RESEARCH ARTICLE

Salmonella enterica persister cells exhibit distinct susceptibility profiles following exposure to human serum and macrophages

Rodrigo Lira Rodrigues[1], Júlia Abreu da Rosa[1], Douglas Diefenbach da Cunha[1], Thaís Lima Nunes[1], Pedro Maria Abreu Ferreira[2], Bruno Kendi Makiyama[3], Florencia María Barbé-Tuana[3], Sílvia Dias de Oliveira[1], Carlos Alexandre Sanchez Ferreira[1]*

1 Laboratório de Imunologia e Microbiologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Rio Grande do Sul, Brazil, **2** Laboratório de Ecologia de Interações, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Rio Grande do Sul, Brazil, **3** Laboratório de Imunobiologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Rio Grande do Sul, Brazil

* cferreira@pucrs.br

OPEN ACCESS

Citation: Rodrigues RL, Rosa JAd, Cunha DDd, Nunes TL, Ferreira PMA, Makiyama BK, et al. (2026) *Salmonella enterica* persister cells exhibit distinct susceptibility profiles following exposure to human serum and macrophages. PLoS One 21(3): e0343532. https://doi.org/10.1371/journal.pone.0343532

Editor: Mohammad Faezi Ghasemi, Islamic Azad University, IRAN, ISLAMIC REPUBLIC OF

Received: October 20, 2025

Accepted: February 6, 2026

Published: March 6, 2026

Data availability statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was carried out with the support of the Coordination for the Improvement of Higher Education Personnel

Abstract

Salmonella enterica, particularly non-typhoidal serovars (NTS), is a leading cause of foodborne illness, with invasive infections posing high mortality risks in developing countries. Fluoroquinolones and third-generation cephalosporins, such as ceftazidime (CAZ), are used to treat severe infections, yet they are facing concerning rates of antimicrobial resistance. Furthermore, recalcitrant and/or persistent infections are often linked to persister cells, a phenotype that enables cells to survive in the presence of high concentrations of antibiotics. Although persisters are associated with chronic infections, their interactions with the human immune system, particularly serum resistance and opsonophagocytosis, are not well understood. Here, three NTS isolates from the food protein chain (S45, S48, and 4SA(2)) were used. Persister cells were selected by exposure to CAZ concentration 100 times higher than the minimum inhibitory concentration and then assessed for serum resistance, opsonophagocytosis, and intracellular survival in primary human macrophages. The isolates exhibited heterogeneous persister fractions (1.06%–39.55% survival after 72h of CAZ exposure). Persisters exhibited equal or greater serum resistance than regular cells. Isolate 4SA(2) proliferated in 100% human serum, with persister-derived cells showing higher growth rates. Following opsonization, serum-resistant persisters of all isolates were phagocytosed at significantly higher rates than serum-resistant regular cells. Intracellular survival varied: S45 persisters proliferated post-internalization; S48 persisters and regulars were eradicated; 4SA(2) showed no phenotype difference. Complement enhanced the intracellular survival of S45 but not S48 or 4SA(2). Despite having different intracellular outcomes, *Salmonella* persisters showed higher

– Brazil (CAPES) – Financing Code 001, CNPq, and FAPERGS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

levels of opsonophagocytosis and serum resistance. These findings suggest that cell surface modifications may facilitate host cell uptake and contribute to antimicrobial treatment failure and long-term infection. The phenotypic diversity among isolates underscores the importance of considering persister heterogeneity and host-pathogen immune interactions in order to understand recalcitrant infection dynamics and design more effective therapeutic strategies.

Introduction

Salmonella spp. is a Gram-negative bacillus taxonomically classified into two species: *Salmonella bongori* and *Salmonella enterica*. The latter has six subspecies and more than 2,500 serovars [1]. In the human gastrointestinal tract, *Salmonella enterica* subsp. *enterica* is the predominant subspecies found, and its serovars are classified as either typhoidal or non-typhoidal [2]. Typhoidal serovars, such as *S.* Typhi and *S.* Paratyphi, are adapted to humans and can cause severe systemic infections [3]. Non-typhoidal serovars (NTS) can cause self-limiting enterocolitis in humans [4] and, less frequently, they can also be found in systemic infections known as invasive NTS (iNTS), which has a high mortality rate in developing countries [5]. The most common NTS and iNTS serovars are *S.* Typhimurium and *S.* Enteritidis [6,7], although *S.* Agona has shown increasing rates, especially from contaminated food [8]. In 2021, 510,000 cases of iNTS were reported worldwide, resulting in 62,000 deaths [9].

Third-generation cephalosporin antibiotics, such as ceftazidime (CAZ), are preferred for treating severe gastrointestinal and systemic infections in children and during pregnancy. This strategy avoids the DNA effects caused by fluoroquinolones, which are also used to treat systemic infections [10,11]. Therefore, the worldwide prevalence of Enterobacterales resistant to third-generation cephalosporins raises great concern in the context of public health, with these bacteria being categorized as a critical priority in the WHO's 2024 Bacterial Priority Pathogens List. Additionally, fluoroquinolone-resistant NTS is categorized as a high priority [12]. In addition to the development of antimicrobial resistance, the presence of persister cells has been implicated in recalcitrant and/or persistent infections in the host [13,14]. Persisters are cells with a non-heritable phenotype that can survive high concentrations of antimicrobials. They usually present slow or no growth and are typically found in small subpopulations within an isogenic cellular culture [15,16]. Therefore, the failure to clear infections during antimicrobial treatments is possibly due, at least in part, to the presence of such cells. Once the stressor, the antimicrobial, is removed, the persister cell can regrow and repopulate the infection site [17]. Persisters have been described as a point of concern in the emergency crisis of antibiotic resistance, as they have been shown to acquire and spread resistance plasmids via horizontal gene transfer [18].

Salmonella may be considered a professional intracellular pathogen, as it can infect cells such as enterocytes, macrophages, dendritic cells, neutrophils and B lymphocytes [19–23]. iNTS cells can infect macrophages and spread through the bloodstream to the liver and spleen. The intracellular lifestyle involves modulating host

cell physiology and evading phagolysosomes [24]. Once inside the host cell, the vacuole environment can stimulate the bacteria to adopt a persister phenotype. These cells can then subvert phagocytic cells, creating a favorable niche in which they can survive and potentially replicate [25,26]. Outside of host cells, these bacteria must cope with mammalian immune serum components, such as the complement system and antimicrobial peptides (AMPs). The complement system is a proteolytic cascade that is recognized for its role in surveillance against pathogenic invasion. Its canonical mechanisms in mammalian host defense involve extracellular actions, primarily the direct lysis of microorganisms, opsonization, and the induction of inflammation with concurrent phagocyte attraction and activation [27]. On the other hand, non-canonical complement functions include intracellular activation and modulation of processes such as autophagy and cellular metabolism [28]. AMPs are usually composed of 12–50 amino acids and can interact with the bacterial membrane via electrostatic attraction, many times causing injuries at the site of deposit [29]. Mechanisms of serum immune evasion include the presence of lipopolysaccharide (LPS) variants [30], capsule [31], Outer Membrane Vesicles (OMVs) [32], and the ability to recruit host's complement regulators and produce enzymes that cleave its components [33]. In order to move to an intracellular environment, *Salmonella* cells change their evasion mechanisms. The Type 3 Secretion Systems (T3SS) encoded in *Salmonella* Pathogenicity Islands – type I (SPI-I) enables the penetration of the host cells, reaching the cytoplasm, and releasing effector proteins that remodel the local cytoskeleton [34,35]. SipA (SPI-I) is responsible for *Salmonella* invasion by actin-binding and influences phagosome maturation [36,37]. SifA (*Salmonella* Pathogenicity Islands – type II; SPI-II) helps in the production of microtubules that extend from the *Salmonella*-Containing Vacuole (SCV) to enable nutrient collection [38,39]. SptP (SPI-I) and SpvC (SPI-II) may suppress the innate immune response and proinflammatory cytokine secretion by downregulating MAPK and NF-κB, respectively [40–42]. Therefore, iNTS cells must adapt to intracellular and extracellular challenges [43], possibly with serum opsonization as an interconnection point.

Although persister cells have been associated with chronic *Salmonella* infections, how these cells deal with the innate humoral human immune system has been poorly characterized. Additionally, the influence of this phenotype on opsonophagocytosis, intracellular survival, and replication is a worthwhile question. In fact, it has been shown that regular NTS cells are phagocytosed faster than the membrane attack complex (MAC) of the complement system can effectively kill them, suggesting an immune system exploit strategy [44]. Understanding how serum-resistance and antibiotic-persistence mechanisms trade off with opsonic uptake and intracellular fate remains to be achieved, ultimately considering whether bacteria are cleared, persist nonreplicating, or later resuscitate. Taken together, these gaps highlight the need to elucidate how persister cells influence host–pathogen interactions. Here, we investigated three NTS isolates collected from the food protein production chain that presented different levels of persister fractions regarding human serum activity, macrophage uptake and intracellular survival.

Results

Isolates present a significant degree of heterogeneity when comparing persister fraction levels

The three ceftazidime-susceptible isolates (S45, S48, and 4SA(2); see **Table 1**) were treated with 100-fold the concentration of their respective ceftazidime MIC for up to 72 h, resulting in different survival curves (**Fig 1**). S45 and S48 presented

Table 1. *Salmonella enterica* subsp. *enterica* isolates.

Salmonella enterica* subsp. *enterica			**MIC (μg/mL)**
SOROVAR	**CODE**	**ORIGIN**	**CAZ**
Salmonella Enteritidis	**S45**	Meat meal	0.25
Salmonella Enteritidis	**4SA(2)**	Swine feces	0.25
Salmonella Agona	**S48**	Feathers meal	0.06

Isolates with their respective codes, origin and MIC values (μg/mL). MIC breakpoints for CAZ: ≤ 1 μg/mL, susceptible; > 4 μg/mL, resistant. MIC cut-off values were interpreted according to the European Committee on Antimicrobial Susceptibility Testing [74].

https://doi.org/10.1371/journal.pone.0343532.t001

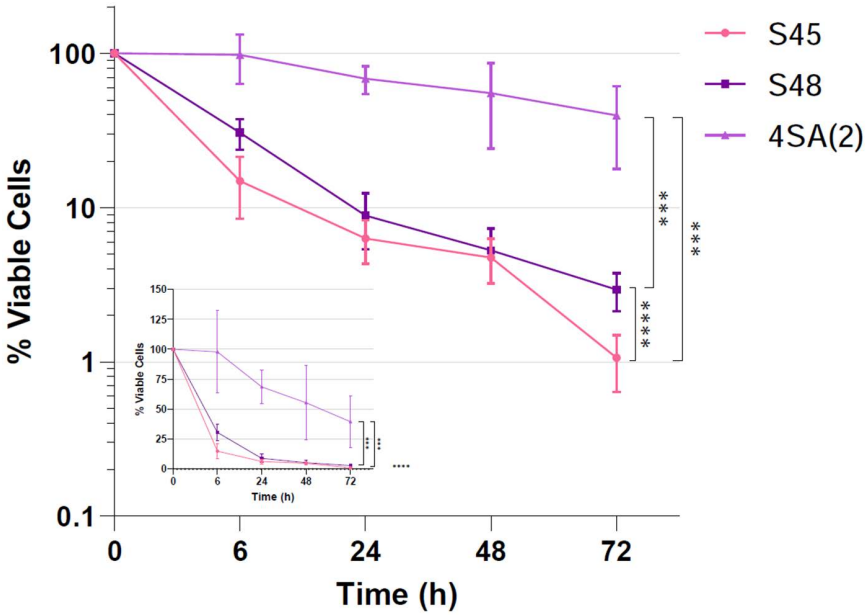

Fig 1. Ceftazidime 100-fold MIC survival curves. Mid-log bacterial cultures were exposed to ceftazidime at 100-fold MIC for each isolate at room temperature for 72 **h.** Pink circles represent isolate S45, purple squares indicate isolate S48 and lilac triangles show isolate 4SA(2). Major and small graphic show data in logarithmic and linear scale, respectively. ***, $p < 0.005$; ****, $p < 0.0001$. Data is represented by means and standard error of percentages from three biological and technical triplicates.

https://doi.org/10.1371/journal.pone.0343532.g001

survival fractions of 6.31% and 8.89% after 24 h of exposure to the drug, with a subsequent drop to 1.06% and 2.94% after 72 h, respectively. 4SA(2) presented higher levels of surviving cells in all time points evaluated (**Supplementary** S1 Table), presenting 68.57% and 39.55% surviving cell fractions after 24 and 72 h, respectively. All isolates presented different levels of surviving cells after 72 h of ceftazidime exposure, and these cells were considered persisters for the following experiments.

Isolates show distinct surviving patterns for both regular and persister cells when exposed to human sera

Persister and regular cells of the three isolates were incubated with human sera, revealing heterogeneous serum resistance profiles (Fig 2). Incubation of all cell groups with buffer or iS did not result in a reduction in cell numbers. Regular and persister cells of S45 and S48 presented a reduction in cell numbers following serum exposure. In contrast, 4SA(2) showed an increase in cell numbers at the evaluated time points.

Persisters presented similar or higher levels of cell numbers compared to regular cells when exposed to 100% (S) or 50% (S50) sera for the three isolates (Fig 2A-2F). S45 persister cells showed higher survival rates when exposed for 1 h to S or S50, while they were not significantly different in the remaining time points. S48 persister cells showed higher counts than regular cells after exposure to S50 for 1 and 2 h. Although the differences were not statistically significant after exposure to S50 for 3h and to S for all time points, a clear non-significant trend towards higher serum resistance in persisters can be seen (**see Supplementary** S2 Table). Different from S45 and S48, 4SA(2) showed cell multiplication of both regular and persister cells. After 2 and 3 h of S and 3 h of S50 exposure, persisters exhibited higher growth rates than regulars. No significant differences were observed in the remaining experimental groups. Thermically inactivated serum (iS) exposure resulted in contrasting results comparing the isolates. S45 presented higher levels of regular cells than persisters after 1 h and 3 h, and after 2 h there is also a non-significant trend ($p = 0.076$; **see Supplementary** S2 Table) for higher regular

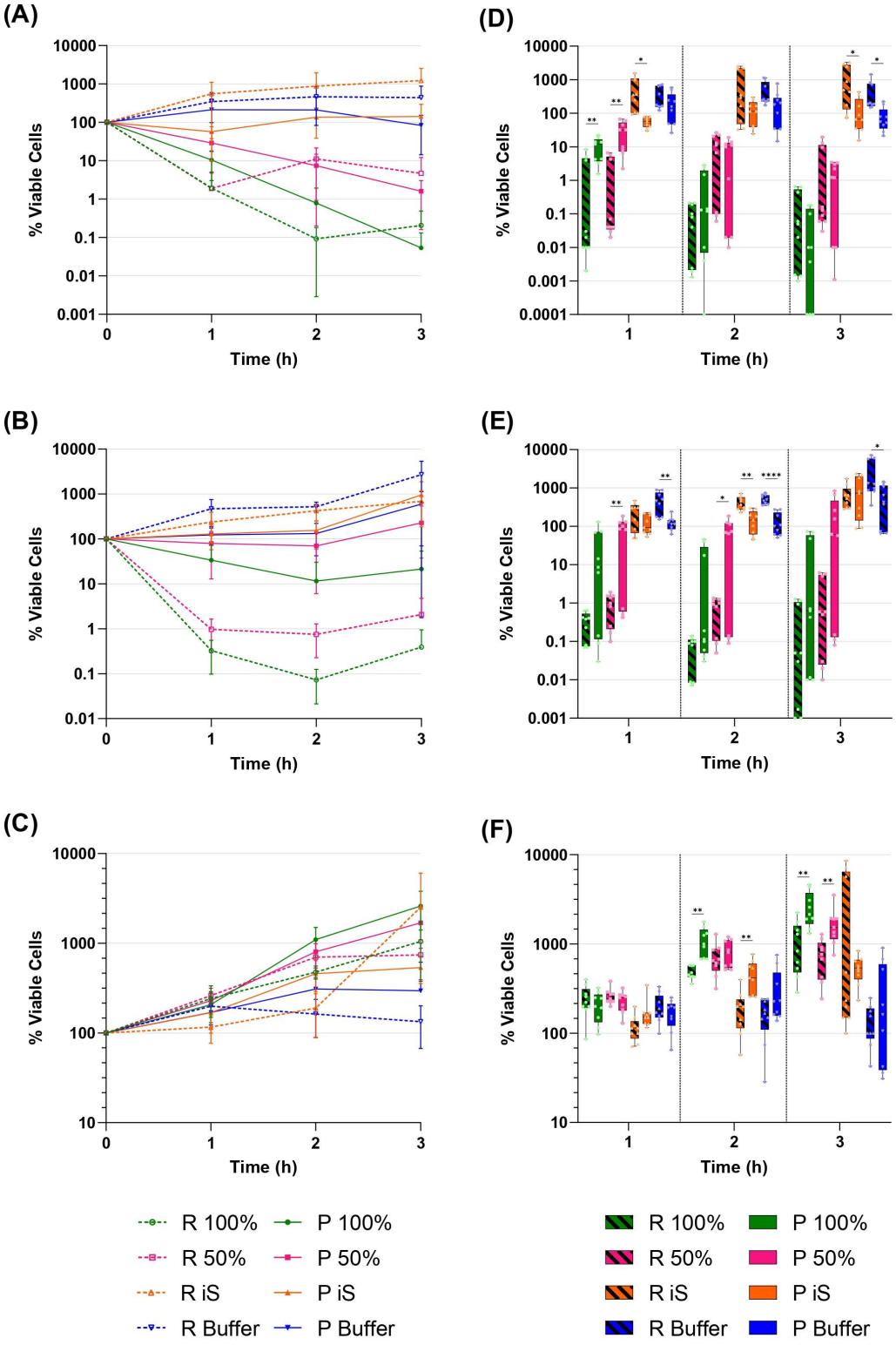

Fig 2. Isolates serological susceptibility profiles. ~10^6 CFU (10 μL) of each cell group were added to human serum (100% Serum, **S)**, human serum 1:1 PMHC (50% Serum, S50), thermically inactivated serum **(iS)**, or PMHC buffer (Buffer, **B)**, and incubated at 37ºC for 3 **h.** Images **A, B,** and

C represent the serum susceptibility curves, and images **D**, **E** and **F** represent the data dispersion of S45, S48 and 4SA(2), respectively. Dashed lines and striped bars represent the regular cells, while solid lines and smooth bars represent the persister cells. Dots show data dispersion (**D**, **E** and **F)**. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. Data shown as means and standard errors of percentages of three biological and technical triplicates.

https://doi.org/10.1371/journal.pone.0343532.g002

derived multiplication (Fig 2A, 2D). S48 presented higher levels of regulars than persisters after 2 h of exposure, but no differences were detected in the other time points (Fig 2B, 2E). 4SA(2), on the other hand, presented higher levels of persister derived cells than regulars after 2 h of exposure, but no differences were detected in the other time points (Fig 2C, 2F). Exposure of cells to physiological buffer (B) led to two different profiles comparing regulars and persister cells: S45 and S48 regular cells presented higher or a non-significant trend to higher multiplication rates than persisters, whereas 4SA(2) showed no differences between them (Fig 2A-2F; **see Supplementary** S2 Table).

Persister and regular cells present variable profiles of macrophage internalization and intracellular survival

Regular and persister cells that can survive in human serum may be opsonized by serum components and/or alter their phenotype in response to the new conditions, possibly influencing phagocyte recognition and intracellular bacterial viability. Therefore, in order to evaluate the influence of opsonization in serum-resistant regular and persister cells, as well as their capacity of survival/proliferation within phagocytes, S, iS and B treated cells were used to infected primary cultures of human macrophages (Fig 3). The three isolates exhibited different profiles when comparing regular and persister cells, but serum-resistant persisters showed higher levels of phagocytosis than regular cells in all isolates.

Following sera exposure, the S45 isolate (Fig 3A) presented higher levels of phagocyted persister cells than the regular group, which was not observed following iS and B treatments. The number of viable bacteria within macrophages did not differ between regular and persister cells after 30 min of incubation. However, after 24 h of incubation, persister cells showed higher numbers for all treatments. Interestingly, S45 regular and persister cells were phagocyted at higher levels after iS and B than S treatment, although resulted in lower numbers of surviving cells, except for iS-treated persister cells after 24 h.

The S48 isolate (Fig 3B) showed higher numbers of internalization ($p < 0.0001$; **see Supplementary** S3 Table) of S-treated persisters than regular cells, whereas B-treated persisters were less phagocyted than regulars ($p < 0.0001$; **see Supplementary** S3 Table). On the other hand, iS-treated regular cells seemed to be not internalized. No serum-resistant bacterial cells of this isolate could be rescued from macrophages. iS-treated regular cells were present at higher levels than persisters after 24 h, although no differences could be seen after 30 min and in the internalization levels. B-treated regular cells showed higher levels of internalization as well as of rescued cells after 24 h of macrophage incubation than persisters. In contrast to regular cells, which showed a 2-log difference when comparing each treatment (S > iS > B), persister cells of all treatments were internalized in similar proportions.

The 4SA(2) isolate (Fig 3C) exhibited higher levels of phagocytosis for S-treated persisters compared to regular cells, whereas the iS treatment produced the opposite pattern. No significant differences were obtained with the B treatment. Persisters exhibited higher levels of internalized viable cells than regular cells after the iS and B treatments after 24 h of incubation, though no differences were observed after the S treatment. All treatments of both regular and persister cells resulted in an increase in viable cell numbers after 24 h compared to 30 min of macrophage incubation ($p < 0.005$; **see Supplementary** S3 Table).

Discussion

Foodborne illnesses affect an estimated 600 million people annually, and NTS/iNTS are among the main associated infections. *Salmonella* spp. have the longest persistence among infectious bacteria in food samples [45,46]. Around 100 *Salmonella* serovars may cause disease in humans, though *S.* Typhimurium and *S.* Enteritidis are the primary causative agents of NTS and iNTS. However, the relative frequency of serovar infections has been changing, with *S.* Agona climbing

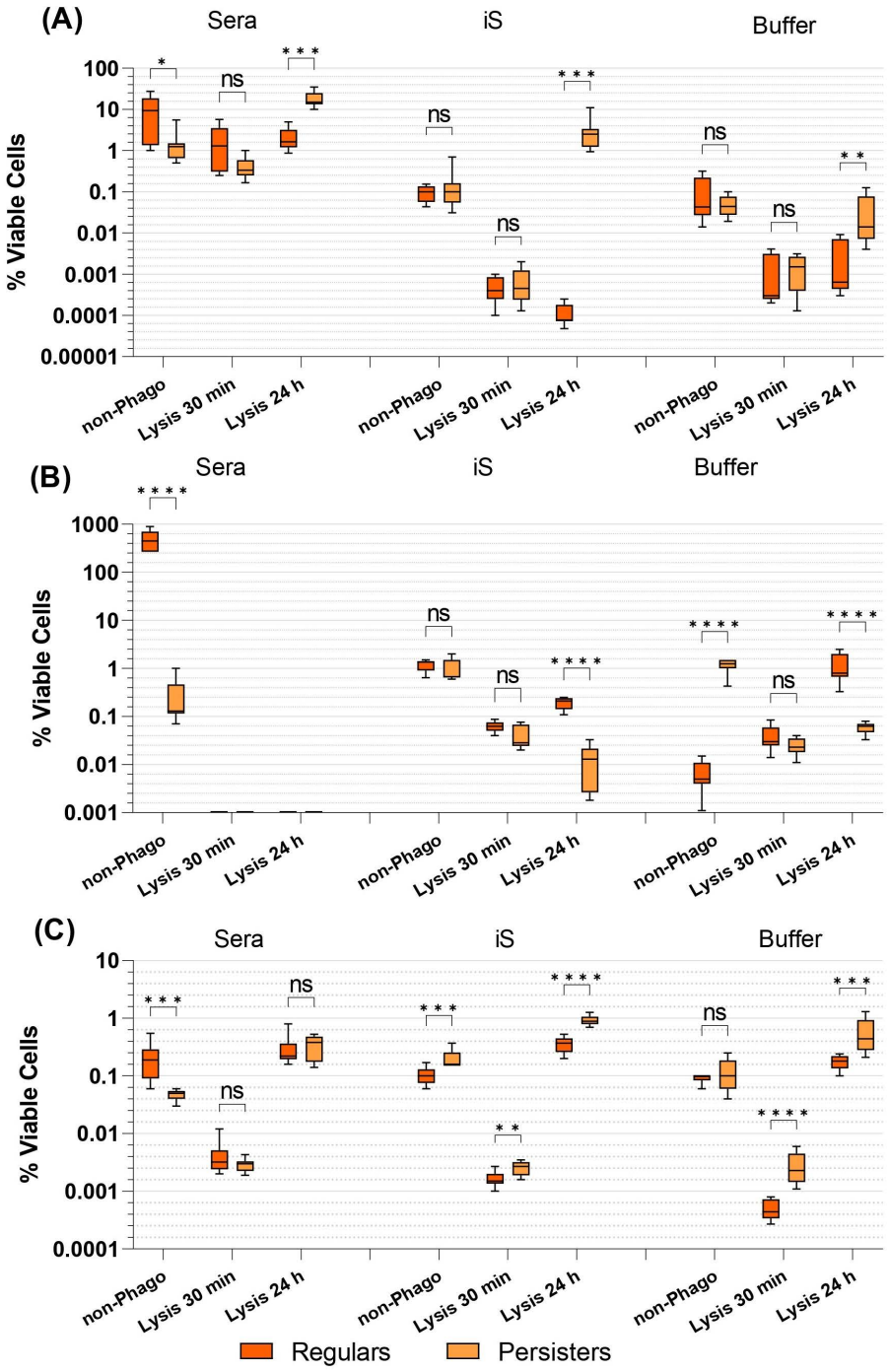

Fig 3. Percentages of *Salmonella* viable cells after incubation with human macrophages. *Salmonella* surviving cells after 1 h of incubation with S (100% Sera), iS (thermically Inactivated Sera), or B (Buffer) treatments were used to infect human macrophages, and non-phagocytosed and phagocytosed viable bacterial cells were quantified. Images **(A)**, **(B)** and **(C)** represents the isolates S45, S48 and 4SA(2), respectively. Dark orange and light orange boxplots indicate regular and persister cells, respectively. The middle line indicates data medians. Non-Phago: non-phagocytosed cells; Lysis 30 min and Lysis 24 h: cells from macrophages lysed after 30 min and 24 h from the start of incubation, respectively; ns: no significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ****: $p < 0.0001$. Data shown as percentage of the initial inoculum used for infection (% Viable Cells), and as means and standard error of three biological replicates.

https://doi.org/10.1371/journal.pone.0343532.g003

up in the last decades. It has been suggested that it can persist for long periods in biofilms and in viable but non-culturable state, specially in dry food [8,47]. *S*. Typhimurium exhibits higher levels of macrophage uptake and intracellular survival than *S*. Enteritidis, therefore indicating that serovar differences may account for alternative courses of disease [6,7,48]. After invading enterocytes or being uptaken by M cells, *Salmonella* cells are engulfed by phagocytes. During the infection, they are released into the bloodstream and/or lymphatics and come into contact with the complement system [33]. Therefore, iNTS must be able to resist both phagocytosis and humoral immune components, facing an additional constraint when antimicrobials are used.

Cephalosporins are among the treatment options for iNTS and severe NTS, although considerable resistance rates have been reported [49]. Furthermore, persistent infections may arise from asymptomatic carriers, and recurrence of infections may originate from persister cells. In this context, studies have examined the impact of *Salmonella* persisters to understand their formation mechanisms [50], impact on bacterial biofilms [51], development of adaptation strategies [52], and survival in treated infections, including evasion of the host's immune system [27]. From a One Health perspective, this study examined these cells by comparing three isolates recovered from the animal food chain within the context of the human immune system. Furthermore, we evaluated isolates with different levels of persisters and serum resistance, including a high-persister isolate (4SA(2)).

Although the behavior and mechanisms of serum evasion by *Salmonella* cells have been extensively characterized [53–55], few studies have specifically addressed how persisters interact with human serum and phagocytes. *Pseudomonas aeruginosa* persisters have been described as more resistant to serum than regular cells [56]. Our data indicate that persisters from the three isolates present equal or higher serum resistance compared to regular cells, but with striking differences. The high-persister isolate 4SA(2) showed full serum resistance, and both persisters and regular cells were able to multiply in the human serum. Interestingly, persisters from the high-persister isolate 4SA(2) exhibited similar or higher multiplication rates than regular cells, indicating that serum components modulated differently the metabolism of these cells. Considering that the regular population is a mixture of persister and non-persister cells, and approximately 40% of the 4SA(2) regular population may consist of persisters, the multiplication difference between persisters and non-persister cells must be significantly greater for this isolate. Thermically inactivated serum has already been shown to induce the growth of persisters from *Escherichia coli*, *Staphylococcus aureus* and *P. aeruginosa* [56], corroborating that serum components may trigger resuscitation of persister cells. As expected, persisters exposed to physiological buffer alone, therefore not subjected to any major stress, showed equal or lower levels of proliferation than regular cells in the three isolates. Mechanisms underlying *Salmonella* serum resistance are associated with outer membrane configuration and/or composition [27,57], and many proposed modifications explaining the persister phenotype also frequently involve changes at the cell surface [58–61]. Indeed, the term "evaders" was coined from analyses of serum-resistant Gram-negative blood pathogens because serum-resistant cells presented similarities to persisters, such as biphasic killing curves and transient, non-heritable tolerance. Furthermore, evaders were shown to have active metabolism and to resume growth [62]. Nevertheless, it is important to highlight that the genotypic comparison of serum-resistant and -susceptible NTS strains showed no obvious correlation between genetic and phenotypic variations. On the other hand, susceptible strains presented upregulation of *wca* locus genes, implying higher production of a colonic acid-containing exopolysaccharide. Meanwhile, resistant strains increased *fep*E, which regulates LPS production [63]. This apparent emphasis on regulatory and/or epigenetic networks is another parallel that mirrors evader and persister lifestyles and may help explain therapeutic eradication failure and chronicity. The possible connection between evaders and persisters in *Salmonella* still needs to be clarified, but our data are consistent with the presence of varying mechanisms involving the three isolates.

In addition to the MAC and AMPs, opsonophagocytosis is another mechanism by which the innate immune system clears infections caused by microorganisms. Several *Salmonella* pathogen-associated molecular patterns (PAMPs) have been identified as being associated with Toll-like receptor (TLR) recognition by phagocytes and enterocytes [64–66]. Concerning persisters, *P. aeruginosa* and *S. aureus* showed lower macrophage phagocytosis levels compared to regular cells

[56,67]. Serum-resistant, opsonized *P. aeruginosa* persisters also showed lower engulfment [56], contrary to the *Salmonella* persisters described here. All three isolates evaluated presented higher levels of internalization of serum-resistant, opsonized persisters than of serum-resistant, opsonized regular cells by primary human macrophages. This difference possibly relies on the different strategies of intracellular survival and/or infection these bacteria deploy when facing phagocytes. The remaining evaluated parameters comparing the isolates facing macrophage exposure displayed different, sometimes contrasting, profiles. For instance, serum-resistant S45 persisters showed higher proliferation than regular cells after 24 h whereas serum-resistant S48 persisters and regular cells could not be rescued from the macrophages, and the high-persister isolate 4SA(2) did not present significant differences. Additionally, for S45 cells, complement components seem to be essential to increase survival within macrophages, and serum components are important for boosting intracellular proliferation. However, these characteristics are absent in S48 and the high-persister isolate 4SA(2) cells. Therefore, isolates from a single serovar may present highly discrepant behaviors when dealing with environmental stresses, including the human immune system. This is not surprising, especially when compared to the degree of variation found among *Salmonella* isolates of the same or different serovars concerning persisters, including phenotypes and transcriptome profiles [16,68].

Evaluations based on a single strain of a serovar should be taken with caution as they may not represent the outcomes of serovar infection by different isolates. Taking this into account, the fact that only serum opsonophagosized *S*. Agona isolate (S48) persister cells could not be rescued from the macrophages indicates that serotype differences may be present. As iS-treated persisters cells from S48 were rescued from macrophages, complement system-derived opsonization seems to be a major player in this outcome. Additionally, LPS composition may be another important factor, as it has already been shown to influence phagocyte intracellular fate in *Salmonella*, modulating type I IFN responses [69]. Contrasting to persisters and iS- and B-treated regular cells, serum-treated S48 regular cells were hardly phagocytosed, also pointing to complement or other thermically labile components triggering phagocytosis resistance. Therefore, variations in the surface of regular and persister serum-resistant cells probably modulate opsonization efficiency and/or macrophage receptors recognition. In fact, serum resistance in *S*. Typhimurium has been shown to do not result from C3b deposition avoidance, but suggested as dependent on C3b localization in the bacterial surface [70]. Protective antibodies, able to activate the classical complement pathway, were shown to be generated against trimeric, but not monomeric, *Salmonella* OMPs. This difference was suggested to come from a larger opening in the LPS layer, which allows for antibody access [71]. On the other hand, both persister and regular cells of the *S*. Enteritidis isolates (S45 and the high-persister isolate 4SA(2)) were phagocytosed and able to survive and proliferate intracellularly. So, for the *S*. Agona isolate (S48), phagocytosis seems to be avoided by serum resistant cells, whereas for the two *S*. Enteritidis isolates (S45 and the high-persister isolate 4SA(2)) higher phagocytosis levels may lead to higher numbers of infected cells and intracellular proliferation. The degree of mismatch between macrophage polarization and the bacterial evasion scaffold has been suggested as an explanation for these contrasting scenarios [72]. Taking together, these data suggest that different isolates/serovars employ different strategies, favouring either phagocytosis avoidance or the “Trojan Horse” strategy, the latter enabling enhanced intracellular persistence and proliferation. Furthermore, the phenotypic variation developed by *Salmonella* strains in host tissues influences decisively the delayed bacterial clearance by antibiotics [73] and starvation may be a major trigger of *Salmonella* antibiotic resilience [74]. Therefore, the presence of a common pattern of higher phagocytosis levels of opsonized, serum-resistant persisters compared to their regular counterparts was unexpected and deserves further attention.

Salmonella growth arrest inside phagocytic vacuoles and the formation of persisters in this environment has been described [26,75,76]. Intracellular persisters are not dormant, but active metabolically and able to modulate the immune response [77]. This condition is associated with the Stringent Response, which has the alarmone Guanosine (penta)-tetraphosphate ((p)ppGpp) as a bacterial molecular signalling, stimulated by ATP depletion and amino acid starvation, accounting for the development of the persister phenotype inside the host cell [75,78]. Herein, the growth of persister and

regular cells of the three isolates does not appear to be fully interrupted within the 24-h evaluation period in the vacuoles. The connection of this characteristic to serum resistance may help to explain persistent bacterial carriage and recalcitrant infections [79].

Variations in LPS have been associated with serum resistance or susceptibility in *Salmonella*. LPS length is categorized as short, long, or very long based on the number of carbohydrate repeats of the O-antigen, which is correlated with serum resistance [70,80,81]. However, studies have also shown that composition influences antibody binding and complement evasion, particularly when associated with longer LPS chains that anchor the components of the complement system and protect the bacterial membrane against the formation of the MAC [81–84]. Furthermore, overall LPS heterogeneity influences phagocytosis effectiveness and bacterial intracellular survival, possibly by modulating inflammasome triggering and IFN-γ responses [48]. OMPs play important roles in *Salmonella*'s ability to resist serum. PgtE, a promiscuous protease, degrades complement proteins [85,86] and contributes to intracellular survival in neutrophils [87], while Rck binds to complement inhibitors [53,88]. TraT binds to factor H and CD46, which enables both complement inhibition and cellular invasion [89]. Interestingly, these three OMP genes are not upregulated when NTS is exposed to serum [63]. However, *pgt*E has been shown to be upregulated inside phagocytes. This suggests that LPS is the primary protective agent against the complement system extracellularly, whereas the protease is more significant within the SCV [87]. Therefore, variations in the outer membrane directly deal with extracellular challenges, whether they come from MAC assembly or opsonophagocytic-mediated killing mechanisms. In this sense, OMPA and OMPW upregulation have been described for persisters and correlated with increased virulence in an *in vivo* model [60]. OMPA maintains the stability of the *Salmonella* membrane in the presence of ceftazidime [90] and inhibits macrophage autophagy and bacterial lysosome degradation [91]. This makes OMPA one of the possible connections between antimicrobial persistence, serum resistance, and intracellular survival. Comparing the transcriptomes of three NTS isolates, ceftazidime- or ciprofloxacin-persisters presented only four commonly differently regulated genes, indicating a small shared physiological scaffold. Among these genes, *mur*G was downregulated, while *psp*A was upregulated. This suggests that an altered cell wall and increased membrane protection are essential for the persistence phenotype [68]. PspA has recently been demonstrated to induce membrane remodeling, including the formation of double-membrane vesicles [92,93]. Considering that *Salmonella* extracellular vesicles (EVs) have been shown to protect bacterial surfaces by acting as traps for the complement system and that this protection is dose-dependent [55], PspA could contribute to serum resistance by enhancing EV production. Taken together, these findings suggest that antimicrobial persistence adaptations likely impact serum evasion and intracellular bacterial fitness.

As mentioned above, evaluations based on a single isolate should be interpreted with caution because they may not represent the full range of *Salmonella* infection scenarios. Therefore, a limitation of this study is that the evaluation was restricted to three isolates. We also analyzed bacteria that were initially under exponential growth. However, stationary or biofilm-grown *Salmonella* may exhibit different behaviors. Additionally, different MOIs in macrophage exposure could provide important insights into the impact of persisters, which could not be addressed here.

Conclusion

Bacterial persisters have been associated with antibiotic treatment resilience, but their concomitant relationship with other stresses is what may result in a successful infection and/or reinfection. As seen here, the protein food chain carries *Salmonella* isolates that exhibit different persister behaviours when dealing with the human immune system, as expected given their phenotypic diversity. We demonstrated that isolates with different ceftazidime persister fractions exhibited varying serum survival and opsonophagocytosis patterns when comparing regular and persister cells. Despite these variations, the persister cells of the three isolates exhibited higher or equal levels of serum resistance compared to their regular counterparts. Additionally, the high-persister isolate 4SA(2), which is fully resistant to human serum, exhibited equal or higher multiplication of persister-derived cells than regular cells when incubated in serum. Regarding macrophage exposure, serum-resistant persisters were phagocytosed at higher levels than regular cells, though intracellular survival varied

greatly among isolates. The higher opsonophagocytosis of serum-resistant persisters from isolates presenting contrasting persister fractions and serum-resistance profiles may point to a relationship between evaders, persisters, outer membrane composition and host intracellular uptake and viability. Designing more effective therapeutics that avoid recalcitrant infections may benefit from a deeper understanding of how persister cells influence host-*Salmonella* interactions.

Methods

Isolates

Three ceftazidime-susceptible NTS isolates of *Salmonella enterica* subsp. *enterica* were selected from the collection of the Laboratory of Immunology and Microbiology of PUCRS, and named as S48 (*S*. Agona), S45 and 4SA(2) (*S*. Enteriditis) (**Table 1**). Original collection and identification were obtained between 1995 and 2012 from Southern of Brazil.

Minimum Inhibitory Concentration

The ceftazidime (CAZ) (Sigma-Aldrich, St. Louis, USA) minimum inhibitory concentrations (MIC) were determined by broth microdilution method, in triplicate and are shown in **Table 1**. The cut-off values were interpreted according to the European Committee on Antimicrobial Susceptibility Testing [94].

Persistence Test

The survival curves of planktonic cell cultures after exposure to ceftazidime were determined based on the methodology of Drescher, S. P. M. *et al*, 2019 [16], with modifications. Overnight cultures were diluted 1:30 in Lysogeny Broth (LB) and incubated at 37 °C until the mid-exponential growth phase is achieved (approximately 10^8 CFU/mL). Before ceftazidime exposure, the initial cell density was determined by diluting a 100 µL-aliquot until 10^{-6} in 0.85% saline, spotting 10 µL of each dilution in triplicate on nutrient agar, and then incubating at 37 °C for 24 h. Cultures were exposed to ceftazidime at 100-fold MIC for each isolate at room temperature for 72 h. To determine the surviving fractions, aliquots of 300 µL were removed after 6, 24, 48 and 72 h of incubation, and centrifuged at 5225.5 *g* for 7 min. Pellets were resuspended with 300 µL of saline, diluted and each dilution quantified as described above. All analyses were performed on biological triplicates. Survival cell fractions were calculated by dividing the number of colonies counted after each time point by the number of colonies before the antibiotic treatment. To confirm the persistence phenotype and discard the possible selection of resistant cells, persisters were regrown on nutrient agar and then re-exposed to the antimicrobial at the previously determined MIC. A schematic overview of all procedures can be seen in **Fig 4**.

Serum Preparation

Between 10 and 12 volunteers agreed and signed a written consent term approved by the Ethical Research Committee of PUCRS (CEP-PUCRS, CAEE: 69255123.6.0000.5336) between 25/07/2023 and 12/03/2024. 20 mL of peripheral venous blood were collected and distributed into five 5 mL tubes to coagulate at room temperature. The tubes were centrifugated at 400 *g* for 15 min and the serum without haemolysis or fibrin were transferred to another tube. The pool of sera with final volume of ~50 mL was distributed in microtubes and stored at −80 ºC.

Human serum aliquots were thawed according to the demand for experiments, and the unused volume was discarded. For serum inactivation, the microtubes were heated at 56 ºC for 30 min.

Serum Susceptibility Assay

Regular cells and persisters obtained after 72 h of exposure to ceftazidime were immediately centrifuged at 5000 *g* for 30 min and resuspended in PMHC buffer (1x PBS, 1 mM HEPES, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3) at a density of ~10^8 CFU/mL. ~ 10^6 CFU (10 µL) of regular or persister cells were then incubated at 37ºC for 3 h with one of the following

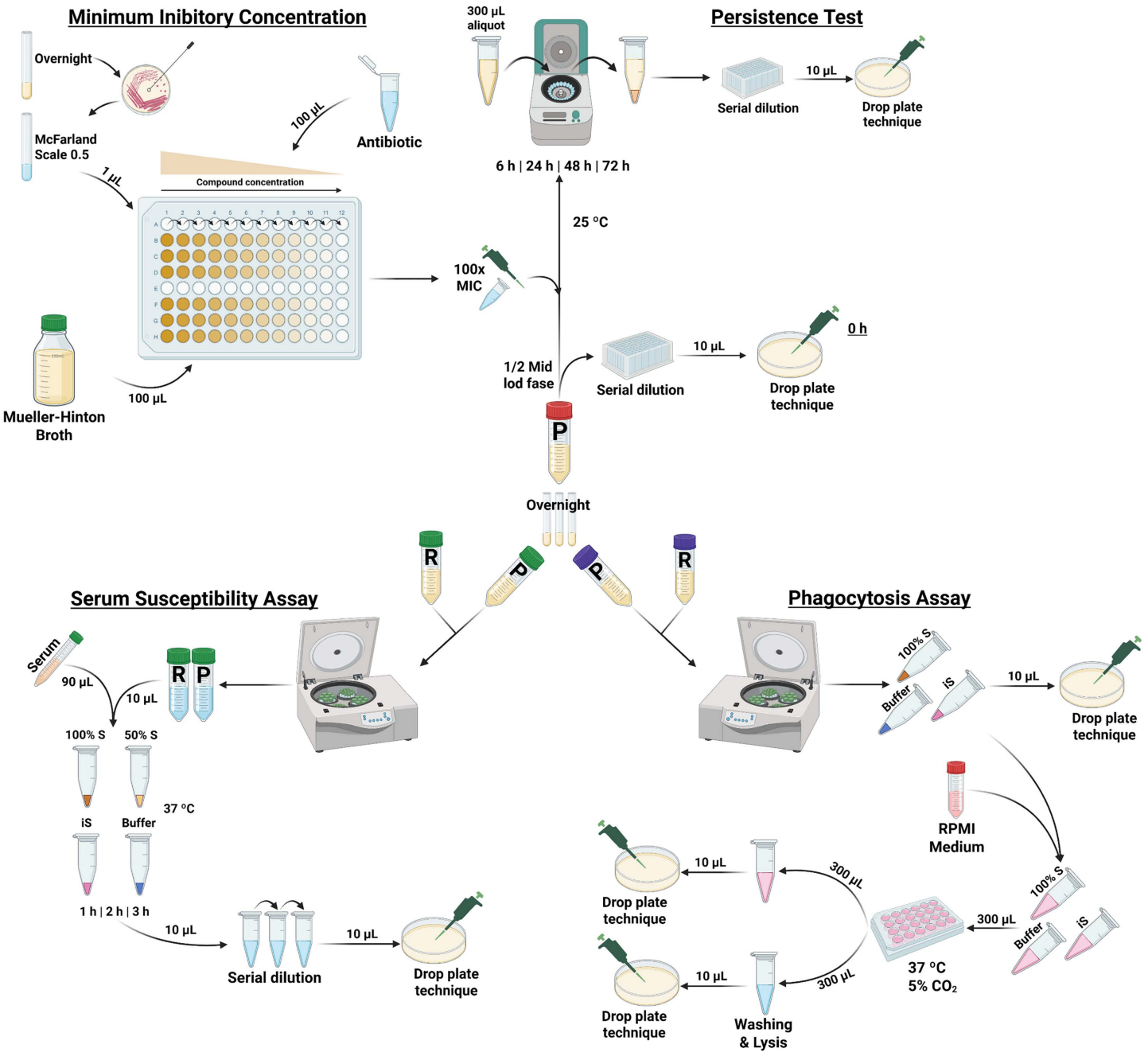

Fig 4. Diagram showing the methodology used to evaluate the susceptibility of regular and persister cells to serum and opsonophagocytosis. Flow diagram of all experimental design. Created in BioRender: Lira, **R.** (2024).

https://doi.org/10.1371/journal.pone.0343532.g004

treatments: 1- S (100% Serum), addition of 90 µL of human serum; 2- S50 (50% Serum), addition of 45 µL of serum and 45 µL of PMHC buffer; 3- iS (inactivated serum), addition of 90 µL of thermically inactivated serum; 4- B (PMHC Buffer), addition of 90 µL of PMHC buffer. After 1, 2, and 3 h, 10 µL aliquots from each group were taken and used for counting as described above. A schematic overview of all procedures can be seen in Fig 4.

Phagocytosis Assay

Peripheral venous blood was collected from three healthy donors in EDTA tubes. After centrifugation at 400 *g* for 15 min, the plasma was removed and added the same plasma volume of 1x PBS and homogenized. 4 mL were then placed carefully into 4 mL (v/v) of histopaque (Sigma-Aldrich) and centrifuged at 400 *g* for 30 min at 4 ºC without break. Four layers were visible (plasma, a mononuclear fraction cloud, histopaque and red blood components), and the phagocytic cells were collected and washed with 15 mL of cold PBS. To determine cell amounts, 10 µL was added to 90 µL of methylene blue, and 10 µL was placed in Neubauer chamber, reaching ~ $5x10^5$ cells. Finally, the cells were plated in a 24-well flat-bottomed polystyrene plate with RPMI 1640 media supplemented with L-glutamine and phenol red (Sigma-Aldrich, R8758), 1 mM sodium pyruvate (Sigma-Aldrich, S8636), 25 mM HEPES solution (Sigma-Aldrich, H0887), 1x non-essential amino acid (Sigma-Aldrich, M7145), 1% penicillin-streptomycin solution (Sigma-Aldrich, P4333), 40 µg/mL gentamicin (Chemitec), 0.25% amphotericin B (Thermo Fisher, 15290018), 10% Autologous Human Serum (AHS) and 50 ng/mL macrophage colony-stimulating factor human (M-CSF, PeproTech, 300−25), a stimulator for maturation, for one week at 37 ºC and 5% CO_2.

Following the serum susceptibility assay, surviving cells after 1 h of incubation with 100%, iS, and B treatments were used to infect macrophages. Based on previous surviving cell levels obtained after the serum susceptibility assay, estimated bacterial numbers were adjusted at a Multiplicity of Infection (MOI) of 10 diluted in RPMI medium and incubated with the macrophages for 30 min at 37 ºC/5% CO_2, with no antibiotics. Before incubation, 10 µL aliquots were taken to measure the actual initial bacterial cell density. Gentamicin (1 µL/mL) was then added and cultures were incubated until 24 h at 37 ºC/5% CO_2. For phagocytosis quantification, the supernatants were collected and kept on ice until used for bacterial number enumeration as described above. Plate wells were washed 2x with PBS, pH 7.4 and macrophages were mechanically lysed immersed in PBS at 4 ºC. Contents were aspirate and preserved in ice until used for bacterial number enumeration as described above. A schematic overview of all procedures can be seen in **Fig 4**.

Statistical analysis

For the Persistence Test, we used the Generalized Linear Models (GLM) with quasibinomial error family and a 'logit' link [95]. Model syntax was built as follows: i) for cell type comparisons, the survivor fraction was included as the dependent variable and the cell type as the independent variable, and ii) for treatment comparisons, the survivor fraction was included as the dependent variable and the treatment as the independent variable. Models were built and tested in the R platform (R Core Team, Vienna, Austria) with the 'glm' function. The significance of the models was assessed based on the comparison with null models, using the likelihood ratio test. Significant models were subjected to post-hoc testing for pairwise comparisons between cell types or treatments. For this purpose, we used Fisher's LSD test, using the 'PostHocTest' function of the 'DescTools' package [96].

For the Serum Susceptibility Assays, we used two-way ANOVA with Geisser-Greenhouse correction and Fisher's LSD test for multiple comparisons, as described above.

Phagocytosis Analysis was set using Multiple Test T for nonparametric data, Mann-Whitney tests using Holm-Sidak method for multiple comparison corrections and Two-Way ANOVA with Tukey test for multiple comparison corrections.

All analyses were performed using GraphPad Prism version 9.5.1 and R Studio, both for Windows 11. Results with $p \leq 0.05$ were considered statistically significant.

Supporting information

S1 Table. *p* values of the comparison of the survival curves against ceftazidime of the three *Salmonella enterica* isolates. The comparisons of the survival curves time points results of isolates 4SA(2), S48, and S45 were performed by Two-Way ANOVA based in GLM model with Fisher's LSD test. $p < 0.05$ was considered statistically significant; significant results are highlighted in bold.
(DOCX)

S2 Table. *p* value of the comparison between regular and persister cells after exposure to human serum. The analyses of the four treatment groups (100% Serum, S; 50% Serum, S50; thermically inactivated serum, iS; Buffer, B) in the different time points was performed by Two-Way ANOVA based in GLM model with Fisher's LSD test. $p < 0.05$ was considered statistically significant; significant results are highlighted in bold.
(DOCX)

S3 Table. *p* values of the comparison between regular and persister cells after exposure to primary human macrophages. The analyses of bacterial uptake by primary macrophages and intracellular survival after 30 min or 24 h of incubation were performed by the Multiple t test with Mann-Whitney test and Holm-Šídák method for correct multiple comparisons. Non-Phago: non-phagocytosed bacterial cells; Lysis 30 min: bacterial cells rescued after 30 minutes of incubation with macrophages; Lysis 24 h: bacterial cells rescued after 24 h of incubation with macrophages; 100% Serum, S; 50% Serum, S50; thermically inactivated serum, iS; Buffer, B. $p < 0.05$ was considered statistically significant; significant results are highlighted in bold.
(DOCX)

Acknowledgments

We thank Samara P. M. Drescher for addressing our concerns about the persistence data. We thank Maila P. Dias e Vanessa Fey for technical assistance.

Author contributions

Conceptualization: Florencia María Barbé-Tuana, Sílvia Dias de Oliveira, carlos alexandre sanchez ferreira.

Data curation: Rodrigo Lira Rodrigues, Pedro Maria Abreu Ferreira, Florencia María Barbé-Tuana, Sílvia Dias de Oliveira, carlos alexandre sanchez ferreira.

Formal analysis: Rodrigo Lira Rodrigues, Júlia Abreu da Rosa, Pedro Maria Abreu Ferreira, Florencia María Barbé-Tuana, Sílvia Dias de Oliveira, carlos alexandre sanchez ferreira.

Funding acquisition: Sílvia Dias de Oliveira, carlos alexandre sanchez ferreira.

Investigation: Rodrigo Lira Rodrigues, Júlia Abreu da Rosa, Douglas Diefenbach da Cunha, Thaís Lima Nunes, Bruno Kendi Makiyama, carlos alexandre sanchez ferreira.

Methodology: Rodrigo Lira Rodrigues, Júlia Abreu da Rosa, Douglas Diefenbach da Cunha, Thaís Lima Nunes, Bruno Kendi Makiyama.

Project administration: carlos alexandre sanchez ferreira.

Resources: Rodrigo Lira Rodrigues, carlos alexandre sanchez ferreira.

Software: Rodrigo Lira Rodrigues, Pedro Maria Abreu Ferreira.

Supervision: carlos alexandre sanchez ferreira.

Validation: Rodrigo Lira Rodrigues, Florencia María Barbé-Tuana, carlos alexandre sanchez ferreira.

Visualization: Rodrigo Lira Rodrigues.

Writing – original draft: Rodrigo Lira Rodrigues, carlos alexandre sanchez ferreira.

Writing – review & editing: Rodrigo Lira Rodrigues, Júlia Abreu da Rosa, Douglas Diefenbach da Cunha, Thaís Lima Nunes, Pedro Maria Abreu Ferreira, Bruno Kendi Makiyama, Florencia María Barbé-Tuana, Sílvia Dias de Oliveira, carlos alexandre sanchez ferreira.

References

1. Chlebicz A, Śliżewska K. Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. Int J Environ Res Public Health. 2018;15(5):863. https://doi.org/10.3390/ijerph15050863 PMID: 29701663
2. Kurtz JR, Goggins JA, McLachlan JB. Salmonella infection: Interplay between the bacteria and host immune system. Immunol Lett. 2017;190:42–50. https://doi.org/10.1016/j.imlet.2017.07.006 PMID: 28720334
3. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive salmonella infections. Clin Microbiol Rev. 2015;28(4):901–37. https://doi.org/10.1128/CMR.00002-15 PMID: 26180063
4. Ferrari RG, Rosario DKA, Cunha-Neto A, Mano SB, Figueiredo EES, Conte-Junior CA. Worldwide Epidemiology of Salmonella Serovars in Animal-Based Foods: a Meta-analysis. Appl Environ Microbiol. 2019;85(14):e00591-19. https://doi.org/10.1128/AEM.00591-19 PMID: 31053586
5. Jajere SM. A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. Vet World. 2019;12(4):504–21. https://doi.org/10.14202/vetworld.2019.504-521 PMID: 31190705
6. Mather AE, Phuong TLT, Gao Y, Clare S, Mukhopadhyay S, Goulding DA, et al. New Variant of Multidrug-Resistant Salmonella enterica Serovar Typhimurium Associated with Invasive Disease in Immunocompromised Patients in Vietnam. mBio. 2018;9(5):e01056-18. https://doi.org/10.1128/mBio.01056-18 PMID: 30181247
7. Williamson DA, Lane CR, Easton M, Valcanis M, Strachan J, Veitch MG, et al. Increasing Antimicrobial Resistance in Nontyphoidal Salmonella Isolates in Australia from 1979 to 2015. Antimicrob Agents Chemother. 2018;62(2):e02012-17. https://doi.org/10.1128/AAC.02012-17 PMID: 29180525
8. Waters EV, Lee WWY, Ismail Ahmed A, Chattaway M-A, Langridge GC. From acute to persistent infection: revealing phylogenomic variations in Salmonella Agona. PLoS Pathog. 2024;20(10):e1012679. https://doi.org/10.1371/journal.ppat.1012679 PMID: 39480892
9. Invasive non-typhoidal Salmonella (iNTS) - Level 3 cause. Institute for Health Metrics and Evaluation (IHME). https://www.healthdata.org/research-analysis/diseases-injuries-risks/factsheets/2021-invasive-non-typhoidal-salmonella-ints. 2024. Accessed 2025 April 24.
10. Jiang M, Wang H-M, Zhou G-L, Chen Y-S, Deng J-K. Invasive salmonella infections among children in Shenzhen, china: a five-year retrospective review. Pediatr Infect Dis J. 2022;41(9):684–9. https://doi.org/10.1097/INF.0000000000003588 PMID: 35622427
11. Acar S, Keskin-Arslan E, Erol-Coskun H, Kaya-Temiz T, Kaplan YC. Pregnancy outcomes following quinolone and fluoroquinolone exposure during pregnancy: a systematic review and meta-analysis. Reprod Toxicol. 2019;85:65–74. https://doi.org/10.1016/j.reprotox.2019.02.002 PMID: 30738954
12. Sati H, Carrara E, Savoldi A, Hansen P, Garlasco J, Campagnaro E, et al. The WHO Bacterial Priority Pathogens List 2024: a prioritisation study to guide research, development, and public health strategies against antimicrobial resistance. Lancet Infect Dis. 2025;25(9):1033–43. https://doi.org/10.1016/S1473-3099(25)00118-5 PMID: 40245910
13. Gollan B, Grabe G, Michaux C, Helaine S. Bacterial Persisters and Infection: Past, Present, and Progressing. Annu Rev Microbiol. 2019;73:359–85. https://doi.org/10.1146/annurev-micro-020518-115650 PMID: 31500532
14. Rana K, Nayak SR, Bihary A, Sahoo AK, Mohanty KC, Palo SK, et al. Association of quorum sensing and biofilm formation with Salmonella virulence: story beyond gathering and cross-talk. Arch Microbiol. 2021;203(10):5887–97. https://doi.org/10.1007/s00203-021-02594-y PMID: 34586468
15. Vergoz D, Dé E, Loutelier-Bourhis C, Alexandre S. Antibiotic persister cells in acinetobacter baumannii: overview of molecular mechanisms and removal strategies. Environ Microbiol. 2025;27(11):e70207. https://doi.org/10.1111/1462-2920.70207 PMID: 41271570
16. Drescher SPM, Gallo SW, Ferreira PMA, Ferreira CAS, Oliveira SD de. Salmonella enterica persister cells form unstable small colony variants after in vitro exposure to ciprofloxacin. Sci Rep. 2019;9(1):7232. https://doi.org/10.1038/s41598-019-43631-7 PMID: 31076596
17. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, et al. Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol. 2019;17(7):441–8. https://doi.org/10.1038/s41579-019-0196-3 PMID: 30980069
18. Bakkeren E, Herter JA, Huisman JS, Steiger Y, Gül E, Newson JPM, et al. Pathogen invasion-dependent tissue reservoirs and plasmid-encoded antibiotic degradation boost plasmid spread in the gut. Elife. 2021;10:e69744. https://doi.org/10.7554/eLife.69744 PMID: 34872631
19. Schwan WR, Huang XZ, Hu L, Kopecko DJ. Differential bacterial survival, replication, and apoptosis-inducing ability of Salmonella serovars within human and murine macrophages. Infect Immun. 2000;68(3):1005–13. https://doi.org/10.1128/IAI.68.3.1005-1013.2000 PMID: 10678900
20. Swart AL, Hensel M. Interactions of Salmonella enterica with dendritic cells. Virulence. 2012;3(7):660–7. https://doi.org/10.4161/viru.22761 PMID: 23221476
21. Luk CH, Valenzuela C, Gil M, Swistak L, Bomme P, Chang Y-Y, et al. Salmonella enters a dormant state within human epithelial cells for persistent infection. PLoS Pathog. 2021;17(4):e1009550. https://doi.org/10.1371/journal.ppat.1009550 PMID: 33930101
22. Zaldívar-López S, Herrera-Uribe J, Bautista R, Jiménez Á, Moreno Á, Claros MG, et al. Salmonella Typhimurium induces genome-wide expression and phosphorylation changes that modulate immune response, intracellular survival and vesicle transport in infected neutrophils. Dev Comp Immunol. 2023;140:104597. https://doi.org/10.1016/j.dci.2022.104597 PMID: 36450302
23. Cruz-Cruz AD, Pérez-Lara JC, Velázquez DZ, Hernández-Galicia G, Ortiz-Navarrete V. B Cells as a Host of Persistent Salmonella Typhimurium. Immunology. 2025;175(3):292–9. https://doi.org/10.1111/imm.13928 PMID: 40223577
24. Ehrhardt K, Becker A-L, Grassl GA. Determinants of persistent Salmonella infections. Curr Opin Immunol. 2023;82:102306. https://doi.org/10.1016/j.coi.2023.102306 PMID: 36989589

25. Hoffman D, Tevet Y, Trzebanski S, Rosenberg G, Vainman L, Solomon A, et al. A non-classical monocyte-derived macrophage subset provides a splenic replication niche for intracellular Salmonella. Immunity. 2021;54(12):2712-2723.e6. https://doi.org/10.1016/j.immuni.2021.10.015 PMID: 34788598
26. Schulte M, Olschewski K, Hensel M. The protected physiological state of intracellular Salmonella enterica persisters reduces host cell-imposed stress. Commun Biol. 2021;4(1):520. https://doi.org/10.1038/s42003-021-02049-6 PMID: 33947954
27. Krukonis ES, Thomson JJ. Complement evasion mechanisms of the systemic pathogens Yersiniae and Salmonellae. FEBS Lett. 2020;594(16):2598–620. https://doi.org/10.1002/1873-3468.13771 PMID: 32170725
28. Kemper C, Ferreira VP, Paz JT, Holers VM, Lionakis MS, Alexander JJ. Complement: The Road Less Traveled. J Immunol. 2023;210(2):119–25. https://doi.org/10.4049/jimmunol.2200540 PMID: 36596217
29. Wiesner J, Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system. Virulence. 2010;1(5):440–64. https://doi.org/10.4161/viru.1.5.12983 PMID: 21178486
30. Heesterbeek DAC, Angelier ML, Harrison RA, Rooijakkers SHM. Complement and Bacterial Infections: From Molecular Mechanisms to Therapeutic Applications. J Innate Immun. 2018;10(5–6):455–64. https://doi.org/10.1159/000491439 PMID: 30149378
31. Xiong K, Zhu C, Chen Z, Zheng C, Tan Y, Rao X, et al. Vi Capsular Polysaccharide Produced by Recombinant Salmonella enterica Serovar Paratyphi A Confers Immunoprotection against Infection by Salmonella enterica Serovar Typhi. Front Cell Infect Microbiol. 2017;7:135. https://doi.org/10.3389/fcimb.2017.00135 PMID: 28484685
32. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol. 2015;13(10):605–19. https://doi.org/10.1038/nrmicro3525 PMID: 26373371
33. Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. Nat Rev Microbiol. 2008;6(2):132–42. https://doi.org/10.1038/nrmicro1824 PMID: 18197169
34. Park D, Lara-Tejero M, Waxham MN, Li W, Hu B, Galán JE, et al. Visualization of the type III secretion mediated Salmonella-host cell interface using cryo-electron tomography. Elife. 2018;7:e39514. https://doi.org/10.7554/eLife.39514 PMID: 30281019
35. Lou L, Zhang P, Piao R, Wang Y. Salmonella Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. Front Cell Infect Microbiol. 2019;9:270. https://doi.org/10.3389/fcimb.2019.00270 PMID: 31428589
36. Niedzialkowska E, Runyan LA, Kudryashova E, Egelman EH, Kudryashov DS. Stabilization of F-actin by Salmonella effector SipA resembles the structural effects of inorganic phosphate and phalloidin. Structure. 2024;32(6):725-738.e8. https://doi.org/10.1016/j.str.2024.02.022 PMID: 38518780
37. Liu X, Liu Y, Zhao X, Li X, Yao T, Liu R, et al. Salmonella enterica serovar Typhimurium remodels mitochondrial dynamics of macrophages via the T3SS effector SipA to promote intracellular proliferation. Gut Microbes. 2024;16(1):2316932. https://doi.org/10.1080/19490976.2024.2316932 PMID: 38356294
38. Jennings E, Thurston TLM, Holden DW. Salmonella SPI-2 Type III secretion system effectors: molecular mechanisms and physiological consequences. Cell Host Microbe. 2017;22(2):217–31. https://doi.org/10.1016/j.chom.2017.07.009 PMID: 28799907
39. Liss V, Swart AL, Kehl A, Hermanns N, Zhang Y, Chikkaballi D, et al. Salmonella enterica remodels the host cell endosomal system for efficient intravacuolar nutrition. Cell Host Microbe. 2017;21(3):390–402. https://doi.org/10.1016/j.chom.2017.02.005 PMID: 28238623
40. Choi HW, Brooking-Dixon R, Neupane S, Lee C-J, Miao EA, Staats HF, et al. Salmonella typhimurium impedes innate immunity with a mast-cell-suppressing protein tyrosine phosphatase, SptP. Immunity. 2013;39(6):1108–20. https://doi.org/10.1016/j.immuni.2013.11.009 PMID: 24332031
41. Yang Z, Soderholm A, Lung TWF, Giogha C, Hill MM, Brown NF, et al. SseK3 Is a Salmonella Effector That Binds TRIM32 and Modulates the Host's NF-κB Signalling Activity. PLoS One. 2015;10(9):e0138529. https://doi.org/10.1371/journal.pone.0138529 PMID: 26394407
42. Johnson R, Byrne A, Berger CN, Klemm E, Crepin VF, Dougan G, et al. The Type III Secretion System Effector SptP of Salmonella enterica Serovar Typhi. J Bacteriol. 2017;199(4):e00647-16. https://doi.org/10.1128/JB.00647-16 PMID: 27920299
43. Schultz BM, Melo-Gonzalez F, Salazar GA, Porto BN, Riedel CA, Kalergis AM, et al. New Insights on the Early Interaction Between Typhoid and Non-typhoid Salmonella Serovars and the Host Cells. Front Microbiol. 2021;12:647044. https://doi.org/10.3389/fmicb.2021.647044 PMID: 34276584
44. Siggins MK, O'Shaughnessy CM, Pravin J, Cunningham AF, Henderson IR, Drayson MT, et al. Differential timing of antibody-mediated phagocytosis and cell-free killing of invasive African Salmonella allows immune evasion. Eur J Immunol. 2014;44(4):1093–8. https://doi.org/10.1002/eji.201343529 PMID: 24375424
45. W. H. O. WHO. Food safety. https://www.who.int/news-room/fact-sheets/detail/food-safety. 2024. Accessed 2025 July 29.
46. Donkor ES, Sosah FK, Odoom A, Odai BT, Kunadu AP-H. How Long Do Microorganisms Survive and Persist in Food? A Systematic Review. Microorganisms. 2025;13(4):901. https://doi.org/10.3390/microorganisms13040901 PMID: 40284737
47. Hoffmann M, Miller J, Melka D, Allard MW, Brown EW, Pettengill JB. Temporal dynamics of salmonella enterica subsp. enterica serovar agona isolates from a recurrent multistate outbreak. Front Microbiol. 2020;11:478. https://doi.org/10.3389/fmicb.2020.00478 PMID: 32265893
48. Heffernan LM, Lawrence A-LE, Marcotte HA, Sharma A, Jenkins AX, Iguwe D, et al. Heterogeneity of Salmonella enterica lipopolysaccharide counteracts macrophage and antimicrobial peptide defenses. Infect Immun. 2024;92(10):e0025124. https://doi.org/10.1128/iai.00251-24 PMID: 39225472

49. Saggin BF, Borges KA, Furian TQ, da Rosa Fünkler G, Mollerke R, Cenci MM, et al. Highly resistant Salmonella Heidelberg circulating in broiler farms in southern Brazil. Braz J Microbiol. 2025;56(1):723–9. https://doi.org/10.1007/s42770-024-01555-z PMID: 39500827
50. Ronneau S, Helaine S. Clarifying the link between toxin-antitoxin modules and bacterial persistence. J Mol Biol. 2019;431(18):3462–71. https://doi.org/10.1016/j.jmb.2019.03.019 PMID: 30914294
51. Fernandes S, Gomes IB, Sousa SF, Simões M. Antimicrobial susceptibility of persister biofilm cells of bacillus cereus and pseudomonas fluorescens. Microorganisms. 2022;10(1):160. https://doi.org/10.3390/microorganisms10010160 PMID: 35056610
52. Rishi P, Bhagat NR, Thakur R, Pathania P. Tackling Salmonella Persister Cells by Antibiotic-Nisin Combination via Mannitol. Indian J Microbiol. 2018;58(2):239–43. https://doi.org/10.1007/s12088-018-0713-5 PMID: 29651185
53. Ho DK, Jarva H, Meri S. Human complement factor H binds to outer membrane protein Rck of Salmonella. J Immunol. 2010;185(3):1763–9. https://doi.org/10.4049/jimmunol.1001244 PMID: 20622116
54. Marshall JM, Gunn JS. The O-Antigen capsule of salmonella enterica serovar typhimurium facilitates serum resistance and surface expression of FliC. Infect Immun. 2015;83(10):3946–59. https://doi.org/10.1128/IAI.00634-15 PMID: 26195553
55. Dehinwal R, Cooley D, Rakov AV, Alugupalli AS, Harmon J, Cunrath O, et al. Increased production of outer membrane vesicles by salmonella interferes with complement-mediated innate immune attack. mBio. 2021;12(3):e0086921. https://doi.org/10.1128/mBio.00869-21 PMID: 34061589
56. Hastings CJ, Himmler GE, Patel A, Marques CNH. Immune response modulation by pseudomonas aeruginosa persister cells. mBio. 2023;14(2):e0005623. https://doi.org/10.1128/mbio.00056-23 PMID: 36920189
57. Futoma-Kołoch B, Godlewska U, Guz-Regner K, Dorotkiewicz-Jach A, Klausa E, Rybka J, et al. Presumable role of outer membrane proteins of Salmonella containing sialylated lipopolysaccharides serovar Ngozi, sv. Isaszeg and subspecies arizonae in determining susceptibility to human serum. Gut Pathog. 2015;7:18. https://doi.org/10.1186/s13099-015-0066-0 PMID: 26185527
58. Zhang R, Xia A, Ni L, Li F, Jin Z, Yang S, et al. Strong shear flow persister bacteria resist mechanical washings on the surfaces of various polymer materials. Adv Biosyst. 2017;1(12):e1700161. https://doi.org/10.1002/adbi.201700161 PMID: 32646157
59. Zhang P, Qiu Y, Wang Y, Xiao L, Yu S, Shi M, et al. Nanoparticles promote bacterial antibiotic tolerance via inducing hyperosmotic stress response. Small. 2022;18(19):e2105525. https://doi.org/10.1002/smll.202105525 PMID: 35398987
60. Schmitt BL, Leal BF, Leyser M, de Barros MP, Trentin DS, Ferreira CAS, et al. Increased ompW and ompA expression and higher virulence of Acinetobacter baumannii persister cells. BMC Microbiol. 2023;23(1):157. https://doi.org/10.1186/s12866-023-02904-y PMID: 37246220
61. Huang W-S, Lee Y-J, Wang L, Chen H-H, Chao Y-J, Cheng V, et al. Copper affects virulence and diverse phenotypes of uropathogenic Proteus mirabilis. J Microbiol Immunol Infect. 2024;57(3):385–95. https://doi.org/10.1016/j.jmii.2024.02.007 PMID: 38453541
62. Pont S, Fraikin N, Caspar Y, Van Melderen L, Attrée I, Cretin F. Bacterial behavior in human blood reveals complement evaders with some persister-like features. PLoS Pathog. 2020;16(12):e1008893. https://doi.org/10.1371/journal.ppat.1008893 PMID: 33326490
63. Ondari EM, Klemm EJ, Msefula CL, El Ghany MA, Heath JN, Pickard DJ, et al. Rapid transcriptional responses to serum exposure are associated with sensitivity and resistance to antibody-mediated complement killing in invasive Salmonella Typhimurium ST313. Wellcome Open Res. 2019;4:74. https://doi.org/10.12688/wellcomeopenres.15059.1 PMID: 31231691
64. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001;410(6832):1099–103. https://doi.org/10.1038/35074106 PMID: 11323673
65. Salazar-Gonzalez R-M, Srinivasan A, Griffin A, Muralimohan G, Ertelt JM, Ravindran R, et al. Salmonella flagellin induces bystander activation of splenic dendritic cells and hinders bacterial replication in vivo. J Immunol. 2007;179(9):6169–75. https://doi.org/10.4049/jimmunol.179.9.6169 PMID: 17947692
66. Tam MA, Rydström A, Sundquist M, Wick MJ. Early cellular responses to Salmonella infection: dendritic cells, monocytes, and more. Immunol Rev. 2008;225:140–62. https://doi.org/10.1111/j.1600-065X.2008.00679.x PMID: 18837781
67. Mina EG, Marques CNH. Interaction of Staphylococcus aureus persister cells with the host when in a persister state and following awakening. Sci Rep. 2016;6:31342. https://doi.org/10.1038/srep31342 PMID: 27506163
68. Mattiello SP, Barth VC Jr, Scaria J, Ferreira CAS, Oliveira SD. Fluoroquinolone and beta-lactam antimicrobials induce different transcriptome profiles in Salmonella enterica persister cells. Sci Rep. 2023;13(1):18696. https://doi.org/10.1038/s41598-023-46142-8 PMID: 37907566
69. Avraham R, Haseley N, Brown D, Penaranda C, Jijon HB, Trombetta JJ, et al. Pathogen cell-to-cell variability drives heterogeneity in host immune responses. Cell. 2015;162(6):1309–21. https://doi.org/10.1016/j.cell.2015.08.027 PMID: 26343579
70. Guerra FE, Karlinsey JE, Libby SJ, Fang FC. Evasion of serum antibodies and complement by Salmonella Typhi and Paratyphi A. PLoS Pathog. 2025;21(5):e1012917. https://doi.org/10.1371/journal.ppat.1012917 PMID: 40315236
71. Domínguez-Medina CC, Pérez-Toledo M, Schager AE, Marshall JL, Cook CN, Bobat S, et al. Outer membrane protein size and LPS O-antigen define protective antibody targeting to the Salmonella surface. Nat Commun. 2020;11(1):851. https://doi.org/10.1038/s41467-020-14655-9 PMID: 32051408
72. Dadole I, Blaha D, Personnic N. The macrophage-bacterium mismatch in persister formation. Trends Microbiol. 2024;32(10):944–56. https://doi.org/10.1016/j.tim.2024.02.009 PMID: 38443279
73. Claudi B, Spröte P, Chirkova A, Personnic N, Zankl J, Schürmann N, et al. Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. Cell. 2014;158(4):722–33. https://doi.org/10.1016/j.cell.2014.06.045 PMID: 25126781

74. Fanous J, Claudi B, Tripathi V, Li J, Goormaghtigh F, Bumann D. Limited impact of Salmonella stress and persisters on antibiotic clearance. Nature. 2025;639(8053):181–9. https://doi.org/10.1038/s41586-024-08506-6 PMID: 39910302
75. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science. 2014;343(6167):204–8. https://doi.org/10.1126/science.1244705 PMID: 24408438
76. Hill PWS, Helaine S. Antibiotic Persisters and Relapsing Salmonella enterica Infections. Persister Cells and Infectious Disease. Cham: Springer International Publishing. 2019. p. 19–38. https://doi.org/10.1007/978-3-030-25241-0_2
77. Stapels DAC, Hill PWS, Westermann AJ, Fisher RA, Thurston TL, Saliba A-E, et al. Salmonella persisters undermine host immune defenses during antibiotic treatment. Science. 2018;362(6419):1156–60. https://doi.org/10.1126/science.aat7148 PMID: 30523110
78. Irving SE, Choudhury NR, Corrigan RM. The stringent response and physiological roles of (pp)pGpp in bacteria. Nat Rev Microbiol. 2021;19(4):256–71. https://doi.org/10.1038/s41579-020-00470-y PMID: 33149273
79. Giorgio RT, Helaine S. Antibiotic-recalcitrant Salmonella during infection. Nat Rev Microbiol. 2025;23(5):276–87. https://doi.org/10.1038/s41579-024-01124-z PMID: 39558126
80. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet. 2005;366(9496):1561–77. https://doi.org/10.1016/S0140-6736(05)67629-5 PMID: 16257344
81. Mylona E, Pereira-Dias J, Keane JA, Karkey A, Dongol S, Khokhar F, et al. Phenotypic variation in the lipopolysaccharide O-antigen of Salmonella Paratyphi A and implications for vaccine development. Vaccine. 2024;42(26):126404. https://doi.org/10.1016/j.vaccine.2024.126404 PMID: 39383552
82. Kintz E, Heiss C, Black I, Donohue N, Brown N, Davies MR, et al. Salmonella enterica Serovar Typhi Lipopolysaccharide O-Antigen Modification Impact on Serum Resistance and Antibody Recognition. Infect Immun. 2017;85(4):e01021-16. https://doi.org/10.1128/IAI.01021-16 PMID: 28167670
83. Pawlak A, Rybka J, Dudek B, Krzyżewska E, Rybka W, Kędziora A, et al. Salmonella O48 serum resistance is connected with the elongation of the lipopolysaccharide o-antigen containing Sialic Acid. Int J Mol Sci. 2017;18(10):2022. https://doi.org/10.3390/ijms18102022 PMID: 28934165
84. Krzyżewska-Dudek E, Kotimaa J, Kapczyńska K, Rybka J, Meri S. Lipopolysaccharides and outer membrane proteins as main structures involved in complement evasion strategies of non-typhoidal Salmonella strains. Mol Immunol. 2022;150:67–77. https://doi.org/10.1016/j.molimm.2022.08.009 PMID: 35998438
85. Ramu P, Tanskanen R, Holmberg M, Lähteenmäki K, Korhonen TK, Meri S. The surface protease PgtE of Salmonella enterica affects complement activity by proteolytically cleaving C3b, C4b and C5. FEBS Lett. 2007;581(9):1716–20. https://doi.org/10.1016/j.febslet.2007.03.049 PMID: 17418141
86. Riva R, Korhonen TK, Meri S. The outer membrane protease PgtE of Salmonella enterica interferes with the alternative complement pathway by cleaving factors B and H. Front Microbiol. 2015;6:63. https://doi.org/10.3389/fmicb.2015.00063 PMID: 25705210
87. Lee MH, Perez-Lopez A, Knodler LA, Nguyen G, Walker GT, Behnsen J, et al. PgtE protease enables virulent Salmonella to evade C3-mediated serum and neutrophil killing. mBio. 2025;16(8):e0380224. https://doi.org/10.1128/mbio.03802-24 PMID: 40767554
88. Ho DK, Tissari J, Järvinen HM, Blom AM, Meri S, Jarva H. Functional recruitment of human complement inhibitor C4B-binding protein to outer membrane protein Rck of Salmonella. PLoS One. 2011;6(11):e27546. https://doi.org/10.1371/journal.pone.0027546 PMID: 22102907
89. Li M, Wu M, Sun Y, Sun L. Edwardsiella tarda TraT is an anti-complement factor and a cellular infection promoter. Commun Biol. 2022;5(1):637. https://doi.org/10.1038/s42003-022-03587-3 PMID: 35768577
90. Chowdhury AR, Mukherjee D, Singh AK, Chakravortty D. Loss of outer membrane protein A (OmpA) impairs the survival of Salmonella Typhimurium by inducing membrane damage in the presence of ceftazidime and meropenem. J Antimicrob Chemother. 2022;77(12):3376–89. https://doi.org/10.1093/jac/dkac327 PMID: 36177811
91. Roy Chowdhury A, Hajra D, Mukherjee D, Nair AV, Chakravortty D. Functional OmpA of salmonella typhimurium provides protection from lysosomal degradation and inhibits autophagic processes in macrophages. J Infect Dis. 2025;231(3):716–28. https://doi.org/10.1093/infdis/jiae376 PMID: 39078938
92. Herianto S, Lee H-M, Chen C, Ho J-AA, Tu H-L. Functional reconstitution of bacterial ESCRT-III protein PspA identifies key regions in membrane binding and remodeling. Protein Sci. 2025;34(10):e70309. https://doi.org/10.1002/pro.70309 PMID: 40970465
93. Nachmias D, Frohn BP, Sachse C, Mizrahi I, Elia N. ESCRTs - a multi-purpose membrane remodeling device encoded in all life forms. Trends Microbiol. 2025;33(6):665–87. https://doi.org/10.1016/j.tim.2025.01.009 PMID: 39979199
94. EUCAST. European Society of Clinical Microbiology and Infectious Diseases. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_14.0_Breakpoint_Tables.pdf. 2024.
95. Dunn PK, Smyth GK. Generalized Linear Models With Examples in R. Springer New York. 2018. https://doi.org/10.1007/978-1-4419-0118-7
96. Signorell A. DescTools: Tools for Descriptive Statistics. https://cran.r-project.org/web/packages/DescTools/index.html