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Adaptation of *Salmonella* to Antimicrobials in Food-Processing Environments

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1. Introduction

Salmonella remains an important concern in food processing environments as it causes salmonellosis, a major public health problem throughout the world (Anonymous, 2004; Mead et al., 1999). This review deals with the ability of *Salmonella* to survive and adapt to the antimicrobial stresses encountered in food processing environments. It illustrates how bacteria can develop greater tolerance to these stresses and cross-protection with other environmental stresses, thus increasing their persistence throughout the food chain. Two types of antimicrobial agents are primarily encountered in food-processing environments, food preservatives and disinfectants.

Food preservatives are directly included in food products. Their use is the most common method to guarantee microbial food safety until the end of shelf-life. Among these substances, traditional chemical preservatives should be distinguished from naturally-occurring antimicrobial compounds. The former group includes officially approved compounds such as organic acids (lactic, acetic, sorbic, benzoic acids) and their salts, nitrites and nitrates, or sulfites (Roller, 2003). The latter group includes compounds which come from a natural source: whether they are produced by a microorganism, e.g. several weak acids or bacteriocins, or whether they are extracted from a plant or animal product, e.g. essential oil compounds, lactoperoxidase or lactoferrin (Roller, 2003). In most cases, these natural antimicrobials are used in foods for their acidifying or flavoring properties, but they also play an important role in maintaining microbiological food safety. Indeed, it has been long recognized that numerous natural compounds have antimicrobial properties (Naidu, 2000; Roller, 2003), among them, weak organic acids (Doores, 2005) and essential oil compounds (Burt, 2004). The inhibition of Gram-negative bacteria by bacteriocins produced by lactic acid bacteria is limited because of the impermeability of the outer membrane (Abee et al., 1995), even though treatment with EDTA (which permeabilizes the membrane) can render these bacteria sensitive to nisin but not to pediocin (Schved et al., 1994). Nevertheless, microcin L, a bacteriocin produced by *E. coli,* is highly efficient against *S.* Typhimurium (Morin et al., 2011).

Disinfectants are chemical agents used on inanimate surfaces to inactivate all recognized pathogenic microorganisms (Centers for Disease Control and Prevention, USA). They are

applied regularly in food processing environments in order to control surface biocontamination, and must be officially approved (Anonymous, 1998). Numerous disinfectants with different mechanism of action are widely employed by the food industry, such as peroxygens, quaternary ammonium compounds, halogen-realizing agents or aldehydes (McDonnell & Russell, 1999). But regulations are currently being revised (Reach, biocide directive) and some of these molecules would no longer be permitted in the future years (Anonymous, 2006; Anonymous, 2009).

Antimicrobial properties of a compound can be characterized with respect to its bacteriostatic and/or bactericidal activities. The bacteriostatic activity of an antimicrobial is determined by its minimum inhibitory concentration (MIC); i.e. the minimum concentration that can completely inhibit growth of the target pathogen. The bactericidal activity can be evaluated using a survival curve (a single concentration is applied and survivors are enumerated over time) or by establishing the Minimum Bactericidal Concentration (MBC: a range of concentrations are applied for a specific period of time and survivors are then numbered). Food antimicrobials are intended to eradicate or inhibit the growth of pathogenic microorganisms, whereas disinfectants are only designed to kill the microbial population rapidly.

Although bacterial resistance can be characterized regularly using one of these techniques, it is important to know that, like in numerous pathogens, the resistance of *Salmonella* to antimicrobials can evolve as a function of its living conditions. If bacteria are subjected to stressful conditions, they can increase their survivability under conditions that would normally be lethal. Induced tolerance was first demonstrated in *Salmonella* after a heat pre-shock at 48°C which induced an increase in the further thermotolerance of the strain (Mackey & Derrick, 1986). Preservative factors can impose non-lethal stresses upon bacteria in foods, potentially eliciting stress tolerance. Moreover, consumers today are looking for additive-free, fresh and more natural foodstuffs. In this context, combinations of treatments may be a way to enable their use at low doses while maintaining microbial food safety. The term "hurdle technology" is widely employed to describe these combinations of technologies with preservative effects (Leistner, 1995) and, because these treatments are often applied at the same time, the term multifactorial preservation has been proposed as being more appropriate (Roller, 2003). However, because these cumulative processes are all applied at sub-inhibitory concentrations, they may promulgate an adaptive stress response and enable the survival of a greater fraction of the bacterial population.

Moreover, in food processing or farming environments, *Salmonella* biofilms can settle on surfaces, despite disinfection procedures (Vestby et al., 2009). A biofilm is a bacterial community that adheres to a surface and is embedded in a matrix of microbial extracellular polymeric substances (Costerton et al., 1995; Hoiby et al., 2010). Protected by this matrix, *Salmonella* cells in the deeper layers of the biofilm thus become less accessible to the disinfectant because of diffusion limitations and can develop adaptive stress responses to sub-lethal concentrations of disinfectant.

The ability of the pathogen to respond to stressful conditions has been described as bacterial stress adaptation, stress adaptive response, habituation, induced tolerance, acclimatization or stress hardening (Yousef & Courtney, 2003). Numerous physiological responses are

implicated in this increase in bacterial survival and tolerance in harsh environments (Wesche et al., 2009). Many physiological modifications occur concomitantly in the cell, including protein up- or down-regulation, modifications to the cell membrane composition and altered morphology. In addition, these physiological changes following stressful conditions can induce cross resistance to other stressful environmental conditions, modifications to colonization or virulence (Figure 1). In all cases, bacterial stress will exert a considerable impact on the persistence of *Salmonella* throughout the food chain because of the modifications they induce to cell physiology, reactivity and tolerance. This is therefore a major concern in the area of food microbiology because it may constitute an emergent microbiological hazard.

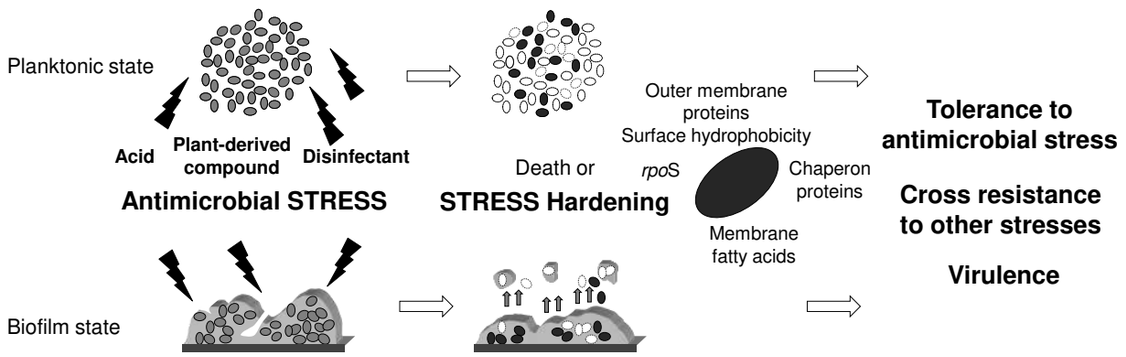

Fig. 1. Adaptation of *Salmonella* to antimicrobial stresses in food processing environments and consequences

Adaptation of *Salmonella* to both food antimicrobials and disinfectants will be discussed in the next three parts. Among the former group, we will focus on the two main groups of natural compounds which have demonstrated their efficiency against *Salmonella* and many other Gram-negative bacteria; i.e. weak acids and essential oil compounds.

2. Adaptation of *Salmonella* to weak organic acids

This section reviews the ability of *Salmonella* to grow and survive under acidic conditions. We will also discuss its capacity to develop acid adaptation and the consequences relative to its cross resistance to other stresses and virulence.

Salmonella may encounter a variety of acidic stress situations in both natural and industrial environments, as well as during pathogenesis. In foodstuffs, weak organic acids (e.g. acetic, lactic, citric acids, etc.) are either present naturally as constituents of the food, are produced by fermentation or are added during food formulation in order to protect against deterioration. For example, acetic acid is widely employed as a food acidulant to inhibit microbial growth and extend the shelf-life of food. Within the human digestive system, *Salmonella* must endure an extreme pH in the stomach and survive in the presence of weak acids in the intestinal environment before reaching the epithelial cells (Baik et al., 1996).

2.1 Growth or survival under acidic conditions

The optimum pH for *Salmonella* growth is generally said to be between 6.5 and 7.5, but the minimum pH value depends on many factors such as the strain and type of acid. For example, *Salmonella* Typhimurium can grow at 25-37°C at pH 4.5 when the pH is adjusted with citric acid, while it has to be over 5.4 with lactic acid and 6.4 with acetic acid (Alvarez-Ordonez et al., 2010a). These findings can be explained because the undissociated form is that which is primarily responsible for antimicrobial activity, as it is able to penetrate into the cell and reduce the cytoplasmic pH by intracellular dissociation. For a given acid, the lower the pH, the higher the proportion of the undissociated form (Dziezak, 1986). However, the activity of each acid will depend on its pKa; the higher the pKa, the higher is the proportion of undissociated form at a given pH. For example, at pH 5, 34.9% of acetic acid is undissociated, as opposed to 0.41% of citric acid at the same pH. Inhibitory efficiency, thus, decreases as follows: acetic acid (pKa 4.75) > lactic acid (pKa 3.08) > citric acid (pKa1 3.14; pKa2 4.77; pKa3 6.39) (Doores, 2005). Acid preservatives are therefore more efficient in acid or acidified foods. Lactic, acetic, and benzoic acids are authorized in foodstuffs at *quantum satis* concentrations ("the amount which is needed"). They are generally added to foodstuffs at a rate of 1% or higher. Above the pH mode of action, some acids exert specific toxicity; for example propionic, sorbic or benzoic acids, which should therefore be used at lower concentrations such as 0.05% to 0.2% (Mescle & Zucca, 1996).

The minimum growth pH of *Salmonella* is also temperature-dependent. The minimum growth pH is increased by about 1 pH unit at high (45°C) or low (10°C) temperatures, compared to the minimum pH at 25-37°C (Alvarez-Ordonez et al., 2010a).

Above the minimum pH level, growth kinetics can be characterized by modeling the growth rate versus the concentration of acid (Guillier et al., 2007). Inhibitory activity will depend on the type of acid, its concentration and the pH. Beyond the MIC determination, one parameter is particularly useful for characterizing acid inhibition: the Non-Inhibitory Concentration (NIC) which is the higher concentration at which no inhibition is observed. Different models can be utilized in this respect, including a square-root model based on the Lambert and Pearson model (Lambert & Pearson, 2000). The growth rate (μmax) as a function of acid concentration (c) is expressed as follows, where MIC, NIC and μmax(c=0) are the model parameters (Guillier et al., 2007):

$$\sqrt{\mu_{\max}(c)} = \sqrt{\mu_{\max}(c=0)\cdot g(c)} \text{ where } g(c) = \exp\left[-\left(\frac{c}{MIC \Big/ \exp\left(\frac{\ln(NIC/MIC)}{-e}\right)}\right)^{\left(\frac{-e}{\ln(NIC/MIC)}\right)}\right]$$

Depending on the acid, different growth rate inhibition profiles have been generated while acid concentration increases in the medium (Figure 2). There was no pH adjustment. For example, the inhibitory effect of lactic acid on *S.* Typhimurium starts above 36.6 mM, while citric acid is effective up to 9.9 mM. The MIC values for citric, lactic and acetic acids in these conditions are respectively 30 mM, 40 mM and 52 mM (Guillier et al., 2007). In addition to

the MIC, determining the NIC is of great importance because for some compounds, the difference between NIC and MIC values is very small. Thus a small error made when adjusting the concentration in a food product can lead to a level that is the wrong side of the growth-no growth limit.

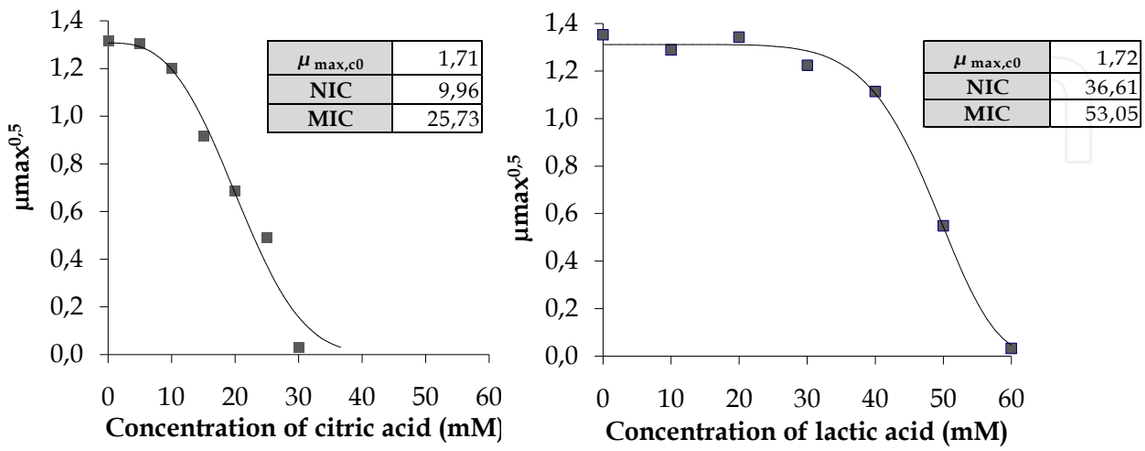

Fig. 2. *S.* Typhimurium growth rate inhibition versus concentration of citric acid or lactic acid (data from Guillier et al., 2007).

Below the minimum growth pH, *Salmonella* can nevertheless survive. The minimum survival pH has usually been stated to be at about 3.0 for *Salmonella*, unlike *E. coli* or *Shigella* that can survive at pH 2 or 2.5 in complex media (Lin et al., 1995). However, different strains of *Salmonella* have been shown to survive at pH 2.5 for prolonged periods of time (de Jonge et al., 2003). The emergence of these acid-resistant strains suggests a preconditioning in acidic conditions, most probably within the intestinal tract of ruminants fed with a carbohydrate-rich diet (de Jonge et al., 2003) as it will be described in the next paragraph.

Furthermore, acid compounds can also have a bactericidal activity against *Salmonella*. This can be evaluated in the same way as other inactivation treatments, using a survival curve or by determining *D*-values (number of minutes required to reduce the number of viable organisms by a factor of 10).

2.2 Onset of acid tolerance response (ATR)

Some early studies showed that the exposure of *Salmonella* to mild acidic conditions could protect it against extreme acid conditions (Huhtanen, 1975). When log-phase cells grown at pH 7.6 were shifted to mildly acidic conditions (pH 5.8) for one generation, they were able to develop 100- to 1,000-fold higher tolerance to extreme acid conditions (pH 3.3) than non-adapted cells shifted directly from pH 7.6 to 3.3 (Foster & Hall, 1990). The increased tolerance to a low pH following acid habituation was referred to as the Acid Tolerance Response (ATR) (Foster & Hall, 1990). Even if the majority of studies investigating acid resistance of *Salmonella* were first conducted with inorganic acids, it was later shown that organic acids also have the ability to induce resistance to many other

stresses. Acid shock confers protection on *Salmonella* against the lethal effect of a low pH under different conditions (see some examples in Table 1) obtained with different types of acids (citric, lactic, malic, acetic acids, HCl, etc.), challenge media (laboratory media or foodstuffs), or growth conditions during adaptation. More than just the pH value, it has been shown that the type of acid can influence the amplitude of the ATR. For *S.* Typhimurium, higher ATR values are obtained in order using citric, acetic, lactic, malic acids (Alvarez-Ordonez et al., 2009). Moreover, food composition can influence the stress responses and subsequent tolerance properties of adapted cells. For example, acid-adapted *S.* Typhimurium was more resistant to extreme acidic conditions when meat extract was used as a challenge medium rather than BHI (Alvarez-Ordonez et al., 2009). Recently, growth temperature has also been shown to be an important factor affecting the *Salmonella* ATR. Acid tolerance was increased 2-fold in cells grown at 10°C while it increased more than 3.5-fold at a temperature higher than 25°C (Alvarez-Ordonez et al., 2010a). Moreover, higher ATR obtained with citric acid occurred only at 25-37°C but not at 10°C or 45°C (Alvarez-Ordonez et al., 2010a). More than preventing growth, maintaining a low temperature may therefore constitute a means of limiting induction of the *Salmonella* ATR. In addition, the ATR may emerge with some strains more than others. For example, *S.* Typhimurium demonstrated a higher degree of acid tolerance than *S.* Typhi (Arvizu-Medrano & Escartin, 2005).

The acid tolerance response is a major concern in terms of food safety as it could permit *Salmonella* survival in acidic environments such as fermented foods, following acid cleaning treatments of industrial surfaces or the gastrointestinal tract. For example, acid-adapted cells were able to survive for a period of two months in cheddar, Swiss and mozzarella cheeses stored at 5°C (Leyer & Johnson, 1992).

2.3 ATR mechanism

The ATR mechanism of *Salmonella* has been widely described (Bearson et al., 1997; Foster, 1991; Foster & Hall, 1990; Lee et al., 1994). *Salmonella* possesses both log-phase and stationary-phase ATR systems. The log-phase ATR appears to be a two-stage inducible system (Foster, 1991) with complex regulation systems detailed below. The higher level of acid tolerance provided in stationary-phase appears to differ from log-phase ATR. It has been attributed to a pH-dependant system called stationary-phase ATR, together with a generalized stress response (Lee et al., 1994).

The two-stage system of log-phase ATR consists of pre-acid and post-acid shocks. Pre-acid shock, activated at pH 6, involves an emergency pH homeostatic system, designed to maintain the intracellular pH higher than 5. It induces several amino acid decarboxylases, such as lysine decarboxylase, which decarboxylates intracellular lysine to cadaverine while consuming a proton. Cadaverine is then exchanged for another lysine from the medium *via* a CadB antiporter (Bearson et al., 1997). A higher relative expression of the lysine and arginine decarboxylase systems was demonstrated in acid-adapted *Salmonella* Typhimurium (Alvarez-Ordonez et al., 2010b). The existence of these homeostatic systems, that can use the extracellular amino acids present in foodstuffs such as meat extract, explains the higher acid tolerance observed in these media. However, prolonged exposure (6h) to mild acid stress causes a loss of homeostasis, resulting in more susceptibility to lethal stress (Greenacre et al., 2003).

Post-acid shock is induced when the pH falls below 4.5. Acid shock proteins (ASP) are required for the adaptive acid tolerance response. Their synthesis is dependent on several regulatory genes: the alternative sigma factor σ^S encoded by *rpoS*, the iron regulator Fur and the two-component signal transduction system PhoPQ (Baik et al., 1996; Bearson et al., 1997; Foster, 1991; Foster, 1999). The alternative sigma factor σ^S has been recognized as a key factor in increasing the stress resistance of *Salmonella* cells in stationary phase but it can also be induced by stresses that bacteria may encounter during processing, such as acid stress (Dodd & Aldsworth, 2002). It controls the expression of about 40 genes/operons involved in generating the physiological changes associated with survival processes, among them ASP synthesis. For example, *S.* Typhimurium produces forty-three ASPs at pH 4.5 (Foster, 1993). At pH values lower than 2.6, the addition of chloramphenicol (which inhibits protein synthesis) has been found to reduce subsequent acid resistance, although the cells were not as sensitive as control cultures grown at pH 7.0 (Humphrey et al., 1993). Some ASPs are chaperon proteins which may protect internal proteins from denaturation (Foster, 1991). The pre-acid shock stage offers the cell an enhanced ability to synthesize APSs following acidification of the medium. The long-term adaptation of *S.* Enteritidis to propionate acid induces the differential expression of over twenty proteins. Of the five over-expressed proteins, two were clearly found to be implicated in acid induced resistance: Dps, a DNA-binding protein from starved cells and CpxR, a transcriptional regulatory protein (Calhoun et al., 2010). These two proteins are normally associated with virulence and pathogenesis.

Moreover, acid adaptation in log-phase has been shown to induce increased production of specific outer membrane proteins and enhanced surface hydrophobicity without lipopolysaccharide alteration (Leyer & Johnson, 1993). These surface modifications may be of considerable concern because they may affect the ability of *Salmonella* to adhere to inert surfaces in food processing environments, and then to form biofilms. Alongside proteome modifications, some other physiological modifications appear following acid adaptation, such as modifications of fatty acid profiles. The proportions of membrane cyclopropane fatty acid (CFA) in *S.* Typhimurium were found to be higher at a pH lower than 6 when compared with those of cells grown at pH 7.5 (de Jonge et al., 2003). CFA levels were also found to be 1.5-fold higher in acid-adapted cells (grown in the presence of acids at pH values of 6.4, 5.4, or 4.5 in BHI) than in non-adapted cells (Alvarez-Ordonez et al., 2008). It could be speculated that acid-induced RpoS in the exponential phase might increase CFA synthase activity in *S.* Typhimurium (Kim et al., 2005). CFAs are produced by the addition of a methyl group from *S*-adenosyl methionine across a *cis*-double bond of unsaturated fatty acids (UFAs). They are generally known to be preferentially synthesized when cells enter the stationary phase in *Salmonella,* as in numerous other bacteria (Kim et al., 2005). The conversion of UFAs into CFAs induces a decrease in membrane fluidity. This may be a way for stressed cells to limit exchanges with the external medium and conserve energy. The membrane fluidity of *Salmonella* adapted to citric acid was measured by fluorescence anisotropy of a fluorescent probe (DPH) and the results showed that it was lower than that of control cells (Alonso-Hernando et al., 2010). The over-synthesis of CFA is considered to be a major factor in the acid resistance of Gram-negative bacteria (Brown et al., 1997; Chang & Cronan, 1999).

Another concern is that, following acid adaptation, some injured cells may shift into a viable but non-cultivable (VBNC) state, which is one of the current mechanisms that

allows non-sporulating cells to survive. These cells cannot grow on the selective media used to detect pathogens in foods, typically xylose lysine desoxycholate agar for *Salmonella*. They can escape the microbiological controls, but can slowly repair themselves and then re-grow in foodstuffs while being able to maintain their metabolic activity and pathogenicity (Xu et al., 2008).

Acid adaptation appears to be a multiple adaptive response related to the synthesis of several regulatory systems and leading to major modifications of cytoplasmic proteins, together with modifications to membrane protein and fatty acid contents. It is essential to understand these physiological adaptations as they can induce bacterial resistance and hence the persistence of pathogens in the food chain.

2.4 Cross resistance in acid-adapted *Salmonella*

In foods or food processing environments, *Salmonella* can encounter multiple subsequent stressor treatments. Numerous studies have demonstrated that acid adaptation can ensure cross-resistance (or cross-protection) to stresses other than acid stress, such as heat, biocide damage or high osmolarity.

Heat tolerance was the first cross-protection which was demonstrated after *Salmonella* was exposed to acid. For example, a 10-fold difference in survivors was seen between acid-adapted or non-adapted populations after a 20 min challenge at 50°C (Leyer & Johnson, 1993). Heat tolerance was also shown after acid adaptation in orange or watermelon juices (Mazzotta, 2001; Sharma et al., 2005). Several acid shock proteins, including chaperon proteins, are similar to those induced by heat shock, such as GroEL or Dnak (Foster, 1993). This may be one explanation for the cross resistance observed.

Acid adaptation has also been described to enhance tolerance towards osmotic stress (Leyer & Johnson, 1993; Tosun & Gönül, 2003) but it was dependent upon the acid used for adaptation. Acetic acid adaptation provided cells with protection against both NaCl and KCl stresses, while lactic acid adaptation did not protect against osmotic stressors (Greenacre and Brocklehurst, 2006). In the same way, *S.* Enteritidis adapted to acid in marinades did not display any increased resistance to drying processes (Calicioglu et al., 2003).

Acid adaptation provoked dramatic sensitization (~10,000-fold) of *Salmonella* to halogen-based sanitizers including chlorine and iodine (Leyer & Johnson, 1997). This was explained because hypochlorous acid oxidized a higher percentage of cell surface sulfhydryl groups in acid-adapted cells than in non-adapted cells, and sulfhydryl oxidation was correlated to cell inactivation. In the same way, *Salmonella* adapted with lactic acid displays sensitivity to hydrogen peroxide which is concomitant with down-regulation of the OxyR regulon (Greenacre et al., 2006). These results could have an interesting application in maintaining microbial food safety. The weakening of *Salmonella* defenses by acid shock could constitute a potential strategy to enhance the action of halogen-based sanitizers. However, it has been shown that the long-term adaptation of *Salmonella* to propionate induces strong tolerance to the *in vitro* oxidative and nitrosative stresses that may be encountered in mammalian hosts (Calhoun & Kwon, 2010).

Salmonella serotype	Acid pretreatment conditions	Exposure to acid shock	ATR (versus non adapted cells)	Reference
S. Typhimurium	From mid-log phase cells, one cell doubling in Minimal E glucose pH 5.7 (HCl) (vs control at pH 7.0)	Challenge at pH 3.2 - 45 min	16% survival vs 0.25%	(Foster & Hall, 1990)
S. Enteritidis	From mid-exponential phase cells, Lemco broth for 3h at pH 3 (HCl) vs pH 7 (control)	Survival curves at pH 2.5 D-value is the number of minutes required to reduce the number of viable organisms by a factor of 10	D-value 1.9 min vs 0.4 min	(Humphrey et al., 1993)
S. Typhimurium	TSB 37°C 1h in aerobic conditions 100mM propionate vs NaCl (control)	TSB pH 3.0 1h	42% survival vs 0.2%	(Kwon & Ricke, 1998)
S. Typhimurium	2h at 20°C in TSBG in mid-exponential phase Acetic acid pH 5.5 vs TSBG pH 7 (control) Acetic acid pH 5.8 vs TSBG pH 7 Lactic acid pH 5.5 vs TSBG pH 7 Lactic acid pH 5.8 vs TSBG pH 7	Survival curves at pH 3.0 Kmax is the slope of the survival curve	Death rate Kmax 1 h^{-1} vs 28.5 h^{-1} Kmax 1.7 h^{-1} vs 28.5 h^{-1} Kmax 2.4 h^{-1} vs 28.5 h^{-1} Kmax 5.4 h^{-1} vs 28.5 h^{-1}	(Greenacre et al., 2003)
S. Typhimurium	90 min at 30°C in TSB without dextrose lactic acid pH 4.5 vs TSB pH 7.0 (control) lactic acid pH 5.0 vs TSB pH 7.0	Survival curves at pH 3.5 Kmax is the slope of the survival curve	Death rate Kmax 4.6 h^{-1} vs 9.4 h^{-1} Kmax 7.7 h^{-1} vs 9.4 h^{-1}	(Koutsoumanis & Sofos, 2004)
S. Typhimurium	4h at 25°C in PBS pH 5.5 (HCl)	Survival curves in commercial yoghurt at 5°C for 10 days	Reduction of the population 0.6 log vs. 1.34	(Shen et al., 2007)
S. Enteritidis	Adaptation at pH 5.0 for 2h vs control pH 7.3	Survival curves at pH 4.0 and 20°C obtained using the Weibull model $logN=logN_0-b.t^n$	b: 0.93 vs 2.96; n: 0.86 vs 0.42	(Xu et al., 2008)
S. Enteritidis	Adaptation at pH 5.0 for 2h vs control pH 7.3	Survival curves at pH 4.0 and 4°C obtained using the Weibull model $logN=logN_0-b.t^n$	b: 0.11 vs 0.75; n: 0.99 vs 0.74	(Xu et al., 2008)
S. Typhimurium	Growth until stationary phase in: BHI acetic pH 6.4 - 37°C vs non acidified BHI pH 7.4 BHI citric pH 6.4 - 37°C vs non acidified BHI pH 7.4 BHI HCl pH 6.4 - 37°C vs non acidified BHI pH 7.4 BHI citric pH 4.5 - 37°C vs non acidified BHI pH 7.4 BHI HCl pH 4.5 - 37°C vs non acidified BHI pH 7.4	Survival curve at pH 3 (HCl) The negative reciprocal of the survival curve was used for the D-value	D-value : 15.7 min vs 11.8 min D-value : 22.0 min vs 11.8 min D-value : 15.5 min vs 11.8 min D-value : 24.6 min vs 11.8 min D-value : 22.2 min vs 11.8 min	(Alvarez-Ordonez et al., 2010a)

Table 1. Examples of conditions for acid adaptation and tolerance in *Salmonella*

2.5 Virulence of acid-adapted *Salmonella*

The infectivity of *Salmonella* is based on its ability to overcome numerous lethal environments in order to reach the site of infection. These include the acid barrier of the stomach, the physical barrier of epithelial cells and various immune defenses. To survive in these acidic environments, *Salmonella* has developed elaborate systems to sense stresses and adaptive response to acid stresses. The connection between ATR and virulence is a matter of debate. On the one hand, an acid tolerant *S.* Enteritidis isolate was found to be more virulent in mice than an acid-sensitive isolate (Humphrey et al., 1996). ATR is known to be regulated by RpoS, which also influences the expression of specific virulence factors (Fang et al., 1992; Gahan & Hill, 1999). Mutations in the *rpoS* gene of virulent *S.* Typhimurium strains render them incapable of developing a full ATR and significantly reduce their virulence potential (Fang et al., 1992; Lee et al., 1995). On the other hand, one study demonstrated that low pH environments could select persistent phenotypes of *Salmonella* with increased acid tolerance, but these were less virulent (Karatzas et al., 2008a). Similarly, the long-term adaptation of *Salmonella* to propionate reduced the overall infectivity of the adapted cells by inhibiting the colonization ability of the organism (Calhoun & Kwon, 2010). Moreover, flagella motility, which is fundamental to the virulence process, was shown to be repressed at a low pH in order to allow the conservation of ATP for survival processes (Adams et al., 2001).

3. Adaptation of *Salmonella* to plant-derived antimicrobials

Among naturally occurring antimicrobials that can be used in foods, we will focus here on phyto-antimicrobials which have seen a considerable resurgence of interest during the past 10 years among both consumers and industry. Essential oils and their compounds are known to have antimicrobial properties against numerous bacteria and fungi, including *Salmonella* (Burt, 2004; Kim et al., 1995b). Most of these compounds are "generally recognized as safe" (GRAS) and have been registered by the European Commission for their use as flavoring compounds in food products.

3.1 Growth inhibition of *Salmonella* by plant-derived antimicrobials

The antibacterial activity of essential oils and their components is due to the permeabilization of the cytoplasmic membrane that leads to a loss of cellular constituents (Burt, 2004; Helander et al., 1998). Its characterization has been widely reported in laboratory media. As previously described, the mimimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) can be determined by modeling growth rate as a function of the antimicrobial concentration (Guillier et al., 2007). As for acid compounds, the growth rate curves versus concentrations have different profiles depending on the compound. For example, menthol is efficient above 0.91 mM for a MIC of 3.3 mM, while the inhibitory effect of carvacrol starts at 0.73 mM for a MIC at 0.90 mM (Guillier et al., 2007). Among 14 natural antimicrobials, phenolic compounds were shown to be the most efficient in inhibiting the growth of *S.* Typhimurium. The MICs of carvacrol, thymol, eugenol, geraniol, menthol, α-terpineol and *trans*-cinnamaldehyde in BHI at 37°C after 24h were respectively 0.9, 0.9, 3.0, 2.9, 3.3, 4.1 and 3.7 (Guillier et al., 2007). Similar MICs have been found in various media for thymol and carvacrol (Cosentino et al., 1999; Helander et al., 1998; Kim et al., 1995a; Olasupo et al., 2003).

Plant-derived compounds have also been shown to retain a degree of efficacy in foods, but they generally need to be used at higher concentrations to achieve the same level of inhibition. Carvacrol at 3% succeeded in eradicating *Salmonella* in fish cubes after 4 days at 4°C while citral and geraniol were less efficient (Kim et al., 1995b). Basil oil at 50 ppm reduced the number of bacteria in food from 5 to 2 log cfu/g after storage for 3 days (Rattanachaikunsopon & Phumkhachorn, 2010). The susceptibility of bacteria to the antimicrobial effect of essential oils appears to be dependent on the composition and pH of the food, and the storage temperature (Juven et al., 1994; Tassou et al., 1995). Proteins and lipids, among other food constituents, can interact with essential oils, thus limiting their activity. For example, neutralization of the antibacterial effect of thymol was demonstrated by the addition of bovine serum albumin, which probably binds thymol and thus prevents it from penetrating through the bacterial membrane (Juven et al., 1994). Moreover, the activity of thymol on *Salmonella* is greater under anaerobic conditions (Juven et al., 1994).

The concentrations used in food products are to a great extent governed by their effect on the organoleptic properties. The concentrations thus needed to inhibit bacteria often exceed the flavor threshold acceptable to consumers. In that context, multifactorial preservation is highly appropriate for these compounds. Characterization of the synergy between two compounds can be achieved using the isobologram method or by calculating the fractional inhibitory concentration (Najjar et al., 2007), but different experimental designs are necessary if the combinations involve numerous compounds. Some combinations have been shown to be highly effective against the growth of *Salmonella,* such as the use of two plant-derived compounds (cinnamaldehyde-thymol, cinnamaldehyde-carvacrol, thymol-carvacrol) (Zhou et al., 2007b), three compounds (thymol-carvacrol-citral) (Nazer et al., 2005), or plant-derived compounds associated with EDTA, acetic or citric acid (Zhou et al., 2007a).

Some of these compounds can be also used in the vapor phase. For example, allyl isothiocyanate (8.3 µL/liter of air), carvacrol (41.5 µL/liter) or cinnamaldehyde (41.5 µL/liter) were able to inactivate *Salmonella* (decrease of population > 5 log) on sliced tomatoes at 4°C in 10 days (Obaidat & Frank, 2009).

3.2 The tolerance response of *Salmonella* to plant-derived compounds

In the context of multifactor preservation, when bacteria are subjected to sub-inhibitory concentrations of antimicrobials, attention should be paid to the potential for bacterial adaptation and the induction of cross-resistance to other treatments. Little is known today about bacterial adaptation to plant-derived compounds, notably in the case of *Salmonella*. Nevertheless, the presence of thymol during the growth of *S.* Thompson induced the up- or down-regulation of many proteins (Di Pasqua et al., 2010). Different chaperon proteins were up-regulated or *de novo* synthesized, such as GroEL and Dnak which are key proteins in protecting cells against stress. Outer membrane proteins were also up-regulated in the presence of thymol (Di Pasqua et al., 2010). Moreover, the membrane fatty acid composition was markedly modified during growth in the presence of plant-derived terpenes such as carvacrol, thymol, citral or eugenol (Dubois-Brissonnet et al., 2011). The saturated fatty acids became more abundant in the cell membrane of *Salmonella* grown in the presence of these terpenes. Membrane saturation appears to be a primary response of bacteria in order to maintain both membrane integrity and functionality. Moreover, compared with control

cells, the cyclization of unsaturated fatty acids (UFA) to cyclopropane fatty acids (CFA) was markedly reduced when cells entered the stationary phase. It was hypothesized that terpenes, that have log $P_{o/w}$ values of about 3, probably accumulate between the acyl chains of fatty acids, thus limiting the accessibility of *S*-adenosylmethionine to the UFA *cis*-double bond (Dubois-Brissonnet et al., 2011).

A few studies have demonstrated cross-resistance induced by plant-derived compounds. *Salmonella* exposed to sublethal concentrations of tea tree oil displayed reduced susceptibility to a range of antibiotics when compared to non-habituated cultures (McMahon et al., 2007). For example, with a MIC of mupirocin of 64 mg/L in control *S.* Enteritidis, 1024 mg/L was not efficient against adapted cells (McMahon et al., 2007). *S.* Typhimurium cells adapted to carvacrol, thymol, citral or eugenol have all displayed higher tolerance to the bactericidal activity of two disinfectants used in industrial and medical environments, peracetic acid and didecyl dimethyl ammonium bromide (Dubois-Brissonnet et al., 2011). Because these two antimicrobials have different modes of action, it was hypothesized that this induced tolerance could be a general response to the stress induced by slow growth rates and by the reduction in membrane permeability caused by membrane saturation and terpene intercalation (Dubois-Brissonnet et al., 2011).

4. *Salmonella* adaptation to disinfectants – The biofilm implication

Disinfectants are chemical agents used to decontaminate inanimate surfaces. Several groups of disinfectants are regularly used in food processing environments; e.g. halogen-realizing agents, aldehydes, peroxygens or quaternary ammonium compounds (McDonnell & Russell, 1999). The mode of action of these disinfectants has been described extensively in numerous reviews (McDonnell & Russell, 1999; Russell, 2003). Unlike antibiotics, they have a broad spectrum of antimicrobial activity and generally act on several targets in microbial cells, such as outer and cytoplasmic membranes, functional and structural proteins, DNA, RNA and other cytosolic constituents (Russell, 2003).

4.1 *Salmonella* tolerance response to disinfectants

As disinfectants are intended to inactivate rapidly the biocontamination of surfaces, bacterial resistance to a disinfectant is generally evaluated by determining the minimum bactericidal concentration. The MBCs of *Salmonella* to three widely used disinfectants (peracetic acid, benzalkonium chloride and ortho-phthalaldehyde) were evaluated comparative to those of seven other bacterial species (Bridier et al., 2011a). The MBCs, defined here as the concentrations enabling a 5-log reduction in the initial population within 5 minutes at 20°C, were 8.2, 42 and 175 mg/L for the three disinfectants, respectively. Compared with other species, *Salmonella* did not display a remarkable resistance to these three disinfectants. There were only slight intra-specie variations among the ten different *Salmonella* strains, whatever their serotype (Bridier et al., 2011a).

Nevertheless, *Salmonella* is frequently isolated from food-processing environments. For example, *Salmonella* was isolated in 5.3% of 3485 samples of pork and 13.8% of 3573 environmental samples from seven slaughterhouses in four European countries (Hald et al., 2003). In Canada, 37.5% of the 1295 samples collected from 65 abattoirs were positive for *Salmonella* (Bohaychuk et al., 2009). The link between a persistence of *Salmonella* in the

food-processing environment and its adaptation to the disinfectants used in that environment has been discussed. On the one hand, some authors have demonstrated that there was no obvious association between the susceptibility of isolates to disinfectants, their tendencies to persist and the previous use of biocides (Gradel et al., 2005). A few serotypes isolated from Danish broiler houses tended to have higher MICs to some disinfectants, but not necessarily those they had encountered previously (Gradel et al., 2005). Similarly, some Norwegian isolates found to be persistent in fish feed factories were no more resistant to nine disinfectants used in these than isolates from other sources (Moretro et al., 2003). On the other hand, *Salmonella* is known to be able to adapt itself to some disinfectants and to demonstrate increased resistance to disinfection procedures. For example, the benzalkonium chloride (BKC) MIC of *S.* Virchow was shown to rise from 4 to 256 μg/mL after repeated exposure to sublethal concentrations (Braoudaki & Hilton, 2004). This strain was also able to adapt to chlorhexidine. Moreover, the benzalkonium adapted-strain displayed elevated tolerance to both BKC and chlorhexidine; however, the chlorhexidine-adapted strain did not display reciprocal cross-resistance to benzalkonium chloride, suggesting specific resistance mechanisms (Braoudaki & Hilton, 2004). Growth in the presence of increasing sub-inhibitory concentrations of tri-sodium phosphate or acidified sodium chlorite also caused a significant increase in their MICs for *Salmonella* (Alonso-Hernando et al., 2009). Furthermore, 7-day passages of *S.* Typhimurium in sub-inhibitory concentrations of an ammonium quaternary compound containing formaldehyde and glutaraldehyde (AQCFG) selected variants with reduced susceptibility to antibiotics (Karatzas et al., 2007).

Cross resistance to other environmental stresses was also demonstrated following disinfectant adaptation. Significant increase in thermotolerance and resistance to high pH occurred after 1h shock with 1.5% tri-sodium phosphate (TSP) (Sampathkumar et al., 2004). But sensitivity to acid and hydrogen peroxide concomitantly increased.

The impact of disinfectant adaptation on virulence is dependent on the type of biocide and conditions of stress. AQCFG-adapted variants displayed an altered expression of several virulence proteins and reduced invasiveness in an epithelial cell line (Karatzas et al., 2008b). Conversely, *S.* Typhimurium LT2 retained its adhesive and invasive abilities after treatment with 5 mg/L peracetic acid for 1h in sewage effluent (Jolivet-Gougeon et al., 2003).

Like for many other stresses, differential protein expression seems concomitant to increased tolerance. In AQCFG-variants, it has been demonstrated higher levels of the different proteins that protect against stressors such as oxidants or peroxides (Karatzas et al., 2008b). Following chlorine shock, *Salmonella* genes that were associated with stress response, biofilm formation or energy metabolism were also over-expressed (>1.5-fold) (Wang et al., 2010) and hydrogen peroxide treatment induced the synthesis of 30 proteins in *Salmonella,* including that of Dnak, a chaperon protein (Morgan et al., 1986). Moreover, the resistance of BKC-adapted *Salmonella* is related to the up-regulation of an active efflux system (Braoudaki & Hilton, 2005). Similarly, in pre-mentioned variants, bacterial adaptation to disinfectants coincides with the up-regulation of the AcrAB efflux pump, which is responsible for producing the multiple-antibiotic-resistance *mar* strains that are resistant to many agents (Karatzas et al., 2007).

In addition, membrane composition can sometimes be altered with disinfectant adaptation. *S.* Enteritidis grown in the presence of three sanitizers, including a chlorinated product, an alkaline cleaner and a phenolic solution, was shown to increase its short-chain polysaccharide fractions of the LPS (Venter et al., 2006). A reduction in cell hydrophobicity was induced following the adaptation of *S.* Enteritidis to BKC at sub-inhibitory concentrations, although no change to the LPS or outer membrane composition was noticed (Braoudaki & Hilton, 2005). When *Salmonella* cells were exposed to 1.5% TSP for 1 h, significant changes were seen to affect the membrane fatty acid composition: the saturated and cyclic to unsaturated fatty acid ratio increases which leads to a increase in membrane saturation (Sampathkumar et al., 2004). But modifications to membrane fluidity were not related with the induction of resistance following growth in increasing sub-inhibitory concentrations of tri-sodium phosphate (TSP) (Alonso-Hernando et al., 2010).

Because the mechanisms of *Salmonella* adaptation to disinfectants are not fully understood, it is wise to continue the widely accepted practice of rotating the use of disinfectants in the food industry to prevent the development of adaptation and increased resistance.

4.2 Implication of biofilms in the tolerance response of *Salmonella* to disinfectants

The persistence of *Salmonella* in food processing environments is often related to their survival on the surface of equipment. For example, an *S.* Enteritidis strain isolated from an egg conveyor belt was found to be a source of persistent infection in poultry (Stocki et al., 2007). Similarly, *Salmonella* was able to persist in food bowls that had contained raw meat contaminated by this pathogen (Weese & Rousseau, 2006). The survival of microorganisms in food-processing environments is frequently linked to the presence of three-dimensional biofilm structures on surfaces. *Salmonella* produces a biofilm matrix that is mainly composed of fimbriae (curli) and cellulose (Solomon et al., 2005). A study of 111 *Salmonella* strains isolated in Norwegian feed and fish meal factories demonstrated that persistent strains were those that could produce more biofilm than presumably non-persisting strains (Vestby et al., 2009). Images of *Salmonella* biofilms (Figure 3) were acquired using scanning electron microscopy at the MIMA2 microscopy platform (http://voxel.jouy.inra.fr/mima2).

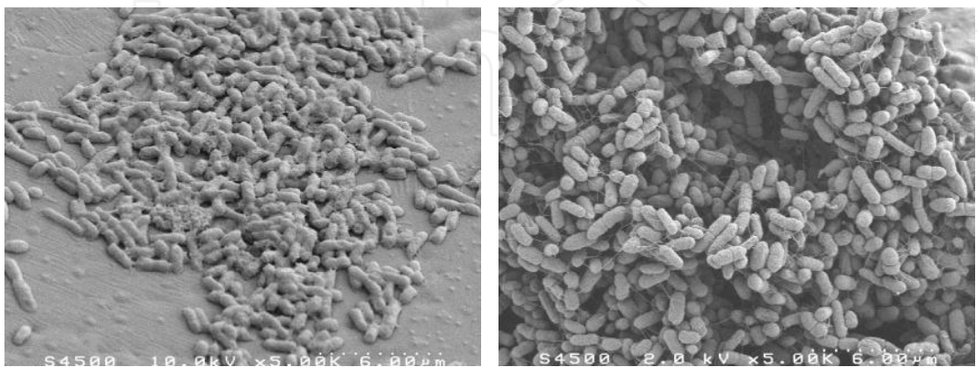

Fig. 3. Images of *Salmonella* biofilms obtained by scanning electron microscopy

Like many other biofilms, *Salmonella* biofilms display specific properties that include increased resistance to biocide treatment (Wong et al., 2010). Reductions in surface-attached

Salmonella using 256 ppm of sodium hypochlorite and BKC were respectively decreased 2.1 and 3-fold compared to the planktonic population (Riazi & Matthews, 2011). Sodium hypochlorite was shown to be more efficient than BKC and hydrogen peroxide against *Salmonella* biofilms formed in the Calgary Biofilm Device (Rodrigues et al., 2011). *S.* Typhimurium is more resistant to chlorine treatments when associated with *Pseudomonas fluorescens* in a mixed biofilm (Leriche & Carpentier, 1995).

Rather than a true resistance, biofilm insusceptibility is sometimes referred to as a tolerance because it is mainly induced by physiological adaptation to the biofilm mode of life (Russell, 1999). Biofilm resistance is multifactorial, resulting from the addition of different mechanisms such as diffusion and/or reaction problems affecting the sanitizers in the structure, the appearance of resistant biofilm-specific phenotypes (persister cells), of physiological and genetic heterogeneity, and adaptation to sanitizers (Bridier et al., 2011b). In deeper regions of the three-dimensional structures of biofilms, *Salmonella* may be protected against biocide activity because of the limited reaction-diffusion penetration of antimicrobial agents (Figure 4). Only sub-lethal concentrations of disinfectants can reach the bacteria which may thus develop adaptation mechanisms.

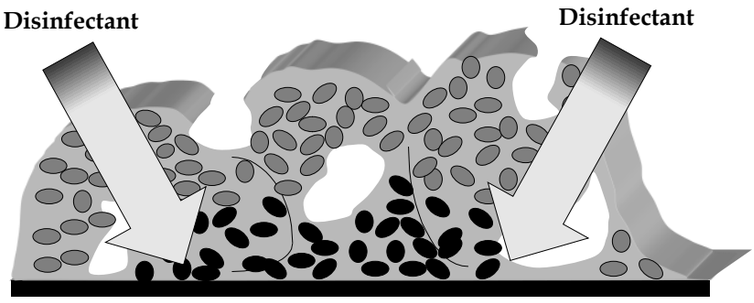

Fig. 4. Limited penetration of antimicrobial agents within the biofilm architecture due to diffusion-reaction problems (black cells can survive and adapt themselves to sub-lethal concentrations of disinfectants)

A few studies have described the adaptation of biofilm cells to disinfectants. One early study showed that when *S.* Typhimurium biofilm was disinfected daily, the proportion of viable but nonculturable cells increased in a 4-day biofilm (Leriche & Carpentier, 1995). Chlorine consumption of such biofilm was increased, suggesting that adaptation to chlorine stress induces a hyper-production of exopolymeric substances that can protect the viable cells. *Salmonella* biofilm cells were also reported to display a greater adaptation than their planktonic counterparts to BCK after continuous exposure to 1 µg/mL of this disinfectant (Mangalappalli-Illathu & Korber, 2006). Specific proteins involved in energy metabolism, protein biosynthesis, nutrient binding, cold shock and detoxification were up-regulated. Conversely, proteins involved in proteolysis, cell envelope formation, universal stress, heat shock response (Dnak) and broad regulatory functions (Hns) were down-regulated following adaptation (Mangalappalli-Illathu & Korber, 2006).

However, surface-attached *Salmonella* exposed sequentially to 100 ppm chlorhexidine digluconate remained susceptible to the action of the sanitizer. The bacteria were not able to adapt neither develop induced resistance (Riazi & Matthews, 2011). During this experiment, the disinfectant is applied at a much higher concentration than that used by Mangalappali-

Illathu et al., and it can more easily reach the surface-attached cells which are not embedded in an exopolymeric matrix. This therefore can explain why, in this case, the attached cells were not able to adapt and develop increased resistance.

Very little is known about cross-resistance and virulence abilities of biofilm cells adapted to disinfectant. A recent study has shown that following a disinfection treatment, surviving biofilm *Salmonella* cells demonstrated significantly up-regulated virulence genes (Rodrigues et al., 2011).

Therefore, in order to guarantee microbial food safety, cleaning procedures must be applied regularly in order to prevent biofilm formation, and the concentrations used should be high enough to eradicate surface-attached bacteria. Under these conditions, biofilm formation and adaptation to disinfectants should be limited.

5. Conclusion

Because antimicrobials are currently used to ensure microbiological food safety, the potential for the development of bacterial adaptation and induced tolerance to antimicrobial stresses must be taken into account. Although the mechanisms of acid adaptation are now well understood, knowledge on the adaptation of *Salmonella* to naturally occurring compounds and disinfectants needs to be improved. Research must be carried out to monitor the evolution of stress-tolerant pathogens in foods and to generate appropriate methods to detect and control them. Studies should focus on food matrix systems or food-processing plants because these should be considered as highly stressful environments. The ultimate purpose should be to predict and control the behavior of *Salmonella* in terms of its stress tolerance, cross resistance to other stresses, persistence in the environment and virulence in the host.

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