UNIVERSIDADE FEDERAL DA PARAÍBA
CENTRO DE CIÊNCIAS AGRARIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

ALAN DOUGLAS DE LIMA ROCHA

GENOMA COMPLETO DE CEPAS DE *SALMONELLA ENTERICA* ISOLADAS DE CORPOS D'ÁGUA DA PARAÍBA SOB IMPACTO AGROPECUÁRIO: DIVERSIDADE E RESISTÊNCIA ANTIMICROBIANA

AREIA
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Tese apresentada ao Programa de Doutorado da Pós-Graduação em Zootecnia da Universidade Federal da Paraíba como parte dos requisitos para obtenção do título de Doutor em Zootecnia.

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Prof Dr. Celso José Bruno de Oliveira

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RESUMO

A *Salmonella enterica*, um agente patogénico zoonótico difundido de origem alimentar, continua a ser uma preocupação significativa de saúde pública e um fardo económico a nível mundial. Estima-se que *Salmonella* spp. causem 93,8 milhões de casos de gastroenterite em todo o mundo anualmente, levando a 59.100 mortes. Nos Estados Unidos (EUA), estima-se que a gastroenterite causada apenas por *Salmonella* não tifóide afete aproximadamente um milhão de pessoas anualmente, resultando em aproximadamente 3,7 bilhões de dólares em custos médicos. Apesar dos esforços históricos de contenção centrados na transmissão oral-fecal e no controlo de qualidade na produção de alimentos, a incidência de *Salmonella* persiste, com surtos preocupantes associados a alimentos à base de plantas nos últimos anos. Uma subestimação da *Salmonella* em ambientes aquáticos e a sua potencial persistência a longo prazo levanta preocupações sobre o papel da água na epidemiologia da *Salmonella*. Estudos recentes veem mostrando que a capacidade da *Salmonella* de permanecer viável em ambientes não entéricos é consideravelmente maior do que se acreditava anteriormente. Além disso, a *Salmonella* não é um alvo microbiano para os métodos normalmente utilizados para avaliar a qualidade da água. Esses aspectos podem apontar para a possibilidade de que a presença e persistência de *Salmonella* em ambientes aquáticos possam estar subestimadas. Este estudo investigou os fatores ambientais que influenciam a *Salmonella* em bacias hidrográficas naturais utilizadas para fins agrícolas e pecuários na Paraíba, Brasil. Além disso, realizou o levantamento dos principais sorovares envolvidos na contaminação destes recursos hídricos.

Palavras-chave: recursos hídricos; fatores de risco; sequenciamento genômico.

COMPLETE GENOME OF SALMONELLA ENTERICA STRAINS ISOLATED FROM WATER BODIES IN PARAÍBA UNDER AGRICULTURAL IMPACT: DIVERSITY AND ANTIMICROBIAL RESISTANCE

ABSTRACT

Salmonella enterica, a widespread zoonotic foodborne pathogen, remains a significant public health concern and economic burden worldwide. It is estimated that *Salmonella* spp. cause 93.8 million cases of gastroenteritis worldwide annually, leading to 59,100 deaths. In the United States (US), gastroenteritis caused by non-typhoid *Salmonella* alone is estimated to affect approximately one million people annually, resulting in approximately $3.7 billion in medical costs. Despite historic containment efforts focused on oral-fecal transmission and quality control in food production, the incidence of *Salmonella* persists, with concerning outbreaks associated with plant-based foods in recent years. An underestimation of *Salmonella* in aquatic environments and its potential long-term persistence raises concerns about the role of water in *Salmonella* epidemiology. Recent studies have shown that *Salmonella*'s ability to remain viable in non-enteric environments is considerably greater than previously believed. Furthermore, *Salmonella* is not a microbial target for methods typically used to assess water quality. These aspects may point to the possibility that the presence and persistence of *Salmonella* in aquatic environments may be underestimated. This study investigated the environmental factors that influence *Salmonella* in natural watersheds used for agricultural and livestock purposes in Paraíba, Brazil. Furthermore, it carried out a survey of the main serovars involved in the contamination of these water resources.

Keywords: water resources; risk factors; genomic sequencing.

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LISTA DE ABREVIAÇÕES

AESA Agência Executiva de Gestão de Águas

AMP Ampicillin

AMR Aminoglycosides resistance

AOAC Association of Official Agricultural Chemists.

ARGs Antimicrobial resistance genes

ARM Average rain month

ARW Average rain week

AST antimicrobial susceptibility testing

AUG2 Amoxicillin/clavulanic acid 2:1

AXO Ceftriaxone

BAM Bacteriological Analytical Manual

BC-PCR Broth cultivation-polymerase chain reaction

BHI brain heart infusion broth

BS Bismuth sulfite agar

CFX Cefuroxime

CHL Chloramphenicol

CIP Ciprofloxacin

CLSI Clinical and Laboratory Standards Institute

CO Conductivity

DNA Deoxyribonucleic acid

DO Dissolved oxygen

DR Day rain

ENO Enrofloxacin

ERT Ertapenem

FLF Florfenicol

FOM Figure of merit

GEN Gentamicin

HDI Human development index

HE Hektoen enteric agar

LASSO Least Absolute Shrinkage and Selection Operator

LIA Lysine iron agar

MeSH Medical Subject Headings

MFX Moxifloxacin

MMS Modified Moore swab technique

MR Monthly rain

NAL Nalidixic acid

OFX Ofloxacin

PCR Polymerase chain reaction

PDR Previously day rain

PFGE Pulsed-field gel electrophoresis

pH Hydrogen potential

Ph.D. Philosophy Doctor

PICO Population, intervention, comparison, and outcome method

PRISMA Preferred Reporting Items for Systematic Reviews and Meta-Analyses

RE Resistivity

RMLE Restricted maximum-likelihood estimator

RV Rappaport-Vassiliadis Broth

SA Salinity

STs International sequence types

SYBR Synergy Brands

SXT Trimethoprim/sulfamethoxazole

TDS Total dissolved solids

TE Temperature

TET Tetracycline

TSA Tryptic soy agar

TSI Triple sugar iron agar

TT Tetrathionate broth

TU Turbidity

US United States of America

USA United States of America

UV Ultraviolet

VBNC Viable but nonculturable bacteria

WR	Weekly rain
XLT-4	Xylose Lactose Tergitol 4
XNL	Ceftiofur

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

A *Salmonella enterica* é um dos principais patógenos zoonóticos transmitidos por alimentos globalmente, causando sérios ônus econômico e de saúde pública. Ela ocupa o segundo lugar entre as doenças alimentares mais frequentes nos EUA, atrás apenas da campilobacteriose. Estima-se ser causa de 93,8 milhões de casos de gastroenterite anualmente em todo o mundo, resultando em 59.100 mortes. Nos Estados Unidos (EUA), a gastroenterite causada por *Salmonella* não tifoide afeta aproximadamente um milhão de pessoas anualmente, resultando em aproximadamente US$ 4,1 bilhões em custos médicos.

Durante o século XX, tornou-se consenso que a salmonelose é transmitida principalmente pela via oral-fecal, ou seja, através da ingestão de água ou alimentos contaminados por *S. enterica* originados de esgoto e resíduos animais. Portanto, políticas de controle de qualidade na produção, manipulação e armazenamento de alimentos de origem animal, assim como a conscientização sobre o consumo de água tratada, foram estabelecidas como meio para mitigar a salmonelose e outras doenças transmitidas por alimentos em consumidores. Essas medidas certamente contribuíram para a redução a longo prazo do número de casos observados no último século. No entanto, não foram observadas reduções significativas adicionais na incidência de salmonelose no século XXI. Pelo contrário, há uma incidência constante de salmonelose, afetando cerca de um milhão de pessoas por ano apenas nos EUA. Além disso, ao longo das últimas duas décadas, vários surtos de salmonelose foram rastreados até o consumo de alimentos de origem vegetal, levantando uma bandeira vermelha sobre a eficácia das medidas tradicionais de controle de *Salmonella* que costumavam ser aplicadas à indústria agroalimentar em todo o mundo.

A condição microbiológica da água utilizada na agricultura, independentemente da fonte, é crucial para a segurança dos produtos agroalimentares. Surto de salmonelose têm sido associados ao uso de água contaminada em ambientes agrícolas. Considera-se geralmente que o trato gastrointestinal de vertebrados é o habitat natural da *Salmonella enterica*, e o uso de água reciclada de sistemas de produção animal é considerado um importante fator de risco para a contaminação de produtos agrícolas. No entanto, a ocorrência de *Salmonella* em fontes de água pode ir além de descobertas acidentais de curto prazo determinadas pela presença transitória de bactérias como resultado de eventos de contaminação fecal dispersa. Mecanismos de viabilidade podem permitir que a *Salmonella* sobreviva com sucesso em ambientes aquáticos naturais por vários meses. Portanto, uma atenção maior tem sido dada tanto pela comunidade científica quanto pelos interessados na indústria agroalimentar ao papel potencial da água utilizada na irrigação e processamento de alimentos na epidemiologia da salmonelose.

Apesar do aumento no número de estudos relatando a presença de *Salmonella* em ambientes aquáticos naturais, não há acordo sobre sua frequência média e representação relativa de sorovares em fontes de água, bem como possíveis condições ambientais envolvidas na permancia da *Salmonella* em ambientes aquáticos. Esta tese de doutorado focou em tentar trazer respostas e dados que auxiliem nesta busca.

2 CAPÍTULO 1: REFERENCIAL TEÓRICO

Revisiting the biological behavior of *Salmonella enterica* in hydric resources: a meta-analysis study addressing the critical role of environmental water on food safety and public health

2.1 Abstract

The increasing number of studies reporting the presence of *Salmonella* in environmental water sources suggests that it is beyond incidental findings originated from sparse fecal contamination events. However, there is no consensus on the occurrence of *Salmonella* as its relative serovar representation across non-recycled water sources. We conducted a meta-analysis of proportions by fitting a random-effects model using the restricted maximum-likelihood estimator to obtain the weighted average proportion and between-study variance associated with the occurrence of *Salmonella* in water sources. Moreover, meta-regression and non-parametric supervised machine learning method were performed to predict the effect of moderators on the frequency of *Salmonella* in non-recycled water sources. Three sequential steps (identification of information sources, screening and eligibility) were performed to obtain a preliminary selection from identified abstracts and article titles. Questions related to the frequency of *Salmonella* in aquatic environments, as well as putative differences in the relative frequencies of the reported *Salmonella* serovars and the role of potential variable moderators (sample source, country and sample volume) were formulated according to the population, intervention, comparison, and outcome method (PICO). The results were reported according to the Preferred Reporting Items for Systematic Review and Meta-Analyzes statement (PRISMA). A total of 26 eligible papers reporting 148 different *Salmonella* serovars were retrieved. According to our model, the *Salmonella* frequency in non-recycled water sources was 0.19 [CI: 0.14; 0.25]. The source of water was identified as the most import variable affecting the frequency of *Salmonella*, estimated as 0.31 and 0.17% for surface and groundwater, respectively. There was a higher frequency of *Salmonella* in countries with lower human development index (HDI). Small volume samples of surface water resulted in lower detectable *Salmonella* frequencies both in high and low HDI regions. Relative frequencies of the 148 serovars were significantly affected only by HDI and volume. Considering that serovars representation can also be affected by water sample volume, efforts towards the standardization of water samplings for monitoring purposes should be considered. Further approaches such as metagenomics could provide more comprehensive insights about the microbial ecology of fresh water and its importance for the quality and safety of agricultural products.

Keywords: agriculture; epidemiology; foodborne pathogens; one health; salmonellosis; systematic review.

2.2 Resumo

O número crescente de estudos que relatam a presença de *Salmonella* em fontes de água ambientais sugere que está além de descobertas incidentais originadas de eventos esparsos de contaminação fecal. No entanto, não há consenso sobre a ocorrência de *Salmonella* quanto à sua representação relativa de sorovares em fontes de água não recicladas. Conduzimos uma meta-análise de proporções ajustando um modelo de efeitos aleatórios usando o estimador de máxima verossimilhança restrito para obter a proporção média ponderada e a variância entre estudos associada à ocorrência de *Salmonella* em fontes de água. Além disso, foram realizados meta-regressão e método não paramétrico de aprendizado de máquina supervisionado para prever o efeito dos moderadores na frequência de *Salmonella* em fontes de água não recicladas. Três etapas sequenciais (identificação das fontes de informação, triagem e elegibilidade) foram realizadas para obter uma seleção preliminar a partir dos resumos e títulos dos artigos identificados. Perguntas relacionadas à frequência de *Salmonella* em ambientes aquáticos, bem como supostas diferenças nas frequências relativas dos sorovares de *Salmonella* relatados e o papel dos potenciais moderadores de variáveis (fonte da amostra, país e volume da amostra) foram formuladas de acordo com a população, intervenção, comparação e método de resultado (PICO). Os resultados foram relatados de acordo com a declaração Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA). Um total de 26 artigos elegíveis relatando 148 sorovares diferentes de *Salmonella* foram recuperados. De acordo com nosso modelo, a frequência de *Salmonella* em fontes de água não reciclada foi de 0,19 [IC: 0,14; 0,25]. A fonte de água foi identificada como a variável mais importante que afeta a frequência de *Salmonella*, estimada em 0,31 e 0,17% para águas superficiais e subterrâneas, respectivamente. Houve maior frequência de *Salmonella* em países com menor índice de desenvolvimento humano (IDH). Amostras de pequeno volume de águas superficiais resultaram em frequências detectáveis de *Salmonella* mais baixas, tanto em regiões de alto como de baixo IDH. As frequências relativas dos 148 sorovares foram significativamente afetadas apenas pelo IDH e pelo volume. Considerando que a representação dos sorovares também pode ser afetada pelo volume da amostra de água, devem ser considerados esforços para a padronização das amostragens de água para fins de monitoramento. Outras abordagens, como a metagenómica, poderiam fornecer conhecimentos mais abrangentes sobre a ecologia microbiana da água doce e a sua importância para a qualidade e segurança dos produtos agrícolas.

Palavras-chave: agricultura; epidemiologia; patógenos de origem alimentar; uma saúde; salmonelose; revisão sistemática.

2.3 Introduction

Salmonellosis is a cosmopolitan disease caused by *Salmonella enterica*, a major pathogen causing human foodborne illness worldwide (MAJOWICZ et al., 2010; HENDRIKSEN et al., 2011; EUROSURVEILLANCE EDITORIAL TEAM, 2016; TACK et al., 2019). *Salmonella* species are estimated to cause 93.8 million cases of gastroenteritis worldwide annually, leading to 59,100 deaths (MAJOWICZ et al., 2010; ROTH et al., 2018). In the United States (USA), gastroenteritis caused by non-typhoidal *Salmonella* only was estimated to affect approximately one million people annually resulting in approximately US$ 3.7 billion medical costs (MAJOWICZ et al., 2010; BATZ et al., 2012). *Salmonella* was associated with 33% of the foodborne illness cases registered in 2018 in ten sites covering 15% of the USA population and has been cited as the second most prevalent foodborne pathogen, preceded only by *Campylobacter* spp. (TACK et al., 2019).

The microbiological condition of water used in agriculture, regardless of the source, is crucial for the safety of agri-food products. Salmonellosis outbreaks have been associated with the use of contaminated water in agricultural settings (HARRIS et al., 2003; WALSH et al., 2014; LIU et al., 2018). As the gastrointestinal tract of vertebrates is generally considered the natural habitat of *Salmonella enterica*, the use of recycled water from animal production systems is usually considered a major risk factor for produce contamination (ABULREESH, 2012). However, *Salmonella* occurrence in water sources might go beyond short-term accidental findings determined by the transient presence of bacteria as a result of scattered fecal contamination events. Viability mechanisms can enable *Salmonella* organisms to successfully survive in natural aquatic environments for several months (DOMINGO et al., 2000; LIU et al., 2018). In laboratory, however, *Salmonella* has been observed to survive for up to 5 years in phosphate-buffered solution at room temperature (LIAO AND SHOLLENBERGER, 2003).

Although *Salmonella* can survive in a wide range of pH (4.05 - 9.5) and temperature (7 - 48°C) under controlled laboratory conditions (COX et al., 2014), the natural environment associated with irrigation water sources such as rivers or lakes may impose challenging conditions for the long-term viability of *Salmonella.* Variations in physicochemical properties (temperature, salts, pH, oxygen), nutrient availability, interaction with other microorganisms, and exposure to UV radiation (WILKES et al., 2011; WANJUGI AND HARWOOD, 2013) have been shown to reduce *Salmonella* viability in water over time, generally up to 30 days (STEELE AND ODUMERU, 2004). On the other hand, the production biofilm can facilitate the survival of *Salmonella* in water and aquatic invertebrates, such as free-living protozoa and vertebrate hosts (SHA et al., 2011; LIU et al., 2018; CHEN et al., 2019). Furthermore, re-introduction of *Salmonella* into irrigation ponds should be also considered, as previously demonstrated for *Salmonella* Newport (LI et al., 2015a). Re-introduction events are usually caused by animal waste contamination through sewage discharges, rainfall, or associated surface run-off events. Therefore, natural or non-recycled water sources such as rivers and irrigation canals have been shown to act as reservoirs of viable *Salmonella* (BAUDART et al., 2000; LI et al., 2014; MARTÍNEZ et al., 2017) and play a critical role as contamination sources of *Salmonella* and other microbes to fresh produce (HANNING et al., 2009), circulating back to humans and other animals (LI et al., 2015a).

Despite the increasing number of studies reporting the presence of *Salmonella* in natural aquatic environments, there is no agreement on its average frequency and relative serovar representation across water sources. Because of this knowledge gap and the great importance of water for the sustainability of food production worldwide, this meta-analysis aimed at determining the weighted average proportion and between-study variance of *Salmonella* frequency in non-recycled water environments and the role of putative moderators affecting both the frequency and relative representation of serovars.

2.4 Materials and Methods

Three sequential steps were performed by the authors in order to obtain a preliminary selection from identified abstracts and article titles: Identification of information sources, Screening and Eligibility. The selected articles were finally included in the study.

Identification of information sources

The identification of putative information sources was guided by questions that were formulated according to the population, intervention, comparison, and outcome method (PICO) (SANTOS et al., 2007). The following questions were asked: What is the occurrence of *Salmonella* in aquatic environments? Are there differences in the presence of *Salmonella* between surface and groundwater? Which serovars are most prevalent in surface water? Which serovars are most prevalent in groundwater? Which serovars are present in both surface and groundwater? Are there differences in frequency and diversity of *Salmonella* serovars among countries? Could differences in the frequency and diversity of *Salmonella* be attributed to sample volume? Are there differences in presence and abundance related to seasonality?

A literature search was performed using Medical Subject Headings (MeSH) terms on Pubmed, Web of Science, and Embase databases. The search components are described below. The initial screening process was performed from April to November 2020. Further directed searches were carried out by checking the reference lists of relevant articles.

Search component 1 (SC1) - population: water OR groundwater OR lake OR pond OR river

Search component 2 (SC2) - intervention: *Salmonella* spp. OR *Salmonella enterica* OR *Salmonella**.

After retrieving the search components results, the Boolean operator "AND" was used to combine SC1 and SC2.

Screening

The research considered only papers in English published between 2015 and 2020 and duplicate articles were excluded. Editorials, letters, and Ph.D. thesis were also excluded. Based on the title and abstract contents, only articles presenting proper identification of the serovars isolated from surface or groundwater sources were selected.

Eligibility

The eligibility assessment was performed after the complete analysis of the entire manuscript. For serotyping characterization, publications using the standard Kauffmann-Le Minor scheme were first selected, but some articles using serotyping through pulsed-field gel electrophoresis (PFGE) were also included. The exclusion of publications using rapid methods of *Salmonella* detection was justified by two key reasons. Firstly, publication reporting serovar identification provides more information for biological interpretation and therefore fit better the purpose of our study, as these publications can be used to respond all the focus questions. For instance, the assessment of the frequencies of *Salmonella* serovars could be biased by the inclusion of articles using primers targeting a small group of serovars. Secondly, although some rapid tests could provide higher sensitivity values for *Salmonella* detection compared to conventional microbiological culture (COX et al., 2014), the comparative analysis could be biased by the large methodological variation represented by the numerous available tests, including commercial and in-house methods. Therefore, the publications considered in the present study described microbiological isolation methods performed according to standard methodologies such as BAM and AOAC, although minor differences existed, mainly in terms of types of media. Importantly, as the large number of serovars usually requires the use of a combination of culture media (COX et al., 2014), there is possibility of bias in the comparative analysis of the serovars across the different studies. Finally, the results were reported according to the Preferred Reporting Items for Systematic Review and Meta-Analyses Statement (PRISMA) (MOHER et al., 2015).

Risk of bias assessment

Possible sources of bias included study inclusion/exclusion criteria and the impact of missing data, missing primary results, the chosen database, date, language, number of articles, and article type selected for this study.

Statistical analyses

Information regarding the identification of manuscripts (authors, publication year, country), total number of collected samples, number of positive samples, number of *Salmonella* serovars, sample source (superficial or ground water), and water sample volume were obtained and kept in excel spreadsheets. Frequencies of *Salmonella* serovars were calculated by dividing the number of positive samples of each serovar by the total number of collected samples. Spreadsheets containing the data used in this meta-analysis are available as supplementary material. Because of the existence of proportions outside the range of 0.2 to 0.8, the frequency values were logit-transformed before analysis. The summary effect size (i.e., the weighted average proportion) was obtained by fitting a random-effects model using the restricted maximum-likelihood estimator (RMLE), assuming there are within- and between-study variances across the studies. The estimates of summary proportions and their confidence intervals were visualized according to forest plot as proposed by Lewis & Clarke (2001). In order to assess the true variation in effect sizes (between-study variance), the study heterogeneity (τ^2) was calculated and tested for significance according to Q-test at 95% probability. Heterogeneity was also quantified by I^2 statistics as proposed by Higgins et al. (2003). When the effect sizes had high heterogeneity, we conducted a moderator analysis by means of meta-regression in order to investigate potential sources of systematic variation between the studies. Three potential moderators were investigated: 1) Sample source: surface water or groundwater; 2) Water sample volume: small (<999 mL) or large (≥1,000 mL); and 3) Sample origin: samples from countries with low (< 0.8) or high (≥ 0.8) human development index (HDI), according to the latest Human Development Index Ranking (UNDP, 2020). We used China´s HDI for the reports from Taiwan. The analyses were performed using metafor package in R (VIECHTBAUER, 2010; RSTUDIO TEAM, 2019; R CORE TEAM, 2020). In addition, a decision tree was built by supervised machine learning using rpart package in R (THERNEAU et al., 2013), and the Pearson correlation coefficient between observed and estimated frequencies was calculated.

The influence of the moderators on the relative frequency of the reported *Salmonella* sorovars was verified with canonical correspondence analysis, using the vegan package in R (OKSANEN et al., 2011). Diversity indexes Shannon and Pielou were also calculated for richness and evenness estimates, respectively.

The relative frequencies of serovars were submitted to cluster analysis of row and columns, based on binary distance and hierarchical clustering. Hierarchical clustering of the 26 articles condiering the relative frequencies of the serovars was built using average linkage was built from a binary distance matrix in R 4.11. The optimal number of clusters was defined according to the FOM (figure of merit) index using the clValid package in R (BROCK et al., 2008). Heatmaps were obtained using the ComplexHeatmap package in R (GU et al., 2016).

2.5 Results

Literature search

A total of 1,723 articles were identified at PubMed, 1,277 at Web of Science, and 2,194 at Embase, totaling 5,194 papers. Of these, 1,972 duplicates or triplicates were detected and excluded. A total of 3,222 remaining publications were obtained after the exclusion of redundant papers. Most articles (n = 101) were excluded for not informing the proper identification of serovars.

After titles and abstracts were read, 26 papers addressing both *Salmonella* and non-recycled environmental water were considered adequate and included in the present study (Figure 1). For statistical analysis, these 26 publications resulted in 29 observations as three papers reported *Salmonella* occurrence for both surface and groundwater.

A total of 148 different serovars were reported. Of these, 139 originated from surface water, from which 123 serovars were reported only in this water source. A total of 25 serovars were reported in groundwater, including nine serovars that were exclusively reported in this source. Only 16 serovars were reported in both sources.

The United States was identified as the country with the highest number of papers reporting the identification of *Salmonella* serovars (13), followed by Canada (3), and Taiwan (2). The remaining articles originated from Burkina Faso, China, Croatia, Ghana, Mexico, Spain, Sri Lanka, and Uganda.

Table 1 presents the relative frequency ranges of *Salmonella* serovars observed in non-recycled surface water and groundwater. Only serovars that were reported in at least five different studies were included in this Table. Supplementary Tables 1 and 2 (Tables S1 and S2) provide detailed information about the occurrence of all reported *Salmonella enterica* serovars in the 26 publications.

The frequency of *Salmonella enterica* in water samples varied from 1.14 (STOKDYK et al., 2020) to 100% (Maurer et al., 2015; Kovačić et al., 2017) as observed in Figure 2. The highest number of isolates reported in a single study (n=247) was associated with surface water samples (KADYKALO et al., 2020), while the highest serovar diversity (35 different serovars) was observed by Jokinen et al. (2015).

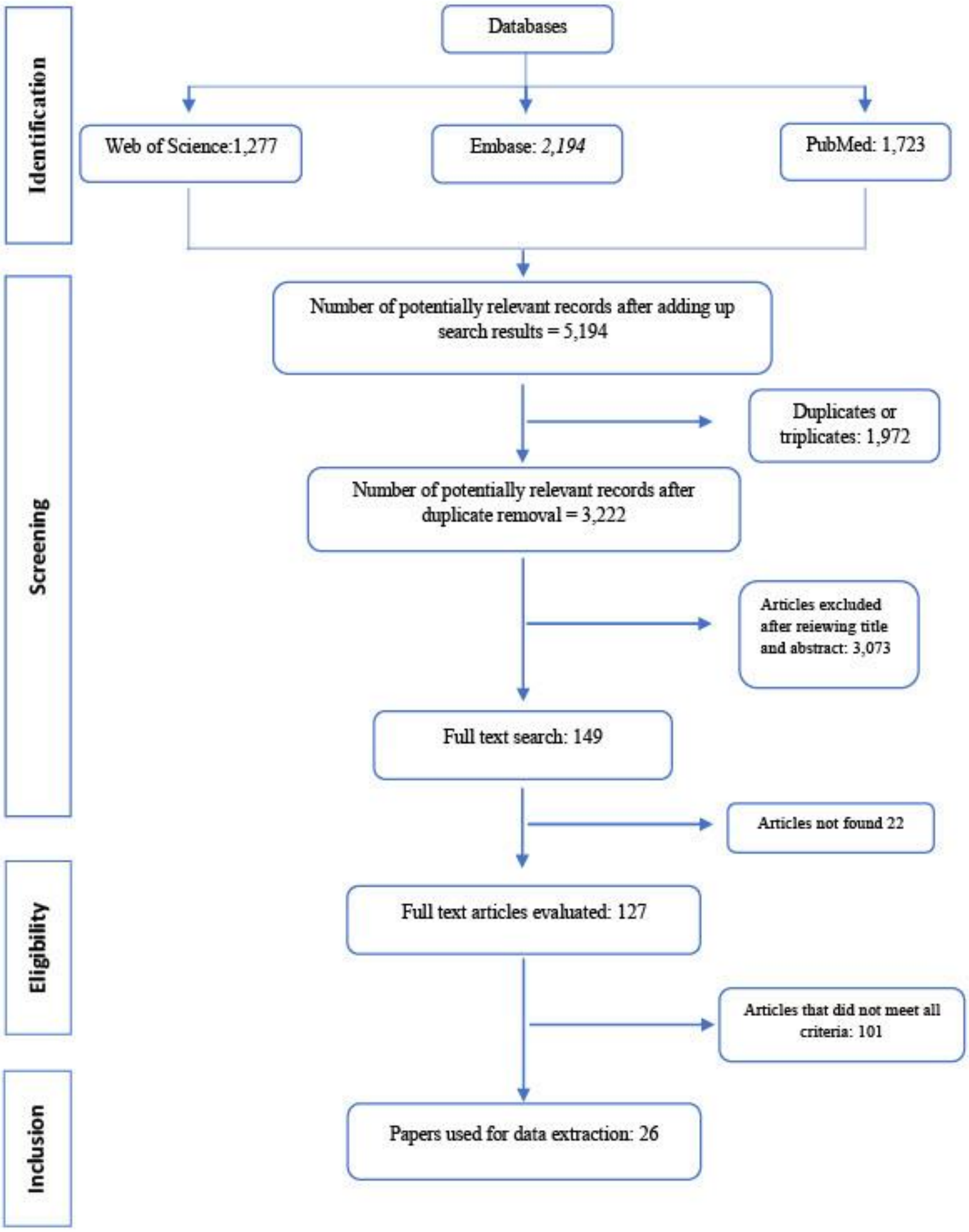

Figure 1 - PRISMA flow diagram showing the sequential steps for articles selection and inclusion in the meta-analysis.

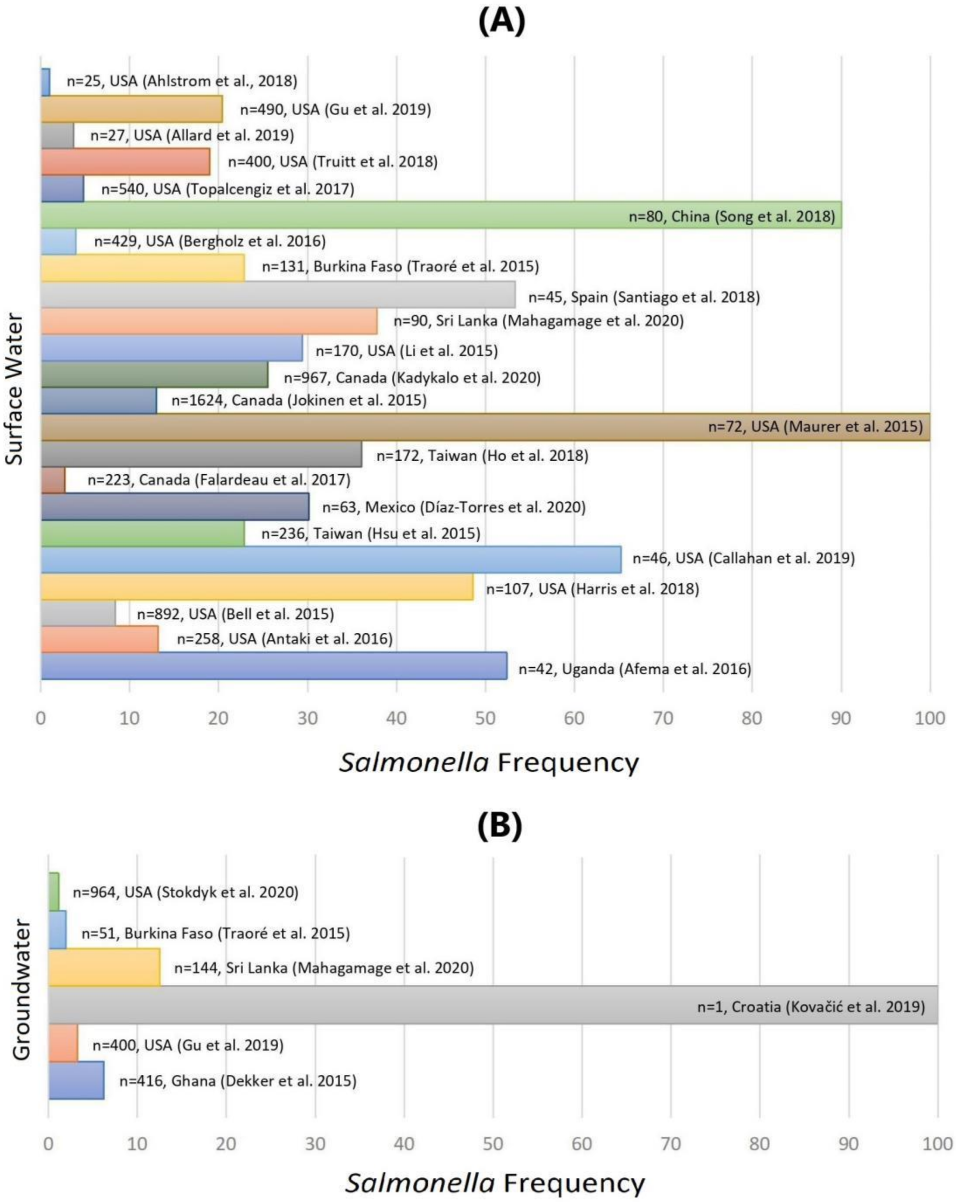

Figure 2 - Frequency of *Salmonella enterica* serovars detected in non-recycled surface water (A) and groundwater samples (B) as per reported in 26 peer reviewed scientific publications between the years 2015 and 2020.

Considering groundwater only (Table 1), the relative frequency of *Salmonella enterica* varied from 3.85 to 100%. The higher number of isolates for this type of water (n=26) was reported by Dekker et al. (2015). Except for Kovačić et al. (2017), which reported the occurrence of *Salmonella* Enteritidis in a single sample (100%), the highest frequency of *Salmonella* and the greatest diversity of serovars were observed in a study conducted in Sri Lanka (MAHAGAMAGE et al., 2020). Importantly, although the study from Kovačić et al. (2017) refers to a single sample related to an outbreak investigation, no minimum sample size was predetermined as inclusion criteria for the present meta-analysis and therefore that study has been included in the present investigation.

The Forest plot showing the summary effect size of the *Salmonella* proportions in water is shown in Figure 3. According to our results, *Salmonella* frequency in non-recycled water sources was 0.19 [CI: 0.14; 0.25]. Although a significant ($P < 0.0001$) and high heterogeneity ($\tau^2 = 0.0711$; $I^2 = 99.72\%$) was observed, only source was identified as a significant mediator ($P < 0.10$) in the meta regression analysis. The descriptive average frequencies were 31.97% and 20.85% in surface water and groundwater samples, respectively, as shown in supplementary Tables S1 and S2.

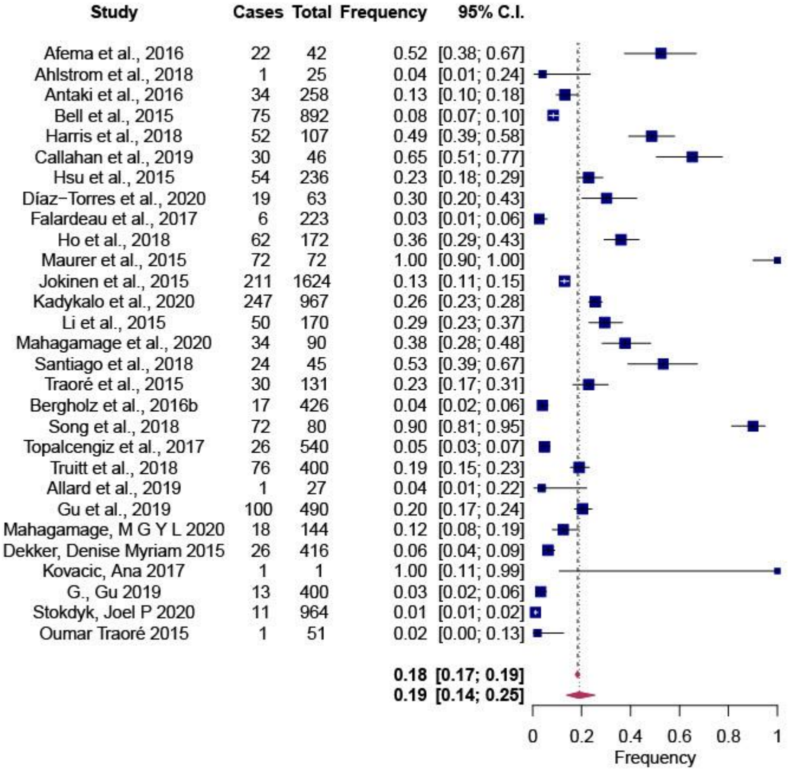

Figure 3 - Forest plot showing the summary effect size of proportions of *Salmonella* frequencies in non-recycled water sources using 26 selected articles and 29 observations. This summary effect size was obtained in R 4.11 (package metafor) by fitting a random-effects model using the restricted maximum-likelihood estimator (RMLE). Heterogeneity parameters and statistics are indicated in the model.

The decision tree (Figure 4) obtained by supervised machine learning resulted in a 0.48 Pearson correlation coefficient between observed and estimated frequencies. All three moderators (water source; HDI, and sample volume) were shown to affect *Salmonella* frequency in water but source was identified as the most relevant one.

Estimate frequencies of 0.31 and 0.17% for surface and groundwater were obtained, respectively. Considering surface water only, samples from countries with lower HDI resulted in a higher *Salmonella* frequency (0.42) compared to developed regions (0.26). Sampling of small water volumes resulted in lower detectable *Salmonella* frequencies in both high and low HDI regions. The water sampling technique reported by the majority of the studies consisted of transporting determined volume of water to the laboratory for filtering. The use of less than 1L water samples was reported by seventeen studies (65.38%), while other six studies (23.08%) described the use of 1 to 4 L water samples. Only two publications (7.69%) reported the use of *in situ* water filtration (10L) by means of the modified Moore swab technique (MMS) (ALLARD et al., 2019; CALLAHAN et al., 2019) and only a single study reported the use of *in situ* ultrafiltration (728 L) with commercial dialyzers (STOKDYK et al., 2020). Detailed information is shown in Tables S1 and S2.

According to the canonical correspondence analysis, both HDI and sample volume significantly ($P < 0.0001$) affected the relative frequencies of the 148 *Salmonella* serovars across the studies. The hierarchical clustering of the 26 publications considering the relative frequencies of the 148 *Salmonella* serovars is shown in supplementary material (Figure S1). Five distinct clusters were observed. The larger cluster (2) is comprised only by studies from countries with high HDI and the majority of these studies reported the use of water samples with less than 1L. On the other hand, all but one (16) study in the Cluster 1 originated from countries with low HDI. Two manuscripts (17 and 25) were not grouped in any cluster.

The heatmap representing the relative frequencies of *Salmonella* serovars according to the cluster analysis of rows (*y*) and columns (*x*) is shown in Figure 5. Cluster 1 (*x*) included the 135 less representative serovars, while cluster 5 (*x*) was comprised the most frequent serovars: *S.* Newport and *S.* Typhimurium, which were identified in 19 and 16 studies, respectively. Other frequent serovars were grouped in Clusters 2 (*S.* Barelly, *S.* Mbandaka, *S.* 4,[5],12:i:-, *S.* Braenderup), 3 (*S.*Rubislaw, *S.* Muenchen, *S.* Give, *S.* Hartford) and 4 (*S.* Kentucky, *S.* Stanleyville). Considering that the relative frequencies of the *Salmonella* serovars across studies are affected by HDI and volume, individualized heatmaps according to these moderators are shown in Figures 6 and 7, respectively. The higher frequencies of some serovars such as *S.* Rubislaw, *S.* Muenchen, *S.* Give, *S.* Hartford, *S.* Rissen, *S.* Saintpaul, and *S.*

Thompson across studies from high HDI countries is shown in Figure 6. According to Figure 7, some serovars were more frequently observed in studies using larger water samples, such as *S.* Newport, *S.* Typhimurium, *S.* Mbandaka, *S.* Braenderup, and *S.* Kentucky. On the other hand, *S.* Agona, *S.* Derby, and *S.* Virchow were more frequently observed in some studies using small volume water samples (8, 16, 19) than in studies using greater water volume samples.

There were no differences ($P > 0.05$) in the diversity of *Salmonella* serovars across water sources, HDI and volume as measured by Shannon and Pielou indexes for richness and evenness, respectively.

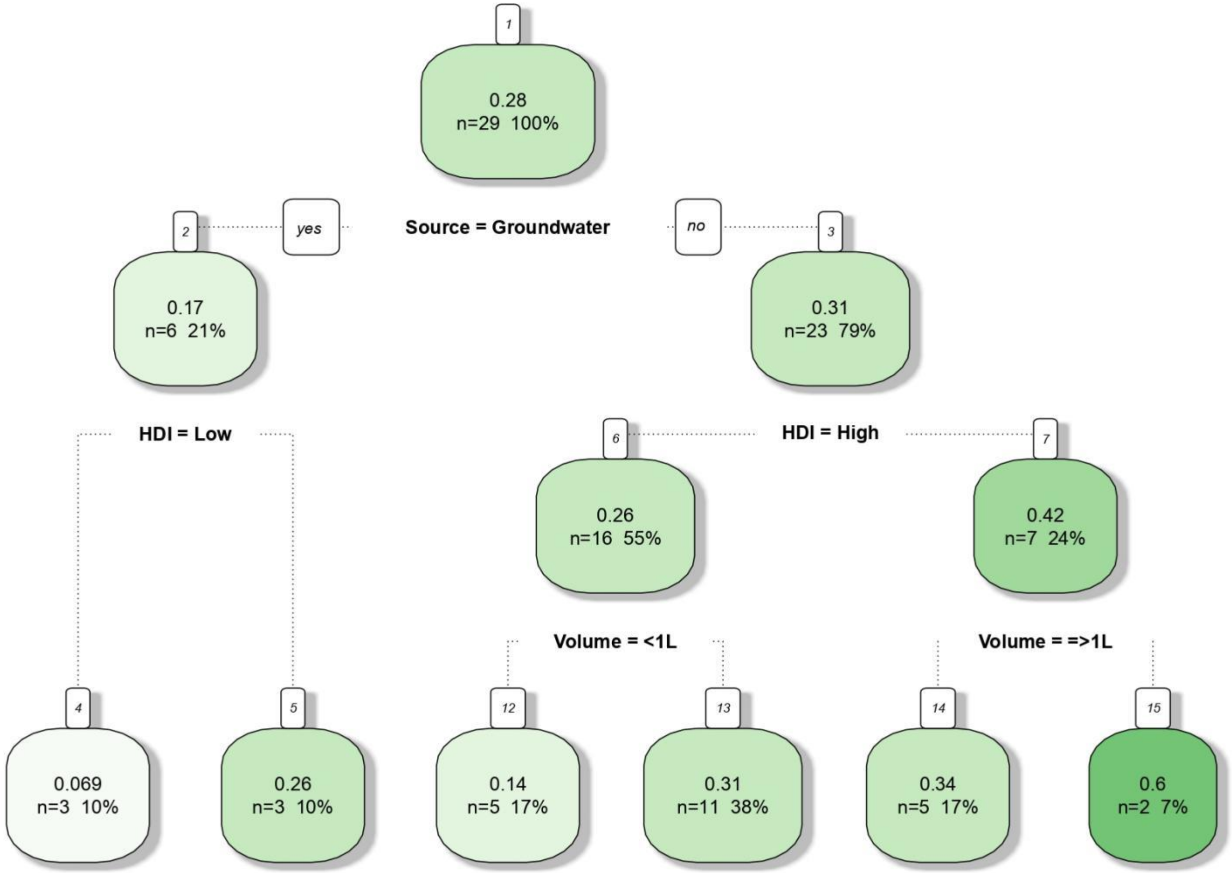

Figure 4 - Decision tree predicting the frequency *of Salmonella* in non-recycled water sources in function of the moderator variables source (surface or groundwater), human development index (HDI) of the country from which the samples originated (high or low) and water samplevolume (< 1 L or ≥ 1 L). The predictive algorithm has been built in R (package rpart) using meta-analysis data of 26 peer reviewed scientific publications between the years 2015 and 2020.

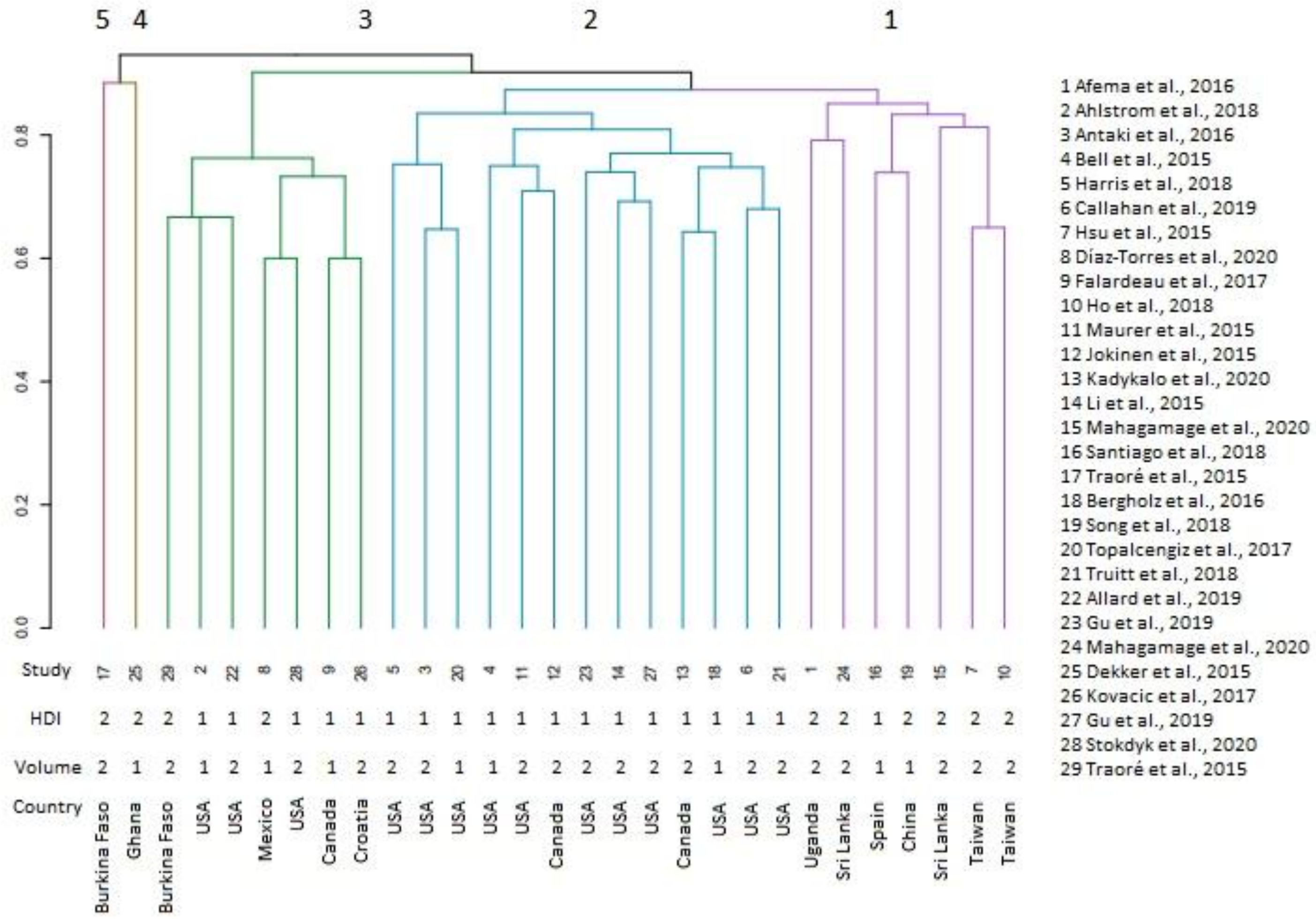

Figure S1 - Hierarchical cluster analysis of 29 peer reviewed scientific publications between the years 2015 and 2020 considering the relative freuquencies of reported *Salmonella* serovars. Hierarchical clustering using average linkage was built from a binary distance matrix in R 4.11.

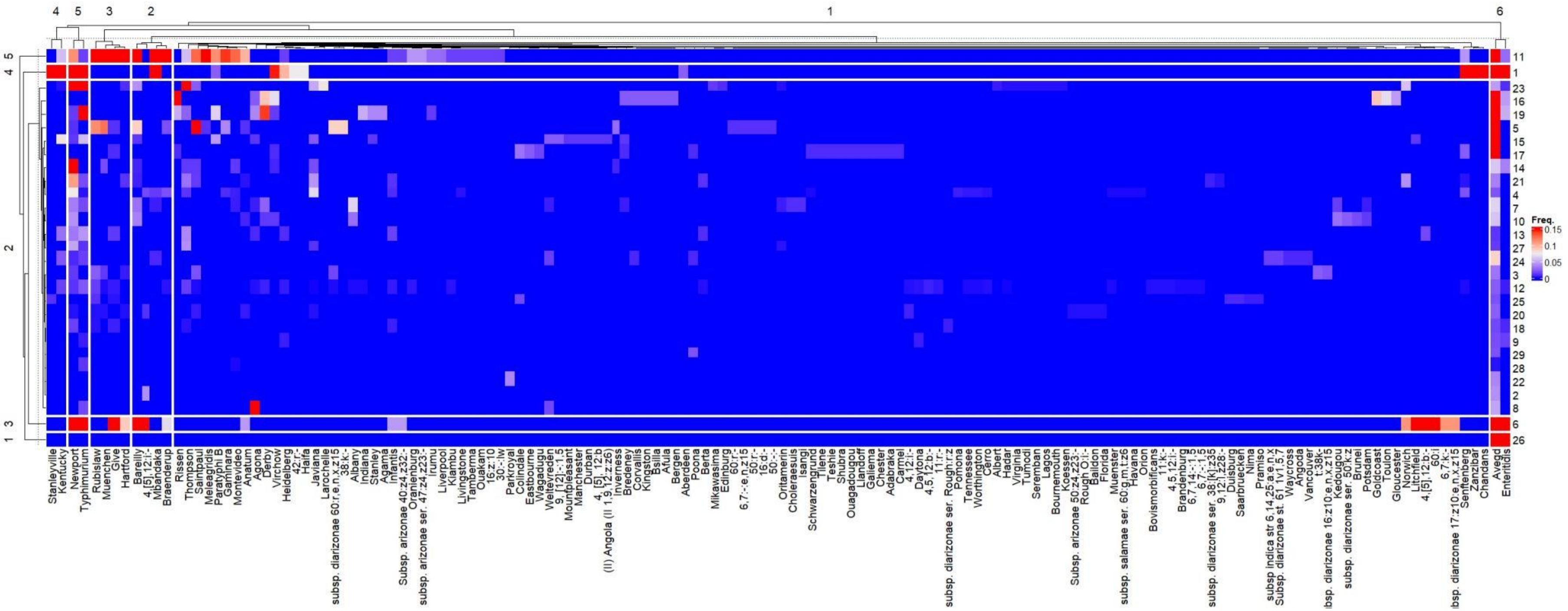

Figure 5 - Heatmap of the relative frequencies of *Salmonella enterica* serovars isolated from surface and groundwater sources as per reported in 26 peer-reviewed scientific publications between the years 2015 and 2020. The heatmap was built in R (package ComplexHeatmap).

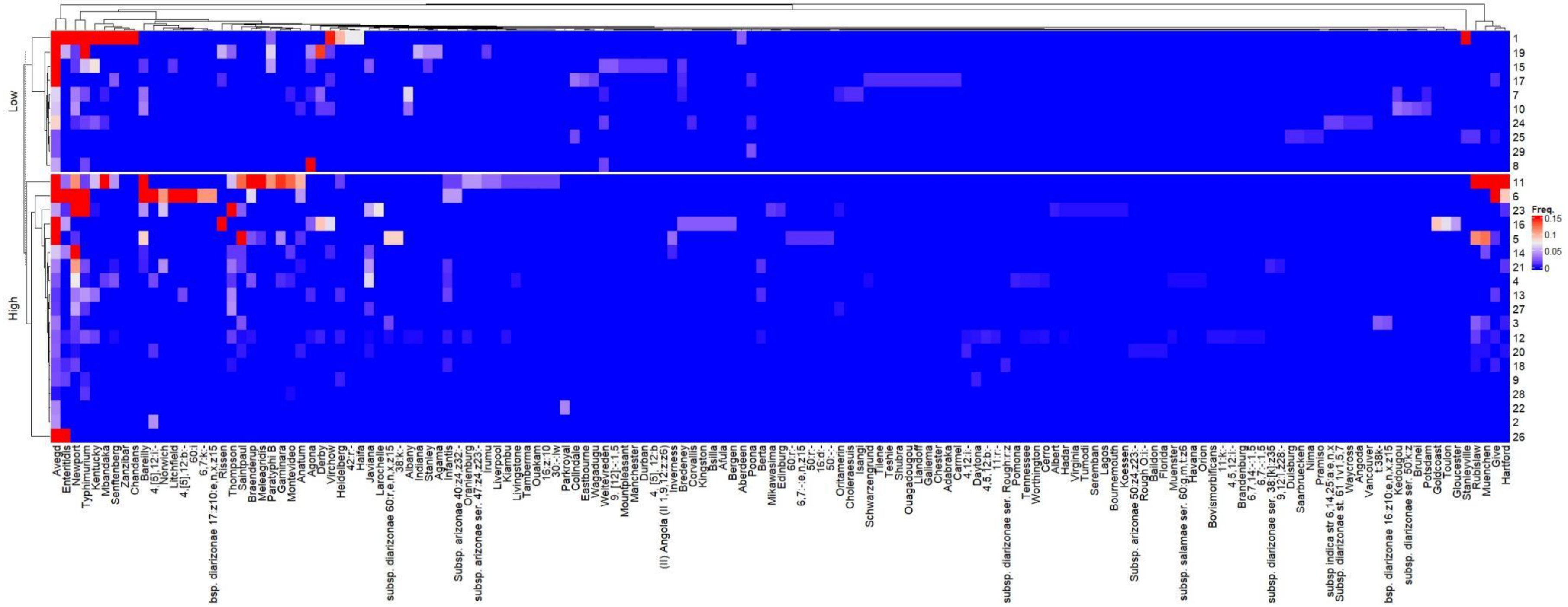

Figure 6 - Heatmap of the relative frequencies of *Salmonella enterica* serovars isolated from non-recycled water sources according to the human development index (HDI) of countries associated with 26 peer-reviewed scientific publications between the years 2015 and 2020. The heatmap was built in R (package ComplexHeatmap).

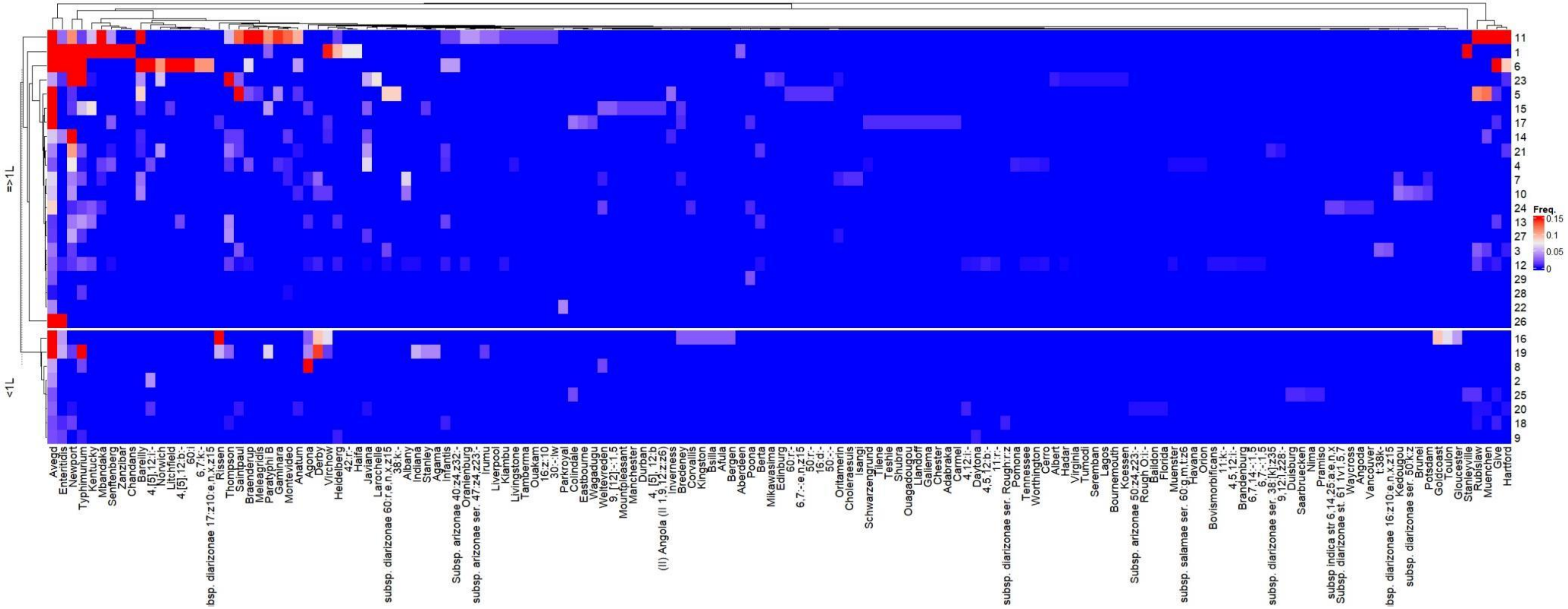

Figure 7 - Heatmap of the relative frequencies of *Salmonella enterica* serovars isolated from non-recycled water sources according to the water sample volume used in 26 peer-reviewed scientific publications between the years 2015 and 2020. The heatmap was built in R (package ComplexHeatmap).

Table 1 - Relative representation of *Salmonella enterica* serovars associated with surface and groundwater sources that have been reported in peer-reviewed scientific publications addressing the occurrence of *Salmonella* in aquatic environments between the years 2015 and 2020 (Only serovars reported in at least five different studies are considered).

Serovar	Source	Relative representation, %	References
S. Newport	Surface water	1.14% to 58%	(Afema et al., 2016), (Antaki et al., 2016), (Bell et al., 2015), (Harris et al., 2018), (Callahan et al., 2019), (Hsu et al., 2015b), (Ho et al., 2018), (Maurer et al., 2015), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Li et al., 2015a), (Mahagamage et al., 2020), (Bergholz et al., 2016), (Song et al., 2018), (Topalcengiz et al., 2017), (Truitt et al., 2018), (Gu et al., 2019)
	Groundwater	5.55% and 43.18%	(Gu et al., 2019), (Mahagamage et al., 2020).
S. Typhimirium	Surface water	0.29% to 37.31%	(Afema et al., 2016), (Bell et al., 2015), (Callahan et al., 2019), (Hsu et al., 2015b), (Díaz-Torres et al., 2020), (Falardeau et al., 2017), (Maurer et al., 2015), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Mahagamage et al., 2020), (Song et al., 2018), (Truitt et al., 2018), (Gu et al., 2019).
	Groundwater	9.09% to 90.91%	(Gu et al., 2019), (Mahagamage et al., 2020), (Stokdyk et al., 2020).
S. Enteritidis	Surface water	0.75% to 50%	(Afema et al., 2016), (Callahan et al., 2019), (Falardeau et al., 2017), (Maurer et al., 2015), (Jokinen et al., 2015), (Li et al., 2015a), (Santiago et al., 2018), (Bergholz et al., 2016), (Song et al., 2018), (Gu et al., 2019).
	Groundwater	100%	(Kovačić et al., 2017).

S. Bareilly	Surface water	1.69% to 16.21%	(Harris et al., 2018), (Callahan et al., 2019), (Hsu et al., 2015b), (Ho et al., 2018), (Maurer et al., 2015), (Li et al., 2015a), (Mahagamage et al., 2020), (Truitt et al., 2018), (Gu et al., 2019).
	Groundwater	0%	
S. Thompson	Surface water	1.17% to 18.3%	(Bell et al., 2015), (Maurer et al., 2015), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Li et al., 2015a), (Bergholz et al., 2016), (Song et al., 2018), (Truitt et al., 2018), (Gu et al., 2019).
	Groundwater	36.36%	(Gu et al., 2019).
S. Infantis	Surface water	0.29% 11.76%	(Bell et al., 2015), (Callahan et al., 2019), (Maurer et al., 2015), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Bergholz et al., 2016), (Truitt et al., 2018).
	Groundwater	0%	
S. Saintpaul	Surface water	0.89% to 19.54%	(Antaki et al., 2016), (Harris et al., 2018), (Maurer et al., 2015), (Jokinen et al., 2015), (Li et al., 2015a), (Topalcengiz et al., 2017), (Truitt et al., 2018), (Gu et al., 2019).
	Groundwater	0%	
S. Agona	Surface water	1.83% to 86.67%	(Hsu et al., 2015b), (Díaz-Torres et al., 2020), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Mahagamage et al., 2020), (Santiago et al., 2018), (Song et al., 2018).
	Groundwater	0%	
S. Give	Surface water	1.15% to 12.31%	(Harris et al., 2018), (Callahan et al., 2019), (Maurer et al., 2015), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Traoré et al., 2015), (Bergholz et al., 2016).

	Groundwater	3.85%	(Dekker et al., 2015).
S. Javiana	Surface water	0.44% to 24.89%	(Bell et al., 2015), (Jokinen et al., 2015), (Li et al., 2015a), (Mahagamage et al., 2020), (Topalcengiz et al., 2017), (Truitt et al., 2018), (Gu et al., 2019).
	Groundwater	9.1%	(Gu et al., 2019)
S. Anatum	Surface water	0.85% to 9.52%	(Harris et al., 2018), (Callahan et al., 2019), (Ho et al., 2018), (Maurer et al., 2015), (Topalcengiz et al., 2017), (Truitt et al., 2018).
	Groundwater	0%	
S. Hartford	Surface water	0.75% to 10.85%	(Antaki et al., 2016), (Callahan et al., 2019), (Maurer et al., 2015), (Topalcengiz et al., 2017), (Truitt et al., 2018), (Gu et al., 2019).
	Groundwater	0%	
S. Kentucky	Surface water	0.19% to 21.21%	(Afema et al., 2016), (Maurer et al., 2015), (Jokinen et al., 2015), (Kadykalo et al., 2020), (Mahagamage et al., 2020), (Gu et al., 2019).
	Groundwater	16.67%	(Mahagamage et al., 2020).
S. Muenchen	Surface water	0.89% to 14.84%	(Antaki et al., 2016), (Harris et al., 2018), (Maurer et al., 2015), (Jokinen et al., 2015), (Li et al., 2015a), (Topalcengiz et al., 2017).
	Groundwater	0%	
S. Rubislaw	Surface water	4.72% to 20.52%	(Antaki et al., 2016), (Harris et al., 2018), (Maurer et al., 2015), (Jokinen et al., 2015), (Bergholz et al., 2016), (Topalcengiz et al., 2017).
	Groundwater	15.38%	(Dekker et al., 2015).

S. Senftenberg	Surface water	0.85% to 11.11%	(Afema et al., 2016), (Bell et al., 2015), (Maurer et al., 2015), (Jokinen et al., 2015), (Traoré et al., 2015), (Truitt et al., 2018).
	Groundwater	0%	
S. Virchow	Surface water	1.49% to 8.1%	(Afema et al., 2016), (Ho et al., 2018), (Li et al., 2015a), (Santiago et al., 2018), (Traoré et al., 2015), (Song et al., 2018).
	Groundwater	0%	

Table 2 - Frequency of serovars of *Salmonella enterica* subsp. *enterica* associated with groundwater sources that have been reported in peer reviewed scientific publications between the years 2015 and 2020.

Serovar	**Country**	**Prevalence (%)**	**Volume of water analyzed**	**References**
Colindale; Duisburg; Give; Nima; Pramiso; Rubislaw; Saarbruecken; Stanleyville	Ghana	26/416 (6,25%)	200 mL	(Dekker et al., 2015)
Javiana; Newport; Oritamenrin; Thompson; Typhimurium	USA	13/400 (3,25%)	4 L	(Gu et al., 2019)
Enteritidis	Croatia	1/1 (100%)	1 L	(Kovačić et al., 2017)
Subsp. diarizonae st. 61 1v1,5,7; Angoda; Corvallis; Kentucky; Mbandaka; Newport; Poona; subsp indica str 6,14,25:a:e,n,x; Typhimurium; Vancouver; Waycross; Weltevreden	Sri Lanka	18/144 (12,5%)	2,5 L	(Mahagamage et al., 2020)
Poona	Burkina Faso	1/51 (1,96%)	1 L	(Traoré et al., 2015)
Montevideo; Typhimurium	USA	11/964 (1,14%)	728 L	(Stokdyk et al., 2020)

2.6 Discussion

Occurrence of Salmonella in aquatic environments

The overall weighted average proportion was 0.19 [CI: 0.14; 0.25] for positive water samples, suggesting that viable *Salmonella* organisms are frequently found in non-recycled water sources worldwide. The increasing number of human salmonellosis outbreaks associated with the consumption of fresh produce or industrialized plant-based products, including fruits, vegetables, legumes, grains, nuts and seeds, has posed the question whether environmental water could play a role as potential *Salmonella* contamination source. The 127 peer-reviewed studies retrieved after the initial screening test of this systematic review confirms the increasing interest of the scientific community on this topic. These publications reported the occurrence of *Salmonella* from different aquatic environments under a wide range of conditions (DOMINGO et al., 2000; BELL et al., 2015; LI et al., 2015a; LIU et al., 2018). Obviously, the capacity of *Salmonella* to survive and to adapt to challenging environmental conditions is a basic principle for the bacteria to fulfill their biological cycle (fecal-oral route of transmission), suggesting the existence of mechanisms to overcome stressors in aquatic and terrestrial microcosms. Long-term persistence of *S. enterica* in irrigation ponds has been previously reported (GREENE et al., 2008; LUO et al., 2015), indicating that this pathogen is able to adapt to stressors in hydrological niches and highlighting the importance of assessing the quality of irrigation water regularly (WINFIELD AND GROISMAN, 2003). However, little is known about the real ability of *Salmonella* to adapt and evolve in natural environments such as surface and groundwater sources.

Are there differences in the presence of Salmonella between surface and groundwater?

The amount of true heterogeneity observed in our study, represented by the between-study variance and expressed by the parameter (τ^2), (BORENSTEIN et al., 2005) was large and statistically significant, indicating the existence of systematic differences in effects across 26 articles used in the present meta-analysis. Interestingly, the I^2 parameter varies from 0% to 100% and allows comparisons of the estimated heterogeneity across different meta-analysis studies. In the case of the present study, the observed I^2 value was 99.72%. Therefore, there is evidence that the variance is determined by the existence of importance variable moderators. That said, we highlight the limited number of moderators (n=3) investigated in the present meta-analysis that could act as sources of heterogeneity, as reflected by the lack of important information across all studies in the meta-analysis, for instance, the proximity of the sampled water of potential contamination sources (agriculture, livestock, sewage), psychochemical properties of water that might play an important role in the survival of *Salmonella* and competing organisms, the presence of other contaminants, characterization of wildlife in the proximity of the water bodies, among others.

The fact that the source of water was identified as a significant moderator for *Salmonella* frequency according to the meta regression analysis and was also identified as the most relevant moderator in the regression tree corroborates the hypothesis that viable *Salmonella* is more frequently recovered from surface water sources than groundwater. This finding was expected, considering the greater exposure of surface water sources to contaminants in general. Indeed, surface water is more easily exposed to discharge of sewage, inadequate agricultural, livestock and industrial run-offs, climatic events and visit of wild animals (BERGHOLZ et al., 2016; KARKEY et al., 2016; TORO et al., 2016; GU et al., 2019; JECHALKE et al., 2019). The latter is particularly noteworthy, since a wide range of *S. enterica* serovars have been extensively reported in wildlife (MAURER et al., 2015; TORO et al., 2016; DE SOUZA et al., 2020).

The lower frequency of *Salmonella* in groundwater compared with surface water (0.17 *vs.*0.31, respectively) observed in our study corroborates previous findings (ABULREESH, 2012; GU et al., 2019). Underground reservoirs have long been considered excellent sources of drinking water to human and animal populations, mainly because it is naturally filtered by the soil underlying rock formations. Although they usually provide superior microbial quality associated with lower microbial loads, the belief that groundwater is pure and no treatment is needed before consumption has been questioned (LI et al., 2018; LIU et al., 2018; STOKDYK et al., 2020) by the increasing number of studies reporting *Salmonella* contamination in groundwater (DEKKER et al., 2015; LI et al., 2015b; PALAMULENI et al., 2015). There are several possibilities of contamination of groundwater, even though some of them are sporadic. Quality can be compromised by insufficient well depth or during construction (LIU et al., 2018) and well pollution may result from events such as improperly functioning sewer systems, contaminated stormwater and agricultural run-off, especially after storms and floods (GU et al., 2019). Contamination events can be sporadic or one-off, nevertheless, the water sources can become compromised for longer periods (DEKKER et al., 2015). Some experimental studies showed that *Salmonella* can remain viable for periods longer than 100 days in water, and that viability is mainly affected by ambient temperature (DOMINGO et al., 2000; IBRAHIM et al., 2019).

Importantly, there was a considerably higher number of studies on the occurrence of *Salmonella* in surface water (n=23) compared with groundwater (n=6). Possibly, the greater interest in assessing surface water is related to its relevance and economic importance for both rural and urban settings worldwide. In fact, the majority of reports in the present study originated from regions where surface water sources have been commonly used for irrigation purposes in agri-food production systems.

In summary, the occurrence of *Salmonella* in groundwater should not be neglected. Further studies addressing *Salmonella* contamination in groundwater are warranted as they could be particularly important in regions where irrigation practices depend on this type of water, such as semiarid settings.

Which serovars are most prevalent in surface water and ground water?

Between 2015 and 2020, *S.* Newport was the most frequent serovar identified in both surface (464 isolates; 18.33%) and groundwater (20 isolates; 0.78%). Furthermore, Callahan et al. (2019) reported *S.* Newport as the most isolated serovar throughout the year. *S.* Newport infection rates have been stable over the decades, with approximately 750 confirmed cases per year in Europe (EUROSURVEILLANCE EDITORIAL TEAM, 2016). Wild birds are considered important reservoirs as recurrent *S.* Newport outbreaks have been reported due to direct contamination of vegetables such as tomatoes, soil or irrigation water (BELL et al., 2015). The factors causing variations in *S.* Newport rates in the United States remain unknown (CRIM et al., 2018).

Salmonella Typhimurium was the second most frequent serovar contaminating both surface water (9.56%) and groundwater (0.63%). This serovar has been one of the two leading serovars associated with human salmonellosis since 1990 (HERIKSTAD et al., 2002). The persistence of this pathogen in freshwater microcosms has been associated with the expression of the *hil*A gene, a regulatory system for the expression of invasive *Salmonella* phenotypes, including the expression of the *ssp*C, *inv*F and *org*A invasion genes (NUTT et al., 2003). Therefore, it is possible that strains circulating in environmental water sources could present increased virulence.

S. Thompson, also a frequent serovar, has been associated with sporadic salmonellosis outbreaks every year in different countries (FRIESEMA et al., 2012; GAULIN et al., 2017; SUIJKERBUIJK et al., 2017; EUN et al., 2019). Under laboratory conditions, a 3 ppm chlorine water treatment induced the viable but not cultivable state in *S.* Thompson (HIGHMORE et al., 2018), raising concerns about the efficacy of chlorine-based treatment of water for human consumption. Therefore, *S.* Thompson may be a potential pathogen of treated water for human consumption.

S. Javiana, *S.* Kentucky, and *S.* Rubislaw serovars have been also identified as frequent serovars contaminating non-recycled water sources. These serovars have been shown to play a role in human salmonellosis. The number of cases of *S.* Javiana has been dramatically increasing in the USA in the last decades (CDC, 2013). It is worth noting that drinking water has been reported as an important source of human infection by *S.* Javiana (CLARKSON et al., 2010; MUKHERJEE et al., 2019). *S.* Kentucky is involved in approximately 100 cases of human salmonellosis yearly in the USA (CDC, 2016). Although it is not one of the leading serovars causing human salmonellosis, there is increasing concern with the emergence of multidrug resistance particularly associated with this serovar (MILTON et al., 2018; AL-GALLAS et al., 2021a; AL-GALLAS et al., 2021b). On the other hand, *S.* Rubislaw has been mainly detected in environmental samples (MAURER et al., 2015), and various free-living animals (POTTER et al., 2011; RUSH et al., 2020; HERNANDEZ et al., 2021).

Are there differences in the relative frequency of Salmonella serovars among regions?

According to our findings, there are indications that the origin of samples, as determined by the HDI index related to the country of origin, might contribute to both overall isolation frequency and relative distribution of *Salmonella* serovars. Based on the decision tree (Figure 4), the frequencies of *Salmonella*-positive samples in surface water were higher in countries with low HDI compared with countries with higher HDI (0.42 *vs.* 0.26, respectively). This finding could be explained by contamination events that are probably more frequent in developing regions as a result of improper sewage treatment and disposal. However, the opposite was observed for groundwater samples and frequency estimates were 0.069 and 0.26 for low and high HDI, respectively.

Further investigations should be conducted to address the differences in the relative frequencies of serovars between high and low HDI countries. Some serovars such as *S.* Muenchen, *S.* Give, *S.* Hartford, *S.* Rissen, *S.* Saintpaul, *S.* Rubislaw, and *S.* Thompson were highly frequent across studies from high HDI countries while others (*S.* Agona, *S.* Derby, *S.* Anatum) were more frequently observed in studies from low HDI countries. It is plausible to admit that the relative serovar frequencies across the regions depend on natural, social and economical drivers impacting the epidemiological and evolutionary aspects of *Salmonella enterica*, and therefore very difficult to be predicted.

Although meta-analysis indicated *Salmonella* Agona as a frequent serovar present in water samples from the low HDI countries included in our study, it is among the ten leading serovars associated with human salmonellosis in European countries, with 378 to 582 cases per year (POPA AND POPA, 2021). Outbreaks of non-typhoidal salmonellosis associated with this serovar has been linked to fresh food consumption (EHUWA et al., 2021; HASSAN et al., 2019; ESTRADA-ACOSTA et al., 2014), such as papaya (HASSAN et al., 2019) and tomato (ESTRADA-ACOSTA et al., 2014). Moreover, irrigation water is considered a major contamination source in agricultural settings (ESTRADA-ACOSTA et al., 2014). However, salmonellosis cases attributed to *S.* Agona have also been attributed to the consumption of contaminated processed foods such as peanut butter and infant formulae (EHUWA et al., 2021).

Can differences in the frequency and diversity of Salmonella be attributed to sample volume?

Interestingly, water sample volume was shown to significantly affect the relative frequency of *Salmonella* serovars across the different studies. According to the decision tree (Figure 4), higher frequency of *Salmonella* was seen in larger water samples (≥ 1L) from both high and low HDI countries. In high HDI countries, the frequencies were 0.31 *vs*. 0.14, while in low HDI countries, a greater difference was observed (0.34 *vs.* 0.6). Although water sample volume has been referred as critical factor for the recovery of *Salmonella enterica* from water, there are no previous reports directly assessing the role of water sample volume on *Salmonella* isolation frequency. This meta-analysis study suggests that water volume might play an important role on the recovery of viable *Salmonella* serovars in environmental water. Moreover, the relative frequency distribution findings reported in our study and visualized as a heatmap (Figure 7) indicate a higher recovery frequency of public health relevant *Salmonella* serovars when large water samples are used (≥ 1L), such as *S.* Typhimuiurm, *S.* Newport, and *S.* Enteritidis.

Important aspects indicate that the occurrence of *S. enterica* in natural water sources is underestimated. Firstly, a considerable number of the studies in our investigation (27.58%) reported using small-volume samples (< 1 L), which may compromise the microbiological recovery. Although there is a consensus towards the use of larger water samples to detect microorganisms present in low densities (BISHA et al., 2011; MCEGAN et al., 2013; SBODIO et al., 2013), there is a lack of studies comparing the real effect of water volume on the recovery of *Salmonella* serovars. Furthermore, conventional microbiological isolation is limited in terms of detection of viable but nonculturable bacteria (VBNC), i.e., organisms presenting a very low metabolic rate or state of dormancy (LIN et al., 2016). Problems in VBNC *Salmonella* cultivation and identification have been well documented (OLIVER, 2005; MORISHIGE et al., 2017). This condition might be of particular importance for *Salmonella* organisms in natural water environments, as bacteria may be subjected to many stressors.

Considering how the number of viable organisms might affect the accuracy of the conventional culture method, alternative techniques have been proposed to overcome cost and logistic problems associated with the transport of large volumes of water to laboratories. Among these, the modified Moore swab (MMS) stands out as a high efficient and low operating cost method alternative for *in-situ* filtration of large sample volumes (usually 10 liters or more) (SBODIO et al., 2013; SHARMA et al., 2020; SIKORSKI AND LEVINE, 2020).

Are there differences in presence and abundance related to seasonality?

Due to the very limited number of publications with serovar identification covering long periods of time, no statistical analysis was performed to assess the relationship between *S. enterica* frequency and season or climatic condition. Seventeen of the twenty-six articles reported isolation of *S. enterica* from all or most of the samples collected during the entire experimental period. There is no substantial variation regarding the frequency of serovars throughout the different seasons of the year (BELL et al., 2015; JOKINEN et al., 2015; MAURER et al., 2015; TRAORÉ et al., 2015; DEKKER et al., 2015; AFEMA et al., 2016; BERGHOLZ et al., 2016; TOPALCENGIZ et al., 2017; FALARDEAU et al., 2017; SANTIAGO et al., 2018; TRUITT et al., 2018; HARRIS et al., 2018; HO et al., 2018; CALLAHAN et al., 2019; GU et al., 2019; STOKDYK et al., 2020; DÍAZ-TORRES et al., 2020).

Four articles performed a single sampling per site (HSU et al., 2015a; LI et al., 2015a; KOVAČIĆ et al., 2017; ALLARD et al., 2019). Despite having made multiple samplings from the same sites over time, one study still analyzed the data as a single set, because it focused on reporting the incidence of antibiotic resistance in the isolated strains and did not assess the variation of isolates over time (KADYKALO et al., 2020).

Two studies carried out in Colorado and Georgia (USA) showed higher isolation rates in different seasons, spring and autumn, respectively (ANTAKI et al., 2016; AHLSTROM et al., 2018). One study showed higher frequency in the rainy season, between spring and early summer (SONG et al., 2018). Interestingly, Mahagamage et al. (2020) reported increased frequencies of *S. enterica* isolation from surface water in rainy seasons, while the contrary was observed for groundwater. Overall, the relationship between *Salmonella* isolation frequency and seasons of the year or dry or rainy period seems to depend on several local variables. Factors such as average temperature, predominant type of exploitation in the region (agriculture, livestock or industry), availability of water (scarcity or abundance, regardless of the season), type of source and location of the source (level of preservation or urbanization of the surroundings) seem to have a strong influence on water contamination levels throughout the year.

To better assess these relationships, it is necessary to include further studies on the effects of climatic factors over long periods of time.

In summary, this meta-analysis investigation established the expected frequency of *Salmonella* recovery from water samples. There is a higher recovery rate from surface water compared with ground water. The serovar representation across those samples can be affected by the investigated region and collected water sample volume, mainly for those serovars that are relevant in public heath. Further conclusions about other putative important moderators were not possible because of the lack of information in the accessed studies. In this sense, we encourage longitudinal study designs and thorough serotyping that enable conclusions on seasonal variations or the effects of factors such as physicochemical parameters of water and special-temporal information. Furthermore, high throughput approaches such as metagenomics could provide invaluable information about complex relationships between *Salmonella* and other biotic factors. Given the importance of water quality for agri-food systems and the public health importance of *Salmonella*, it is extremely important to better understand this dynamics, so that more effective strategies to control and mitigate salmonellosis can be envisioned and designed.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

A.D.L.R.: Literature search, Data analysis, Manuscript writing. R.G.F.: Conceptualization, Literature search, Data analysis, Manuscript writing. W.E.P: Data analysis, Manuscript revision. L.A.L.: Data analysis, Manuscript writing. P.E.N.G: Manuscript writing, Manuscript revision. A.I.M.: Manuscript writing, Manuscript revision. M.T.: Manuscript writing, Manuscript revision. E.J.D.S.: Manuscript writing, Manuscript revision. J.M.: Conceptualization, Manuscript revision. C.J.B.O.: Conceptualization, Manuscript writing, Manuscript revision

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Reference styles

We used Vancouver system for in-text citations.

Supplementary materials

Tables S1 and S2. The Supplementary Material for this article can be found online at:

Data Availability Statement

The data that support the findings of this study are available upon reasonable request from the authors

Contribution to the Field Statement

Salmonellosis is a major foodborne disease worldwide and can be caused by a wide range of serovars of *Salmonella enterica* subspecies *enterica*. Considering the increasing number of studies reporting the occurrence of *Salmonella* in natural (non-recycled) environmental water sources, we aimed to estimate the frequency of this pathogen in water, and to investigate the representation of *Salmonella* serovars across different studies. We also investigated how the relative frequencies of those serovars can be affected by factors such as the type of water source (surface and groundwater), the human development index of the country of origin, and the water sample volume. This meta-analysis resulted in novel information that can be used to improve *Salmonella* monitoring programs in agricultural settings. The presence of *Salmonella* in natural water sources should no longer be neglected considering the importance of water for irrigation purposes in agri-food production systems.

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3 CAPÍTULO 2 - Predictors of *Salmonella enteric*a contamination in natural watersheds under agricultural and livestock impact

Predictors of *Salmonella enteric*a contamination in natural watersheds under agricultural and livestock impact

3.1 ABSTRACT

Salmonella enterica (*S. enterica*), a ubiquitous zoonotic foodborne pathogen, remains a significant public health hazard and economic burden globally. Despite historical containment efforts that focused on the mitigation of its oral-fecal transmission and improved quality control in agrifood systems, the incidence of salmonellosis persists, with concerning outbreaks associated with plant-based foods in recent years, many of them probably caused by irrigation water. This study investigated anthropogenic and environmental factors influencing *S. enterica* occurrence in natural watersheds impacted by agricultural and livestock purposes in Paraiba, Brazil. A longitudinal 10-month sampling from multiple sampling sites across the three major river basins was conducted. Water samples were obtained by Modified Moore Swabs (MMS) and processed by conventional *S. enterica* isolation methodologies. Physicochemical parameters, climate, and human activities near the water sources were recorded. A logistic regression model was fitted using GLM and further adjusted according to the selected variables using the LASSO method. A non-statistical decision tree model was also fitted using the *rpart* package in R. Rainfall emerged as a pivotal factor influencing *S. enterica* recovery, potentially due to leaching from soil. Seasonal effects were observed, with higher *S. enterica* prevalence in autumn, but variability across regions poses challenges for generalization. Non-ruminant farming positively correlated with higher *S. enterica* occurrence, while higher water temperature, salinity, and resistivity were linked to decreased contamination. The complexity of multivariate conditions driving *S. enterica* contamination in surface waters underscores the need for region-specific investigations.

KEYWORDS: risk factors; microbial persistence; environmental factors; waterborne transmission.

3.2 RESUMO

Salmonella enterica (*S. enterica*), um agente patogénico zoonótico omnipresente de origem alimentar, continua a ser um perigo significativo para a saúde pública e um fardo económico a nível mundial. Apesar dos esforços históricos de contenção que se centraram na mitigação da sua transmissão oral-fecal e na melhoria do controlo de qualidade nos sistemas agroalimentares, a incidência da salmonelose persiste, com surtos preocupantes associados a alimentos de base vegetal nos últimos anos, muitos deles provavelmente causados pela água de irrigação. Este estudo investigou fatores antrópicos e ambientais que influenciam a ocorrência de *S. enterica* em bacias hidrográficas naturais impactadas por fins agrícolas e pecuários na Paraíba, Brasil. Foi realizada uma amostragem longitudinal de 10 meses em vários locais de amostragem nas três principais bacias hidrográficas. Amostras de água foram obtidas por swabs de Moore modificados (MMS) e processadas por metodologias convencionais de isolamento de *S. enterica*. Foram registrados parâmetros físico-químicos, clima e atividades humanas próximas às fontes de água. Um modelo de regressão logística foi ajustado usando GLM e posteriormente ajustado de acordo com as variáveis selecionadas usando o método LASSO. Um modelo de árvore de decisão não estatística também foi ajustado usando o pacote rpart em R. A precipitação emergiu como um fator essencial que influencia a recuperação de S. enterica, potencialmente devido à lixiviação do solo. Foram observados efeitos sazonais, com maior prevalência de *S. enterica* no outono, mas a variabilidade entre regiões coloca desafios à generalização. A criação de não ruminantes correlacionou-se positivamente com a maior ocorrência de S. enterica, enquanto a maior temperatura da água, salinidade e resistividade foram associadas à diminuição da contaminação. A complexidade das condições multivariadas que conduzem à contaminação por *S. enterica* nas águas superficiais sublinha a necessidade de investigações específicas da região.

Palavras-chave: fatores de risco; persistência microbiana; fatores ambientais; transmissão pela água.

3.2 Introduction

Salmonella enterica (*S. enterica*) is a leading zoonotic foodborne pathogen globally, causing serious economic and public health burden. It ranks second among the most frequent foodborne diseases in the US, only behind campylobacteriosis (TACK et al., 2019). The estimated costs caused by salmonellosis in the US increased from 3.7 billion dollars in 2012 (HOFFMANN et al., 2015) to 4.1 billion in 2018 (U.S. DEPARTMENT OF AGRICULTURE (USDA), 2022). Although some *S. enterica* serovars are restricted to specific host species, in which they can cause systemic clinical disease, the vast majority of the more than 2,600 different serovars that have been already identified can be usually found in the intestinal tract of a wide range of hosts, including domestic and wild animals (ABULREESH, 2012; CHENG et al., 2019).

During the 20th century, it became a consensus that salmonellosis is transmitted mainly by the oral-fecal route, i.e., through the ingestion of water or foodstuff contaminated by *S. enterica* originated from sewage and animal waste (COSTALUNGA AND TONDO, 2002; GUARD-PETTER, 2001). Therefore, quality control policies in the production, handling and storage of animal foods, as well as awareness of treated water consumption, were established as the means for mitigating salmonellosis and other foodborne diseases in consumers (MAIJALA et al., 2005). These measures have certainly contributed to the long-term reduction in the number of cases observed in the last century (CDC, 2013). However, no further significant reductions in the incidence of salmonellosis have been observed in the 21st century. Conversely, there is a steady incidence of salmonellosis, affecting around one million people per year in the US alone (TACK et al., 2019). Furthermore, over the past two decades, several salmonellosis outbreaks have been traced back to the consumption of plant-based foods (LIU et al., 2018; WALSH et al., 2014), raising a red flag about the effectiveness of the traditional *Salmonella* control measures that have been usually applied to the agri-food industry worldwide. Therefore, greater attention has been paid by both the scientific community and stakeholders of the agrifood industry to the potential role of water used in irrigation and food processing on the epidemiology of salmonellosis.

The ability of *S. enterica* to remain viable in extra-intestinal environments is considerably greater than previously believed (DOMINGO et al., 2000; LIAO AND SHOLLENBERGER, 2003). Furthermore, the presence of this pathogen is not among the microbiological parameters of water quality assessment, and the use of *E. coli* as an indicator of *S. enterica* contamination seems not to be accurate (BENJAMIN et al., 2013). These aspects may point out the possibility that the presence and persistence of *S. enterica* in aquatic environments may be underestimated.

Although the occurrence of *S. enterica* in natural water sources has usually been attributed to sporadic events involving waste contamination, the long-term persistence of *S. enterica* strains in natural waters suggests that natural water sources might be potential reservoirs of *S. enterica* (BAUDART et al., 2000; LI et al., 2014). Previous studies reported physicochemical parameters, such as turbidity (HAVELAAR et al., 2017) and conductivity (WELLER et al., 2020) to be significantly associated with *S. enterica* contamination in water. However, there is a lack of knowledge on comprehensive investigations addressing potential drivers affecting the occurrence of viable *S. enterica* in aquatic environments.

The aim of this study was to investigate physicochemical, climate, and non-climatic environmental factors associated with the occurrence of *Salmonella enterica* in natural watersheds impacted by agricultural and livestock production in Paraiba, Northeastern Brazil.

3.3 Materials and Methods

Study design and geographic characterization

We performed a longitudinal study over a period of ten months in five large dams (A to E) associated with the three largest hydrographic basins in Paraíba state in Brazil: Piranhas, Paraíba, and Mamanguape rivers (Table 1) (Figure 1). Three sampling sites were selected in each dam based on the presence of agricultural and livestock activities close to the water margins. Water samples were collected in triplicate from each sample site monthly, totaling 150 samples (450 swabs). The sampling visits in each sampling site were performed in the following months: February, March, April, May, August, November and December of 2022 and January, February and Mach of 2023. Therefore, samplings covered all the four different seasons (Summer, Autumn, Winter and Spring).

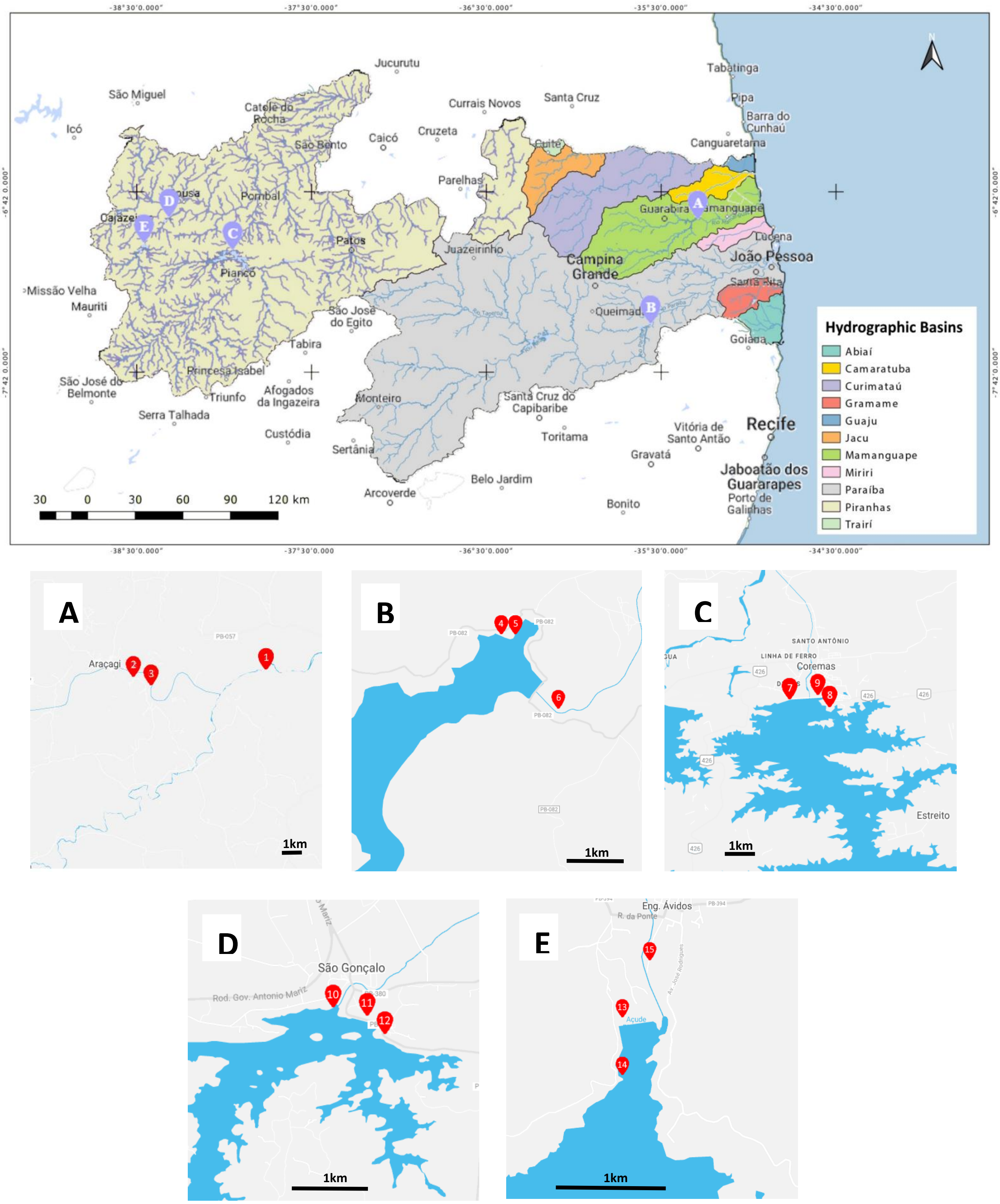

Figure 8 - Map of the river basins in the state of Paraíba with indication of the sampled points. (A) Araçagí, (B) Itatuba, (C) Coremas, (D) Sousa and (E) Cajazeiras.

Table 3 - Identification, total water capacity and location of the dams in Paraiba State, Brazil, from which water sources were collected over a 10-month period for *Salmonella enterica* isolation.

Dam ID	Dam name/City	River	Capacity (m³)	Number of sampling sites	Site Coordinate	Water Flow Condition
A	Araçagi/Araçagi	Mamanguape	63,289,037	3	6°51'14.9"S 35°18'03.0"W	Stagnant
					6°51'29.0"S 35°22'04.0"W	Flowing
					6°51'45.9"S 35°21'32.0"W	Flowing
B	Acauã/Itatuba	Paraíba	253,000,000	3	7°26'02.9"S 35°33'58.8"W	Stagnant
					7°26'05.4"S 35°33'51.7"W	Stagnant
					7°26'39.6"S 35°33'32.0"W	Flowing
C	Coremas/Coremas	Piancó	744,144,694	3	7°01'26.8"S 37°57'12.4"W	Stagnant
					7°01'35.1"S 37°56'36.8"W	Stagnant
					7°01'25.6"S 37°56'47.5"W	Stagnant
D	São Gonçalo/Souza	Piranhas	40,582,277	3	6°50'39.5"S 38°18'56.2"W	Stagnant
					6°50'44.2"S 38°18'40.3"W	Stagnant
					6°50'51.2"S 38°18'33.9"W	Stagnant
E	Engenheiro ávidos/Cajazeiras	Piranhas	293,617,376	3	6°59'12.1"S 38°27'18.3"W	Stagnant
					6°59'27.5"S 38°27'19.2"W	Stagnant
					6°58'57.8"S 38°27'12.3"W	Flowing

The Piranhas river basin is the largest in the state, occupying an area of 26,047.49 km². It rises in the southwest of the state and flows northeast into the Atlantic Ocean in the north of the state of Rio Grande do Norte (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS (AESA), 2022a, 2017). The largest dams in the state belong to this basin (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS (AESA), 2022b). Samples were taken from a total of nine sampling sites in this basin. Three sampling sites were selected in the Coremas-Mãe d'água complex in the course of the Piancó river, the largest water reservoir in the state, with a total capacity of 1,289 million m³ of water. Two other sampling sites were located in Engenheiro Ávidos dam, in the course of the Piranhas River, which stands as the third largest dam with a capacity of 293 million m³. One sampling site was set at the Piranhas river, and the three remaining sampling sites were defined on the São Gonçalo dam, also in the Piranhas river.

The Paraíba river basin occupies an area of 20,071.83 km². It rises in the south scepter of the state and runs eastward, flowing into the Atlantic Ocean (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS (AESA), 2022a, 2017). Although it is the second largest dam in terms of extension, it is of utmost economic importance as it represents the source of water to 52% of the state's population (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS (AESA), 2017). Among the three sampling sites in this basin, two were located in Acauã dam, the fourth largest reservoir in the state with a capacity for 253 million m³ of water, and the last sampling point was located in the Paraíba River.

The Mamanguape river basin occupies an area of 3,522.69 km². It rises in the center of the state and runs northeast, flowing into the Atlantic Ocean (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS (AESA), 2022a, 2017). Three sampling sites were established in this basin, including two on the Jacaré river and one in the Araçagi dam in the course of the Mamanguape river. The basins of the Paraíba River and Mamanguape River are artificially interconnected through the Acauã-Araçagi canal (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS (AESA), 2017) as a result of the water distribution project known as the São Francisco river transposition. The Paraíba river receives water from the São Francisco river through the eastern canal, which further transfers water to the Mamanguape river through the Acauã-Araçagi canal.

Samplings and physical-chemical analysis of water

Water samplings were performed by the Modified Moore Swab (MMS) technique, consisting of sterilized polyvinyl chloride cassettes containing internal cheese cloth (swab) as filtering matrix constructed as previously reported (Sbodio et al., 2013). Individual MMS pieces were unwrapped at the sampling site and attached to a portable peristaltic pump (CPD-201-3, MS Tecnopon Equipamentos Especiais LTDA, SP, Brazil) using a sterile latex tube. A total 10 L-water volume was filtered at a 500 mL per minute filtering speed for 20 minutes. After samplings, individual swabs were aseptically transferred into a sterile flask containing 250 mL of modified buffered peptone water (added 1.25g of sodium chloride, 0.875g of disodium hydrogen phosphate and 0.375g of potassium dihydrogen phosphate) and properly refrigerated at 4ºC for transport to the laboratory. Triplicates from each sampling site were obtained by repeating the sampling procedure consecutively at each sampling site using individual sterile cassettes.

Physicochemical parameters were determined *in situ* by means of a portable multiparameter metering equipment (Akso Electronic Products LTDA, RS, Brazil), including temperature (TE), hydrogen potential (pH), conductivity (CO), salinity (SA), resistivity (RE), total dissolved solids (TDS), and dissolved oxygen (DO). Turbidity (TU) was determined with the digital turbidimeter (TU430v.2, Akso Produtos Eletrônicos LTDA, RS, Brazil).

The geographic coordinates of the sampling sites were recorded using the Epicollect application on an Android device (Aanensen et al., 2014). Rainfall and water reservoir volume data were collected from records made available by the Executive Water Management Agency (AGÊNCIA EXECUTIVA DE GESTÃO DE ÁGUAS - AESA), a state agency that is part of the Integrated Water Resources Planning and Management System in Brazil, through a publicly available database (http://www.aesa.pb.gov.br/aesa-website/meteorologia-chuvas/).

Characterization of anthropogenic agricultural and livestock activities and occurrence of wild fauna

The characterization of the activities close to the water margins was determined by visual inspection at each sampling visit. We considered the typology of the rural properties observed on the main road leading to the sampling sites, in addition to the identification of the agrifood production activities on the visible water body margins. The sampling sites were classified as: 1. Extractive activity of fishing (fish and/or shrimp); 2. Intensive fish and/or shrimp farming; 3. Ruminant farming; 4. Non-ruminant farming; 5. Cultivation of plants/produces/vegetables for human consumption; 6. Cultivation of fodder for livestock feeding; 7. Presence of wild animals; 8. Human recreational activities.

Determination of water flow condition, rainfall regime and water levels in dams

Water flow condition was classified as flowing (under a steady unidirectional flow) or as stagnant water (without unidirectional flow) according to visual inspection. Information on precipitation and water levels in the dams was obtained from the publicly available AESA database. We reported the total precipitation at the sampling site within the sampling month (MR), in the week before sampling (WR), on the day before sampling (PDR), and on the day of sampling (DR). Furthermore, we observed the effect of average rainfall on the entire river basin in the week before sampling (ARW) and within the month of sampling (ARM). Data regarding water levels in the dams was obtained from records available for each dam on the dates of their respective samplings (http://www.aesa.pb.gov.br/aesa-website/monitoramento/).

Sample processing and Salmonella* ssp. *isolation

Salmonella isolation was performed according to Andrews et al. (2014) with minor modifications. Swabs were pre-enriched in modified buffered peptone water at 37 ± 1°C for 24 hours. After the incubation, 0.1 mL aliquots were inoculated into 9.9 mL of Rappaport-Vassiliadis Broth (RV; Oxoid, Thermo Fisher Scientific, Loughborough, UK), and 1mL aliquots inoculated into 9 mL tetrathionate broth (TT; Oxoid). After incubation at 42.5 ± 1°C for 18 hours by means of a water bath.

TT and RV-enriched samples were streaked onto XLT-4 Agar (Oxoid). In parallel, broth cultivation-polymerase chain reaction (BC-PCR) targeting the *InvA* gene was performed for both TT and RV-enriched samples. Aliquots from positive samples in the BC-PCR test were also streaked onto Hektoen enteric agar (HE; Oxoid) and bismuth sulfite agar (BS; Oxoid). Culture plates were incubated at 37 ± 1ºC for 24 hours. Up to three typical *Salmonella* colonies from each plate were inoculated into triple sugar iron agar (TSI; Oxoid) and lysine iron agar (LIA; Oxoid) and incubated at 37 ± 1ºC for 16 hours. Presumptive *Salmonella* isolates were inoculated on tryptic soy agar (TSA; Oxoid), followed by molecular confirmation by PCR. The DNA template for PCR confirmation of isolates was prepared using a loopful of bacterial mass expanded into 100 µl of DNase/RNase-free ultrapure water (Thermo Fisher Scientific, MA, USA).

The remaining steps were identical to the enrichment broth-PCR (EB-PCR) method described in subheading 2.6. The PCR-confirmed isolates were incubated in brain heart infusion broth (BHI; Oxoid) added 20% glycerol (Neon Comercial Reagentes Analíticos LTDA, Susano, Brazil) and stored at -80°C.

Enrichment-broth Polymerase Chain Reaction (EB-PCR)

Total DNA extraction was performed by a boiling-centrifugation method (FRESCHI et al., 2005). Shortly, cultured broth (1,5 mL) was centrifuged at 12,000 *g* for 2 minutes. The supernatant was discarded, the pellet was resuspended in 800µl ultrapure water, and centrifuged again at 12,000 *g* for 2 minutes. The supernatant was discarded, and the pellet resuspended in 200µl ultrapure water and heated at 95°C for 10 min. A final centrifugation step (12,000 *g* for 2 minutes) was performed, and 100µl supernatant was transferred to a sterile microtube to be used as a DNA template.

The master mix was prepared in a 25 µl final volume containing 1.4 mM $MgCl_2$, 1.25 U Taq DNA Polymerase (Platus, Sinapse, Brazil), 2 mM of each dNTP, 10 µM of each primer, 1 µg DNA template and 10X Taq Buffer. We used the primers F: 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' and R: 5'-TCA TCG CAC CGT CAA AGG AAC C-3' targeting the *InvA* gene.

The PCR was performed in a Thermocycler (Biometra T-Advanced, Analytik Jena AG, Jena, Germany) under the following conditions: initial denaturation at 95°C for 3 minutes, followed by 35 cycles consisting of a denaturation step at 95°C for 30 seconds, an annealing step at 53°C for 30 seconds, and an extension step at 72°C for 2 minutes and 20 seconds, and a final extension at 72 °C for 5 minutes. PCR amplicons were electrophorized on a 1.5% agarose gel at 80 V for 40 minutes in 1x Tris Acetate EDTA buffer, stained with SYBR Safe DNA Gel staining solution (ThermoFisher Scientific) and visualized under UV light o Felipe(Gel Logic 212 PRO, Carestream Health, NY, USA).

Statistical analysis

The complete dataset was tabulated in Microsoft Excel (Microsoft, NM, USA) and available as Supplementary material (Table S1). Statistical analyses were performed in R (RSTUDIO TEAM, 2019).

The LASSO (Least Absolute Shrinkage and Selection Operator) method was used for feature selection. A logistic regression model with L1 penalty was fitted using the *glmnet* package (FRIEDMAN et al., 2010). The coefficients were plotted in relation to the regularization parameter lambda. The best lambda was selected based on cross-validation. Non-zero coefficients were identified as selected variables. A logistic regression model was fitted with all independent variables using GLM. The model summary was generated with test statistics for each coefficient. Analysis of variance (ANOVA) was used to evaluate the statistical significance of each independent variable. A new logistic regression model was adjusted only with the variables selected using the LASSO method. The marginal effects of the model's independent variables were plotted using the *ggeffects* package (LÜDECKE, 2018). Odds Ratios (OR) and their 95% confidence intervals were calculated for each independent variable in the model.

A decision tree model was fitted using the *rpart* package in R (THERNEAU et al., 2013). The decision tree model was visualized using *rpart.plot* package and the correlation between the response variable (*Salmonella* presence) and the variables predicted by the decision tree model was also calculated. The rattle library (WILLIAMS, 2011) was used to build the decision tree.

3.5 Results

Physicochemical parameters of water

Table 2 shows the maximum and minimum values of the physicochemical parameters measured in the water samples from each investigated dam according to the *S. enterica* status, i.e., positive or negative. It also shows overall maximum and minimum for each parameter considering all sampling sites. Detailed information about the physicochemical parameters in each individual sample is available as supplementary material (Table S1).

Characterization of anthropogenic, agricultural, and livestock activities, and occurrence of wild fauna

The anthropogenic activities in the hydrographic basins observed during water samplings are shown as supplementary material (Table S1). The most observed activities were CTR1 (fish/shrimp farming) and CTR 8 (human recreational activity), with 296 and 295 observations, respectively. On the other hand, CTR6 (fodder planting for animal feeding) was the lowest observed activity (n=29). The other activities (CTR2, CTR3, CTR4, CTR5 and CTR7) were observed 89 +/- 1 times on average.

Rainfall regime and reservoir volume levels

The rainfall regime and respective water volume levels in the dams are shown in the Supplementary material Table S1. The monthly rainfall observed in the sampling sites over the study period varied from 4.8 to 297.3 mm (Table 3). These values were similar to those observed for the whole basins, ranging from 7.2 to 293.4 mm. Precipitation over the months in each municipality can be seen in Table 3. Seasonal precipitation ranged from 4.8 to 273.2 mm in Summer, 58.2 to 297.3 mm in Autumn, 12.2 to 102.6 mm in Winter, and 8.3 to 140.2 mm in Spring. Detailed information about water levels over the sampling period is shown in Table 3.

Salmonella* spp. *detection frequencies

Out of the 443 MMS samples collected from the 15 different sampling points during the 10 month-sampling period, 314 (70.9%) were positive for *S. enterica*. Seven swabs were missed over the 10-month sampling. The frequencies of *S. enterica* isolation in each sampling site over the 10-month sampling period are shown in Figure 2A. All but one dam (C) had at least one positive sample in every sampling month. Nevertheless, we detected at least one positive sample in 9 out of 10 samplings performed in this dam. The *S. enterica* frequencies varied from 42.5% (Dam C) to 97.8% (Dam E). Regarding the seasonal variation, the highest frequency of *S. enterica* positive samples was observed in Autumn (83.3%; 75/90), followed by Spring (74.4%; 67/90), Summer (69.7%; 152/218) and Winter (44.4%; 20/45), as shown in Figure 2B.

Table 4 - Maximum and minimum values of the physicochemical parameters temperature, pH, salinity, conductivity, dissolved oxygen, total dissolved solids, and resistivity measured in the water samples from each investigated dam according to the *S. enterica* status, i.e., positive (+) or negative (-).

Dam	***Salmonella* status**	**Temp (ºC)**		**pH**		**Salinity (PPT)**		**Conductivity (µS)**		**Turbidity (NTU)**		**Dissolved Oxygen (mg/L)**		**TDS (mg/L)**		**Resistivity (Ω)**	
		Min.	**Max.**	**Min.**	**Max.**	**Min.**	**Max.**	**Min.**	**Max.**	**Min.**	**Max.**	**Min**	**Max.**	**Min.**	**Max.**	**Min.**	**Max.**
A	+	28.90	41.00	6.70	9.24	0.34	0.86	747.67	1652.67	12.02	262.33	0.68	15.99	522.67	1220.00	579.67	7583.33
	_	27.80	39.70	7.10	8.99	0.36	0.88	718.00	1710.33	19.81	262.33	2.39	15.41	519.00	1270.00	568.67	1360.00
B	+	26.30	40.00	6.85	9.30	0.53	0.75	1117.67	1548.67	0.00	335.33	0.50	8.41	786.33	1083.33	650.67	912.67
	_	27.30	40.00	6.95	9.13	0.59	0.75	1117.67	1548.67	0.59	335.33	0.44	6.97	854.00	1083.33	650.67	831.67
D	+	28.00	39.20	6.97	9.00	0.05	0.13	103.83	294.67	0.00	42.57	0.59	8.98	74.20	178.33	3733.33	9540.00
	_	28.00	38.20	6.97	9.71	0.05	0.12	100.70	240.00	0.00	28.50	0.59	10.53	72.50	174.00	4116.67	9723.33
C	+	29.30	42.60	6.78	9.30	0.05	0.12	107.83	246.00	0.00	83.63	1.42	10.24	76.80	174.00	3970.00	9263.33
	_	29.30	38.80	6.84	9.30	0.05	0.14	108.63	281.33	0.00	155.43	1.40	10.53	76.80	206.00	3366.67	9263.33
E	+	28.60	39.20	7.38	8.85	0.04	0.10	81.37	215.67	0.00	169.03	3.59	9.55	61.80	153.00	1090.00	5340.00
	_	28.60	28.60	8.24	8.34	0.04	0.09	81.37	190.53	2.15	9.00	7.32	8.12	61.80	136.00	1150.00	5206.67
Total	+	26.30	42.60	6.70	9.30	0.04	0.86	81.37	1652.67	0.00	335.33	0.50	15.99	61.80	1220.00	579.67	9540.00
	_	27.30	40.00	6.84	9.71	0.04	0.88	81.37	1710.33	0.00	335.33	0.44	15.41	61.80	1270.00	568.67	9723.33

Table 5 - Monthly rainfall (mm) and water levels expressed as percentage of the total capacity in the sampled dams observed in a 10-month longitudinal sampling study.

Sampling month	A (Araçagí)		B (Itatuba)		C (Sousa)		(D) Coremas		(E) Cajazeiras	
	Precipitation (mm)	Dam Volume	Precipitation (mm)	Volume	Precipitation (mm)	Volume	Precipitation (mm)	Volume	Precipitation (mm)	Volume
Feb/22	22.5	46.08%	4.8	14.52%	70.6	51.67%	40.0	46.59%	85.5	35.89%
Mar/22	154.9	43.91%	135.4	14.71%	194.0	101.75%	222.2	52.81%	184.2	35.54%
Apr/22	133.4	59.51%	58.2	17.09%	171.3	102.00%	144.6	57.40%	171.4	35.23%
Mai/22	297.3	72.29%	212.0	18.73%	60.9	83.14%	76.1	57.14%	84.3	34.49%
Ago/22	64.1	100.58%	35.0	65.84%	33.7	99.19%	102.6	54.15%	12.2	34.39%
Nov/22	60.2	95.61%	8.3	63.16%	74.9	77.98%	67.0	49.25%	140.2	33.80%
Dez/22	23.1	93.00%	28.7	62.09%	40.7	69.88%	47.0	48.02%	26.1	33.66%
Jan/23	162.8	92.40%	72.1	60.10%	127.4	49.63%	58.7	45.14%	126.0	33.85%
Feb/23	74.9	91.08%	35.4	58.72%	161.3	58.85%	114.3	44.67%	136.7	34.14%
Mar/23	142.0	95.61%	66.1	57.27%	230.5	58.96%	242.3	42.81%	273.2	33.95%

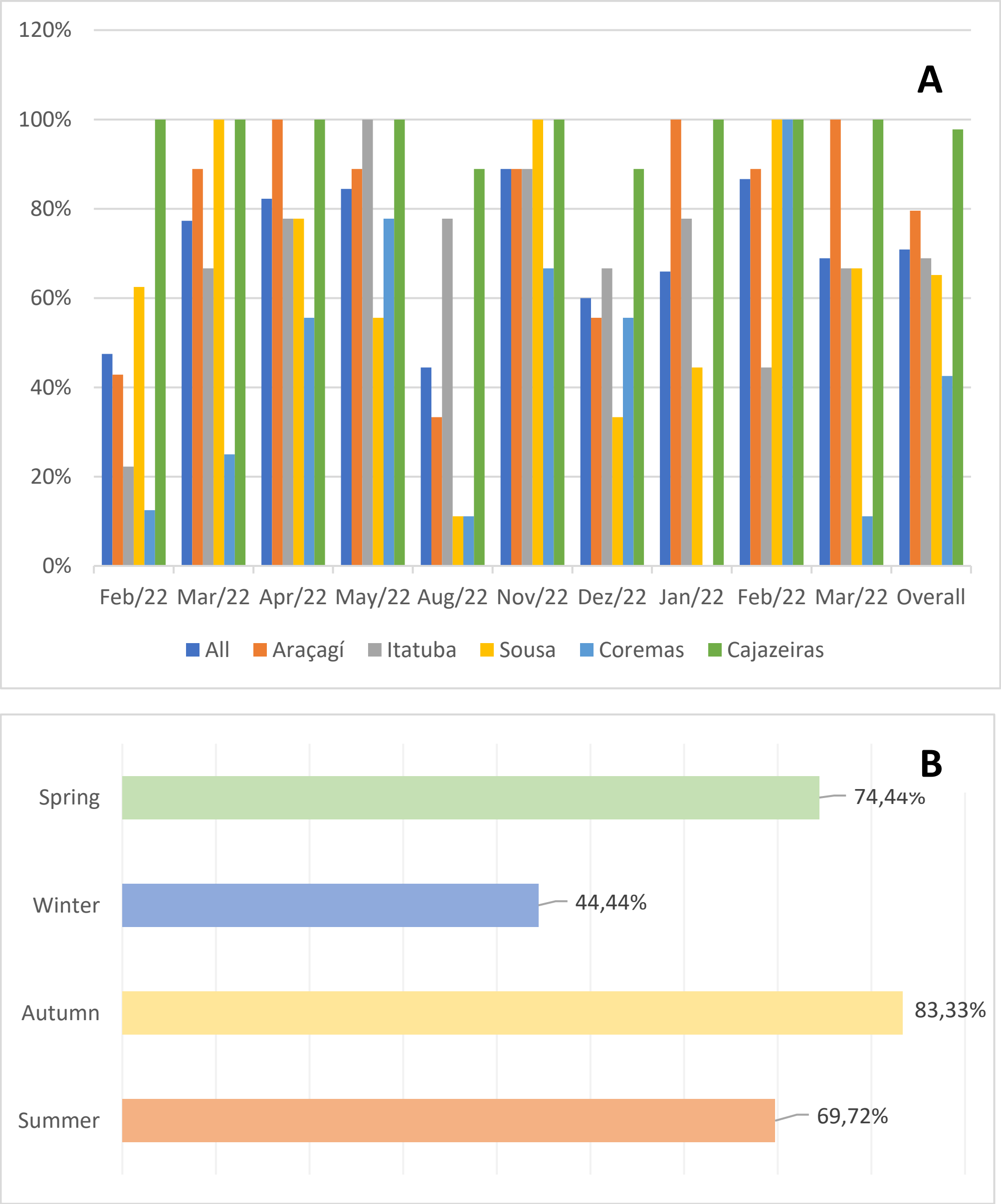

Figure 9 - *Salmonella enterica* isolation rate over a 10-moth sampling period in each of the five investigated dams (A) and total frequency of *Salmonella enterica* positive samples observed in each season (B).

Predictors of Salmonella contamination

According to the regression model (Figure 3), we identified season, rainfall regime, some physicochemical features, and anthropogenic agricultural variables to be significantly associated with *S. enterica* contamination in water. Autumn and Spring seasons (Figure 4A), flowing water (Figure 4B), rainfall within the sampling month (Figure 4C), and the presence of non-ruminant animals (Figure 4D) were identified by feature selection using LASSO as variables associated with increased *S. enterica* occurrence (Figure 3). On the other hand, Winter (Figure 4A), high water temperature

(Figure 5A), higher water salinity (Figure 5B), high water resistivity (Figure 5C), higher water pH (Figure 5D) and proximity to fodder planting areas (Figure 5E), were significantly associated with decreased *S. enterica* occurrence (Figure 3).

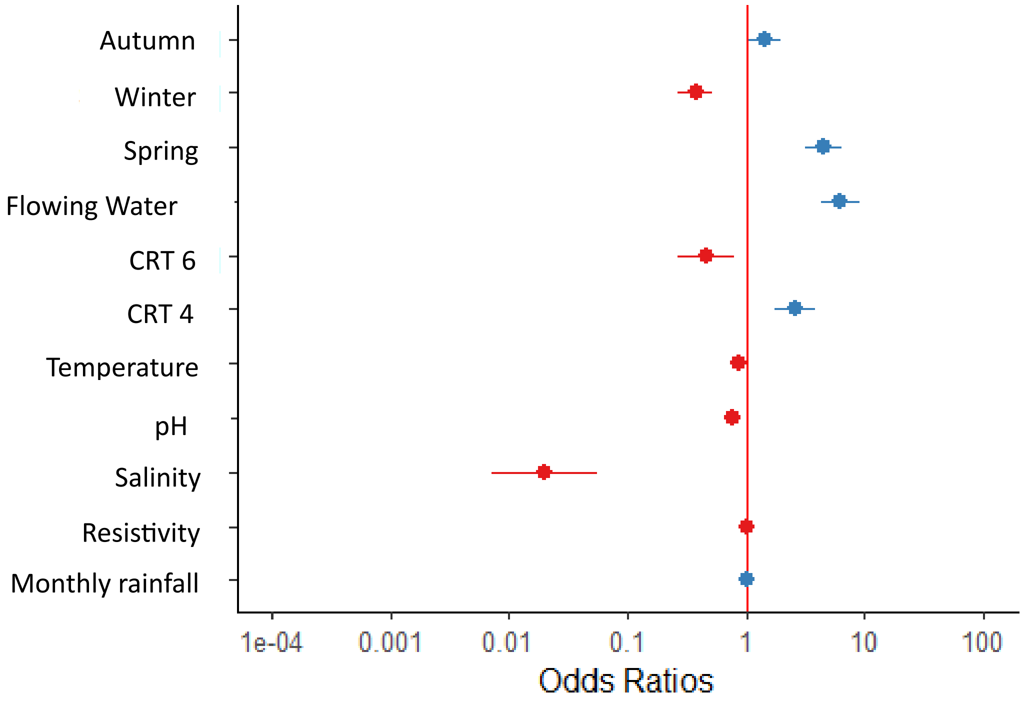

Figure 10 - Variables significantly associated with *Salmonella enterica* contamination in natural waters in Northeastern Brazil. Odds-ratio (OR) values and their respective 95% confidence intervals for positively blue (Autumn: 1.438207e+00, Spring: 4.464933e+00, Flowing Water: 6.225849e+00, CRT4 (Non-ruminant farming): 2.567683e+00 and Monthly rainfall: 1.006626e+00) and negatively red(Winter: 3.723249e-01, CRT6 (Cultivation of fodder for livestock feeding): 4.554419e-01, Temperature: 8.659572e-01, pH: 7.657033e-01, Salinity: 2.005134e-02, and Resistivity: 9.997191e-01).

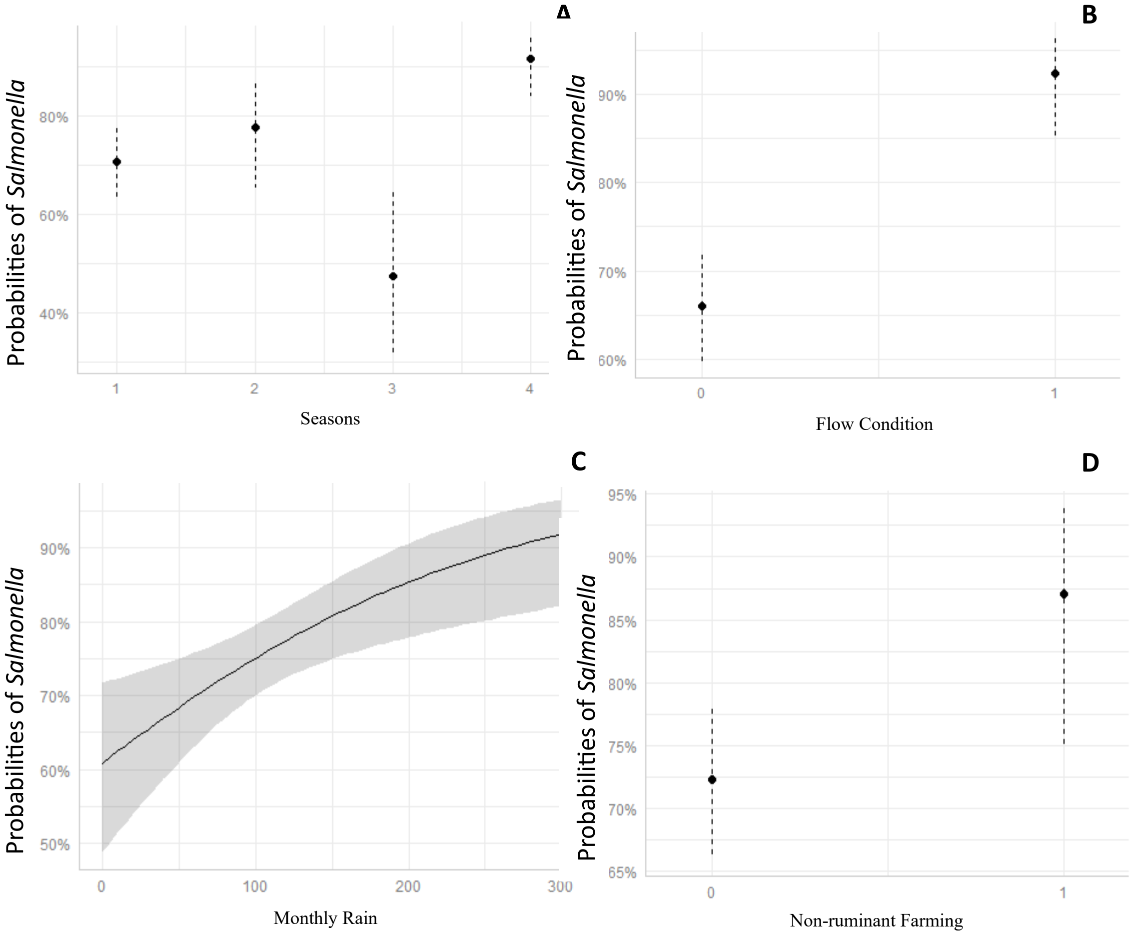

Figure 11 - Predicted probability of occurrence of *Salmonella enterica* according (A): Season (1-Summer,2 - Autumn,3 - Winter ,4 - Spring); (B): Flow condition (0=stagnant water; 1=flowing water); (C): Monthly Rain; (D): Non-ruminant farming (CRT4) close to the water margins (0 - Absence of *Salmonella*. 1 - Presence of *Salmonella*).

Strength of association and decision tree

The strength of association between factors and *S. enterica* recovery from the natural water sources was determined by means of both statistical (OR) values from regression modelling) and non-statistical (decision tree based on the supervised learning approach) methods.

According to the decision tree (Figure 6), total precipitation at the sampling site within the sampling month (MR) played a key role in *S. enterica* recovery, followed by a combination of factors, including temperature and seasonality. The correlation between the variables predicted by the decision tree and *S*. enterica status was moderate.

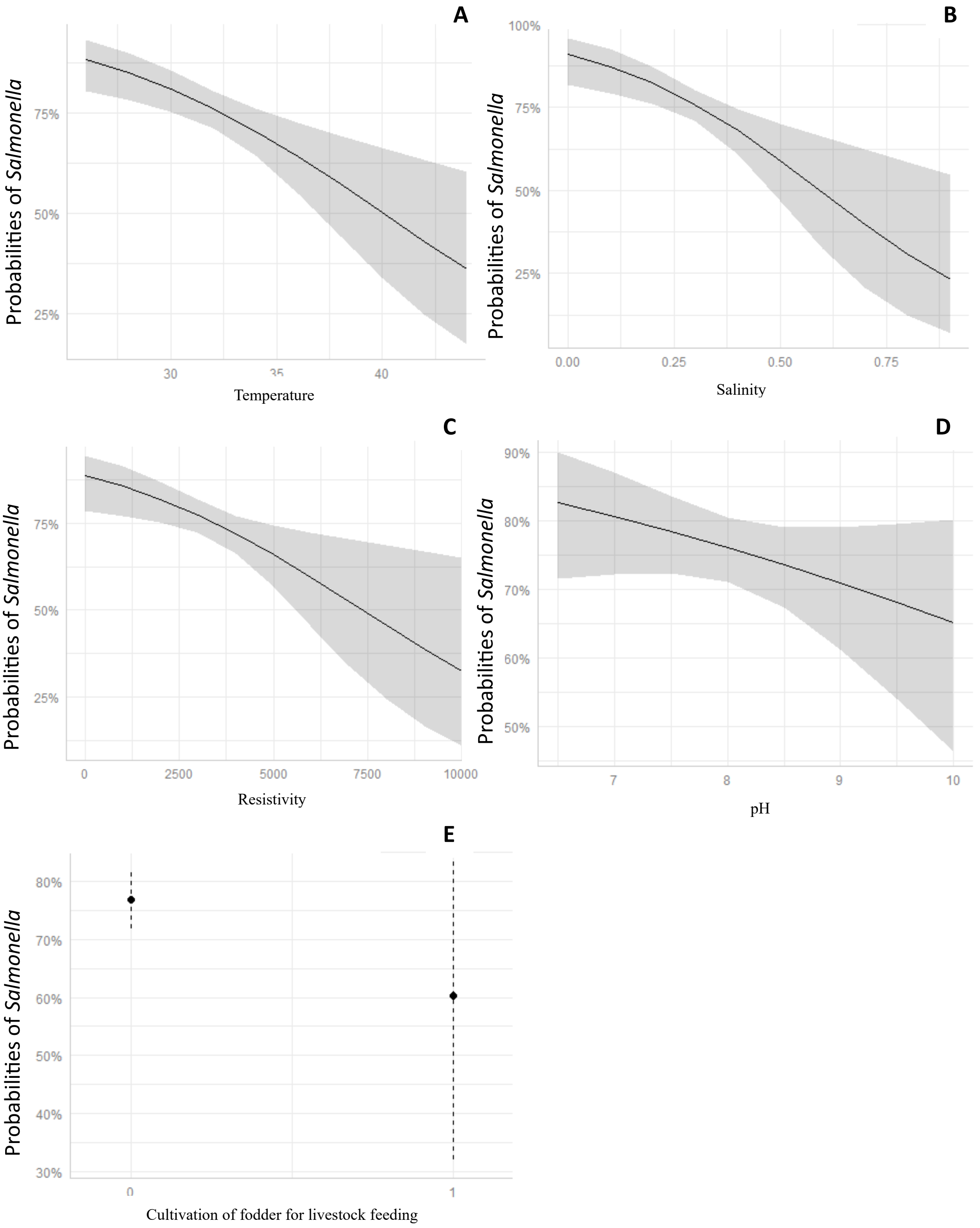

Figure 12 - Predicted probability of occurrence of *Salmonella enterica* as a function of (A) temperature variation (°C); (B) Salinity (PPT); (C) Resistivity (Ω); (D) pH and (E) Cultivation of fodder for livestock feeding (CRT6) in areas close to water margins (0 - Absence of *Salmonella*. 1 - Presence of *Salmonella*).

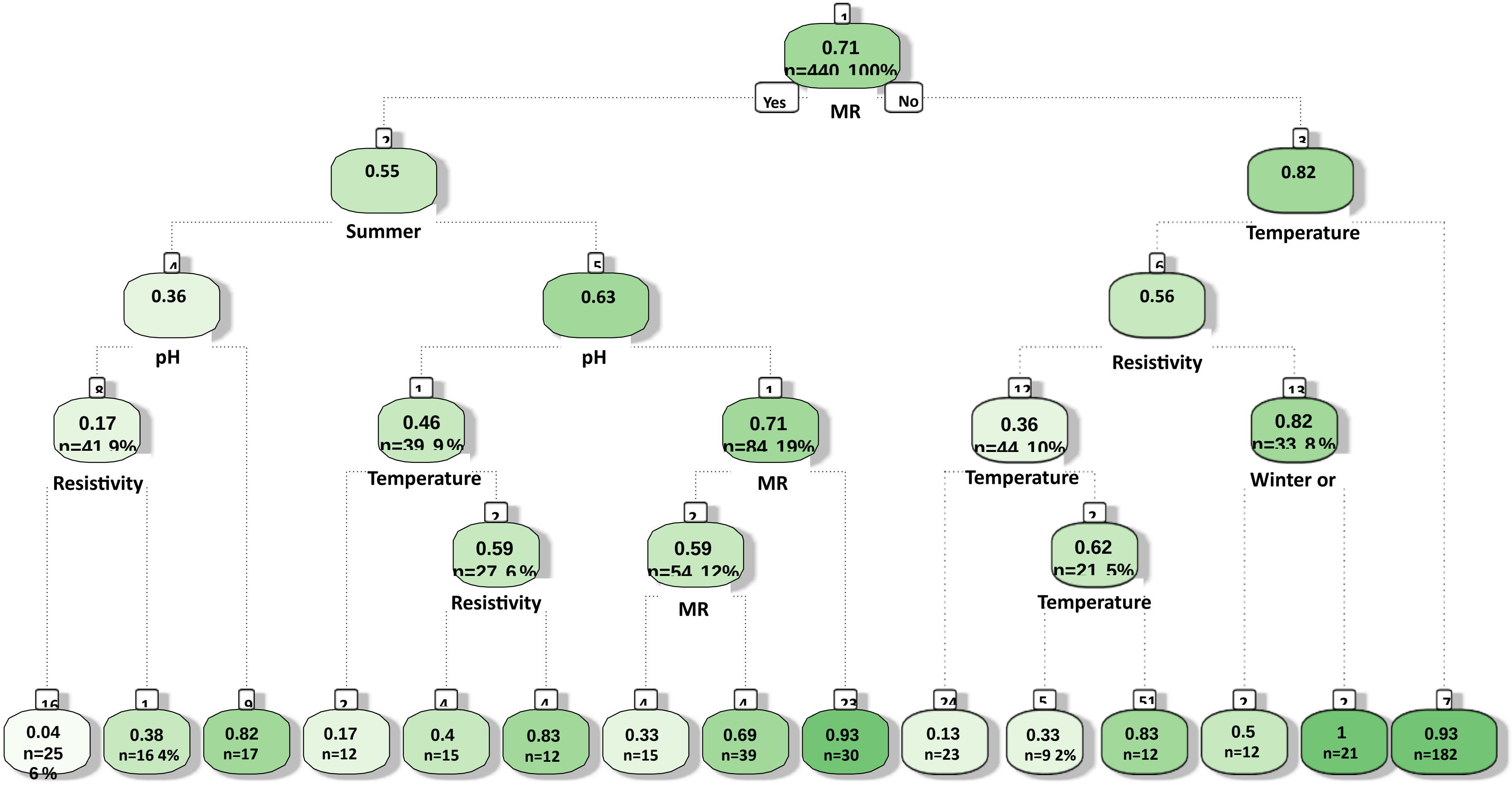

Figure 13 - Decision tree predicting the correlation between the response variable (*Salmonella* presence) and the variables Total monthly precipitation (MR), Seasons (Summer, Autumn and Winter) and physical-chemical factors (Temperature, pH e Resistivity). The predictive algorithm has been built in R (package *rpart*) using the complete experiment dataset.

3.6 Discussion

Our results indicated total precipitation within the sampling month as a key factor associated with *S. enterica* recovery from water samples over the 10-month investigated period, corroborating a previous study in Mexico (SIMENTAL AND MARTINEZ-URTAZA, 2008). Interestingly, we observed no statistical differences regarding other rainfall-related variables included in this study, such as rainfall in the week before sampling, on the day before sampling, or on the day of sampling. These results are in line with those reported in the USA (WELLER et al., 2020). On the other hand, Luo et al. (2015) observed a positive association between rainier weeks and *S. enterica* recovery rates. Considering variables related to different rainfall periods, Haley et al. (2009) observed increased *S. enterica* isolation rates only in samplings performed up to 3 days after rain. Although there are some variations regarding the specific rain periods affecting *S. enterica* recovery, our results highlight the evidence that rainfall could play an important role in the occurrence of *S. enterica* in natural surface waters, which is probably related to leaching (FREY et al., 2015). Moreover, the rise of water levels caused by rainfall can reach areas previously dry or disconnected from the main body of water, favoring the transference of microbes from soil to the water body. Lastly, sudden large rainfall volumes can lead to the overflow of pits and sewage galleries, spreading organisms previously restricted to these structures.

The lowest rate of *S. enterica* recovery (44.4%) was observed in winter. Although there are studies from different countries observing no effects of seasonality on *S. enterica* recovery (CHÁVEZ-DÍAZ et al., 2020; COOLEY et al., 2018; FALARDEAU et al., 2017), our results support previous investigations indicating a lower *S. enterica* detection rate in winter (LUO et al., 2015; TORO et al., 2022). The highest *S. enterica* detection frequency observed in autumn in our study is also supported by previous studies: Strathmann et al. (2016), who observed a 54.2% *S. enterica* frequency in autumn and 43.8% in winter; Zhou et al. (2019) observed a 57.1% *S. enterica* frequency in autumn and 52.4% in summer; as well as Marine et al. (2015) comparing *S. enterica* isolation in Autumn and Spring. Overall, there is a wide variation across studies reporting seasonal effects on *S. enterica* recovery from water. This is expected since the variation in *S. enterica* recovery rates throughout the year does not seem to be solely attributed to the season. It is more likely that the season is part of a set of complex interplaying variables. Moreover, the wide variation in climatic and non-climatic variables associated with seasonality across different parts of the world makes it difficult for the results from different studies to be directly compared. These variables include temperature ranges, rainfall variation, migratory fauna, temporary agricultural activity, and recreational water use, among others. This complex scenario requires a modeling approach as presented herein.

Considering the geographical and administrative characteristics of the region's water resources investigated herein, we were able to compare water samples exposed to the same general conditions but taken from flowing (rivers) or stagnant (dams along the river) conditions in the same hydrographic basin. In this aspect, our results indicated a higher contamination rate in flowing water (101/117; 86.3%) compared with stagnant water (213/326; 65.3%). In our survey, we did not find other studies that carried out this comparison. It is possible that flowing water reduces competitiveness between microorganisms, favoring *S. enterica*, which does not have the ability to compete with more adapted microorganisms. Therefore, flowing water could theoretically benefit *S. enterica* to survive longer in natural surface waters.

As for activities carried out near bodies of water, only sites close to non-ruminants farming, such as swine and poultry, seemed to be significantly associated with higher *S. enterica* contamination, while sites close to fodder planting had reduced *S. enterica* contamination. The higher rate of isolation in areas close to non-ruminants farming may be due to the lack of adequate treatment of animal waste from intensive systems. Furthermore, waste can be carried away by rain towards water bodies. On the other hand, water bodies close to areas designated for pasture plantations were less prone to *S. enterica* contamination probably because there were no animals or recurring human activity. These areas show reduced wild animal fauna. Moreover, whenever ruminant farming occurs in such areas, animals usually have seasonal access or no access at all, as the pasture is exclusively used for hay or silage production.

Among the physicochemical factors, temperature, pH, salinity, and resistivity showed an inverse correlation with *S. enterica* contamination (Figure 4A, 4B, 4C, and 4D). Our findings showed an inverse effect of temperature on *S. enterica* recovery compared with previous studies showing increased *S. enterica* isolation rates from warmer waters (GONZÁLEZ-LÓPEZ et al., 2022; ZHOU et al., 2019). However, it is noteworthy that the water temperature reported by these studies ranged from 1ºC to 36ºC, while the water temperatures in our study ranged from 26.3ºC to 42.6ºC, considering that our study was performed in a tropical semiarid region. Those are relevant differences that should be considered. It is possible that the higher temperature intervals observed in our study led to increased stress conditions to *S. enterica* and possibly other mesophilic bacteria. We observed a weak negative correlation between pH and *S. enterica* contamination, supporting previous findings (GONZÁLEZ-LÓPEZ et al., 2022). On the other hand, Bradshaw et al. (2016) observed that the combination of water pH and dissolved oxygen levels below 6.65 and 11.3 mg L-1, respectively, favored the contamination by *S. enterica* and *E. coli*. We observed that increased water salinity levels reduced the *S. enterica* recovery. This finding is supported by previous studies (GONZÁLEZ-LÓPEZ et al., 2022; MARTINEZ-URTAZA AND LIEBANA, 2005). We also showed that increased water resistivity was associated with decreased *S. enterica* contamination levels. As far as we know, there are no other studies investigating the role of this variable in *S. enterica* contamination.

Although we found no significant association for other physicochemical variables, total dissolved solids, turbidity, conductivity, and dissolved oxygen have been previously identified as factors associated with *S. enterica* contamination (BRADSHAW et al., 2016; HAVELAAR et al., 2017; WELLER et al., 2020; ZHOU et al., 2019). These conflicting results are probably a reflection of the complexity of interplaying variables affecting *S. enterica* contamination. Therefore, we believe that direct comparisons across different studies should be made with caution, especially by decision-makers proposing measures to reduce *S. enterica* contamination. We strongly suggest that regional investigations are required to understand the dynamics of *S. enterica* contamination in water, providing scientific-based evidence that could be used to mitigate *S. enterica* contamination in agrifood products in a regional context.

Conclusions

Rainfall seems to play a significant role in *Salmonella enterica* recovery from natural surface water bodies, probably due to the carry-over of microorganisms from soil. Although a seasonal effect was observed, with higher *S. enterica* recovery in autumn, the consideration of seasonality across different geographical regions complicates the utility of of this variable in comprehending *S. enterica* contamination dynamics in water. Our findings indicate the non-ruminant farming close to water margins was associated with increased *S. enterica* contamination, while higher water temperature (> 36ºC), salinity (> 0.60 ppt) and resistivity (>6000Ω) were linked to reduced *S. enterica* occurrence. Additionally, a higher *Salmonella* frequency was observed in flowing water compared to stagnant water. The complexity multivariate condition driving *S. enterica* contamination in surface waters should be considered. Further research, including other potential factors, such as biological co-contamination, and detection methods, such as quantitative approaches, could shed further light on the dynamics of *S. enterica* contamination in natural water bodies. However, it remains challenging to directly compare studies performed in different geographical settings. Consequently, we encourage that regional studies must be performed to understand better the status and dynamics of *S. enterica* in water to support direct measures towards the reduction of contamination in the agrifood chain.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

A.D.L.R.: Literature search, Data analysis, Manuscript writing. L.A.L.: Conceptualization, Literature search, Data analysis, Manuscript writing. W.E.P.: Data analysis, Manuscript writing. P.E.N.G.: Manuscript writing, Manuscript revision. M.T.: Manuscript writing, Manuscript revision. J.M.: Conceptualization, Manuscript revision. C.J.B.O.: Conceptualization, Manuscript writing, Manuscript revision

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Supplementary materials

Tables. The Supplementary Material for this article can be found online at:

Data Availability Statement

The data that support the findings of this study are available upon reasonable request from the authors.

Contribution to the Field Statement

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4 CAPÍTULO 3 - Environmental water as a source of rare *Salmonella enterica* serovars

Environmental water as a source of rare *Salmonella enterica* serovars

4.1 ABSTRACT

Although *Salmonella enterica* remains a major foodborne pathogen globally, *little attention has been paid to infrequent serovars.* Herein we reported the occurrence of 30 rare *Salmonella enterica* serovars isolated from environmental water sources between 2021 and 2022 in the northeast of Brazil. Among them, *Salmonella* Carrau (*n*= 14), *S*. Oran (*n*= 9), *S*. Gaminara (*n*= 5), and *S*. Urbana (*n*= 4) were the most frequent serovars. Whole-genome sequencing analysis revealed the presence of antimicrobial resistance genes (ARGs) in all isolates*. The highest AMR gene abundance was observed for* aminoglycosides [*aac(6')-Iaa (100%)],* quinolones [*parC:p.T57S (98.1%) and qnrB19 (3.77%)], and* fosfomycin [*fosA7 (3.77%)].* Additionally, some isolates carried plasmids [IncX3, IncFII(S), IncFII(Cf) Col(pHAD28), and IncFII(SARC14)] that could facilitate the spread of antimicrobial resistance. Phylogenetic analysis indicated the presence of distinct clades for each serovar. Interestingly, twenty serovars are endemic lineages circulating in Brazil, with the exception of *S*. Kiambu, which belongs to an international lineage. These findings underscore the importance of monitoring and understanding the distribution of *Salmonella* in water sources to safeguard public health and prevent the spread of antimicrobial resistance.

Keywords: antimicrobial resistance; *Salmonella* serovars; dam water; river.

4.2 RESUMO

Embora a *Salmonella enterica* continue a ser um importante agente patogénico de origem alimentar a nível mundial, pouca atenção tem sido dada aos serovares pouco frequentes. Aqui relatamos a ocorrência de 30 sorovares raros de *Salmonella enterica* isolados de fontes de água ambientais entre 2021 e 2022 no Nordeste do Brasil. Dentre eles, *Salmonella* Carrau (n= 14), *S*. Oran (n= 9), *S*. Gaminara (n= 5) e S. Urbana (n= 4) foram os sorovares mais frequentes. A análise de sequenciamento do genoma completo revelou a presença de genes de resistência antimicrobiana (ARGs) em todos os isolados. A maior abundância de genes AMR foi observada para aminoglicosídeos [aac(6′)-Iaa (100%)], quinolonas [parC:p.T57S (98,1%) e qnrB19 (3,77%)] e fosfomicina [fosA7 (3,77%)]]. Além disso, alguns isolados continham plasmídeos [IncX3, IncFII(S), IncFII(Cf) Col(pHAD28) e IncFII(SARC14)] que poderiam facilitar a propagação da resistência antimicrobiana. A análise filogenética indicou a presença de clados distintos para cada sorovar. Curiosamente, vinte sorovares são linhagens endêmicas que circulam no Brasil, com exceção de *S*. Kiambu, que pertence a uma linhagem internacional. Estas conclusões sublinham a importância de monitorizar e compreender a distribuição de *Salmonella* nas fontes de água para salvaguardar a saúde pública e prevenir a propagação da resistência antimicrobiana.

Palavras-chave: resistência antimicrobiana; sorovares de *Salmonella*; água da represa; rio.

IMPORTANCE

This research emphasizes the importance of monitoring and understanding the distribution of *Salmonella* in water sources, providing crucial insights into the diversity, antimicrobial susceptibility, and phylogenetic relatedness of rare serovars. The study underscores the need for continued surveillance, risk assessment, and water management strategies to safeguard public health and prevent the spread of antimicrobial resistance. The findings contribute significantly to the epidemiological understanding of *Salmonella enterica* in environmental settings, highlighting potential risks to agrifood systems and public health.

4.3 Introduction

Non-typhoidal *Salmonella enterica* (*S. enterica*) is a leading foodborne pathogen causing gastrointestinal infections in both humans and animals. While *S. enterica* has been commonly associated with contaminated food, it has been frequently reported in environmental water sources, including lakes, rivers, streams, ponds, and groundwater (LIU et. al., 2018; ROCHA et al., 2022). *S. enterica* can enter water bodies through different pathways such as contaminated runoff, sewage and wastewater discharge, agricultural practices, and wildlife (LI et al., 2015; TORO et. al., 2016; HARRIS et al., 2018), where it can survive for extended periods, especially in freshwater environments (DOMINGO et al., 2000; LIU et. al., 2018).

Some *Salmonella* serovars have been frequently reported in natural water sources globally, such as *S*. Newport, *S*. Typhimurium, *S*. Thompson, *S*. Javiana, *S*. Kentucky, and *S*. Rubislaw (ROCHA et al., 2022). However, water may be a source of a variety of serovars rarely or poorly described in the literature. For instance, despite *S*. Typhimurium and *S*. Enteritidis being the two most prevalent serovars related to foodborne diseases, foodstuff has been described as a source of many other rare antibiotic resistance *S. enterica* serovars that could not be neglected (MONTE et al., 2021). Therefore, investigations on the diversity and characterization of *S. enterica* serovars contaminating natural waters are necessary for the successful assessment of the putative risks to agrifood systems and public health.

In this context, we reported herein the occurrence, antimicrobial susceptibility, and phylogenetic relatedness of 30 rare *S. enterica* serovars isolated from environmental water sources in northeastern Brazil between 2021 and 2022.

4.4 Results

Salmonella enterica serovars

As showed in Table 1, we obtained a total of 68 *S. enterica* isolates belonging to 30 infrequent serovars: *S*. Carrau (n= 14), *S*. Oran (n= 9), *S*. Gaminara (n= 5), *S*. Urbana (n= 4), *S*. I 16:e,h:e,n,z15 (n=4), *S*. II 43:z4,z23:- or IIIa 43:z4,z23:- or Farmingdale or IV 43:z4,z23:- (n=3), *S*. I 18:d:- (n=3), *S*. I 7:l,v:- (n=3), *S*. I 4:b:- (n=2), *S*. Lille (n=1), *S*. Freetown (n=1), *S*. Businga (n=1), *S*. Bullbay (n=1), *S*. Molade or Wippra (n=1), *S*. Kiambu (n=1), *S*. Lomita (n=1), *S*. Mikawasima (n=1), *S*. Langenhorn (n=1), *S*. Othmarschen (n=1), *S*. Adelaide (n=1), *S*. I 4:-:1,5 (n=1), *S*. I 7:k:- (n=1), *S*. I 7:-:1,5 (n=1), *S*. I 16:r:e,n,z15 (n=1), *S*. I,v:e,n,z15 (n=1), *S*. IV [1],53:g,z51:- (n=1), *S*. IV 50:z4,z23:- (n=1), *S*. I 3,10:d:- (n=1), *S*. Somone or IV 6,7:z4,z24:- (n=1), and *S*. IV 45:g,z51:- (n=1). Among the 68 isolates obtained from 2021 to 2022, 46 originated from dams and 22 from rivers.

Antimicrobial susceptibility testing

All strains were susceptible to amoxicillin/clavulanic acid, ceftiofur, ceftriaxone, cefuroxime, ampicillin, trimethoprim/sulfamethoxazole, chloramphenicol, florfenicol, ciprofloxacin, moxifloxacin, tetracycline, and ertapenem. On the other hand, 44 isolates (64.7%) were resistant to gentamicin, two (2.9%) to nalidixic acid, and only one (1.4%) isolate was resistant to enrofloxacin. Only three resistance patterns were observed among the isolates: GEN (42/68), NAL-GEN (1/68), and NAL-GEN-ENO (1/68). The remaining 25 isolates were pan-susceptible.

Whole-genome sequencing analysis

All strains from rare serotypes (n= 68; 100%) harbored at least two antimicrobial resistance determinants (Table 1). The antimicrobial resistance genes (ARGs) qnrB19, aac(6’)-Iaa, and fosA7, encoding resistance to quinolones, aminoglycoside, and fosfomycin respectively, were identified among the isolates. In addition, it is worth noting that 60 (88.2%) isolates harbored mdsA and mdsB determinants, encoding multidrug and metal efflux proteins.

Out of 68 isolates, 67 (98.5%) isolates displayed a mutation in the codon 57 (Threonine → Serine) of the quinolone resistance-determining region (QRDR) parC region. No mutations in gyrA, gyrB and parE were observed. Additionally, incompatibility group plasmids such as IncFII(S), IncFII(Cf) Col(pHAD28), and IncFII(SARC14) were identified. Two *S*. Carrau and two *S*. Urbana isolates were found to harbor the IncX3 plasmid (Table 1), known to harbor clinical important ARGs such as blaNDM-1 and blaKPC-2.

Multi-locus sequence typing (MLST)

Genomic prediction revealed the occurrence of 22 international sequence types (STs) as summarized in Table 1. *S*. Carrau (ST226), *S*. Oran (ST965), and *S*. Urbana (ST754) showed consistent profiles, particularly in the serovar-ST ration. To the best of our knowledge, only ST226 has been previously reported in Brazil, with all other STs being described for the first time in this study.

Phylogenetic analysis

Each serovar was represented by a monophyletic clade on the reconstructed phylogeny (Fig. 1). We observed that the main drivers for cluster analysis were serovar and sequence types, since all isolates were clustered together by serovar and ST, and not by resistance profile, year of isolation, source or geographic location. These findings are in agreement with previous studies (MONTE et al., 2019; MONTE et al., 2021). *S*. Carrau, *S*. Oran, *S*. Urbana, *S*. Gaminara, *S*. Othmarschen, *S*. Businga, *S*. Bullbay, *S*. Molade, *S*. Lomita, *S*. Mikawasima, *S*. Langenhorn, *S*. I 16:e,h:e,n,z15, *S*. II 43:z4,z23:- or IIIa 43:z4,z23:- or Farmingdale or IV 43:z4,z23:-, *S*. I 18:d:-, *S*. I 7:l,v:- , *S*. I 7:k:-, *S*. I 7:-:1,5, *S*. I 16:r:e,n,z15, *S*. l,v:e,n,z15, and *S*. IV 45:g,z51:- belongs to endemics lineages circulating in Brazil, given these isolates were not clustered with international clones across the phylogeny being independent lineages. Conversely, *S*. Kiambu nested with strains from different countries (USA, Canada, Mexico, China, UK, Northern Ireland, and Netherlands) and sources (clinical and non-clinical) (PDG000000002.2708).

Table 6. Genomic features of rarely isolated *Salmonella enterica* serovars.

Accession number	Serovar by WGS	Source	Resistance genes	Sequence type	R-type	Location	Plasmid
GCA_023495885.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	7°25'56.5"S 35°33'41.7"W	IncX3
GCA_023733395.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	6°51'16.4"S 35°18'03.9"W	None
GCA_025402575.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	6°39'33.8"S 35°40'34.9"W	None
GCA_025401895.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	6°39'30.6"S 35°40'39.7"W	None
GCA_025512195.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	6°59'13.3"S 38°27'19.0"W	IncFII(S)
GCA_029753455.1	Carrau	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	7°26'38.2"S 35°33'36.7"W	None
GCA_029673985.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	6°54'53.3"S 37°35'04.1"W	None
GCA_029673945.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	6°59'29.6"S 38°27'23.0"W	IncFII(S)
GCA_029667365.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	6°55'17.8"S 37°35'25.2"W	None

GCA_029668105.1	Carrau	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	6°51'47.1"S 35°21'32.1"W	None
GCA_029744325.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	7°25'56.5"S 35°33'41.7"W	IncX3
GCA_029746395.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	7°01'26.4"S 37°57'11.1"W	None
GCA_029748545.1	Carrau	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	GEN	6°51'27.8"S 35°22'02.5"W	None
GCA_029751595.1	Carrau	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	226	Pan-susceptible	6°51'16.4"S 35°18'03.9"W	None
GCA_029755455.1	Oran	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	Pan-susceptible	6°59'29.6"S 38°27'23.0"W	None
GCA_029754375.1	Oran	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°50'45.1"S 38°18'40.1"W	None
GCA_029756695.1	Oran	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	Pan-susceptible	6°59'13.3"S 38°27'19.0"W	None
GCA_029754235.1	Oran	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°59'13.3"S 38°27'19.0"W	None
GCA_029755175.1	Oran	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°58'57.6"S 38°27'12.1"W	None
GCA_029746255.1	Oran	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°59'29.6"S 38°27'23.0"W	None

GCA_029745175.1	Oran	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°59'29.6"S 38°27'23.0"W	None
GCA_029745155.1	Oran	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°58'57.6"S 38°27'12.1"W	None
GCA_029744645.1	Oran	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	965	GEN	6°58'57.6"S 38°27'12.1"W	None
GCA_023800495.1	Urbana	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	754	GEN	6°51'22.5"S 38°21'05.5"W	IncFII(Cf)
GCA_025513125.1	Urbana	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	754	Pan-susceptible	6°59'13.3"S 38°27'19.0"W	IncX3
GCA_023495205.1	Urbana	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	754	GEN	6°51'22.5"S 38°21'05.5"W	IncFII(Cf)
GCA_029667725.1	Urbana	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	754	Pan-susceptible	7°01'34.9"S 37°56'36.7"W	IncX3
GCA_029673145.1	Gaminara	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	239	GEN	6°39'35.5"S 35°40'34.0"W	None
GCA_029672805.1	Gaminara	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9398	GEN	7°25'57.0"S 35°33'39.2"W	IncFII(S)
GCA_029747085.1	Gaminara	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9398	GEN	7°25'57.0"S 35°33'39.2"W	IncFII(S)
GCA_023495185.1	Gaminara	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	239	GEN	7°26'38.2"S 35°33'36.7"W	None

GCA_023494005.1	Gaminara	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9398	GEN	7°25'57.0"S 35°33'39.2"W	IncFII(S)
GCA_025512175.1	Lille	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9792	Pan-susceptible	6°54'28.3"S 37°35'07.3"W	None
GCA_025511735.1	Freetown	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	2940	Pan-susceptible	7°01'27.4"S 37°57'11.8"W	IncFII(S)
GCA_025511215.1	Businga	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9793	GEN	6°50'45.1"S 38°18'40.1"W	None
GCA_025550195.1	Bullbay	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9402	GEN	6°50'45.1"S 38°18'40.1"W	None
GCA_029674085.1	Molade or Wippra	river	*qnrB19, parC:p.T57S, fosA7, mdsA, mdsB*	544	NAL-GEN	7°01'00.3"S 37°59'07.8"W	Col(pHAD28)
GCA_029668905.1	Kiambu	river	*qnrB19, aac(6')-Iaa, mdsA, mdsB*	309	NAL-GEN-ENO	7°01'00.3"S 37°59'07.8"W	Col(pHAD28)
GCA_029745735.1	Lomita	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°51'27.8"S 35°22'02.5"W	None
GCA_029745815.1	Mikawasima	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	6°51'27.8"S 35°22'02.5"W	None
GCA_029671665.1	Langenhorn	river	*aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	7°26'38.2"S 35°33'36.7"W	IncFII(SARC14)
GCA_023802655.1	Othmarschen	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9457	GEN	6°58'57.6"S 38°27'12.1"W	None

GCA_029753435.1	Adelaide	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	440	GEN	6°50'51.6"S 38°18'33.7"W	None
GCA_029673665.1	I 16:e,h:e,n,z15	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°39'30.3"S 35°40'39.4"W	None
GCA_029667845.1	I 16:e,h:e,n,z15	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	6°39'30.3"S 35°40'39.4"W	None
GCA_029673125.1	I 16:e,h:e,n,z15	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	6°39'30.3"S 35°40'39.4"W	None
GCA_029673065.1	I 16:e,h:e,n,z15	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°39'30.3"S 35°40'39.4"W	None
GCA_029746135.1	II 43:z4,z23:- or IIIa 43:z4,z23:- or Farmingdale or IV 43:z4,z23:-	dam water	*aac(6')-Iaa, parC:p.T57S*	762	Pan-susceptible	6°51'22.5"S 38°21'05.5"W	None
GCA_029752115.1	II 43:z4,z23:- or IIIa 43:z4,z23:- or Farmingdale or IV 43:z4,z23:-	river	*aac(6')-Iaa, parC:p.T57S*	3942	GEN	6°51'27.8"S 35°22'02.5"W	None
GCA_029755295.1	II 43:z4,z23:- or IIIa 43:z4,z23:- or Farmingdale or IV 43:z4,z23:-	dam water	*aac(6')-Iaa, parC:p.T57S*	3942	GEN	6°51'16.4"S 35°18'03.9"W	None
GCA_029668585.1	I 18:d:-	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°54'28.3"S 37°35'07.3"W	None

GCA_029663955.1	I 18:d:-	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°55'17.8"S 37°35'25.2"W	None
GCA_029667445.1	I 18:d:-	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	6°54'28.3"S 37°35'07.3"W	None
GCA_023496145.1	I 7:l,v:-	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9397	GEN	6°51'27.8"S 35°22'02.5"W	None
GCA_025402055.1	I 7:l,v:-	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°39'33.8"S 35°40'34.9"W	None
GCA_025537495.1	I 7:l,v:-	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9397	GEN	6°51'27.8"S 35°22'02.5"W	None
GCA_029668525.1	I 4:b:-	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	7°01'00.3"S 37°59'07.8"W	None
GCA_029663845.1	I 4:b:-	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	7°01'00.3"S 37°59'07.8"W	None
GCA_023729115.1	I 4:-:1,5	dam water	*aac(6')-Iaa, parC:p.T57S, fosA7, mdsA, mdsB*	9456	Pan-susceptible	6°54'28.3"S 37°35'07.3"W	None
GCA_029663575.1	I 7:k:-	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°55'17.8"S 37°35'25.2"W	None
GCA_029744205.1	I 7:-:1,5	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	7°25'57.0"S 35°33'39.2"W	None
GCA_025401955.1	I 16:r:e,n,z15	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	Unknown	GEN	6°39'30.6"S 35°40'39.7"W	None

GCA_025540175.1	I,v:e,n,z15	dam water	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	9402	GEN	7°25'56.5"S 35°33'41.7"W	None
GCA_023496625.1	IV [1],53:g,z51:-	river	*aac(6')-Iaa, parC:p.T57S*	9399	Pan-susceptible	7°26'38.2"S 35°33'36.7"W	None
GCA_029670665.1	IV 50:z4,z23:-	dam water	*aac(6')-Iaa, parC:p.T57S*	433	Pan-susceptible	6°39'30.3"S 35°40'39.4"W	None
GCA_029744485.1	I 3,10:d:-	river	*mdsA, mdsB, aac(6')-Iaa, parC:p.T57S*	2742	GEN	6°51'27.8"S 35°22'02.5"W	None
GCA_029751555.1	Somone or IV 6,7:z4,z24:-	river	*aac(6')-Iaa, parC:p.T57S*	162	GEN	7°26'38.2"S 35°33'36.7"W	None
GCA_029755335.1	IV 45:g,z51:-	river	*aac(6')-Iaa, parC:p.T57S*	Unknown	Pan-susceptible	7°26'38.2"S 35°33'36.7"W	None

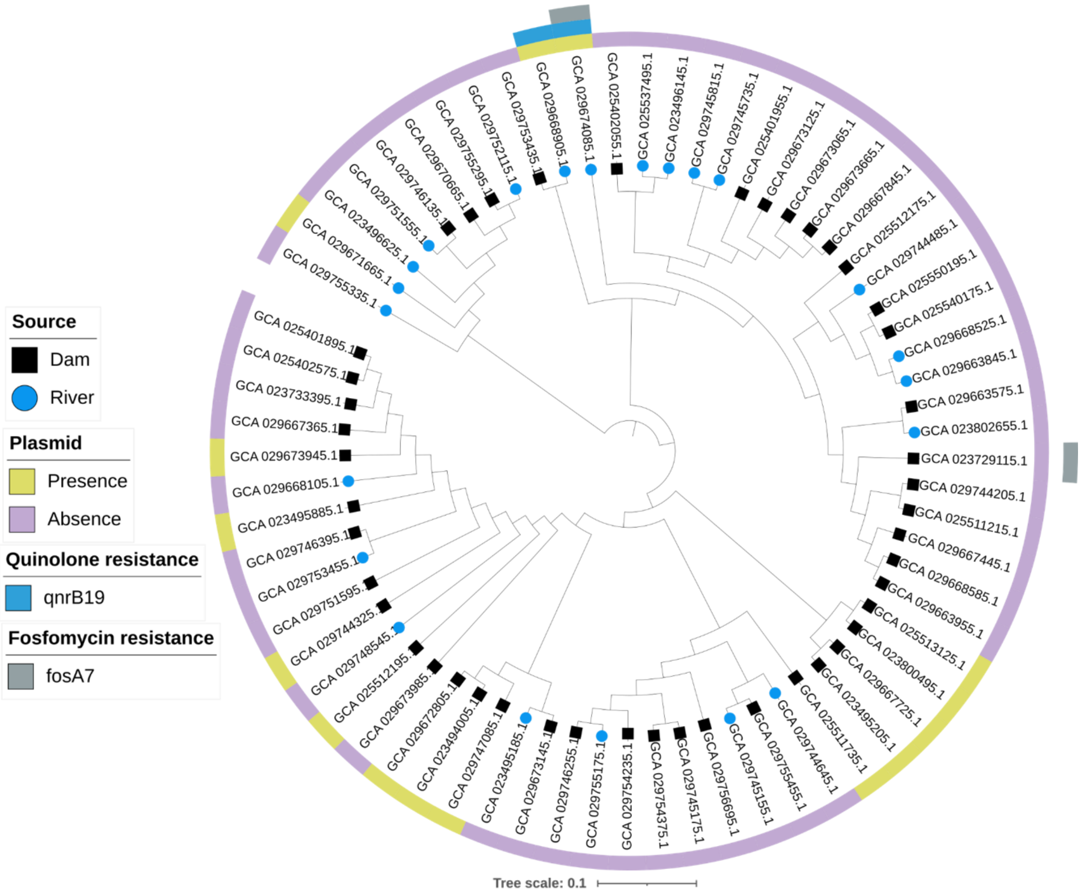

Figure 14 - Maximum-likelihood phylogenetic tree of 68 rare *S. enterica* strains isolated from environmental water. The resulting phylogeny was rooted at midpoint.

4.5 Discussion

Although the isolation of uncommon *Salmonella* serovars continues to be reported (TANKSON et al., 2006; OCTAVIA et al., 2019; MONTE et al., 2021), there are relatively few studies regarding these serovars in Brazil and abroad.

Salmonella Carrau was primarily identified in swine in 1944 (HORMAECHE et al., 1944). Nearly six decades apart, this serovar was reported in Brazil from asymptomatic dogs (MACIEL et al., 2004), human (LOUREIRO et al., 2010), turtle (SOUSA et al., 2011), and swine plasma (MONTE et al., 2021). Besides, *S*. Carrau strains were isolated from ham, sausage, meat, and cheese in Colombia (KARCZMARCZYK et al., 2010). The diversity of sources from which *S*. Carrau has been isolated demonstrates its ability to adapt to new environments and potentially infect different hosts. Most of reported isolates also harbored the same parC substitution (Thr57→Ser) that we detected in this study and belonged to the same ST226 previously reported in Brazil by MONTE et al. (2021), indicating a common genetic lineage or clonal group circulating in Brazil.

Most *Salmonella* Urbana strains have been described in human clinical cases, mostly in children (DEVI AND MURRAY 1991; SIRINAVIN et al., 1991; RIMHANEN-FINNE et al., 2010; KOCIANOVÁ et al., 2010; SABA et al., 2013; WILLIAMS et al., 2015). Additionally, there are reports of this serovar in turtle tank water (KOCIANOVÁ et al., 2010), retail meat samples in Lagos, Nigeria (SMITH et al., 2016), and raw cashews linked to a salmonellosis outbreak associated with a fermented cashew Brie analog in the USA (LOUVAU AND HARRIS 2023).

The presence of *Salmonella* Gaminara is limited to a few reports, including an outbreak in individuals who consumed unpasteurized orange juice in USA (PARISH 1998). Gopee et al. (2000) cultured resistant *S*. Gaminara strains from captive wildlife in Trinidad, while Durango et al. (2004) isolated 9% of *S*. Gaminara from fast food outlets in Colombia. Pantozzi et al. (2010) reported susceptible strains from domestic animals in Argentina and most recently, Bonardi et al. (2019) detected *S*. Gaminara from wild boars in Italy.

The first report of *Salmonella* Lille was made from chicken in 1954 (KAUFFMANN et al., 1954), followed by its report in Iraq (AL-HINDAWI AND RISHED 1979). In 2007, a *S*. Lille strain was isolated from ground beef (GUPTA et al., 2016), while in 2012 from peripheral lymph nodes of healthy cattle at slaughter (WEBB et al., 2017). More recently, Rodríguez et al. (2018) reported *S*. Lille in backyard chickens in Argentina.

To date, there is only one study reporting *S*. Freetown, which relates to an isolate from a stool sample of a child in Argentina (CAFFER et al., 1996). Similarly, there is only one report of *S*. Businga isolated in the Belgian Congo (VAN OYE AND LUCASSE 1957).

Although *S*. Molade is rarely isolated, this serovar has been described abroad in different sources such as pasturma beef sausages (ABBAR AND TAHIR 1989), eggshell (SURESH et al., 2006), and chicken and duck (KUMAR et al., 2019).

There are only few studies reporting *Salmonella* Kiambu. Curiously, there is a case of transmission of *S*. Kiambu from feral pigeons to human (LACASSIN et al., 1995). The remaining studies indicate the presence of this serovar in foodstuff

(BOUCHRIF et al., 2009), western grey kangaroos (POTTER et al., 2011), cattle (GUPTA et al., 2016), wastewater (MHONGOLE et al., 2017), papayas (HASSAN et al., 2019), human feces (ASAKURA et al., 2020), dairy heifer calves (ALERI et al., 2022), and poultry production (QUINN et al., 2023). Consistent with our findings, *S*. Kiambu isolates from wastewater belonged to ST309 (ALERI et al., 2022) and were resistance to multiple antimicrobials (MHONGOLE et al., 2017). There is much to be deciphered about this serovar, given lineages of these isolates seem to be circulating overseas.

The first case of *Salmonella* Lomita appears to be related to a cause of thoracic empyema (BARTOS AND HEJZLAR 1959). The remaining reports point to the presence of this serovar in poultry (KUMAR AND SAWHNEY 1970), spondylodiscitis (CHEVALIER et al., 1993), badgers (WILSON et al., 2003), retropharyngeal abscess in a child (SU et al., 2003), pigeon (OSMAN et al., 2013), squabs (OSMAN et al., 2014), and fecal sample of a male patient (LI et al., 2020). The isolation of antimicrobial resistant *S*. Lomita isolates from clinical samples highlights the pathogenicity of this this serovar for both humans and animals.

Salmonella Mikawasima has been reported in human sources (BARTLETT et al., 1978), freshwater aquarium snail (BARTLETT et al., 1978), human outbreaks (SYNNOTT et al., 1993; MYŠKOVÁ et al., 2014; FREEMANET al., 2016), freshwater reservoirs (POLO et al., 1999), hospital (NAVARRO et al., 2001), patients with sporadic diarrhea (KANEKO et al., 2007), and tilapia (BUDIATI et al., 2016).

Although *Salmonella* Othmarschen has not been commonly isolated, some reports highlight its occurrence in calves (ALLSUP et al., 1969), nosocomial outbreak (MOROSINI et al., 1996), humans (ERDEM et al., 2005), funeral outbreak (KIM et al., 2007), ostrich (KEOKILWE et al., 2015), and iliacus abscess (JHA et al., 2016).

Rarely isolated, *Salmonella* Adelaide was first reported in 1943 from a fatal case of enteritis in Adelaide (ATKINSON 1943). Shortly after, Atkinson et al. (1952) reported its occurrence in humans, rats, and lizards. Yet, there are studies reporting *S*. Adelaide from infantile diarrhea (VARGHESE et al., 1969), feral goats (MCORIST AND MILLER 1981), meat products (MEHRABIAN AND JABERI 2007), dead keet caecum (BOKO et al., 2013), and egg farms (MCWHORTER AND CHOUSALKAR 2015).

Salmonella Oran ST965 is an exceptionally rare serovar, as it has not been deposited in the Enterobase database and has not been previously described in Brazil or any other location. Our results indicate that the ST965 is conserved within this serovar, and the presence of AMR genes encoding resistance to aminoglycosides and quinolones demonstrates its potential to adapt by acquiring resistance mechanisms. Similarly, there is no report of *Salmonella* Bullbay ST9402, with the exception of two genomes deposited in Enterobase. As an extremely rare serovar, it is difficult to predict its potential role as a foodborne pathogen.

While there are no reports of *Salmonella* Langenhorn in current literature, the presence of this serovar is limited to a single genome deposited in Enterobase reporting a strain collected from a human in Germany in the year of 1960. Lastly, there are no reports for the remaining detected serovars *S*. I 16:e,h:e,n,z15, *S*. II 43:z4,z23:- or IIIa 43:z4,z23:- or Farmingdale or IV 43:z4,z23:-, *S*. I 18:d:-, *S*. I 7:l,v:-, *S*. I 4:b:-, *S*. I 4:-:1,5, S. I 7:k:-, *S*. I 7:-:1,5, *S*. I 16:r:e,n,z15, *S*. I,v:e,n,z15, *S*. IV [1],53:g,z51:-, *S*. IV 50:z4,z23:-, *S*. I 3,10:d:-, *S*. Somone or IV 6,7:z4,z24:-, and *S*. IV 45:g,z51:-.

Conclusions

The detection of a diversity of rare *Salmonella enterica* subsp. *enterica* strains from rivers and dams highlights the importance of monitoring and understanding the presence and distribution of these potentially pathogenic bacteria in water sources. It emphasizes the need for continued surveillance, risk assessment, and effective water management strategies to safeguard public health and prevent the spread of antimicrobial resistance. This study contributes to our knowledge of the epidemiology of *Salmonella enterica* in environmental settings.

The significance of the serovar lies not only in its infrequent isolation, but also on the emergence of antimicrobial resistance, particularly to quinolones, which was mostly identified in this study. Besides vertical AMR transmission, the presence of plasmid-mediated quinolone resistance gene (qnrB19) highlights a potential concern in public health since this plasmid is horizontally disseminated. Finally, the occurrence of rare serovars carrying important virulence factors reveals the pathogenic potential of these uncommon *S. enterica* serovars. Despite the uncertainty about their true relevance to public health, our findings suggest that the occurrence of antimicrobial-resistant *Salmonella* serovars in environmental water should be further monitored and addressed.

4.6 Material and methods

Study design, sampling, and microbiological procedures

We conducted a longitudinal study spanning 12 months, focusing on 10 dams linked to the three largest hydrographic basins in Paraíba state, Brazil: Piranhas, Paraíba, and Mamanguape rivers. Within each dam, we selected three independent sampling sites near water margins where agricultural and livestock activities were prevalent. Water samples were collected in triplicate from each sampling site.

The water sample procedure involved the use of Modified Moore Swabs (MMS), which are sterile PVC (polyvinyl chloride) cassettes containing a cheesecloth swab that was tightly sealed within (SBODIO et al., 2013). At the sampling site, individually packaged MMS were unwrapped and attached to a portable peristaltic pump (CPD-201-3, MS TECNOPON Equipamentos Especiais LTDA, SP, Brazil) using a sterile latex tube. A volume of 10 liters of water was filtered for a period of 20 minutes at a rate of 500 mL per minute. The filtration matrices were transferred aseptically into sterile contains with 250 mL of modified buffered peptone water (1.25g of sodium chloride, 0.875g of disodium hydrogen phosphate, and 0.375g of potassium dihydrogen phosphate). The containers were refrigerated at 4ºC and transported to the laboratory. The triplicates from each sampling site were acquired by doing three successive samplings at each site utilizing individual sterile cassettes. The sampling sites' geographic coordinates were documented using the Epicollect application on an Android device (AANENSEM et al., 2014).

Salmonella isolating was performed according to the FDA/BAM protocol (ANDREWS et al., 2014) with minor modifications. Shortly, pre-enrichment was performed at 37 ± 1°C for 24 hours. Aliquots (0.1 mL) were transferred into 9.9 mL Rappaport-Vassiliadis Broth (RV; Oxoid, Thermo Fisher Scientific, Loughborough, UK), while 1 mL-aliquots were transferred into 9 mL Tetrathionate Broth (TT; Oxoid, Thermo Fisher Scientific, Loughborough, UK). Broths were enriched at 42.5 ± 1°C for 18 hours in a water bath. A loopful of TT and RV enriched broths were streaked onto XLT-4 Agar (Oxoid, Thermo Fisher Scientific, Loughborough, UK).

In parallel, we performed an enrichment broth-polymerase chain reaction (be-PCR) targeting the InvA gene for detecting *Salmonella* positive samples. DNA was extracted by the boiling-centrifugation method as previously reported (FRESCHI et al., 2005). Enriched broth (1.5 mL) was centrifuged at 12,000 g for 2 minutes. The resulting supernatant was discarded, and the pellet was resuspended in 800 µl of ultrapure water, followed by a further centrifugation step at 12,000 g for 2 minutes. Subsequently, the supernatant was removed, and the pellet was resuspended in 200 µl of ultrapure water and heated at 95°C for 10 minutes. A final centrifugation step (12,000 rpm for 2 minutes) was performed, and 100 µl supernatant was transferred into a DNAse-free sterile microtube.

For the PCR assay, we used the species-specific primers (Forward: 5' GTG AAA TTA TCG CCA CGT TCG GGC AA 3' and Reverse: 5' TCA TCG CAC CGT CAA AGG AAC C 3') targeting the InvA gene in *Salmonella* spp. PCR was performed in a 25 µl master mix final volume containing 1.4 mM MgCl2, 1.25 U DNA Polymerase (Platus Taq, Sinapse Inc, Brazil), 2 mM of each dNTP, 10 µM of each primer, 1 µg DNA, and 10X Taq Buffer (KCl). The DNA amplification was performed in a thermal cycler (Biometra T-Advanced , Analytik Jena AG, Jena, Germany) with the following cycling conditions: an initial denaturation (95°C; 3 minutes), followed by 35 cycles consisting of a denaturation step (95°C for 30 seconds), annealing (53°C for 30 seconds), and extension (72°C for 2 minutes and 20 seconds), and a final extension step (72°C for 5 minutes). The resulting PCR products were electrophorized on a 1.5% agarose gel at 80 V for 40 minutes in 1x tris-acetate EDTA buffer, stained with SYBR Safe DNA Gel Stain, and visualized under ultraviolet light (Gel Logic 212 PRO, Carestream Health, NY, USA).

Positive broths in the be-PCR assay were further streaked onto Hektoen Enteric Agar (HE; Oxoid) and Bismuth Sulfite Agar (BS; Oxoid). The agar plates were incubated at 37 ± 1ºC for 24 hours. Up to three typical *Salmonella* colonies from each plate were selected, giving priority to morphological diversity. Selected colonies were inoculated on triple sugar iron (TSI; Oxoid) agar and lysine iron agar (LIA; Oxoid) slants for biochemical tests, which were further incubated at 37 ± 1ºC for 16 hours. Presumptive *Salmonella* isolates were streaked onto Tryptic Soy Agar (TSA; Oxoid) followed by PCR confirmation, which was performed using a loopful of bacterial mass expanded in 100 µl of DNase/RNase-Free water as a DNA template.

The PCR-confirmed isolates were incubated in brain heart infusion broth (BHI; Oxoid) supplemented with 20% glycerol (Neon Comercial Reagentes Analíticos LTDA, Susano, Brazil) and stored at -70°C.

Salmonella strains and serotyping

We obtained a total of 68 *S*. enterica isolates belonging to infrequent serovars in this study, including 46 isolates from dams and 22 isolates from rivers between 2021 and 2022 (Table 1). For the selection criteria, we defined as rare or infrequent the serovars not listed in “An Atlas of Salmonella in the United States, 1968-2011” the CDC Salmonella Atlas as well as all serovars not ranked among the most frequent serovars associated with human salmonellosis in Brazil from 2011 to 2020 (SANTOS et al., 2002).

S. enterica isolates (n=68) were whole-genome sequenced at the Center for Food Safety and Applied Nutrition (CFSAN), FDA. All isolates were serotyped in silico using default settings in SeqSero 1.2 (http://www.genomicepidemiology.org/).

Antimicrobial susceptibility testing

All *S. enterica* strains were tested for antimicrobial susceptibility testing (AST) against critically important antimicrobials, including amoxicillin/clavulanic acid 2:1 ratio (AUG2), ceftiofur (XNL), ceftriaxone (AXO), cefuroxime (CFX), ampicillin (AMP), trimethoprim/sulfamethoxazole (SXT), chloramphenicol (CHL), florfenicol (FLF), ciprofloxacin (CIP), ofloxacin (OFX), nalidixic acid (NAL), moxifloxacin (MFX), enrofloxacin (ENO), tetracycline (TET), gentamicin (GEN), ertapenem (ERT). Zone diameters were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2023) and the VET01S (CLSI 2023). Multidrug resistance was defined as resistant to three or more classes of antimicrobials.

Genomic sequencing and data analysis

S. enterica isolates were whole-genome sequenced in Illumina Miseq plataform at the Center for Food Safety and Applied Nutrition (CFSAN), FDA. DNA extraction was performed using a commercial kit (DNA Blood and Tissue kit, Qiagen, Germantown) according to manufacturer’s guidelines. Genomic DNA of isolates were sequenced at a 300-bp paired-end-read using the Illumina DNA Prep library preparation kit in the NextSeq 2000 platform (Illumina, San Diego, CA). Reads were submitted as FASTQ files to the Sequence Read Archive (SRA), NCBI, Bioprojects PRJNA186035 and PRJNA560080 (Suppl. Table 1). Fastq files were uploaded into CLC Genomics Workbench (CLC Bio, Qiagen, Aarhus, Denmark), to check the quality of the sequences and ensure the non-contamination of the reads. Afterwards, de novo assembly was performed using the same software. All sequences have been deposited at the NCBI, and their accession numbers are listed in Table 1.

All isolates were serotyped in silico using default settings in SeqSero 1.2 (http://www.genomicepidemiology.org/). For the selection criteria, we defined as rare or infrequent the serovars not listed in “An Atlas of *Salmonella* in the United States, 1968-2011,” the CDC *Salmonella* Atlas, as well as all serovars not ranked among the most frequent serovars associated with human salmonellosis in Brazil from 2011 to 2020 (SANTOS et al., 2002).

Additionally, we used AMRFinderPlus version 3.11.11 with database version 2023-04-17.1 to identify antimicrobial resistance genes and point mutations in the assemblies (FELDGARDEN et al., 2021). Plasmidome, and multi locus sequence typing profiles were determined using PlasmidFinder 2.1, and MLST 2.0 databases, respectively, which are available at the Center for Genome Epidemiology (http://www.genomicepidemiology.org/).

Phylogenetic analysis

A maximum likelihood phylogenetic tree of the 68 rare *S. enterica* strains was reconstructed using default settings of CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny). Whole-genome sequences were retrieved from GenBank database. The resulting phylogeny was visualized and annotated using iTol version 6 as per Letunic and Bork (2016).

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5 CONSIDERAÇÕES FINAIS

Com base em tudo que foi visto anteriormente, é possível inferir uma série de considerações finais que contribuem significativamente para o entendimento da dinâmica da contaminação por *Salmonella enterica* em ambientes naturais e sua relevância para a saúde pública. Primeiramente, a influência dos fatores climáticos, especialmente a precipitação, demonstrou ser um elemento crucial na recuperação de *S. enterica* de amostras de água, destacando a importância da sazonalidade e da variação geográfica na compreensão dessa dinâmica. Além disso, a presença de atividades humanas, como a criação de animais não ruminantes próximos a corpos d'água, foi identificada como um fator significativo na contaminação por *S. enterica*, ressaltando a necessidade de políticas de gestão ambiental e sanitária eficazes.

A diversidade de sorotipos de *Salmonella enterica* detectados, incluindo cepas raras, evidencia a complexidade dessa bactéria e sua capacidade de se adaptar a diferentes ambientes e hospedeiros, representando um desafio adicional para a prevenção e controle da salmonelose. A detecção de resistência antimicrobiana, particularmente a quinolonas, entre essas cepas ressalta a importância da vigilância epidemiológica e do uso responsável de antimicrobianos para mitigar o surgimento e disseminação de resistência.

Além disso, a análise dos fatores físico-químicos da água revelou correlações variadas com a presença de *S. enterica*, destacando a complexidade dos mecanismos subjacentes à sua sobrevivência e persistência no ambiente aquático. Esses achados destacam a necessidade de abordagens integradas e multidisciplinares para entender e enfrentar efetivamente o problema da contaminação por *S. enterica* em recursos hídricos.

Em última análise, a compreensão abrangente dos fatores que influenciam a ocorrência e disseminação de *S. enterica* em ambientes naturais é essencial para o desenvolvimento de estratégias de intervenção e controle mais eficazes. Essas estratégias devem ser baseadas em evidências científicas robustas e considerar a diversidade geográfica e sazonal, bem como as interações complexas entre fatores ambientais, atividades humanas e características biológicas da bactéria. Somente por meio de uma abordagem holística e colaborativa será possível mitigar efetivamente os riscos à saúde pública associados à contaminação por *Salmonella enterica* em recursos hídricos.