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Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal *Salmonellae*

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ABSTRACT

The ability of *Salmonella* to resist and adapt to harsh conditions is one of the major features that have made this microorganism such a relevant health hazard. However, the impact of these resistance responses on other aspects of *Salmonella* physiology, such as virulence and growth ability, is still not fully understood. The objective of this study was to determine the maximum growth rates (in three different media), virulence (adhesion and invasion of Caco-2 cells), and other phenotypic characteristics (biofilm-forming ability and antimicrobial resistance) of 23 *Salmonella* strains belonging to different serovars, and to compare them with their previously determined stress resistance parameters. Significant differences ($p < 0.05$) in growth rates, virulence, and biofilm-forming ability were found among the 23 strains studied. Nevertheless, whereas less than 3-fold change between the lowest and the highest growth rate was observed, the percentage of cells capable of invading Caco-2 cells varied more than 100-fold, that to form biofilms more than 30-fold, and the antibiotic MICs varied up to 512-fold, among the different strains. Results indicate that those strains with the highest cell adhesion ability were not always the most invasive ones and suggest that, in general terms, a higher stress resistance did not imply a reduced growth ability (rate). Similarly, no association between stress resistance and biofilm formation ability (except for acid stress) or antibiotic resistance (with minor exceptions) was found. Our data also suggest that, in *Salmonella*, acid stress resistance would be associated with virulence, since a positive correlation of that trait with adhesion and a negative correlation with invasion was found. This study contributes to a better understanding of the physiology of *Salmonella* and the relationship between bacterial stress resistance, growth ability, and virulence. It also provides new data regarding intra-specific variability of a series of phenotypic characteristics of *Salmonella* that are relevant from the food safety perspective.

1. Introduction

The relevance of *Salmonella* as a foodborne pathogen is undisputed. Together with *Campylobacter*, it has been at the top of the ranking of the most commonly reported causes of foodborne outbreaks and cases for the last 40–50 years in the United States and Europe (Dewey-Mattia et al., 2018; EFSA, 2019; Gould et al., 2013; Omer et al., 2018). The success of *Salmonella* seems to depend on multiple factors, including its ability to withstand multiple stresses encountered in the environment and in the digestive tract, to invade gut cells and survive intracellularly, and to compete for nutrients such as iron, but also to rapidly adapt and evolve (Dandekar et al., 2012; Nilsson et al., 2005; Petrovska et al., 2016; Spector and Cubitt, 1992; Waldner et al., 2012; Winfield and Groisman, 2003).

Nowadays, more than 2500 serovars of *Salmonella* have been described, but those responsible for most human infections are a smaller group. Thus, less than 20 serovars are responsible of more than 80% of all the cases reported (CDC, 2018; EFSA, 2019). The causes underlying this phenomenon have been explored but not fully elucidated. Thus, it is well known that some serovars are host-specific -or have a very narrow range of potential hosts; the epidemiological studies carried out to date have revealed that the incidence of diverse serovars in animals and food products varies widely, also depending on the type of product (Foley et al., 2013; Sabbagh et al., 2010). However, it is also well known that other factors can be even more relevant for explaining this phenomenon such as, for instance, the ability of *S.* Enteritidis to contaminate eggs through the trans-ovarian route (Gantois et al., 2009). Furthermore, it is quite plausible that further factors might also be determining the

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differences in prevalence observed among *Salmonella* serovars, such as differences in resistance to stress, or differences in terms of growth fitness and/or competition for nutrients.

The genotypic and phenotypic diversity of *Salmonella* has been widely studied, especially regarding virulence and antibiotic resistance (Gerlach and Hensel, 2007; Jajere, 2019), but also regarding stress resistance (Abdullah et al., 2018; Guillén et al., 2020a, 2020b; Lianou and Koutsoumanis, 2013). Although its ability to resist and adapt to harsh conditions is one of the major features that have made *Salmonella* such a relevant health hazard, the impact of these resistance responses on other aspects of *Salmonella* physiology, such as virulence and growth fitness, are much less known (Guillén et al., 2021). Relevant exceptions should be noted such as, for instance, the proven relationship between bile resistance and expression of virulence factors (Prouty and Gunn, 2000; Urdaneta et al., 2019), between oxidative stress and proliferation or survival in macrophages (Golubeva and Slauch, 2006; Krishnakumar et al., 2004), or the role of heat-shock proteins in pathogenesis (Behrens-Kneip, 2010; Humphreys et al., 2003). By contrast, the trade-off between survival potential and nutritional competence (Notley-McRobb et al., 2002), which has been proven to exist in *Salmonella*'s close relative *E. coli*, still remains to be demonstrated.

In view of the above, it is clear that more in-depth studies that specifically deal with the impact of microbial stress resistance responses on other relevant aspects of microbial physiology, such as growth fitness and/or virulence are still required. The objective of our study was to determine growth fitness (in 3 different growth media), virulence (adhesion and invasion of Caco-2 cells) biofilm-forming ability and antimicrobial resistance of 23 *Salmonella* strains belonging to different serovars, and to compare them with their stress resistance as previously determined in Guillén et al. (2020a, 2020b).

2. Material and methods

2.1. Bacterial strains

23 strains belonging to 15 serovars of *Salmonella enterica* subsp. *enterica* were selected to carry out this investigation. The rationale behind the choice of these strains has already been discussed in previous works (Guillén et al., 2020a, 2020b). The strains of *S.* Typhimurium (STCC 443, STCC 4594, STCC 7162 and STCC 722), *S.* Enteritidis (STCC 4300, STCC 4155, STCC 4396, STCC 7160 and STCC 7236), *S.* Derby STCC 4397, *S.* Infantis STCC 4373, *S.* Virchow STCC 4154, *S.* Gallinarum STCC 4883, *S.* Senftenberg 775W STCC 4565, *S.* Saintpaul STCC 4153, and *S.* Stanley STCC 4141 were supplied by the Spanish Type Culture Collection. The strains of *S.* Hadar NCTC 13033, *S.* Newport NCTC 129, *S.* Kentucky NCTC 5799, *S.* Mbandaka NCTC 7892, and *S.* Livingstone NCTC 9125 were supplied by Public Health England. *S.* Heidelberg DMS 9379 was supplied by German Collection of Microorganisms, and the strain of *S.* Typhimurium SL1344 was kindly provided by Tim Brocklehurst from the Institute of Food Research, Norwich. The source of the strains (for which it is known) is included in Supplementary Material (Table S1). The strains were maintained frozen at −80 °C in cryovials for long-term preservation.

2.2. Growth conditions

Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (TSB-YE, Oxoid) in 96-well microtiter plates (Thermo Scientific, Roskilde, Denmark), and incubated at 37 °C under static conditions as described in Guillén et al. (2020b).

2.3. Maximum growth rate determination assays

The growth rates of the 23 *Salmonella* strains were calculated in three different media: TSB-YE at 37 °C, Luria-Bertani (LB) broth supplemented with 100 μM 2-2′dipyridyl (DPY), an iron chelator, at 37 °C, and minimal medium, M9-broth, supplemented with 20 mM gluconate, which is the principal carbon source in the intestine (Bleibtreu et al., 2013). Precultures of each of the strains were diluted 1: 100 into 100 μL of prewarmed media placed in 96-well microtiter plates. These plates were sealed (under anaerobic conditions for LB-DYP and M9-Gluconate growth curves) with a polyester impermeable film (VWR) and incubated under static conditions at 37 °C for 24 h. Samples were taken at preset intervals, adequately diluted in buffered peptone water (Oxoid), and plated in tryptic soy agar (Oxoid) supplemented with 0.6% w/v yeast extract (Oxoid, TSA-YE). These plates were incubated for 24 h at 37 °C and then colonies were manually counted. Growth curves were obtained by plotting the decimal logarithm of the number of cells (Log_{10} CFU/mL) against time, and were then fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000).

$$Y_t = Y_0 + \mu_{max}\cdot A_t - \frac{Y_{max}-Y_0}{M}\cdot ln\left[1 - e^{-M} + \left(e^{-M\cdot\frac{Y_{max}-Y_0-\mu_{max}\cdot A_t}{Y_{max}-Y_0}}\right)\right] \quad (1)$$

$$A_t = t - \lambda\cdot\left[1 - \frac{1}{h_0}\cdot ln\left(1 - e^{-h_0\cdot\frac{t}{\lambda}} + e^{-h_0\cdot\left(\frac{t}{\lambda}-1\right)}\right)\right] \quad (2)$$

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL); Y_0 is the Log_{10} of the initial cell concentration (CFU/mL); Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the Lag time (h); and M and h_0 are the curvature parameters, that in this study were fixed at a constant value of 10 (Baranyi and Roberts, 2000). Curve fitting was carried out using GraphPad PRISM® (GraphPad Software, San Diego, CA, USA) statistical software.

2.4. Virulence assays

2.4.1. Caco-2 cell maintenance and preparation

The human colon carcinoma Caco-2 cell line (TC7 clone) was kindly provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie-Paris 6, UMR S 872, Les Cordeliers, France) at Passage 25 and used in experiments at Passage 30-35. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in 75 cm^2 flasks. Cells were grown in Dulbecco's Modified Eagle's Medium + Gluta-MAX™ (DMEM, Invitrogen, France) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, France), 1% Minimal Essential Medium with Non-Essential Amino Acids (MEM NEAA 100×, Invitrogen, France), and 1% antibiotics (penicillin/streptomycin, Invitrogen). Once the cells reached 80% confluence, they were dissociated with 0.05% Trypsin-1 mM EDTA (Invitrogen) and seeded at a density of approximately 15,000 cells per well in 96-well tissue culture plates (Nunc, France) containing 200 μL of complete medium per well. Plates were incubated in humidified atmosphere containing 5% CO_2 at 37 °C for 15–17 days to attain fully differentiated cell layers. Culture medium was replaced every 2 days, and cell confluence was confirmed by optical microscopy.

2.4.2. Adhesion and invasion in Caco-2 cells

Prior to use for virulence assays, cell layers were washed three times in DPBS (Dulbecco's Phosphate Buffered Saline); 200 μL of complete medium without antibiotics were added. For adhesion assay, suspensions of different *Salmonella* strains were added at an initial concentration of 10^6 CFU/mL on washed Caco-2 cells. Cells were incubated with bacteria for 30 min in humidified atmosphere containing 5% CO_2 at 37 °C. After incubation, non-adhered bacteria were removed by washing the cell cultures twice with DPBS, and the cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. These lysates were adequately diluted and then plated in Xylose Lysine Desoxycholate Agar (XLD, Oxoid). Plates were incubated at 37 °C for 24 h before manual counting of growing colonies. For invasion assays, bacterial inoculation was performed as described for

the adhesion assay, and plates were maintained in 5% CO_2 at 37 °C for 30 min. The infected cells were washed twice with DPBS, after which they were maintained during 1 h in DMEM containing 100 μg/mL of gentamicin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) per well to inactivate extracellular bacteria. After incubation, cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. Lysates were processed for determination of *Salmonella* counts as described above. The adhesion and invasion rates were calculated as percentages of adhered or invading bacteria to initial bacteria added. These percentages were calculated and represented with GraphPad software.

2.5. Biofilm formation ability assay

Biofilm formation ability of the 23 studied strains was evaluated in a 96-well microtiter plate by adapting the protocol of Patel and Sharma (2010). Briefly, overnight pre-cultures of *Salmonella* strains were diluted 1:100 in 100 μL TSB-YE media in wells of a sterile 96-well polystyrene microtiter plate (Fisher Scientific, Newark, DE) and incubated under static conditions at 37 °C. After 24, 48 and 72 h incubation in microplate culture, media was completely removed, and the wells were washed three times by immersing the plate in sterile distilled water tempered to 37 °C. The plates were air-dried for 30 min, and 125 μL crystal violet solution (0.1% w/v, Fisher Scientific) was added per well and incubated at room temperature during 20 min. Crystal violet solution was removed by washing as indicated above. To quantify biofilm formation, 125 μL of acetic acid (30% v/v) were added to each well, and the absorbance of each well at 580 nm was measured (Genios, Tecan, Männedorf, Switzerland). Thus, the concentration of crystal violet remaining in each well is proportional to the number of biofilm forming cells. For each replicate experiment, four wells were inoculated for each strain. According to the criteria suggested by Stepanovic et al. (2000) and based on the OD produced by bacterial films, strains were classified into the following categories: strong, moderate, weak, or no biofilm producers. In order to establish meaningful comparisons the area under the curve (AUC) was calculated as described in Espina et al. (2015). Briefly, the absorbance at 580 nm vs time (up to 72 h; with measurements every 24 h) was plotted for each strain and the AUC values were calculated using GraphPad software and following the trapezoid rule, where the total area is the sum of all rectangular trapezoids, each defined by two adjacent absorbance values with respect to the ground (in the y axis) and the time between those measurements (in the x axis). The formula we applied was:

$$AUC = \sum_{i=1}^{n-1} \frac{x_i \cdot (y_i + y_{i+1})}{2} \quad (3)$$

where x_i is the time between measurements in hours, y_i is the absorbance value at 580 nm for each measurement, and n is the total number of measurements.

2.6. Antibiotic resistance assays

The minimum inhibitory concentration (MIC) of seven antibiotics representative of different classes (ampicillin, chloramphenicol, nalidixic acid, oxytetracycline, rifampicin, streptomycin, and sulfanilamide) against the 23 strains under investigation was determined by Broth Dilution Susceptibility Tests. Briefly, 1 μL of bacterial pre-culture was inoculated into 100 μL of fresh TSB-YE (yielding an initial concentration of approx. 10^7 CFU/mL) with increasing concentrations of the corresponding antibiotic, and incubated for 24 h at 37 °C. The range of concentrations used to determine the MICs of antibiotic was 0 to 512 μg/mL, except for sulfonamides, for which the range was 0 to 4096 μg/mL. MICs were then determined as the lowest concentration of antibiotic that completely inhibited growth (optical absorbance equal or lower than non-inoculated wells) of each strain after 24 h of cultivation at 37 °C.

2.7. Statistical analysis

All the determinations were carried out in triplicate on different working days. Standard deviations (SD) and Pearson's and Spearman correlation coefficients were calculated using GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA). The same software was used to carry out the Iterative Grubbs's test (Alpha = 0.05) and the statistical analyses (Welch's *t*-test, ANOVA, and Tuckey tests; *p*-value < 0.05). In order to quantify and compare experimental, intra-serovar and intra-species variability the Coefficient of Variation (CV, in %) was used (CV = standard deviation × 100 / mean) as described in Lianou and Koutsoumanis (2012).

3. Results and discussion

3.1. Growth rates in different media

Growth curves of the 23 *Salmonella* strains under study were obtained in three different media: in TSB-YE, a nutrient-rich medium, in LB medium with iron limitation caused by the addition of DPY, and in a minimal medium containing gluconate as the sole carbon source. The latter two media were tested because they simulate pathophysiological conditions in the intestine, and therefore anaerobic conditions were used (Bleibtreu et al., 2013). Growth curves obtained were fitted with the Baranyi model (Baranyi and Roberts, 2000). The μ_{max} (Log_{10}/h) values calculated for each strain in the three growth media are shown in Fig. 1 (growth parameters and goodness of the fit parameters are included in Supplementary Table 2). In TSB-YE, the average μ_{max} (Log_{10}/h) for the 23 strains was 0.966 ± 0.204; the highest growth rate was that of *S.* Saintpaul (1.293 ± 0.064), and the lowest that of *S.* Gallinarum (0.457 ± 0.054). In LB-DPY, the average μ_{max} (Log_{10}/h) was 0.697 ± 0.112; the highest growth rate was determined for *S.* Senftenberg (0.862 ± 0.067), and the lowest for *S.* Gallinarum (0.342 ± 0.021). Finally, in M9-Gluconate, the average μ_{max} (Log_{10}/h) was 0.549 ± 0.092; the highest growth rate was that of *S.* Typhimurium (0.713 ± 0.033) and the lowest, as in the case of the other two media tested, that of *S.* Gallinarum (0.310 ± 0.047). None of the strains displayed a statistically significant Lag phase (h) (different from 0; $p > 0.05$) in any of the three media tested, except for *S.* Gallinarum in TSB-YE (1.05 h ± 0.433).

ANOVA analysis of the calculated μ_{max} values revealed a significant effect ($p < 0.05$) of the strain studied in the three growth media/conditions assayed (Supplementary Fig. 1). The obtained data also indicate that, among the three media tested, *Salmonella* strains displayed a higher growth rate ($p < 0.05$) in TSB-YE, a rich medium with no nutrient limitation. In LB-DPY, growth rates were reduced by 26% on average, because in this medium, iron, which is essential for bacterial growth, particularly during infection (Costa et al., 2016; Tan et al., 2019), is chelated by DPY, making it less bioavailable. Growth in M9-Gluconate imposed an even higher fitness cost (also statistically significant; $p < 0.05$), leading to an average reduction of 42% in *Salmonella* growth rates (Fig. 1). Apart from that, a significant correlation ($p < 0.05$) was observed between the maximum growth rates in TSB-YE and those in M9-Gluconate, with a Pearson correlation coefficient of 0.536 ($p = 0.008$) and a Spearman correlation coefficient of 0.437 ($p = 0.037$). A significant correlation was also observed when comparing the μ_{max} values obtained in TSB-YE and LB-DYP, with a Pearson correlation coefficient of 0.593 ($p = 0.003$) although in this latter case the significance of the Spearman correlation test only indicated a trend ($p < 0.1$) ($r_s = 0.382$, $p = 0.072$). Similarly, a significant correlation was found between the maximum growth rates calculated in LB-DPY and M9-Gluconate (Pearson r = 0.522, $p = 0.011$), but according to the Spearman rank correlation coefficient it was only a trend ($r_s = 0.388$, $p = 0.067$). These results would suggest that those strains that display a higher growth rate under non-limiting conditions would also display a higher growth rate in

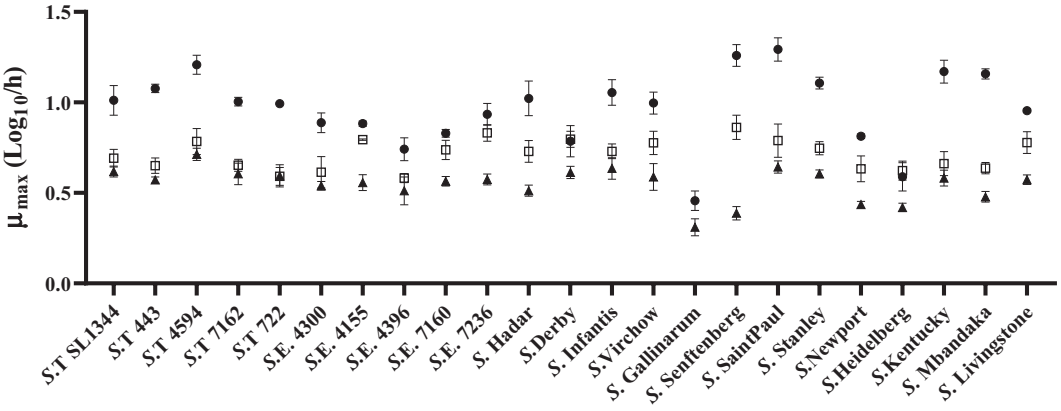

Fig. 1. Maximum growth rates (μ_{max} (Log_{10}/h)) of the 23 *Salmonella enterica* strains studied in TSB-YE (●), LB supplemented with 100 μM 2-2′dipyridyl (DPY) (□) and M9-broth supplemented with 20 mM gluconate (▲) at 37 °C. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

media with reduced amounts of Fe or with gluconate as the sole carbon source. However, it should be noted that a high degree of experimental variability was observed, and that correlations were not significant if *S.* Gallinarum was excluded from the analysis. These conclusions should be therefore taken with care.

On the other hand, no significant differences were found when comparing the average μ_{max} value in TSB-YE of either the *S.* Enteritidis strains (0.855 ± 0.074; n = 5) or the *S.* Typhimurium strains (1.059 ± 0.090; n = 5) with the average μ_{max} value of the other 13 strains tested (non-Enteritidis and non-Typhimurium strains; 0.974 ± 0.252; n = 13) or with the overall average value of all the 23 strains (0.967 ± 0.204 n = 23). Similar results were obtained for the other two media tested (Fig. 2). Still, in TSB-YE and M9-Gluconate, the average μ_{max} values of *S.* Typhimurium were significantly higher than those of *S.* Enteritidis strains. It should also be noted that inter-serovar variability (CV = standard deviation × 100 / mean) tended to be slightly higher (up to 2.8 times) than intra-serovar variability in these latter two media. These conclusions should nevertheless be taken with caution too, since the number of strains per serovar was low (5), and comparisons were established among groups with a different number of strains (5 vs 13 vs 23). Further work would therefore be required to validate them.

Most of the available studies dealing with the growth ability of *S. enterica* strains/serovars have reported a small variability in growth parameters. Thus, Juneja et al. (2003) concluded that slight variations in kinetic parameters among *Salmonella* strains were not associated with any serovar effect, but merely reflected an experimental variability, while Lianou and Koutsoumanis (2011) observed that differences in growth rate among 60 *Salmonella* strains mainly depended on composition of growth medium; furthermore, this variability was not related to the *Salmonella* serovar under any of the growth conditions tested. Díez-García et al. (2012) observed an up to 4-fold change among the growth kinetic parameters determined for a total of 69 *S. enterica* strains belonging to 10 serovars growing in TSB at 37 °C, conditions very close to one of those studied herein; even in this case, however, these differences can be considered small if compared to the variability of other phenotypical characteristics. Mutations conferring a growth advantage have already been described for *Salmonella* and the closely related *E. coli*. Thus, competitive fitness and/or growth rates can be increased through mutations in genes affecting general gene expression, e.g., mutations in *rpoA/B/S* and *arcA* (Knöppel et al., 2018; Saxer et al., 2014), or in genes directly associated with the use of certain resources, e.g., mutations in pyruvate kinase I (PykF) or *glpK*, which allow a better utilization of glucose or glycerol as a source of carbon and energy. This suggests that, some strains displaying increased growth rates would at least sporadically appear, and/or that some specific serovars might, in the same way, display an increased fitness under very specific growth conditions (e.g. those governing in their particular niche), which, in turn, would contrast with the low variability in growth rate observed in most studies. It should nevertheless be noted that most of these studies have been carried out in non-selective media and under very favorable growth conditions. In addition, this low variability might also be explained, at least partially, because bacteria would make use of several strategies, rather than a single strategy, to optimize their fitness, as suggested by Knöppel et al. (2018).

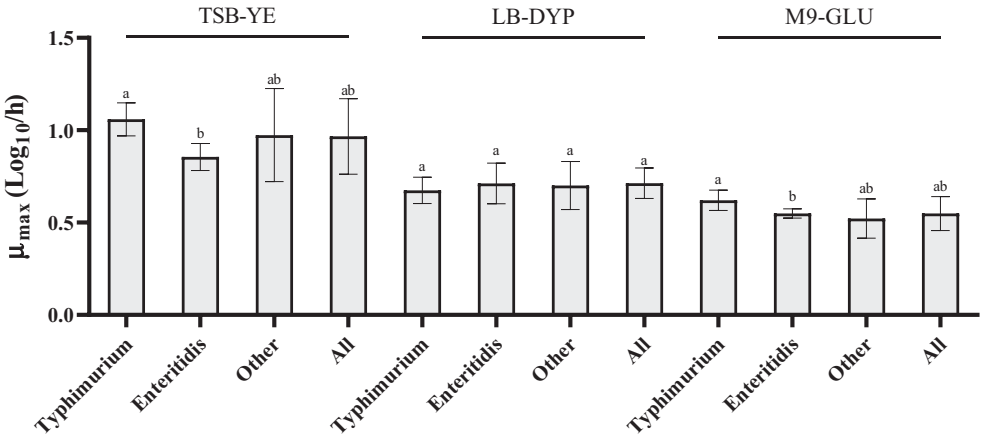

Fig. 2. Average μ_{max} (Log_{10}/h) values of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella enterica* strains studied (All) in TSB-YE, LB supplemented with 100 μM 2-2′dipyridyl and M9 supplemented with 20 mM gluconate at 37 °C. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups.

3.2. Caco-2 cell adhesion and invasion

The percentage of cells capable of adhering to the Caco-2 layer ranged from 0.47% to 6.95%, with *S.* Newport showing the lowest capacity to adhere to Caco-2 cells and *S.* Saintpaul the highest (Fig. 3A). On the other hand, invasion ability varied between <0.01% and 1.02%. *S.* Gallinarum and *S.* Newport showed the lowest capacity to invade Caco-2 and *S.* Enteritidis 7160 the highest (Fig. 3B). Statistical analysis (ANOVA) revealed that the strain under study had a significant effect on the rates of adhesion and invasion ($p < 0.05$) (Fig. 3A and B), and also that significant differences existed between the rate of adhesion of *S.* Enteritidis and *S.* Typhimurium and between the rate of invasion of *S.* Typhimurium and of those strains not belonging to serovars Enteritidis or Typhimurium (Fig. 4). It should also be noted that considerable variation in the rate of adhesion and invasion among strains belonging to the same serovar was observed. Thus, up to 4-fold differences in adhesion ability were found among the five different strains of the Typhimurium serovar, and almost 3-fold changes were found for the five strains of the serovar Enteritidis. On the contrary, the highest intra-serovar variability in invasivity was displayed by the serovar Enteritidis (CV = 124%), which included the strains with the highest and lowest ability to invade enterocytes among all the strains tested, excluding *S.* Gallinarum and Newport.

Nevertheless, it should be noted that experimental variability among biological replicates was very high, with a Coefficient of Variation (3 biological replicates) for invasion assays of 56% on average, and up to 91% for certain strains such as *S.* Enteritidis 7160, a phenomenon that might be masking actual differences among strains/serovars.

The Grubbs's test detected as a possible outlier the invasion value obtained for *S.* Enteritidis 7160; it was therefore eliminated to perform the correlation analysis. No association was found between adhesion and invasion ($r = -0.109$, $p = 0.630$, $r_s = -0.115$, $p = 0.610$). The process of adhesion and invasion of cells by *Salmonella* spp. has been widely studied using the intestinal epithelial cell line Caco-2 (Dostal et al., 2014; Gagnon et al., 2013; McWhorter et al., 2015). The variability in adhesion ability to Caco-2 cells among *Salmonella* strains

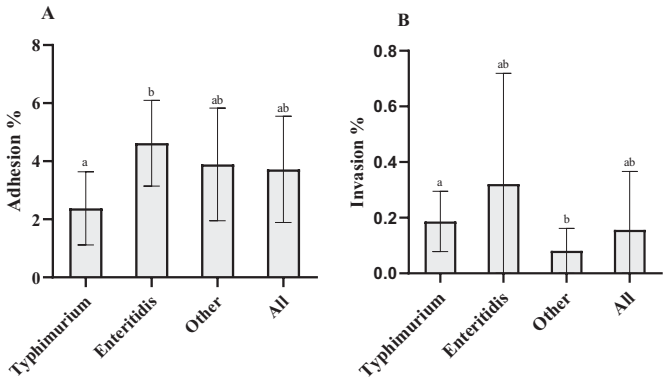

Fig. 4. Adhesion (A) and invasion capacity (B) to Caco-2 cells of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella* strains studied (All). Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups.

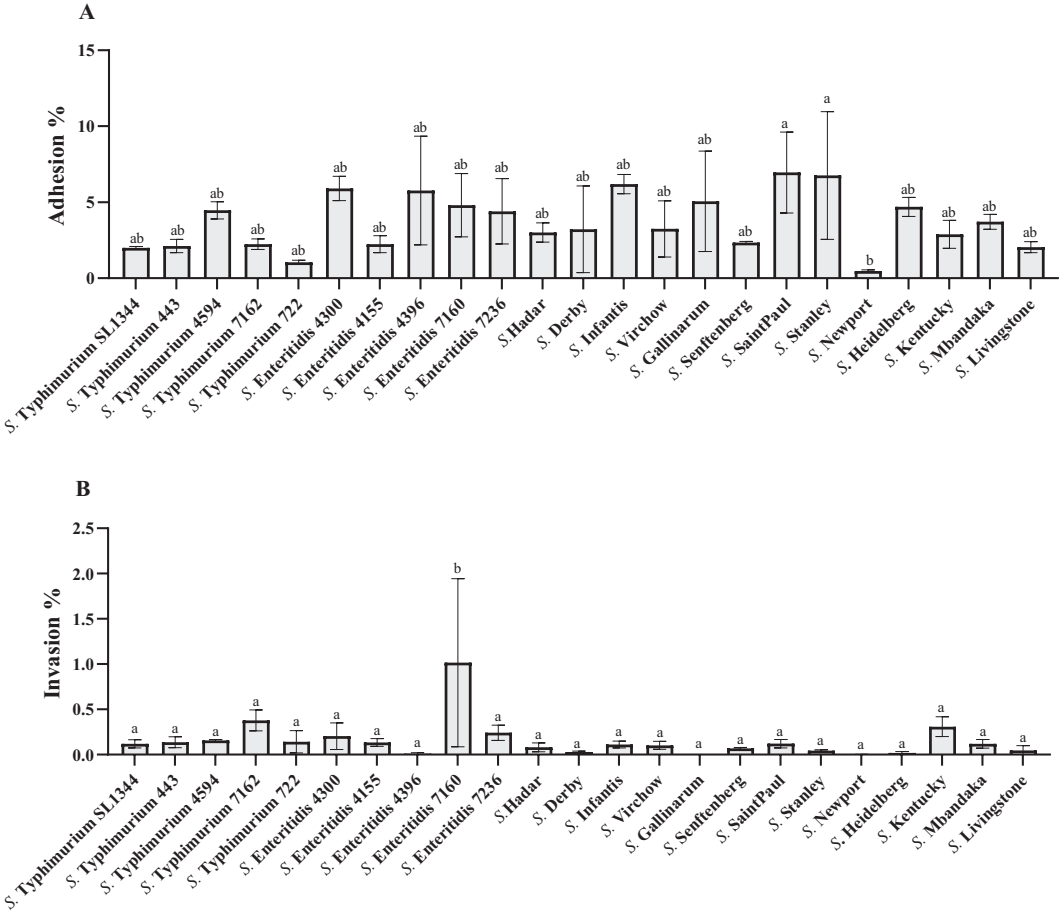

Fig. 3. Adhesion (A) and invasion (B) capacity to CaCo-2 cells of the 23 strains of *Salmonella enterica* studied. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

observed in this study is in accordance with those reported in the work of Gagnon et al. (2013), who indicated that such differences in adhesion were highly dependent on the serovar and, at the same time, dependent on the expression of genes encoding protein secretion systems, effector proteins and chaperones, and/or transcriptional regulators. Differences in adhesion ability among serovars might be explained by the fact that the majority of *Salmonella* strains possess serotype-specific virulence plasmids, which are low-copy-number plasmids (1 to 2 copies per cell) ranging from 50 to 100 kb, depending on the serovar (van Asten and van Dijk, 2005). In addition, and as observed here, considerable variation in the rate of adhesion and invasion among strains belonging to the same serovar and even possessing the same virulence genes has also been reported (Dostal et al., 2014; McWhorter et al., 2015).

On the other hand, the invasion capacity of the strains included in this study was low, similarly to that observed by McWhorter et al. (2015). Although it has been demonstrated that the adhesion and invasion processes are coordinated, different pathways modulate these separate virulence mechanisms (Velge et al., 2012), which would explain why strains displaying a high adhesion ability are not always the most invasive ones (e.g. *S.* Stanley). Possession of serotype-specific virulence plasmids would explain, at least partially, the differences in invasion ability among serovars, as indicated above for adhesion. Substantial differences in virulence among strains of the same serovar (Enteritidis) have also been reported (Shah, 2014). Transcriptional analysis has revealed that *S.* Enteritidis strains with low pathogenicity displayed reduced expression of several transcriptional regulators, reduced expression of genes involved in virulence (e.g., *Salmonella* pathogenicity island 1 (SPI-1), SPI-5, as well as fimbrial and motility genes), and protection against osmotic, oxidative, and other stresses, such as iron-limiting conditions commonly encountered within the host. It should also be noted that environmental conditions are known to influence the expression of *Salmonella* virulence genes; therefore, growth, pre-invasion and invasion conditions can significantly affect the invasiveness of *S.* Typhimurium as well as the ability of the bacteria to replicate intracellularly (Dostal et al., 2014; Foster et al., 2001; Ibarra et al., 2010; Kortman et al., 2012; Tan et al., 2019). As a way of example, McWhorter et al. (2015) observed substantial differences in the invasion ability of *Salmonellae* depending on the environment in which the *Salmonella* cells were grown – normal saline or LB broth – with greater invasion capacity observed in the latter. Similarly, it has been demonstrated that the concentration of iron, both before and during adhesion and invasion assays, can significantly affect the rates of *Salmonella* adhesion and invasion (Dostal et al., 2014; Foster et al., 2001; Kortman et al., 2012; Tan et al., 2019). Further work will thus be required to determine if the differences in adhesion and invasion ability among strains and serovars reported herein would also exist in more complex media that simulate gut conditions more closely.

Finally, it should be noted that a lower invasion capacity of *S.* Gallinarum, as compared to *S.* Enteritidis, has also been previously reported in chicken and human epithelial cell lines (Rossignol et al., 2014). By contrast, although data regarding the adhesion and invasion ability of *S.* Newport have been reported (Deekshit et al., 2015), further work would be required to elucidate whether the low adhesion and invasion abilities (at least in relative terms) of the *S.* Newport strain used in our study are specific of this strain, or whether they are a common feature of the whole serovar.

3.3. Static biofilm formation ability

The ability to form biofilms is a well-known phenotypic characteristic of *Salmonella* cells. The results of the static biofilm formation assay are shown in Fig. 5. It should be noted that, in order to establish meaningful comparisons, the AUC values calculated as described in materials and methods were compared. The average value for this parameter of the 23 tested strains of *Salmonella* spp. was 4.7 ± 6.0: The *S.* Gallinarum strain was the one with the lowest biofilm formation capacity (0.92 ± 0.94), while the *S.* Senftenberg strain showed maximum biofilm formation capacity (28.9 ± 4.2). This biofilm formation ability of the *S.* Senftenberg strain is remarkable and will be discussed below. Thus, the biofilm formation ability value of *S.* Senftenberg was 6 times higher than the average value of all the 23 *Salmonella* strains, and twice that of the strain with the second highest formation capacity (*S.* Typhimurium 722). These two strains can also be classified – as likewise described in "Materials and methods"– as strong biofilm producers. *S.* Typhimurium STCC 7162, *S.* Derby, *S.* Saintpaul, *S.* Stanley, *S.* Newport, and *S.* Livingstone strains were moderate biofilm producers, and the rest of the strains were weak biofilm producers. On the other hand, intra-serovar variability in biofilm formation was higher for *S. Typhimurium* strains (more than 9-fold difference between the strain with the highest and the lowest ability; CV = 99.2%) than for *S.* Enteritidis strains (less than 2-fold; CV = 21.1%), but still lower than the overall inter-serovar variability (almost 16-fold; CV = 126%, even if *S.* Senftenberg is excluded from the analysis). In spite of this high variability, Welch's *t*-test indicated that Enteritidis strains displayed (on average) a lower biofilm ability than strains belonging to serovars other than Enteritidis and Typhimurium (Fig. 6).

The ability of *Salmonellae* to form biofilms on polystyrene surfaces (a hydrophobic material) is well documented, as is the above-described existence of wide differences among strains and serovars. Furthermore, various authors have already suggested the existence of serovar-

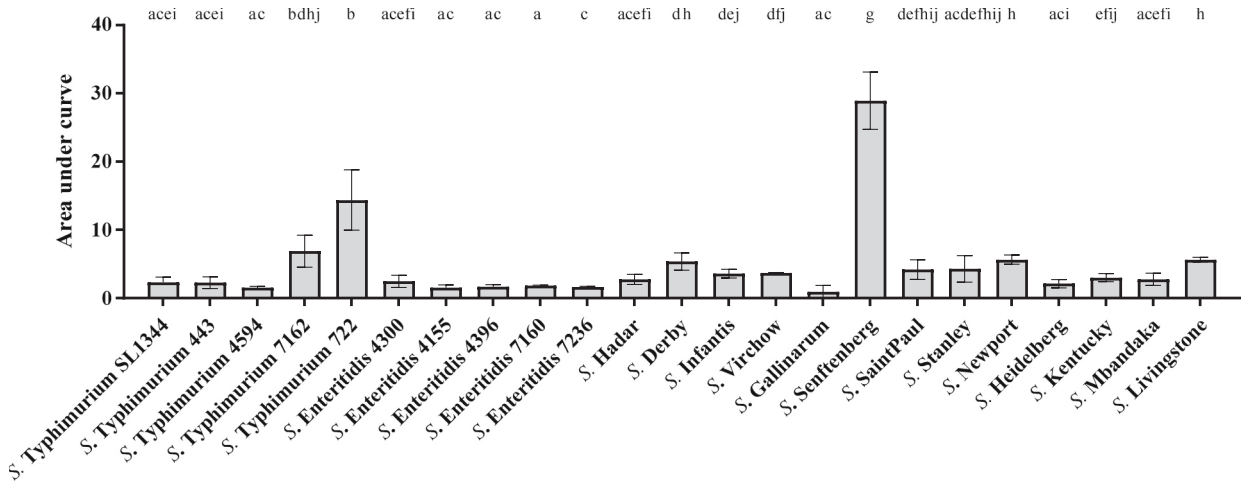

Fig. 5. Biofilm-forming ability of the 23 strains of *Salmonella enterica* studied. Values correspond to the Area under the Curve calculated as described in Material and methods. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

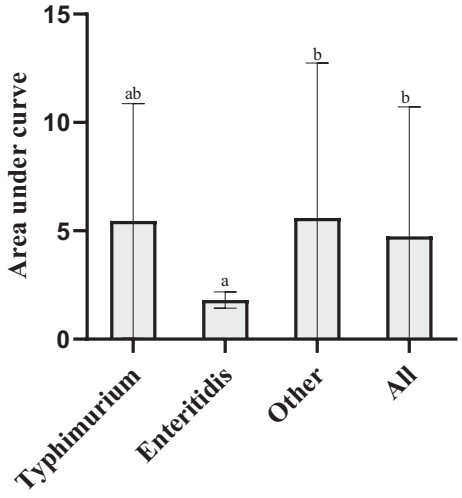

Fig. 6. Biofilm-forming ability of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella* strains studied (All). Values correspond to the Area under the Curve calculated as described in Material and methods. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups.

specific attachment mechanisms (Berger et al., 2009; Klerks et al., 2007), which is the first step of the biofilm formation process, and Patel and Sharma (2010) indicated that attachment to lettuce leaves and subsequent biofilm formation by *Salmonella* strains may differ depending on the specific properties of the serovars. Similarly, Díez-García et al. (2012) observed substantial differences in terms of biofilm forming ability among the *Salmonella* serovars they tested, and reported a variability similar to that found here in the biofilm forming ability of Typhimurium and Enteritidis strains. Furthermore, Vestby et al. (2009) found a clear difference in terms of biofilm-forming capability among 116 strains belonging to four serovars. Their results also suggested that the type of serovar would exert an important influence on biofilm formation. Nevertheless, although the latter authors determined that strains of the Typhimurium serovar were relatively poor biofilm producers, our results indicate that Typhimurium strains display a high variability in biofilm formation capacity, with strain 722 being a strong producer, and 7162 a moderate producer. This wide intra-serovar variability might explain why some other authors did not find any relationship between the biofilm-forming ability of strains and their serovar (Lianou and Koutsoumanis, 2012). On the other hand, and as pointed out above, the strain of Senftenberg used in this study was classified as a strong biofilm former, ranking above all the other strains studied. Unluckily, although currently only limited information is available regarding the ability of *S.* Senftenberg to form biofilms, it should be noted that Xia et al. (2009), who studied biofilm formation in 16 *Salmonella* isolates from retail foods, observed that Senftenberg isolates were most prolific in biofilm formation, and Vestby et al. (2009), using a microtiter plate assay over prolonged incubation periods, reported that serovar Senftenberg strains were the only ones that displayed a significant increase in OD values from day two to four. Our results are also consistent with previous investigations indicating that *S.* Gallinarum, together with other host-specific serovars, is a weak biofilm producer (MacKenzie et al., 2017).

3.4. Resistance to antibiotics

As can be observed in Table 1, substantial differences were observed in MIC values against the seven tested antibiotics depending on the strain, although these differences were considerably more marked for certain groups of antibiotics. *S.* Typhimurium 7162 generally showed the highest resistance to all antibiotics under study: it was the most resistant to ampicillin, chloramphenicol, and oxytetracycline. In contrast, *S.* Enteritidis 4396 can be considered the most sensitive to the antibiotics included in our study. If the results corresponding to each antibiotic are analysed separately, one can observe that all strains except *S.* Typhimurium 7162 had MICs below 20 μg/mL for ampicillin (between 2 and 16 μg/mL). Resistances to chloramphenicol and oxytetracycline laid between 4 and 16 μg/mL. For chloramphenicol, however, three strains had higher MIC values (*S.* Infantis and *S.* Senftenberg, 32 μg/mL and *S.* Typhimurium 7162, 128 μg/mL), while a further three strains had higher MIC values for Oxytetracycline (*S.* Infantis and *S.* Enteritidis 4300 32 μg/mL and *S.* Typhimurium 7162 128 μg/mL). For nalidixic acid, the range of observed MICs was wider (between 1 and 64 μg/mL), with *S.* Typhimurium 722 and *S.* Enteritidis 7160 not inhibited, even at the higher concentration tested (64 μg/mL). The MICs

Table 1
Minimum inhibitory concentrations (MIC) for the different antibiotics of the 23 *Salmonella enterica* strains tested. Units in μg/mL.

	Ampicillin	Chloramphenicol	Nalidixic acid	Oxytetracycline	Rifampicin	Streptomycin	Sulfonamide
*S.*T SL1344	4	4	8	1	16	256	4096
*S.*T 443	8	8	32	8	16	64	4096
*S.*T 4594	8	16	64	8	16	>512	4096
*S.*T 7162	>512	128	64	512	32	512	4096
*S.*T 722	8	8	>64	8	16	>512	4096
S. E. 4300	8	8	8	32	16	128	4096
S. E. 4155	2	4	4	2	16	16	>4096
S. E. 4396	2	4	1	1	8	16	>4096
S. E. 7160	1	4	>64	1	16	16	>4096
S. E. 7236	4	4	16	8	16	16	>4096
S. Hadar	16	8	64	8	16	>512	2048
S. Derby	16	8	64	8	16	256	4096
S. Infantis	16	32	64	32	>64	>512	4096
S. Virchow	8	16	16	4	16	128	4096
S. Gallinarum	2	4	64	2	16	>512	4096
S. Senftenberg	16	32	32	8	32	128	4096
S. SaintPaul	8	8	64	8	16	>512	4096
S. Stanley	8	8	16	8	32	128	4096
S. Newport	2	4	4	2	8	128	4096
S. Heidelberg	8	4	8	4	8	>512	4096
S. Kentucky	8	8	8	4	>64	64	>4096
S. Mbandaka	8	8	8	4	16	128	4096
S. Livingstone	8	8	32	8	>64	64	4096

T: Typhimurium. E: Enteritidis.

determined for rifampicin ranged from 8 to 64 μg/mL, with a particular higher resistance to this antibiotic of Kentucky, Livingstone and Infantis strains; against streptomycin, most of the strains showed values higher than 128 μg/mL, whereby the most sensitive strains belonged to the Enteritidis serovar (STCC 4155, 4396, 7160 and 7236). All strains displayed a MIC equal or superior to 4096 μg/mL against sulfonamides, except for *S.* Hadar, with a MIC of 2048 μg/mL. Regarding the comparison between serovars, and the comparison between intra-serovar and inter-serovar variability, and excluding *S.* Typhimurium 7162 from the analysis because of its high resistance levels, statistical analysis indicates that resistance to all the antibiotics studied would generally be comparable among *S.* Typhimurium, *S.* Enteritidis and all the other strains, as would be intra- and inter-serovar variability. In any case, it should be noted that resistance to ampicillin, chloramphenicol, and oxytetracycline on the part of the *S.* Enteritidis and Typhimurium strains featured in this study tended to be lower than that of the other serovars (analysed together) (Fig. 7).

It is well known that bacterial antibiotic resistance is determined by many factors, such as membrane permeability, the level of expression of various proteins, or the presence and expression of certain genes, among others (Beceiro et al., 2013). The resistance of microbial cells to a particular antibiotic would thus be due to the sum of the strain's intrinsic resistance, the resistance it has developed to attempt to adapt to the new stimulus, and the resistance it may have acquired by horizontal gene transfer (Schwarz et al., 2005). Although this also implies that the presence of a certain antibiotic resistance gene in two different strains does not mean that both will have the same degree of resistance to that antibiotic, the impact exerted by that presence (and expression) of certain genes/mutations upon microbial antibiotic resistance is more than well known, in any case, and has been extensively studied. Therefore, in order to try to determine if the differences in antibiotic resistance among strains were associated with the presence of any of those antibiotic genes, we decided to perform a basic *in-silico* analysis in which we searched for the presence of certain of those *Salmonella* resistance genes and/or mutations conferring resistance to the groups of antibiotics tested in this study, which are the same as those included in Li et al. (2019), in the publicly available genome sequences (NCBI and ENA) of the strains studied here. Unfortunately, to the best of our knowledge, the complete genome sequence (chromosome + plasmids if found) is only publicly available for seven of the 23 strains included in our study: *S.* Typhimurium SL1344, *S.* Typhimurium 443, *S.* Typhimurium 4594, *S.* Typhimurium 722, *S.* Saintpaul, *S.* Stanley and *S.* Newport. Our analysis revealed that none of the β-lactam, chloramphenicol, quinolone, tetracycline, or sulfonamide resistance genes studied was present in any of those seven strains. Similarly, no mutations in the *rpoB* gene, which have been linked to rifampicin resistance, were found (Brandis and Hughes, 2018). Only genes conferring resistance to aminoglycosides (*aac(6′)-Ib* and *aadA1*) were found in some of the strains, but no clear relationship between their presence and an increased resistance to streptomycin was ascertained (Supplementary Table 3). This almost complete absence of antibiotic resistance genes might be related to the fact that all of the strains used in this study were obtained from collections, and it would suggest that the differences in antibiotic resistance of these 7 strains would be more closely associated with differences in their intrinsic resistance. On the other hand, the sizeable differences between *S. Typhimurium* 7162 and all the other strains in terms of resistance to several of the antibiotics studied suggest either that this strain would have acquired this multiple resistance through horizontal gene transfer, or that its physiology/phenotypic characteristics would differ widely from all the other strains. Further work would be required to verify these two hypotheses.

Finally, multiple correlations have been obtained between the MIC values of the different strains (Table 2 and Supplementary Table 4). Multiple antibiotic resistance is usually attributed to the fact that genes conferring antimicrobial resistance in *Salmonella* are usually transported in integrons and plasmids (Chen et al., 2004; Schwarz et al., 2005); however, as previously pointed out, this does not seem to be the case, at least for some of the strains studied here. Despite the interest inherent in these obtained correlations, they lie outside of the scope of this article and will not be further discussed here.

3.5. Relationship between growth rates, virulence, phenotypic characteristics, and resistance to food processing-related stresses

We correlated the growth, virulence, biofilm-forming ability, and antibiotic resistance parameters ascertained in this study with one another and with the resistance parameters (*2D*-values) to different environmental stresses and food technologies as previously determined in Guillén et al. (2020a, 2020b) for the same set of strains. The rationale behind the choice of the *2D*-value parameter (time required to inactivate the first 2-Log10 cycles) was already discussed in Guillén et al. (2020a). The iterative Grubbs's test was applied to identify potential outliers that could exert a disproportionate influence on further data analysis and lead to non-valid conclusions. Grubbs's test detected multiple outliers:

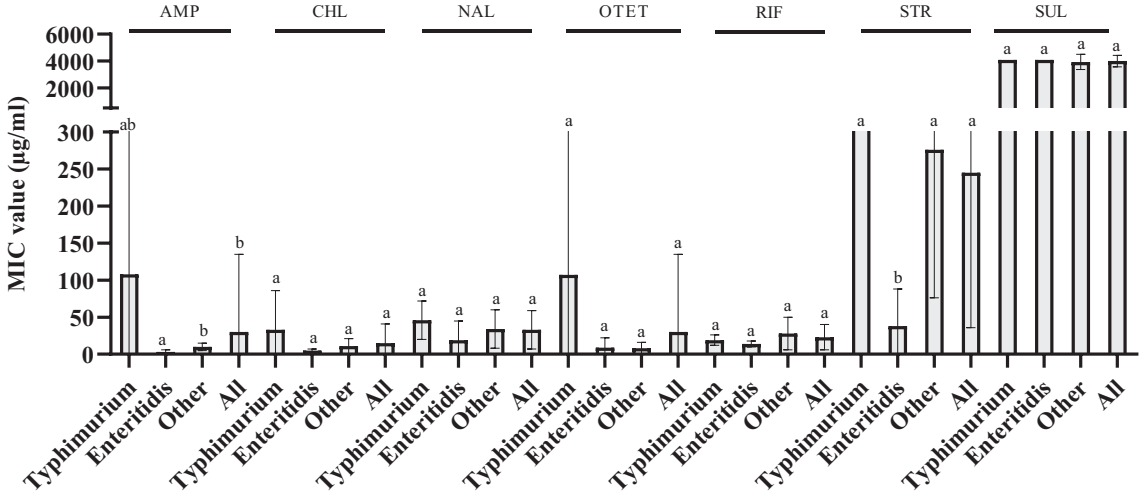

Fig. 7. Minimum inhibitory concentrations (MIC) for the different antibiotics of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella* strains studied (All). Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups. Ampicillin (AMP); Chloramphenicol (CHL); Nalidixic acid (NAL); Oxytetracycline (OTET); Rifampicin (RIF); Streptomycin (STR); Sulfonamide (SUL).

Table 2
Spearman's correlation coefficient values calculated for the *2D* resistance values (obtained in Guillén et al., 2020a, 2020b), μ_{max} values in the 3 media studied (see text), adhesion and invasion ability (%), biofilm formation capacity (AUC) and MIC values of each antibiotic of the 23 *Salmonella enterica* strains studied. Values in parentheses correspond to the *p*-value.

	pH	H_2O_2	NaCl	Heat	HHP	PEF	UV	TSB-YE	LB-DPY	M9-gluconate	Adhesion	Invasion	Biofilm	AMP	CHL	NAL	OTET	RIF	STR
pH		0.139	0.160	−0.251	**0.454**	0.134	−0.070	−0.194	0.279	−0.138	**0.433**	**−0.484**	**−0.447**	−0.117	−0.256	0.268	−0.163	−0.290	−0.049
		(0.526)	(0.467)	(0.260)	**(0.030)**	(0.541)	(0.750)	(0.376)	(0.197)	(0.530)	**(0.039)**	**(0.022)**	**(0.037)**	(0.603)	(0.250)	(0.215)	(0.467)	(0.179)	(0.826)
H_2O_2	0.139		0.311	−0.041	0.018	0.103	−0.042	−0.042	0.071	−0.282	−0.260	−0.018	0.012	−0.179	−0.294	−0.033	−0.242	0.002	−0.150
	(0.526)		(0.149)	(0.857)	(0.936)	(0.641)	(0.851)	(0.847)	(0.749)	(0.193)	(0.231)	(0.938)	(0.956)	(0.426)	(0.185)	(0.882)	(0.279)	(0.992)	(0.494)
NaCl	0.160	0.311		−0.256	0.009	**0.643**	0.282	0.156	**0.431**	**0.415**	−0.098	0.334	−0.115	−0.417	−0.313	0.009	−0.248	0.109	−0.380
	(0.467)	(0.149)		(0.250)	(0.968)	**(0.001)**	(0.193)	(0.478)	**(0.040)**	**(0.049)**	(0.656)	(0.128)	(0.610)	(0.054)	(0.156)	(0.967)	(0.267)	(0.621)	(0.074)
Heat	−0.251	−0.041	−0.256		−0.133	−0.281	0.324	0.227	−0.253	−0.157	−0.293	0.096	0.143	0.342	0.307	0.196	0.223	0.373	0.185
	(0.260)	(0.857)	(0.250)		(0.554)	(0.206)	(0.142)	(0.310)	(0.256)	(0.484)	(0.185)	(0.678)	(0.526)	(0.129)	(0.176)	(0.381)	(0.330)	(0.088)	(0.411)
HHP	**0.454**	0.018	0.009	−0.133		0.128	−0.140	−0.238	0.171	−0.154	−0.126	−0.290	−0.062	−0.226	−0.347	0.178	−0.216	−0.261	0.103
	(0.030)	(0.936)	(0.968)	(0.554)		(0.559)	(0.523)	(0.274)	(0.434)	(0.484)	(0.567)	(0.191)	(0.785)	(0.312)	(0.113)	(0.417)	(0.334)	(0.228)	(0.641)
PEF	0.134	0.103	**0.643**	−0.281	0.128		0.258	0.021	**0.430**	0.337	0.022	0.350	−0.191	−0.233	−0.163	0.164	0.218	0.239	−0.271
	(0.541)	(0.641)	**(0.001)**	(0.206)	(0.559)		(0.235)	(0.925)	**(0.041)**	(0.116)	(0.920)	(0.111)	(0.393)	(0.296)	(0.468)	(0.455)	(0.329)	(0.272)	(0.210)
UV	−0.070	−0.042	0.282	0.324	−0.140	0.258		0.226	0.255	0.270	−0.049	**0.426**	−0.097	0.187	0.128	0.028	0.193	0.404	−0.191
	(0.750)	(0.851)	(0.193)	(0.142)	(0.523)	(0.235)		(0.299)	(0.240)	(0.212)	(0.823)	**(0.048)**	(0.667)	(0.406)	(0.570)	(0.900)	(0.390)	(0.056)	(0.382)
TSB-YE	−0.194	−0.042	0.156	0.227	−0.238	0.021	0.226		0.382	**0.437**	0.024	**0.455**	0.233	**0.507**	**0.697**	0.222	**0.429**	**0.541**	0.168
	(0.376)	(0.847)	(0.478)	(0.310)	(0.274)	(0.925)	(0.299)		(0.072)	**(0.037)**	(0.914)	**(0.034)**	(0.297)	**(0.016)**	**(0.000)**	(0.309)	**(0.046)**	**(0.008)**	(0.443)
LB-DPY	0.279	0.071	**0.431**	−0.253	0.171	**0.430**	0.255	0.382		0.388	0.004	0.170	0.014	0.296	0.333	0.227	0.289	0.371	−0.192
	(0.197)	(0.749)	**(0.040)**	(0.256)	(0.434)	**(0.041)**	(0.240)	(0.072)		(0.067)	(0.986)	(0.449)	(0.950)	(0.181)	(0.130)	(0.298)	(0.191)	(0.081)	(0.381)
M9-gluconate	−0.138	−0.282	**0.415**	−0.157	−0.154	0.337	0.270	**0.437**	0.388		0.093	**0.436**	0.343	0.254	0.389	0.403	0.352	0.374	0.265
	(0.530)	(0.193)	**(0.049)**	(0.484)	(0.484)	(0.116)	(0.212)	**(0.037)**	(0.067)		(0.674)	**(0.042)**	(0.118)	(0.254)	(0.074)	(0.056)	(0.108)	(0.078)	(0.221)
Adhesion	**0.433**	−0.260	−0.098	−0.293	−0.126	0.022	−0.049	0.024	0.004	0.093		−0.115	−0.315	0.036	0.087	0.143	0.206	−0.021	0.115
	(0.039)	(0.231)	(0.656)	(0.185)	(0.567)	(0.920)	(0.823)	(0.914)	(0.986)	(0.674)		(0.610)	(0.154)	(0.874)	(0.702)	(0.515)	(0.358)	(0.924)	(0.601)
Invasion	**−0.484**	−0.018	0.334	0.096	−0.290	0.350	**0.426**	**0.455**	0.170	**0.436**	−0.115		0.020	0.100	0.256	0.125	0.371	0.338	−0.067
	(0.022)	(0.938)	(0.128)	(0.678)	(0.191)	(0.111)	**(0.048)**	**(0.034)**	(0.449)	**(0.042)**	(0.610)		(0.931)	(0.665)	(0.263)	(0.578)	(0.098)	(0.124)	(0.768)
Biofilm	**−0.447**	0.012	−0.115	0.143	−0.062	−0.191	−0.097	0.233	0.014	0.343	−0.315	0.020		**0.487**	**0.439**	0.206	0.362	0.357	0.273
	(0.037)	(0.956)	(0.610)	(0.526)	(0.785)	(0.393)	(0.667)	(0.297)	(0.950)	(0.118)	(0.154)	(0.931)		**(0.025)**	**(0.047)**	(0.357)	(0.107)	(0.103)	(0.220)
Ampicillin	−0.117	−0.179	−0.417	0.342	−0.226	−0.233	0.187	**0.507**	0.296	0.254	0.036	0.100	**0.487**		**0.814**	0.420	**0.758**	**0.471**	**0.481**
	(0.603)	(0.426)	(0.054)	(0.129)	(0.312)	(0.296)	(0.406)	**(0.016)**	(0.181)	(0.254)	(0.874)	(0.665)	**(0.025)**		**(0.000)**	(0.052)	**(0.000)**	**(0.027)**	**(0.023)**
Chloramphenicol	−0.256	−0.294	−0.313	0.307	−0.347	−0.163	0.128	**0.697**	0.333	0.389	0.087	0.256	**0.439**	**0.814**		**0.433**	**0.697**	**0.598**	0.329
	(0.250)	(0.185)	(0.156)	(0.176)	(0.113)	(0.468)	(0.570)	**(0.000)**	(0.130)	(0.074)	(0.702)	(0.263)	**(0.047)**	**(0.000)**		**(0.044)**	**(0.000)**	**(0.003)**	(0.134)
Nalidixic acid	0.268	−0.033	0.009	0.196	0.178	0.164	0.028	0.222	0.227	0.403	0.143	0.125	0.206	0.420	**0.433**		**0.459**	0.350	**0.566**
	(0.215)	(0.882)	(0.967)	(0.381)	(0.417)	(0.455)	(0.900)	(0.309)	(0.298)	(0.056)	(0.515)	(0.578)	(0.357)	(0.052)	**(0.044)**		**(0.032)**	(0.101)	**(0.005)**
Oxytetracycline	−0.163	−0.242	−0.248	0.223	−0.216	0.218	0.193	**0.429**	0.289	0.352	0.206	0.371	0.362	**0.758**	**0.697**	**0.459**		**0.474**	0.329
	(0.467)	(0.279)	(0.267)	(0.330)	(0.334)	(0.329)	(0.390)	**(0.046)**	(0.191)	(0.108)	(0.358)	(0.098)	(0.107)	**(0.000)**	**(0.000)**	**(0.032)**		**(0.026)**	(0.134)
Rifampicin	−0.290	0.002	0.109	0.373	−0.261	0.239	0.404	**0.541**	0.371	0.374	−0.021	0.338	0.357	**0.471**	**0.598**	0.350	**0.474**		0.019
	(0.179)	(0.992)	(0.621)	(0.088)	(0.228)	(0.272)	(0.056)	**(0.008)**	(0.081)	(0.078)	(0.924)	(0.124)	(0.103)	**(0.027)**	**(0.003)**	(0.101)	**(0.026)**		(0.932)
Streptomycin	−0.049	−0.150	−0.380	0.185	0.103	−0.271	−0.191	0.168	−0.192	0.265	0.115	−0.067	0.273	**0.481**	0.329	**0.566**	0.329	0.019	
	(0.826)	(0.494)	(0.074)	(0.411)	(0.641)	(0.210)	(0.382)	(0.443)	(0.381)	(0.221)	(0.601)	(0.768)	(0.220)	**(0.023)**	(0.134)	**(0.005)**	(0.134)	(0.932)	

Ampicillin (AMP); Chloramphenicol (CHL); Nalidixic acid (NAL); Oxytetracycline (OTET); Rifampicin (RIF); Streptomycin (STR).
Significant correlations are indicated in bold.

the *2D*-value to heat of *S.* Senftenberg 775W, invasion percentage of *S.* Enteritidis 7160, biofilm percentage of *S.* Senftenberg 775W, and the MIC values of *S.* Typhimurium 7162 for ampicillin, chloramphenicol, and oxytetracycline. These values, together with the MICs for sulfonamides, were therefore excluded from subsequent analysis. Several correlations ($p < 0.05$) and trends ($p < 0.10$) were found using the Pearson's and Spearman's tests (Table 2 and Supplementary Table 4); the most relevant ones will be discussed below.

It is widely assumed that stress resistance implies a fitness cost for bacteria, although its magnitude seems to depend on the nature of the stressing agent/microbial response triggered (Karatzas et al., 2008a, 2008b; Urdaneta et al., 2019). However, in our case, no significant (inverse) correlation between stress resistance and fitness cost was observed for the 23 strains studied here. Moreover, a correlation was found between Pulsed Electric Fields (PEF) and NaCl resistance and the growth parameters in LB-DYP and M9-Gluconate, but not in TSB-YE (Table 2 and Supplementary Table 4). Besides the fact that the results obtained here -with only 23 strains- cannot be directly extrapolated to the whole *Salmonellae*, we should also recall that variability in stress resistance and in growth rates among the strains of *Salmonella* were both low (less than 3-fold). This, together with the experimental variability inherent to these types of determinations, might be hindering the existence of such a relationship or might be leading to the appearance of casual, but not causal, relationships. Thus, further work would be required to elucidate the actual nature of such relationships, especially regarding the relationship between PEF, NaCl resistance, and growth rates in limited but non-selective media. In addition, it should be noted that although it has been demonstrated that deletion or overexpression of certain genes involved in stress resistance does have an impact on growth ability/rates (Sabater-Muñoz et al., 2015; Shetty et al., 2019; Spector and Cubitt, 1992), and, similarly, since a fitness cost has been observed in stress-resistant strains selected through repetitive exposure to stressing agents (Karatzas et al., 2008a, 2008b; Licciardello et al., 1969), one can expect that strains in real food scenarios with mutations causing a high fitness cost would soon be outcompeted if such genetic changes were not counterbalanced by compensatory mutations (Levin et al., 2000), unless the bacteria found a particular niche in which the alteration turned out to be profitable on a medium and long-term basis.

Regarding the relationship between stress resistance and virulence, results concerning acid resistance stand out. Thus, a positive correlation was found between acid resistance and adhesion rates (Spearman $r_s = 0.433$, $p = 0.039$), as well as a negative correlation with invasion rates (Spearman $r_s = -0.484$, $p = 0.022$). Exposure to acidic conditions results in the induction of different regulons, including Fur, PhoPQ, OmpR, RpoE, and RpoS, some of which play a role in the regulation of *Salmonella* virulence (Álvarez-Ordóñez et al., 2011; Muller et al., 2009), leading to the down-regulation of certain SPI-1 genes such as *SirA*, *HilA*, *HilC*, *HilD*, and *InvF* (Ellermeier et al., 2005; Kim et al., 2014; Ryan et al., 2015). Previous studies have reported that, as observed herein, exposure to acidic conditions can lead to a decreased invasion ability of *Salmonella* cells (Kim et al., 2014), which would probably be linked to that down-regulation of SPI-1 genes. On the other hand (as mentioned above), despite the fact that these virulence mechanisms are co-regulated, this latter phenomenon would not exert an influence on adhesion, which could be mediated by adhesion factors including pili, fimbriae, flagella, non-fimbrial adhesins such as SiiE, or other adhesins (Barlag and Hensel, 2015; Horstmann et al., 2020). Similarly, Karatzas et al. (2008a) observed that sustainable *Salmonella enterica* acid resistant variants, obtained through repeated cycles of acid challenge and growth, displayed an increased expression of SEF17 fimbriae, but a reduced virulence. Nevertheless, *S.* Enteritidis strains possessing a low pathogenicity (both in vitro and in vivo) as well as a low acid resistance have also been reported (Shah, 2014). Conversely to acid resistance, a correlation was found between *Salmonella* resistance to PEF ($r = 0.363$, $p = 0.097$) and to UV ($r_s = 0.426$, $p = 0.048$), on the one hand, and its invasion ability, on the other hand, although in the former case statistical analysis only indicated a trend. The resistance mechanisms of *Salmonella* to these technologies are still not very well known; therefore, it is difficult to hypothesize about the potential causes underlying this correlation. From the few data available, it can be remarked that RpoS has been suggested to play a role in *Salmonella* PEF and UV resistance (Child et al., 2002; Rice et al., 2015; Sagarzazu et al., 2013) and virulence, which might explain the data we have obtained. A more detailed review on the relationship between stress resistance, growth ability and virulence in non-typhoidal *Salmonellae* can be found elsewhere (Guillén et al., 2021).

Apart from acid stress, it is remarkable that no correlation between any of the studied stressing agents or growth ability and biofilm formation was observed. Similarly, Lianou and Koutsoumanis (2012) did not observed any relationship between the biofilm-forming ability and the growth kinetic behavior of the *Salmonella* strains they studied. No correlation among resistance to any of the food-related stresses and any of the studied antibiotics was found using Spearman's test. We found a negative correlation, however, between acid resistance and biofilm formation ability (Spearman $r_s = -0.447$, $p = 0.037$). It has been demonstrated that the acid-induced PhoPQ system would be a repressor of biofilm formation, which would explain the results obtained herein, although the precise role of PhoPQ in PhoPQ is still not fully understood (Prouty and Gunn, 2003; Steenackers et al., 2012; Tsai et al., 2020). By contrast, other authors have reported opposite results regarding the relationship between acid resistance and biofilm formation in *Salmonella*. Thus, Shah et al. (2011, 2012) found that, within a set of six *S.* Enteritidis strains, those not capable of forming biofilms were also the most acid-sensitive ones. Further studies will be required to explain these apparently contradictory results.

In addition to the comparisons established between *Salmonella* resistance to different food-related stresses and the other phenotypic characteristics, other relevant correlations were observed. We found a positive correlation between invasion and maximum growth rates in TSB-YE (Spearman $r_s = 0.455$, $p = 0.034$) and in M9-Gluconate ($r_s = 0.436$, $p = 0.042$; $r = 0.447$, $p = 0.037$); surprisingly, however, no association was found between maximum growth rates in LB-DYP and virulence, even though the relationship between Fe availability (both after and during invasion) and *Salmonella* virulence ability has already been demonstrated, and even though, in our study, a correlation between growth rates in the three media was observed (see above). It is well known that in a nutrient-rich medium, *Salmonella* activates the expression of genes encoding effector proteins such as HilD and HilC, thereby activating *hilA* expression, or InvF, which controls the expression of SPI-1 and SPI-2 (Ellermeier et al., 2005); it is also well known that nutrient limitation is a key regulatory signal for certain virulence genes in *Salmonella* (O'Neal et al., 1994). Thus, for instance, the expressions of SPI-1 and SPI-2 are induced in vitro by limiting concentrations of potassium or magnesium (Bustamante et al., 2008; Kröger et al., 2013). Although it seems clear that an increased growth rate and/or ability to compete for nutrients would provide *Salmonella* cells with an advantage once inside the gut and would therefore make these strains more virulent, we know of no previous study indicating a correlation between growth ability/rates and invasivity in vitro.

A negative correlation between adhesion rate and biofilm formation capacity ($r = -0.434$, $p = 0.043$) was also found. Even though adhesion to Caco-2 cells and to a polystyrene surface are analogous processes, the characteristics of the surface and the structures/metabolic pathways involved are not the same (although some participate in both phenomena); in addition, the formation of biofilms is a multi-step process in which adhesion is only one of the phases (Peng, 2016). Other correlations were found, such as the one between ampicillin ($r_s = 0.487$, $p = 0.025$) or chloramphenicol ($r_s = 0.439$, $p = 0.047$) and biofilm formation capacity, or the one between growth rates and antibiotic resistance (Table 2 and Supplementary Table 4), but the discussion thereof lies outside the scope of this article.

Finally, it should be noted that the results obtained would be similar if the outliers are included in the analysis, with only some minor

exceptions (Supplementary Table 5). The most relevant consequence from including them is, probably, that a correlation between heat and hydrogen peroxide resistance and biofilm formation would be found ($r = 0.875$, $p = 0.000$; $r = 0.490$, $p = 0.048$, respectively). This is due to the fact that the *S.* Senftenberg 775W strain here studied is the most heat and hydrogen peroxide resistant and also the one with the highest ability to form biofilms. The high heat resistance of this particular *S. Senftenberg* 775W strain is well known (Ng et al., 1969), but this is a strain-specific and not serovar-specific trait, whereas the high ability to form biofilms seems to be a serovar-specific characteristic (Xia et al. (2009)) what suggest that this correlation –between heat resistance and biofilm formation ability- is just casual. In any case, the mechanisms responsible for the high heat and hydrogen peroxide resistance of this strain, as well as for the high capability to form biofilms of the serovar Senftenberg deserve further study. Including this strain in the analysis also changed the significance of the correlations between acid pH resistance and biofilm forming ability (from $r_s = -0.447$, $p = 0.037$ to $r_s = -0.365$, $p = 0.087$) and between the later and adhesion (from $r_s = -0.434$, $p = 0.043$ to $r_s = -0.346$, $p = 0.106$). On the other hand, the highly invasive *S.* Typhimurium STCC 7160 strain is also one of the most acid-resistant ones and, therefore, including it in the analysis results in the disappearance of the correlation between acid resistance and invasion ability. Also some changes in the correlations between antibiotic resistance and 1) stress resistance (appearance of a significant correlation between ampicillin and heat resistance), 2) growth ability (a positive correlation between oxytetracycline resistance and growth in M9-broth supplemented gluconate) and 3) biofilm forming ability (a positive correlation between resistance to oxytetracycline and biofilm forming ability) occurred –mainly- due to the inclusion in the analysis of the antibiotic multi-resistant strain *S.* Typhimurium STCC 7162.

4. Conclusions

In this study we determined the maximum growth rates in different media, the ability to adhere to and invade Caco-2 cells, biofilm formation capacity, and antibiotic resistance of 23 *Salmonella* strains belonging to 15 different serovars, and compared these traits with the resistance of the 23 strains to different food-related stresses.

Significant differences in growth rates among strains, as well as depending on the growth medium/condition assayed, were observed. Our results suggest that those strains displaying a higher growth rate under non-limiting conditions also display a higher growth rate in media with reduced amounts of Fe, or with gluconate as the only carbon source. However, less than a 3-fold difference between the lowest and the highest growth rate (determined in the same medium) was observed. Statistical analysis also revealed that the type of strain under study had a significant effect on the rate of adhesion and the rate of invasion; however, no correlation was found, i.e. strains with a high adhesion ability were not always the most invasive ones.

Conversely to that described for growth rates, the ability to form biofilms varied widely: more than 30-fold among the different strains studied. Finally, regarding antibiotic resistance, and if *S.* Typhimurium 7162 is excluded from the analysis, variability among strains in terms of resistance depended on the antibiotic studied, with 8-fold differences in the MIC of ampicillin, chloramphenicol, and rifampicin, but more than 64-fold for nalidixic acid.

On the other hand, our results suggest that, in general terms, the higher stress resistance of some strains/serovars did not impose a fitness cost to them. Similarly, no association was found between stress resistance and biofilm formation ability (except for acid stress) or antibiotic resistance (except for two cases: UV-rifampicin and H_2O_2-oxytetracycline, inverse in the latter case). Our data also suggest that acid stress resistance is associated with virulence in *Salmonella*, since a positive correlation of acid stress resistance with adhesion and a negative one with invasion was found. In any case we remind the reader that this is only an observational study, and that further work would be required to verify the existence (or absence) of these relationships, along with associated underlying mechanisms.

Data reported herein would also be helpful in developing predictive models of *Salmonella* growth, and for improving quantitative microbiological risk assessments (QMRA) of *Salmonella* in food products. Thus, results here reported not only provide an estimation of the intra and inter-serovar variability in growth, stress resistance and virulence within non-typhoidal *Salmonellae*, which is of the highest relevance for QMRA, but they also help to identify strains that might potentially suppose a higher risk for food safety because of their higher fitness, stress resistance or virulence (e.g. *S.* Senftenberg 775w because of its high heat resistance and biofilm forming ability). Further work will be required in order to determine the mechanisms responsible for the differences in fitness, stress resistance and virulence among *Salmonellae,* especially those conferring some strains very particular characteristics, and to determine if these strains suppose a higher risk or not.

CRediT authorship contribution statement

Silvia Guillén: Investigation, Methodology, Formal Analysis, Writing-Original draft preparation. **María Marcén**: Investigation, Writing - Review & Editing. **Ester Fau**: Investigation, Writing - Review & Editing. **Pilar Mañas**: Conceptualization, Writing - Review & Editing. **Guillermo Cebrián**: Conceptualization, Writing - Review & Editing, Supervision.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109462.

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