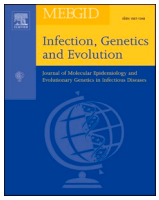
Infection, Genetics and Evolution 116 (2023) 105519

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid



Invasive non-typhoidal *Salmonella* (iNTS) aminoglycoside-resistant ST313 isolates feature unique pathogenic mechanisms to reach the bloodstream

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ARTICLE INFO

Keywords:
Salmonella Typhimurium ST313
Phylogeny
Virulence and resistance genes
Galleria mellonella
RNA-seq

ABSTRACT

Invasive non-typhoidal Salmonella (iNTS) from the clonal type ST313 (*S. Typhimurium* ST313) is the primary cause of invasive *salmonellosis* in Africa. Recently, in Brazil, iNTS ST313 strains have been isolated from different sources, but there is a lack of understanding of the mechanisms behind how these gut bacteria can break the gut barrier and reach the patient's bloodstream. Here, we compare 13 strains of *S. Typhimurium* ST313, previously unreported isolates, from human blood cultures, investigating aspects of virulence and mechanisms of resistance. Initially, RNAseq analyses between ST13-blood isolate and SL1344 (ST19) prototype revealed 15 upregulated genes directly related to cellular invasion and replication, such as *sopD2*, *sifB,* and *pipB*. Limited information is available about *S. Typhimurium* ST313 pathogenesis and epidemiology, especially related to the global distribution of strains. Herein, the correlation of strains isolated from different sources in Brazil was employed to compare clinical and non-clinical isolates, a total of 22 genomes were studied by single nucleotide polymorphism (SNPs). The epidemiological analysis of 22 genomes of *S. Typhimurium* ST313 strains grouped them into three distinct clusters (A, B, and C) by SNP analysis, where cluster A comprised five, group B six, and group C 11. The 13 clinical blood isolates were all resistant to streptomycin, 92.3% of strains were resistant to ampicillin and 15.39% were resistant to kanamycin. The resistance genes *acrA*, *acrB*, *mdtK*, *emrB*, *emrR*, *mdsA*, and *mdsB* related to the production of efflux pumps were detected in all (100%) strains studied, similar to pathogenic traits investigated. In conclusion, we evidenced that *S. Typhimurium* ST313 strains isolated in Brazil have unique epidemiology. The elevated frequencies of virulence genes such as *sseJ*, *sopD2*, and *pipB* are a major concern in these Brazilian isolates, showing a higher pathogenic potential.

1. Introduction

Salmonella enterica serovar Typhimurium is a common case of gastroenteritis worldwide (Haraga et al., 2008; Ashton et al., 2017). The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* causes approximately 1.2 million illnesses and 450 deaths every year in the United States (CDC (Centers for Disease Control and Prevention), 2021). Sequence type (ST) 19 is the most studied and prevalent ST amongst the *S. Typhimurium* strains, followed by ST34 and ST313 (Achtman et al., 2012; Almeida et al., 2017). Recently, the *S. Typhimurium* ST313 strains were described in sub-Saharan Africa as associated with antimicrobial resistance and high mortality, with very high prevalence in the African continent (Feasey et al., 2014; Ley et al., 2014).

Groups at the highest risk for severe salmonellosis include people with weakened immune systems, adults older than 65 years, children

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https://doi.org/10.1016/j.meegid.2023.105519
Received 19 July 2023; Received in revised form 19 October 2023; Accepted 24 October 2023
Available online 27 October 2023

younger than 5, and under cancer treatment (CDC (Centers for Disease Control and Prevention), 2021). *Salmonella* may cause severe diarrhea, hospitalization, and even death in these groups. Another worldwide concern has been the increase in the number of *Salmonella* strains multidrug-resistant strains to different classes of antibiotics. Antibiotic resistance may be closely associated with the development of bloodstream infection and/or treatment failure (CDC (Centers for Disease Control and Prevention), 2021). The current perspectives on iNTS can be alarming because its worldwide distribution has grown mainly in people with comorbidities, including HIV and malaria in Africa since many multidrug-resistant strains are being isolated in this continent (Haselbeck et al., 2017).

S. Typhimurium ST313 strains are iNTS generally isolated from human blood and considered more invasive for their ability to evade the gastrointestinal tract and reach the bloodstream (Singletary et al., 2016). In Brazil, our group has recently isolated *S. Typhimurium* ST313 strains from foods, human feces, and blood (Almeida et al., 2017; Perez-Sepulveda et al., 2021).

The pathogenesis of *S. Typhimurium* is a complex, multifactorial and orchestrated mechanism to cause gastroenteritis (Haraga et al., 2008). Several pathogenicity islands have been described in the literature and were better elucidated with the sequencing of *S. Typhimurium* LT2 and SL1344 strains, including islands that classically are associated with infectious processes, such as *Salmonella* Pathogenicity Islands 1 and 2 (SPI-1 and SPI-2). These islands are required for the invasion and survival of this pathogen in epithelial and phagocytic cells and systemic infection in mice (Galan and Curtiss 3rd, 1989; Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998; Moreira et al., 2010). Moreover, SPI-1 and SPI-2 encode distinct Type III Secretion System (T3SS), molecular needle-like structures responsible for injecting essential effector proteins during these two distinct extra and intracellular moments of infection (Galan and Curtiss 3rd, 1989; Groisman and Ochman, 1993; Shea et al., 1996; Hensel et al., 1998; Moreira et al., 2010).

Only a few *Salmonella* serovars harbor specific virulence plasmids. In the case of *S. Typhimurium*, a major virulence plasmid has approximately 95 Kb, and is denominated as pSLT. Specifically, in this plasmid, there is a highly conserved genomic region denominated *spv* (*Salmonella* plasmid virulence), which encodes for four structural SpvA, SpvB, SpvC, and SpvD proteins and the SpvR regulator (Fàbrega and Vila, 2013). In addition, the SpvB and SpvC effectors are encoded within the SPI-2, participating in the replication within SCV, macrophage apoptosis, and host-decreasing inflammatory response (Brown et al., 1986; Gulig and Doyle, 1993; Lobato-Márquez et al., 2016; Hiley et al., 2019).

Different phylogenetic approaches can be applied using sequencing data, such as the construction of phylogenetic trees from single copy marker genes and from single nucleotide polymorphism (SNPs), whole genome multilocus sequence typing (wgMLST) and confirmation of the sequence type (ST) through multilocus sequence typing (MLST) (Wang et al., 2015; Alikhan et al., 2018; Wu, 2018; Seribelli et al., 2021a).

Recent studies employed RNAseq analyses to elucidate possible transcriptional differences between *S. Typhimurium* ST313 and ST19 strains. Recently, a collaborative work performed by this research group showed that *S. Typhimurium* ST313 isolated from human feces established better colonization and invasion in the murine colon and higher expression of genes related to pathogenesis in comparison to *S. Typhimurium* ST19 prototype strain SL1344 (Seribelli et al., 2021b).

An alternative manner to assess the pathogenicity of these pathotypes is using an alternative infection model, *Galleria mellonella* that was employed since they have a primitive innate immune response with a certain degree of homology with the mammalian systems, the hemocytes present in the hemolymph behave similarly to phagocytic cells showing robust oxidative burst in response to microbial targets (Marmaras and Lampropoulou, 2009; Bender et al., 2013; Viegas et al., 2013). Furthermore, in contrast to other invertebrate models, experiments with *G. mellonella* worms can be performed at 37 °C, to mimic the human pathogens (Bender et al., 2013). Studies with fungi and several microbial pathogens, including *S. Typhimurium*, demonstrated a positive correlation in the data obtained in virulence assays using a mouse model compared to the findings in the *G. mellonella* model (Brennan et al., 2002; Slater et al., 2011; Seribelli et al., 2020).

Salmonella Typhimurium epidemiological information is scarce and almost null when linked to food products in Brazil; therefore, an approach with strains isolated from different sources was compared here, especially to alert about the risk of contamination by drug-resistant strains. Moreover, given the importance of clinical isolates of *S. Typhimurium* ST313 from blood cultures, we performed transcriptomics, gene expression assays and *in vivo* infection to better understand circulating lineages in the country and their pathogenic mechanisms. Herein, we have compared *S. Typhimurium* ST313 strains isolated from clinical and non-clinical sources in Brazil using comparative genomics and transcriptomic analysis, besides assessing the virulence of the strains *via* the *G. mellonella* infection model.

2. Material and methods

2.1. Bacterial strains

The 13 *S. Typhimurium* ST313 strains isolated from human blood cultures in the São Paulo State in Brazil between 1998 and 2011 were selected from the collection of the UNESP Hospital (Table 1), following the relevant guidelines and regulations of UNESP, as previously reported (Perez-Sepulveda et al., 2021). Another nine *S. Typhimurium* ST313 genomes isolated from humans and food in the São Paulo State in Brazil, previously sequenced and described by *in silico* analysis (Almeida et al., 2017), were included here to compare genomic SNP tree analysis. The reference strain *S. Typhimurium* SL1344 prototype (ST19) was originally isolated from the intestine of a calf with salmonellosis. The *S. Typhimurium* ST313 African lineage two reference strain D23580 was also included here (Kingsley et al., 2009).

2.2. Antimicrobial resistance profile of S. Typhimurium ST313 strains

The antimicrobial susceptibility test was performed by the Disk Diffusion Method (DDM) for 11 antimicrobials, including ampicillin, streptomycin, chloramphenicol, gentamicin, kanamycin, tetracycline, nalidixic acid, sulfonamides, trimethoprim-sulfamethoxazole, and ciprofloxacin following all recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2022). The *Escherichia coli* ATCC 25922 strain was used as a control.

2.3. Virulence plasmids and resistance genes

The virulence plasmid genes were identified for all 13 *S. Typhimurium* isolates ST313 from blood using the Virulence Factors Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) with a threshold of ≥70% identity and ≥ 70% coverage comparing plasmid *S. enterica* subsp. *enterica* serovar Typhimurium str. LT2 pSLT, 93,939 bp, (Chen et al., 2005).

The presence of plasmids was determined *in silico* using Plasmid Finder (Center for Genomic Epidemiology, https://cge.cbs.dtu.dk/services/PlasmidFinder/) with a threshold set for a minimum of 95% identity and minimum coverage of 60% (Carattoli et al., 2014). BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the location, percentages of identity, and coverage between the *S. enterica* subsp. *enterica* serovar Typhimurium str. LT2 pSLT, 93,939 bp, NC_003277 reference sequences of the pSLT, pBT1, pBT2, and pBT3 plasmids, and the *spvABCDR*, *pefABCD*, *rck*, and *mig-5* genes of the positive and negative genomes for IncFIIs.

The resistance genes were detected using ResFinder 4.1 - Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ResFinder/) with settings of threshold of 90% and minimum length of 60% and Comprehensive Antibiotic Resistance Database (CARD) with high

Table 1
Identification and year of isolation of the 24 *S. typhimurium* genomes ST313 used in the study.

GenBank Access Number	Identification	Sources	Year of isolation	Reference or source
014250355.1	78/98	Human Blood	1998	Perez-Sepulveda et al., 2021
014250355.1	220/98	Human Blood	1998	Perez-Sepulveda et al., 2021
014250355.1	522/98	Human Blood	1998	Perez-Sepulveda et al., 2021
014250355.1	2366/98	Human Blood	1998	Perez-Sepulveda et al., 2021
014250355.1	31,217/04	Human Blood	2004	Perez-Sepulveda et al., 2021
014250355.1		32,714/04 Human Blood	2004	Perez-Sepulveda et al., 2021
014250355.1	339/07	Human Blood	2007	Perez-Sepulveda et al., 2021
014250355.1	1880/08	Human Blood	2008	Perez-Sepulveda et al., 2021
014250355.1	2081/08	Human Blood	2008	Perez-Sepulveda et al., 2021
014250355.1	3956/10	Human Blood	2010	Perez-Sepulveda et al., 2021
014250355.1	4270/10	Human Blood	2010	Perez-Sepulveda et al., 2021
014250355.1	5339/10	Human Blood	2010	Perez-Sepulveda et al., 2021
014250355.1	1435/11	Human Blood	2011	Perez-Sepulveda et al., 2021
LVGE00000000	CFSAN033876	Human Feces	1989	Almeida et al., 2017
LVGD00000000	CFSAN033877	Human Feces	1990	Almeida et al., 2017
LVGA00000000	CFSAN033881	Human Feces	1993	Almeida et al., 2017
LVFZ00000000	CFSAN033882	Human Feces	1995	Almeida et al., 2017
LVFX00000000	CFSAN033884	Raw Pork Sausage	1996	Almeida et al., 2017
LUJB00000000	CFSAN033886	Human Feces	1998	Almeida et al., 2017
LUJA00000000	CFSAN033887	Lettuce	1998	Almeida et al., 2017
LVFU00000000	CFSAN033891	Human Feces	2000	Almeida et al., 2017
LUIX00000000	CFSAN033894	Human Feces	2003	Almeida et al., 2017
000210855.2	SL1344 ASM21085v2	Cattle ST19 UK	–	–
000027025.1	D23580 ASM2702v1	Human Blood ST313	2004	Kingsley et al., 2009

quality/coverage (includes contigs >20,000 bp and excludes prediction of partial genes) that is available at https://card.mcmaster.ca/analyze/rgi (Camacho et al., 2009; Mcarthur et al., 2013; Bortolaia et al., 2020).

2.4. RNA-seq

S. Typhimurium ST313 522/98 was isolated from human blood in 1998 at UNESP Hospital and SL1344 (ST19), according the guidelines and regulations from UNESP Hospital, as previously reported (Perez-Sepulveda et al., 2021). The strains were cultured for 16–18 h in LB broth (Difco) at 37 °C. The cultures were diluted into LB broth (Difco) and incubated under shaking at 37 °C for 3 h until reaching an optical density of 1.0 at 600 nm (Seribelli et al., 2021b). In order to disrupt cells and inactivate nucleases, 500 μL of TRIzol (Life Technologies) was used and RNA was isolated using the RNA RiboPure® Bacteria kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The concentration and purity of the total RNA were verified by NanoDrop 2000 Spectrophotometer (Thermo Scientific) at 260 nm and 280 nm (Seribelli et al., 2021b). Extracted with an A260 nm/A280 nm ratio between 2.0 and 2.2 were considered adequate, in the same way, were used for quantification and verification of sample quality the Bioanalyzer RNA 6000 Nano (Agilent Technologies) and Bioanalyzer DNA 1000 kits (Agilent Technologies) (Seribelli et al., 2021b). The ribosomal RNA was depleted by kit QIAseq FAST Select (Qiagen®) and was converted into cDNA libraries using the TruSeq® Stranded mRNA kit (Illumina). After preparing the library, it was quantified by QIAseq Library Quant System (Qiagen®), using the standard curve method to perform the sequencing with the MiSeq® v3 kit (Illumina). The sequencing was performed using a MiSeq® sequencer (Illumina), and the biological triplicate was prepared according to the manufacturer's instructions (Illumina).

2.5. Mapping of RNA-seq libraries and differential gene expression analysis

Initially, raw reads served as input for Trimmomatic (Bolger et al., 2014), which performed quality filtering, removing Illumina adapters sequences, low-quality bases (phred score quality>20), and reads shorter than 35 bp. Trimming was followed by read error correction by the SGA *k*-mer-based algorithm (Simpson and Durbin, 2012). The preprocessed reads were compared to the raw reads through FastQC analysis (Andrews, 2010) to evaluate the trimming and quality control performed previously.

Next, the reads were mapped against the *Salmonella enterica* serovar Typhimurium SL1344 reference genome (Kröger et al., 2012) Genbank access code GCA_000210855.2, using Bowtie2 software (Langmead and Salzberg, 2012), following previously described optimization (Baruzzo et al., 2017).

The gene differential expression calling was achieved initially by counting the number of reads in each transcript through HTSeq (Anders et al., 2015). Finally, the count data were directed to differential analysis with the DESeq2 R package (Love et al., 2014).

2.6. Galleria mellonella infection model

S. Typhimurium ST313 and *S. Typhimurium* SL1344 (ST19) strains were grown overnight, harvested by centrifugation, and resuspended in 1000 μL PBS. The experiments were performed and adapted as described by Scalfaro et al. (2017).

The susceptibility of *G. mellonella* to bacterial infection was used in this study. Groups of ten larvae weighing ~200–250 mg were used for all experiments and were inoculated with 10^8, 10^6, and 10^4 *S. Typhimurium* CFU/larva by injections of 10 μL bacterial suspension using a Hamilton micro syringe into the hemocoel through the last left pro-leg. As controls, PBS injected animals were included in each experiment. After infection, larvae were placed in sterile Petri dishes and incubated in the dark at 37 °C, and the number of dead larvae was counted and recorded daily for three days. All experiments were performed in triplicate, and only the experiments where the larvae survived up to 3 dpi (days post-infection) were included in the analysis.

2.7. Whole genome sequencing

Whole genome sequencing of 13 Typhimurium ST313 strains

isolated from human blood in Brazil was performed on the HiSeq 4000 (Illumina) at the University of Liverpool as part of the 10 K Salmonella Genome Consortium deposited in Enterobase (https://enterobase.warwick.ac.uk/species/index/senterica) (Perez-Sepulveda et al., 2021). The *S. Typhimurium* ST313 genomes were assembled as previously described (Seribelli et al., 2021c; Silva et al., 2021). Briefly, raw reads were processed by a5 pipeline (August 2016 version) (Coil et al., 2015), starting with trimming with Trimmomatic (Bolger et al., 2014), performing quality filtering, removing adaptor sequences, low-quality bases (phred score quality<28), and short reads (>35 bp). The read error correction was performed by SGA (Simpson and Durbin, 2012), following the IDBA-UD algorithm (Peng et al., 2012) for *de novo* assembling succeeded by SSPACE (Boetzer et al., 2011) scaffolding. Next, it was executed reference-assisted scaffolding through MeDuSa 1.6 (Bosi et al., 2015) using the deposited genomes of close-related *S. Typhimurium* strains LT2, SL1344, ST4–74, 14028S, and D23580 to guide the scaffolds in the correct order and orientation, building a single scaffold. The final scaffolds were passed through up to 20 iterations in Gapfiller 1.10 (Nadalin et al., 2012), which used the error-corrected reads to fulfill the gaps between the contigs sequences, improving L_{50}contig count.

2.8. Phylogeny

The genomes of all the 22 *S. Typhimurium* ST313 strains described in item 2.1 were used in the phylogenetic analyses.

The phylogenetic tree based on SNPs of the whole genome sequencing was performed by CSI Phylogeny 1.4 (Call SNPs & Infer Phylogeny) of the Center for Genomic Epidemiology at https://cge.cbs.dtu.dk/services/CSIPhylogeny/ - following the parameters: select min. Dep that SNP positions 10×, select min. Relative dep that SNP positions 10%, select minimum distance between SNPs (prune) 10 bp, select min. SNP quality 30, select min. Read mapping quality 25 and select min. *Z*-score 1.96 (Kaas et al., 2014). The SNPs matrix included was a maximum of 818 SNPs amongst all *S. Typhimurium* ST313 strains studied.

2.9. Statistical analysis

All statistical analyses were performed using the GraphPad Prism v7.04 (GraphPad Software, San Diego, CA, USA) by one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons post-test. Data were considered significant when $p < 0.05$. Graphical and statistical analyses of the virulence assay in *Galleria mellonella* were performed by the Long-rank method (Mantel-Cox).

3. Results

3.1. Antimicrobial resistance profile of S. Typhimurium ST313 blood-isolated strains

All 13/13 (100%) blood-isolated *S. Typhimurium* ST313 strains were resistant to streptomycin, 12/13 (92.3%) strains were resistant to ampicillin, and 2/13 (15.39%) strains were resistant to kanamycin (Table 2). All the strains were susceptible to other antibiotics assayed, such as chloramphenicol, gentamicin, tetracycline, nalidixic acid, sulfonamides, sulfamethoxazole-trimethoprim, and ciprofloxacin. The resistance genes *acrA*, *acrB*, *mdtK*, *emrB*, *emrR*, *mdsA*, and *mdsB* related to the production of efflux pumps were detected in all (100%) *S. Typhimurium* ST313 strains with identity for all genes between 91.6 and 100% (Table 2). Nevertheless, the strains have not shown resistance to antimicrobials related to these genes. Resistance genes related to enzyme production were also investigated but were not detected in any of these *S. Typhimurium* ST313 strains.

Table 2
Genotypic and phenotypic antimicrobial resistance profiles of the 13 *S. typhimurium* ST313 strains studied isolated from bloodstream.

Barcode	Identification	Efflux Pump Resistance genes (Identity%)	Phenotypic Resistance profiles
FD01848810	78/98	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM
FD01848812	220/98	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848813	522/98	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP-KAN
FD01848818	2366/98	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848828	31,217/04	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848833	32,714/04	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848836	339/07	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM- AMP
FD01848843	1880/08	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-KAN
FD01848844	2081/08	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848852	3956/10	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848853	4270/10	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848858	5339/10	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP
FD01848859	1435/11	*acrA, acrB, mdtK, emrB, emrR, mdsA, mdsB* (91.69–100%)	SM-AMP

SM- streptomycin; AMP- ampicillin; and KAN- kanamycin.

3.2. Virulence genes were detected in sequenced isolates

The *spvABCDR* operon, *pefABCD* operon, *rck*, and *mig-5* genes were detected in 12/13 *S. Typhimurium* ST313 isolates from human blood. In order to confirm the location of genes, Plasmid Finder was used to document the presence of the IncFIIs plasmid incompatibility group, which belongs to the pSLT plasmid. Specifically, BLASTn analysis for all isolates confirmed the presence of the pSLT plasmid carrying these regions in the 12 isolates with coverage of 100% and 99–100% of identity

Table 3
Characteristics of the plasmid genes of 13 *Salmonella Typhimurium* isolates from human blood that presented these genes by Virulence factors database (VFDB).

Genes	Proportion of isolates	Query cover (%)	Identity (%)
spvA	12/13	100	100
spvB	12/13	100	100
spvC	12/13	100	100
spvD	12/13	100	99–100
spvR	12/13	100	100
pefA	12/13	100	100
pefB	12/13	100	100
pefC	12/13	100	99–100
pefD	12/13	100	100
rck	12/13	100	100
mig-5	12/13	100	100

IncFIIs and pSLT were confirmed in these genomes.

(Table 3). The pBT1, pBT2, and pBT3 plasmids were not detected in any of the *S. Typhimurium* ST313 genomes studied.

3.3. Virulence traits differently expressed in ST313 blood-isolate transcript

Interestingly, transcriptome studies were performed between the *S. Typhimurium* ST313 and SL1344 (ST19) strains, revealed 399 genes were differentially expressed (168 upregulated and 231 down-regulated). *S. Typhimurium* ST313 showed upregulated genes linked to various biological processes, including ethanolamine, primary alcohol, and amine metabolism, also pathogenesis. Conversely, the down-regulated genes expression was mainly linked to cellular general processes, metabolism, transport, biological adhesion, and response to stimulus (Fig. 1). Specifically, 15 SPI-2 encoded genes were upregulated in ST313 compared to SL1344 (ST19) strain, directly linked in *S. Typhimurium* invasion, such as *sopD2*, *sifB, pipB*, *ssaB*, *ssaI*, *ssaG*, *ssaJ*, *ssaM*, *ssaR*, *ssaT*, *ssaK*, *sseJ*, *spvC*, *spvA*, and *srcA*. SPI-2 effector proteins and regulators are essential for intracellular survival and replication (Srikanth et al., 2011). The higher levels of gene expression for the SPI-2-encoded effectors *sopD2* (3.6-fold), *sifB* (2.9-fold) and *pipB* (2.1-fold) are related to *Salmonella* inducing filaments (Sifs) formation, an important structure to intracellular fitness and replication. The *ssaB* gene was >5.2-fold increased, the SsaB is required for SPI-2 chaperone protein SpiC for the injectosome. Moreover, the *ssaJ* gene was >3.2-fold increased, which it is required for T3SS- effector function, and the *sseJ* gene was 2.0-fold upregulated, another key effector in the T3SS SPI-2-encoded (Fig. 2).

Furthermore, it was observed that *spvA* (3.0-fold) and *spvC* (3,3-fold) genes were upregulated in the *Salmonella* virulence plasmid. In addition, there was an increase of 3.8-fold for the *katE* gene, which encodes for a catalase and it is important to protect the bacteria from oxidative stress, as illustrated by the volcano plot for these pathogenesis-related traits in the *S. Typhimurium* ST313 (Fig. 2).

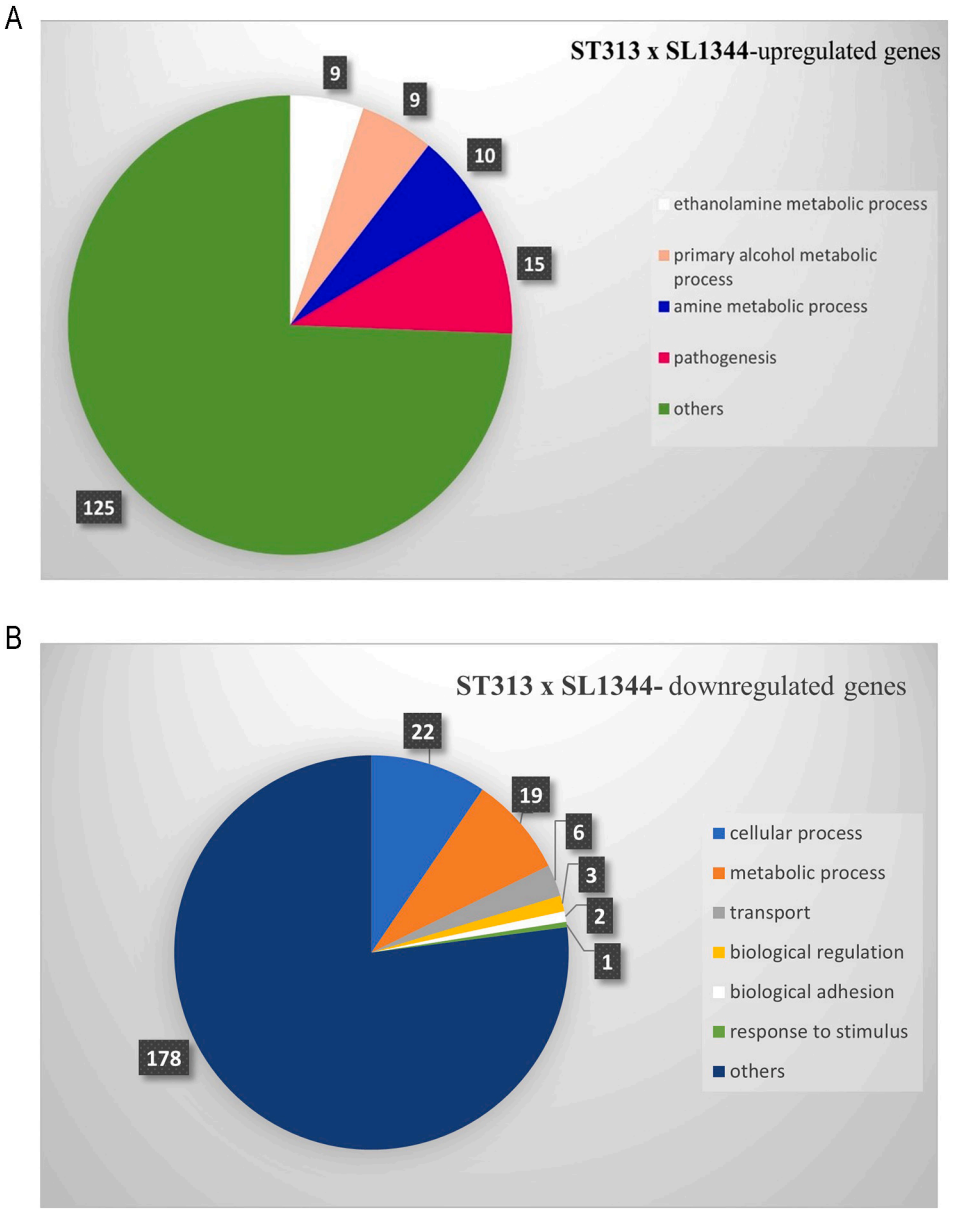

Fig. 1. Transcriptome analyses of *S. typhimurium* SL1344 (ST19) and ST313 in LB-37 °C. (A) *S. typhimurium* ST313 522/98 × SL1344 (ST19) – 168 upregulated genes. (B) *S. typhimurium* ST313 522/98 × SL1344 (ST19) – 231 downregulated genes.

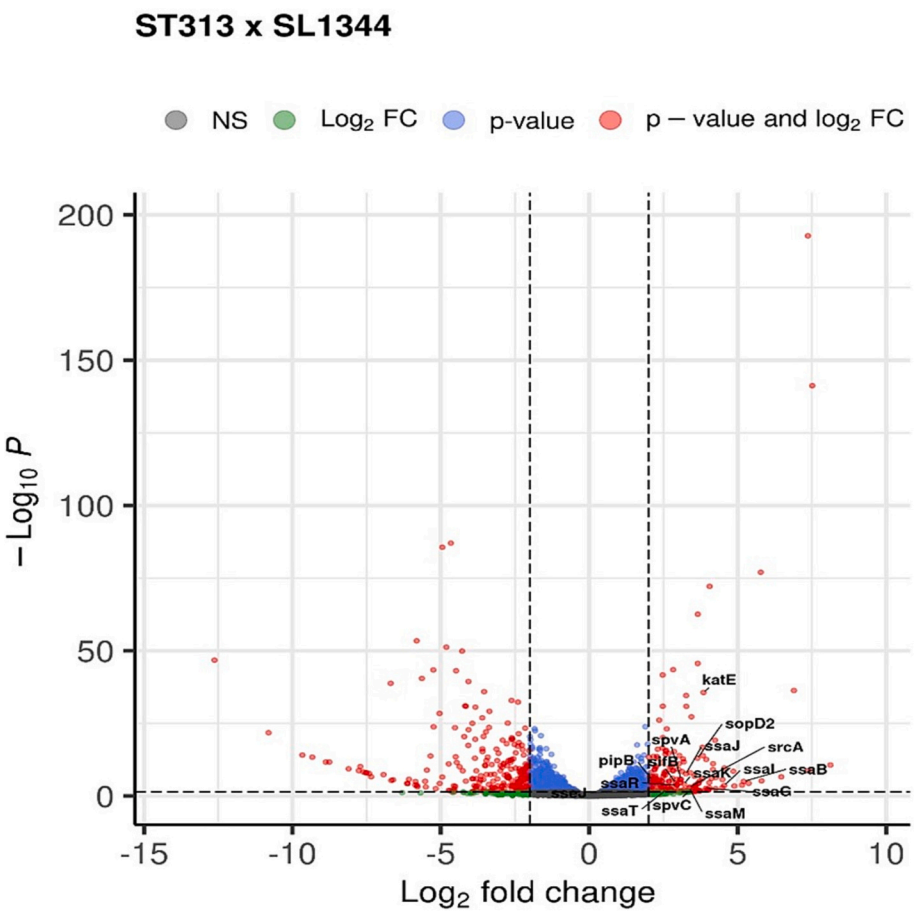

Fig. 2. Volcano plot of *S. typhimurium* SL1344 (ST19) and ST313 upregulated genes in LB-37 °C.

3.4. S. Typhimurium ST313 blood-isolates infectivity in Galleria mellonella model

Infectious diseases *in vivo* models are essential to evaluate pathogens. Herein, we employed the survival within the *Galleria mellonella* model to better compare the virulence of distinct clinical strains for three days post-infection. We assessed distinct concentrations of 10^8, 10^6, and 10^4 CFU/ larva inoculated with *S. Typhimurium* ST313 compared to SL1344 (ST19) were tested. At 10^8 CFU/larva concentration, all larvae inoculated with SL1344 (ST19) (100%) and 90% with ST313 deceased after 1 dpi. On the other hand, the 10^6 CFU/larva concentration showed 30% survival on the 1dpi with SL1344 (ST19) and 40% for ST313, on the 2nd and 3rd day, survival remained at 20% for inoculation SL1344 (ST19) and 30% for ST313 (Fig. 3). However, the percentage of survival was 100% during the 3 dpi in the larvae inoculated with the concentration of 10^4 CFU/larva inoculated with SL1344 (ST19) or ST313 (data not shown). Infection by *S. Typhimurium* caused higher melanization at concentrations above 10^6 CFU/larva, and the results demonstrated that mortality was dose-dependent. In addition, the data shown represent three independent experiments which obtained similar results.

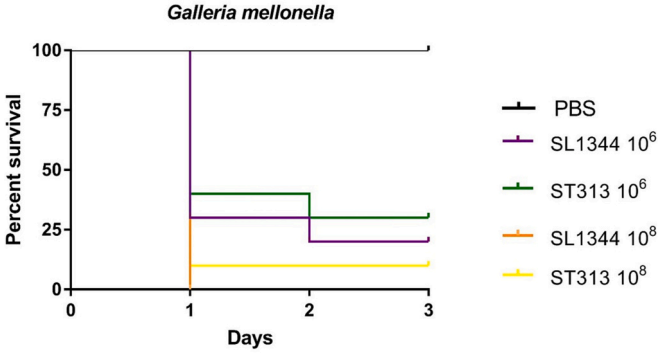

Fig. 3. Survival percentages of *Galleria mellonella* larvae infected with *S. typhimurium* ST19 (SL1344) and *S. typhimurium* ST313 522/98 in a three-day period.

3.5. Phylogenetic comparisons between these geographically distinct Brazilian areas

The phylogenetic analysis based on SNPs grouped the 24 *S. Typhimurium* genomes isolated in Brazil into three groups denominated A, B, and C. Specifically, cluster A comprised five *S. Typhimurium* ST313 genomes, including four isolated from blood and only one isolated from human feces. Group B comprised six *S. Typhimurium* ST313 genomes isolated from blood. Group C comprised 13 *S. Typhimurium* ST313 genomes, and they were subdivided into C1 and C2. Group C1 grouped four *S. Typhimurium* ST313 genomes isolated from blood and group C2 comprised nine *S. Typhimurium* ST313 genomes isolated from human feces, food, and the two reference strains, SL1344 from ST19 and D23580 from African ST313 (Fig. 4).

4. Discussion

Emergent pathogens surveillance in public health should be a constant concern. In Brazil, few studies have been conducted on *Salmonella Typhimurium* ST313, an important intestinal pathogen recently associated with more invasive, serious infections. Together, the results of the present study of *S. Typhimurium* ST313 isolates from the bloodstream

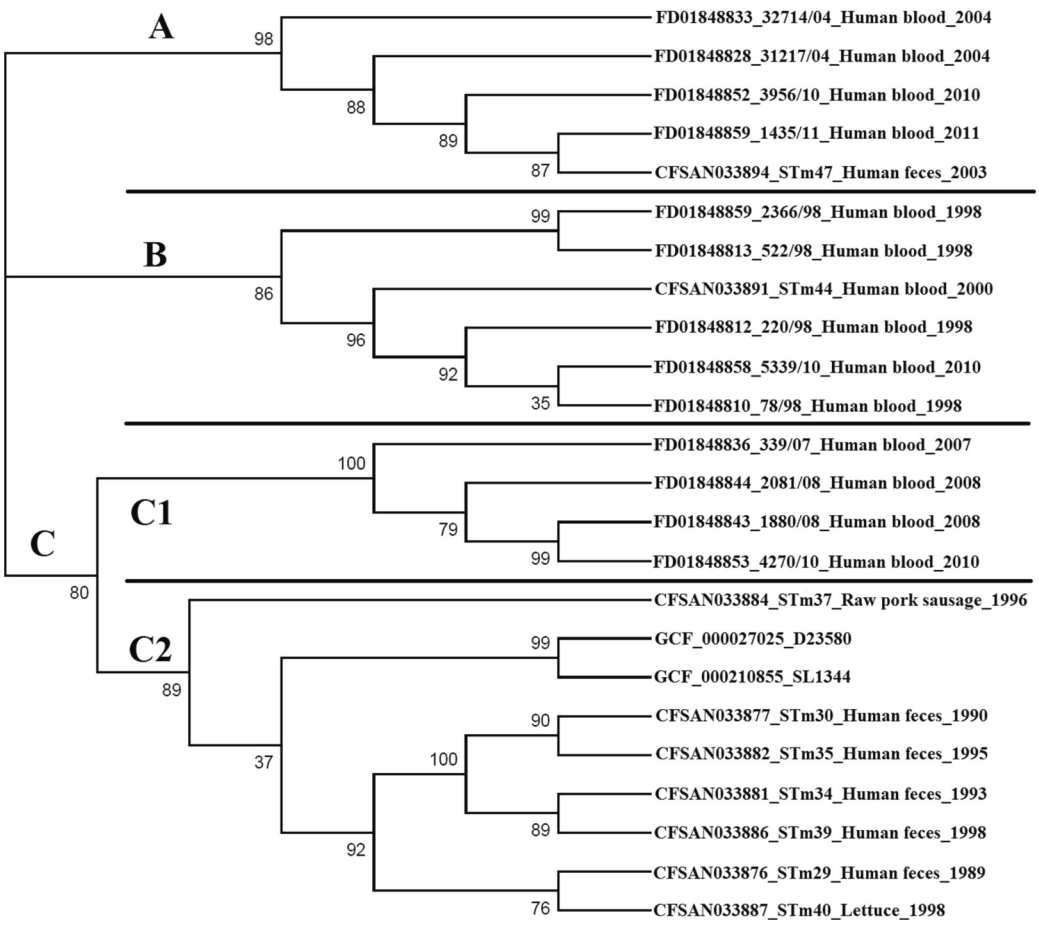

Fig. 4. Phylogenetic analysis based on SNPs from whole genome sequencing by CSI Phylogeny 1.4 (Call SNPs & Infer Phylogeny) for 22 *Salmonella Typhimurium* genomes ST313 isolated from human blood ($n = 14$), human feces ($n = 6$) and food ($n = 2$) in Brazil.

between 1998 and 2011 corroborate the other ST313 isolated from different sources between 1989 and 2003 in Brazil.

Herein, 13 clinical *S. Typhimurium* ST313 strains from human blood-isolated cultures in Brazil were evaluated and fully characterized, including antimicrobial resistance profiles, virulence plasmid, transcriptome analysis, *in vivo* infection, and phylogenetic diversity compared to nine other distinct *S. Typhimurium* ST313 isolates from clinical and non-clinical sources.

All 13 *S. Typhimurium* ST313 strains isolated from patients at the University Hospital in Brazil were resistant to streptomycin. Streptomycin is not often used to treat infections caused by *Salmonella enterica*; however, it has been previously used as a growth promoter in avian production, an important concerning factor due to the possibility of resistance transmission amongst bacterial strains *via* food and zoonotic contact (McDermott et al., 2016; Almeida et al., 2018).

The ampicillin resistance was found in 12 (92.3%) *S. Typhimurium* ST313 strains and two (15.4%) were also resistant to kanamycin. Antimicrobial resistance of *S. Typhimurium* strains in the United States has increased in recent decades, Wang and colleagues used CDC and National Antimicrobial Resistance Monitoring System (NARMS) data from 1996 to 2016 from human, animal, and retail meats isolates to demonstrate the increased resistance profile to ampicillin, streptomycin, sulfonamides, and tetracycline for this serovar (Wang et al., 2019).

The multidrug resistance observed for *S. Typhimurium* ST313 strains in the African continent has been a serious public health problem, being responsible for a high-mortality rate of 20.6% in patients infected with this iNTS (Uche et al., 2017; Canals et al., 2019). In contrast, Almeida et al. (2018) observed low antimicrobial resistance in *S. Typhimurium* ST313 isolated from different sources such as food, feces, and human blood in Brazil. Increasing drug resistance in foodborne pathogens is an alarming concern, especially nontyphoidal *Salmonella* in the last 13 years in the US (CDC, 2021).

The resistance genes related to the production of efflux pumps were detected in the *S. Typhimurium* studied. Efflux pumps are organized in structures covering the width of the Gram-negative cell envelope and selectively communicate the cytoplasm with the external environment. The AcrAB-TolC system has been one of the most studied, transporting a wide range of substrates and conferring resistance to several antibiotics, including some β-lactams (Munita and Arias, 2016). In addition, Buckley et al. (2006) showed in *S. Typhimurium* SL1344, the AcrAB-TolC system may also exert the ability to efflux toxic compounds from the lysosome, supporting intracellular survival. It has been observed in mutants of the system that have had reduced adhesion and/or invasion abilities (Buckley et al., 2006).

Moreover, all 13 *S. Typhimurium* ST313 genomes were investigated by the Center for Genomic Epidemiology and CARD on their resistance genes database. No resistance gene related to the production of beta-lactamases were found in any analyses performed, which is in agreement with the phenotypic disc diffusion tests (data not shown), once the *S. Typhimurium* ST313 strains were resistant to few antibiotics. These analyses are essential to know more about the epidemiology of this important ST in Brazil.

The *S. Typhimurium* virulence plasmid (pSLT) was detected in 92.3% of the strains, confirming the pathogenic potential of the strains studied and their differences compared to other ST313 strains isolated in other locations as previously described (Pulford et al., 2021). Furthermore, the *spv* operon has been associated with the survival and multiplication of *Salmonella spp.* in host macrophages (Rychlik et al., 2006). On the

other hand, the *pef* fimbrial operon (plasmid-encoded fimbriae) is essential for the adhesion of *Salmonella spp.* to the small intestine in infant mice, resulting in the accumulation of fluid in the lumen (Baumler and Heffron, 1995; Ledeboer et al., 2006). The *rck* and *mig*-5 plasmid genes have been associated with the resistance of *S. Typhimurium* to the host complement system and the neutralization of toxic compounds produced by macrophages, respectively (Rychlik et al., 2006).

Recently, a genomic study with the D23580 *S. Typhimurium* ST313 strain isolated in Malawi showed pSLT lack and the presence of the pSLT-BT plasmid carrying the Tn*21*-like locus, associated with resistance to different antibiotics including chloramphenicol, ampicillin, kanamycin, streptomycin, sulfonamides, and trimethoprim (Kingsley et al., 2009; Singletary et al., 2016). The pSLT plasmid was also directly linked with *S. Typhimurium* ST313 strains isolated from humans and food in Brazil, with the presence of the *spv*ABCDR locus, *pef*ABCD locus, *rck,* and *mig*-5 genes in all isolates (Seribelli et al., 2020). Pulford et al. (2021) have studied phylogenetic clades of *S. Typhimurium* ST313 genomes isolated from distinct locations and their relationship with different plasmids. Our analyses here show that Brazilian *S. Typhimurium* ST313 strains are genetically similar to the UK isolates, where it was found the presence of pSLT and the absence of pBT1, pBT2, and pBT3 in comparison to ST313 L1, L2, and L3 strains isolated from Africa (Pulford et al., 2021).

Herein, the *S. Typhimurium* ST313 strains expression was upregulated for the *ssa* (secretion system apparatus) genes compared to SL1344 levels, such as *ssaB*, *ssaI*, *ssaG*, *ssaJ*, *ssaM*, *ssaR*, and *ssaT,* responsible for encoding the T3SS structural component of SPI-2 (Hensel et al., 1997). In general, most of the upregulated genes were related to the pathogenesis of this important pathogen present mainly *via* SPI-1 and SPI- 2-encoded effectors during *S. Typhimurium* infection (Shea et al., 1999; Heijden and Finlay, 2012). The SPI-2 effectors are essential to internalize *S. Typhimurium* and are required for the *Salmonella*-containing vacuole (SCV) maturation (Ruiz-Albert et al., 2002; Freeman et al., 2003). Here, the upregulated SPI-2 encoded genes were evident, such as the *sseJ* gene, an acyltransferase/lipase that is associated with SCV biogenesis in human epithelial cells (Ruiz-Albert et al., 2002; Freeman et al., 2003). Some studies suggested that the protein SseJ is necessary for the full virulence of *S*. Typhimurium in mice and proliferation into human cell culture (Ohlson et al., 2005; Trombert et al., 2010). The increased expression of the *sseJ* gene in *S. Typhimurium* ST313, compared to SL1344 (ST19), was verified in this study.

Our data corroborate with a recent study, which observed that the *sopD2* and *pipB* genes were upregulated in *S. Typhimurium* ST313 isolated from human feces after transcriptomic analysis compared to *S. Typhimurium* SL1344 (ST19) (Seribelli et al., 2021b). Altogether, the data indicated that ST313 was more efficient in colonizing and invading the large intestine of C57BL/6 J mice due to its increased expression of the *sopD2*, *pipB hilA*, and *ssaS* genes, which are found or effectors of SPI-1 and SPI-2 (Seribelli et al., 2021b). Similarly, we demonstrated that *sopD2* and *pipB* were also upregulated in the ST313 isolates from human blood compared to SL1344 (ST19), showing that the pathogenesis of these isolates is a major concern. Conversely, previously characterized African-isolated iNTS ST313 strains invaded non-phagocytic cells less efficiently and stimulated less inflammasome activation than ST19 isolates associated with gastroenteritis (Carden et al., 2015).

Interestingly, a study showed that decreased KatE catalase activity in *S. Typhimurium* ST313 L1, L2, and L3 isolates compared to SL1344 (ST19) this phenotype may be associated with adaptation to a restricted host range and loss of characteristics related to environmental persistence (Singletary et al., 2016). The KatE catalase protects *S. Typhimurium* from oxidative stress environments and has shown maximum activity in stationary phase cultures (Pulford et al., 2021). Here, we observed increased expression of *katE* in *S. Typhimurium* ST313 compared with SL1344(ST19).

In vivo studies such as the *G. mellonella* are an important alternative model to evaluate the virulence of distinct pathogenic microorganisms (Ramarao et al., 2012). The model consists of *G. mellonella* larvae that are easily cultivated in large numbers and at low cost and produce human-like components of the innate immune response (Scorzoni et al., 2015). Here, we found a difference in larvae survival about the bacterial concentration tested but no difference in virulence between the ST19 and ST313 strains after three days of incubation. Recently, Bender and colleagues observed 100% mortality of larvae in <10 h after inoculation of 10^7 CFU/larva with *S. Typhimurium* NCTC 12023 *(*Bender et al., 2013). Moreover, according to Viegas et al. (2013), the concentration of 10^8 CFU/larva of *S. Typhimurium* resulted in 100% larval mortality in *G. mellonella* on the first day after infection, and it was also observed that using concentrations lower than 10^5 CFU/larva, the mortality was almost null or close from zero.

The study with Brazilian *S. Typhimurium* ST313 strains in the *G. mellonella* model demonstrated a difference in virulence between strains isolated from humans and foods but no differences between STs (Seribelli et al., 2020). On the other hand, another study reported that Brazilian ST313 isolated from humans demonstrated to be more virulent than ST19 in C57BL/6 J mice treated with streptomycin (Seribelli et al., 2021b). Recently, studies have highlighted the similarity between the genome of *S. typhi*, which is restricted to humans and causes systemic disease, and *S. Typhimurium* ST313. It was also observed that ST313 occupies a phenotypically intermediate state between *S. Typhimurium* ST19 and *S. typhi* for both host cell invasion and inflammasome activation, suggesting that ST313 is becoming phenotypically closer to systemic disease-causing serovars; this may justify that trials with models distant from humans do not have the expected difference (Carden et al., 2017; Puyvelde et al., 2019; Pulford et al., 2021).

However, *G. mellonella* allows experiments with large samples without compromising ethical standards, maintaining genetic homogeneity, and testing diverse bacterial concentrations (Lacharme-Lora et al., 2019). Future studies with other animal models can be carried out to observe the differences.

The phylogenetic comparison tree based on SNPs grouped the *S. Typhimurium* ST313 genomes, showing that ST313-isolated strains from human blood in Brazil are genetically distinct from most strains isolated from human feces and food in this country and from *S. Typhimurium* ST19 and ST313 reference genomes (Fig. 1).

5. Conclusions

The transcriptomic analyses and further characterization showed that *S. Typhimurium* ST313 strains isolated from human blood in Brazil are different from what has been described for ST313 strains isolated from human blood in Africa in recent years by other articles and authors. The pathogenic potential was evidenced in the *S. Typhimurium* ST313 strains studied due to the presence or higher expression of crucial virulence genes related to invasion and survival in host cells. Our work here contributed to a better understanding of these invasive strains and their diversity, which present a serious emergent public health problem.

Ethics approval and consent to participate

Not applicable (All previous published bacterial strains).

Consent for publication

Not applicable.

CRediT authorship contribution statement

Isabela Mancini Martins: Formal analysis, Writing - original draft. **Amanda Aparecida Seribelli:** Formal analysis. **Tamara R. Machado Ribeiro:** Formal analysis, Methodology. **Patrick da Silva:** Resources. **Bruna Cardinali Lustri:** Formal analysis, Methodology. **Rodrigo T. Hernandes:** Resources. **Juliana Pfrimer Falcão:** Supervision, Writing -

review & editing, Funding acquisition. **Cristiano Gallina Moreira:** Supervision, Writing - review & editing, Funding acquisition.

Funding

São Paulo Research Foundation (FAPESP) (Proc. 2019/19338–8, 2019/03049–7, 2019/26696–8, and 2022/05030–4) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) — Brasil Finance Code 001 for financial support. During this work, Seribelli, A.A. was supported by a scholarship from São Paulo Research Foundation (FAPESP) Proc. 2017/06633–6). Falcão, J. P. received a productive fellowship from Council for Scientific and Technological Development (CNPq) Grant 304399/2018–3.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

The data from 22 S. Typhimurium genomes released under the BioProjects, PRJNA656707 and PRJNA186035, available at (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA656707) (https://www.ncbi.nlm.nih.gov/bioproject/186035).

Acknowledgements

All financial Support

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