High contamination rates can be associated to different extra-insecure factors, such as inadequate practices during milking, storage and transport, as well as the lack of control systems (Agarwal et al., 2012). The data suggest a possible link between *Salmonella* spp. contamination and warmer seasons, though this may be influenced by other unmeasured factors (Table 1). This is similar to what has been previously described in studies conducted in Egypt, Ethiopia, Spain and Colombia, where bacterial proliferation in raw milk (highlighting the appearance of *Salmonella* species) more than doubled during the summer (warm seasons), compared to the cold season (Sospedra et al., 2009; Omar et al., 2018; Tobar-Delgado et al., 2020; Admasu et al., 2024). This may be due to the affinity of *Salmonella* spp. which, in spite of having the capacity to grow between 5° and 47°C, has a higher growth rate at

temperatures above 30°C (Cabello et al., 1993; Daigle, 2021). It is essential to enhance control measures and bio-sanitary monitoring aimed at specific pathogens that tend to proliferate more rapidly during the warmer seasons.

The predominance of serotypes Enteritidis (26.66%) and Typhimurium (27.55%) isolates for *Salmonella* spp. (Table 3) is concordant with studies previously conducted in China, Brazil and Ethiopia where these serovars of *Salmonella enterica* were the most common in samples of raw milk and its derivatives (Degenhardt et al., 2023; Wang et al., 2023a; Beyene et al., 2024). In 2018, a study on the sources of *Salmonella* spp. contamination, which also highlighted these serovars as the most commonly found, indicated that foods of animal origin are the main source of *Salmonella* spp. isolation (42.76% of cases) (Ferrari et al., 2019). These cases of contamination were mainly associated to noncompliance with good animal farming practices, including deficiencies in milking, storage and transportation of milk (Bedassa et al., 2023; Wang et al., 2023b; Antoszewska et al., 2024). A higher number of *Salmonella* spp. positive samples was found in small producers, although this may reflect sampling bias or confounding variables (Table 1). In 2021, it was described that small farmers in Ecuador have deficiencies in the implementation of good animal farming practices, which may be influencing the proliferation of this microorganism (Lowenstein et al., 2016). To respond to these deficiencies, regulatory agencies such as AGROCALIDAD should establish monitoring and control measures to reduce the high rates of contamination and consumption of contaminated foods that pose a public health risk, in addition to previously indicated guidelines (AGROCALIDAD, 2012).

The presence of the serovars Typhi and Paratyphi (Table 3), although found to a lesser extent than non-typhi serovars, are a public health concern because they are causative agents of typhoid and paratyphoid fever (Bhandari, 2024). As these serovars are only associated with human infections and have no animal reservoirs, their presence in raw milk is most likely due to human contamination, although contamination during sample collection cannot be ruled out (Agarwal et al., 2012; Soyer et al., 2013; LeBoa et al., 2023). While contamination of raw milk with typhoid serovars is uncommon, recent studies have demonstrated the presence of *Salmonella* Typhi in raw milk. This presence has been attributed to inadequate hygienic conditions during handling and storage (Kaur et al., 2018; Bai et al., 2019). The worrying appearance of these pathogens is a wake-up call to regulatory entities to raise quality standards and sanitary control in small and medium sized dairy farms to avoid future outbreaks associated with these bacteria. When differentiating the serovars of *Salmonella enterica* based on the climatic season of collection, a peculiar pattern was observed, in which the few samples found in the months grouped as “rainy” correspond mostly to serovar enteritidis. Heat stress, increased fly activity, and enhanced biofilm formation in warm conditions can all contribute to greater *Salmonella* spp. prevalence by promoting shedding, transmission, and environmental persistence (Traub-Dargatz et al., 2006; Borges et al., 2018; Shahanaz et al., 2025). Since the serovars studied here share similar growth capacities in terms of temperature, there is not enough evidence to associate it to any specific phenomenon (Cabello et al., 1993; Daigle, 2021). The differentiation method is applied for bacterial isolates, therefore it is necessary to use assays capable of detecting the simultaneous presence of more than one serovar in the same sample (such as enriched milk) by applying qPCR methods like the one already used in this study to identify the presence of *Salmonella* spp. directly from milk (Postollec et al., 2011).

Given that it was not possible to assign a serovar in 19.11% of the samples positive for *Salmonella* spp. (Table 3), the need for future studies that address serovars that are fundamental for both animal health and possible zoonotic events is highlighted. These unidentified samples may correspond to a wide variety of serovars, among them: *Salmonella* Dublin, mainly associated with infections in cattle, *Salmonella* Newport, associated with fatal cases due to food consumption, or *Salmonella* Heidelberg, found in dairy products in a few cases (Clegg et al., 1983; Self et al., 2023; Velasquez-Munoz et al., 2024). Considering account that about 2,500 serotypes of *Salmonella enterica* have been proposed, where <100 are responsible for human infections; the analysis of all these is only feasible using metagenomic sequencing techniques (Cabello et al., 1993; Nichols et al., 2020). Given the serious pathogenic effect of typhoid serovars, and that serovars Enteritidis and Typhimurium comprise up to 75% of salmonellosis cases, these are a priority for diagnosis (WHO, 2018). It is important to note that in 2019, typhoid *Salmonella* serovars revealed 110,000 associated deaths around the world, while, despite their lower pathogenicity, 155,000 deaths from non-typhi *Salmonella* are estimated each year (Majowicz et al., 2010; WHO, 2023). There is no reliable data on mortality due to salmonellosis in Ecuador, however in 2023 a study in Colombia indicated that *Salmonella* spp. continued to be a cause of mortality due to foodborne infection (Rosso et al., 2023). It is important to assess the impact on public health of *Salmonella* spp.

contamination in food, both as reported in this study and possibly present in other foods.

Analyzing the *16S* gene sequences of the 10 bacterial isolates, as expected, all the sequences obtained clustered with sequences of *Salmonella enterica* previously reported in the NCBI (Figure 3). These results reaffirm the presence of this pathogen in the selected raw milk samples. Among the sequenced samples, those for which the serovar could not be identified also showed high levels of similarity with the other sequences obtained (Supplementary material). Nevertheless, the use of *16S* to assign a serovar is not reliable, and more specific techniques such as qPCR or NGS are necessary (Weisburg et al., 1991; Postollec et al., 2011). Although the sequencing of bacterial isolates serves as a form of specific identification of bacteria genus, its high cost and execution time (including the bacterial isolation process) make it inefficient compared to updated methods such as qPCR assays. Early and specific detection allows adequate control and containment measures to minimize the risk to public health that contaminants such as *Salmonella* spp. represent. Further research is required to implement simultaneous detection and quantification of prevalent serovars directly from contaminated materials, to assess the extent of the issue in agriculture, hence reducing costs and application time.

5 Conclusion

The presence of *Salmonella* spp. in raw milk highlights the significant public health risks associated with its consumption, particularly in regions where proper heat treatment is not practiced. The application of qPCR methods demonstrated high sensitivity and specificity. This enabled the direct detection of *Salmonella* spp. without the need for time-consuming bacterial isolation. The identification of serovars associated with both animal and human contamination underscores the importance of improving hygiene practices during milking, storage, and transportation. Strengthening regulatory surveillance and adopting molecular diagnostic tools are critical steps to reduce contamination rates and prevent foodborne illnesses. These findings provide valuable evidence to influence in national food safety policies and support the development of targeted surveillance programmes aimed at reducing the risk of *Salmonella* transmission through raw milk.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was approved by Committee on the Care and Use of Laboratory and Domestic Animal resources of the Agency of Regulation and Control of Phytosanitary and Animal Health of Ecuador (AGROCALIDAD), under number INT/DA/019. The study

was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AL-G: Data curation, Formal analysis, Writing – original draft, Conceptualization, Investigation. CS-C: Data curation, Methodology, Writing – review & editing. MR-C: Investigation, Writing – review & editing, Methodology. BP-T: Conceptualization, Writing – review & editing, Methodology. SS-P: Conceptualization, Writing – review & editing. LN: Writing – review & editing, Investigation, Conceptualization, Methodology, Funding acquisition, Project administration, Validation.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2025.1593266/full#supplementary-material>

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