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Comparative stress response to food preservation conditions of ST19 and ST213 genotypes of *Salmonella enterica* serotype Typhimurium

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ABSTRACT

The replacement of the most prevalent *Salmonella enterica* genotypes has been documented worldwide. Here we tested the hypothesis that the current prevalent sequence type ST213 of serotype Typhimurium in Mexico has a higher resistance to stressful food preservation conditions than the displaced sequence ST19. ST19 showed higher cell viability percentages than ST213 in osmotic (685 mM NaCl) and acidic (pH 3.5) stress conditions and in combination with refrigeration (4 °C) and ambient (≈22 °C) temperatures. Both genotypes showed the same poststress recovery growth. ST213 formed biofilm and filamentous cells (FCs) under stress, whereas ST19 did not. ST213 cells also showed higher motility. The capacity of ST213 to form FCs may explain its lower viability percentages when compared with ST19, i.e., ST213 cells divided less under stress conditions, but FCs had the same recovery capacity of ST19 cells. ST213 presented a higher unsaturated/saturated fatty acids ratio (0.5–0.6) than ST19 (0.2–0.5), which indicates higher membrane fluidity. The transcript levels of the *rpoS* gene were similar between genotypes under the experimental conditions employed. Biofilm formation, the generation of FCs, cell motility and membrane modification seem to make ST213 more resistant than ST19 to food preservation environments.

1. Introduction

Salmonella enterica is one of the main food-borne pathogens that affects humans and farm animals. In humans, *S. enterica* causes gastroenteritis and other diseases, such as bacteremia and endovascular and localized infections (Crum-Cianflone, 2008); thus, *S. enterica*-related diseases continue to have a significant impact on morbidity, mortality and economic costs worldwide (Kirk et al., 2015). It is estimated that approximately 94 million cases of salmonellosis occur each year, and Typhimurium and Enteritidis are the most frequent serotypes associated with food contamination (Majowicz et al., 2010). During its infection cycle, this bacterial pathogen lasts for prolonged periods outside the host and is exposed to harsh environmental conditions (Spector and Kenyon, 2012). In particular, food matrices constitute complex microenvironments where bacteria face extreme pH, osmolarity, and temperature, or sudden changes in physicochemical variables that may be lethal (Álvarez-Ordóñez et al., 2015). However, *S. enterica* has the ability to adequately overcome these adverse surroundings through a wide variety of genetic, biochemical and physiological responses that allows it to adapt and survive. Within populations that are submitted to stress, the bacterial cells that survive are usually able to colonize and invade the host intestinal mucosa via contaminated food (Spector and Kenyon, 2012). Among the stress survival strategies described, *S. enterica* induces biofilm formation (Lianou and Koutsoumanis, 2012; Steenackers et al., 2012), generates filamentous cells (Mattick et al., 2000), and modifies both its membrane composition and fluidity (Álvarez-Ordóñez et al., 2008). The switch in transcription levels of adequate gene sets go along with these cellular and biochemical responses to stress. Within the set of genes that participate in different stress environments, the alternative sigma factor RpoS/σ^S

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Table 1
Bacterial strains used in this study.

Strain	Source	Serotype	Sequence Type (ST)	Location of isolation
ATCC 14028	Tissue, animal-pools of heart and liver from 4-week-old chickens	Typhimurium	19	–
SAL004	Cheese	Typhimurium	19	Ciudad Hidalgo, Michoacán, Mexico
SAL115	Cheese	Typhimurium	213	Tanhuato, Michoacán, Mexico

and σ^S-dependent genes are associated and work to coordinate stress resistance (McMeechan et al., 2007; Robbe-Saule et al., 2003). Furthermore, some stress responses are involved in the severity of damage to the host during the infection process and result in crosstalk between the pathogen's stress resistance and virulence (Altier, 2005; Álvarez-Ordóñez et al., 2012).

Globally, the epidemiological data of *S. enterica* shows a dynamic pathogen with continuous regional turnover in serotype prevalence (Galanis et al., 2006; Herikstad et al., 2002; Olsen et al., 2001). Recent findings have documented the pathogen dynamics at a more subtle level by describing the replacement of genotypes as determined by sequence type (ST) within prevalent serotypes associated with different geographical areas. For instance, the ST313 genotype has the highest incidence among invasive non-typhoidal *Salmonella* (iNTS) in sub-Saharan Africa; replacing the ST19 genotype, prevalent in other geographical areas (Feasey et al., 2012; Okoro et al., 2012). Recently, the change in genotype prevalence occurred in Guangdong, China, where ST34 is one of the most prevalent in this region (Sun et al., 2014). The advantages of assessing the epidemiological dynamics of *S. enterica* at the genotype level using STs instead of serotypes have been recently highlighted (Achtman et al., 2012). Furthermore, the ST genotype of *S. enterica* using a 7 loci protocol has high congruence with whole genome typing (Bale et al., 2016; Larsen et al., 2012).

Genotype replacement of the *S. enterica* serotype Typhimurium has also been observed in Mexico. Even though ST19 strains were prevalent in clinical and food samples in 2002, ST213 genotype strains became prevalent in 2005 (Wiesner et al., 2009). We observed this same replacement tendency in contaminated food samples in 2008–2011 (unpublished data). Far from being only a local public health concern, such replacement has global consequences because ST19 is the most abundant genotype of the Typhimurium serotype worldwide and seems to be the founder genotype of the remaining Typhimurium strains. Although the ST213 genotype was initially reported in Mexico as a predominant genotype (Achtman et al., 2012; Wiesner et al., 2009), it has also begun to be found at low incidence in other countries in North America, Europe and Asia (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Furthermore, it has been recently documented to have significant differences in virulence among clinical strains of both genotypes (Wiesner et al., 2016).

Despite growing evidence on the worldwide replacement of formerly prevalent serotypes/genotypes in different countries or geographical areas, the underlying genetic, biochemical and physiological differences between strains involved in such dynamics have not been evaluated. This lack of knowledge hampers optimization of strategies for control of food contamination and public health surveillance programs because exposure to environmental stress and antimicrobial conditions generated by the host defenses, provide selective advantages for the survival of resistant strains. This also drives horizontal gene transfer that leads to the evolution of virulence, which contributes to the occurrence of outbreaks caused by a particular serotype or genotype of a pathogen (Arnold et al., 2007).

We hypothesize that the replacement of ST19 by ST213 as the predominant genotype isolated from contaminated food in Mexico and its recent spread worldwide might be partially explained by its superior stress resistance. Particularly, the ST213 genotype might resist and survive better than the ST19 genotype in food preservation conditions such as low water activity (a_w), acidic pH, and refrigeration temperatures (4 °C). Thus, this study might help to better understand *S. enterica* adaptive responses and evolution associated with food chains and man-made generated environments with relevance to public health.

2. Materials and methods

2.1. Bacterial strains

The strains of serotype Typhimurium were provided by the State Public Health Laboratory of Michoacán and include ST213 (SAL115) and ST19 (SAL004). Sequence typing assignation was performed by 7 loci MLST and according to the protocols described at the MLST website (http://mlst.ucc.ie/mlst/dbs/Senterica/documents/primersEnterica_html). The numbers for alleles and sequence types (STs) were assigned according to the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The reference *S. enterica* strain (ATCC14028) serotype Typhimurium was used as the control. Relevant information for each strain is shown in Table 1.

2.2. Culture conditions

The strains were cultured overnight at 37 °C and 180 rpm in 10 ml Luria-Bertani (LB) broth to obtain the inoculum for stress assays and as control conditions to contrast stress responses. The culture conditions to simulate stress associated with food preservation, including reduced water activity (ra_w), low pH and refrigeration temperature (4 °C), were performed as previously described (Humphrey et al., 2011), and their combinations were also tested. Stress broth were prepared by modifying LB medium with NaCl and 1 M HCl until we achieved the desired salt concentration and pH as follows: NaCl 685 mM (LB ra_w), pH 3.5 (LB ac) and the combination of NaCl 685 mM and pH 3.5 (LB ra_w-ac). Bacterial cultures were prepared by inoculating a single colony in 10 ml of LB broth and incubating overnight at 37 °C and 180 rpm. The culture was centrifuged at $1360 \times g$ for 4 min and the pellet was resuspended in 10 ml of the corresponding stress broth. The cultures were statically incubated at room temperature (≈22 °C) or at refrigeration temperature (4 °C) for 8 days.

2.3. Viability count

The cell viability was determined using the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA). This kit contains a 1:1 (v/v) mixture of SYTO-9/propidium iodide markers capable of staining nucleic acids. The strains were stained according to the manufacturer's instructions. The percentage of live and dead bacteria was evaluated using a BD Accuri™ C6 flow cytometer.

2.4. Poststress recovery assay

After incubation under stress conditions, the cultures were centrifuged ($1360 \times g$) for 10 min, the cell pellet was resuspended and diluted 1:200 (v/v) in 10 ml LB broth and incubated at 37 °C at 180 rpm for 6 h to allow cell recovery. To determine the number of colony-forming units (CFUs), serial dilutions of the culture were made every 2 h and 100 µl of the culture was spread on LB agar plates and incubated for 24 h at 37 °C. After the incubation period, the CFUs were determined. All of the recovery assays were conducted six times.

2.5. Cell filamentation observations

The studied strains were submitted to reduced water activity (ra_w) stress conditions as previously described and incubated statically both at room and refrigeration temperature for 8 days. After the stress period, the changes in cell morphology were assessed by Gram staining of the cells viewed in a Leica microscope equipped with a Leica ICC50 HD digital camera. The images were captured and processed using Leica Application Suite 3.00 software. The percentage of filamentous cells in each genotype were quantified by counting 100 cells in nine different microscopic fields for control and each stress condition. The sizes of the elongated cells were viewed at 100× magnification. Cells that were longer than three normal cell lengths (2–3 µm) were considered filamentous.

2.6. Biofilm formation and viability

The biofilm formation was quantified using a modified crystal violet staining colorimetric assay (Burton et al., 2007) as follows. Bacteria were incubated in 96-well plates under stress conditions, and after 8 days, the broth was gently removed, and each well was washed three times with 200 µl of PBS and then allowed to dry for 15 min. Bound cells in each well were stained with 200 µl of crystal violet (0.4% v/v) for 15 min at room temperature. The unbound dye was removed, and wells were washed gently three times with 200 µl of PBS. The plate was dried for 15 min and the crystal violet in each well was solubilized by adding 200 µl of 33% (v/v) acetic acid. The plate was read at 595 nm using a Microplate Reader iMark (Bio-Rad, USA).

To determine the biofilm bacterial viability, an MTT colorimetric assay based on the reduction of tetrazolium salts was used according to Trafny et al. (2013) with minor modifications. Briefly, the strains were incubated in 96-well plates under stress conditions, and after 8 days, the medium was gently removed, and the wells were washed three times with 200 µl of PBS and allowed to dry for 15 min. A total of 190 µl of PBS and 10 µl of 5 mg/ml of 3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma, USA) solution was added and then incubated 2 h at 37 °C in darkness. After incubation, the MTT solution was removed, and 200 µl of isopropanol/HCl (19:1 v/v) was added to dissolve the formazan crystals and then incubated for 15 min at room temperature. The plate was shaken for 60 s and read at 595 nm using a Microplate Reader iMark (Bio-Rad, USA). Biofilm formation and viability assays were conducted nine times.

2.7. Cellulose and curli production

The production of cellulose and curli was determined as described by Aya-Castañeda et al. (2015). The production of curli was evaluated using LB agar without NaCl and supplemented with 40 mg/l of Congo red and 20 mg/l brilliant blue. A total of 10 µl of bacterial LB culture with an OD_{600} of 0.6 was used to inoculate agar plates and incubated for 8 days at 28 °C; afterwards, the colony morphology was observed. To confirm the production of cellulose, bacteria were inoculated onto LB agar plates without NaCl containing 200 mg/l of calcofluor and incubated at 28 °C for 48 h. The fluorescence emitted under UV light (366 nm) indicates cellulose production by the strains. This assay was conducted six times.

2.8. Cell motility

To evaluate cell motility, a swimming assay was performed for each strain. LB agar plates (0.3% agar) were inoculated in the center with overnight cultures. The inoculated plates were incubated at 37 °C for 8 h. The visible region of the colony propagated in the agar was measured in millimeters (Martins et al., 2013). This assay was conducted nine times.

2.9. Fatty acid analysis

The fatty acid analyses were conducted from bacterial cultures obtained as previously described only in cultures without stress and those stressed with pH 3.5 and incubated statically at room temperature or refrigeration for 8 days. After the stress period, the cultures were centrifuged at 1360 × *g* for 4 min, and the pellet was resuspended in 1 ml of distilled water. Fatty acid profiles were obtained using the four-step fatty acid extraction procedure: i) saponification, ii) methylation, iii) extraction and iv) base wash (Jensen et al., 2013). Fatty acid methyl ester analyses were performed with an Agilent 6890 Plus Chromatograph (Agilent, Palo Alto, CA, USA) and Sherlock System Software 4.0.

2.10. Quantitative reverse transcriptase PCR (qRT-PCR)

The RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and used as a template for reverse transcription-PCR. Complementary cDNA was synthesized using random hexamer primers (Invitrogen, USA). The expression analysis of the *rpoS* gene was performed with qPCR using the comparative Ct method (ΔΔCt) in a StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and according to the manufacturer's instructions. The reactions were conducted with a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The primers were synthesized by Elim Biopharmaceuticals Inc. (Hayward CA, USA), and their specificity was determined by endpoint PCR. The 16S rRNA gene was used as an internal control. The primer sequences were 16S rRNA: 5′ CAGAAGAAGCACCGGCTAAC 3´/5′ GACTCAAGCCTGCCAGTTTC 3´ (Yang et al., 2014) and *rpoS*: 5′ GTTGGACGCGACTCAGCTTT 3´/5′ TTTTACCACCAGACGCAGGTT 3´ (Wang et al., 2016). The results were normalized using the *S. enterica* (ATCC14028) strain in stationary-phase (calibrator treatment).

2.11. Data analysis

Data were obtained from three independent experiments and compared by analysis of variance (ANOVA) with Tukey's multiple comparisons test. The results were reported as the mean ± standard error (SE). *P*-values of < 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism V6.0 software.

3. Results

3.1. Viability under stress and poststress recovery of S. enterica genotypes

All of the tested stress conditions decreased the viability of ST19, ST213 and the reference strain when compared with incubation in nonstress conditions (Fig. 1). Under simulated refrigeration (4 °C), the combination of LB ra_w-ac strongly affected the viability of the ST213 strain (38.15%), whereas the ST19 strain was the most affected with the LB ac condition (65%). When incubated at room temperature, strain ST213 had a lower percentage of viability in each of the tested stress conditions (LB ra_w: 52%, LB ac: 29.7%, and LB a_w-ac: 60.5%) compared to ST19 (LB ra_w: 67.6%, LB ac: 75.5%, and LB a_w-ac: 86.8%). The cell viability of the ATCC 14028 strain was higher than that of ST19 and ST213 in LB ra_w (82%) and LB ac (90%) at room temperature. However, it decreased significantly for LB a_w-ac (28%), whereas in refrigeration it was similar to the ST19 strain except for the LB pH condition, where it showed better viability (79%) than ST19 (65%). After 8 days of stress, the ST213 strain had lower viability values than the ST19 strain in all of the tested conditions, except for the refrigeration conditions at low a_w, where there was no difference, and at pH 3.5, where the ST213 strain had higher viability values than the ST19 strain.

Subsequently, the ability of the strains to recover from stress in LB liquid medium was evaluated during a 6 h period (Fig. 2). The ATCC 14028, ST19 and ST213 strains showed similar recovery kinetics under

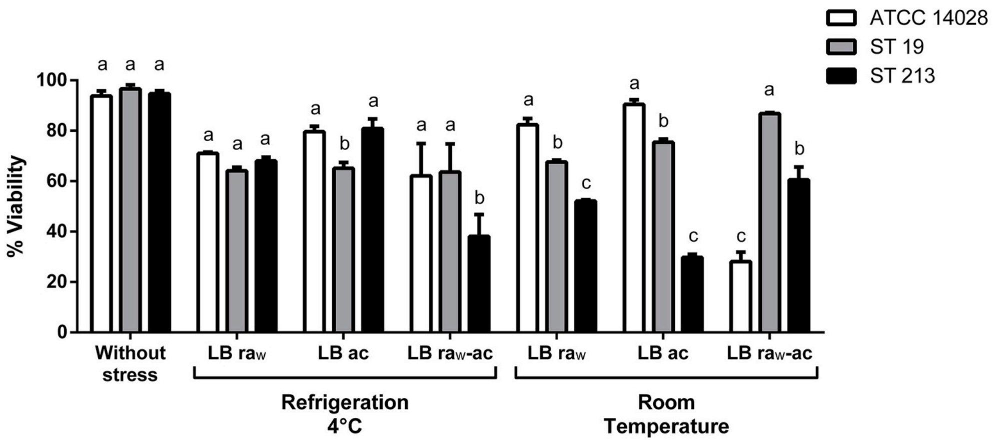

Fig. 1. Viability of genotypes of *Salmonella enterica* serotype Typhimurium after incubation in stress conditions. The *S. enterica* strains ATCC 14028, ST19 and ST213 were exposed to low water activity (LB ra_W), pH 3.5 (LB ac) and their combination (LB ra_W-ac) and incubated at refrigeration (4 °C) or room temperature (≈22 °C) for 8 days. The data represent the mean ± S.E. (n = 6). ANOVA with Tukey's multiple comparisons was used. Different letters represent significant difference in each condition tested ($p < 0.05$).

all stress conditions, and no statistically significant differences were found ($p < 0.05$), except for the LB ra_w-ac at room temperature. In such conditions the ST213 genotype strain showed lower recovery than both ST19 strains (Fig. 2g). All of the recovery kinetics showed growth after 2 h, but they were slower in cells of both environmental strains (ST19 and ST213) coming from 4 °C under LB ra_w-ac (Fig. 2f) than in cells coming from room temperature at the same conditions (Fig. 2g). The ATCC 14028 strain showed slower recovery growth in LB ac at the refrigeration temperature (Fig. 2d).

3.2. Cell filamentation in response to stress

The capacity of both genotypes to form filamentous cells at low a_w and at both at 4 °C and room temperature was evaluated. The results show that the ST19 genotype was not able to form filamentous cells under any of the tested conditions, whereas the ST213 strain formed filaments at low a_w in both temperatures and in normal LB at 4 °C (Fig. 3). The ATCC 14028 reference strain is also able to form filamentous cells at low a_w at both temperatures, which showed a similar percentage of filamentous cells as ST213 under the same conditions (i.e., below 10% for both strains). There was an increase in filamentous cells in the LB a_w condition compared to the condition with no osmotic stress for the ST213 and ATCC 14028 strains, independent of temperature.

3.3. Ability of ST19 and ST213 genotypes to establish viable biofilm under stress

The ST213 strain was able to form biofilms under all of the evaluated incubation conditions and formed the largest amount at 4 °C compared with the other evaluated strains (Fig. 4a). However, under LB a_w-ac stress in both temperatures, its ability to establish a biofilm was significantly diminished. However, ST19 was not able to form biofilm under any of the conditions evaluated, whereas the reference strain formed biofilm only in LB and LB ac at room temperature, which indicated that both refrigeration and NaCl affects its ability to establish biofilm. To determine if the cells within formed biofilms were viable under stress, their metabolic activity was evaluated by the MTT assay (Fig. 4b). Biofilm cells from the ST213 strain were metabolically active, although at 4 °C, the metabolic activity was lower in comparison to room temperature. The reference strain was metabolically active in LB and LB ac at room temperature. The Pearson correlation coefficient of 0.7646 ($p < 0.001$) clearly indicates that those strains able to establish the biofilm under the stress tested conditions remained viable after 8 days of incubation.

3.4. Colony morphotype and cell motility of S. enterica genotypes

The cellulose synthesis and curli fimbriae expression are important components in biofilm formation, which might be identified by determining the morphotype of the strains. The bdar (brown, dry and rough) morphotype was observed in all three strains, which indicated their capability to produce only curli fimbriae. In addition, the production of cellulose can be visualized by growing strains on agar containing calcofluor, but no fluorescence was observed under UV light for any of the three strains, which confirmed their inability to produce cellulose (Fig. 5). Other cellular structures related to biofilm formation are the flagellum components and strain motility. In this regard, a greater motility was observed in the ST213 strain (37.6 mm) in comparison with ST19 (2.1 mm) and reference (4.5 mm) strains (Fig. 6).

3.5. Plasma membrane fatty acid modifications in response to stress

The fatty acid composition of the studied strains grown in control conditions and in LB ac at 4 °C and room temperature for 8 days was evaluated. Nine major peaks were found for three strains in all of the tested conditions (Table 2), which corresponds to approximately 90% of the total fatty acids detected. Such shared fatty acids were identified as dodecanoic acid (C12:0), tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), hexadecenoic acid (C16:1), heptadecanoic acid (C17:0), methylene hexadecanoic acid (C17:0 cyc), octadecanoic acid (C18:0), octadecenoic acid (C18:1) and methylene octadecenoic acid (C19:0 cyc). Under nonstress conditions, both the reference strain and the ST19 genotype showed a higher percentage of saturated fatty acids (SFA) when compared to ST213. The reference and ST19 strains showed no internal differences in fatty acid composition between the nonstress and LB ac at 4 °C incubation conditions. However, when both strains were incubated in LB ac at room temperature, their percentage of SFA decreased, whereas the content of unsaturated fatty acids (UFA) increased. In the case of the ST213 genotype, LB ac at the 4 °C stress level induced a significant decrease in the content of SFA, but no fatty acids composition change was shown in acid stress at room temperature compared to the nonstress condition. Interestingly, it was observed that the increases in C17:0 cyc and C19:0 cyc percentages were associated with the decrease in the C16:1 and C18:1 percentages, respectively. Finally, the UFA/SFA ratio was used as an indirect indicator of membrane fluidity. In this sense, strain ST213 showed higher values of the UFA/SFA ratio (Fig. 7) with only acid stress at 4 °C, which decreased this rate.

3.6. Expression of the rpoS gene

All of the studied strains significantly increased *rpoS* gene transcription under the evaluated stress conditions when compared with the

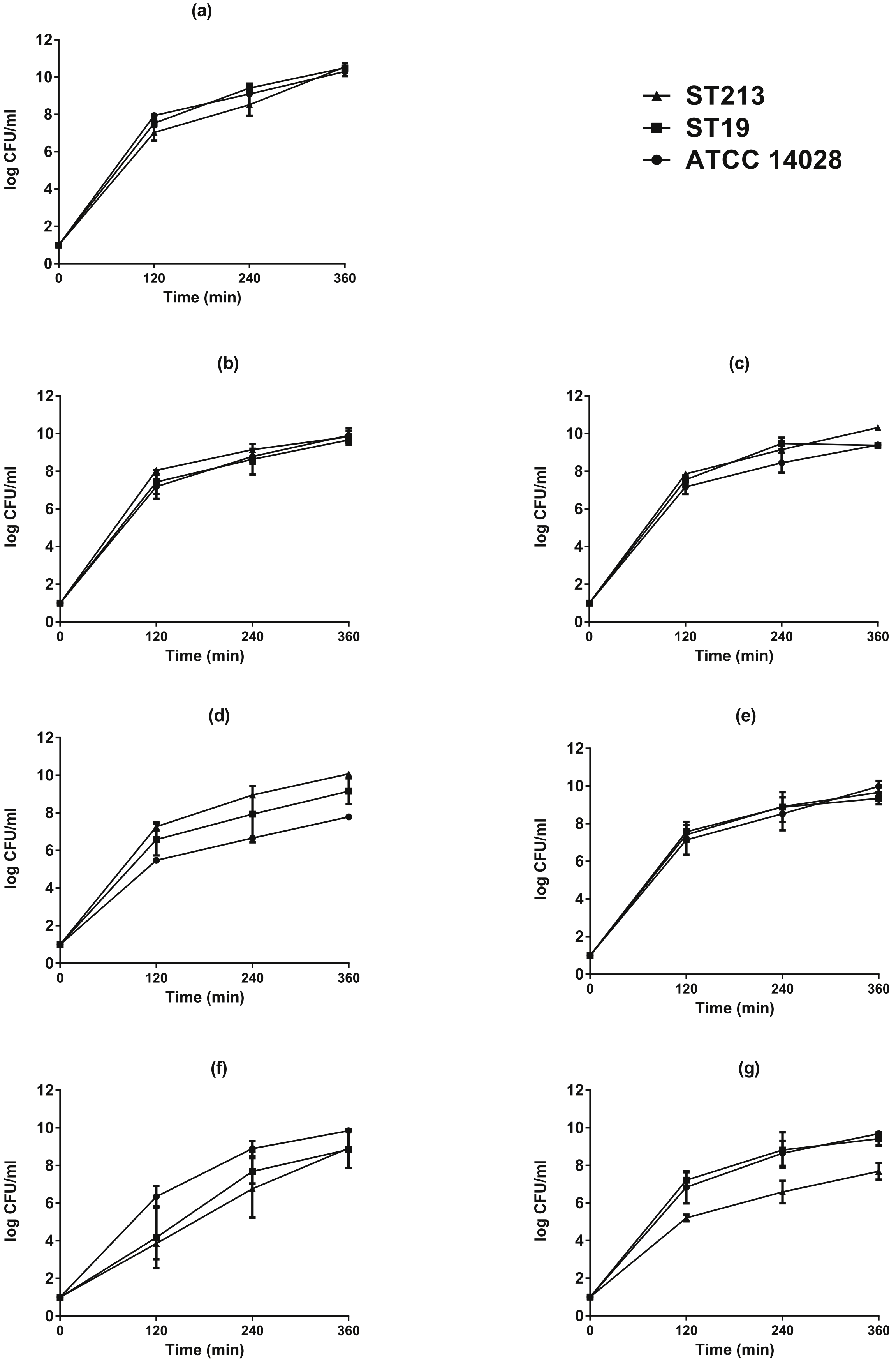

Fig. 2. Recovery of ST19 and ST213 *Salmonella enterica* serotype Typhimurium genotypes after stress. After the stress conditions: (a) non-stressed, (b) low aw at 4 °C, (c) low a_w at room temperature, (d) pH 3.5 at 4 °C, (e) pH 3.5 at room temperature, (f) low a_w-pH 3.5 at 4 °C and (g) low a_w-pH 3.5 at room temperature, recovery on liquid LB medium of *Salmonella enterica* serotype Typhimurium strains ATCC 14028, ST19 and ST213 for 6 h was monitored. The data represent the mean ± S.E. (n = 6).

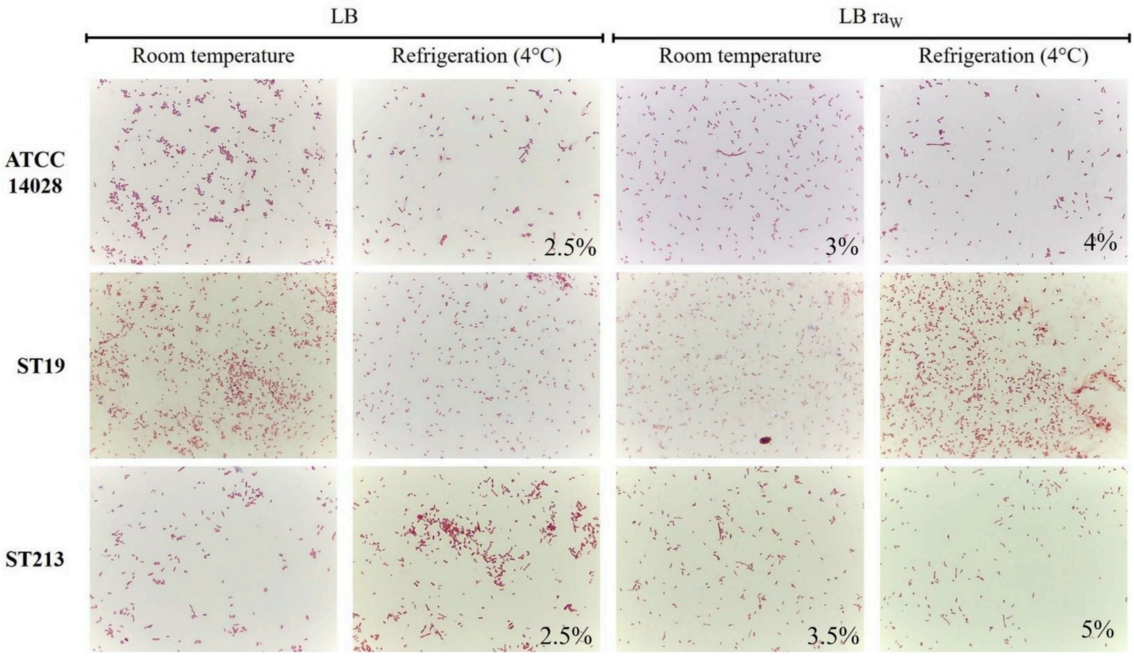

Fig. 3. Cell filamentation of ST19 and ST213 genotypes under stress conditions. *S. enterica* serotype Typhimurium strains, ATCC 14028, ST19 (SAL004) and ST213 (SAL115) were subjected to low a_w in both refrigeration and room temperature for 8 days. Changes in cell morphology were assessed by gram staining. Cells longer than three normal cell lengths were considered filaments. The percentage of filamentous cells in each tested condition is indicated in its respective image. The images are representative of nine independent assays (n = 9).

respective stationary phase cultures used as inoculum of the stress assays (Fig. 8). These high expression levels remained in the three strains after 2 h of poststress recovery. The expression of the *rpoS* gene in the stress conditions was higher in the reference strain at 1748-fold for acid pH at room temperature and 1461-fold induction for refrigeration when compared to the nonstress and stationary phase cultures. Regarding ST19, the induction was 114- and 315-fold, whereas ST213 obtained induction at 402- and 210-fold for acid pH at room temperature and refrigeration, respectively. In overnight culture in the stationary phase, both the ST19 (7-fold) and ST213 (9-fold) strains show higher mRNA levels of this gene compared with the reference strain. At 2 h of poststress growth, the transcription levels of this gene in the reference strain were higher than those observed in the stationary phase (241-fold), whereas in the case of the ST19 and ST213 strains, the values obtained were 306- and 342-fold, respectively. The fact that the *rpoS* gene showed high transcription levels, particularly in the reference strain at acidic conditions both under refrigeration and at ambient temperatures, agrees with the high viability percentages observed in this strain. Conversely, both ST19 and ST213 genotypes had higher transcription levels of the *rpoS* gene in the stationary phase.

4. Discussion

All of the studied cellular responses were associated with stress resistance in *S. enterica*. However, to the best of our knowledge, cellular differences in the stress responses between the involved genotypes in prevalent genotype replacement within a geographic area have not been previously reported.

Even though it was initially unexpected, the ST213 strain presented the lowest viability in most of the tested stress conditions. *S.* Typhimurium cells can enter a viable but nonculturable (VBNC) state when subjected to osmotic (Kusumoto et al., 2012), thermal (Reissbrodt et al., 2002), and acidic (Xu et al., 2010) stress. This might help explain the low viability values of the ST213 genotype. The possible superior capability of ST213 over ST19 to enter into the VBNC under stress conditions requires further examination.

The greatest survival variability among the three studied strains was observed at room temperature. In addition, it was observed that the ST213 strain had better viability percentages at pH 3.5 and 4 °C compared to incubation at room temperature. The survival variability among *S. enterica* strains has been attributed to differential physiological and metabolic adaptations in response to unfavorable environmental conditions (Xu et al., 2008). The possible protective effect of refrigeration temperature during acid stress in *S. enterica* has been previously reported. Shah et al. (2013) documents that a preadaptation in cold stress increases the survival of *S. enterica* due to the transcription induction of multiple genes associated with amino acid metabolism, oxidative stress and DNA repair, whereas very few of these genes were induced when only exposed to acid stress. However, Xu et al. (2008) observed that *S. enterica* cells preadapted to pH 5.0 increased their resistance to poststress exposure at pH 4.0 and 4 °C and produced a cross-protection between acid and cold temperature, which was possibly mediated by the acid tolerance response (ATR). Additionally, *S. enterica* is able to tolerate high osmolarity through a biphasic response involving potassium uptake followed by an increase in the cytoplasmic concentration of compatible solutes such as glycine, betaine, carnitine, ectoine, proline and trehalose (Alvarez-Ordonez et al., 2011). The ATR response and cellular responses to osmotic shock need be evaluated in detail in genotypes ST213 and ST19.

The results show that the SAL004 and ATCC 14028 strains have significantly different responses to stress associated with food preservation conditions despite both being ST19; such intra-genotype differences might be partially explained by the archival character of the latter strain. Studies on the *S.* Typhimurium ATCC 14028 strain span more than 40 years, and since then, it has been preserved in stock cultures. Once this strain has been provided to a scientific laboratory, it is subjected to continuous serial passages in diverse culture media. It has been widely documented that both archiving and serial passage of reference strains of human pathogenic bacterial species cause the strain to undergo biochemical, physiological and genetic changes (Fux et al., 2005). Significant genetic differences have been found between laboratory reference strains and their clinical counterparts for *Escherichia*

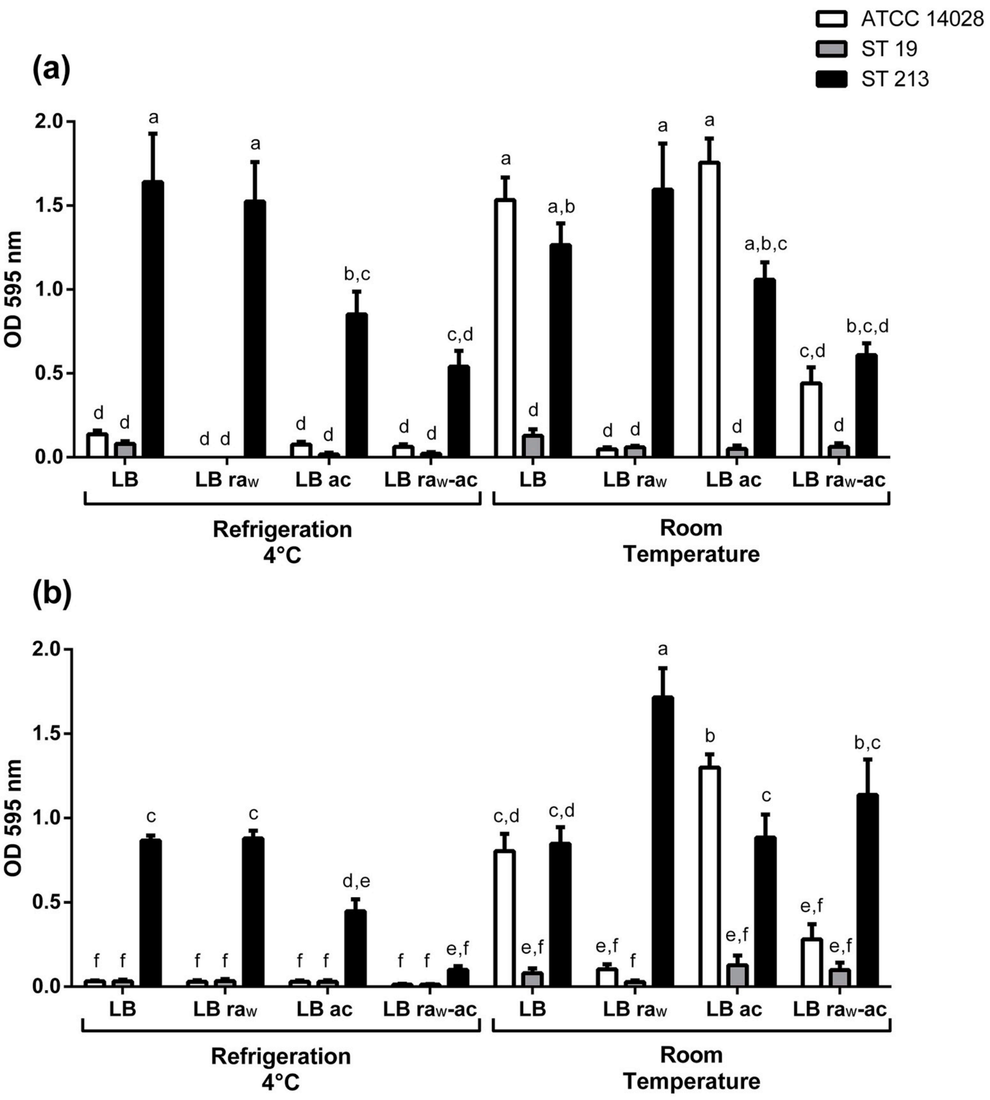

Fig. 4. Biofilm formation by *Salmonella enterica* strains under stress conditions. (a) Biofilm-forming ability and (b) biofilm metabolic activity was evaluated using crystal violet staining and MTT, respectively. The *S. enterica* serotype Typhimurium strains ATCC 14028, ST19 and ST213 were subjected to stress at low a_w (LB ra_w), pH 3.5 (LB ac) and the combination of low a_w and pH 3.5 (LB ra_w-ac) for 8 days, incubated at refrigeration (4 °C) or room temperature (≈22 °C). The data represent the mean ± S.E. (n = 9). ANOVA with Tukey's multiple comparisons test was used. Different letters represent significant difference in each condition tested ($p < 0.05$).

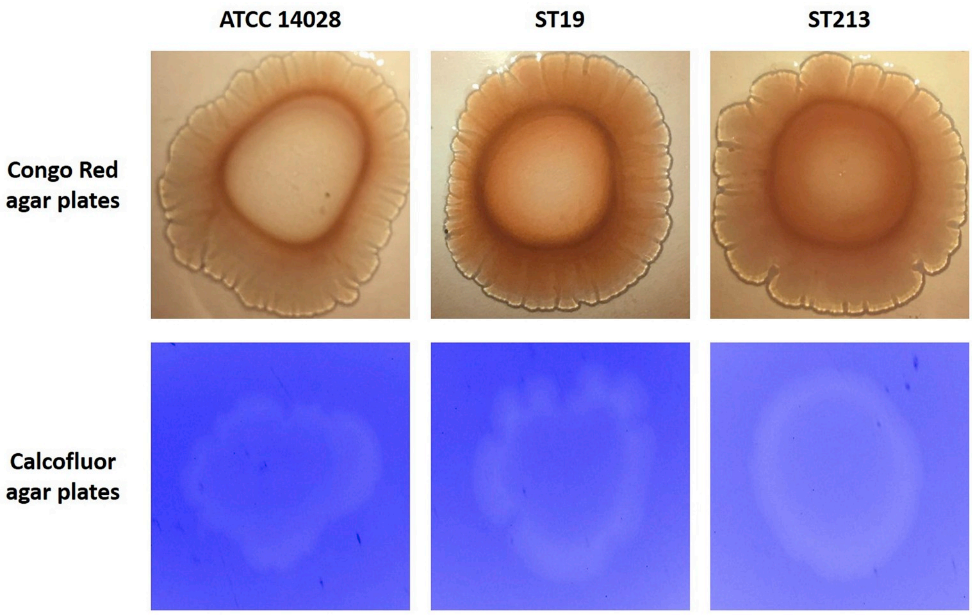

Fig. 5. Morphotype analysis of *Salmonella enterica* strains. The morphotype and the ability to produce cellulose of *S. enterica* serotype Typhimurium strains ATCC 14028, ST19 and ST213 was evaluated with Congo red agar and calcofluor agar, respectively. The images are representative of six independent assays (n = 6). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

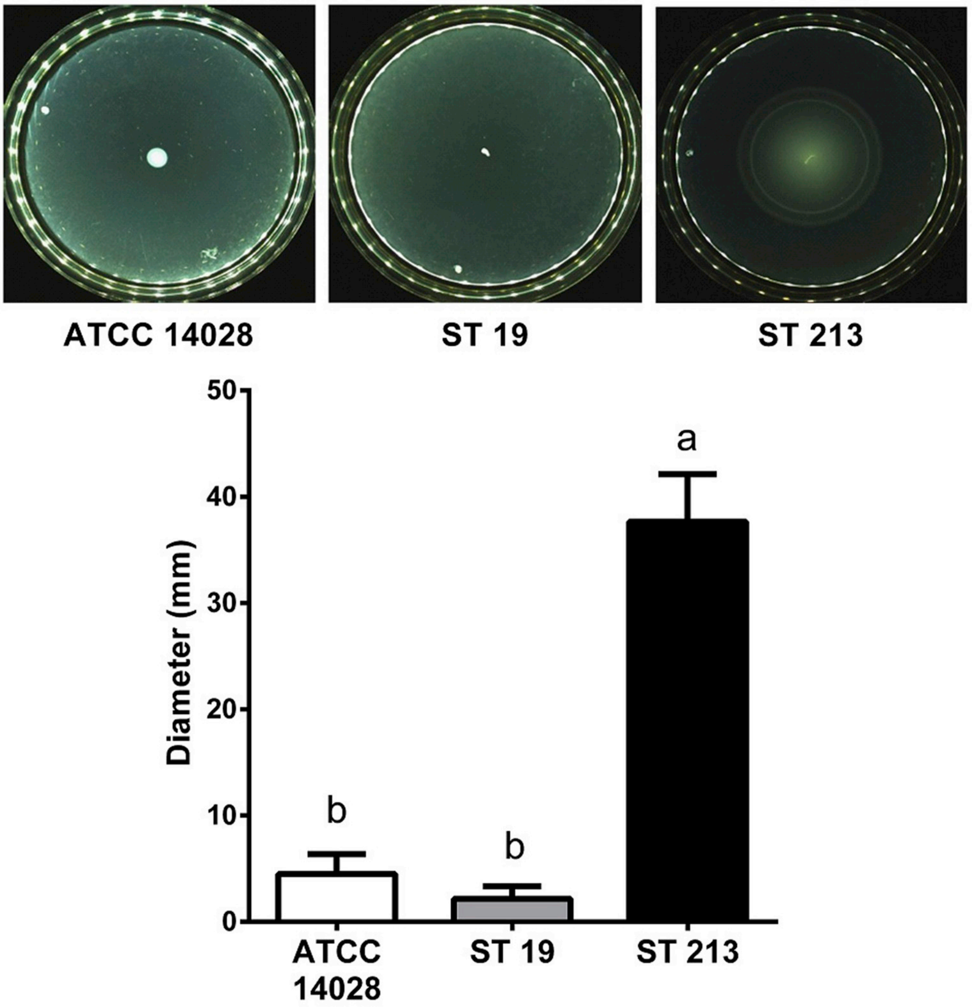

Fig. 6. Swimming motility assessment of *Salmonella enterica* strains. Swimming motility assays were performed in *S. enterica* serotype Typhimurium strains ATCC 14028, ST19 and ST213 cultured on LB 0.3% agar plates. The images are representative of three triplicate independent experiments. Data represent the mean ± S.E. (n = 9). ANOVA with Tukey's multiple comparisons test was used. Different letters represent significant difference ($p < 0.05$).

coli, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Fux et al., 2005). The phenotypic reported changes in pathogenic bacteria include the loss of virulence (Hopkins et al., 1996; Somerville et al., 2002) and flagella (Sellek et al., 2002), as well as physiological changes *in vitro* assays (Cafini et al., 2015). Archival strains of *S.* Typhimurium show genomic rearrangements, including small deletions and insertions and large duplications (Porwollik et al., 2004). Phenotypic changes in *S.* Typhimurium archival strains include rough/smoothness colony morphology, motility, auxotrophic reversion, phage types and prophage-to-phage releases (Eisenstark, 2010). In the specific case of the *S. enterica* ATCC 14028 strain, laboratory passage has induced the loss of the rdar morphotype and has generated a high mutation rate in the *rpoS* gene (Davidson et al., 2008). Furthermore, genomic analysis reveals that strain 60-6516, which is the putative ancestor of the 14,028 strain, lacks a 2.3 kbp region that expands the genes *dcuA* (an anaerobic C4-dicarboxylate transporter), *aspA* (aspartate ammonia lyase), and *fxsA* (F exclusion of bacteriophage T7), as well as STM14_5203, which encodes a hypothetical protein of unknown function; this loss presumptively occurred in culture or during recent cultivation of the 60–6516 strain (Jarvik et al., 2010).

The ability of *S. enterica* cells to form filaments has been previously associated with sublethal stress conditions (Humphrey et al., 2011; Kieboom et al., 2006; Mattick et al., 2000). Filamentation in bacteria occurs when cell growth continues in the absence of cell division and results in the formation of elongated bacteria that have multiple copies of chromosomes (Justice et al., 2008). It has been suggested that osmotic stress causes inhibition of the production or action of proteins involved in cell division (e.g., FtsZ) in *S. enterica* and thus blocks septation, but allows the function of proteins involved in biomass formation, which could provide a survival advantage in unfavorable environments. Once conditions are favorable, septation can be resumed, and it rapidly results in a large number of viable *S. enterica* cells that are ready to continue replicating (Kieboom et al., 2006; Mattick et al., 2000; Stackhouse et al., 2012). This might be one of the reasons why, despite having a lower percentage of viability than the SAL004 and ATCC 14028 strains, the ST213 strain has similar recovery curves to these ST19 strains in some of the analyzed stress conditions.

Foodborne bacteria submitted to stress directs the cellular metabolism for survival and avoids the use of ATP for cell division (Jones et al., 2013). Under acid stress, bacteria use their available energy to change: (1) the membrane fatty acid composition to make it less permeable to protons, (2) the synthesis of DNA binding proteins to avoid acid damage of the genetic material, and (3) the activation of metabolic pathways that consume protons. Under reduced water availability, bacterial cells maintain turgor pressure by incorporating compatible soluble metabolites via active transport or by synthesizing them, if they are not available, and limiting the energy available for cell division. At low temperatures, the proteins required for energy generation, such as the tricarboxylic acid (TCA) cycle and electron transport, are upregulated in the context of low energy availability for

Table 2
Fatty acid composition of *S. enterica* Typhimurium strains grown under different conditions.

Strain	Growth conditions	Fatty acid (%)					
		C12:0	C14:0	C16:0	C16:1	C17:0	C17:0 cyc
ATCC	Without stress	2.6 ± 0.8a	9.2 ± 0.6 a	39.6 ± 1.1 a	5.8 ± 1.5 e	0.9 ± 0.4 e,f	19.3 ± 0.9 a
	pH 3.5 4 °C	2.7 ± 0.5 a	9.1 ± 0.5 a	40.3 ± 1.1 a	7.2 ± 0.3 e	1.0 ± 0.3 e,f	15.3 ± 2.5 b
	pH 3.5 RT	2.4 ± 0.0 a	7.2 ± 0.0 b,c	36.9 ± 0.0 b	8.3 ± 0.0 c,d,e	0.8 ± 0.0 f	14.3 ± 0.0 b,c
ST19	Without stress	2.7 ± 0.6 a	8.3 ± 0.2 a,b	35.4 ± 0.8 b,c	10.2 ± 1.0 b,c,d	2.1 ± 0.0 b,c,d	16.2 ± 1.4 b
	pH 3.5 4 °C	3.1 ± 0.7 a	8.6 ± 1.5 a,b	36.5 ± 0.5 b	7.5 ± 0.7 d,e	2.8 ± 0.9 a	17.1 ± 2.1 a,b
	pH 3.5 RT	3.0 ± 0.4 a	6.6 ± 0.4 c,d	32.6 ± 1.9 d	14.8 ± 2.4 a	1.5 ± 0.2 d,e	12.7 ± 1.2 c,d
ST213	Without stress	2.8 ± 0.7 a	5.4 ± 0.7 d,e	33.3 ± 1.3 c,d	15.1 ± 1.1 a	2.4 ± 0.2 a,b,c	8.8 ± 0.2 e
	pH 3.5 4 °C	2.5 ± 0.0 a	5.3 ± 0.4 e	35.2 ± 1.4 b,c	11.0 ± 0.9 b,c	2.6 ± 0.2 a,b,c	10.4 ± 0.3 d,e
	pH 3.5 RT	3.4 ± 1.0 a	5.7 ± 0.4 d,e	32.2 ± 3.1 d	13.2 ± 4.5 a,b	1.9 ± 0.4 c,d	9.5 ± 2.0 e

Strain	**Growth conditions**	**Fatty acid (%)**					
		C18:0	C18:1	C19:0 cyc	Minor FA	SFA	UFA
ATCC	Without stress	1.4 ± 0.2 b	7.8 ± 1.7 f	5.7 ± 1.5 a	7.7 ± 0.2 c,d	86.2 ± 3.3 a	13.8 ± 3.3 f
	pH 3.5 4 °C	2.0 ± 0.6 a	10.7 ± 2.2 e,f	4.5 ± 0.6 a,b	7.2 ± 0.4 d	81.9 ± 2.0 a,b	18.1 ± 2.0 e,f
	pH 3.5 RT	1.2 ± 0.0 a,b	16.0 ± 0.0 c,d	5.5 ± 0.0 a	7.5 ± 0.0 c,d	74.8 ± 1.9 d	25.2 ± 1.9 c
ST19	Without stress	1.3 ± 0.2 b,c	12.5 ± 1.0 d,e	2.4 ± 0.3 c,d	9.0 ± 0.4 a,b	76.5 ± 1.8 c,d	23.5 ± 1.8 c,d
	pH 3.5 4 °C	1.1 ± 0.3 b,c	10.8 ± 3.8 e,f	3.5 ± 0.8 b,c	9.0 ± 0.5 a,b	81.1 ± 3.3 b,c	18.9 ± 3.3 d,e
	pH 3.5 RT	0.5 ± 0.2 d	17.1 ± 2.3 b,c	2.2 ± 0.7 c,d	9.0 ± 0.6 a,b	67.1 ± 3.8 e,f	32.9 ± 3.8 a,b
ST213	Without stress	1.2 ± 0.1 b,c	21.1 ± 1.2 a	1.6 ± 0.2 d	8.4 ± 0.4 b,c	62.9 ± 0.2 f	37.1 ± 0.2 a
	pH 3.5 4 °C	1.5 ± 0.1 b	20.4 ± 0.1 a,b	3.2 ± 0.5 b,c	8.0 ± 0.3 b,c,d	68.1 ± 1.1 e	31.9 ± 1.1 b
	pH 3.5 RT	0.9 ± 0.4 c,d	20.3 ± 1.5 a,b	3.0 ± 1.5 c,d	9.8 ± 1.8 a	65.6 ± 4.4 e,f	34.4 ± 4.4 a,b

Minor FA: Minor fatty acids. SFA: Total saturated fatty acids. UFA: Total unsaturated fatty acids. The data represent the mean ± S.D. (n = 3). ANOVA with Tukey's multiple comparisons was used. Different letters represent significant difference in each fatty acid column ($p < 0.05$).

protein synthesis, which indicates that cellular filamentation is a result of the stringent response or starving response (Jones et al., 2013). All of these cytological, biochemical and physiological processes are under the control of global transcription regulators and, in some cases, include *rpoS* and consume large amounts of ATP, which limits their availability for cell division. Thus, the available data suggest that cellular filamentation is a consequence of cellular processes that consume energy rather than a direct bacterial cytological strategy to resist stress. However, filamentation in *S. enterica* and other foodborne bacteria is relevant from a public health perspective because filamentous cells form single colonies when plated on solid agar medium and cause lower CFU counts in a food samples and the underestimation of bacterial numbers in current assessments and predictive models (Jones et al., 2013). Thus, the ST213 strain represents a higher public health risk than ST19. Additionally, slightly higher filamentous cells were observed in ST213 than in ATCC 14028 and might contribute to lower counts in some of the recovery kinetics.

Another strategy employed by *S. enterica* in adverse conditions is biofilm formation, which is defined as a community of bacterial cells immersed in a polymeric matrix that adheres to inert or living surfaces and gives protection to bacteria against environmental stress, disinfectants and the host immune system (Steenackers et al., 2012). It has been reported that biofilm formation by *S. enterica* is strongly strain-dependent and is affected by environmental conditions (Lianou and Koutsoumanis, 2011), but present work documents genotype significant differences to form a biofilm under stress conditions associated with food preservation. Vestby et al. (2009) screened 148 *S. enterica* strains, and 23% displayed bdar morphology. Their results indicate that cellulose is not a major component of the *S. enterica* biofilm matrix. In this sense, other components, such as curli fimbriae, which is the large secreted BapA protein (proteinaceous fraction), and the O-antigenic capsule (exopolysaccharide fraction) could be involved in biofilm formation (Steenackers et al., 2012).

It should be noted that motility is also important for biofilm formation (Mireles et al., 2001), whereas other data suggest that flagellin, not motility *per se*, is necessary for biofilm formation (Kim and Wei, 2009; Prouty and Gunn, 2003; Römling, 1999), thus the motility observed by the ST213 strain could be involved in the formation of biofilm. Interestingly, the motility data of the ATCC 14028 strain are heterogeneous in the literature, with several studies reporting a higher swimming capability than the present study (Ballesteros et al., 2018; Birhanu et al., 2018; Marshall and Gunn, 2015), and at least one other work reported lower motility (Kim and Kwon, 2013) than our data. Despite all of the cited references and present work that used the same motility assay in LB medium, such discordance might be partially explained by differences in the incubation time before reading the

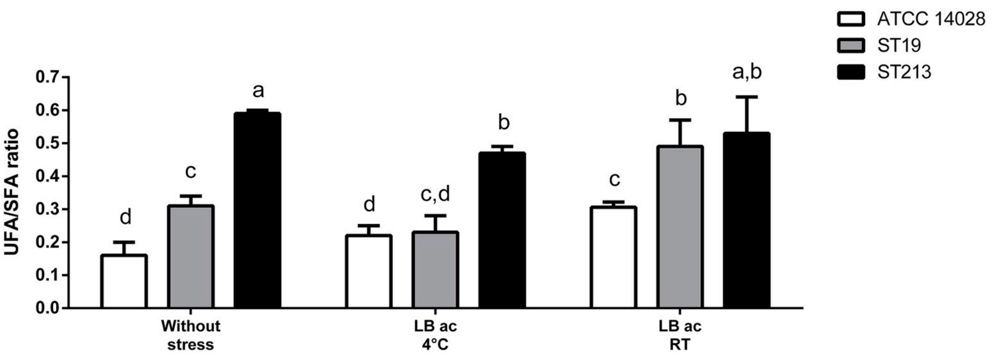

Fig. 7. The UFA/SFA ratio of *Salmonella enterica* under different conditions. Influence of acid pH on the UFA/SFA ratio of *S. enterica* serotype Typhimurium strains incubated at refrigeration (4 °C) or room temperature (≈22 °C) for 8 days. The data represent the mean ± S.D. (n = 3). ANOVA with Tukey's multiple comparisons was used. Different letters represent significant difference ($p < 0.05$).

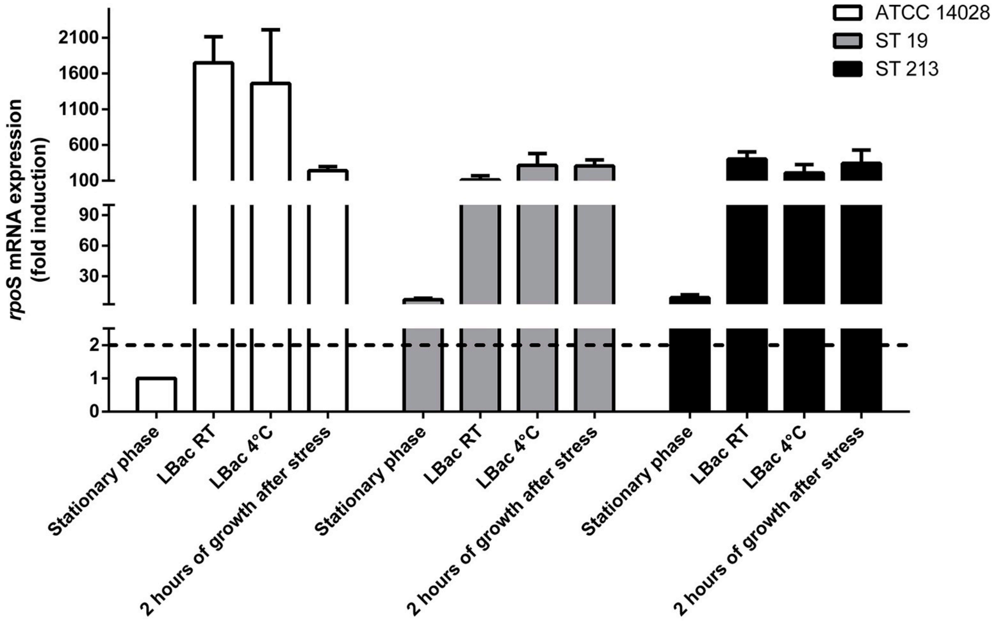

Fig. 8. Relative fold change in the *rpoS* gene expression. The relative expression of stressed *S. enterica* serotype Typhimurium strains in LB medium at pH 3.5 (LB ac) incubated for 8 days under refrigeration (4 °C) or at room temperature (RT, ≈22 °C) and strains with 2 h of growth in standard LB medium after stress in LB ac at 4 °C was compared with strains at stationary phase (overnight growth) in standard LB medium. The results were normalized using ATCC strain in stationary phase.

swimming halo. Additionally, LB medium includes peptone in its formulation, and it has been documented that different peptone sources cause significant differences in the motility results of the ATCC 14028 strain (Gray et al., 2006). In addition, previously discussed storage and medium passages of the ATCC 14028 strain might also account for the motility variations among studies. Like motility, filamentation has also been associated with biofilm formation. As a complex multicellular structure, the division of labor among different cell types (Claessen et al., 2014), and filamentous cells has been observed within biofilms of pathogenic bacteria (Justice et al., 2008). To the best of our knowledge, no filamentous cells have been described in *S. enterica* biofilms that were generated as a response to stress. In enterobacteria, filamentous cells are present during biofilm formation in cold adapted *E. coli*, but it does not appear to enhance the attachment to food surfaces compared to biofilms with non-filamentous cells (Visvalingam and Holley, 2013). Thus, biofilm cellular complexity in *S. enterica*, particularly among ST19 and ST213 genotypes, remains a pending task to be addressed in future studies.

In addition to its relevance in stress tolerance, diverse lines of evidence relate biofilm formation with virulence in *S. enterica*. For instance, biofilm cells highly overexpress *stn* (enterotoxin) and *invA* genes at pH 6 compared to planktonic cells (Xu et al., 2010). Further work demonstrates that the *spiA* gene product has a role in biofilm formation in addition to its participation as a component of the virulence factor SPI-2 type III secretion system (Dong et al., 2011). Physiological evidence shows that biofilm cells show higher adhesive capability in the spleens of mice than planktonic ones (Turnock et al., 2002). However, Borges et al. (2018) do not find a relationship among *in vivo* pathogenicity and the capability of biofilm formation of Typhimurium strains isolated from salmonellosis outbreaks and poultry. Further work needs to be conducted to assess the possible relationship between the biofilm formation capability of the ST213 genotype and its virulence. Furthermore, ST213 swarming capability and biofilm formation are associated with higher antibiotic resistance (Baugh et al., 2012; Butler et al., 2010; Turnbull and Surette, 2008). Thus, a detailed analysis of the relationships between the stress response and antibiotic resistance of ST19 and ST213 is necessary to better understand replacement of the former by the latter genotype and its implications on public health.

Fatty acid composition of the plasma membrane determines its fluidity, and several studies have suggested a relationship between the degree of membrane fluidity and stress tolerance (Álvarez-Ordóñez et al., 2012). For instance, at an acidic pH, the *S. enterica* plasma membrane reduces UFA and increases SFA contents, which is mainly cyclopropane fatty acids (CFA). Such changes diminish membrane fluidity, which has been associated with higher resistance to an acidic environment (Álvarez-Ordónez and Prieto, 2010; Álvarez-Ordóñez et al., 2008; Kim et al., 2005; Yang et al., 2014). Furthermore, our results agree with previously documented significant changes in fatty acid, protein, and carbohydrate profiles in the *S.* Typhimurium biofilm cells exposed to acidic conditions (Xu et al., 2010), which clearly shows specific metabolic responses and changes in cell surface hydrophobicity related to acidic stress.

Most of the previously discussed cellular responses to stress are closely related to regulation by alternative sigma factor RpoS. For example, entrance in the stationary phase by *S. enterica* and other gram-negative bacteria involves several metabolic, biochemical, and genetic change characteristics of this state, which is fine-tuned by alternative sigma factor RpoS (Navarro-Llorens et al., 2010). Because cells in this study used for the stress tests were from overnight growth and reached a stationary phase, this might explain the lack of a lag phase of the studied genotypes in recovery growth. Higher transcriptional levels at the stationary phase for both evaluated genotypes indicate that they were better prepared to face stressful conditions than the reference strain, which explains their lower transcriptional increase when entering into such conditions. It will be interesting to conduct further work to analyze the differences in poststress recovery of log-phase cells between both studied genotypes. Additionally, strain differences in the stress response have been associated with point or larger mutations in key genes, such as *rpoS*, but might also be caused by differential expression of RpoS (σ^S)-dependent genes due to mutations that affect the transcript translation of this gene or the generation of an unstable σ^S protein whose function is affected (Lianou and Koutsoumanis, 2013).

Some of the genes involved in the biphasic response to tolerate high osmolarity are regulated by the σ^S factor (Spector and Kenyon, 2012), which, along with the E (σ^E) factor, plays a relevant role in the response to osmotic stress. McMeechan et al. (2007) demonstrated that *rpoS* and *rpoE* defective mutants of *S. enterica* had a low survival in a hyperosmotic environment; it is of further interest to obtain these kinds of defective mutants in the evaluated genotypes to evaluate the survival in stress conditions. Low transcription levels of the *rpoS* gene after 2 h poststress recovery might explain the similar recovery kinetics shown by the ST19 and ST213 strains, even though the ST213 strain in most stress conditions presented lower percentages of viability. This could be due to a possible competition between σ^{70} and σ^S for the binding site in

RNA polymerase, which establishes a balance between the resources that an organism dedicates to growth/replication (σ^{70}) and maintenance/repair (σ^{S}) (Aertsen and Michiels, 2004). A subtle overproduction of σ^{70} stops the transcription of genes requiring σ^{S}, and cells become sensitive to stress; conversely, the overexpression of σ^{S} attenuates the expression of genes requiring σ^{70}. This antagonism between sigma factors has been shown to be highly regulated and is related to the nutritional quality of the environment and the hormone-like nucleotide ppGpp (Jishage, 2002; Nyström, 2002). Interestingly, Kim et al. (2005) found that σ^{S} expression reduces C19:0 cyc production but not C17:0 cyc in congruence with our observation of high σ^{S} expression and low C19:0 cyc content in the studied strains.

Beyond physiological characteristics (biochemical and genetic characteristics associated with archival and serial passages of the ATCC 14028 reference strain previously stated), intra-genotype variation of ST19 clinical strains have been documented, particularly regarding antibiotic profile resistance, *in vitro* human macrophage invasion, and motility (Panzenhagen et al., 2018). Comparative genomic analysis reveals that clinical ST19 strains from Brazil have split into two linages, one of which showed genome degradation affecting genes coding for proteins related to membrane, SOS response, and transcriptional regulation (Panzenhagen et al., 2018). It is still unknown whether the ST19 (SAL004) environmental strain in this study is undergoing the same or a similar process of genome degradation that will aid in explaining their differences with the ATCC 14028 reference strain. Besides the present work, there is only one previous experimental characterization of an ST of the Typhimurium serotype involved in the replacement of a prevalent genotype in one country/geographical region, which corresponds to the ST313 genotype first detected in sub-Saharan Africa (Feasey et al., 2012; Okoro et al., 2012), which have also started to replace the ST19 genotype as iNTS. Genomic analysis revealed the presence of two lineages among the ST313 African strains (Okoro et al., 2012) and subsequent work documented a clear differentiation among Brazilian and African lineages (Panzenhagen et al., 2018). Despite this, initial studies conducted to characterize the ST313 genotype strains showed no significant differences in non phagocytic cell invasion, Caspase-1-dependent macrophage death induction, IL-1β release, and transcription levels of *sopE2* and *fliC* genes; however, all ST313 strains showed significant differences in such characteristics with all ST19 studied strains (Carden et al., 2015). In contrast, recent work comparing ST313 strains from Brazil and Africa found significant phenotypical differences among them (Almeida et al., 2017; Panzenhagen et al., 2018). Based on these antecedents of clinical strains of the ST19 and ST313 genotypes, we anticipate that further studies characterizing new clinical and environmental isolates of ST19 and ST213 genotypes from co-occurrence areas will document high intra-(ST) genotypic and physiological variability.

It is important to emphasize that the ST313 genotype was exclusively isolated from asymptomatic carriers and systemic invasive disease in Africa, and there is a lack of knowledge of its relevance on gastroenteritis (Feasey et al., 2012), whereas the ST213 genotype in this study was isolated from contaminated food and stool samples (Wiesner et al., 2009). Such differences in the sample provenance might determine genotype adaptation to host interactions and make it relevant from a public health perspective; it appears that genomic and physiological particularities of the ST313 genotype evolved to become human adapted and were spread by person-to-person transmission to cause iNTS (Feasey et al., 2012). If the food and gastroenteritis associated origin of ST213 strains demand a similar or different set of genomic, biochemical and physiological changes than ST313, then this is an interesting issue to address in further studies.

5. Conclusions

Although ST19 has high survival percentages, and recovery and σ^{S} transcription are shown to be similar between tested genotypes, ST213 displays higher capability to cell elongation, biofilm formation, motility, and membrane modification in stress conditions. Such characteristics might confer to ST213 the capability to survive and disperse outside the human host. This may partially help to explain the increase in food and clinical prevalence in Mexico, replacement of the ST19 genotype, and its recent dispersion to other countries. Collectively, this work shows that differential cell responses might subtly occur between the ST19 and ST213 genotypes of the Typhimurium serotype at the intraspecific level. Studies of the genotypes involved in turnover at other geographical areas still need to be conducted to determine if these cellular modifications are also present in current genotypes over displaced ones or if other stress resistance mechanisms are present.

Further studies are necessary to have a clearer picture of cellular differences between the STs analyzed and how differences emerge. Additionally, complementary work is needed to evaluate if ST213 stress resistance mechanisms increase its virulence. These kinds of data will aid in improving food production chain conditions and surveillance programs to avoid outbreaks by new circulating variants of *S. enterica* serotype Typhimurium.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.010.

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