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Acid and low temperature treatments on *Salmonella* Enteritidis inoculated in pork and its subsequent survival in simulated gastric fluid

Ácido e baixas temperaturas sobre *Salmonella* Enteritidis inoculada em carne suína e sua sobrevivência subsequente ao fluido gástrico simulado

Simone Quintão Silva[I] Miriam Teresinha dos Santos[II] Simone Albino Paes[II] Maria Cristina Dantas Vanetti[II]

ABSTRACT

*The objective of this study was to evaluate the acid resistance of **Salmonella enterica** serovar Enteritidis (**S.** Enteritidis) in stored pork and in simulated gastric fluid (SGF). A culture of **S.** Enteritidis was subjected to acid treatment prior to inoculation into pork, stored under refrigeration at frozen temperatures and exposed to SGF. The **S.** Enteritidis CCS3 and ATCC 13076 strains previously subjected to acid treatment (at pH 4.0-5.0) were inoculated in pork and stored at 4°C and -18°C. Storage at 4ºC did not affect the populations of both **S.** Enteritidis strains. After 84 days at -18°C, the mean population of both CCS3 and ATCC strains were reduced by 0.8 and 1.5 log cycles, respectively. Prior acid treatment did not enhance the survival of both strains at low temperatures. After acid treatment and low temperature storage, **S.** Enteritidis ATCC 13076 lost culturability after being exposed to SGF for 10 minutes. In contrast, **S.** Enteritidis CCS3 was tolerant until three hours of SGF exposure. **S.** Enteritidis CCS3 submitted to pH 4.0 was more tolerant to SGF exposure than when submitted to pH 4.5, 5.0 and without acid treatment. Therefore, this study indicates that exposure to an acidic and cold environment during processing enhanced the ability of **S.** Enteritidis to survive in the gastric environment of the human stomach, possibly increasing the risk of a **Salmonella** infection after consumption of pork.*

Key words: *acid resistance, SGF, freezing survival, storage survival, cross-resistance.*

RESUMO

*O objetivo deste estudo foi avaliar a resistência ao ácido de **Salmonella enterica** serovar Enteritidis (**S.** Enteritidis) previamente submetidas a tratamento ácido e inoculadas em carne suína armazenada em temperaturas de refrigeração e congelamento ao fluido gástrico simulado (FGS). As linhagens de **S.** Enteritidis CCS3 and ATCC 13076 previamente submetidas a tratamento ácido variando de pH 4.0 a 5.0 foram inoculadas em carne de porco e armazenadas a 4 e −18°C. A estocagem por sete dias a 4°C não afetou as populações das duas linhagens de **S.** Enteritidis. Após 84 dias a -18°C, as reduções médias das populações das linhagens foram de 0,8 e 1,5 ciclos logarítmicos, respectivamente. O tratamento ácido prévio não aumentou a sobrevivência das duas culturas sob baixas temperaturas. Após tratamento ácido e estocagem em temperaturas baixas, **S.** Enteritidis ATCC 13076 perdeu a culturabilidade após 10 minutos de desafio ao FGS. Contrariamente, **S.** Enteritidis CCS3 mostrou-se tolerante à exposição por três horas ao FGS. **S.** Enteritidis CCS3 submetidas a tratamento ácido prévio em pH 4,0 mostraram-se mais tolerantes à exposição por 180 minutos ao FGS que células submetidas aos tratamentos ácidos em pH 4,5 e 5,0 e células sem tratamento. Portanto, este estudo indica que **S.** Enteritidis submetida a um ambiente ácido e frio durante o processamento pode melhorar a sua capacidade de sobreviver à barreira gástrica em humanos, possivelmente, aumentando o risco de surto por **Salmonella** após consumo de carne de porco.*

Palavras-chave: *resistência a ácido, FGS, sobrevivência ao congelamento, sobrevivência à estocagem, resistência-cruzada.*

INTRODUCTION

Salmonella enterica is a pathogen that infects both animals and humans, causing gastroenteritis or systemic infections. To colonize the host, ***S. enterica*** must overcome the acidity of the stomach, the microbial flora of the gut, and the intestinal barrier in the host (ÁLVAREZ-ORDÓÑEZ et al., 2012). This study evaluated ***S. enterica*** serovar Enteritidis under various stress conditions, as it is the

[I]Departamento de Microbiologia, Universidade Estadual Paulista (UNESP), Campus de São José do Rio Preto, 15054-000, São José do Rio Preto, SP, Brasil. E-mail: simonequintaosilva@gmail.com. Corresponding author.
[II]Universidade Federal de Viçosa (UFV), Viçosa, MG, Brasil.

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most prevalent serovar among the brazilian human population (ROWLANDS et al., 2014). Although the main source of ***Salmonella*** are eggs and egg products (GANTOIS et al., 2009), other foods such as raw milk, beef and pork was associated to ***S. enterica*** infection outbreaks (ROWLANDS et al., 2014; CDC, 2015).

Several reports documented the presence of ***S.*** Enteritidis in swine abattoirs in Brazil (BESSA et al., 2004; KICH et al., 2011). Strategies used in abattoirs to prevent ***S. enterica*** growth include lower storage temperatures and surface decontamination using acids (SUN et al., 2003; PIPEK et al., 2006). Although these strategies create environments of moderate stress, ***S. enterica*** is capable of adapting to these adverse conditions, not only in natural environments in animal or human hosts, but also in commercial ones such as abattoirs and food industries (WINFIELD & GROISMAN, 2003; ÁLVAREZ-ORDÓÑEZ et al., 2012).

However, stress factors encountered during processing and storage of food as well as during passage through host physiological barriers (For e.g. gastric fluid) had a significant effect on the survival of ***S. enterica***. The physiological pH of human fluid gastric can range from 1.5 to 3.5 (MARIEB & HOEHN, 2010). Studies reported that ***S. enterica*** is resistant to acid and low temperatures conditions (MÜLLER et al., 2012). Bacteria adapted to mild acid stress survived in similar or different stress conditions due to a cross protection effect (SPECTOR & KENYON, 2012). Moderate acidic conditions could trigger resistance to gastric fluid in ***S. enterica***, increasing the risk and severity of illness (YUK & SCHNEIDER, 2006).

There was significant increase in the prevalence of ***S.*** Enteritidis and incidence of human salmonellosis in recent years. Moreover, acid tolerance is an important virulence factor related to survival of foodborne pathogens at low pH conditions of the gastric barrier. Studying the stress factors, therefore contributes to the resistance development in ***S.*** Enteritidis, which became increasingly relevant. The objective of this study was to evaluate the effect of pH and low storage temperatures on ***S.*** Enteritidis and its subsequent growth in simulated gastric fluid (SGF).

MATERIALS AND METHODS

Bacterial strains

Salmonella enterica serovar Enteritidis strains (ATCC 13076 and CCS3) were used in this study. The CCS3 strain was isolated from a swine carcass in a abattoir in Brazil (LIMA et al., 2004).

Acid treatment

S. Enteritidis strains were grown in Trypticase Soy Broth (TSB; pH 7.2) at 37°C for 12h. For acid treatment, the cells were suspended at a concentration of 10^8CFU mL^{-1} in TSB, adjusted to different pH (4.0, 4.5, and 5.0) with hydrochloric acid (5mol L^{-1}), for 1h at 37°C. After the acid treatment, the non-challenged and acid-challenged cells were centrifuged at 1275 x *g* for 10min at 4°C. Pellets were resuspended in 0.85% sterile saline solution at a concentration of 10^8CFU mL^{-1} for subsequent experiments.

Inoculation in cold and frozen pork

Pieces of pork loin (*Longissimus dorsi*) were first exposed to ultraviolet radiation for 30min for surface decontamination. Pork samples (10g each) were removed aseptically from the inside of the muscle and placed in sterile plastic bags. Aliquots of 1mL of non-challenged and acid-challenged ***S.*** Enteritidis ATCC 13076 and CCS3 (as described above) were transferred to the pork samples and spread uniformly. Pork samples were stored at 4±1ºC for up to 7 days and at -18±1ºC for up to 84 days. Non-inoculated pork samples were used as controls. All assays performed simultaneously, used the same equipment. The temperature of the equipment was monitored during the experiment.

Viable counts

The viable counts were determined by the drop plate method (MORTON, 2001). First, 90 mL of 0.85% saline solution were added to a portion of pork loin and homogenized in a stomacher (Laboratory Blender Stomacher 400, Seward) for 2min. Aliquots of the homogenate were serially diluted (1:10) and plated on Trypticase Soy Agar (TSA). The procedure was performed in triplicates and the plates were incubated for up to 10 hours at 37°C.

Evaluation of acid resistance in SGF

After different periods of storage at 4°C and -18°C, the surviving populations of ***S.*** Enteritidis were evaluated for resistance to SGF using a protocol adapted from CAMPBELL et al. (2004). First, aliquots of 5mL of pork homogenate were transferred to 10mL of SGF (pH 1.5) and incubated at 37°C. The SGF was prepared according to BEUMER et al. (1992) and heated to 37°C prior to mixing with homogenate samples. The bacterial count was performed using the drop plate technique on TSA in triplicates.

Statistical analysis

To evaluate the effect of acid treatment on the survival of cultures at low temperatures, the experiment conducted used a randomized 4x2x5 factorial design, replicated thrice. This included four treatments (one without acid treatment and three with acid treatment at pH 4.0, 4.5, and 5.0), two strains (ATCC 13076 and CCS3) and five periods of storage at 4°C (0, 1, 3, 5, and 7 days) and -18°C (0, 14, 28, 56, and 84 days). Data were analyzed using variance and

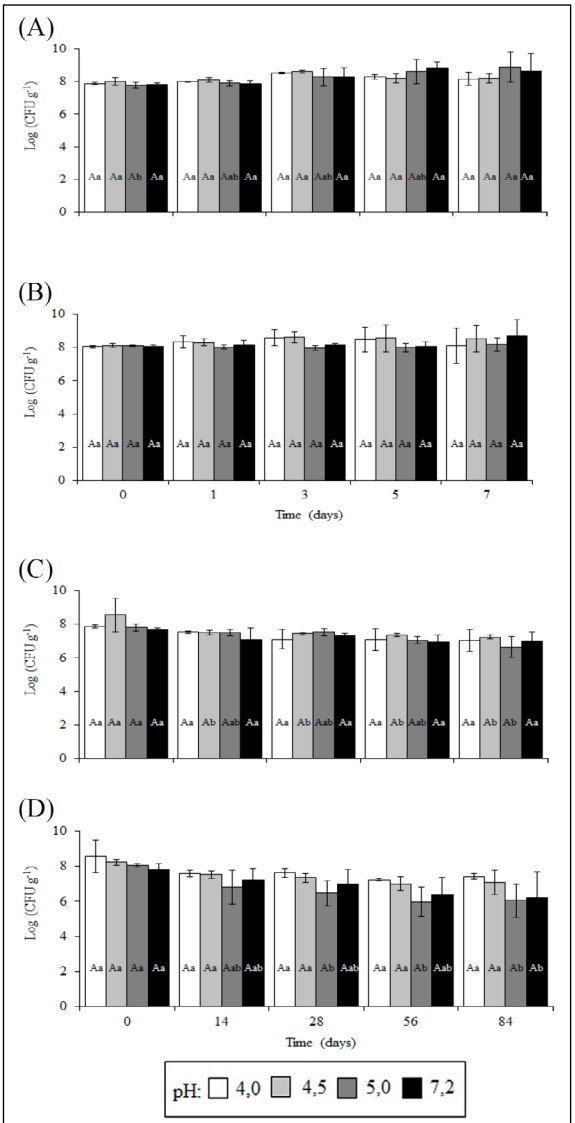
regression analysis. For factors like acid treatment and cultures, the means were compared using the F test and p values less than 0.05 were considered statistically significant. For factors such as storage period at 4°C and time of exposure in SGF, models were based on the significance of regression coefficients, using the t-test, adopting 10% for probability and magnitude of determination coefficient determination (R2). Statistical analyses were performed using the SAEG software, version 8.0 (UFV, 2000).

RESULTS AND DISCUSSION

Survival of ***Salmonella*** Enteritidis in chilled and frozen pork

Experimental conditions used in this study simulated conditions such as pH gradient (4.0 to 5.0), cool temperatures (4°C), frozen storage (-18°C), and host environment (SFG) that may occur during pork processing. The survival of ***S.*** Enteritidis CCS3 and ATCC 13076 in chilled pork is shown in figure 1A and 1B, respectively. Factors such as bacterial strain ($P<0.05$) and storage time ($P<0.001$) significantly impacted survival of ***S.*** Enteritidis in refrigerated stored pork, but not after acid treatment ($P>0.05$). We observed that the population of the CCS3 strain increased by an average of 0.6log cycle during storage. However, the population of the ATCC 13076 strain remained almost unchanged (variation of only 0.1log cycle). This suggested that ***S.*** Enteritidis ATCC 13076 is more sensitive to low or freezing temperatures than the CCS3 strain. These results; whereas, also confirmed that refrigeration is not sufficient to inhibit e growth of all ***S.*** Enteritidis strains in pork. KINSELLA et al. (2007) also observed the survival and increased growth of ***S. enterica*** serovar Typhimurium DT104 after 72 hours of beef storage at 4°C.

The survival rate of ***S.*** Enteritidis stored at -18°C was significantly different between strains ($P<0.01$) and between periods of storage ($P<0.001$) (Figure 1C and 1D). It was also observed that acid treatment at pH 4.0 to 5.0, prior to storage, did not affect the survival of ***S.*** Enteritidis in frozen pork ($P>0.05$). There was a decrease in the populations of both strains during the storage period. The mean reduction in viable cells of CCS3 and ATCC 13076 strains was approximately 0.8 and 1.5log cycles, respectively. ***S.*** Enteritidis ATCC 13076 had a lower survival rate than CCS3 strain during storage at freezing temperatures. These observations supported the hypothesis that the ATCC 13076 strain is more sensitive to low temperatures than the CCS3 strain. ESCARTÍN et al. (2000) reported that the decrease in the population of ***S. enterica*** serovars in naturally contaminated pork, stored at -15°C for 78 weeks, was approximately 3log cycles.



Means denoted as capital letters differ in the conditions of the acid treatments whereas the ones denoted as lowercase letters differ in the evaluation time, according to Tukey test ($P<0.05$).

Figure 1 - Logarithm of the number of colony forming units per gram (CFU g^{-1}) of ***Salmonella*** Enteritidis CCS3 strain (A) and ATCC 13076 strain (B) inoculated in pork meat kept at 4ºC for up to seven days; and survival of *S.* Enteritidis CCS3 strain (C) and ATCC 13076 strain (D) inoculated in pork meat kept at -18ºC for up to 84 days.

The differences observed in this study, between the survival of the CCS3 and ATCC 13076 strains at low temperatures could be due to the induction of cold shock proteins that protect cell components, as well as changes in the lipids of the

cell membrane, DNA supercoiling, and ribosome function (SPECTOR & KENYON, 2012).

In this research, prior acid treatment of pork did not affect the survival rate of both ***S.*** Enteritidis CCS3 and ATCC 13076 during storage at refrigeration or freezing temperatures, indicating that there was no cross-resistance. Conversely, cross-resistance was observed for ***S.*** Enteritidis previously exposed to acidic conditions in culture medium (XU et al., 2008) and for ***Salmonella*** Typhimurium in fermented dairy products (SHEN et al., 2007) at low temperatures. This phenomenon investigated food pathogens acquiring resistance to adverse conditions after moderate stress conditions during food processing.

Survival of ***Salmonella*** Enteritidis in SGF

S. Enteritidis CCS3 after acid treatment at pH 4.0, 4.5, 5.0, and 7.2 for 1h after challenged at low temperature (4°C and -18°C) survived in SGF (pH 1.5) for up to 3 hours (Figure 2), whereas ATCC 13076 strain challenged under the same acidic conditions and low temperature was not detected after 10min of exposure in SFG (Table 1). For ***S.*** Enteritidis CCS3, we observed a significant association ($P<0.001$)

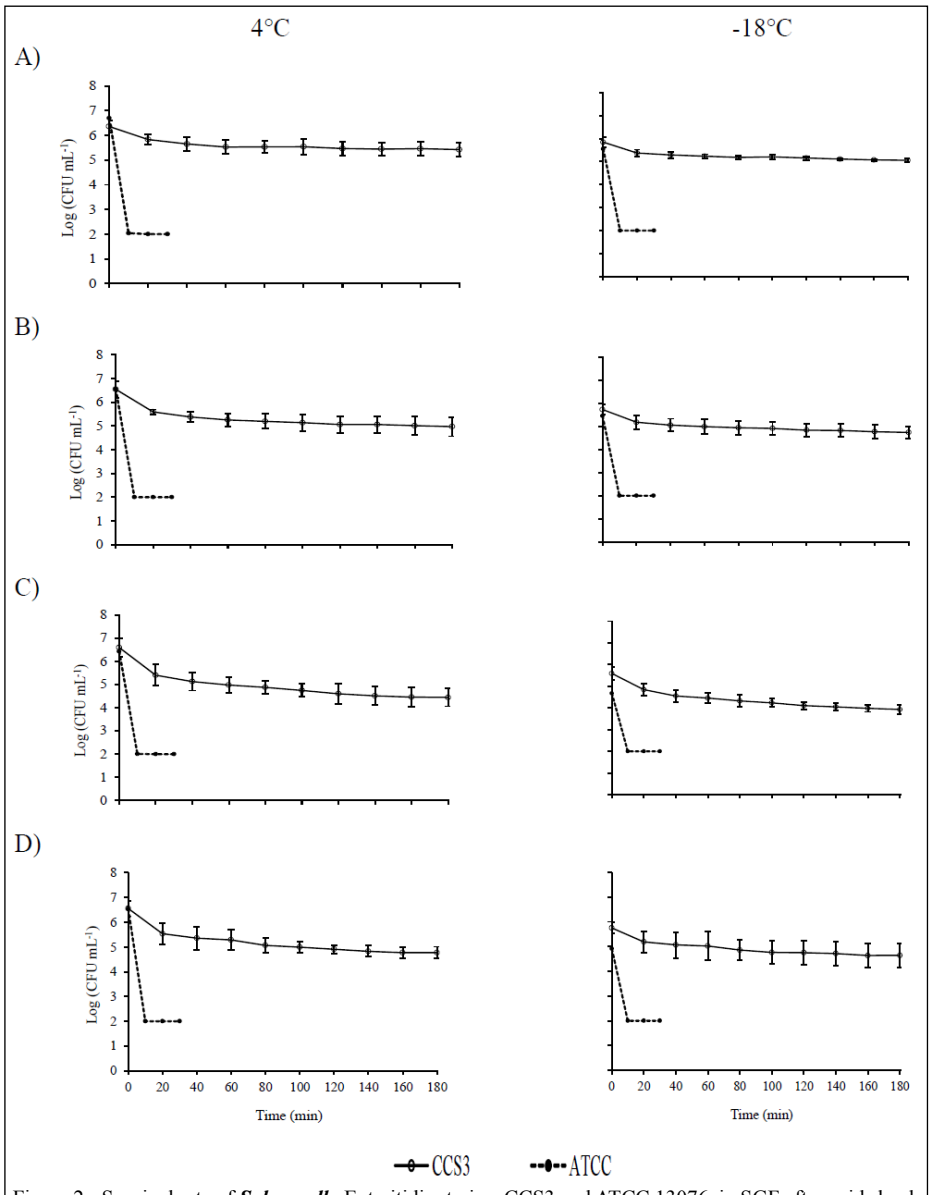


Figure 2 - Survival rate of ***Salmonella*** Enteritidis strains, CCS3 and ATCC 13076, in SGF after acid shock treatment at different pH values for 1h and storage at either 4ºC for 7 days or at -18ºC for 84 days. Cells underwent acid treatment at (A) pH 4.0 (B) pH 4.5 (C) 5.0 (D) pH 7.2 (control).

Table 1 - Survival rate of ***Salmonella*** Enteritidis ATCC 13076 (Number of Log CFU mL^{-1}) in SGF after acid treatment at pH 4.0, 4.5, 5.0, and 7.2 for 1h (P>0.05), and subsequent storage at either 4ºC for 7 days or at -18ºC for 84 days (P<0.001).

pH of acid treatment	Survival Rate (4°C)		Survival Rate (-18°C)	
	0min	10min	0min	10min
4.0	6.48	= 2.0*	5.30	= 2.0
4.5	6.38	= 2.0	4.98	= 2.0
5.0	6.55	= 2.0	4.25	= 2.0
7.2	6.70	= 2.0	4.40	= 2.0

*Values correspond to the detection limit of the drop plate method.

between exposure time and pH value of acid treatment and acid resistance in the SGF. Decrease in the population of the CCS3 strain was more substantial during the first 20 minutes of exposure. After this period, the reduction in the number of viable cells was gradual, indicating the development of tolerance in ***S.*** Enteritidis CCS3 to acidic conditions. Before exposure to SGF, population of CCS3 strain was approximately 6.5log cycles (Figure 1A and 1B) and 5.8log cycles after 7 days of refrigeration and 84 days of freezing (Figure 1C and 1D), respectively. After 3 hours of exposure in the SGF, final population of strains stored for 7 days and 84 days reduced to 4.9 and 4.5 log cycles, respectively (Figure 2).

The average reduction of the population of ***S.*** Enteritidis CCS3 was approximately 1.4log cycle after challenge in SGF. The CCS3 strain previously challenged at pH 4.0 (Figure 2A) showed only a 15% decrease in the number of log cycles at the end of 180 minutes of exposure to SGF. Therefore, a decrease of approximately 30% was observed in the CCS3 strain population during the exposure time, after acid treatments at pH 4.5 and 5.0, and in the absence of acid treatment (control) (Figure 2B, 2C, and 2D). This behavior indicated that acid shock at pH 4.0 followed by refrigeration increased the survival rate of CCS3 strain in more acidic environments such as SGF.

The increase in exposure time in SGF significantly reduced (P<0.001) the population of ***S.*** Enteritidis ATCC 13076 inoculated in pork stored at refrigeration and freezing temperatures (Table 1). The ATCC 13076 population strain showed a reduction of at least 4 log cycles after 10 minutes of exposure to SGF, indicating high sensitivity to the acidic environment. The survival rate of ATCC 13076 strain did not show significant differences (P>0.05) between acid treated and control pork stored at refrigeration and freezing temperatures. There was a difference between the survival of ***S.*** Enteritidis ATCC 13079 and CCS3 in SGF; whereas, the majority of the population of the CCS3 strain remained viable for over three hours of SGF exposure, the population of the ATCC 13076 strain showed a drastic decrease in viability after only 10 minutes of exposure.

In this study, the population of ***S.*** Enteritidis CCS3 was exposed to acidic conditions (pH 4.0) survived better at low temperatures as well as in simulated gastric fluid, which suggested cross-resistance. YUK & SCHNEIDER (2006) also observed that the adaptation of ***Salmonella*** serovars present in refrigerated juices with moderate pH resulted in increased survival of these cells in SGF.

Furthermore, the results showed that ***S.*** Enteritidis ATCC 13076 and CCS3 overcame low temperatures in pork after being subjected to acid treatment. However, it is less likely that the ATCC 13076 strain survive the acidic environment created by the gastric juices of the stomach. These results demonstrated that ***S.*** Enteritidis CCS3 populations exposed to acid solutions such as those used for decontamination of animal carcasses and sanitizing areas in abattoirs developed tolerance to the gastric environment.

CONCLUSION

Refrigeration of pork did not inhibit the growth of ***Salmonella*** Enteritidis CCS3 and ATCC 13076. Although, the ATCC 13076 strain was not detected after 10 minutes, ***S.*** Enteritidis CCS3 survived for up to 3 hours in SGF. Thus, the ***S.*** Enteritidis CCS3 strain that was isolated from swine carcass poses a potential risk to the health of consumers due to contamination of undercooked meat as well as cross contamination of ot her food products.

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