Phenotypic analyses of *Salmonella enterica* serovar Enteritidis strains isolated in the pre- and post-epidemic period in Brazil

Fábio Campioni¹ · Carolina Nogueira Gomes¹ · Dália dos Prazeres Rodrigues² · Alzira Maria Morato Bergamini³ · Juliana Pfrimer Falcão¹

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Abstract

Salmonella Enteritidis has caused, since the 1980s, a sustained epidemic of human infections in many countries. This study analyzed *S. Enteritidis* strains isolated before and after the epidemic period in Brazil regarding their capacities to survive to acid, oxidative, and high-temperature stresses, and capacity to grow in egg albumen. Moreover, the ability to invade human epithelial cells (Caco-2) and to survive inside human (U937) and chicken (HD11) macrophages was checked. Post-epidemic strains showed a better ability to survive after 10 min under acid stress at 37 °C ($P \leq 0.05$). However, both groups of strains showed similar ability to survive after 1 h under acid stress at 37 °C and at 42 °C independently of the time of exposure. Similar ability was verified in both groups of strains regarding oxidative stress, growth in egg albumen, high-temperature stress, invasion to Caco-2 cells, and invasion and survival in macrophages. In conclusion, post-epidemic *S. Enteritidis* strains showed a better ability to survive under the acid stress found in the stomach, which might be an advantage to reach the intestine and colonize chickens and humans. However, both groups of strains did not differ significantly in the majority of the phenotypic tests analyzed in this study.

Keywords *Salmonella* Enteritidis · Epidemic · Stress · Cell invasion · Macrophage survival

Introduction

Salmonella enterica serovar Enteritidis (*S. Enteritidis*) is a major *Salmonella* serovar causing, since the 1980s, a sustained epidemic of human infections in several countries linked mostly to the consumption of raw or undercooked chicken or eggs, becoming one of the top *Salmonella* serovars isolated in the globe [1].

The precise date of the epidemic beginning is not known, as well as the exact reasons have not been well established. However, the existence of a *S. Enteritidis* epidemic clone, harboring genes for chicken colonization, has been shown in some countries [2–5]. In Brazil, the introduction of an epidemic clone was noticed after 1994, increasing the isolation of *S. Enteritidis* from less than 1 to 65% of the total *Salmonella* isolates [3, 6, 7].

In young chickens less than 2 weeks old, *S. Enteritidis* usually causes gastroenteritis and systemic disease with several mortality degrees. Adult chickens can be colonized by *S. Enteritidis* usually becoming asymptomatic carriers, with intermittent fecal shedding [1, 8].

Inside the chickens, *S. Enteritidis* has to survive to the poultry gastric acidity (pH 2.6), to invade the intestinal epithelium, and to survive to the oxidative stress inside the heterophils. After that, these strains disseminate in the internal tissues of the chickens, including the oviduct, consequently contaminating eggs in formation [8–11]. Inside the eggs, *S. Enteritidis* has to survive to the egg compounds, such as lysozyme, ovotransferrin, and avian β -defensins, which is indispensable for an eventual transmission of the bacteria to humans [12–16].

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✉ Juliana Pfrimer Falcão
jufalcao@fcfrp.usp.br

- ¹ Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto - USP, Av. do Café, s/n. Bloco S – Sala 41, Ribeirão Preto, SP 14040-903, Brazil
- ² Laboratório de Enterobactérias, Fundação Oswaldo Cruz – FIOCRUZ, Av. Brasil, 4365, Pavilhão Rocha Lima, Rio de Janeiro, RJ 21040-900, Brazil
- ³ Laboratório de Microbiologia de Alimentos, Instituto Adolfo Lutz – Centro de Laboratórios Regionais - Ribeirão Preto VI, Rua Minas 877, Ribeirão Preto, SP 14085-410, Brazil

In humans, after the bacteria ingestion, *S. Enteritidis* has to survive to the acid stress of the stomach, intestinal mucus, and to compete with the host microbiota. When the colonization of the intestine is established, these bacteria can invade the intestinal epithelium, usually by M cells, reaching the basal lamina and being phagocytized by macrophages. However, some strains are able to survive to these cells killing them [17–20].

The capacity to survive to all the steps above mentioned depends primarily on the virulence of a specific strain, which is independent of the phage type or of the genotypic similarity of a specific strain [8, 21–24].

In a previous study, we sequenced and analyzed the whole genome of 256 *Salmonella enterica* serovar Enteritidis strains isolated over a 48-year period in Brazil and found a genomic shift in strains isolated after 1994 [3]. These strains isolated after the epidemic period in Brazil presented the prophage SE20, which is considered a recent acquisition in the PT4 strain P125109 and might have conferred a better ability to the strains to colonize chickens and might be related to the emergence of *S. Enteritidis* worldwide in comparison to the strains isolated before this period and without this phage region [2, 3].

In light of these facts, we decided to phenotypically analyze *S. Enteritidis* strains isolated before and after the epidemic period in Brazil in order to check their capacity to invade epithelial cells and to survive to the acid and oxidative stresses, egg albumen, and macrophage phagocytosis. The obtained results may help to understand the possible phenotypic differences between strains isolated in these two periods in Brazil.

Materials and methods

Bacterial strains

A total of 40 *S. Enteritidis* strains isolated in the pre-epidemic period (20) from humans (15), chickens (4), and food (1), between 1980 and 1993, and post-epidemic period (20) from humans (9), chickens (3), and food (8) between 1994 and 2013 in Brazil were studied (Table 1). These strains were selected according to the pre- and post-epidemic clusters generated by whole-genome SNP analysis, previously published [3].

The strains were provided by the Adolfo Lutz Institute of Ribeirao Preto (IAL-RP), Oswaldo Cruz Foundation (FIOCRUZ-RJ), and by AVIPA (Avicultura Integral e Patologia S/A) from Brazil.

Survival to the acid stress

Survival to the acid stress was compared between the 20 *S. Enteritidis* strains isolated in the pre- and 20 isolated in the post-epidemic period (Table 1), according to the protocol described by Humphrey et al. and Shah et al. [8, 25]. In early

Table 1 Year and source of isolation of the 40 *Salmonella enterica* serovar Enteritidis strains studied

Strain	Year of isolation	Source	Period
SE 17/80	1980	Chicken	Pre-epidemic
SE 3494/82	1982	Human	Pre-epidemic
SE 172/85	1985	Human	Pre-epidemic
SE 4638/86	1986	Human	Pre-epidemic
SE STAL/86	1986	Human	Pre-epidemic
SE 4258/86	1986	Human	Pre-epidemic
SE 337/86	1986	Human	Pre-epidemic
SE 4	1986	Human	Pre-epidemic
SE 144/88	1988	Food	Pre-epidemic
SE A65/88	1988	Human	Pre-epidemic
SE 92/89	1989	Human	Pre-epidemic
SE 96/89	1989	Human	Pre-epidemic
SE 97/89	1989	Human	Pre-epidemic
SE 98/89	1989	Human	Pre-epidemic
SE 103/89	1989	Human	Pre-epidemic
SE 108/89	1989	Human	Pre-epidemic
SE 5	1992	Human	Pre-epidemic
SE 881/93	1993	Chickens	Pre-epidemic
SE 1328/93	1993	Chickens	Pre-epidemic
SE 1329/93	1993	Chickens	Pre-epidemic
SE 11	1994	Human	Post-epidemic
SE 18	1995	Food	Post-epidemic
SE 76	1996	Human	Post-epidemic
SE 85	1997	Food	Post-epidemic
SE 100	1998	Human	Post-epidemic
SE 126	1999	Food	Post-epidemic
SE 150	2000	Food	Post-epidemic
SE 153	2001	Human	Post-epidemic
SE 183	2002	Human	Post-epidemic
SE 201	2003	Food	Post-epidemic
SE 298/04	2004	Chickens	Post-epidemic
SE 241	2005	Food	Post-epidemic
SE 249	2006	Human	Post-epidemic
SE 131/07	2007	Chickens	Post-epidemic
SE 257/08	2008	Chickens	Post-epidemic
SE 263	2009	Human	Post-epidemic
SE 269	2010	Food	Post-epidemic
SE 285	2012	Human	Post-epidemic
SE 76/2013	2013	Human	Post-epidemic
SE 221/2013	2013	Food	Post-epidemic
Total			40 strains

stationary phase (16–18 h), each *S. Enteritidis* strain studied was inoculated in Luria-Bertani (LB) medium (pH ~7.0) at 37 °C. Their optical density (OD) was adjusted to an OD₆₀₀ = 0.2 and 1 mL, which was previously shown to correspond to

approximately 1×10^8 colony-forming units (CFU) [26] centrifuged at $8000 \times g$ for 3 min. An aliquot was plated to verify the number of viable cells. The pellet was resuspended in 10 mL of LB medium at pH 2.6, acidified with hydrochloric acid at 5 M, followed by incubation at 37 °C (human body temperature) and 42 °C (chicken body temperature).

Aliquots were collected after 10 min and 1 h of incubation in each temperature, and serial dilutions were done followed by cultivation in LB plates at pH 7.0 incubated at 37 °C during 18–24 h for viable CFU counting. The experiments were done in triplicates.

Survival to the oxidative stress

Survival to the oxidative stress was compared between the 20 *S. Enteritidis* strains isolated in the pre- and 20 isolated in the post-epidemic period (Table 1), according to the protocol described by Robbe-Saule et al. and Shah et al. [8, 27]. In early stationary phase (16–18 h), each *S. Enteritidis* strain studied was inoculated in Luria-Bertani (LB) medium (pH ~7.0) at 37 °C. Their OD was adjusted to an $OD_{600} = 0.2$ and 1 mL, which was previously shown to correspond to approximately 1×10^8 CFU [26] centrifuged at $8000 \times g$ for 3 min. An aliquot was plated to verify the number of viable cells. The pellet was resuspended in 10 mL of normal saline (0.9% NaCl), after addition of H_2O_2 to a final concentration of 15 mM followed by incubation at 37 °C (human body temperature) and 42 °C (chicken body temperature).

Aliquots were collected after 10 min and 1 h of incubation in each temperature, and serial dilutions were done followed by cultivation in LB plates at pH 7.0 incubated at 37 °C during 18–24 h for viable CFU counting. The experiments were done in triplicates.

Growth in egg albumen

The ability to survive in egg albumen was compared between the 20 *S. Enteritidis* strains isolated in the pre- and 20 isolated in the post-epidemic period (Table 1), according to the protocol described by Lu et al. and Shah et al. [8, 28]. For this, non-fertilized and antibiotic-free eggs were commercially acquired. For each experiment, 2–3 eggs were disinfected by immersion in 70% ethanol and aseptically broken to collect the egg albumen in a sterilized recipient. Aliquots of 1 mL of egg albumen were distributed in a 24-well plate followed by inoculation of approximately 500 CFU of each strain studied. The plates were incubated at 25 °C for 24 h, serial dilutions of each well were done, followed by cultivation in LB plates at pH 7.0 incubated at 37 °C during 18–24 h for viable CFU counting.

The experiments were done in triplicates, and wells with egg albumen but without the bacterial strains were used as negative controls.

Survival to high-temperature stress

The ability to survive to the preconized temperature to cook food to avoid *Salmonella* was compared between the 20 *S. Enteritidis* strains isolated in the pre- and 20 isolated in the post-epidemic period (Table 1). The temperature used was the one defined by the United States Centers for Disease Control and Prevention, 165 °F/~75 °C [29]. For this, the strains were grown in LB medium pH ~7.0 under shaking at 37° until the early stationary phase (16–18 h). The OD was adjusted to an $OD_{600} = 0.2$ and 1 mL, which was previously shown to correspond to approximately 1×10^8 CFU and centrifuged at $8000 \times g$ for 3 min. An aliquot was plated to verify the number of viable cells. The pellet was inoculated in 10 mL of a new LB medium previously heated to 75 °C and incubated for 10 min and 1 h. Serial dilutions were made in 0.9% normal saline for each strain, followed by plating in LB agar incubated at 37° for 18 h for colony-forming units counting. Each strain was tested in triplicates.

Invasion to human epithelial cells

The ability to invade human colon adenocarcinoma cells (Caco-2) was compared between the 20 *S. Enteritidis* strains isolated in the pre- and 20 isolated in the post-epidemic period (Table 1), according to the protocol described by Shah et al. [24]. Briefly, Caco-2 cells were grown in DMEM (Dulbecco's Modified Eagle Medium—Thermo Scientific) supplemented with 10% of fetal bovine serum (Thermo Scientific) and incubated in 5% CO_2 at 37 °C. A total of 1×10^6 Caco-2 cells were seeded on a 12-well plate for 12 days to obtain well-differentiated cells. The medium was changed every other day.

The bacterial strains were grown overnight in LB medium under shaking at 37 °C according to that described in “Survival to the acid stress.” After OD adjustment, an aliquot was plated to check the viable cells and 1 mL was centrifuged at $8000 \times g$ for 3 min and resuspended in 1 mL of DMEM cell culture medium without antibiotics and fetal bovine serum. The strains were added to the monolayers of Caco-2 cells with multiplicity of infection (m.o.i.) ratio of 10:1 (bacterial/epithelial cell). The plates were centrifuged at $450 \times g$ for 10 min in order to bring the bacteria in contact to the cells and then incubated in 5% CO_2 at 37 °C for 2 h. After incubation, the cells were washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria and were incubated another 2 h with DMEM containing 100 µg/mL of gentamicin. The cells were washed three times with PBS followed by cell lysis with 1% triton X-100 for 10 min. Serial dilutions were made in 0.9% normal saline for each well followed by plating in LB agar incubated at 37° for 18–24 h for colony-

forming units counting. Each strain was tested in triplicates, and wells with cells but without bacteria were used as negative controls.

Invasion and survival to human (U937) and chicken (HD11) macrophages

The ability to survive to U937 (humans) and HD11 (chickens) macrophage phagocytosis was compared between the 20 *S. Enteritidis* strains isolated in the pre- and 20 isolated in the post-epidemic period (Table 1), according to the protocol described by Shah et al. [30].

For this, U937 and HD11 cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (Thermo Scientific) and incubated in 5% CO₂ at 37 °C. A total of 1×10^6 cells were seeded on a 24-well plate for 48 h. For U937 cells, 10 nM of phorbol myristate acetate (PMA) was added to the media in order to activate the cells and promote adherence to the plates.

The bacterial strains were prepared as described in “Invasion to human epithelial cells”, and 1 mL was centrifuged and resuspended in 1 mL of RPMI cell culture medium without antibiotics and fetal bovine serum. Each strain was added to one well in two different plates, with m.o.i. ratio of 50:1 (bacterial/macrophage). The plates were centrifuged at 450×g for 10 min in order to bring the bacteria in contact to the cells and then incubated in 5% CO₂ at 37 °C for 30 min. After, the cells were washed three times with PBS to remove the extracellular bacteria and incubated with RPMI containing 100 µg/mL of gentamicin. After 30 min, one of the plates was washed three times with PBS followed by cell lysis with 1% triton X-100 for 10 min. The other plate was kept incubated for a total of 8 h in order to observe intra-macrophage survival. Serial dilutions were made in 0.9% normal saline for each well followed by plating in LB agar incubated at 37° for 18 h for colony-forming units counting. Each strain was tested in triplicates, and wells with cells but without bacteria were used as negative controls.

Statistical analysis

The statistical analysis was done using the Mann-Whitney *U* test; *P* values ≤ 0.05 were considered significant.

Results

Survival to the acid stress

A total of 19 out of 20 pre-epidemic strains survived to the acid stress at 37 °C/10 min with a mean survival of 3.4×10^5 CFU/mL. On the same way, 19 out of 20 post-epidemic strains survived to the same condition and time of exposition with a mean survival of 2.1×10^6 CFU/mL (Fig. 1). The difference of the

survival means between pre- and post-epidemic strains in 10 min was statistically significant (*P* ≤ 0.05).

After 1 h of exposition, none of the 20 pre-epidemic strains survived to the acid stress at 37 °C while 4 out of 20 post-epidemic strains survived to the same conditions with a mean survival of 6.5×10^1 CFU/mL (Fig. 1). This difference of the survival means between pre- and post-epidemic strains in 1 h was not statistically significant (*P* > 0.05).

At 42 °C, 15 out of 20 pre-epidemic strains survived to 10 min in acid stress condition with a mean survival of 4.5×10^4 CFU/mL, and 15 out of 20 post-epidemic strains survived to the same time and conditions with a mean survival of 1.4×10^5 CFU/mL (Fig. 1). This difference of the survival means between pre- and post-epidemic strains was not statistically significant (*P* > 0.05). In 1 h of acid stress at 42 °C, none of the strains survived (Fig. 1).

Survival to the oxidative stress

All the 20 pre- and 20 post-epidemic strains survived to the oxidative stress at 37 °C/10 min with a mean survival of 2.4×10^7 CFU/mL for the pre-epidemic strains and 2.5×10^7 CFU/mL for the post-epidemic strains (Fig. 2). This difference of the survival means between pre- and post-epidemic strains in 10 min was not statistically significant (*P* > 0.05).

After 1 h of exposition, 18 out of the 20 pre-epidemic strains survived to the acid stress at 37 °C with a mean survival of 7.5×10^6 CFU/mL while 16 out of 20 post-epidemic strains survived to the same conditions with a mean survival of 7.0×10^6 CFU/mL (Fig. 2). This difference of the survival means between pre- and post-epidemic strains in 1 h was not statistically significant (*P* > 0.05).

At 42 °C, all the 20 pre-epidemic and 20 post-epidemic strains survived to the oxidative stress of 10 min with a mean survival of 2.1×10^7 CFU/mL for the pre-epidemic strains and 2.0×10^7 CFU/mL for the post-epidemic strains (Fig. 2). This difference in the survival means between pre- and post-epidemic strains was not statistically significant (*P* > 0.05). After 1 h of oxidative stress at 42 °C, 17 out of 20 pre-epidemic strains survived with a mean survival of 3.8×10^6 CFU/mL and 15 out of the 20 post-epidemic strains survived with a mean survival of 4.0×10^6 CFU/mL (Fig. 2). This difference of the survival means between pre- and post-epidemic strains was not statistically significant (*P* > 0.05).

Growth in egg albumen

All the 20 pre- and 20 post-epidemic strains survived and multiplied in egg albumen. Beginning with 500 CFU, the pre-epidemic strains multiplied to a mean of 7.7×10^4 CFU/mL and the post-epidemic strains to a mean of 6.2×10^4 CFU/mL. This difference of the multiplication rates between pre- and post-epidemic strains was not statistically significant (*P* > 0.05).

Fig. 1 Survival of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil to acid stress. **a** 10 min and 1 h of exposition at 37 °C. **b** 10 min and 1 h of exposition at 42 °C. * $P \leq 0.05$

Survival to high-temperature stress

All the 20 pre-epidemic strains survived to the temperature of 75 °C/10 min with a mean survival of 1.0×10^3 CFU/mL, while 19 out of the 20 post-epidemic strains survived to the same condition and time with a mean survival of 1.2×10^3 CFU/mL (Fig. 4). After 1 h of high temperature, 14 out of the 20 pre-epidemic strains and 15 out of the post-epidemic strains survived to the stress with a mean survival of 3.4×10^2 CFU/mL and 4.5×10^2 CFU/mL, respectively (Fig. 4). In both cases, the difference of the survival rates between pre- and post-epidemic strains was not statistically significant ($P > 0.05$).

Invasion to human epithelial cells

All the 20 pre-epidemic strains invaded Caco-2 cells with a mean invasion of 3.5×10^3 CFU/cm², while 18 out of the 20 post-epidemic strains invaded Caco-2 cells with a mean invasion of 5.6×10^3 CFU/cm² (Fig. 5). This difference in the invasion rates between pre- and post-epidemic strains was not statistically significant ($P > 0.05$).

Invasion and survival to human (U937) and chicken (HD11) macrophages

All the 20 pre- and 20 post-epidemic strains invaded U937 macrophages in 30 min with a mean invasion of 2.8×10^3 CFU/cm² for pre- and 1.3×10^3 CFU/cm² for post-epidemic strains (Fig. 6). After 8 h of incubation, 7.0×10^1 CFU/cm² of pre-epidemic strains survived inside the macrophage, while 7.1×10^1 CFU/cm² for the post-epidemic strains (Fig. 6). This difference in the invasion rates between pre- and post-epidemic strains was not statistically significant ($P > 0.05$).

On the same way, all the 20 pre- and 20 post-epidemic strains invaded HD-11 macrophages in 30 min with a mean invasion of 2.5×10^3 CFU/cm² for pre- and 3.4×10^3 CFU/cm² for post-epidemic strains (Fig. 7). After 8 h of incubation, 1.1×10^4 CFU/cm² of pre- and post-epidemic strains survived inside the macrophage (Fig. 7).

Discussion

In a previous study, we identified by whole genome sequencing analyses that the genomic profile of *S. Enteritidis* strains in

Fig. 2 Survival of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil to oxidative stress after. **a** 10 min and 1 h of exposition at 37 °C. **b** 10 min and 1 h of exposition at 42 °C

Brazil that shifted after 1994 was replaced by a global epidemic group of strains [3]. Based on those previous results, this study comparatively analyzed phenotypic traits of a set of *S. Enteritidis* strains isolated in the pre- and post-epidemic period in Brazil.

When ingested even by chickens or humans, *S. Enteritidis* suffer the acid stress in the stomach of the hosts. Our results showed that post-epidemic strains survived better after 10 min of exposition to the acid stress at 37 °C ($P \leq 0.05$) than pre-epidemic strains (Fig. 1), which is a crucial factor to reach the human intestine. In a similar study conducted with *S. Enteritidis* strains isolated from North America, the authors divided the *S. Enteritidis* in high- and low-pathogenic strains according to some phenotypic characteristics and found a higher survival of the high-pathogenic strains in the acid stress either after 10 min and 1 h at 37 °C [8]. In our study, after 1 h of acid stress at 37 °C, none of the pre-epidemic strains and only 4 post-epidemic strains were recovered (Fig. 1). At 42 °C, similar to the groups from North America [8], we were able to recover both groups of strains in 10 min of acid stress.

However, after 1 h, none of the strains survived, showing a high sensitivity of both groups of strains to acid stress in chicken body temperature (Fig. 1).

Resistance to hydrogen peroxide produced by avian and human macrophages is essential for *S. Enteritidis* survival and dissemination inside the host. In the present study, pre- and post-epidemic strains were observed to survive in a similar manner independent of the temperature to be 37 °C or 42 °C either in 10 min or in 1 h of oxidative stress (Fig. 2). The same was observed by Shah et al. comparing high- and low-pathogenic *S. Enteritidis* strains isolated in North America, which showed similar behaviors under the oxidative stress [8].

In the study of Humphrey et al., the authors found reduced susceptibility to acid and oxidative stress in *S. Enteritidis* strains from phage type 4 (PT4) isolated from chickens in comparison to the PT4 ones isolated from humans, showing a variation of traits in strains belonging to the same phage type [31]. On the other hand, we found different behaviors when the strains were analyzed individually independently of the source of isolation (Table 1 and Figs. 1 and 2).

Fig. 3 Growth of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil in egg albumen for 24 h at 25 °C

The ability to contaminate internal compounds of the egg is essential for *S. Enteritidis* strains to be transmitted to humans [12, 13, 32]. In the present study, all the strains analyzed were able to survive and multiply in the egg albumen independently even if the strain was isolated in the pre- or in the post-epidemic period and the source of isolation (Fig. 3). On the other hand, in the study of Shah et al., the authors found high-pathogenic strains surviving and multiplying in the egg albumen, while 2 out of 3 low-pathogenic strains did not survive in the egg albumen [8]. In the study of Yim et al., the authors analyzed pre- and post-epidemic *S. Enteritidis* strains from Uruguay and found reduced ability of pre-epidemic strains to grow in the egg albumen. Moreover, the authors noticed this diminished ability in non-human strains in comparison to the human gastroenteritis strains [22].

The Centers for Disease Control and Prevention (CDC) recommends cooking egg dishes and poultry at a temperature of at least 70–75 °C [29]. In the present study, pre- or post-epidemic strains survived to 10 min and 1 h of high-temperature stress, with some strains from both groups surviving only for 10 min (Fig. 4). This fact shows that the heat tolerance among the *S. Enteritidis* strains in this study was strain dependent, independently of the period of isolation, reinforcing the resistance of this pathogen to high temperatures for long periods. In the study by Humphrey et al., the authors found a better heat tolerance at 52 °C in two *S. Enteritidis* strains PT4 from humans than the two isolated from chickens. Moreover, the strains with more heat and acid tolerance showed to be more virulent in mice and more invasive in laying hens, particularly in reproductive tissues, which

Fig. 4 Survival of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil to high-temperature stress (75 °C) after 10 min and 1 h of exposition

Fig. 5 Invasion rates of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil in human epithelial cells (Caco-2)

can help spread this pathogen to humans through eggs and chicken meat [31, 33].

The ability to invade human epithelial cells was analyzed in differentiated Caco-2 cells. Either pre- or post-epidemic strains invaded Caco-2 cells similarly. In both groups, invasive or less invasive strains were verified (Fig. 5). The same variation in the invasion profiles of *S. Enteritidis* strains was verified in other studies [22–24].

Both groups of *S. Enteritidis* strains survived inside human (U937) and chicken (HD11) macrophages similarly (Figs. 6 and 7). However, while in human macrophages, the number of colony-forming units decreased after 8 h of incubation (Fig. 6), in chicken macrophages,

this number increased, showing that besides surviving inside these macrophages, the strains were able to multiply inside them (Fig. 7). The decreasing number of *S. Enteritidis* strains from both groups in human macrophages after 8 h of incubation was expected once this serovar does not typically cause disseminated infection in humans but gastroenteritis [34, 35].

The better survival of *S. Enteritidis* strains in chicken macrophages reinforces the capacity of these strains to infect chickens [36]. The prophage region SE20 has been reported as a recent acquisition of some *S. Enteritidis* strains that provide a better ability to colonize chickens [2]. In a previous study, we identified the prophage region SE20 only in strains

Fig. 6 Invasion and survival rates of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil in human macrophages (U937)

Fig. 7 Invasion and survival rates of 40 *Salmonella* Enteritidis strains isolated in the pre- and post-epidemic period in Brazil in chicken macrophages (HD11)

from the post-epidemic cluster [3]. However, our results showed similar survival capacity in chicken macrophages either in pre-epidemic strains, which did not contain the SE20 region, or in post-epidemic strains, which contain that region (Fig. 7). In a study by He et al., the authors found a lower reduction of *S. Enteritidis* strains inside HD11 macrophages after 24 h of incubation, in comparison to other chicken-related *Salmonella* serovars [37]. In the study of Shah et al., the authors verified a less invasive profile in HD11 macrophages among low-pathogenic strains in comparison to the high-pathogenic ones [24].

In conclusion, *S. Enteritidis* post-epidemic strains showed a better ability to survive to the acid stress of the stomach, which might be an advantage in order to reach the intestine and colonize chickens and humans. Our study showed that post-epidemic strains did not differ significantly from pre-epidemic strains in the majority of the phenotypic tests analyzed in this study. Further studies analyzing other genotypic and phenotypic traits are required to elucidate why this new subtype has been prevailing in the gastroenteritis cases caused by *S. Enteritidis* in Brazil.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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