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Influence of lactic acid and acetic acid on *Salmonella* spp. growth and expression of acid tolerance-related genes

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A B S T R A C T

Salmonella spp. is an important foodborne pathogen, often associated with meat products. This pathogen presents a complex tolerance mechanism in the presence of organic acids, which is regulated by a diversity of genes, including *rpo*S, *nlp*D and *clp*P. The present study aimed to measure the expression of such genes by *Salmonella* strains subjected to acid stress conditions, and associate these data with microbial growth. A culture collection composed of 79 strains of *Salmonella* spp. obtained from bovine and swine production chains was subjected to PFGE using *Xba*I, and 3 strains (serovars Derby, Typhimurium and Meleagridis) were selected for acid tolerance trials. The selected strains were inoculated in meat extract broth (MEB) added to lactic or acetic acids at a final pH of 4.0, 5.0 or 6.0, and incubated at 37 °C for 6, 12, 24 and 48 h. As controls, *Salmonella* strains were inoculated in MEB at pH 7.0, and incubated in the same conditions. Bacterial populations were monitored by direct plating and gene expression using qPCR. *Salmonella* presented similar populations to controls and evident expression of *rpo*S at pH 5.0 and 6.0. However, *Salmonella* populations were not detectable after 6 h at pH 4.0. The adaptability of *Salmonella* to pH 5.0 and 6.0 emphasizes the importance of adequate monitoring of pH reduction during cleaning procedures in food industries, such as organic acid spraying in bovine carcasses. The data obtained demonstrated the relevance of *rpo*S in the acid tolerance mechanism of *Salmonella* strains, prompting further studies to investigate its expression in meat systems.

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1. Introduction

Decontamination of bovine carcasses by organic acid spraying has been routinely used in slaughterhouses located in the United States and Canada, with the aim of reducing contamination by foodborne pathogens and spoilage microorganisms (Adams & Hall, 1988; Delmore, Sofos, Schmidt, & Smith, 1998; Goddard, Mikel, & Conner, 1996). However, this procedure is not allowed in slaughterhouses located in other countries, such as Brazil and countries in the European Union, but it can be considered an attractive alternative for the reduction of microbial contamination due to poor hygiene conditions during processing (Del Río, Panizo-Morán, Prieto, Alonso-Calleja, & Capita, 2007).

The main organic acids used for the decontamination of animal carcasses are lactic acid, acetic acid and citric acid, and their concentrations can range from 1% to 5%, depending on the acid and the meat processing step (USDA, 1996, 2013). The bactericide activity of organic acids occurs due to their undissociated forms, which target the metabolic functions of microorganisms, such as protein production, the inhibition of ATP and an increase in osmotic pressure (Lues & Theron, 2011).

Organic acids are being routinely used as sanitizers by the food industry, demanding for proper studies to demonstrate their activities in target pathogens. It is necessary to verify the specific factors involved in bactericidal activity, and the selection of potential resistant strains that may pose a relevant risk to consumers (Smulders & Greer, 1998). Based on this information, organic acids could be employed by food industries at ideal conditions, aiming at the specific and proper control of target microorganisms.

Food industries usually employ organic acids with the aim of controlling foodborne pathogens and microorganisms that present variable behavior under stress conditions (Dubal et al., 2004; Greer & Dilts, 1995; Hwang & Beuchat, 1995; Tinney, Miller, Ramsey, Thompson, & Carr, 1997; Wolf et al., 2012). As an example, under acid stress, *Salmonella* presents a complex tolerance mechanism of survival that involves multiple genes: *rpo*S and *nlp*D are responsible for protein expression, which protects the bacterial cell against damage caused by acid stress, and *clp*P is involved in the regulation of these proteins inside the cell (Foster, 2001; Hengge-Aronis, 2002; Lange, Fischer, & Hengge-Aronis, 1995; Lues & Theron, 2011; Paesold & Krause, 1999).

Therefore, this study aimed to evaluate the behavior of *Salmonella* strains under acid stress conditions created by lactic and acetic acids in order to assess variations in their microbial populations and the expression of genes involved in acid tolerance.

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Table 1
Serotypes and sources of isolates considered in the present study.

Salmonella serotype	*N*	Source (number of isolates)
4,5, 12:i	9	Field samples—swine production (5), Feaces from swine slaughterhouse (1), Swine carcass (3)
Agona	1	Field samples—swine production (1)
Dublin	7	Bovine carcass (7)
Derby	16	Bovine carcass (8) Field samples—swine production (6), Swine carcass (1) Feaces from swine slaughterhouse (1)
Give	2	Bovine carcass (2)
Infantis	1	Bovine carcass (1)
Mbandaka	7	Field samples—swine production (5), Feaces from swine slaughterhouse (2)
Meleagridis	1	Feaces from swine slaughterhouse (1)
Panama	3	Swine carcass (1), Field samples—swine production (1), Feaces from swine slaughterhouse (1)
Typhimurium	28	Field samples—swine production (20), Feaces from swine slaughterhouse (4), Swine carcass (3), Residual water/washing swine carcass (1)
Worthington	1	Feaces from swine slaughterhouse (1)
S. enterica subs. *salamae*	3	Bovine carcass (3)

2. Materials and methods

2.1. Microorganisms

A culture collection composed of 79 *Salmonella* isolates was considered in the present study. All isolates were obtained from previous studies from different steps of the beef and pork processing chain (Table 1; Bersot, 2005; Cossi et al., 2013; Cossi et al., 2014). All isolates were previously identified by serological reactions (Fiocruz, Rio de Janeiro, RJ, Brazil) and stored at −20 °C in tripticase soya broth (TSB, Oxoid Ltd., Basingstoke, England) added to glycerol 20% (v/v).

2.2. Pulsed field gel electrophoresis (PFGE)

Cultures from all isolates were diluted in NaCl 0.85% (w/v) until absorbance 1.0 ($\lambda = 660$ nm), and 400 μL aliquots were transferred to micro-tubes containing 20 μL of proteinase K (20 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and 400 μL of 1% agarose (w/v; Bio-Rad Laboratories, Hercules, CA, USA). Cell lysis and plug washing steps followed the PulseNet protocol for molecular subtyping of *Escherichia coli* O157:H7, *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri* (PulseNet, 2013).

Plug fragments were transferred to micro-tubes containing 5 μL of *Xba*I solution (10 U/μL; Promega, Madison, WI, USA), 20 μL of restriction solution 10× (Promega) and 175 μL of sterile water, and incubated at 37 °C for 2 h. Then, the digested fragments were separated using agarose gel (Promega) at 1% (w/v) by PFGE (CHEF DR-III; Bio-Rad Laboratories), with the following parameters: 6 V/cm, 120°, 19 h. PulseMarker (50–1.000 kb; Sigma-Aldrich) was used as a reference. Gels were stained in a GelRed™ bath (Biotium, Inc.; Hayward, CA, USA) and the genetic profiles were visualized by a transilluminator. Analyses of restriction profiles and dendrograms were performed using BioNumerics 6.6 (Applied Maths, Ghent, Belgium), considering 3% of tolerance for similarity.

2.3. Detection of genes involved in Salmonella acid tolerance

Based on the PFGE grouping, 35 isolates were selected and subjected to PCR reactions to detect genes involved in acid tolerance (*rpo*S, *nlp*D and *clp*P), as well as a housekeeping gene (*gm*K).

Table 2
Primer sequences, PCR product's expected sizes, and annealing temperatures considered for PCR reactions targeting acid tolerance-related genes and *gm*K in *Salmonella* isolates.

Gene	Primer sequences	Fragment sizes (pb)	Annealing	Reference
*rpo*S	F:GGTGAGATTGGGTATTCACC R:TTCTCGACTGCACGGATAAGC	213	50.0 °C	NC_011294
*nlp*D	F:TATGGCTGGCAGGTTGTACC R:CCCATTTTCCATCTGCACG	237	50.0 °C	NC_011294
*clp*P	F:GATGGTCATTGAACAGACC R:GTGTCATAGATGGACATCC	226	50.4 °C	NC_011294
*gm*K	F:TTGGCAGGGAGGCGTTT R:GCGCGAAGTGCCGTAGTAAT	101	52.1 °C	Botteldoorn et al., 2006

DNA was extracted using the Wizard® Genomic DNA Purification (Promega). The primer sequences for *rpo*S, *nlp*D and *clp*P were designed using DNAMAN 8.0 (Lynnon Corp., Pointe-Claire, Quebec, Canada), and *gm*K primer sequences were obtained from Botteldoorn et al. (2006) (Table 2). PCR reactions were composed of 12.5 μL of GoTaq Green Master Mix (Promega), 2.0 μL DNA, 1.0 μL from each primer (10 pmol/μL) and 8.5 μL ultrapure DNA-free water (Promega). PCR conditions were: initial denaturation at 94 °C for 1 min; 30 cycles of denaturation at 93 °C for 1 min, annealing at different temperatures for 1 min, and extension at 72 °C for 2 min; this was followed by a final extension at 72 °C for 5 min (Table 2). PCR products were electrophoresed in agarose gels at 1% (w/v; Promega), stained in a GelRed™ bath (Biotium) and visualized by a transilluminator. The specific sizes of PCR amplification products for each gene are presented in Table 2.

2.4. Expression of genes involved in Salmonella acid tolerance

2.4.1. Microorganisms

Based on the obtained genetic profiles and on the serovars identified, three strains were selected: *S.* Derby (S1), *S.* Typhimurium (S2) and *S.* Meleagridis (S3; Fig. 1). The strains were streaked onto plates containing TSB (Oxoid) supplemented with agar at 1.5% (w/v), and incubated at 37 °C for 24 h. Isolated colonies were transferred with a sterile swab to tubes containing 9 mL of meat extract broth (MEB), prepared according to Freney et al. (1999), in order to obtain cultures with turbidity similar to tube 4 of the MacFarland scale (corresponding to 1.2 $\times 10^9$ CFU/mL).

2.4.2. Treatments

Aliquots of 1 mL from each culture were transferred to tubes containing 9 mL of MEB at different pH values (4.0, 5.0 and 6.0), and adjusted with lactic acid (at 4%, v/v) and acetic acid (at 4% v/v). As a control, cultures were transferred to tubes containing MEB at pH 7.0. After this step, each treatment had the inoculum adjusted to 1.2×10^8 CFU/mL. The seven treatments that were obtained for each strain were incubated at 37 °C for 48 h, and after 0, 6, 24 and 48 h of incubation, aliquots of the cultures were obtained and used to estimate the bacterial population by direct plating; the expression of genes involved in acid tolerance were assessed using qPCR.

2.4.3. Monitoring populations of Salmonella strains

Aliquots of 1 mL from each culture and treatment were obtained at the specified intervals and diluted ten-fold in 0.85% NaCl (w/v). Selected dilutions were pour-plated in duplicate in plate count agar (Oxoid), and incubated at 37 °C for 24 h. After incubation, colonies were counted and the results were expressed in CFU/mL.

2.4.4. Monitoring the expression of acid tolerance-related genes of Salmonella strains

Aliquots of 250 μL from each culture and treatment were obtained at the specified intervals and subjected to extraction of total RNA, using

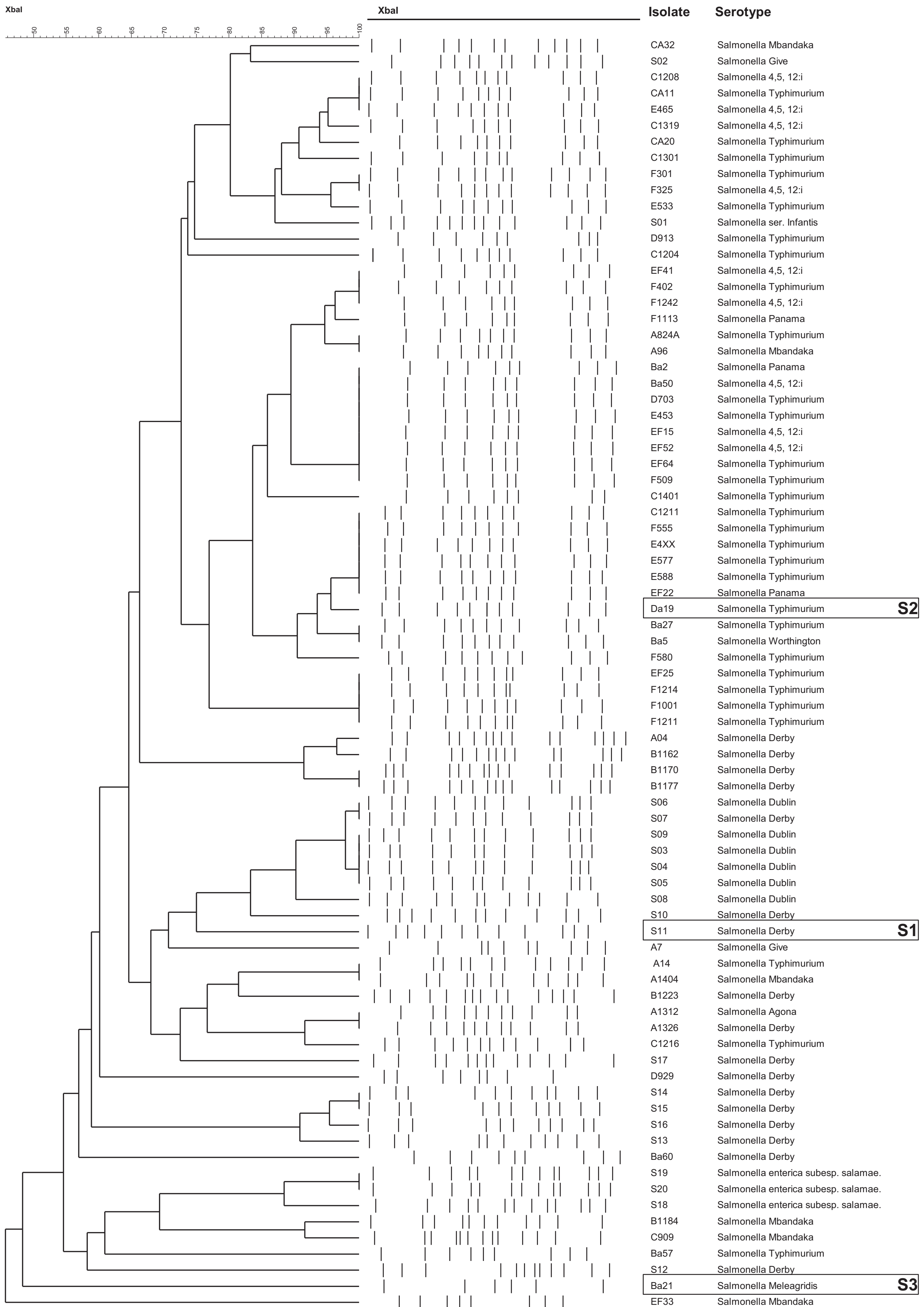
XbaI
50 55 60 65 70 75 80 85 90 95 100
XbaI
Isolate
Serotype
CA32 Salmonella Mbandaka
S02 Salmonella Give
C1208 Salmonella 4,5, 12:i
CA11 Salmonella Typhimurium
E465 Salmonella 4,5, 12:i
C1319 Salmonella 4,5, 12:i
CA20 Salmonella Typhimurium
C1301 Salmonella Typhimurium
F301 Salmonella Typhimurium
F325 Salmonella 4,5, 12:i
E533 Salmonella Typhimurium
S01 Salmonella ser. Infantis
D913 Salmonella Typhimurium
C1204 Salmonella Typhimurium
EF41 Salmonella 4,5, 12:i
F402 Salmonella Typhimurium
F1242 Salmonella 4,5, 12:i
F1113 Salmonella Panama
A824A Salmonella Typhimurium
A96 Salmonella Mbandaka
Ba2 Salmonella Panama
Ba50 Salmonella 4,5, 12:i
D703 Salmonella Typhimurium
E453 Salmonella Typhimurium
EF15 Salmonella 4,5, 12:i
EF52 Salmonella 4,5, 12:i
EF64 Salmonella Typhimurium
F509 Salmonella Typhimurium
C1401 Salmonella Typhimurium
C1211 Salmonella Typhimurium
F555 Salmonella Typhimurium
E4XX Salmonella Typhimurium
E577 Salmonella Typhimurium
E588 Salmonella Typhimurium
EF22 Salmonella Panama
Da19 Salmonella Typhimurium S2
Ba27 Salmonella Typhimurium
Ba5 Salmonella Worthington
F580 Salmonella Typhimurium
EF25 Salmonella Typhimurium
F1214 Salmonella Typhimurium
F1001 Salmonella Typhimurium
F1211 Salmonella Typhimurium
A04 Salmonella Derby
B1162 Salmonella Derby
B1170 Salmonella Derby
B1177 Salmonella Derby
S06 Salmonella Dublin
S07 Salmonella Derby
S09 Salmonella Dublin
S03 Salmonella Dublin
S04 Salmonella Dublin
S05 Salmonella Dublin
S08 Salmonella Dublin
S10 Salmonella Derby
S11 Salmonella Derby S1
A7 Salmonella Give
A14 Salmonella Typhimurium
A1404 Salmonella Mbandaka
B1223 Salmonella Derby
A1312 Salmonella Agona
A1326 Salmonella Derby
C1216 Salmonella Typhimurium
S17 Salmonella Derby
D929 Salmonella Derby
S14 Salmonella Derby
S15 Salmonella Derby
S16 Salmonella Derby
S13 Salmonella Derby
Ba60 Salmonella Derby
S19 Salmonella enterica subesp. salamae.
S20 Salmonella enterica subesp. salamae.
S18 Salmonella enterica subesp. salamae.
B1184 Salmonella Mbandaka
C909 Salmonella Mbandaka
Ba57 Salmonella Typhimurium
S12 Salmonella Derby
Ba21 Salmonella Meleagridis S3
EF33 Salmonella Mbandaka

Table 3
Microbial counts of S1 (*S.* Derby), S2 (*S.* Typhimurium) and S3 (*S.* Meleagridis) after inoculation in meat extract broth (MEB) added to lactic acid or acetic acid and adjusted at different pH values, and incubated at 37 °C until 48 h. Controls obtained in MEB inoculated with the same cultures and pH 7.0, incubated at 37 °C until 48 h. Values in log CFU/mL.

Strain	Organic acid	pH	Incubation time			
			0 h	6 h	24 h	48 h
S1	Control	7.0	8.1	8.4	8.4	8.6
	Lactic acid	6.0	8.1	7.9	8.5	8.9
		5.0	8.2	8.0	8.6	8.7
		4.0	7.9	5.3	<2.0	<2.0
	Acetic acid	6.0	8.2	8.2	8.5	8.7
		5.0	8.4	8.0	8.3	8.2
		4.0	7.8	<2.0	<2.0	<2.0
S2	Control	7.0	8.3	8.9	9.3	8.8
	Lactic acid	6.0	8.6	8.9	8.7	9.1
		5.0	8.3	9.7	9.6	9.1
		4.0	8.1	<2.0	<2.0	<2.0
	Acetic acid	6.0	8.3	8.7	8.7	9.3
		5.0	8.3	8.3	8.5	9.1
		4.0	<2.0	<2.0	<2.0	<2.0
S3	Control	7.0	8.8	9.1	9.3	9.2
	Lactic acid	6.0	8.6	9.0	9.0	9.2
		5.0	8.6	8.9	9.0	9.3
		4.0	8.6	5.6	2.2	<2.0
	Acetic acid	6.0	8.6	8.8	9.5	9.1
		5.0	8.6	8.6	8.4	8.4
		4.0	8.0	3.9	<2.0	<2.0

Trizol (Life Technologies, Grand Island, NY, USA) and according to the manufacturer's recommendations. The extracted RNA was quantified by spectrophotometry (NanoDrop Lite; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The cDNA was obtained using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen, Washington, DC, USA), following the manufacturer's specifications. The reactions were performed in triplicate using the Eco™ Real-Time PCR System (Illumina Inc., San Diego, CA, USA) and Maxima® SYBR® Green/ROX qPCR Master Mix (Fermentas, Maryland, USA), according to the manufacturer's instructions. The genes (targets and housekeeping gene) and oligonucleotides are listed in Table 2; *gm*K was used as the endogenous control (Botteldoorn et al., 2006). The reactions were performed simultaneously for the target genes and endogenous control, following the protocol: 2 min at 50 °C, activation of the polymerase at 95 °C for 10 min, and 40 cycles at 95 °C for 20 s and 60 °C for 1 min. Then, the melting curve was analyzed to check the reaction specificity for dissociation: 95 °C for 15 s, 55 °C for 15 s and 95 °C for 15 s. The mean values of curve thresholds (CT_S) were considered to calculate the relative expression of target genes by the comparative method using the $2^{-\Delta\Delta Ct}$ equation (Livak & Schmittgen, 2001).

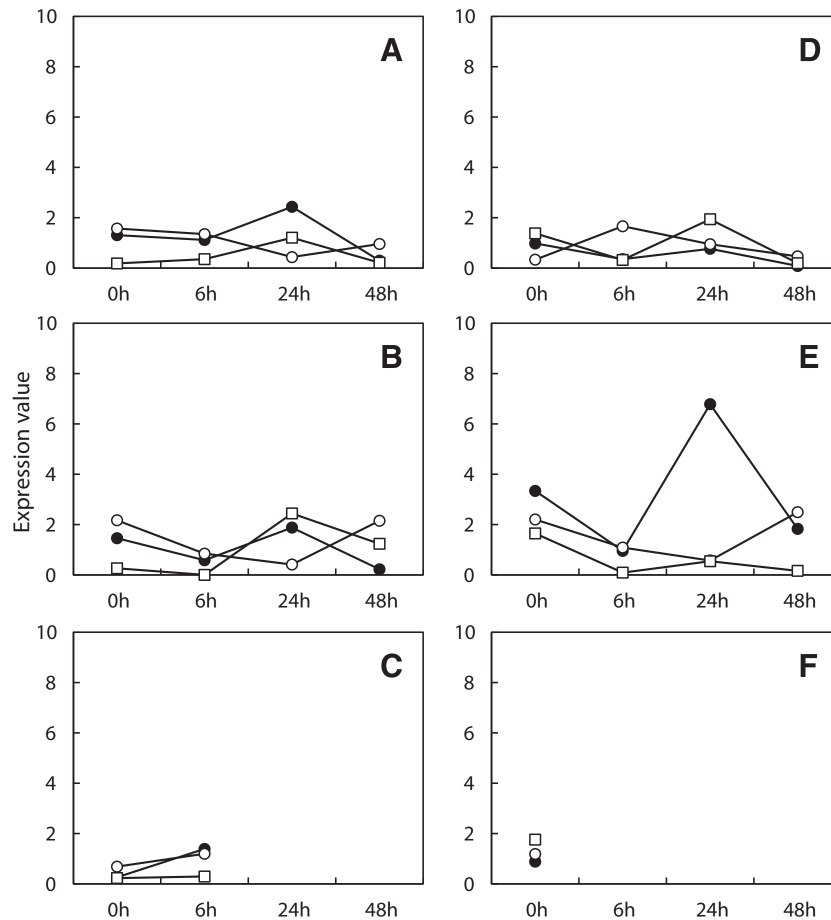

Fig. 2. Relative expression of genes enrolled in acid tolerance of S1 (*S.* Derby) after incubation at 37 °C for 0, 6, 24 and 48 h in distinct acid stress treatments. A, B and C: trials performed with meat extract broth added to lactic acid with pH at 6.0, 5.0 and 4.0, respectively. D, E and F: trials performed with meat extract broth added to acetic acid with pH at 6.0, 5.0 and 4.0, respectively. *rpo*S gene (—●—), *nlp*D gene (—○—), *clp*P gene.

Fig. 1. Dendrogram and pulsotypes obtained by *Xba*I macrorestriction and PFGE of *Salmonella* isolates obtained from beef and pork production chains. Similarity between pulsotypes was obtained considering a tolerance of 3%. Isolates marked with rectangles were selected for sequential studies to check the *Salmonella* behavior at acid stress conditions.

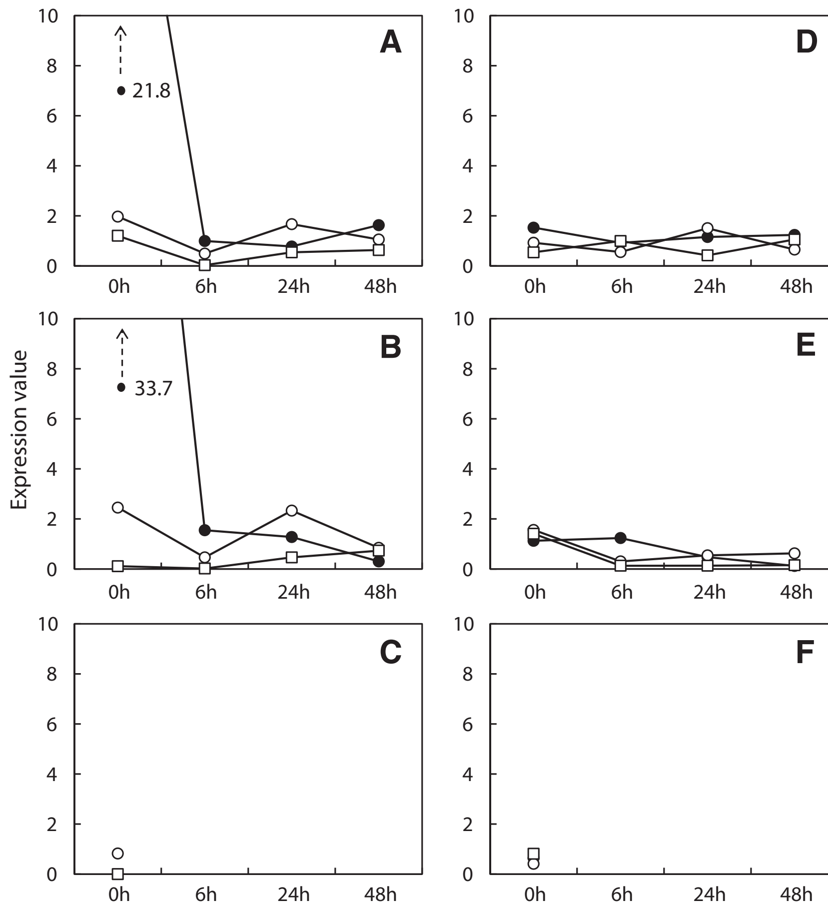

Fig. 3. Relative expression of genes enrolled in acid tolerance of S2 (*S.* Typhimurium) after incubation at 37 °C for 0, 6, 24 and 48 h in distinct acid stress treatments. A, B and C: trials performed with meat extract broth added to lactic acid with pH at 6.0, 5.0 and 4.0, respectively. D, E and F: trials performed with meat extract broth added to acetic acid with pH at 6.0, 5.0 and 4.0, respectively. *rpo*S gene (—●—), *nlp*D gene (—○—), *clp*P gene (—□—).

3. Results and discussion

Salmonella pulsotypes obtained by PFGE are presented in Fig. 1. The isolates presented 6 to 15 digested fragments, and 48 pulsotypes were identified. The 35 isolates that were selected for PCR reactions presented positive results for the tested acid tolerance-related genes *rpo*S, *nlp*D and *clp*P.

The 3 isolates that were selected based on the previous results were inoculated in acid treatment media at 10^8 CFU/mL in order to allow proper gene expression and RNA detection by qPCR. Therefore, the changes in *Salmonella* populations could be observed under acid stress conditions promoted by lactic and acetic acids, as demonstrated by Table 3. These changes can be explained by the expression of genes involved in the bacterial acid tolerance mechanisms (Figs. 2, 3 and 4). Three genes investigated in this study (*rpo*S, *nlp*D and *clp*P) produce proteins that regulate or protect the bacteria against cell damage caused by stress (Foster, 2001; Hengge-Aronis, 2002; Lange et al., 1995; Lues & Theron, 2011; Paesold & Krause, 1999). The better efficacy of acetic acid in controlling and reducing micro-organisms can be explained by its lower dissociation capability when compared to lactic acid. In this study, this efficacy can be observed in Table 3, which demonstrates the decrease of *Salmonella* populations in treatments with acetic acid at pH 5.0 and pH 4.0.

*rpo*S expression by S1 occurred mainly after 24 h of exposure, with lactic acid at pH 6.0 (Fig. 2A) and with lactic or acetic acid at pH 5.0 (Figs. 2B and 2E). However, the expression of *rpo*S increased after a decrease of S1 bacterial growth (6 h, Table 3), indicating a response due to the acid conditions. The expression of *rpo*S may have provided a restoration in bacterial growth after 24 h, particularly in the presence of lactic acid at pH 4.0 and 5.0. S2 presented *rpo*S expression just after the first acid contact, both at pH 6.0 and 5.0 (Figs. 3A and 3B).

Salmonella adaptive responses to organic acids have been described for different serotypes (Álvarez-Ordóñez, Prieto, Bernardo, Hill, & López, 2011), and the present study demonstrated an acid tolerance behavior by the three strains tested. These results show that *Salmonella* can adapt to organic acids, particularly at pH 6.0 or pH 5.0. However, when the pH is lower (pH 4.0), bacterial survival is not viable after 6 to 24 h. These conditions may be observed in commercial practice, as animal carcasses that are sprayed with organic acids can present pH levels between 3.3 and 5.8, depending on the specific acid, spraying time, concentration and other factors (Álvarez-Ordóñez, Fernandez, Bernardo, & Lopez, 2009). However, depending on the pH value required to promote adequate bacterial reduction, the sensory quality of meat can be jeopardized (Smulders & Greer, 1998).

The storage time of animal carcasses can also interfere with the activity of organic acids over microbial pathogens. Meat pH can increase during the storage period, promoting a buffering effect over the organic acids (Álvarez-Ordóñez et al., 2009). Considering the results obtained, lactic and acetic acids at pH 4.0 promote complete bacterial elimination after 6 or 24 h (Figs. 2C, F, 3C, F, 4F and C).

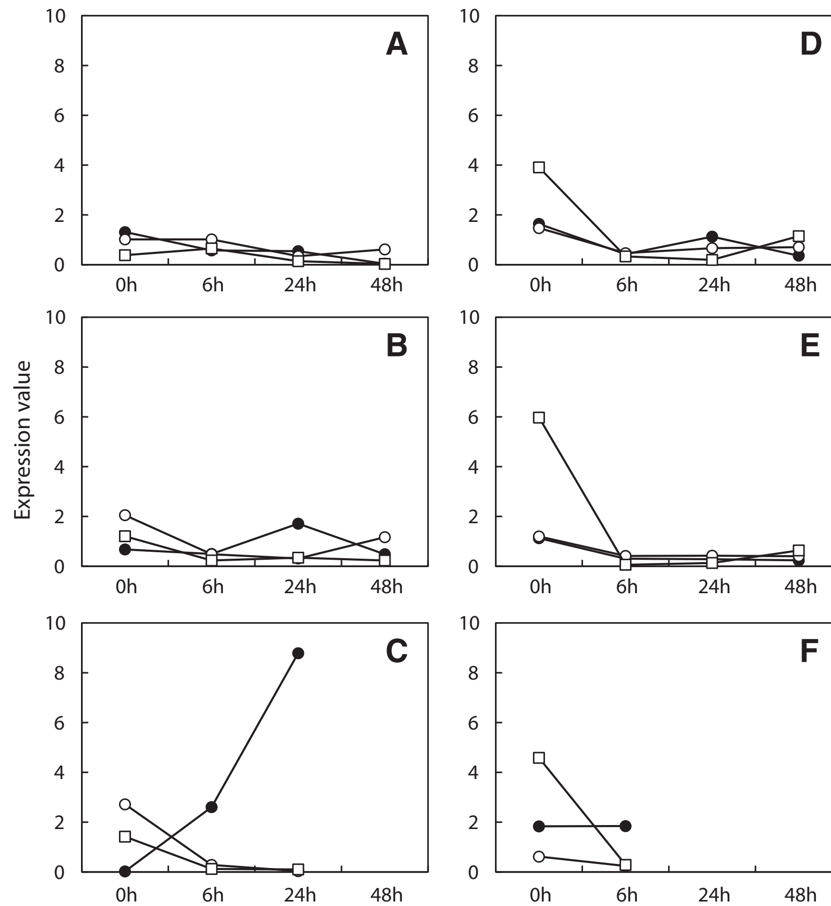

Fig. 4. Relative expression of genes enrolled in acid tolerance of S3 (*S.* Meleagridis) after incubation at 37 °C for 0, 6, 24 and 48 h in distinct acid stress treatments. A, B and C: trials performed with meat extract broth added to lactic acid with pH at 6.0, 5.0 and 4.0, respectively. D, E and F: trials performed with meat extract broth added to acetic acid with pH at 6.0, 5.0 and 4.0, respectively. *rpo*S gene (—●—), *nlp*D gene (—○—), *clp*P gene (—□—).

The ability of *S.* Typhimurium to develop organic acid tolerance increases concerns about food safety, since this serotype is among the most commonly reported in outbreaks and cases of food poisoning (Hendriksen et al., 2011). Another important piece of evidence that may be reflected in public health is that certain genes involved in acid tolerance are regulated by virulence genes located in plasmids (*spv*). These genes may interfere with bacterial growth rates inside the host cells during systemic infections (Chen et al., 1995; El-Gedaily, Paesold, Chen, Guiney, & Krause, 1997; Fang et al., 1992; Heiskanen, Taira, & Rhen, 1994). Audia, Webb, and Foster (2001) stated that *Salmonella* acid stress mechanisms can also provide cross-protection against other environmental stresses, such as oxidative stress, heat, osmolality and DNA damage. In these situations, other sanitizers employed by the food industry do not have the same bactericidal activity, for example, chlorine-based compounds.

Participation of the *nlp*D gene in *Salmonella* acid tolerance mechanisms has not been properly clarified. Some studies indicate that σ^s is produced by a polycistronic RNA portion that comprises the *nlp*D and *rpo*S genes, and deletions in the *nlp*D region can decrease protein production by 40% (Lange et al., 1995; Paesold & Krause, 1999). The results obtained in this study cannot indicate how *nlp*D behavior occurred under tested conditions.

*clp*P is involved in mechanisms that promote σ^s degradation when the bacteria is not under stress conditions (Foster, 2001). Based on this information, *clp*P could be expressed when *rpo*S and/or *nlp*D genes were expressed at low levels. However, the obtained data for the *clp*P gene demonstrated the absence of this behavior pattern

4. Conclusions

The results of this study have demonstrated the adaptability of *Salmonella* spp. when in contact with lactic acid and acetic acid solutions at pH values between 5.0 and 6.0, with clear expression of the *rpo*S gene. However, these findings suggest the need for further studies to investigate this acid tolerance behavior in meat systems in order to predict the possible effects of organic acid spraying in animal carcasses related to the development of resistant *Salmonella* strains.

Acknowledgments

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