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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Antimicrobial resistance and genetic background of non-typhoidal *Salmonella enterica* strains isolated from human infections in São Paulo, Brazil (2000–2019)

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Abstract

Salmonella enterica causes Salmonellosis, an important infection in humans and other animals. The number of multidrug-resistant (MDR) phenotypes associated with *Salmonella* spp. isolates is increasing worldwide, causing public health concern. Here, we aim to characterize the antimicrobial-resistant phenotype of 789 non-typhoidal *S. enterica* strains isolated from human infections in the state of São Paulo, Brazil, along 20 years (2000–2019). Among the non-susceptible isolates, 31.55, 14.06, and 13.18% were resistant to aminoglycosides, tetracycline, and β-lactams, respectively. Moreover, 68 and 11 isolates were considered MDR and Extended Spectrum β-Lactamase (ESBL) producers, respectively, whereas one isolate was colistin-resistant. We selected four strains to obtain a draft of the Genome Sequence; one *S.* Infantis (ST32), one *S.* Enteritidis (ST11), one *S.* I 4,[5],12:i:- (ST19), and one *S.* Typhimurium (ST313). Among them, three presented at least one of the following antimicrobial resistance genes (AMR) linked to mobile DNA: $bla_{TEM\text{-}1B}$, *dfrA1*, *tetA*, *sul1*, *floR*, *aac(6')-Iaa*, and *qnrE1*. This is the first description of the plasmid-mediated quinolone resistance (PMQR) gene *qnrE1* in a clinical isolate of *S.* I 4,[5],12:i:-. The *S.* Typhimurium is a colistin-resistant isolate, but did not harbor *mcr* genes, but it presented mutations within the *mgrB*, *pmrB*, and *pmrC* regions that might be linked to the colistin-resistant phenotype. The virulence pattern of the four isolates resembled the virulence pattern of the highly pathogenic *S.* Typhimurium UK-1 reference strain in assays involving the in vivo *Galleria mellonella* model. In conclusion, most isolates studied here are susceptible, but a small percentage present an MDR or ESBL-producer and pathogenic phenotype. Sequence analyses revealed plasmid-encoded AMR genes, such as β-lactam and fluoroquinolone resistance genes, indicating that these characteristics can be potentially disseminated among other bacterial strains.

Keywords *Salmonella enterica* · Brazilian ST313 · Antimicrobial resistance · β-lactams · Fluoroquinolones · Genetic virulence

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Introduction

Salmonella enterica, a Gram-negative rod belonging to the family *Enterobacteriaceae*, causes Salmonellosis, one of the most prevalent foodborne infections in the world. On the basis of clinical patterns, *S. enterica* can be divided into two groups: typhoidal (TS) or non-typhoidal (NTS) *Salmonella*. TS is restricted to humans, but many NTS serotypes infect humans and food-producing animals. Poultry products, livestock, and swine are among the most common sources of NTS [1]. As a result, infections caused by NTS have high epidemiological impact worldwide. For instance, NTS serotypes account for 39% of the foodborne diseases in Brazil and affect 3.4 million people in Africa [2, 3]. *S. enterica* subsp. *enterica* serovars Enteritidis and Typhimurium (including its flagellar monophasic variant) are among the most prevalent NTS serovars associated with human infections, causing intestinal and extraintestinal infections [4]. In particular, *S.* Enteritidis causes most (87%) human infections in Europe and underlies 37% of infections in Latin America [5]. Other serovars have gained epidemiological importance because the number of isolates has increased around the world. One example is *S.* Infantis, which is now considered the fourth most common serovar in Europe [6]. Another serovar worth mentioning is *S.* Heidelberg which has become one of the most frequently isolated serovars associated with human infections worldwide, including in Brazil [7].

Fluoroquinolone, β-lactams, and trimethoprim-sulfamethoxazole are normally prescribed to treat Salmonellosis, but the growing number of multidrug-resistant (MDR) strains can hamper the treatment of this illness [8]. In this regard, colistin is normally the last treatment choice against MDR strains including *Salmonella* spp. However, the rising number of strains carrying plasmid-encoded colistin resistance genes is a cause of concern to public health and it can curb the treatment against MDR pathogens [9].

Bearing the epidemiological importance of NTS serotypes as human pathogens and the increasing resistance pattern associated with them, we aimed to characterize 789 *Salmonella enterica* isolates from human infections in the state of São Paulo, Brazil, and to evaluate their resistance or susceptibility profile against a panel of antimicrobial drugs. On the basis of the resistance profile, we will select strains to characterize their genotype and to investigate their pathogenic profile by using the in vivo *Galleria mellonella* model. The results presented here will contribute to a better understanding of NTS resistance and pathogenic profiles in Brazil.

Material and methods

Bacterial isolates

The bacterial isolates employed here belong to the collection of the Adolfo Lutz Institute (IAL), Regional Laboratory Center Campinas III, Campinas, São Paulo, or to the University Hospital of the University of São Paulo (USP), São Paulo, Brazil. A total of 789 *Salmonella enterica* isolated between 2000 and 2019 were studied. All isolates from the bacteria collection at USP were stored in ultralow freezers using glycerol as the cryoprotectant substance. Initially, the isolates at Adolfo Lutz Institute were stored in nutrient agar at room temperature; posteriorly, they were transferred to ultralow freezers in nutrient broth with glycerol at the final concentration of 15%. All the samples were isolated from human infections and previously serotyped and isolated by the Laboratories that provided the samples. *S.* Enteritidis (359, 45.50%), *S.* Typhimurium (79, 10.01%), *S.* Typhimurium flagellar monophasic variant (66, 8.36%), *S.* Dublin (30, 3.67%), and *S.* Saint Paul (29, 3.67%) were the most prevalent among the *Salmonella enterica* serovars. Isolates whose antigen O was difficult to determine, possibly due to rough Salmonella variants, or isolates whose flagellar phase (antigen H) could not be determined, were not assigned a serotype; thus, 70 isolates (8.87%) were called *Salmonella* spp. in Fig. 1. The other 156 isolates (19.77%) belong to other serovars as described in Fig. 1. The number of isolates per year that were provided to us is shown in Fig. 1 and the distribution of the most prevalent serovars are displayed in Fig. 1.

Antimicrobial resistance testing

Disk diffusion assay

The antimicrobial resistance profile was assayed by the Kirby-Bauer disk diffusion susceptibility test, and the samples were classified as susceptible or non-susceptible according to the CLSI M02-A12 (2015) [10] instructions. The antimicrobials were chosen according to the CLSI M100 (2020) [11] guidelines. By following this same CLSI document, the susceptibility or non-susceptibility patterns were evaluated after incubation at 37 °C for 18 h. The antimicrobial disks and the respective concentrations were as follows: Penicillins (amoxicillin,10 μg); Cephalosporins (cefoxitin, 30 μg; cefotaxime, 30 μg; and ceftazidime, 30 μg); Carbapenems (imipenem, 10 μg; meropenem, 10 μg; and ertapenem, 10 μg); Chloramphenicol (chloramphenicol, 30 μg); Fluoroquinolones (ciprofloxacin, 5 μg; and enrofloxacin, 5 μg); Tetracyclines (tetracycline,

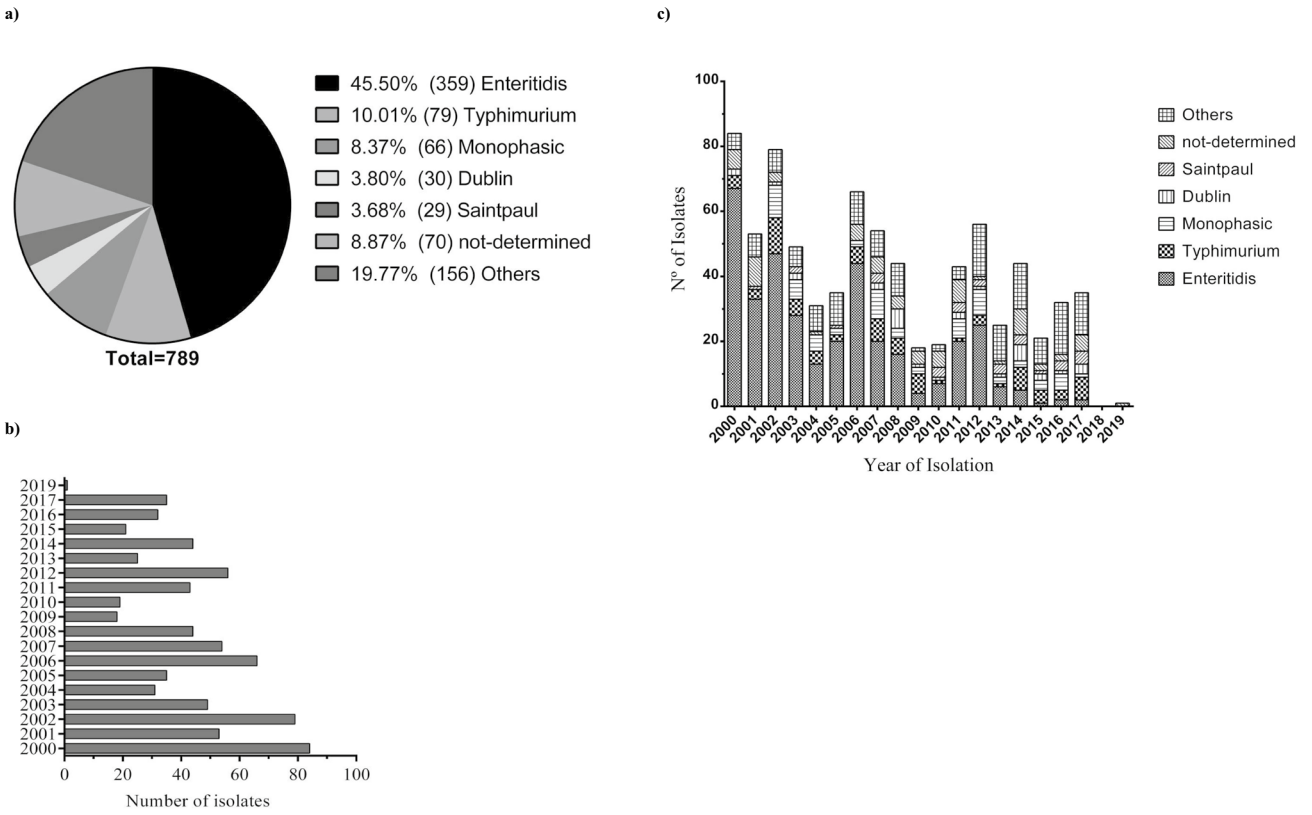

Fig. 1 Metadata of isolates provided for the present study. **A** Diversity of *Salmonella* sp. serovars. **B** Number of isolates per year. **C** Distribution of the most prevalent serovars per year

30 µg); Aminoglycosides (streptomycin, 10 µg; and gentamicin, 10 µg); Sulfonamides/Trimethoprim (trimethoprim/sulfamethoxazole, 25 µg), and Polymyxins (colistin, 10 µg). The *Escherichia coli* ATCC 25,922 strain was used as a reference because it is sensitive to all the tested antimicrobials.

AMPC production test

AmpC beta-lactamase production was detected in the bacterial isolates by using the double-disk synergy test [12]; cefoxitin/cloxacillin (30 µg/200 µg) was employed as previously described [13]. Only the cefoxitin-resistant bacterial isolates were submitted to this test.

ESBL production test

The Extended Spectrum β-Lactamase (ESBL) production assay was performed as described in CLSI M100 (2020) [11]. Only bacterial isolates characterized as resistant to third generation cephalosporins were included. The isolates that were resistant to imipenem, meropenem, or both were further assayed to identify carbapenemase producers. The inhibition zones around these antimicrobial disks with and without EDTA (0.1 M), cloxacillin (75 mg/mL), or phenylboronic acid (40 mg/mL) were compared and classified according to the ANVISA document (2013) [14]. *K. pneumoniae* ATCC 700,603 (Pasteran et al., 2011) [15], an SHV-18 producer, was used as the positive control, and *E. coli* 25,922 was applied as a sensitive, non-ESBL producer control.

Minimal inhibitory concentration (mic)

The disk diffusion (Kirby-Bauer) test was used as a screening method to detect possible colistin-resistant samples. All 789 samples were assayed by this method. The isolates that presented an inhibition zone ≤ 10 mm for colistin were selected for further characterization by the Minimal Inhibitory Concentration (MIC) test according to the CLSI M07-A10 document (2015) [16]. The results were interpreted according to the CLSI M100 document (2020) [11].

Genomic sequencing and bioinformatic analyses

Based on the resistance profile, one isolate resistant to extended-spectrum β-lactams (520/2008), two MDR isolates (NCMO-6928/2005 and 725/2016), and the colistin-resistant

isolate (NCMO-6924/2007) were selected for whole-genome sequencing (WGS).

Genomic DNA was extracted and purified by using Wizard® Genomic DNA Purification (Promega, USA); the manufacturer's guidelines were followed. The DNA libraries were prepared by using the Nextera™ XT DNA kit (Illumina Inc., Hayward, CA), and the sequences were obtained by the 100-bp paired-end-read strategy in the Illumina HiSeq2500 (Illumina Inc., San Diego, CA) platform. All these steps were performed at the Central Laboratory of High-Performance Technologies (LaCTAD) of the University of Campinas (UNICAMP).

The assemblies were obtained with SPAdes v3.15.2 [17] and Newbler 3.0 (unpublished) in collaboration with LaCTAD and the National Laboratory of Scientific Computing. The scaffolds were annotated by using the prokaryotic genome automatic annotation pipeline (PGAAP) available at NCBI. Downstream analyses available at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/) were used to confirm the multi-locus sequence typing, serotype, presence of plasmids, and antimicrobial resistance genes. Additionally, CARD V3.0.8 [18] was used to investigate other antimicrobial resistance genes. The PHASTER [19] program setup with the default parameters was employed to detect phage sequences. Virulence factor sequences were obtained from Virulence Factor Database (VFDB 2019) [20] and compared to our isolates and the reference strains *S.* Typhimurium LT2 and UK-1. The integrity of the most well-characterized *Salmonella* Pathogenicity Islands (SPI-1/2/3/4/5/6) was evaluated using the *S.* Typhimurium LT2 as a reference. Plasmid sequences of the 725/2016 strain were compared to the NCBI prokaryotic database by using the BLASTn tool to confirm whether mobile elements and resistance genes were shared with reference plasmids. Sequence alignment of plasmids and SPIs was performed using the MAUVE 2.1 program adjusted to the default parameters [21].

Galleria mellonella killing assay

The pathogenic profiles of the bacterial strains selected for WGS (the 725/2016, 520/2008, NCMO-6924/2007, and NCMO-6928/2005 strains) were also assayed; the in vivo *Galleria mellonella* model was applied. The *S.* Typhimurium UK-1 strain, a highly pathogenic strain, donated by Professor Roy Curtis III (College of Veterinary Medicine, University of Florida, USA), and *Escherichia coli* HB101 were used as pathogenic and non-pathogenic control strains, respectively. An additional negative control group consisting of *G. mellonella* larvae inoculated with phosphate-buffered saline (PBS-10 mM) was also included.

The assay was performed by following the instructions of a previous study [22]. Larvae weighing approximately 250 mg without signs of illness or melanization were selected for the test. The injections contained 10 µL of each bacterium at 10^4 CFU/larvae (10 larvae/strain), and the same volume was used for the PBS-injected larvae (5 larvae/experiment). The larvae were scored for survival every 24 h for 96 h and were considered dead if they were inert. The experiments were repeated three times, independently, and the results are expressed as the means of the Kaplan–Meier curves.

Results

Antimicrobial resistance profile

Disk diffusion assay

Table 1 lists the number and percentage of resistant bacterial isolates observed in this study. As seen in the columns, we categorized the data on the basis of the percentage (and number between parenthesis) of resistant isolates of serovars. The last column presents the results obtained for each drug; the total number of evaluated isolates (789) is considered, irrespective of the serovar. Therefore, the last column represents the overall resistance profile of the bacterial isolates. We verified the highest percentage of non-susceptible isolates for streptomycin (29.27%), tetracycline (14.07%), and amoxicillin (12.67%). Among the tested isolates, 339 (42.96%) were resistant to at least one antimicrobial, and 68 (0.86%) were resistant to three or more classes of antimicrobials, which classifies them as MDR according to Magiorakos et al. (2012) [23]. When we considered only the MDR isolates, most of them were typed as Typhimurium (16, 23.53% of the MDR), Typhimurium flagellar monophasic variant (15, 22.05% of the MDR), and Enteritidis (13, 19.11% of the MDR). Thus, although Enteritidis was the most frequent serovar in this study, Typhimurium and its flagellar monophasic variant were the most representative for the MDR phenotype, corresponding to almost 50% of the isolates characterized as MDR.

When we considered only the Enteritidis isolates, which were the most frequent serovar (45.5%), resistance to amoxicillin (11.42%) was the most common. The Enteritidis 520/08 strain was resistant to cefotaxime and ceftazidime (third generation cephalosporins) as well as meropenem (carbapenem). The isolates of Typhimurium and its flagellar monophasic variant, which together accounted for over 18% of the isolates, had similar antimicrobial resistance profiles and were resistant to streptomycin. Regarding resistance against cephalosporins, among the Typhimurium isolates, two were resistant against third generation cephalosporins, two against cefotaxime, and one against ceftazidime.

Table 1 Strain ID, year of isolation, Source, MLST, resistance phenotype, antimicrobial resistance genes, intact prophages, and plasmid replicons of the WGS isolates

Antibiotic	Class	Enteritidis %(n)	Typhimurium %(n)	Monophasic %(n)	Dublin %(n)	Other serovars %(n)	Total %(n)
Amoxicillin	Penicillins	11.42% (41)	21.52% (17)	21.21% (14)	20% (6)	8.63% (22)	12.67% (100)
Cefotaxime	3rd Cephalosporins	0.28% (1)	2.53% (2)	0% (0)	0% (0)	0.78% (2)	0.63% (5)
Cefoxitin	2nd Cephalosporins	0% (0)	0% (0)	3.03% (2)	3.33% (1)	0% (0)	0.38% (3)
Ciprofloxacin	Fluoroquinolones	0.28% (1)	1.27% (1)	1.52% (1)	0% (0)	0% (0)	0.38% (3)
Chloramphenicol	Chloramphenicol	2.23% (8)	16.46% (13)	15.15% (10)	3.33% (1)	3.53% (9)	5.20% (41)
Ceftazidime	3rd Cephalosporins	0.28% (1)	1.27% (1)	0% (0)	0% (0)	0.78% (2)	0.51% (4)
Enrofloxacin	Fluoroquinolones	2.79% (10)	5.06% (4)	3.03% (2)	6.67% (2)	5.49% (14)	4.05% (32)
Ertapenem	Carbapenems	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Imipenem	Carbapenems	0% (0)	0% (0)	0% (0)	0% (0)	0.39% (1)	0.12% (1)
Gentamycin	Aminoglycosides	4.18% (15)	11.39% (9)	16.67% (11)	6.67% (2)	4.70% (12)	6.21% (49)
Streptomycin	Aminoglycosides	9.47% (34)	56.96% (45)	46.97% (31)	43.33% (13)	42.35% (108)	29.27% (231)
Meropenem	Carbapenems	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
SXT[1]	Sulfonamides	2.51% (9)	17.72% (14)	12.12% (8)	10% (3)	5.49% (14)	6.08% (48)
Tetracycline	Tetracyclines	7.24% (26)	26.58% (21)	28.79% (19)	40% (12)	12.94% (33)	14.07% (111)
Total		100% (359)	100% (79)	100% (66)	100% (30)	100% (255)	100% (789)

[1] *SXT* sulfamethoxazole/trimethoprim

Moreover, two 4,[5],12:i:- isolates were resistant to cefoxitin, a second generation cephalosporin.

ESBL phenotypic detection

We evaluated all the isolates resistant to carbapenems or cephalosporins by the double-disk synergy test, which detects ESBL producers. Table 2 summarizes information about the 11 samples that we considered ESBL producers according to these analyses. None of the samples tested positive for carbapenemase production, whereas three isolates presented a profile that correlated with *ampC* production (resistant to cefoxitin and inhibited by cloxacillin). Furthermore, eight isolates showed resistance to third generation cephalosporins and were inhibited by clavulanic acid. These results indicated that these isolates were ESBL producers, but further analyses are needed to identify which type or types of ESBL are produced by each isolate.

Minimal inhibitory concentration (mic)

We selected 17 isolates classified as colistin-resistant according to the disk diffusion method to confirm this phenotype through the MIC test. We only confirmed one Typhimurium

Table 2 Meta-data of ESBL-producer isolates according to double disk synergy test results

Strain	Source	Year	Serovar	Inhibitor	Resistance Phenotype
NCMO-6974	Feces	2000	Enteritidis	CLA	CAZ/CTX
NCMO-7025	Feces	2006	Enteritidis	CLA	AML/CTX
NCMO-7017	Blood	2007	1,4,5,12:i:-	CLO	AML/FOX/S/SXT/TE
NCMO-7027	Feces	2007	Typhimurium	CLO	AML/CT/C/FOX/ENR/CN/S/TE
NCMO-7028	Blood	2007	Dublin	CLA	CTX/CAZ/S
520	Feces	2008	Enteritidis	CLA	CTX/CAZ/IPM/CN/S
1061	Feces	2009	Typhimurium	CLA	AML/CTX/C/S/TE
1139	Feces	2009	Typhimurium	CLA	AML/CTX/C/S/TE
NCMO-7124	Blood	2012	Typhimurium	CLO	FOX/TE
709	Feces	2015	Muenchen	CLA	AML/CTX/CAZ/CN/S/TE
382	Blood	2017	*S.* sp	CLA	AML/CTX

AML amoxicillin, *C* chloramphenicol, *CAZ* ceftazidime, *CLA* clavulanic acid, *CLO* cloxacillin, *CN* gentamycin, *CT* colistin, *CTX* cefotaxime, *ENR* enrofloxacin, *FOX* cefoxitin, *IPM* imipenem, *S* streptomycin, *SXT* sulfamethoxazole+ trimethoprim, *TE* tetracycline

strain (NCMO-6924/2007) as colistin-resistant, with an MIC of 8 µg/mL. This strain was isolated from a fecal sample, which also exhibited resistance to aminoglycosides.

Whole-genome sequencing

Antimicrobial resistance genotype

We selected the isolates that were resistant to colistin (NCMO-6924/2007), fluoroquinolone (725/2016), and meropenem (520/2008) as well as one MDR isolate (NCMO-6928/2005) for WGS. Table 3 details the antimicrobial resistance genes detected in these strains. Despite the MDR phenotype, the Infantis NCMO-6928/2005 strain only presented the *aac(6')-Iy* gene, which is correlated with resistance to aminoglycosides and was also detected in Enteritidis 520/2008. This Enteritidis strain exhibited mutations in the *gyrA* gene, which encodes the DNA gyrase protein A subunit, a characteristic that can be associated with resistance to quinolone. However, we only evaluated the resistance to fluoroquinolone of the strains. We also found mutations in *gyrA* in the flagellar monophasic variant 725/2016 strain, which showed a fluoroquinolone resistance profile. In this case, the 725/2016 strain carried an additional quinolone resistance gene called *qnrE1*, which might explain its fluoroquinolone-resistant phenotype. The *qnrE1* gene is a plasmid-associated gene that was first described in Argentina [24]. Comparing the gene context of the 725/2016 strain with the bacterial strain where *qnrE1* was originally described (*K. pneumoniae* Q1130), sequence similarity was high. The exception was *ahp* genes, which probably encoded proteins of different sizes (Fig. 2). In addition, the flagellar monophasic strain (725/2016), which is resistant to amoxicillin, presented the bla_{TEM-1} gene associated with resistance to β-lactam. Within this strain, we also detected two genes associated with resistance to aminoglycosides, *aadA1* and *aac(6')-Iaa*. We also detected the latter gene in the Typhimurium NCMO-6924/2007 strain. Additionally, we detected the gene *sul1*, which is associated with resistance to sulfonamide, in the 725/2016 strain; the trimethoprim resistance gene *dfrA1*; one tetracycline resistance gene *tetA*; and one chloramphenicol resistance gene *floR*.

The colistin-resistant *Salmonella* Typhimurium NCMO-6924/2007 strain did not present any of the *mcr* genes described to date, so we investigated chromosomal mutations linked with resistance to colistin. Mutations in *phoPQ*, *pmrCAB*, and *mgrB* have been linked with resistance to colistin [26–28]; therefore, we aligned these regions by using *Salmonella* Typhimurium LT2 as a reference. The results showed mutations in *mgrB* (Val1Met), *pmrB* (Val1Met), and *pmrC* (Leu128Pro) in the NCMO-6924/2007 strain.

The Enteritidis 520/2008 strain, which presented a cefotaxime, ceftazidime, and meropenem resistance phenotype, did not harbor any ESBL-producing genes with ≥ 95% identity according to CARD. Nevertheless, this strain showed 91% sequence identity in the *ampH* gene according to CARD analyses. *ampH* is intrinsic to the family *Enterobacteriaceae* and, when overexpressed, can result in resistance to beta-lactam. Thus, we decided to investigate this gene in all the sequenced isolates. Comparing the *Salmonella* Typhimurium LT2 *ampH* sequence with the sequence of all four strains obtained by BLASTp analyses, only the Infantis NCMO-6928/2005 and Enteritidis 520/08 strains presented mutations within the region. The Infantis strain presented two mutations (Arg112His and Ile282Thr), whereas the Enteritidis strain presented three mutations—the same two mutations detected in NCMO-6928/2005 and an additional one (Met224Ile). Given that *ampH* has a certain degree of polymorphism, we were unable to associate the allelic variation observed herein with a specific resistance phenotype.

Presence of mobile genetic elements

We investigated the genome of the strains sequenced herein for the presence of mobile elements such as plasmids and prophages. The results are depicted in Table 3. The Enteritidis 520/2008 and Typhimurium NCMO-6924/2007 strains carried plasmids belonging to two (IncFII and IncFIB) incompatibility groups. We detected three different types of plasmid replicons (IncFIA, IncHI2, and IncHI2A) in the 725/2016 strain, the flagellar monophasic variant strain. As for prophages, all the strains carried at least one intact sequence (Table 3). We detected the Sal-3 prophage in both the Enteritidis and Typhimurium strains, whereas the Infantis strain harbored L-413C, a prophage found in *Yersinia* spp. We found the lambdoid Gifsy-1 and Gifsy 2 prophages in the genome of the flagellar monophasic variant and Enteritidis strains, respectively, and we found both phages in the genome of the Typhimurium strain. However, the flagellar monophasic variant 725/2016 did not harbor the intact Gifsy-2 sequence.

Phage sequences are frequently related to virulence. Therefore, we searched for the presence of phage-associated virulence genes by BLASTn analyses. Gifsy-1 harbors the *gogB*, *gogA*, and *gipA* virulence genes, and we detected *gogB* in the 725/2016 and NCMO-6924/2007 strain. We found *sodC*-1, a virulence factor encoded by a gene of the Gifsy-2 prophage, in Enteritidis 520/2008, Typhimurium NCMO-6924/2007, and the flagellar monophasic variant 725/2016 strains even though the latter strain only presented a partial sequence of the Gifsy-2 prophage.

Galleria mellonella *in vivo* assay and virulence genes

All the *G. mellonella* larvae inoculated with the *E. coli* HB101 (10^4 CFU/larvae) strain and with PBS survived during the experiment, which lasted 96 h. We expected these results because *E. coli* is a non-virulent strain used as negative control. However, for the NCMO-6924/2007, 725/2016, 520/2008, and NCMO-6928/2005 strains (10^4 CFU/larvae), we verified 100% death within 24 h, as observed for the highly pathogenic *S.* Typhimurium UK-1 strain (Fig. 3).

All isolates presented intact SPI-1/2/4/5 regions when aligned with *S.* Typhimurium LT2. The *S.* Infantis isolate did not present the gene *rhuM* from SPI-3. The *S.* Typhimurium and *S.* monophasic variant isolates presented an intact SPI-6, whereas *S.* Enteritidis did not present *rhsDE* and presented a smaller *vgrS* (501 bp) compared to the same gene of *S.* LT2 (2190 bp). The *S.* Infantis isolate only presented a deletion of the *rhsE* gene.

The VFDB database was compared to all isolates from this study as well as the reference strains *S.* Typhimurium LT2 and UK-1. The *spvABCDR* and *pefABCD* operons and the gene *rck* were only found in the *S.* Enteritidis 520/08 and *S.* Typhimurium 6924/2007 isolates. The isolate *S.* Typhimurium 6924/2007 also possessed the gene *invA* from *Yersinia* species, which is also harbored by *S.* monophasic variant 725/16. Strain 725/16 was the only strain to harbor the genes *ibeB* and *gtrA*. In addition to the aforementioned genes, there were minor differences between fimbrial adherence determinants, which are expected among isolates from different serotypes. Overall, the isolates presented similar virulence determinants when compared to *S.* Typhimurium LT2 and UK-1.

Discussion

The *Salmonella* isolates studied here are mostly resistant to streptomycin (29.27%), tetracycline (14.07%), and amoxicillin (12.67%). According to a study conducted by the Brazilian National Health Surveillance Agency, *Salmonella* spp. isolated from fowls present elevated resistance to streptomycin (89.3%), an aminoglycoside; sulfonamides (72.4%); and ampicillin (44.8%), a type of penicillin [29]. The high percentage of aminoglycoside- and penicillin-resistant (streptomycin and amoxicillin, respectively) strains reported here corroborates the results reported by the Brazilian National Health Surveillance Agency. The prominent resistance to tetracyclines and penicillins described in this work endorses the results reported in a meta-analysis study evaluating the antimicrobial resistance phenotype of *Salmonella* spp. from poultry and clinical isolates [30]. That study reported 36.9 and 23.6% tetracycline- and ampicillin-resistant isolates, respectively. Nevertheless, resistance to sulfonamide was the most common resistance phenotype (46.4%) described in that study [30]. Here, we only tested sulfonamide combined with trimethoprim, but we did not verify high frequency of resistant isolates (6.42%). The high frequency of resistance to aminoglycosides, tetracyclines, and penicillins among bacteria might be associated with excessive use of these compounds to promote growth in farm animals [31].

Regarding compounds that are clinically important for the treatment of Salmonellosis, e.g., quinolone and fluoroquinolones, our isolates show low resistance (in percentage) to enrofloxacin (3.95%) and ciprofloxacin (0.37%). These results contrast with the findings of studies from other countries showing increased resistance to fluoroquinolone/quinolone among *Salmonella* spp. [32–34]. Despite the low percentage of resistance to fluoroquinolone verified herein, understanding the mechanisms of resistance among the resistant samples is essential, so that mitigation strategies can be adopted to prevent strains from disseminating. Bearing this in mind, we selected the 725/2016 isolate, resistant to ciprofloxacin and enrofloxacin, for WGS.

We detected the *qnrE1*quinolone resistance gene, mediated by plasmids (PMQR), in the 725/2016 isolate. This gene, originally called *qnrB88*, was described for the first time in a *Klebsiella pneumoniae* (GenBank accession no. KY781949) isolated from humans in 2007 in Argentina [24]. Since then, studies have reported that this quinolone resistance gene is present in *K. pneumoniae* strains isolated from a domestic bird [35], a domestic cat [36], and an Amazonian fish [37], *S.* Typhimurium from food sources [38], clinical isolates of *S.* Enteritidis, *S.* Newport, and *S.* Infantis isolated from humans [39], *S.* Typhimurium obtained from food products [40, 41], *S.* I.4,5,12:i: from swine [42], and *Enterobacter cloacae* and *K. pneumoniae* isolated from chicks [43]; all aforementioned *qnrE-1* positive strains were isolated in Brazil. This gene was also reported in *E. coli* isolated from livestock in Uruguay [44]. Despite the apparent prevalence of this resistance gene in Latin America, there are reports of its presence in *Salmonella* spp in China [45], *Enterobacter asburiae* in Thailand [46], and *Enterobacter cloacae* in Japan [47].

It is worth mentioning that in 2009 quinolones were banned as growth-promoters in Brazilian food-producing animals [48]. Notwithstanding this prohibition, AMR genes against quinolones are still being disseminated among clinical and non-clinical isolates, similar to the isolate from 2016 reported here (725/2016) as well as the Brazilian *qnrE1* studies mentioned above which mostly report samples isolated after 2009.

Compared to the genetic surroundings of *qnrE1* detected in the 725/2016 strain, *S.* Typhimurium isolates obtained from food in Brazil [40] present the highest similarity. As shown in Fig. 2, the flagellar monophasic variant 725/2016

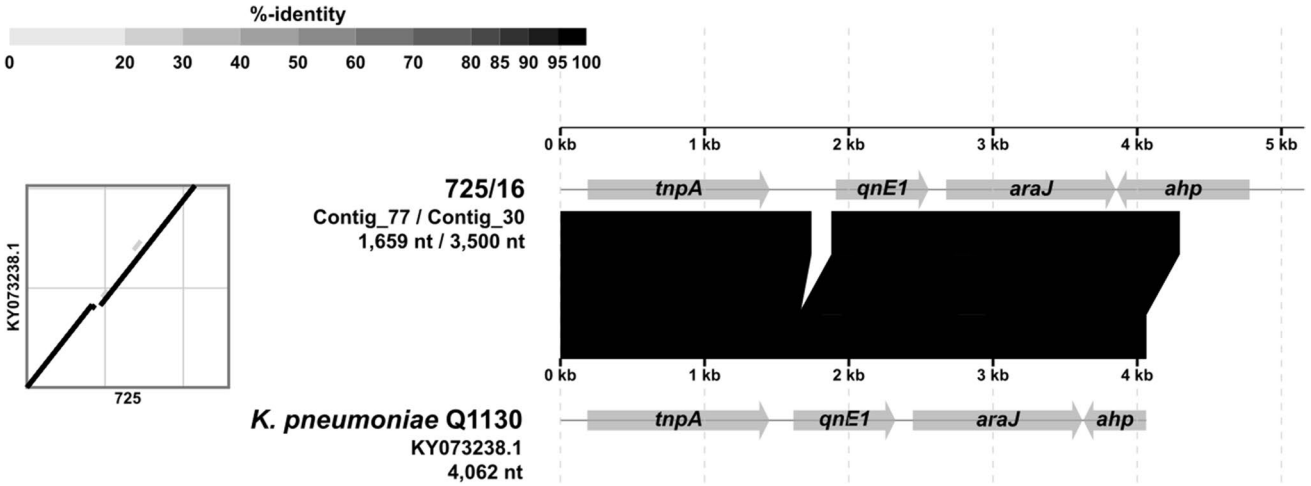

Fig. 2 Structure and arrangement of the *qnrE1* gene and flanking observed in the *S. enterica* flagellar monophasic variant strain 725/16 (**a**) compared to that od *K. pneumoniae* Q1130 (accession number KY073238) (**b**). Gene alignments were constructed using ViPTre version 2.0 [25]

Table 3 Strain ID, year of isolation, Source, MLST, resistance phenotype, antimicrobial resistance genes, intact prophages, and plasmid replicons of the WGS isolates

Isolate/Year	Source	MLST	Resistance Phenotype*	Resistance Genotype	Prophage	Plasmid
Enteritidis NCMO-520/2008	Feces	ST11	CTX/CAZ/MEM/CN/S	*gyrA/ aac(6')-ly*	Sal3[a]/Gifsy-2[b]	IncFIB/IncFII
Infantis NCMO-6928/2005	Feces	ST32	AML/S/SXT/TE	*aac(6')-ly*	*Yersinia* L-413C[a]	-
Monophasic NCMO-725/2016	Feces	ST19	AML/CIP/ENR/C/CN/S/SXT/TE	bla_{TEM-1}*/aadA1/aac(6')-laa/gyrA/qnrE1/dfrA1/sul1/floR/tetA*	Gifsy-1[b]	IncFIA/IncHI2/IncHI2A
Typhimurium NCMO-6924/2007	Feces	ST313	CT/S	*aac(6')-laa*	Gifsy 1-2[b]/Sal3[a]	IncFIB/IncFII

AML amoxicillin, *C* chloramphenicol, *CAZ* ceftazidime, *CIP* ciprofloxacin, *CN* gentamycin, *CT* Colistin

[a]P2-like prophages, [b]Lambda group, CTX cefotaxime, *ENR* enrofloxacin, *MEM* meropenem, *S* streptomycin, *SXT* sulfamethoxazole+ trimethoprim, *TE* tetracycline

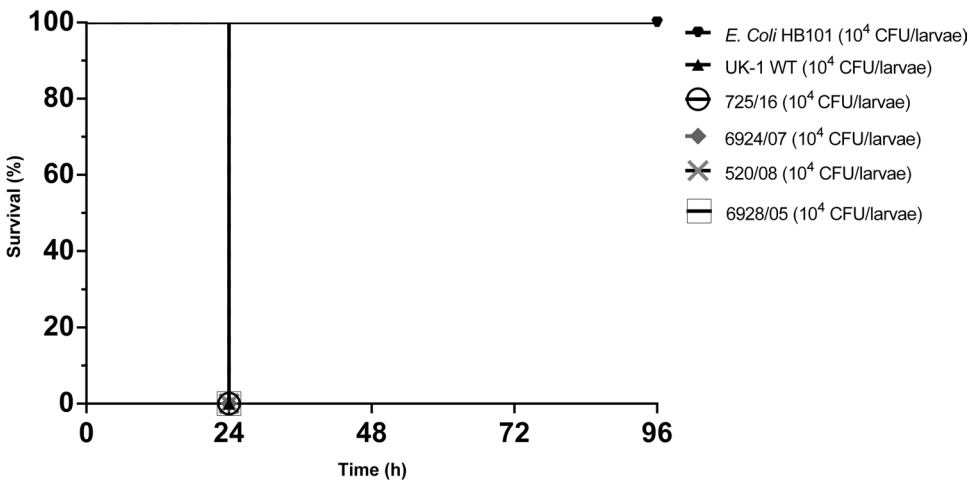

Fig. 3 *Galleria mellonella* Kaplan-Meier survival curve obtained by using *E.coli* HB-101, S.Typhimurium UK-1, S. 4,5,12:i:- 725/16, *S. Typhimurium* NCMO-6924/07, S. Enteritidis 520/08, and S. Infantis NCMO-6928/05 at 104 CFU/larvae for 96 h. The graph shows a representative result of three independent experiments

exhibits a downstream protein called *ahp*, which is the same size (1035 bp) as the Brazilian *S.* Typhimurium strains [40], whereas other studies revealed a 5'-truncated *ahp* downstream of the *qnrE1* gene [24, 35, 39]. In addition, the Brazilian *S.* Typhimurium strains [40] present the same IncH/IncF plasmid replicon type as the 725/2016 strain, which also differs from strains showing a 5'-truncated *ahp* because they only present IncM1 plasmids. Therefore, the *qnrE1* gene might be associated with different types of mobile elements. The 725/2016 strain harbors the same AMR genes as the *qnrE1*-positive *S.* Typhimurium strains reported previously [38, 40]. Hence, the same plasmid could be responsible for disseminating several AMR genes. During IncH/IncF plasmid assembly in our strain, many assembly strategies indicated that the *qnrE1* gene is part of the IncH/IncF plasmid. This result indicated that the *qnrE1* gene is in the IncH/IncF plasmid. To the best of our knowledge, no previous studies have shown that the *qnrE1* gene is disseminated by IncH/IncF plasmids in Brazilian clinical isolates of *Salmonella* spp.

In addition to sharing a similar *qnrE1* genetic context, the aforementioned *S.* Typhimurium strains from food also belong to the same ST of our isolate, the ST19. Additionally, they were isolated in a similar period of time; the 725/2016 was isolated in 2016 whereas the *S.* Typhimurium from food were isolated in 2015 [40]. The *S.* Typhimurium ST19 is considered the most frequently isolated sequence type in gastroenteritis patients worldwide and is also the most prevalent *Salmonella* spp. ST in Brazil [40, 49].

Cephalosporins and carbapenems are the usual choice to treat MDR fluoroquinolone-resistant *Salmonella* spp. isolates [50]. Overall, our samples are highly susceptible to extended-spectrum β-lactams, with only 11 samples being considered ESBL producers. None of the isolates present the KPC-producer phenotype. Following the functional classification scheme [51], three isolates present the phenotype of group-1 cephalosporinase, whereas the other eight isolates show characteristics of group-2 cephalosporinase. Cephalosporins were banned as performance-enhancing substances and food-preservatives in Brazilian livestock starting from 2009 to avoid cross-resistance between clinical and food isolates [48], but our study shows (Table 2) that even after this period, EBLSs are still being disseminated in public health. Therefore, epidemiological studies should be encouraged to understand the spread and possible causes of this resistance phenotype. Cephalosporinases are commonly found among bacterial isolates from the food industry and clinical isolates, and they have been reported in continents such as Africa [52], North America [53], South America [54], and Europe [55]. Given the variety of β-lactamases, molecular studies are needed to characterize the molecular mechanism underlying the detected phenotypes.

The double-disk synergy test revealed that the cephalosporin-resistant Enteritidis 520/2008 presents an ESBL-producer phenotype and because of that we selected this isolate for WGS. Analyzing the MLST of the Enteritidis 520/08 strain, ST11 was detected; this sequence type is widely distributed globally and is the most frequent *S.* Enteritidis sequence type. In Brazil, ST11 has been isolated from human-associated Salmonellosis as well as non-human sources [56]. Regarding the resistance genotype, in silico analyses showed that it does not contain any known ESBL genes associated with mobile elements. However, this isolate bears three amino acid substitutions in a gene that is commonly distributed in the family *Enterobacteriaceae*, the so-called *ampH*, which encodes a protein that binds and hydrolyzes β-lactams, including cephalosporin C, penicillin G, and cefoxitin. Overexpression of this gene is associated with a cephalosporin resistance phenotype [57–59]. Therefore, *ampH* expression levels in Enteritidis 520/2008 must be investigated to confirm whether overexpression of this gene is associated with the cephalosporin resistance phenotype.

Colistin is the last-resort antimicrobial when it comes to fighting MDR strains resistant to fluoroquinolone and ESBL-producers [50]. We detected only one colistin-resistant isolate, *S.* Typhimurium (NCMO-6924/2007), isolated from feces in 2007, which is resistant to only streptomycin and colistin. To further investigate the mechanism of resistance and other molecular characteristics in this isolate, we performed WGS. This isolate belongs to ST313, which is prevalent in sub-Saharan Africa and is commonly isolated from systemic infections. Although this ST is rarely reported outside of sub-Saharan Africa, it has been isolated in different regions of the world and is present in Brazil [49, 60]. Previous studies have indicated differences in phylogenetic analyses, virulence and antimicrobial resistance phenotypes of ST313 isolated in Brazil and in sub-Saharan Africa; Brazilian isolates showed more susceptibility and a less invasive phenotype than African isolates, although data indicated the expression of higher levels of pathogenic genes and induction of inflammation in the mice model of infection [61]. Additionally, the African strains are mostly isolated from blood, whereas the ST313 Brazilian strains reported to date were mostly isolated from feces [49]. This corroborates our results considering that strain NCMO-6924/2007 is isolated from feces and overall presented a susceptible profile.

Additionally, the *S.* Typhimurium NCMO-6924/2007 isolate does not harbor any plasmid-mediated colistin-resistant genes, so we investigated chromosomal mutations in regions correlated with resistance to polymyxin. We detected mutations in the *mgrB* (Val1Met), *pmrB* (Val1Met), and *pmrC* (Leu128Pro) regions. A previous study that used colistin-supplemented medium to select *S.* Typhimurium LT2 mutants described amino acid substitutions linked to resistance to colistin within the *pmrAB* region [27].

Nevertheless, none of the described mutations corresponded to the mutations reported herein. Therefore, we can only speculate that the amino acid substitutions found within the NCMO-6924/2007 isolate are associated with its colistin resistance phenotype, and more studies are needed to confirm this result. Although the isolate reported here did not harbor plasmid-mediated genes that can be disseminated, it is important to stress the importance of studies investigating the prevalence of antimicrobial resistance throughout the country, especially in the case of colistin, which was only banned in 2016 as a growth-promoter in veterinary medicine in Brazil [48].

Besides the aforementioned AMR genes, the four strains submitted to WGS present at least one streptomycin resistance gene (*aac(6')-ly* or *aac(6')-laa*), corroborating the phenotype detected in the disk diffusion assay. Furthermore, the monophasic variant 725/2016 harbors the *dfrA1* gene, which is associated with resistance to trimethoprim. This gene is frequently reported among European isolates [62]. Not only the *dfrA1* gene has been detected in Brazilian isolates of *Salmonella* spp.—in fact, other AMR genes have also been detected within the 725/2016 strain (*tetA*, *floR*, *sul1*, *aadA1*, and *aac(6')-Iaa*) [38, 40]. Their wide distribution among isolates worldwide could be explained by their frequent association with mobile elements, which facilitates their spread [62–67]. The genes reported in the monophasic variant correspond to the phenotype presented in the disk diffusion test.

Despite the multidrug-resistant phenotype of Infantis NCMO-6928/2005 isolate, the in silico analyses did not detect any plasmid-encoded proteins that could explain the tetracycline, sulfamethoxazole + trimethoprim, or amoxicillin resistance phenotype of the isolate. Additionally, we did not detect plasmids within this isolate. Thus, its resistance phenotype might be correlated with overexpressed efflux pumps or any other additional antimicrobial resistance mechanisms. The *S.* Infantis strain NCMO-6928/2005 belongs to a globally distributed sequence type, named ST32. This ST is considered the most frequent sequence type of *S.* Infantis worldwide, and its presence has been reported in the state of São Paulo from a variety of sources [40, 68].

Despite the differences detected among the four strains during the in-silico analysis, all of them present the same killing rate in the *Galleria mellonella* model. We compared the virulence phenotype of the four strains to the virulence phenotype of invasive *Salmonella* Typhimurium UK-1 and the non-virulent *E. coli* HB101. As expected, the non-virulent control did not kill any larvae, but all the other strains at 10^4 CFU/larvae killed 100% of the larvae within 24 h. Thus, the four strains described herein can be as invasive as the UK-1 strain in this model. This ability is correlated with different factors, and different hosts require different mechanisms. Features associated with pathogenicity include virulence factors associated with prophage sequences and plasmid-mediated virulence factors.

Analyzing the presence of SPI-1 through 6 within our isolates, it was possible to detect almost all of the SPIs fully conserved, which might explain the homogenous results of *G. mellonella* among the isolates and reinforces their significance to the pathogenicity of the species. SPI-6 presented deletions in isolates *S.* Enteritidis 520/2008 and *S.* Infantis NCMO-6928/2005. The *rhsDE* region was not fully conserved in either isolate, and a partial deletion of *vgrS*, a gene homologous to *vgrG* in *Pseudomonas* spp., was detected in 520/2008 when compared to *S.* LT2. All these genes have been associated with competition against the host microbiome and deletions within these regions have been associated with a less efficient invasion process and an attenuated phenotype [69]. Nevertheless, these minor deletions in SPI-6 did not affect the virulence in *G. mellonella* larvae of isolates 520/2008 and NCMO-6928/2005 compared to the other isolates tested that harbored an intact SPI-6.

With respect to the presence of virulence plasmids, both the *S.* Enteritidis 520/2008 and *S.* Typhimurium NCMO-6924/2007 strains have the IncFII and IncFIB plasmid types. We did not detect any AMR plasmid-mediated genes within these strains, which contrasts with previous studies showing various AMR genes being disseminated via IncFIB in *Salmonella* spp. [70, 71]. However, the IncFII + IncFIB plasmid types are not only associated with antimicrobial resistance but also play an important role in virulence. *S.* Enteritidis and pathogenic *E. coli* strains frequently harbor virulence plasmids (pSEVs) that are part of the IncFII + IncFIB replicon types. The *pefABCD*, *rck*, and *spvABCDR* genes are virulence factors commonly detected within IncFII + IncFIB pSEV [72]. This corresponds to our findings that all the aforementioned virulence factors are present in the *S.* Enteritidis (520/2008) and *S.* Typhimurium (NCMO-6924/2007) strains. The presence of these genes plays an important role in pathogenicity of *Salmonella* spp.; *spvABCDR* is known for playing a part in intracellular survival and multiplication; the *pefABCD* operon encodes fimbrial genes that adhere to intestinal epithelial cells of mice inducing inflammation; and *rck* has been associated with immune evasion [73, 74]. Despite the presence of this pSV, also found in *S.* UK-1, in only two isolates, this did not result in differences in larval killing rates at 10^4 CFU/larvae.

Along with pSEVs, prophage sequences contribute to the virulence phenotype of bacteria. For instance, previous studies described that Gifsy prophages play an important role in *Salmonella* spp. virulence, and that their absence in *S.* Typhimurium can cause an attenuated phenotype in animal models [75]. Among the virulence factors harbored by the Gifsy-1 prophage, *gogB* was identified in the 725/2016 isolate and *S.* Typhimurium NCMO-6924/2007. This

virulence factor encodes a protein that modulates the host's immune system, presenting an anti-inflammatory role [76]. The Gifsy-2 prophage harbors *sodC-1*, a gene that encodes an antioxidant protein [77] and has been detected in the *S.* Enteritidis 520/2008, *S.* Typhimurium NCMO-6924/2007, and monophasic variant 725/2016 strains. Interestingly, the 725/2016 strain does not present the intact Gifsy-2 sequence, but the detected partial sequence harbors *sodC-1*. The total absence of Gifsy-2 provides an attenuated *Salmonella* phenotype, but when isolates harbor *sodC-1* and the intact Gifsy-1 sequence, their ability to cause an infection in animal models remains the same [75]. Therefore, despite the lack of an intact Gifsy-2, the 725/2016 strain still presents a pathogenic phenotype in the *G. mellonella* model probably due to the presence of *sodC1* and Gifsy-1.

In addition to plasmid-encoded and prophage-encoded virulence factors, some virulence genes absent in the reference strains *S.* Typhimurium LT2 and UK-1 were detected within our isolates. The monophasic variant presented the genes *gtrA* and *ibeB*, which are associated with host immune system escape and invasion of brain endothelial cells, respectively [78, 79]. Moreover, the strains NCMO-6924/2007 and 725/2016 harbored the *invA* gene from *Yersinia* species, which presents homologous virulence factors with *Salmonella* spp., such as *invA*. This gene is part of the type III secretion system playing an important role in the host cells invasion in both species [80]. All isolates studied presented the *invA* gene from *Salmonella* spp., and there is no evidence showing advantages of harboring two copies of this type of invasin.

Despite the minor differences detected among the four strains during the in silico analysis of their virulence genotype, overall the isolates presented all mainly *Salmonella* spp. virulence determinants and SPIs, which correspond with the results in the *Galleria mellonella* model where all of them present the same killing rate within larvae tested.

It is important to note that the genomic analyses performed here were based on draft genomic sequences. Therefore, we cannot exclude that the absence of some sequences can be caused by lower sequence coverage or incomplete assemblies, a common event observed for repeated and phage sequences. Thus, the data we presented regarding pathogenic and phage sequence traits are preliminary and further analyses are required for a complete characterization of the pathogenic genotypes of the strains.

In conclusion, despite the high percentage of susceptible isolates, our study reports an important small percentage of MDR or ESBL-producer and pathogenic phenotypes. The in silico analyses revealed plasmid-encoded AMR genes, such as β-lactam and fluoroquinolone resistance genes, indicating that these characteristics can be potentially disseminated among other bacterial strains. Moreover, our study shows that WGS is important for understanding not only antimicrobial resistance mechanisms, but also virulence factors underlying pathogenic phenotypes.

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Author contribution All the authors contributed to this study conception and design. Bioinformatic analyses were performed by Aline Parolin Calarga, Luiz Gonzaga Paula de Almeida, Ana Tereza Ribeiro de Vasconcelos, and Leandro Costa Nascimento. Material preparation, data collection, in vivo assays, and analysis were performed by Aline Parolin Calarga, Marco Tulio Pardini Gontijo, Taíse Marongio Cotrim de Moraes Barbosa, Thalita Mara de Carvalho Perri, Silvia Regina dos Santos, Eneida Gonçalves Lemes Marques, and Cleide Marques Ferreira. The first draft of the manuscript was written by Aline Parolin Calarga and Marcelo Brocchi. All the authors commented on previous versions of the manuscript and read and approved the final manuscript.

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Declarations

Ethics approval This study was approved by the UNICAMP ethics committee (CAAE number 91276318.2.0000.5404) and by the co-participant institutions: The University of São Paulo (91276318.2.3002.0076) and Adolfo Lutz Institute (91276318.2.3001.0059).

Conflict of interest The authors declare no competing interests.

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