PUTATIVE ROLE OF THE ALTERNATIVE SIGMA FACTOR, SIGB, IN SURVIVAL OF *LISTERIA MONOCYTOGENES* 568 DURING DESICCATION ON STAINLESS STEEL SURFACES

by

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ABSTRACT

Listeria monocytogenes is a foodborne bacterial pathogen that colonises and persists in the food processing environment causing cross-contamination of processed foods, including ready-to-eat meat products. I hypothesised that survival of *L. monocytogenes* during desiccation on stainless steel is influenced by the alternative sigma factor (σ^B), which is a transcriptional regulator of stress responses.

The objective of this research was to test whether σ^B contributes to the survival of *L. monocytogenes* during desiccation, through the characterization of a sigB deletion mutant and its desiccation behaviour.

Deletion of *sigB* reduced *Listeria*'s survival during desiccation in osmolyte- and nutrient-limited conditions. Pre-culturing with osmolytes or adding osmolytes during desiccation, increased survival. RT-PCR results indicated that at high osmolarity transcription of *opuCA* (encoding an osmolyte carnitine transporter) was controlled by σ^B.

I concluded that SigB is partly involved in the short-term survival of *L. monocytogenes* during desiccation, but, σ^B-independent systems mediate survival during long-term desiccation.

LIST OF ABBREVIATIONS AND SYMBOLS USED

amp	Ampicillin
ATP	Adenosine triphosphate
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion Agar
CHP	cumene hydroperoxide
DEPC	Diethylpyrocarbonate-treated
DMF	Dimethylformamide
EB	Ethidium Bromide
g	gram
g	Relative centrifugal force
GABA	γ-aminobutyrate
GAD	glutamate decarboxylase
I.D.	Internal Diameter
Kan	Kanamycin
L. monocytogenes	*Listeria monocytogenes*
Lm568	*Listeria monocytogenes* 568

Lm568 Δ*sigB* mutant	*Listeria monocytogenes* 568 *sigB* deletion mutant
ml/min	milliliter per minute
mM	millimolar
MM	Minimal medium
MPa	Megapascals
PBS	Phosphate-buffered Saline
PPS	Physiological Peptone Saline
RH	Relative Humidity
RTE	Ready-to-eat
RT-PCR	Reverse transcription PCR
SigB (σ^B)	Alternative Sigma Factor B
SS	Stainless Steel
TAE	Tris-acetate-EDTA
TSB	Tryptic Soy Broth
TSB-glu	Tryptic Soy Broth with 1.0% glucose
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1. INTRODUCTION

The genus *Listeria*, which consists of a group of Gram-positive, non-spore-forming, facultatively anaerobic, low G+C, rod-shaped bacteria, includes eight species. Of the eight listerial species (*L. monocytogenes*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. marthii* and *L. rocourtiae*) only *L. monocytogenes* and *L. ivanovii* are facultative intracellular pathogens for both animals and humans (den Bakker, Cummings, Ferreira, Vatta, & Orsi, 2010). Based on cellular O and flagellar H antigens, the serotypes of *L. monocytogenes* are classified into at least 13 types. But, almost all human cases are caused by serotypes 4b, 1/2a and 1/2b (Schuchat, Swaminathan, & Broome, 1991; Swaminathan, 2007).

In the food safety research area, *L. monocytogenes* did not attract the attention of food microbiologists until the early 1980s, where a large outbreak in Halifax, Nova Scotia was linked to contaminated food for the first time (Schlech, Lavigne, Bortolussi, Allen, & Haldane, 1983). Since then the presence of *L. monocytogenes* in the food industry is frequently reported and has resulted in many outbreaks of foodborne listeriosis. A large meat/frankfurter outbreak occurred in the United States in 1998 and resulted in 14 deaths and 4 miscarriages or stillbirths among a total of 108 reported cases (Mead, Dunne, Graves, Wiedmann, & Patrick, 2006). Recently, a multistate outbreak of listeriosis was linked to whole cantaloupes from Jensen Farms, Colorado. The outbreak has involved 28 America States and has killed 29 persons so far from a total of 139 persons infected with outbreak-assicoated strains of *L. monocytogenes* (Centers for Disease Control, November 2, 2011). In Canada, consumption of contaminated deli meat from a Maple Leaf processing plant caused a large outbreak in 2008 and resulted in a total of 53 reported listeriosis cases and 20 deaths (Friscolanti, November 2, 2011). In both cases, retrospective studies indicated that the persistence of *L. monocytogenes* on the surface of processing equipment was most likely the source of infection.

Nowadays, there is an increasing trend in the consumption of ready-to-eat (RTE) foods. RTE foods, which do not undergo further processing prior to consumption, can easily become carriers of pathogens due to cross-contamination in the food processing environment. The ability of *L. monocytogenes* to colonize and persist in that environment may have enabled the bacterium to become an important human foodborne pathogen. *L. monocytogenes* can cause particularly serious diseases in pregnant women, the young, the elderly, and immunosuppressed persons. The early stage of human listeriosis presents as flu-like symptoms including chills, headache and fatigue and may evolve into meningitis, abortions and in more serious cases, death, if patients do not seek medical attention to receive prompt treatments. Scallan et al. (2011) estimated that 31 foodborne pathogens caused 2,612 deaths, of which 1,351 were caused by eating contaminated food in the United States. Of these deaths, 64% were caused by bacteria, 25% by parasites, and 12% by viruses. The leading causes of foodborne-associated deaths were nontyphoidal *Salmonella* spp. (28%), *Toxoplasma gondii* (24%), *L. monocytogenes* (19%), and norovirus (11%). Based on the estimated results by Scallan et al. (2011), *L. monocytogenes* has emerged as a deadly foodborne pathogen that kills approximately 496 Americans every year.

L. monocytogenes has a remarkable ability to grow and survive in a diverse range of environments and has been isolated from soil, water, foods, plants, domestic animals and humans. It can grow at refrigeration temperatures and even at temperatures as low as 1 °C (Chaturongakul, Raengpradub, Wiedmann, & Boor, 2008). It can survive in a wide range of salt concentrations as high as 2 M (12% w/v) and can survive for extended periods in the presence of 3 M (18% w/v) NaCl (Cole, Jones, & Holyoak, 1990). Listeria can grow over a wide pH range from 4.3 to 11.0 (Farber & Farber, 1989; Vasseur, Baverel, Hebraud, & Labadie, 1999) and can tolerate acidic challenges at pH values as low as 2.5 because of a strong adaptive acid tolerance response (Davis, Coote, & O'Byrne, 1996; O'Driscoll, Gahan, & Hill, 1997). Also, it was shown that Listeria survives for more than 151 days on sand (10 °C and 88% relative humidity (RH)) that was used as a model to simulate post-processing contamination of RTE meats by dustlike particulates (De Roin, Foong, Dixon, & Dickson, 2003).

The tolerance of *L. monocytogenes* to adverse environmental conditions is partially attributed to stress genes whose transcription is under the control of the alternative sigma factor, SigB (σ^B). The genome of *L. monocytogenes* encodes a total of five RNA polymerase sigma factors: σ^A, σ^B, σ^C, σ^H, and σ^{54} (σ^L) (Glaser et al., 2001). The expression of σ^B is activated by diverse energy and environmental stresses to direct the synthesis of more than 100 general stress proteins. Early studies demonstrated an involvement in both acid tolerance (Wiedmann, Arvik, Hurley, & Boor, 1998) and osmoregulation (Becker, Cetin, Hutkins, & Benson, 1998). Previous research has shown that *sigB* deletion (Δ*sigB*) mutants express a variety of stress-sensitive phenotypes. For example, survival of Δ*sigB* mutant decreased 1,000- to 5,000-fold compared to the wild type when *L. monocytogenes* stationary phase cells were exposed to pH 2.5 (Wiedmann et al., 1998). Also, a Δ*sigB* mutant exhibited reduced ability to use betaine and carnitine, which are used as osmoprotectants to balance environmental osmolality, in 3% (w/v) NaCl (Becker, Cetin, Hutkins, & Benson, 1998).

An understanding of the role of σ^B in *L. monocytogenes* undergoing osmotic and desiccation stresses may reveal efficient strategies to reduce the presence of this pathogen in the food processing industry. Osmotic stress can be defined as the process where a change in the external osmolality immediately triggers fluxes of water along the osmotic gradient. The movement of water could result either in swelling and bursting of the cell in hypotonic environments or dehydration of the cytoplasm under hypertonic environments (Storz, Hengge, Bremer, & Kramer, 2000). On the other hand, desiccation stress can be defined as the dehydration process caused through losses of water by air-drying (Billi & Potts, 2002b). Both stresses will cause the bacteria to experience water starvation in the cytoplasm and lower water activity in the immediate environment. In order to overcome these devastating scenarios, the cells will use active countermeasures to retain a suitable level of cytoplasmic water and turgor pressure (Galinski & Truper, 1994; Kempf & Bremer, 1998; Poolman & Glaasker, 1998; Wood, 1999b).

1.1 Thesis Objectives

The objectives of this research were to:

a) test the hypothesis that the growth and survival of a *L. monocytogenes* 568 Δ*sigB* mutant would differ from the wild type *L. monocytogenes* 568 (Lm568) during exposure to environmental conditions found in the food processing environments including elevated NaCl levels, different nutritive conditions, and desiccation,

b) determine the presence and concentration of osmoprotectants (glycine betaine, carnitine and proline) in Brain Heart Infusion (BHI), Tryptic Soy Broth with 1% glucose (TSB-glu) and Physiological Peptone Saline (PPS) in order to understand their influence on desiccation survival in the mutant lacking the SigB regulon,

c) investigate if pre-culture with exogenous osmolytes (glycine betaine, carnitine and proline) would increase the desiccation survival of wild type and Δ*sigB* mutant strains on stainless steel (SS) coupons,

d) investigate if the presence of exogenous osmolytes (glycine betaine, carnitine and proline) could improve both strains' survival on SS coupons during desiccation.

The results from this study may contribute to a better understanding of the survival mechanisms expressed by *L. monocytogenes* during desiccation.

CHAPTER 2. LITERATURE REVIEW

2.1 *Listeria monocytogenes* and Food Safety

Listeria monocytogenes is a Gram-positive, food-borne pathogen that is widely distributed in the environment and found in many raw foods. *L. monocytogenes* continues to be a problematic bacterium for the food processing industry and consequently for human health. Several food products have been associated with *Listeria* outbreaks, including milk and dairy products, vegetables, salads, fish and meat products (Autio et al., 1999). The majority of the high risk food products belong to the group of so called "ready-to-eat" (RTE) products which are characterized by being consumed raw or minimally processed or fully cooked at the food processing plant and then consumed without further preparation by the consumer. The initial presence and subsequent growth of *L. monocytogenes* in RTE foods during processing and storage is a significant risk factor since listeriosis, a serious illness, is primarily caused by the ingestion of high levels of *L. monocytogenes* in RTE foods (Luber, Crerar, Dufour, Farber, & Datta, 2011). Consumption of RTE products with high levels of *L. monocytogenes* can cause listeriosis in people belonging to risk groups such as the young, old, pregnant and immunocompromized individuals (Luber et al., 2011). As previously mentioned listeriosis accounts for an estimated 19% of the annual deaths attributable to food-borne pathogens (Scallan et al., 2011).

A number of studies have demonstrated a high prevalence of *L. monocytogenes* in a variety of food processing plants, including poultry production plants (Lawrence & Gilmour, 1995; Ojeniyi, Wegener, Jensen, & Bisgaard, 1996; Wenger, Swaminathan, Hayes, Green, & Pratt, 1990), meat processing plants (Giovannacci, Ragimbeau, Queguiner, Salvat, & Vendeuvre, 1999; Nesbakken, Kapperud, & Caugant, 1996), ice cream plants (Miettinen, Bjorkroth, & Korkeala, 1999), shrimp peeling plants (Destro, Leitao, & Farber, 1996) and plants in which gravad (Autio et al., 1999) and smoked trout (Rorvik, 2000) are produced. These studies have shown that the food processing environment represents a significant contamination source for *L. monocytogenes* in finished products. Other studies have found that the food equipment surfaces are also

important sources of *L. monocytogenes* contamination especially for RTE foods, which often are produced using equipment such as slicing machines (Autio et al., 1999; Miettinen et al., 1999; Tompkin, 2002) and conveyor belts (Krysinski, Brown, & Marchisello, 1992; Lindsay & vonHoly, 1997; Poulis, Depijper, Mossel, & Dekkers, 1993). The association between contaminated surfaces and transmission of *L. monocytogenes* to food, especially RTE food, in the food processing plant has been suggested to be caused by the ability of *L. monocytogenes* to adhere to and to persist on food contact surfaces for months or even years after cleaning and disinfection (Frank & Koffi, 1990; Reij & Den Aantrekker, 2004; Wulff, Gram, Ahrens, & Vogel, 2006). During food manufacturing and sanitation procedures in the food processing plant, *L. monocytogenes* frequently encounters combined stresses, such as acid, heat, cold or freezing temperatures, drying and osmotic pressure changes. Hence, enhanced tolerance for this pathogen to any of these stress factors could result in a decreased susceptibility to disinfectants and an increased survival on common food contact surfaces for longer periods, leading to the possibility for subsequent cross-contamination of food products.

2.2 General Stress Response in *L. monocytogenes* and Other Related Gram-Positive Bacteria

The general stress response of Gram-positive bacteria contributes to their abilities to reproduce or survive in a wide variety of environmental conditions. For example, to establish a food-borne infection in a human host, a bacterium must first persist on food contact surfaces, transfer onto RTE foods when RTE foods contact contaminated surfaces and then survive in contaminated foods which are commonly kept at refrigerated temperatures. Following ingestion, the bacterium must survive in the gastrointestinal tract as the bacterium is exposed to gastric acid stress during gastrointestinal infection and osmotic stress caused by bile salts from the gall bladder (Gahan & Hill, 2005; van Schaik & Abee, 2005). Survival under these extreme and rapidly changing conditions requires the appropriate expression and induction of stress genes and proteins in a bacterial cell in order to respond to stimuli signalling these new environmental conditions. The σ factor is a dissociable subunit of the RNA polymerase holoenzyme and responsible for the holoenzyme's recognition of specific DNA sequences, which represent the promoter

region(s) for a given gene and/or operon, and initiation of transcription at the promoter site. Thus, differential associations between alternative σ factors and the core RNA polymerase essentially reprogram the enzyme with promoter-recognition specificities to enable expression of new sets of target genes required under different environmental conditions (Kazmierczak, Mithoe, Boor, & Wiedmann, 2003).

In Gram-positive bacteria, the general stress-responsive alternative sigma factor, σ^B, directs transcription of genes contributing to bacterial survival under a variety of diverse environmental stresses (Kazmierczak, Mithoe, Boor, & Wiedmann, 2003). Although σ^B was detected more than 20 years ago by Richard Losick and William Haldenwang as the first alternative sigma factor (initially called σ^{37}) of *Bacillus subtilis* (*B. subtilis*), σ^B was not the subject of intense studies after it was realized that σ^B is not involved in sporulation (Hecker & Volker, 2001). It later turned out that σ^B is responsible for the induction of the genes encoding general stress proteins in response to two broad classes of stresses: (1) environmental stress including acid, ethanol, heat and/or salt stress and (2) energy stress including starvation for glucose, phosphate or oxygen (Storz, Hengge, & Price, 2000). The definition of general stress proteins is "a common group of very prominent proteins, which are produced in response to heat, ethanol, salt or acid stress or starvation of glucose, phosphate or oxygen, and may have a rather non-specific, but nevertheless essential, protective function under stress, regardless of the specific growth-restricting factor" (Hecker & Volker, 2001, p.38). The increase in activity of σ^B in response to numerous environmental stresses is not only found in *B. subtilis*, but also in other Gram-positive bacteria such as *L. monocytogenes* and *Staphylococcus aureus* (*S. aureus*). SigB has been extensively studied in *B. subtilis*. The σ^B regulon in *B. subtilis* contains at least 127 genes contributing to stress resistance, transcriptional regulation and membrane transport (Petersohn, Brigulla, Haas, Hoheisel, & Volker, 2001; Price, Fawcett, Ceremonie, Su, & Murphy, 2002).

Compared to research performed on the activity of σ^B of *B. subtilis* in response to changing environmental conditions, less is known about the system in *L. monocytogenes*. However, the regulatory components of the σ^B-system of *L. monocytogenes* have been found to be very similar to those in *B. subtilis* (Figure 1) (O'Byrne & Karatzas, 2008). Since the σ^B operons show a high degree of sequence similarity in *L. monocytogenes* and

B. subtilis, the basic interactions among those operons will probably be very similar (Becker, Cetin, Hutkins, & Benson, 1998; Wiedmann et al., 1998). In both *B. subtilis* and *L. monocytogenes*, *sigB*, which encodes σ^B, is the seventh open reading frame in an operon containing eight genes involved in σ^B regulation (*rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, *sigB*, and *rsbX*) (Ferreira, Sue, O'Byrne, & Boor, 2003a; Wise & Price, 1995).

All eight genes, including *sigB*, are under the dual control of σ^A and σ^B. A housekeeping sigma factor (σ^A)-dependent promoter (P_A) is located in the upstream of *rsbR*. A σ^B-dependent promoter (P_B), which is located upstream of *rsbV*, controls the enhanced transcription of *rsbV*, *rsbW*, *sigB*, and *rsbX* under conditions that stimulate σ^B activity (Becker, Cetin, Hutkins, & Benson, 1998; Benson & Haldenwang, 1992; Kalman, Duncan, Thomas, & Price, 1990).

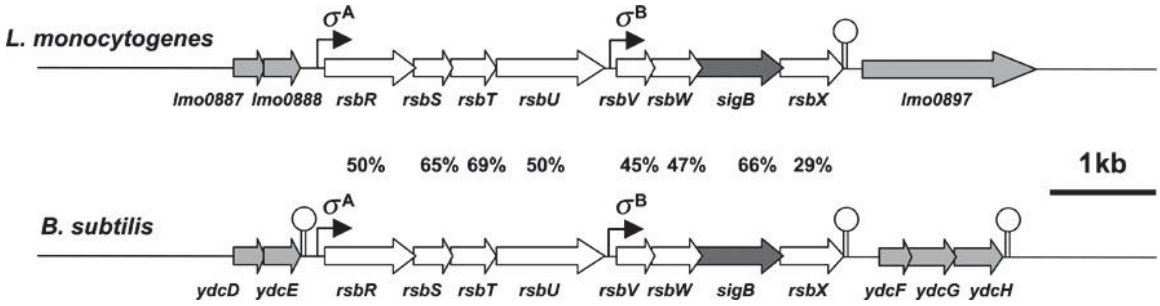


Figure 1. The conserved *sigB* operon sequence in *L. monocytogenes* and *B. subtilis*. Genes belonging to the *sigB* operon are indicated as open arrowheads, with *sigB* itself shown in dark grey. Genes in light grey lie outside of the *sigB* operon. Promoters are indicated as angled arrowheads and putative transcriptional terminators are indicated as stem-loop structures. Transcription of the *sigB* operon is controlled by two promoters, the σ^A-dependent promoter P_A and an internal, σ^B-dependent promoter P_B. The percentages of amino acid sequence identity between homologues are indicated. The schematic is drawn to scale. (From O'Byrne & Karatzas, 2008 with permission of Elsevier Inc. The permission letter is attached in the appendix).

In *B. subtilis*, the σ^B regulatory mechanism is called "partner switching" since it involves RsbW switching between two binding partners, RsbV and σ^B. Also, the activity of σ^B is regulated primarily at the post-translational level by modulating its interaction with RsbW (Hecker, Pane, & Volker, 2007). RsbW, a so-called anti-sigma factor, can interact with σ^B and this association prevents productive interactions between σ^B and the RNA polymerase core enzyme. An anti-anti-sigma-factor RsbV can bind to RsbW and this interaction liberates σ^B to interact with RNA polymerase and participate in the initiation of transcription. The phosphorylation state of RsbV is the principal determinant of whether the σ^B regulon is expressed or not. The reason is that the overall phosphorylation state of RsbV is regulated by the actions of two dedicated phosphatases, called RsbU and RsbP, and the kinase activity of RsbW. Under normal growth conditions, RsbV exists in the phosphorylated form, which allows the anti-sigma factor RsbW to bind σ^B and prevent its association with the RNA polymerase. Under these conditions the kinase activity of RsbW out-competes the phosphatase activities of RsbU and RsbP. However, under conditions of stress, the phosphatase activities of RsbU and RsbP increase, resulting in the unphosphorylated RsbV competing for binding to RsbW. As the relative concentration of the RsbW-RsbV complex increases, the concentration of free σ^B also increases, thus allowing σ^B to bind to core RNA polymerase to trigger the activity of σ^B regulon (O'Byrne & Karatzas, 2008). In *B. subtilis*, both environmental and energy stress signals induce dephosphorylation of RsbV via the RsbU phosphatase (Yang, Kang, Brody, & Price, 1996) and the RsbP phosphatase (Vijay et al., 2000), respectively. However, due to the absence of a close homologue of RsbP in *L. monocytogenes*, both environmental and energy stresses have been shown to activate σ^B of *L. monocytogenes* via RsbU phosphatase activity (Chaturongakul & Boor, 2004).

In *B. subtilis*, the regulatory mechanism detailing how the initial sensory input, such as heat shock, salt, acid and ethanol stress, triggers the downstream activation of σ^B is still not fully understood. In the upstream activation of σ^B, the stress signals trigger RsbU binding with RsbT released from a complex with RsbR and RsbS, thus posing an another partner switching module for RsbT switching between RsbR and RsbS, and RsbU. RsbT, which is needed for the activation of RsbU, is unavailable under normal conditions because it is complexed with non-phosphorylated RsbS. After cells experience

stresses, RsbT displays a kinase activity that can effectively inactivate RsbS by phosphorylation (Hecker & Volker, 2001). Phosphorylation of RsbS occurs at a serine moiety which is located in a similar position as the serine in RsbV (Yang et al., 1996). Consequently, free RsbT can interact and activate RsbU. The complex of RsbT and RsbU increases the phosphatase activity of RsbU and subsequently, results in dephosphorylating of RsbV. The accumulation of unphosphorylated RsbV results in the binding of RsbW as explained above for the first partner-switching module, and activation of the σ^B regulon. *B. subtilis* mutant studies conducted by Kang and other researchers (1998) revealed that the kinase activity of RsbT plays an essential role for stress sensing. Delumeau and other researchers (2006) reported that the RsbRST complex in *B. subtilis* is a large multi-subunit complex that also includes a number of paralogues of RsbR (RsbRB, RsbRC, RsbRD and YtvA). The large 25S complex formed by these proteins has been entitled as a "stressosome" (Delumeau, Murray, Yudkin, & Lewis, 2006). At present, it is not yet clear whether the RsbS, RsbT and RsbR proteins of *L. monocytogenes* are associated with a stressosome-like complex. However, it has been found that the genome of *L. monocytogenes* encodes a number of RsbR paralogues, including Lmo0161, Lmo1642, and Lmo1842, as well as the YtvA paralogues, Lmo0799 (O'Byrne & Karatzas, 2008).

Although post-translational regulation of σ^B activity most likely plays a critical role for controlling σ^B in *L. monocytogenes*, it is clear that regulation can also occur at the transcriptional level (O'Byrne & Karatzas, 2008). Transcription of *sigB*, which lies in an 8-gene operon, is initiated upstream from *rsbV* (Becker, Cetin, Hutkins, & Benson, 1998; Rauch et al., 2005). Transcription of this operon is primarily σ^B-dependent, suggesting that a stress activation of σ^B will rapidly result in an increase in *sigB* transcript (Becker, Cetin, Hutkins, & Benson, 1998). Different environmental stresses, including 4% NaCl, 2% ethanol, acidification to a pH of 5.3, a temperature upshift (from 25 to 48 °C) and entry into stationary phase, induce transcription from the σ^B promoter upstream from *rsbV* (Becker, Cetin, Hutkins, & Benson, 1998). Of these stresses, the biggest increase in *sigB* transcription was observed after osmotic shock (with 4% NaCl) (Becker, Cetin, Hutkins, & Benson, 1998).

Although all seven Rsb proteins (RsbR, RsbS, RsbT, RsbU, RsbV, RsbW, and RsbX) identified in *B. subtilis* and *L. monocytogenes* are not conserved among all Gram-positive bacterial species, RsbV and RsbW are conserved among all species examined to date and thus appear to be essential for regulation of σ^B activity (Ferreira, Sue, O'Byrne, & Boor, 2003a). *S. aureus* was the first pathogenic bacterium in which *sigB* was identified (Kullik & Giachino, 1997; Wu, DeLencastre, & Tomasz, 1996). In *S. aureus*, the *sigB* gene is located in an operon similar to that of *B. subtilis* (Kullik, Giachino, & Fuchs, 1998; Wu et al., 1996). However, *S. aureus* lacks a considerable part of the *sigB* regulon of *B. subtilis*, including the upstream *rsbR*-*rsbS*-*rsbT* genes and the downstream *rsbX* gene. A Blast search revealed that orthologues of *rsbR*, *rsbS*, *rsbT* and *rsbX* are absent from the available *S. aureus* genome sequences (Baba et al., 2002; Kuroda et al., 2001). Despite these differences in *sigB* regulons, the *S. aureus* σ^B activity is regulated by Rsb proteins at post-translational level, and the primary partner-switching module between SigB, RsbV, and RsbW appears to be functional in *S. aureus* (Hecker et al., 2007). The activation of σ^B strongly depends on RsbU in certain stress conditions, such as heat shock, osmotic shock due to addition of $MnCl_2$ or NaCl, and alkaline shock (Pane, Jonas, Foerstner, Engelmann, & Hecker, 2006). In *S. aureus*, mutant studies indicated that loss of σ^B caused a reduced tolerance to acid shock in the exponential phase and to oxidative stress in the stationary phase, as well as to severe heat shock (Chan, Foster, Ingham, & Clements, 1998; Kullik et al., 1998). In one of these mutant studies, the Δ*sigB* mutant displayed reduced pigmentation and accelerated sedimentation (Kullik et al., 1998). However, in contrast to *B. subtilis*, a drop in the cellular adenosine triphosphate (ATP) level and ethanol stress failed to activate σ^B in *S. aureus* (Pane et al., 2006), and the heat shock induction of σ^B in *S. aureus* is moderate and short (Hecker et al., 2007). To better understand the role of σ^B in *S. aureus*, proteomic and transcriptomic analyses identified about 200 genes under positive control of σ^B. A functional classification of the deduced gene products indicated that the genes under positive control of σ^B putatively contribute to cellular processes including cell envelope turnover and biosynthesis, intermediary metabolism and membrane transport processes (Hecker et al., 2007). The transcriptional analyses of σ^B-dependent genes indicated that σ^B is not the sole factor involved in transcription under stresses. Instead it is suggested that the *sigB* regulon is

also under the control of other regulatory factors (Hecker et al., 2007). The overlap between the *S. aureus* and *B. subtilis sigB* regulon is limited. Only around 10% of the genes under the control of σ^B in *S. aureus* are homologous to those in *B. subtilis*; mutants in eight of these common genes displayed increased stress sensitivity in *B. subtilis* (Hoper, Volker, & Hecker, 2005). Because mutants in these genes have not yet been studied in *S. aureus*, the role of σ^B in stress-resistance of *S. aureus* remains to be further investigated (Hecker et al., 2007). Taken together, the regulation of σ^B activity differs significantly between *S. aureus* and *B. subtilis*.

2.3 The Sigma Factors of *Listeria monocytogenes*

2.3.1 Characteristics of the Sigma Factors Found in *L. monocytogenes*

L. monocytogenes responds rapidly to changing environmental conditions. Under laboratory conditions, it can grow in a wide range of environmental conditions, including at temperatures between 1 and 45 °C, a pH range of 4.4 to 9.6 and NaCl concentrations up to 10% w/v (reviewed by Chaturongakul et al., 2008). In host animals, it can persist in a non-growing state under an even wider range of conditions, including pH of 2.5, bile and bile-acid concentrations higher than 0.3% v/v and 5 mM, respectively (Chaturongakul et al., 2008). Moreover, exposing this pathogen to one type of stress followed by another type of stress or the same stress can produce cross-protection to other stresses. The cross-protection can result in robust and persistent strains being present in a given environment (Chaturongakul et al., 2008).

The genome of *L. monocytogenes* encodes five alternative σ factors. They are σ^A, σ^B, σ^C, σ^H, which belong to the σ^{70} family, and σ^L, which belongs to the σ^{54} family. The σ^A, which is encoded by the *rpoD* gene (*lmo1454*), is the primary sigma factor and is very closely related to σ^A in *B. subtilis* responsible for regulating housekeeping gene expression (O'Byrne & Karatzas, 2008). SigB, encoded by the *sigB* gene (*lmo0895*), was first identified and characterized based on its homology to *sigB* in *B. subtilis* (Becker, Cetin, Hutkins, & Benson, 1998; Wiedmann et al., 1998). SigB contributes to the general stress response under rapidly changing environmental conditions and virulence of *L. monocytogenes* in a host. For example, σ^B is involved in acid tolerance, osmoregulation,

oxidative stress, adaption to lower and higher range temperatures and nutrient limitation or energy stress (Becker, Cetin, Hutkins, & Benson, 1998; Becker et al., 2000; Ferreira, O'Byrne, & Boor, 2001; Moorhead & Dykes, 2003; Wiedmann et al., 1998); Evidence, such as microarray analyses and single-gene transcriptional analyses, indicate that several virulence genes are directly under the control of σ^B in *L. monocytogenes*. Examples of virulence genes include *L. monocytogenes* internalins such as *inlAB* that encodes internalin A and B. The internalins A and B play a crucial role for *L. monocytogenes* attachment to and invasion of different host-cell types (Kazmierczak, Mithoe, Boor, & Wiedmann, 2003; McGann, Wiedmann, & Boor, 2007). More importantly, σ^B controls the transcription of *prfA* that encodes the global *L. monocytogenes* virulence gene regulator, PrfA (Nadon, Bowen, Wiedmann, & Boor, 2002; Schwab, Bowen, Nadon, Wiedmann, & Boor, 2005). The *sigC* gene (*lmo0423*) encodes σ^C which belongs to the group of the extracytoplasmic function (ECF) sigma factors (Zhang, Nietfeldt, Zhang, & Benson, 2005). The ECF sigma factors are responsible for responding to extracytoplasmic stress and mediating extracytoplasmic functions (Zhang et al., 2005). Based on phylogenetic analysis of genome composition, σ^C has so far only been found in *L. monocytogenes* strains that belong to the phylogenetic lineage II of the species (Zhang et al., 2005). Serotype 1/2a strains belonging to lineage II are often found in foods (Nadon, Woodward, Young, Rodgers, & Wiedmann, 2001; Piffaretti et al., 1989). Thus, it is possible that σ^C contributes to *L. monocytogenes* survival in the food production environment. Recently, σ^C has been found to impact the regulation of thermal and cold stress adaptation (Chan et al., 2008; Zhang, et al., 2005). SigH, encoded by *sigH* (*lmo0243*), plays a positive role for *L. monocytogenes* growth under alkaline conditions and in a minimal medium, since an *L. monocytogenes* Δ*sigH* mutant showed reduced growth under those two conditions compared with its wild type (Rea, Gahan, & Hill, 2004). Also, σ^H is involved in responding to acidic pH (Phan & Mahouin, 1999). Murine studies indicated that σ^H does not play a dominant role in *L. monocytogenes* pathogenesis. Growth of the Δ*sigH* mutant in the spleens of infected mice was marginally inhibited and may demonstrate an inability to obtain and take up nutrients *in vivo* (Rea, Gahan, & Hill, 2004). SigL (also known as σ^{54}, or RpoN) is encoded by the *sigL* gene (*lmo2461* or *rpoN*) and is closely related (38% sequence identity) to the σ^{54} of *B. subtilis* (Robichon et al.,

1997). *sigL* expression is dependent on growth phase and temperature (Chan et al., 2008; Liu, Graham, Bigelow, Morse, & Wilkinson, 2002). For example, the induction of *sigL* expression occurs in logarithmic-phase cells grown at 10 °C (Liu et al., 2002); however, *sigL* expression is downregulated during stationary phase growth at 4 °C (Chan et al., 2008). The initiation of σ^L-dependent transcription requires σ^L to bind to an activator protein in order to separate the target promoter DNA sequence. This is in contrast to the initiation of σ^{70}-dependent transcription where members of the σ^{70} family factors directly separate target promoter DNA sequences to activate transcription (Raimann, Schmid, Stephan, & Tasara, 2009). Global analysis of gene expression in a *L. monocytogenes* Δ*rpoN* mutant identified that 77 genes are under the control of σ^L and many of these σ^L-dependent genes contribute to carbohydrate and amino acid metabolism (Arous et al., 2004). Twenty-four σ^L-dependent genes were expressed differently during listerial growth in macrophage cells compared to expression levels in BHI (Chaturongakul et al., 2008). Two reports have indicated that σ^L contributes to *L. monocytogenes* adaptation to adverse environmental stresses. Firstly, a *L. monocytogenes* Δ*sigL* mutant was shown to have a reduced ability to utilise carnitine, an osmoprotectant commonly used by the bacteria to overcome osmotic stress (Okada et al., 2006). In the second study, σ^L was shown to play an important role in mediating the stress tolerance of *L. monocytogenes* subjected to different stress conditions associated with RTE food preservation strategies, including low storage temperature, presence of organic acids, and elevated NaCl salt concentrations (Raimann et al., 2009). Moreover, σ^L of *L. monocytogenes* has been involved in the sensitivity to mesentericin Y105, a bacteriocin synthesized by *Leuconostoc mesenteroides* (Robichon et al., 1997), as a *L. monocytogenes* Δ*sigL* mutant displayed increased resistance to mesentericin Y105 (Okada et al., 2006; Robichon et al., 1997). The resistance has been linked to σ^L-dependent expression of the *mptACD* operon, which encodes for the EII^{Man} mannose PTS permease (Raimann et al., 2009).

2.3.2 The Expression of SigB in Response to Changing Environmental Conditions

As described above, σ^B is one of five sigma subunits of the RNA polymerase core enzyme in *L. monocytogenes*, which allows the bacteria to respond to changing environmental conditions by controlling the initiation or enhancement of the transcription of σ^B-dependent genes (Kazmierczak et al., 2003). Early studies demonstrated that σ^B is involved in both acid tolerance (Wiedmann et al., 1998) and osmoregulation (Becker, Cetin, Hutkins, & Benson, 1998). Sue, Fink, Wiedmann, and Boor (2004) found that the induction of σ^B-dependent genes occurs very quickly and can be detected within 5 minutes after the cells are exposed to acid and osmotic stress. Therefore, the σ^B has been garnering the attention of a considerable number of researchers. It has been demonstrated that σ^B contributes to *L. monocytogenes* survival under environmental stress conditions including acid stress, high hydrostatic pressure stress, freeze-thaw stress, cold stress, oxidative stress, osmotic stress, as well as under energy stress conditions including glucose limitation in defined media, entry into stationary phase, and depleted levels of intracellular ATP. Their results are supported by both gene-expression and phenotypic data (Wemekamp, Wouters, de Leeuw, Hain, Chakraborty, & Abee, 2004: Becker, Evans, Hutkins, & Benson, 2000; Chan, Boor, & Wiedmann, 2008; Moorhead & Dykes, 2003; Chaturongakul & Boor, 2004; Raengpradub, Wiedmann, & Boor, 2008; Fraser, Sue, Wiedmann, Boor, & O'Byrne, 2003). A summary of the role of σ^B in *Listeria monocytogenes* subjected to stress conditions is presented in Table 1.

Table 1. Role of σ^B in *Listeria monocytogenes* subjected to stress

Stress condition	Strain	σ^B-dependent genes	Phenotype of *L. monocytogenes sigB* deletion (Δ*sigB*)mutant	Reference
Acid	EGD-e (serotype 1/2a)	*gadB*, *gadC* & *gadD*	The Δ*sigB* mutant was significant less resistant to acid stress (pH 2.5)	Wemekamp et al. 2004
High Hydrostatic Pressure	EGD-e (serotype 1/2a)	Not determined	Survival of the Δ*sigB* mutant was reduced compared to its wild-type strain following exposure of cells to pH 4.5 for 1 hour followed by 350 MPa for 28 minutes	Wemekamp et al. 2004
Freeze-thaw	EGD-e (serotype 1/2a)	Not determined	The level of survival of the wild-type strain after five freeze-thaw cycles was threefold higher than the level of its Δ*sigB* mutant	Wemekamp et al. 2004
Cold	EGD-e, 10403S, and L61 (serotype 1/2a)	*ltrC* & *fri*	σ^B is not required during growth in BHI at 4 °C, but plays a positive role for growth in minimal media and in meats at low temperatures	Wemekamp et al. 2004; Becker et al. 2000; Moorhead and Dykes 2003; Chan et al. 2008

Table 1. Role of σ^B in *Listeria monocytogenes* subjected to stress

Stress condition	Strain	σ^B-dependent genes	Phenotype of *L. monocytogenes sigB* deletion (Δ*sigB*) mutant	Reference
Oxidative	10403S (serotype 1/2a)	*lmo0669* & *lmo1433*	The viability of stationary phase Δ*sigB* cells was one log lower than its wild-type strain after exposure to 13 mM CHP for 15 minutes	Chaturongakul and Boor 2004
Osmotic	10403S (serotype 1/2a)	*opuCA* & *lmo1421*	The Δ*sigB* strain exhibited a reduced ability to use carnitine as osmoprotectants	Fraser et al. 2003
Energy	10403S (serotype 1/2a)	*opuCA* & carbon metabolism genes (e.g. *ldh*)	Survival of the Δ*sigB* strain was reduced compared with the wild-type strain when glucose was limited in defined media, entry into stationary phase delayed and intracellular ATP reduced	Chaturongakul and Boor 2004

SigB partly contributes to *L. monocytogenes* acid resistance. Phenotypic experiments showed that the acid tolerance response of *L. monocytogenes* EGD-e was highly σ^B-dependent when exponential phase cells were exposed to lethal acidic conditions (Wemekamp et al., 2004). The survival of a *L. monocytogenes* EGD-e Δ*sigB* mutant was 10,000-fold lower than the survival of its wild-type strain when exponential phase cells were exposed to pH 2.5 for 1 hour. Upon pre-exposure to a non-lethal pH (pH 4.5) for 1 hour, the wild-type strain subsequently survived well at pH 2.5 for 1 hour. The survival of the pre-exposed Δ*sigB* mutant was 100,000-fold lower, but notably the Δ*sigB* mutant still survived, indicating that σ^B is not solely responsible for the acid resistance. Moreover, Ferreira et al. (2003) studied the contribution of σ^B to the growth-phase-dependent acid resistance and adaptive acid tolerance response in *L. monocytogenes* 10403S. They found that the survival of the Δ*sigB* mutant was consistently lower than the survival of its wild-type strain throughout all growth phases when exposed to BHI broth with pH of 2.5 with and without prior acid adaptation. Also, they found that the acid tolerance response of *L. monocytogenes* is partially dependant on σ^B. For *L. monocytogenes* the ability to increase the intracellular pH following exposure to a low pH is important for survival. The glutamate decarboxylase (GAD) system is one of the acid adaptation systems. It plays an important role in maintaining a relatively high intracellular pH following exposure to acid (Wemekamp et al., 2004). The GAD system involves an antiporter which transports glutamate into the cell, and a glutamate decarboxylase, which converts glutamate into γ-aminobutyrate (GABA) by using up a proton. Then, glutamate and GABA antiporter excludes GABA from the cell (Wemekamp et al., 2004). Several genes encoding components of the GAD system are regulated by σ^B. In *L. monocytogenes*, *gadB* and *gadD* encoding the glutamate decarboxylase enzyme, and *gadC* encoding the associated glutamate and GABA antiporter, are directly regulated by σ^B (Wemekamp et al., 2004).

SigB plays a positive role in *L. monocytogenes* survival after high-hydrostatic-pressure treatment and freeze-thaw cycles. The survival of a *L. monocytogenes* EGD-e Δ*sigB* mutant was 100-fold lower than the survival of its wild-type strain after a treatment with 300 MPa for 20 minutes. Upon pre-exposure to pH 4.5 for 1 hour, the survival of the wild type strain following exposure to a high-hydrostatic-pressure

treatment increased greatly. The survival of the pre-exposed Δ*sigB* mutant was 4-log lower than the survival of the wild-type strain after 28 minutes of exposure to 350 MPa (Wemekamp et al., 2004), indicating the importance of the induction of σ^B-dependent genes during adaptation to a low pH for subsequent survival at a high-hydrostatic-pressure treatment.

The induction of σ^B-dependent genes by pre-exposure to pH 4.5 for 1 hour improved the survival of *L. monocytogenes* after exposure to freeze-thaw cycles. However, the survival of the pre-exposed Δ*sigB* mutant was three-fold lower than the survival of its wild-type strain after five freeze-thaw cycles (Wemekamp et al., 2004). The acid-induced proteins encoded by the σ^B-dependent genes also provide cross-resistance to other stresses such as heat, ethanol, oxidative, and osmotic stress (Ferreira et al., 2003).

The ability of *L. monocytogenes* to survive and grow at low temperatures is partially σ^B-dependent (Chan et al., 2008). *L. monocytogenes* is able to grow at temperatures as low as 1 °C (Chaturongakul, Raengpradub, Wiedmann, & Boor, 2008). This cold adaptation is partly due to its ability to accumulate cryoprotective compounds such as carnitine and betaine (Bayles & Wilkinson, 2000). SigB is involved in efficient accumulation of carnitine and betaine at low temperatures, and consequently the Δ*sigB* mutant grew poorly in comparison with its wild-type strain at low temperatures in minimal medium with the addition of glycine betaine or carnitine (Becker et al., 2000). The transcription of the genes encoding OpuC, which is a carnitine transporter, is under σ^B control (Fraser et al., 2003). However, the transcription of the genes encoding the main glycine betaine transporter (BetL) is σ^B-independent (Fraser et al., 2003). SigB also plays a positive role for growth in minimal media and meats at low temperatures (Becker et al., 2000; Moorhead & Dykes, 2004). However, σ^B is not required for *L. monocytogenes* growth at 4 °C in rich media (BHI) and the transcription of the putative cold stress gene *opuCA* is σ^B-independent during growth at 4 °C in BHI (Chan et al., 2008). Strain variations and methodological differences between laboratories could explain these conflicting observations. The transcription of the cold stress genes *ltrC* (Zheng & Kathariou, 1995) and *fri* (Hebraud & Guzzo, 2000; Olsen et al., 2005) is at

least partially σ^B-dependent (Chan et al., 2008), but roles of these genes in cold tolerance response of *L. monocytogenes* have yet to be established.

Oxidative-stress resistance in *L. monocytogenes* is at least partially contributed to σ^B. Survival studies indicated that the σ^B controlled *lmo0669* and *lmo1433* encode proteins which are responsible for oxidative-stress resistance (Hain et al., 2008; Kazmierczak et al., 2003; Raengpradub et al., 2008). The survival of a *L. monocytogenes* 10403S (serotype 1/2a) Δ*sigB* mutant was reduced significantly compared with its wild-type strain when stationary-phase cells were exposed to 13 mM cumene hydroperoxide (CHP) for 15 minutes (Chaturongakul & Boor, 2004). However, the contribution of σ^B to oxidative-stress resistance in *L. monocytogenes* strains appears to depend on the serotype. A *L. monocytogenes* L61 serotype 1/2a and its isogenic Δ*sigB* mutant did not differ in their resistance to exposure to CHP for 15 minutes while a serotype 4c strain (L99) was significantly less resistant than its isogenic Δ*sigB* mutant (Moorhead & Dykes, 2003).

Exposure to osmotic-stress induces σ^B activity in *L. monocytogenes* and the σ^B-dependent transport systems will import osmoprotectants such as carnitine and glycine betaine, from the environment into the cytoplasm to aid the cell in overcoming osmotic stress (Chaturongakul et al., 2008). Becker et al. (1998) demonstrated that a *L. monocytogenes* 10403S (serotype 1/2a) Δ*sigB* mutant had a reduced ability to use carnitine and glycine betaine as osmoprotectants compared with its wild-type strain. Moorhead and Dykes (2003) demonstrated that a *L. monocytogenes* L61 (serotype 1/2a) Δ*sigB* mutant similary were less able to use carnitine and glycine betaine as osmoprotectants compared with its wild-type strain. But, a *L. monocytogenes* serotype 4c strain (L99) Δ*sigB* mutant grew faster than its wild-type strain during osmotic stress, with the exception of when carnitine and glycine betaine was added to defined media, which then caused the *L. monocytogenes* L99 wild-type strain to grow faster than its Δ*sigB* mutant. It appears that the osmotic stress response of *L. monocytogenes* serotype 1/2a strains 10403S and L61 is under the control of σ^B, whereas the regulation of the osmotic stress response in *L. monocytogenes* L99 is σ^B-independent. This observation indicates that the alternative σ^B-independent systems regulate the osmotic stress response in some strains of *L. monocytogenes* (Moorhead & Dykes, 2003). Fraser et al. (2003) demonstrated that a *L. monocytogenes* 10403S Δ*sigB* mutant was impaired in its

utilization of carnitine, but retained the ability to utilize betaine as an osmoprotectant. Reverse transcriptase PCR (RT-PCR) results showed that the transcription of *opuCA* and *lmo1421*, which encode a carnitine transporter and a compatible solute transporter related closely to known ABC compatible solute transporters, respectively, were strongly σ^B-dependent. Later work showed that *lmo1421* encodes BilE which is a bile exporting system and related to virulence in *L. monocytogenes* (Sleator, Wemekamp, Gahan, Abee, & Hill, 2004). Transcription of *betL* encoding a secondary betaine transporter was σ^B-independent. This result indicated that σ^B plays a major role in the regulation of carnitine utilization in *L. monocytogenes*, but is not involved in betaine utilization (Fraser et al., 2003).

Finally, σ^B is involved in maintaining the cells' viability during exposure to energy stress in *L. monocytogenes*. Energy stress is the limitation of the key metabolites such as carbon, phosphate, or nitrogen during microbial growth (Chaturongakul & Boor, 2004). Specifically, Chaturongakul and Boor (2004) reported how in *L. monocytogenes* 10403S, σ^B positively contributes to the maintenance of viability in defined media supplemented with limiting glucose (0.04% w/v), survival under prolonged static growth into stationary phase in BHI and viability of cells after intracellular ATP depletion. Among the σ^B-dependent genes found to contribute to energy stress were: *opuCA* enco ding a carnitine transporter, *ldh* encoding a carbon metabolic protein (Raengpradub, Wiedmann, & Boor, 2008), *lmo0784-lmo0781* and *lmo0398-lmo0400* encoding mannose-specific and fructose-specific phosphotransferase systems, respectively (Chaturongakul et al., 2008). While the energy stress response of *L. monocytogenes* 10403S were found to be σ^B-dependent by Chaturongakul and colleagues (Chaturongakul & Boor, 2004), the long-term viability of *L. monocytogenes* L61 and L99 during exposure to energy stress was reported to be σ^B-independent by Moorhead and Dykes (2003). This indicates strain differences in the σ^B involvement in overcoming energy stress.

2.3.3 Use of Proteomics and Transcriptomics to Investigate the SigB Regulon

In order to provide further insight into the role of σ^B of *L. monocytogenes* in stress response, a number of groups have made efforts to define the full extent of the σ^B regulon by using proteomic and transcriptomic approaches. It is now clear that the σ^B regulon probably contains about 200 genes that encode proteins with important functions in stress response, carbohydrate metabolism, transport and cell envelope process, transcriptional regulation, and virulence (O'Byrne & Karatzas, 2008; Raengpradub et al., 2008). A proteomic study into identifying components of the σ^B regulon that contributes to acid tolerance showed that the expression of nine acid stress related proteins is under the regulation of σ^B (Wemekamp et al., 2004). Another proteomic study of the σ^B regulon found by using both conventional two-dimensional gel electrophoresis and a novel approach, based on tandem mass spectrometry (Isobaric Tags for Relative and Absolute Quantitation, iTRAQ), that 38 proteins are expressed in a σ^B-dependent manner, including the upregulation of 17 proteins (O'Byrne & Karatzas, 2008). Three members of the universal stress protein (Usp) family, which are found to be upregulated intracellularly, are under the regulation of σ^B (Raengpradub et al., 2008).

Due to the availability of the whole genome sequence for *L. monocytogenes*, global transcriptional studies were undertaken to detect genes with putative σ^B promoter sequences by using a consensus sequence-based search of the *L. monocytogenes* genome (Kazmierczak et al., 2003). This method generated a limited set gene array, carrying 166 putative σ^B-dependent genes (Kazmierczak et al., 2003). From this gene array, 55 genes were identified and confirmed to be expressed differentially in a *L. monocytogenes* Δ*sigB* mutant (Kazmierczak et al., 2003). In this study, genes identified not only included several genes with stress response functions, but also contained several known virulence genes, such as *inlA*, *inlB*, *inlC2*, *inlD*, and *inlE* belonging to the internalin family (Kazmierczak et al., 2003). Also, this study indicated that σ^B is involved in regulating the expression of many genes with cell wall and/or transport-related functions (Kazmierczak et al., 2003). More recently, a whole genome microarray analysis identified that 168 genes are positively regulated by σ^B, with 145 of these being preceded by a potential σ^B promoter sequence (Raengpradub et al., 2008). The size of the *L.*

monocytogenes σ^B regulon identified by microarray analyses is similar in the size of the σ^B regulon identified in *B. subtilis* (127 genes) and *S. aureus* (198 genes) (Raengpradub et al., 2008). Many of the genes identified have functions in the stress response (e.g., *ctc* and *clpC*) but also in the carbohydrate metabolism such as a mannose-specific PTS operon (*lmo0784* to *lmo0781*; *lmo0784*), transcriptional regulation such as *lmo2460* encoding CggR which is a transcriptional regulator highly similarly to the *B. subtilis* central glycolytic gene regulator and virulence such as *inlAB*, *inlD*, *bsh*, and *hfq* (Raengpradub et al., 2008). Milohanic et al. (2003) indicated that a significant subset of PrfA-regulated genes have potential σ^B promoter sequences. As previously mentioned, PrfA is a transcriptional regulator of virulence gene transcription in *L. monocytogenes* (O'Byrne & Karatzas, 2008). It should therefore be noted that σ^B seems to be involved in the control of both the general stress response and virulence-related functions (O'Byrne & Karatzas, 2008).

2.4 Role of SigB in Adaptation to Osmotic Challenges

2.4.1 Microbial Cells in Adverse Osmotic Environments

Maintenance of a positive turgor pressure is a key determinant for the growth of bacteria. The availability of water is one of the most significant factors affecting growth and survival of bacteria. In hyperosmotic environments, bacteria must adjust to fluctuations in the water content of their cytoplasm to remain viable. Changes in the external osmolality induce transmembrane water movements along with the osmotic gradient. These transmembrane water movements can cause either dehydration of the cytoplasm under hypertonic environments or bursting of the cell under hypotonic environments (Storz et al., 2000). Bacteria avoid these devastating stresses by controlling the level of their intracellular compatible solute pool to retain a suitable level of cytoplasmic water and turgor (Storz et al., 2000).

The definition of compatible solutes, also known as osmolytes, are compounds that are soluble in high concentrations and can be accumulated to very high levels in the cellular cytoplasm without disturbing the function of the cell under the high osmotic

conditions (Arakawa & Timasheff, 1985). The types of compatible compounds identified comprise amino acids such as proline, amino acid derivatives including proline betaine, small peptides such as N-acetylglutaminylglutamine amide, methylamines such as glycine betaine, carnitine and their sulfonium analogs such as dimethylsulfonium propionate, sulfate esters such as choline-O-sulfate, polyols such as glycerol, and sugars such as trehalose and sucrose (Storz et al., 2000). It should be noted that glycine betaine, ectoine, proline, trehalose, and carnitine are probably the most widely used compatible solutes in the microbial world (Storz et al., 2000; Patchett, Kelly, & Kroll, 1992). The compatible solutes are used to control turgor, to balance environmental osmolality and to extend the ability of bacteria to colonize more saline environmental conditions (Cunning, Brown, & Elliott, 1998). Some research groups have demonstrated that compatible solutes contribute to membrane integrity and protein folding and stability using *in vitro* experiments (Rudolph & Crowe, 1986). The compatible solutes with their enzyme stabilizing capabilities offer cells protection against heating, freezing, and drying (Lippert & Galinski, 1992).

According to the definition presented by (Storz et al., 2000, p.80): "The osmotic pressure (∏) is defined as the hydrostatic pressure that, in an equilibrium state, balances the difference in chemical potential such that no movement of solvent occurs between the two compartments". In an aqueous solution ∏ can be determined by the following expression: $\prod = (RT/V_w)\ \ln a_w$. This formula is directly related to the water activity (a_w, the ratio of vapour pressure of water (p) to the vapour pressure of pure water (p_0) at the same temperature) and to V_w which represents the partial molar volume of water in the solution (Storz et al., 2000). The term osmolality, which is related to the osmotic pressure (∏) of an aqueous solution, can be defined as the sum of the concentrations of osmotically active solutes (osmolytes) in solution (Wood, 1999a). Osmolality is expressed in units of osmoles (moles of osmolyte) per kilogram of solvent (Storz et al., 2000). When two compartments are separated by a semipermeable membrane, the solvent water has a tendency to flow from the compartment with high chemical potential of water to that with lower chemical potential. For bacteria, the total concentration of osmolytes within a cell is generally higher (and chemical potential thus lower) than outside of the cell (Storz et al., 2000). This causes water to flow from the environments

with high chemical potential of water into the cell. This water influx will increase the cellular volume and subsequently presses the cytoplasmic membrane towards the cell wall. The difference in pressure across the cytoplasmic membrane and the cell wall is defined as the turgor, $\Delta\prod$ ($\Delta\prod = \prod_{in} - \prod_{out}$) (Storz et al., 2000). Turgor balances the difference in osmotic pressure between the inside and outside of the cell and must be maintained through the growth cycle as the driving force for cell extension, growth, and division (Chater & Nikaido, 1999). The turgor ranges from 0.3 to 0.5 MPa and approximately 2MPa for Gram-negative and Gram-positive bacteria, respectively (Storz et al., 2000). This difference indicates that higher cytoplasmic concentrations of solutes in Gram-positive bacteria are needed for expansion of the multilayered peptidoglycan (Storz et al., 2000). By using the Boyle-van't Hoff relationship (cell volume versus reciprocal of external osmolality), which provides a direct determination of turgor pressure, the turgor pressure was determined to be approximately 0.75 osmol/kg (1.9 MPa) in an exponentially growing *B. subtilis* culture in a defined medium (Whatmore & Reed, 1990); for *L. monocytogenes*, the internal osmolality was determined to be approximately 0.55 osmol/kg (1.4 MPa) in the absence of sodium chloride, with an obvious increase to approximately 0.73 to 0.83 osmol/kg (1.8 to 2.1 MPa) at 5% and 7.5% sodium chloride, respectively (Patchett et al., 1992). This osmolality increase indicated that cells accumulated osmolytes in response to the external salt concentration (Patchett et al., 1992).

2.4.2 Transport of Compatible Solutes

2.4.2.1 Compatible Solute Uptake in the Gram-Negative Bacteria

In Gram-negative bacteria, such as *Escherichia coli* and *Salmonella enterica* subsp. *enterica* ser. Typhimurium, the compatible solutes including trehalose, glutamate, glycine betaine, and L-proline have been identified (Gowrishankar & Manna, 1996). In order to take up those compatible solutes into the cellular cytoplasm under hyperosmotic conditions; *E. coli* and *S.* Typhimurium have evolved two transport systems, ProP and ProU (Gowrishankar & Manna, 1996). ProP encoded by *proP* is an integral membrane

protein (Mellies, Wise, & Villarejo, 1995). It is noted that ProP transports glycine betaine and proline under osmotic upshock and amino acid limitation in *E. coli* and *S.* Typhimurium (Mellies et al., 1995). The activity of ProP is regulated at the transcriptional level by two promoters, P1 and P2 (Storz et al., 2000). A study of two different *E. coli proP* promoters indicated that *proP* belongs to the growing class of σ^S-dependent genes which respond to both stationary-phase and high osmotic signals (Mellies et al., 1995). *proU* encodes a transporter (ProU) which was originally found to be involved in uptake of glycine betaine and L-proline at high osmolarity (Gowrishankar & Manna, 1996). The report indicated that the uptake of taurine, ectoine, choline, proline betaine, and dimethylthetin is regulated by ProU as well. *proU* consists of three genes, *proV*, *proW*, and *proX*, and belongs to the large family of periplasmic-binding-protein-dependent transporters (Gowrishankar & Manna, 1996). ProX is a periplasmic glycine betaine- and proline betaine-binding protein in *E. coli* and *S.* Typhimurium (Gowrishankar & Manna, 1996; Storz et al., 2000). Then, ProX delivers glycine betaine and proline betaine to the integral inner membrane component ProW, which transports the osmolytes across the cytoplasmic membrane in a manner dependent on the hydrolysis of ATP by the inner membrane-associated ATPase ProV (Storz et al., 2000). Besides the role of ProU under hyperosmotic conditions, ProU may contribute to virulence in the pathogenic enterobacteria (Gowrishankar & Manna, 1996).

2.4.2.2 Compatible Solute Uptake in the Gram-Positive Bacteria

As discussed above, the availability of uptake systems and compatible solutes in extracellular environments provides a way by which bacteria can respond to osmotic stress. In Gram-positive bacteria, the transport systems have been investigated in *L. monocytogenes* and *B. subtilis* in an attempt to understand these bacteria's ability to respond to a wide range of stress and starvation conditions. Several research groups have demonstrated that the intracellular accumulation of the compatible solutes protects against osmotic, cold, and high pressure stresses, and enhances virulence (probably due to enhanced survival) in both *L. monocytogenes* and *B. subtilis* (Storz et al., 2000).

In *L. monocytogenes,* the compatible solutes including glycine betaine, carnitine, proline, proline betaine, and acetylcarnitine have been detected intracellularly and

among these, glycine betaine and carnitine have been extensively studied and determined to be the most effective osmolytes (Angelidis & Smith, 2003a). Since *L. monocytogenes* cells cannot synthesize glycine betaine and carnitine, the accumulation of those two osmolytes from extracellular environments depends on three transport systems: BetL, Gbu, and OpuC (Angelidis & Smith, 2003a).

BetL and Gbu transport glycine betaine into the cell. The glycine betaine porter I, which is encoded by *betL*, functions as a Na^+ symporter for glycine betaine and is activated by an increase in osmolarity (Angelidis & Smith, 2003a). The glycine betaine porter II, which is encoded by the *gbu* ABC operon, is an ATP-dependent transporter, and this transporter is also osmotically activated (Ko & Smith, 1999). This study indicated that the uptake of glycine betaine is solely dependent on the two transporter (BetL and Gbu) systems in *L. monocytogenes* (Ko & Smith, 1999). A later study by Wemekamp, Wouters, Sleator, Gahan, Hill, & Abee (2002) confirmed this by demonstrating the inability of a double deletion mutant (Δ*betL* and Δ*gbu*) of *L. monocytogenes* to import glycine betaine.

The main carnitine transporter, OpuC, is an ATP-dependent transporter. This transporter system consists of a four gene operon, *opuCABCD*, and is activated to transport carnitine into the cell in response to osmotic and cold shocks (Angelidis & Smith, 2003a). Beside the presence of the known carnitine transporter, OpuC, it was hypothesised that another possible carnitine transporter exists in *L. monocytogenes* (Wemekamp et al., 2002). The hypothesis was proposed based on the study of a triple deletion mutant (Δ*betL*, Δ*gbu*, and Δ*opuC*) of *L. monocytogenes* which was still able to accumulate carnitine during osmotic stress (Wemekamp et al., 2002). This study also indicated that OpuC possibly plays an important role in listerial pathogenesis (Wemekamp et al., 2002).

L-carnitine, crotonobetaine, gamma-butyrobetaine, proline, and glycine betaine have been identified as effective compatible solutes in *B. subtilis* (Hecker & Volker, 2001; Kappes & Bremer, 1998). There are five transport systems found in *B. subtilis*: two are secondary transporters (OpuD and OpuE), whereas three (OpuA, OpuB, and OpuC) are members of the ATP-binding cassette (ABC) superfamily of transporters. OpuE encoded by *opuE* regulates the uptake of proline from extracellular environments

in the presence of high osmolarity (Kappes & Bremer, 1998). The OpuA, OpuC, and OpuD transporters mediate glycine betaine uptake (Storz et al., 2000). OpuD is a secondary transporter that exhibits a significant degree of sequence identity to the carnitine transporter, CaiT (24% identity), and the choline transporter, BetT (35% identity), from *E. coli* (Kappes & Bremer, 1998). The two other *B. subtilis* transporters, OpuA and OpuC, are related to ProU from *E. coli* (Kappes & Bremer, 1998). Each of the three glycine betaine transporters (OpuA, OpuC, and OpuD) has a high affinity for its substrate (Storz et al., 2000). OpuA and OpuD have restricted substrate specificity (Storz et al., 2000), however, OpuC is involved in the osmoregulation by importation of a wide range of compatible solutes with high affinity, including choline, L-carnitine, crotonobetaine, and gamma-butyrobetaine (Kappes & Bremer, 1998; Storz et al., 2000). The other ABC transporter OpuB, which is closely related to OpuC, is also involved in choline uptake by *B. subtilis*, but, unlike OpuC, OpuB only recognizes choline (Kappes & Bremer, 1998; Storz et al., 2000).

2.4.3 SigB and Osmoregulation in *L. monocytogenes*

The ability of *L. monocytogenes* to accumulate compatible solutes under conditions of high osmotic pressure improves its growth and survival in contaminated food products and during passage through the upper gastrointestinal tract of animal and human hosts (Cetin, Zhang, Hutkins, & Benson, 2004). The activity of the general stress sigma factor, σ^B, is stimulated in a variety of stresses, including osmotic shock, cold shock, organic acids, and entry into stationary phase in *L. monocytogenes* (O'Byrne & Karatzas, 2008). Of these stresses, osmotic shock (4% NaCl) induces the biggest increase in transcription from the σ^B-dependent promoter upstream from *rsbV*, suggesting an important role for σ^B in regulating the osmotic response in *L. monocytogenes* (Becker, Cetin, Hutkins, & Benson, 1998). In this osmotic shock study, experiments with the isogenic *L. monocytogenes* and its Δ*sigB* mutant demonstrated that the mutant has an impaired ability to use betaine and carnitine as osmoprotectants (Becker, et al., 1998). In addition, the presence of a putative σ^B promoter binding site upstream of *betL*, *gbu*, and *opuC* suggests that the induction of these genes in response to increased osmolarity is at least partly σ^B-dependent (Angelidis & Smith, 2003a). Although *betL* is preceded by a

consensus σ^B-dependent promoter (Sleator, Gahan, Abee, & Hill, 1999), more recent studies have indicated that its expression is constitutive and σ^B-independent (Cetin et al., 2004; Fraser, et al., 2003). *gbuA* is transcribed from dual promoters, one of which is σ^B-dependent, while the other is σ^A-dependent (Cetin et al., 2004). Therefore, the finding that a Δ*sigB* mutant was impaired in its betaine accumulation (Becker, Cetin, Hutkins, & Benson, 1998; Cetin et al., 2004), is probably due to reduced *gbuA* expression rather than reduced expression of *betL* (O'Byrne & Karatzas, 2008). The transcription of *opuC* relies solely upon σ^B in *L. monocytogenes* (Cetin et al., 2004). This result suggests a mechanism where *L. monocytogenes* prefers to use betaine to maintain osmotic balance under normal conditions, since the activity of σ^B is minimally induced under those conditions (Cetin et al., 2004). However, when the medium's osmolarity increases, σ^B activity increases (Chaturongakul et al., 2008). Subsequently, the expressions of *gbuA* and *opuC* increases, thus providing osmoprotection for *L. monocytogenes* (Cetin et al., 2004).

2.5 Effect of Different Environmental Conditions on Listerial Survival on Stainless Steel Surfaces During Desiccation

2.5.1 Survival on Wet Stainless Steel Surfaces: Surface Attachment and Biofilm Formation

The ability of *L. monocytogenes* to attach to and subsequently form biofilms on various food processing surfaces, including benches, machinery, and floors, poses a serious risk for food producers given the potential health concern for consumers (Blackman & Frank, 1996). This ability is believed to be important for the survival and persistence of *L. monocytogenes* which in some food processing industries has been shown to last for several years (Lunden, Miettinen, Autio, & Korkeala, 2000). Studies of surface adhering *L. monocytogenes* cells and *L. monocytogenes* biofilms have demonstrated that sessile biofilm cells tend to be more resistant to sanitizing agents (Frank & Koffi, 1990; Pan, Breidt, & Kathariou, 2006).

Attachment of *L. monocytogenes* to food contact surfaces is considered to be the first step leading to its survival and persistence. If *L. monocytogenes* is unable to attach to food contact surfaces, the chance of its survival on food contact surfaces is greatly diminished (Ells, 2007). The ability of *L. monocytogenes* to adhere to abiotic surfaces relevant to food processing facilities is dependent on the physicochemical properties of both the cell and contact surface. In terms of physicochemical properties, the charge and hydrophobicity of both surfaces affected the adhesion of *L. monocytogenes* to polystyrene (Tresse, Lebret, Benezech, & Faille, 2006). In this study, *L. monocytogenes* displayed a substantially lower surface hydrophobicity when grown at pH 5 compared to pH 7, greatly reducing the number of the cells attached to polystyrene (Tresse et al., 2006). Other factors affecting attachment include temperature, growth phase, the composition and availability of nutrients in the growth medium (Dickson & Siragusa, 1994; Vatanyoopaisarn, Nazli, Dodd, Rees, & Waites, 2000; Chmielewski & Frank, 2003; Briandet, Meylheuc, Maher, & Bellon, 1999). Many researchers have reported that *L. monocytogenes* is able to adhere to a variety of commonly used materials in the food industry, such as stainless steel, glass, Teflon, nylon, polypropylene, and Buna-N rubber (Chmielewski & Frank, 2003). The type of material affects the attachment of *L. monocytogenes* under different contact time regimes and environmental conditions (Beresford, Andrew, & Shama, 2001; Smoot & Pierson, 1998a; Smoot & Pierson, 1998b). Beresford et al. (2001) investigated the ability of *L. monocytogenes* 10403S to attach to a wide range of food industry materials including stainless steels, polymers, and rubbers and found that shortly after the initial contact, there was no difference in *L. monocytogenes* 10403's ability to adhere to any of these materials at 30 ºC. But after 2 hours at 30 ºC, the numbers of attached cells increased significantly for all materials with the exception of polypropylene. The stainless steel 405 harboured the highest number of the attached cells. Smoot and Pierson (1998a) studied the ability of *L. monocytogenes* Scott A to attach to stainless steel and Buna-N rubber in a wide range of temperature and pH conditions (10-45 °C and pH 4 to 9, respectively). Under those conditions, the rate of attachment to stainless steel was faster than that to Buna-N rubber. A second study done by Smoot and Pierson (1998b) showed that the maximum levels of cells attached to stainless steel and Buna-N rubber were achieved when grown at 30 °C. Furthermore,

significant differences in detachment between stainless steel and Buna-N rubber indicated that the strength of attachment is stronger to Buna-N rubber than to stainless steel (Smoot & Pierson, 1998b). In general, stainless steel surfaces have high free surface energy and are more hydrophilic. Those surfaces allow greater bacterial attachment than hydrophobic surfaces such as Buna-N rubber (Chmielewski & Frank, 2003). Many studies have found that the surfaces of *L. monocytogenes* and stainless steel are both hydrophilic (Barnes, Lo, Adams, & Chamberlain, 1999; Chavant, Martinie, Meylheuc, Bellon, & Hebraud, 2002; Mafu, Roy, Goulet, & Savoie, 1991). Chae and colleagues (2006) studied the variation which occurs among *L. monocytogenes* strains in terms of hydrophobicity and hydrophilicity, and reported most of their test strains to be either moderately hydrophobic or moderately hydrophilic. Silva, Teixeira, Oliveira, & Azeredo (2008) found the hydrophilic cell surfaces of *L. monocytogenes* adhered in larger numbers on stainless steel.

On wet surfaces *L. monocytogenes* has the ability to aggregate, grow into microcolonies and produce biofilms. Chmielewski and Frank (2003) described biofilms, as they occur in nature, as a mixture of viable and nonviable microorganisms attached to a surface and embedded in a matrix consisting of extracellular polymeric substances (EPS). The EPS may contain polysaccharides, proteins, phospholipids, teichoic acids, nucleic acids, minerals and other polymers (Chmielewski & Frank, 2003). The variation in the EPS composition depends on the microbial community, nutrient supply and environmental conditions (Chmielewski & Frank, 2003).

The formation of biofilms takes place in three steps. The first step is the initial attachment of the planktonic cells to a surface. Attachment to a surface can be passive or active depending on the motility of the cells. Passive attachment is controlled by gravity, Brownian motion and fluid dynamics. In active adhesion, bacterial cell surface properties, such as flagella, pili, adhesin proteins, capsules, and surface charge, facilitate initial attachment. The initial attachment caused by the weak interaction of bacteria and a substratum is reversible. This weak interaction involves van der Waals and electrostatic forces and hydrophobic interactions. During this reversible attachment stage, bacteria still exhibit Brownian motion, and mild shearing forces can easily remove bacteria from a surface substrate. It has been reported that this initial attachment of planktonic cells to

a surface often occurs within 5 to 30 seconds, followed by irreversible attachment which is the second step (Mafu, Roy, Goulet, & Magny, 1990).

The second step is mediated by the production of the EPS and the attachment of the bacterial appendages, such as flagella, pili, and adhesin proteins. The interaction between bacterial appendages and a surface substrate involves dipole-dipole interaction, hydrophobic and hydrogen bonds, as well as ionic covalent bonding. The irreversible attachment occurs in a temperature dependent manner within 20 minutes and up to 4 hours after the initial attachment at 4 and 20 °C, respectively (Mafu et al., 1990). Following the irreversible attachment, microcolony formation will proceed given the appropriate growth conditions. Microcolony formation occurs through the simultaneous aggregation and growth of microorganisms along with the production of the EPS.

Studies of bacterial biofilms in natural systems showed that aggregation may involve recruitment of planktonic cells from the surrounding medium through quorum sensing (McLean, Whiteley, Stickler, & Fuqua, 1997; Pesci et al., 1999). Quorum sensing is used by microorganisms as a cell-to-cell communication tool and involves the excretion of autoinducer molecules into the extracellular environment to regulate the behaviour of the bacterial community (McLean et al., 1997; Pesci et al., 1999). Removal of the attached cells from a surface substrate after the second step of biofilm formation is very difficult and requires the use of strong and physical shearing forces, such as scrubbing or scraping, and/or chemical treatments (e.g., enzymes, detergents, surfactants and sanitizers) to break the bond between bacterial appendages and the surface (Chmielewski & Frank, 2003).

The last step in the production of mature biofilms entails continued growth of adhered cells into microcolonies on the surface, recruitment of planktonic cells, and production of EPS. The mature biofilms form heterogeneous 3-dimensional structures and water channels that allow for nutrient flow. The mature biofilms may consist of a single layer of cells surrounded by porous extracellular polymer or multiple layers of cells that are loosely packed within EPS and are interspersed with water channels (Chmielewski & Frank, 2003). They may also consist a single or multiple species (Davey & O'Toole, 2000). Studies of mixed biofilms composed of *L. monocytogenes* together with other bacteria such as *Flavobacterium* spp. or *Pseudomonas* spp. have

indicated that the interaction between multiple species enhances formation of the biofilm (Bremer, Monk, & Osborne, 2001; Sasahara & Zottola, 1993).

During the maturation of natural biofilms an organized structure may develop (Chmielewski & Frank, 2003). A *L. monocytogenes* "honeycomb" structure with holes surrounded by cells was described by Marsh and colleagues (2003). By using laser-scanning confocal microscopy, Rieu et al. (2008) revealed that *L. monocytogenes* under continual nutrient flow conditions forms a network of knitted chains as its dominant biofilm structure on stainless steel. The biofilm's maturation process is influenced by the culture medium. In a chemically defined minimal medium (modified Welshimer's broth, MWB), the initial biofilm development by *L. monocytogenes* Scott A on stainless steel for 12 days at 21 °C was enhanced with increased concentrations of phosphate and amino acids. Replacement of amino acids in MWB with tryptone initially enhanced biofilm development on stainless steel as did the addition of trehalose and mannose (Kim & Frank, 1995). Another study of biofilm formation by eight strains of *L. monocytogenes* on polyvinyl chloride in microtitre well plates found that six strains produced denser biofilms in MWB while the remaining two strains formed thicker biofilms in tryptic soy broth (TSB) (Moltz & Martin, 2005).

The adherence and biofilm formation of *L. monocytogenes* have been found to vary among strains (Borucki, Peppin, White, Loge, & Call, 2003; Kalmokoff et al., 2001; Norwood & Gilmour, 1999). In a study of thirty-six *L. monocytogenes* strains, it was found that all strains were able to attach to stainless steel coupons, however, only one strain of the thirty-six strains was able to produce a biofilm (Kalmokoff et al., 2001). Borucki et al. (2003) examined the ability of eighty *L. monocytogenes* isolates to form a biofilm to determine if there is a link between biofilm formation, phylogenic division, and persistence in the environment. They found serotypes 1/2a and 1/2c, which are isolated from human and animal sources but not normally associated with food-borne outbreaks, generally produced greater biofilms on stainless steel than did other phylogenic groups. Also, persistent strains from bulk milk samples produced higher amounts of biofilm compared to non-persistent strains.

Biofilms can be constantly (re)generated from detached cells migrating from existing biofilms to attach to and colonize new surfaces under dynamic conditions. The

detached cells from biofilms in food processing environments can contaminate food products, especially RTE foods, if coming into contact, resulting in reduction of shelf life of food products and transmission of diseases by human consumption (Chmielewski & Frank, 2003). Also, raw produce may contain bacterial biofilms before entering processing facilities. Morris and co-workers (1997) observed natural biofilms on the leafy surfaces of a variety of plants, including spinach, lettuce, Chinese cabbage, celery, leeks, basil, parsley, and broad leaf endive. A more recent study of microbial biofilms on selected fresh produce confirmed the presence of biofilms on a variety of raw vegetables, including tomatoes, carrots, and mushrooms (Rayner, Veeh, & Flood, 2004). Ells and Truelstrup Hansen (2006) demonstrated that *L. monocytogenes* could form a biofilm on both the waxy intact leaf and cut surfaces of raw cabbage. Therefore, raw produce may contain these pathogenic bacteria, possibly leading to the detachment of biofilm cells from raw produce coming into contact with a surface substrate. Such transferred cells can now attach to and colonize the new surface by forming a biofilm.

Once biofilms become established, it can be very difficult to completely remove or eliminate them from plant surfaces, especially in areas which are not easily accessible, such as conveyor belts, abraded surfaces and drains (Frank & Koffi, 1990; Lee & Frank, 1991). Moreover, growth of bacterial biofilms in food processing environments leads to an increased chance for potential transfer and cross-contamination of processed food products (Chmielewski & Frank, 2003; Rodriguez, Autio, & McLandsborough, 2008).

The surfaces found in food processing facilities are generally low in available nutrients, routinely cleaned and sanitized by chemical and physical treatments, and kept at temperatures which are generally lower than the optimal growth temperature for bacteria. Under these environmentally stressful conditions, biofilm formation is an important adaptive mechanism promoting bacterial survival and persistence (Chmielewski & Frank, 2003).

Many studies have indicated that biofilm cells are generally more resistant to sanitizers than planktonic cells (Bolton, Dodd, Mead, & Waites, 1988; Djordjevic, Wiedmann, & McLandsborough, 2002; Frank and Koffi, 1990; Reid et al., 1993; van der Veen & Abee, 2010). The induction of stress resistance mechanisms in biofilms has been proposed to partly explain the survival mechanisms of biofilm cells subjected to stressful

conditions (van der Veen & Abee, 2010). Evidence has suggested that stress response sigma factors play a critical role for the resistance of Gram-negative and Gram-positive bacterial biofilms to disinfectants and environmental stress conditions (Cochran, Suh, McFeters, & Stewart, 2000; Rachid et al., 2000; van der Veen & Abee, 2010). In *Pseudomonas aeruginosa*, RpoS and AlgT, which are known to protect the cells against environmental stresses, contribute to the resistance of sessile cells to disinfectants (Cochran, Suh, McFeters, & Stewart, 2000). In Gram-positive bacteria, a study of *S. aureus* biofilms under osmotic stress conditions showed that exposure to high osmolarity can induce and increase the production of *S. aureus* biofilms. Under osmotic stress, inactivation of σ^B reduced the ability of *S. aureus* to form a biofilm, indicating that σ^B plays a critical role in the regulation of *S. aureus* biofilm formation in response to environmental stress conditions (Rachid et al., 2000). A later study, which disagreed with these results, indicated that σ^B is not required for biofilm development by *S. aureus* (Valle et al., 2003). However, for *L. monocytogenes* a recent study demonstrated that σ^B is required to obtain wild-type levels of both static and continuous-flow biofilms (van der Veen & Abee, 2010).

2.5.2 Desiccation Survival on Stainless Steel Surfaces: Role of Desiccation Rate, Relative Humidity, Biofilm, Organic Material, and Food Ingredients

Desiccation tolerance can be defined as the ability of cells to undergo nearly absolute dehydration through air-drying, without being killed (Billi & Potts, 2002). It has been proposed that desiccation tolerance is involved in the observed persistence of *L. monocytogenes* in the food processing environment, where it remains viable for years even after cleaning, disinfection, and drying (Vogel, Truelstrup Hansen, Mordhorst, & Gram, 2010). In their recent study, *L. monocytogenes* was observed to survive desiccation under conditions resembling food processing environments for extended periods of time (up to 91 days).

Water plays diverse roles in the bacterial life cycle. Water molecules are critical components of chemical reactions; they contribute to the stability of proteins, DNA, and lipids; confer a structural order upon cells and may play a determinative role in

regulation of gene expression (Potts, 1994; Potts, Slaughter, Hunneke, Garst, & Helm, 2005). The removal of water from a bacterial cell into the surrounding environment can happen due to two different scenarios (Figure 2). In Figure 2 (a) the hyperosmotic stress scenario is depicted where the cells are placed under osmotic stress by being suspended in an aqueous solution of some solute resulting in a water activity that is lower than the cell compartment's. Figure 2 (b) shows the air-drying stress scenario and here the water efflux occurs when the cells are exposed to a gas phase with a water activity that is lower than that found in the cell compartment. Experimentally, cells can be dried either by exposing the cells to an air flow in a biosafety cabinet or cells can be placed into a closed water vapour equilibrium system equipped with a saturated salt solution in order to control the water activity status of bacterial cells (Potts, 1994). The removal of water from a bacterial cell, depending on the amount of water removed, can cause mild, moderate, severe, or extreme water deficit. Most microorganisms grow well at water activities of 0.98 or 98% RH (Potts, 1994). From a study of bound water in relation to biological integrity of a range of desiccation-sensitive and desiccation-tolerant bacteria and viruses, measurements of cellular water contents indicated that when the biological agents were dried at RH 40% and 30%, they contained around 10 g and 3 g of H_2O (per 100 g of solids), respectively (Potts, 1994). The removal of water from cells into the extracellular environments through hyperosmotic and air-drying stresses can occur rapidly or slowly. Figure 2 (b) shows that if there is a big difference in the water activity between cells and the environment, exposure of cells for a limited time to a very low RH may lead to rapid shrinkage of the cytoplasm; however, if the water activity in the environment is sufficient to permit bacterial growth or metabolic activity, even though it might be limited, the cells have time to achieve a suitable water level in cytoplasm and turgor by synthesis or uptake of compatible solutes (Potts, 1994). This microbial survival mechanism for cells exposed to hyperosmotic stress has been discussed in section 2.4.1 and the consequence of rapid and slow water movements from cells under hyperosmotic stress is illustrated in Figure 2 (a). The only difference between air-drying and hyperosmotic stress is the environmental media. Cells under drying (matric) stress are exposed to the atmosphere, whereas cells under hyperosmotic stress are bathed in an

aqueous solution. Therefore, the outcome of hyperosmotic stress is likely to be somewhat similar to that of slow air-dying of cells (Potts, 1994).

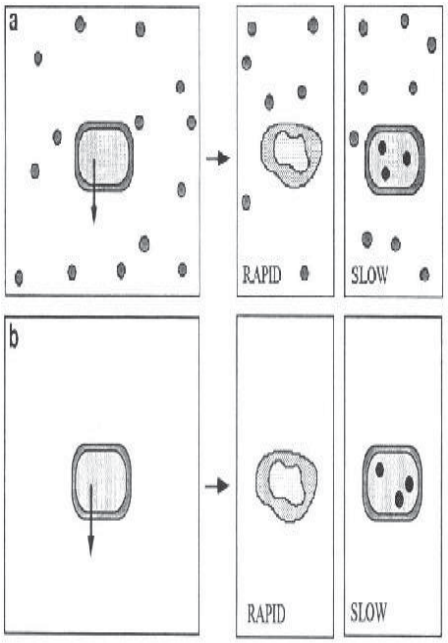


Figure 2. Consequences of the removal of cell water. (a) Hyperosmotic stress. (b) Matric stress (air-drying). The outer and inner membranes of a hypothetical cell are shown. Movement of water (vertical arrows) is either rapid or slow. Hatched circles represent a cosolvent (e.g., a salt). Cross-hatched circles represent a compatible solute (e.g., a carbohydrate) (Potts, 1994) with permission of the American Society for Microbiology. The permission letter is attached in the appendix.

Drying stress is often termed matric water stress and the removal of water from cells through these matric water stresses is called desiccation (Potts, 1994). At the bacterial community level, desiccation brings many changes to cells including a change (usually increase) in surface area, shrinkage, salt precipitation, change in texture, change in shape and change in colour through oxidation of pigments. At the level of the individual cell, changes associated with desiccation include shrinkage of capsular layers, increase in intracellular salt concentrations, crowding of macromolecules, changes in volumes of cell compartments, changes in biophysical properties such as surface tension, reduced fluidity such as increased viscosity, damage to external layers such as pili and

membranes, acquisition of static charge, and change in physiological processes (Potts et al., 2005).

Several studies have investigated the effect of desiccation rate, relative humidity, biofilm, organic material, and food ingredients on listerial survival on stainless steel surfaces during desiccation (Potts, 1994; Vogel et al., 2010; Truelstrup Hansen & Vogel, 2011). The rate at which cells are air dried is critical to cell survival (Potts, 1994). A study on *Listeria* spp. indicated that rapid air-drying results in loss of viability on culinary food units (Cox et al., 1989). Another study reporting on the effect of slow and rapid air-drying of *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *S. aureus* on stainless steel surfaces showed that the rapid air-drying is the most effective treatment to reduce the survival of bacteria (Fuster, Hernadez, & Rodriguez, 2008). This result indicated that the severe water deficit induced by the rapid removal of water from cells probably damaged cell membranes irreversibly leading to their death. In both slow and rapid air-drying treatments, the viability of *S. aureus* declined slower than that of *Ent. cloacae* and *P. aeruginosa*. This result showed that *S. aureus* appears to be more desiccation tolerant than both Gram-negative bacteria that are more susceptible to air-drying stresses (Fuster et al., 2008). In general, the most desiccation tolerant non-spore forming bacteria tend to be Gram-positive (Potts et al., 2005). One possible reason is difference in the cell wall properties between Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a more thick and rigid cell wall than Gram-negative bacteria. The Gram-positive cell wall consists of a single 20 to 80 nm thick homogeneous peptidoglycan layer that is located outside the plasma membrane. In contrast, even though the Gram-negative cell wall is quite complex, it consists of a 2 to 7 nm peptidoglycan layer surrounded by a 7 to 8 nm thick outer lipopolysaccharide membrane (Potts, 1994).

The survival of *L. monocytogenes* under desiccation stress on stainless steel surfaces depends on the RH (Vogel et al., 2010). In this study, survival of *L. monocytogenes* on stainless steel surfaces was tested at relative humidities of 75%, 43%, and 2% and the order of survival at the different RHs was 75% > 43% > 2% RH (Vogel et al., 2010). Part of the explanation for this observation is that the slower drying rates in higher RH environments enhance the bacterial desiccation survival. This observation was

similar to that of Fuster and colleagues' as discussed above. But, an early study by Wilkinson (1966) reported that *Pasteurella* (*Yersinia*) *pestis* A1122, when deposited by aerosolization in growth media on metal surfaces, survived more than 3 days at 11% RH but less than 2 days at 100% RH. A recent study by Kim et al. (2008) found that the order of survival of *Enterobacter sakazakii* in biofilms as affected by relative humidity was 100 > 23 = 43 = 68 > 85% RH on stainless steel. Lin and Beuchat (2007) observed that populations of *Ent. sakazakii* in dry infant cereals decreased more rapidly at a_w = 0.82 to 0.83, compared with a_w = 0.76 or 0.63 to 0.66. Similarly, a study on the behaviour of *Salmonella enterica* subsp. *enterica* ser. Enteritidis and *S.* Typhimurium in chicken manure at different levels of water activity reported that the destruction of *Salmonella* was fastest at a_w = 0.89 (Himathongkham, Nuanualsuwan, & Riemann, 1999). One possible reason for this phenomenon is the accumulation of toxic substances within non-growing cells (Kim, Bang, Beuchat, & Ryu, 2008). Himathongkham et al. (1999) demonstrated that metabolism of *Salmonella* may continue even though cell growth ceases when cells are exposed to a_w ranging from 0.85 to 0.89. The continued metabolism results in accumulation of toxic substances that can damage and kill bacterial cells. Moreover, Rose and colleagues (2003) observed that slow drying as occurs in higher RH environments allows distortion of membrane protein structure and, therefore, causes more damage to the cell membrane. Variability in membrane protein content of different species and strains may explain the variability in response to desiccation (Rose, Donlan, Banerjee, & Arduino, 2003).

The protective role of biofilms in desiccation tolerance was recently documented in *L. monocytogenes* by Truelstrup Hansen and Vogel (2011). They observed that the survival level of *L. monocytogenes* was higher in the low salt biofilm sample than in the low salt non-biofilm sample. This may be caused by the production of EPS along with biofilm formation (Truelstrup Hansen & Vogel, 2011). The EPS matrix has been shown to confer bacteria resistance against desiccation (Kumar & Anand, 1998; McLandsborough, Rodriguez, Perez, & Weiss, 2006; Rodriguez et al., 2008). In the study by Truelstrup Hansen and Vogel (2011), they also observed that the higher number of desiccated biofilm cells were transferred to the surface of cut fresh and cold-smoked salmon tissue in comparison to the number of desiccated non-biofilm cells. The

efficiency of transfer was higher for the desiccated non-biofilm cells. However more biofilm cells were transferred simply because there were more survivors (Truelstrup Hansen & Vogel, 2011). In the end as biofilm formation enhanced the desiccation survival of *L. monocytogenes*, more cells were transferred to fresh and smoked salmon (Truelstrup Hansen & Vogel, 2011). In a similar study, the effect of the transfer of *L. monocytogenes* biofilms grown on stainless steel to bologna and hard salami was evaluated after drying biofilms by equilibration over saturated salt solutions with controlled % RHs at 20 °C for 24 hours (94, 75, 58, and 33% RH) (Rodriguez, Autio, & Mclandsborough, 2007). They observed that as biofilms became drier, the transfer of *L. monocytogenes* from stainless steel surfaces to both foods increased (Rodriguez et al., 2007). Another similar study found that the survival of strong *L. monocytogenes* biofilm formers increased at 78% RH compared with their weak biofilm-forming counterparts. As there were more attached and viable cells on stainless steel knife blades for the strong biofilm formers, transfer to turkey was significantly greater for strong as opposed to weak biofilm formers (Keskinen, Todd, & Ryser, 2008). The transfer has been suggested to happen due to the creation of capillary forces or liquid bridge created between the desiccated cells and the moisture content in the food product (Rodriguez et al., 2007; Truelstrup Hansen & Vogel, 2011). The protective role of biofilms in desiccation tolerance has been also reported in Gram-negative bacteria, such as *Rhizobium leguminosarum* (Vanderlinde et al., 2010) and *Pseudomonas putida* (Chang, Nielsen, de Guzman, Li, & Halverson, 2007). Interestingly, a recent study by Kim et al. (2008) indicated that during desiccation of *Ent. sakazakii* biofilm cells on stainless steel by exposure to 23, 43, 68, and 85% RH at 25 °C, the composition of the desiccation menstruum seemed to be more important for survival than the presence of a biofilm. They observed that reduction in viability of cells was significantly greater in biofilms that had formed in M9 medium, which contains minimal concentrations of nutrients, than in biofilms formed in infant formula, which is a nutrient-rich medium (Kim et al., 2008).

The impact of the presence of organic material (soil) and other compounds in the desiccation matrix for desiccation survival was also investigated for *L. monocytogenes* (Vogel et al., 2010). In their study, *L. monocytogenes* N53-1 survived desiccation better in TSB-glu than in PPS. Also, the number of survivors was higher in TSB-glu than in

fresh salmon juice during desiccation for 91 days. The higher content of desiccation protective nutrients in TSB-glu may protect the cells against desiccation stress. One possible reason that reduced survival was observed in fresh salmon juice could be the production of toxic substances in fresh salmon juice during the processing of the juice where heat is applied and water evaporated. Also during drying, lipids in the salmon juice may become oxidised to produce toxic radicals, which can damage and kill cells (Vogel et al., 2010). It was also observed that the survival level of *L. monocytogenes* N53-1 was higher in TSB-glu with 5% NaCl than in smoked salmon juice (with 5% NaCl) during desiccation for 91 days. This is likely caused by the presence of concentrated smoke (phenol) components in smoked salmon juice during drying, as these components are inhibitory to *L. monocytogenes* (Vogel et al., 2010).

2.5.3 Role of the SigB Factor in the Adherence, Biofilm Formation and Desiccation Resistance of *L. monocytogenes*

As discussed earlier, σ^B plays a central role in regulating *L. monocytogenes* gene expression in response to environmental stresses and nutrient starvation. To investigate whether σ^B affects the surface attachment of this foodborne pathogen, Schwab, Hu, Wiedmann, & Boor (2005) conducted a study that compared the attachment of wild type *L. monocytogenes* and a Δ*sigB* mutant to stainless steel. Results showed that σ^B is not required for initial surface attachment to stainless steel at 24 °C. However, the finding that significantly lower numbers of isogenic Δ*sigB* cells were attached after 48 and 72 hours may indicate the occurrence of changes to the cell envelope of Δ*sigB* cells during prolonged static incubation, probably due to nutrient depletion in the medium. The availability of nutrients influences the biofilm formation by *L. monocytogenes* on stainless steel (Kim & Frank, 1995). Also, σ^B is known to contribute to cellular integrity and viability under conditions of prolonged static incubation (Chaturongakul & Boor, 2004) and carbon starvation (Ferreira et al., 2001). The loss of viability under carbon starvation is most likely due to enhanced lysis of the Δ*sigB* strain (Ferreira et al., 2001). Since σ^B appeared not to be essential for attachment to stainless steel surfaces, another study investigated whether σ^B affects the biofilm formation in *L. monocytogenes*. This

biofilm formation study using both an in-frame Δ*sigB* mutant and its complemented mutant showed that the presence of σ^B is required to obtain wild-type levels of both static and continuous-flow biofilms (van der Veen & Abee, 2010). In the same study, disinfection treatments of planktonically grown cells and cells dispersed from static and continuous-flow biofilms showed that σ^B is involved in the resistance of both planktonic cells and biofilms to the disinfectants benzalkonium chloride and peracetic acid (van der Veen & Abee, 2010). Not only can biofilms enhance the resistance to disinfectants in the food processing environments, but biofilms also improve the desiccation survival of *L. monocytogenes*. However, so far, little is known about the importance of activation and regulation of σ^B, in desiccation resistance of *L. monocytogenes*. In this thesis project, the potential role of the alternative sigma factor, σ^B, in desiccation resistance of *L. monocytogenes* has been investigated.

2.6 Long-Term Survival and Evolution in the Stationary Phase Through the Life Cycle of *L. monocytogenes* in Relation to Food Safety

The exponential growth phase of bacteria, which can only be observed under rich growth conditions in the laboratory, rarely exists in nature. Outside the laboratory, environment stresses, nutrient limitation, and starvation stress almost permanently restrict bacterial growth. It is therefore common that in natural ecosystems, bacteria are more likely to grow at very slow rates, continuously fluctuating from slow-growing to non-growing states or relying on long-term survival strategies in a non-growing state to escape cell death and cell lysis (Hecker & Volker, 2001). This long-term survival state is seldom described in microbiology textbooks that discuss only four phases in bacterial growth curves (lag, exponential, stationary, and death). However, some reports describe a fifth phase, in which bacteria exhibit a long-term survival phase or mode (Wen, Anantheswaran, & Knabel, 2009). This fifth phase has also been described as the "long-term stationary phase" in *E. coli* (Finkel, 2006) or the "senescent phase" in *Serratia* and *Sarcinia* spp. (Steinhaus and Birkeland, 1939). The long-term stationary phase is a highly dynamic period in which the "birth" and "death" rates are balanced. As cells die, cellular integrity is disrupted and the rest of surviving cells can catabolize the biological

detritus from dead cells, including amino acids from proteins, carbohydrates from the cell wall, lipids from cell-membrane material and even DNA to create new cells (Finkel, 2006). As a result of the long-term survival interplay between bacteria and a changing set of growth-restricting stresses, a very complex adaptational network has evolved (Hecker & Volker, 2001).

The expression of alternative metabolic pathways and a wide variety of stress-response genes is essential for long-term survival of bacteria in harsh and natural ecosystems (Finkel, 2006). Moreover, the long-term survival phase is metabolically dynamic with populations changing over time. In this scenario newly created mutants that express a growth advantage during the stationary phase become more competitive than the parent culture and will finally dominate the whole population. This phenomenon occurs reproducibly in the long-term survival phase of *E. coli* (Finkel, 2006).

Entry into stationary phase of *L. monocytogenes* has been reported to involve the alternative sigma factor, σ^B (Schwab, Bowen, Nadon, Wiedmann, & Boor, 2005; Abram, Starr, Karatzas, Matlawska, Boyd, & Wiedmann, 2008). Because the expression of the σ^B-dependent genes were strongly induced during the entry of cells into stationary phase with nutrient-limiting stress or carbon starvation, Boylan and other colleagues (1993) concluded that σ^B controls a large stationary phase regulon (Hecker & Volker, 2001). For example, during prolonged static incubation at 37 °C, the wild-type strain for *L. monocytogenes* survived significantly better following entry into stationary phase compared with its isogenic Δ*sigB* mutant. This result indicated that σ^B, which occurs in a growth phase-dependent manner (Ferreira, Sue, O'Byrne, & Boor, 2003), is important for listerial survival in stationary phase with its associated nutrient limitation or carbon starvation (Chaturongakul & Boor, 2004).

In the natural environment, bacteria have developed a starvation survival response (SSR) to cope with prolonged nutrient-limiting stress (Herbert & Foster, 2001). This study revealed that the SSR of *L. monocytogenes* EGD is under the control of σ^B. Moreover, an increase in cross-resistance to several environmental stresses including acid stress, oxidative stress, and heat shock is found to occur as cells become starved (Herbert & Foster, 2001). *E. coli*, *S.* Typhimurium, and *S. aureus* also show starvation-

associated increases in resistance to environmental stresses (Jenkins, Schultz, & Matin, 1988; Seymour, Mishra, Khan, & Spector, 1996; Watson, Clements, & Foster, 1998).

In addition to the contribution of σ^B in environmental stress response and upon entry into stationary phase, σ^B plays an important role in virulence of *L. monocytogenes*. In *L. monocytogenes*, many virulence genes are regulated by the positive regulatory factor A (PrfA). *L. monocytogenes prfA*, encoding PrfA, is transcribed from three promoters (*prfA*P1, *prfA*P2, and P*plcA*) (O'Byrne & Karatzas, 2008). *prfA*P2-directed expression was confirmed to be under control of σ^B-dependent transcription, and increased when the wild-type *L. monocytogenes* strain entered stationary phase. This indicates the stationary phase cells may be more virulent as well as survive better during gastrointestinal passage (exposure to organic acids, bile salts, and osmotic gradients) and subsequent stages of infection. Recently, a study into the long-term survival phase in *L. monocytogenes* showed that when *L. monocytogenes* enters a long-term survival phase, cells become more barotolerant and thermotolerant due to cytoplasmic condensation when they transition from rods to cocci (Wen et al., 2009). They hypothesised that the enhanced baro- and thermo-tolerance in the long-term survival phase in *L. monocytogenes* results from a lowered water activity in the coccus-shaped cells (Wen et al., 2009), since lowered water acitivity was previously shown to enhance the baro-tolerance of *L. monocytogenes* (Hayman, Kouassi, Anantheswaran, Floros, & Knabel, 2008). Also, it is possible that starvation stress prior to or during the long-term survival phase induces the synthesis of stress proteins that are known to protect cells against both heat (Jenkins et al., 1988) and pressure (Hayman, Anantheswaran, & Knabel, 2008). Due to a larger surface-to-volume ratio, simple or facilitated diffusion for nutrient uptake is enhanced in the smaller cocci compared with the larger rods, thus reducing the need for energy in nutrient transportation during starvation (Wen et al., 2009).

The long-term survival capability of *L. monocytogenes* in food processing environments, especially inside hard-to-clean harbourage sites or biofilms, has brought about serious food safety concerns for food producers. Food safety studies into the mechanisms of the resistance of *L. monocytogenes* cell in the long-term survival state will provide information which may aid in the development of adequate and effective food processing and preservation methods to improve the food safety. Moreover, a

thorough understanding of the activation processes of the alternative sigma factor, σ^B, and the importance of the σ^B regulon in stress conditions may provide effective tools to control and inactivate this food-borne pathogen in the food industry.

CHAPTER 3. MATERIALS AND METHODS

3.1 Bacterial Strains, Plasmids, Media and Culture Conditions

The wild type strain of *Listeria monocytogenes* 568 (Lm568, serotype 1/2a, shrimp plant isolate, Hefford et al., 2005), and its *sigB* deletion (Δ*sigB*) mutant, which was obtained from Dr. Ells (Agriculture and Agri-Food Canada, Kentville, NS), were used in this study. The two strains were stored at -74 °C in brain heart infusion (BHI) broth (Difco, BD Canada, Oakville, ON) with 20% sterile glycerol. Culturing of Lm568 and its Δ*sigB* mutant was carried out in BHI broth at 37 °C for 24 hours. Fresh cultures were streaked onto BHI agar (BHIA) without antibiotics, unless otherwise noted, at 37 °C for 24 hours and then stored at 4 °C for the following experiments. Fresh cultures from the stock cultures on agar were grown every month. When appropriate, antibiotics (all from Sigma-Aldrich, Oakville, ON, Canada) were used in the following concentrations: ampicillin 100 μg/ml and kanamycin 50 μg/ml. Colonies from both strains were randomly selected from BHIA for the routine analysis by polymerase chain reaction (PCR) with appropriate primers to confirm strain identity.

3.2 DNA Sequencing of the *sigB* Gene in *L. monocytogenes* 568 and Its Δ*sigB* Mutant

The sequence for the *sigB* (locus tag: *lmo0895*; Gene ID: 986527 updated on 7_May_2011) gene was obtained from the Genebank database for the sequenced *L. monocytogenes* EGD-e strain (serotype 1/2a) (Glaser et al., 2001) (GeneBank ID NC_003210) and was used to construct primers for the purpose of sequencing the *sigB* gene in Lm568 and the Δ*sigB* mutant. Primers for the *sigB* gene were designed according

to guidelines found in the StrataClone PCR Cloning kit booklet using Primer 3 software (http://primer3.sourceforge.net/e) and were checked using the National Center for biotechnology information (NCBI) Blast program to confirm the total gene specificity of the nucleotide sequences. Nucleotide sequences were synthesized by Sigma Genosys (Sigma-Genosys Canada, Oakville, ON). The PCR using IDTag™ DNA polymerase was run to prepare product for PCR cloning and subsequent sequencing. The following primers were used: *sigB*-F 5'-ATGAAAGCAGGTGGAGGAG-3', *sigB*-R 5'-AAATTATTTGATTCAACTGCCT-3'. Each 25 µl PCR reaction consisted of 2.5 µl 10X PCR buffer, 1.0 µl of 10 mM dNTP stock (10 µM of each dATP, dCTP, dGTP and dTTP), 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 0.2 µl of Taq polymerase (1 unit), and 20.3 µl of diethylpyrocarbonate-treated (DEPC) water (MoBio Laboratories Inc., Carlsbad, CA, USA), and colony mass from a single colony from a fresh BHIA plate for each strain. The steps in the thermocycler program for this PCR reaction were composed of initial denaturation at 94 °C for 3 minutes, amplification for 30 cycles (denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 3 minutes) and a final extension at 72 °C for 10 minutes. The PCR products were then stored at 4 °C. A volume (50 ml) of 1.0% (w/v) Tris-acetate-EDTA (TAE) agarose gel was prepared with addition of 2 µl of 10 mg/ml ethidium bromide (EB) (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). Eight µl of PCR products and 2 µl gel loading buffer (bromophenol blue, 2.5 g/l; sucrose, 400 g/l) were loaded into the wells followed by electrophoresis for 60 minutes at 100 V. A DNA ladder (Sigma-Aldrich) ranging from 50 bp to 3000 bp was also added to a well in order to allow for the sizing of the bands produced from the samples. After electrophoresis, the gel was visualized with UV illumination to verify production of the expected sized fragments. Then, the PCR products were excised from the gel and purified using the GeneClean™ gel purification kit (Q-Biogene, CA, USA) according to manufacturer's instructions. The extracted DNA products were stored at -20 °C until the following experiment. The extracted and purified DNA fragment for each strain was TA cloned using a StrataClone PCR Cloning kit (Stratagene, La Jolla, CA, USA) following manufacturer's instructions. Five µl and 100 µl of the transformation mixtures were spread plated onto LB-ampicillin-X-Gal plates for blue-white colour screening. The LB-ampicillin-X-Gal plates

were prepared by adding 10 ml of 10 mg/ml filter-sterilized ampicillin and 1.6 ml 2% X-Gal (Ambion, Austin, TX, USA) (0.2 g of X-Gal in 10 ml of dimethylformamide (DMF) (Sigma-Aldrich)) into 1 liter of autoclaved LB agar (Oxoid) at 55 °C. The plates were incubated overnight at 37 °C. Colonies harbouring the pSC-A plasmid containing the PCR product inserts are expected to be white. After prolonged incubation, some of the insert-containing colonies may appear light blue according to the manufacturer's instructions (Q-Biogene, CA, USA). Therefore, white or light blue colonies were selected and the presence of the insert identified by PCR analysis. The PCR using IDTag™ DNA polymerase was performed using the following primers: M13-F (5'-GTAAAACGACGGCCAG-3') and M13-R (5'-CAGGAAACAGCTATGAC-3') to amplify the pSC-A-amp/kan *sigB* inserts. The thermocycler program for this PCR reaction was as described above. After electrophoresis and visualization with UV illumination of a small subsample of the PCR product as described above, the remaining PCR products were together with M13 primers sent to Génome Québec's Innovation Centre for DNA sequencing at McGill University. The returned sequences were aligned using BLASTn online software (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) and compared to the complete sequence of Lm EGD-e using the GeneBank database (GeneBank ID NC_003210).

3.3 Phenotypic Comparison of the Wild Type Lm568 and Its Δ*sigB* Mutant.

3.3.1 Gram Staining to Compare the Cell Morphology

Gram staining of Lm568 and the Δ*sigB* mutant was performed with colonies from fresh BHIA plate (15 °C and 3 days). Single colonies were suspended in sterilized distilled water, and a drop of the suspension placed on a glass microscopy slide and fixed by heating. The heat fixed cells were flooded with crystal violet for 1 minute, and then gently rinsed with sterilized distilled water. Then an iodine solution was added for 1 minute and gently rinsed with sterilized distilled water. Decolourization with ethanol was performed for 20 seconds followed by rinsing again with sterilized distilled water. Finally, 1-2 drops of safranin was added for 20 seconds followed by rinsing with sterilized distilled water and drying the excess water off with paper. The Gram stained

cells were viewed using a Nikon Eclipse 50i light microscope under oil immersion (x 1000 magnification) (Nikon Canada, Mississauga, ON). The Gram staining kit was purchased from Oxoid Inc. (Nepean, ON).

3.3.2 Rapid Gram Reaction and Catalase Reaction

For the rapid Gram reaction**,** a loopful of colony mass obtained for each strain from BHIA was placed on a glass microscopy slide. A drop of 5% (w/v) KOH was added onto the slide and mixed in with the colony using an inoculation needle. Gram-negative bacteria will produce "threads" whereas Gram-positive bacteria will remain "watery". Colonies from an *E. coli* strain (ATCC 25922) was used for comparison. For the catalase reaction, a loopful of colony mass for each strain from BHIA plates was placed on a glass microscopy slide, and a drop of 3% (v/v) H_2O_2 was added. Samples, which produced gas bubbles (O_2), were deemed to be catalase positive.

3.3.3 Colony Morphology Assay

To investigate growth and colony morphologies of Lm568 and its Δ*sigB* mutant in environments of increasing osmolality, a loopful of fresh culture of each strain in BHI broth (37 ºC for 24 hours) was streaked onto BHIA supplemented with 5.0%, 10%, and 20% w/v NaCl, respectively, and incubated at 15 ºC for 3, 4, and 5 days, respectively. The colony morphology (round or irregular shape) was viewed using a Nikon Eclipse 50i light microscope (x 40 magnifications) (Nikon Canada, Mississauga, ON). The colony morphology on BHIA (0.5% NaCl) was used as control.

3.3.4 Phase Contrast Microscopy to Determine Cell Size of Lm568 and Its Δ*sigB* Mutant Grown at Different Sodium Chloride Concentrations

To determine the size of Lm568 and its Δ*sigB* mutant cells during growth at 0.5% and 5.0% w/v NaCl, a loopful of fresh culture of each strain incubated in BHI broth with 0.5% or 5.0% NaCl (15 °C for 3 days) was viewed using a Nikon Eclipse 50i light

microscope under oil immersion (x 1000 magnification) (Nikon Canada, Mississauga, ON).

3.4 Comparative study of the growth kinetics of the wild type Lm568 and Its Δ*sigB* mutant

3.4.1 Culture Conditions and Inoculum Preparation

The growth experiments were carried out at 15 °C for the wild type Lm568 and its Δ*sigB* mutant (Table 2).

Table 2. Growth of Lm568 and its Δ*sigB* mutant in different types of media at 15 °C for 3 days.

Growth Medium	Salt Level
Brain Heart Infusion (BHI) broth	0.5%
	5.0%
Tryptic Soy Broth + 1% (w/v) glucose (TSB-glu)	0.5%
	5.0%
Physiological Peptone Saline (PPS)	0.85%
	5.0%
Miminal Medium (MM)	0.5%
	5.0%
MM with 1 mM betaine (B), carnitine (C) or proline (P)	0.5%
	5.0%

Test media included BHI broth, which originally contains 0.5% NaCl, BHI broth with 5.0% NaCl, Tryptic Soy Broth (TSB) (Difco, BD Canada, Oakville, ON) supplemented with 1% glucose (TSB-glu), which originally contains 0.5% NaCl, TSB-glu with 5.0% NaCl, Physiological Peptone Saline (PPS) (0.1% peptone (Oxoid, Nepean, ON), 0.85% NaCl) and PPS with 5.0% NaCl, a chemically defined minimal medium

(MM) based on the work by Premaratne et al. (1991) with addition of NaCl to final concentrations of 0.5% and 5.0% NaCl, respectively. Growth experiments were also carried out in MM with 0.5% or 5.0% NaCl supplemented with osmolytes. Glycine betaine, DL-carnitine and L-proline (all from Sigma-Aldrich, Oakville, ON, Canada) were each added to MM with 0.5% NaCl and MM with 5.0% NaCl as filter-sterilized solutions to a final concentration of 1 mM, unless indicated otherwise. MM contains, per liter, 6.56 g of KH_2PO_4, 30.96 g of $Na_2HPO_4 \bullet 7H_2O$, 0.41 g of $MgSO_4 \bullet 7H_2O$, 0.088 g of ferric citrate, 10 g of glucose, 0.1 g each of L-leucine, L-isoleucine, L-valine, L-methionine, L-arginine, L-cysteine, 0.6 g of L-glutamine, 0.5 mg each of riboflavin and biotin, 1.0 mg of thiamine and 0.005 mg of thioctic acid. After stock solutions of the individual components were combined, pH was adjusted to 6.7 with potassium hydroxide followed by filter sterilization (Becker, Cetin, Hutkins, & Benson, 1998; Premaratne, Lin, & Johnson, 1991).

For each strain, a single colony from a BHIA plate was inoculated in 5 ml of fresh BHI broth, and incubated overnight at 37 °C without agitation. The cultures were harvested by centrifugation at 2,767 × *g*, for 10 minutes, washed twice, pelleted with PPS, and re-suspended in 5 ml of fresh BHI broth. Each sample was diluted to an absorbance value of 1.0 at 450 nm (Abs_{450nm}) (equivalent to approximately 1×10^8 CFU/ml) with fresh BHI broth. Abs_{450nm} was measured by using an Ultrospec™ 1100 *pro* UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, NJ, USA). Subsequently, the adjusted samples were serially diluted in PPS from 10^8 CFU/ml to 10^7 CFU/ml. An aliquot of 400 µl was added to 40 ml of each growth medium described above and gently vortexed to homogeneity to reach a starting concentration of 10^5 CFU/ml. Aliquots of 1.0 ml each were placed into 2.0 ml Eppendorf tubes and incubated at 15 °C under static conditions. Three tubes for each strain were removed every eight hours followed by sampling of 0.5 ml of the culture suspension in each tube and subsequent serial dilution in PPS. Appropriate dilutions were spread plated onto BHIA and incubated at 37 °C for 24 to 48 hours. After incubation, plates with colonies between 30 and 300 colonies were counted and results were recorded as CFU/ml, and then converted into Log_{10} CFU/ml. The obtained Log_{10} CFU/ml data were used to generate growth curves at 15 °C for each strain. At each sampling time, triplicate samples were

analyzed and the entire experiment was repeated twice to yield a total of six observations for each sampling time.

3.4.2 Modelling of Growth Curves Using the Modified Gompertz Equation

The growth curves were fitted to the modified Gompertz equation (Garthright, 1991) in order to mathematically compare the growth rates and patterns of the wild type strain Lm568 and its Δ*sigB* mutant obtained in growth media with varying nutrient compositions and NaCl concentrations. The non-linear equation was fitted to the growth data by using the Gauss-Newton protocol with a maximum of 30 iterations, using the SYSTAT® 11.0 software package (Systat Software Inc., San Jose, CA, USA).

The equation of the modified Gompertz model is as follows:

$$\log_{10} N(t) = A + D \exp\{-\exp[-B(t - M)]\}$$

Where:

A = value of the lower asymptote (initial population at t = 0, Log_{10} CFU/ml)

D = difference in value between the upper and lower asymptote (maximum minus initial populations in Log_{10} CFU/ml)

B = slope factor of the curve (Log_{10} CFU/ml × time)

M = time at which the exponential growth rate is maximal

t = time (hours)

N = number of cells (CFU/ml)

3.5 Comparative Study of the Desiccation Inactivation of the Wild Type Lm568 and Its Δ*sigB* Mutant

3.5.1 Desiccation Conditions, Stainless Steel Coupon Preparation and Cleaning Treatments

To simulate the relatively dry and temperate food production environments, desiccation experiments were carried out at 15 °C and 43% RH. The RH was stabilized at 43% with saturated K_2CO_3 in mini desiccation chambers (V1850-01, Bohlender, Germany). The temperature and RH were continuously recorded by using data loggers (Tinytag, Gemini Data Loggers Ltd., Chichester, UK). Desiccation survival of both strains was investigated on stainless steel (SS 316, type 4 finish) coupons (0.5 × 0.5 cm). SS is widely used as a material in the food processing industry because of its high degree of disinfectibility and resistance to wear, chemical and heat shock (Krysinski et al., 1992) and was here used to mimic the food contact surfaces in the food industry. Before desiccation experiments, the coupons were initially soaked in 5% CiDecon (Decon Labs, Bryn Mawr, PA) for 30 minutes and treated in a sonication bath (Elmo, Singen, Germany) for 5 minutes. Then, the coupons were rinsed with distilled water, followed by another rinse with 100% isopropanol and 95% ethanol to degrease the coupons. The coupons were rinsed once again with distilled water, air dried and sterilized by autoclaving at 121 °C for 15 minutes. All sterilized and dried coupons were stored in a sealed and sterilized glass bottle with cap before use. Used coupons were sterilised at 121 °C for 90 minutes before being subjected to the same cleaning protocol described above.

3.5.2 Influence of Different Positions in a Mini Desiccation Chamber on the Desiccation Inactivation of the Wild Type Lm568 and Its Δ*sigB* Mutant

Experiments were designed to determine whether the position in the mini desiccation chamber of the Petri dishes containing the inoculated SS coupons would influence the desiccation inactivation kinetics of Lm568 and its Δ*sigB* mutant. In the first experiment both strains were cultured in TSB-glu with 0.5% NaCl at 15 °C for 3 days to

obtain cells in the early stationary phase, followed by desiccation of cells in TSB-glu with 0.5% NaCl on SS coupons at 15 °C and 43% RH for 21 days. In the second experiment the same treatment was repeated except cultures were grown and desiccated in TSB-glu with 5.0% NaCl. Petri dishes containing the inoculated SS coupons were placed in four different positions in a mini desiccation chamber (Figure 3) and left at 15 °C and 43% RH for 21 days.

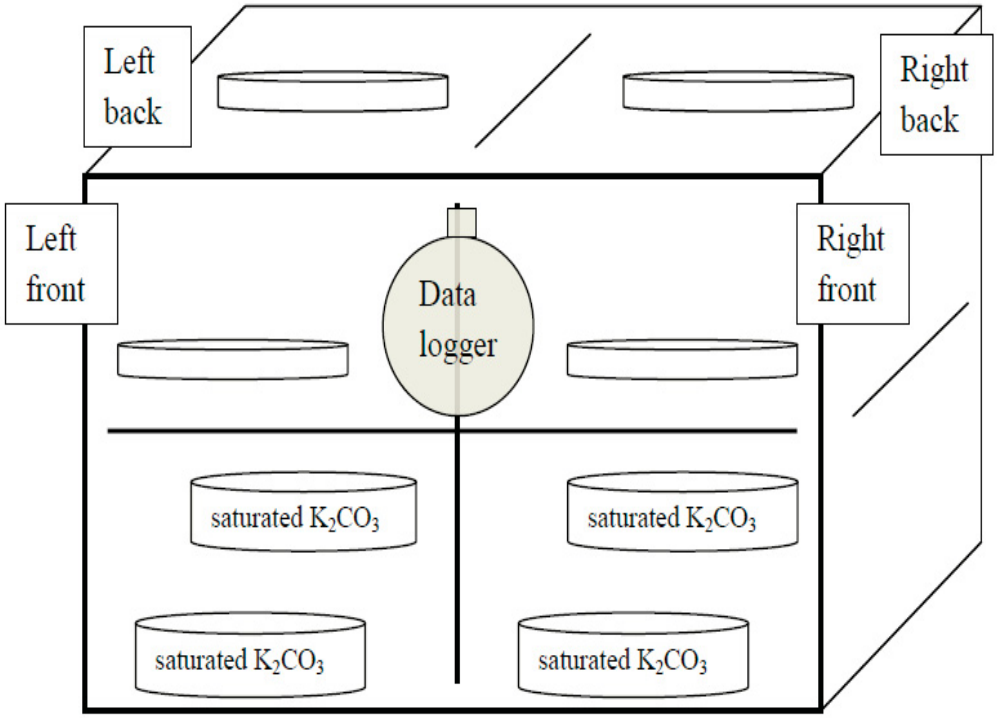


Figure 3. Schematic outline of the desiccation model system: The four Petri dishes with saturated K_2CO_3 solution were placed in the bottom of the mini desiccator to obtain 43% RH. The four open Petri dishes containing stainless steel (SS) coupons inoculated with *L. monocytogenes* cultures were placed on the top shelf of the mini desiccator in four different positions (Left front, Right front, Left back, and Right back). The data logger recording the temperature and RH during the desiccation period was placed in the middle of the top shelf of the mini desiccator.

3.5.3 Culture Conditions and Inoculum Preparations

The initial set of desiccation experiments was conducted in BHI with 0.5% and 5.0% NaCl, TSB-glu with 0.5% and 5.0% NaCl, and PPS with 0.85% and 5.0% NaCl. The second part of the desiccation experiments was conducted in MM with 0.5% and 5.0% NaCl. To determine the effect of external omoslytes, desiccation survival experiments were also carried out in MM with 0.5% NaCl supplemented with 1 mM of glycine betaine, carnitine, or proline, and MM with 5.0% NaCl supplemented with 1 mM of glycine betaine, carnitine or proline. The effect of osmoadaptation was also assessed by pre-culturing cells in each medium supplemented with NaCl to yield a final concentration of 5.0% NaCl.

Prior to desiccation experiments, cultures of Lm568 and the Δ*sigB* mutant were streaked onto BHIA plates and incubated at 37 °C for 24 hours. Colonies were randomly selected from the BHIA plates and grown in each of the growth media described above at 15 °C for 3 days to obtain cells in the early stationary phase. Cells were harvested by centrifugation at 2,767 × *g*, for 10 minutes and resuspended in the corresponding pre-culture medium to an Abs_{450nm} of 1.0 to reach final cell concentrations of approximately 1×10^8 CFU/ml as determined by spread plate counts on BHIA followed by 2 days of incubation at 37 °C.

In the first part of the desiccation experiments, cell suspensions for both strains were prepared to yield the four different treatments (± osmoadaptation and ± high initial desiccation salt level) in each of the complex media (Table 3). Specifically, the following four desiccation treatments were conducted: 1 and 2: Non-osmoadapted cells grown in BHI, TSB-glu, and PPS, respectively, were desiccated in the same medium, which was used during growth, with initial low (0.5%) or high (5.0%) salt level. 3 and 4: Osmoadapted cells grown in BHI, TSB-glu, and PPS with 5.0% NaCl, respectively, were desiccated in the same growth medium with low or high initial salt level.

Table 3. Experimental design for a comparative study of desiccation tolerance of *L. monocytogenes* 568 and its Δ*sigB* mutant in complex media: Cells were desiccated (43% RH and 15 °C) in each of BHI, TSB-glu, and PPS with 0.5% (Low) or 5.0% (High) NaCl with or without prior osmoadaptation (growth with 0.5% or 5.0% NaCl for 3 days at 15 °C).

Treatment	Osmoadaptation	Initial desiccation salt level
1	No	Low
2	No	High
3	Yes	Low
4	Yes	High

In the second part of the desiccation experiments, cell suspensions for both strains were prepared in MM to yield the eight different "pre-culture with osmolyte" treatments (± osmoadaptation, ± pre-culture with each osmolyte, and ± high initial desiccation salt level) (Table 4) and to yield the eight different "desiccation with osmolyte" treatments (± osmoadaptation, ± desiccation with each osmolyte, and ± high initial desiccation salt level) (Table 5).

In the effect of pre-culture with osmolyte experiments (Table 4), the following desiccation treatments were conducted: 1. Non-osmoadapted cells grown in MM were desiccated with low initial salt level. 2: Non-osmoadapted cells grown in MM with 1 mM of glycine betaine, carnitine or proline were desiccated with low initial salt level in MM. 3: Osmoadapted cells grown in MM were desiccated with high initial salt level in MM. 4: Osmoadapted cells grown in MM with 1 mM of glycine betaine, carnitine or proline, were desiccated with high initial salt level in MM.

Table 4. Experimental design to determine the effect of pre-culture with osmolytes on the desiccation tolerance of *L. monocytogenes* 568 and its Δ*sigB* mutant in defined media. After pre-culture in the presence or absence of glycine betaine (B, 1 mM), carnitine (C, 1 mM) and proline (P, 1 mM), cells were desiccated (43% RH and 15 °C) in MM with 0.5% (Low) or 5.0% (High) NaCl with or without prior osmoadaptation.

Treatment	Osmoadaptation	Pre-culture with osmolyte	Initial desiccation salt level
1	No	No	Low
2	No	Yes (B, C or P)	Low
3	Yes	No	High
4	Yes	Yes (B, C or P)	High

Table 5 details the desiccation treatments used in "the desiccation with osmolyte" experiment: 1: Non-osmoadapted cells grown in MM were desiccated in MM with low initial salt level. 2: Non-osmoadapted cells grown in MM were desiccated in MM with low initial salt level and 1 mM of glycine betaine, carnitine or proline. 3: Osmoadapted cells grown in MM were desiccated in MM with high initial salt level. 4: Osmoadapted cells grown in MM were desiccated in MM with high initial salt level and 1 mM of glycine betaine, carnitine or proline.

Table 5. Experimental design to determine the effect of external osmolytes in the desiccation media on the desiccation survival of *L. monocytogenes* 568 and its Δ*sigB* mutant. Cells (± osmoadaptation) were desiccated (43% RH and 15 °C) in MM media (± 5.0% NaCl) without or with 1 mM glycine betaine (B), carnitine (C) or proline (P).

Treatment	Osmoadaptation	Osmolyte in the desiccation medium	Initial desiccation salt level
1	No	No	Low
2	No	Yes (B, C or P)	Low
3	Yes	No	High
4	Yes	Yes (B, C or P)	High

In all desiccation experiments, 10 µl of each sample preparation was spotted evenly on SS coupons which were then placed in Petri dishes without lids. The Petri dishes with the inoculated SS coupons were carefully and quickly transferred to a mini desiccator (V1850-01, Bohlender, Germany). All the desiccation treatments (Tables 3, 4, and 5) were undertaken at a constant temperature (15 °C) and RH of 43% RH, which was obtained by placing four Petri dishes with saturated solutions of K_2CO_3 in the bottom of the desiccator chamber and leaving the chamber at 15 °C for 12 hours prior to the start of the experiments. During all the desiccation experiments, both temperature and RH were continuously monitored using a data logger (Tinytag, Gemini Data Loggers Ltd., Chichester, UK).

3.5.4 Quantification of Surviving Bacteria on Stainless Steel Coupons

All desiccation experiments were performed for 21 days. At specific time intervals, three coupons for each strain and treatment were taken from the desiccator and placed in 1 ml of PPS in an Eppendorf tube (2 ml). The adhering cells were detached from the coupons by sonication for 4 minutes at room temperature in a sonication bath (Elma, Singen, Germany) followed by vortexing for 30 seconds at maximum speed. The sample was then serially diluted in PS, spread plated on BHIA, and enumerated after incubation for 48 hours at 37 °C. This release method follows the procedure applied by Truelstrup Hansen and Vogel (2011). Two independent trials with triplicate samples were performed for each treatment.

3.5.5 Modelling of the Inactivation Curves Using the Weibull Model

The survival of Lm568 and its Δ*sigB* mutant during the desiccation treatments was modelled using the Weibull model (Mafart, Couvert, Gaillard, & Leguerinel, 2001).

The model is as follows:

$$\log_{10} N(t) = \log_{10} N(0) - ((t/delta)^{p})$$

Where:

delta = the time (days) to the first log reduction

p = a curve shape parameter. For downward concave curves p will be greater than 1, while upward concave curves will have p values lower than 1. If p equals 1, then the survival curve follows the traditional first order kinetics (delta = D-value) (Truelstrup Hansen and Vogel, 2011).

N_0 = initial population density (CFU/cm^2)

t = time (days)

N = number of cells (CFU/cm^2)

The model parameters were estimated using the freeware non-linear curve-fitting tool GInaFiT (version 1.5), available at the KULeuven/BioTec–homepage (http://citkuleuven.be/biotec/) and developed by Geeraerd and colleagues (Geeraerd et al., 2005b). Each of the replications was used to obtain model parameter estimates (n=6) to allow for statistical evaluation of the model parameters.

3.5.6 Moisture Loss Measurements

The moisture loss from BHI, TSB-glu, PPS, and MM with 0.5% and 5.0% NaCl was measured over time in order to quantitatively compare the media in relation to the media composition, initial salt content, placement in the desiccator (the front and the back of the chamber), and to determine the final moisture content in all the media. A volume of 2 ml of each medium was pipetted into an aluminum foil pan and quickly transferred into a mini desiccator. At specific time intervals (6 hours), three pans for each medium were taken from the desiccator and the weight of the pan with the increasingly drier medium was measured on a scale (Denver Instrument GmbH, Göttingen, Germany). The measurements were stopped when the desiccator reached a stable RH of 43% RH (at

15 °C) as recorded by the data logger (Tinytag, Gemini Data Loggers Ltd., Chichester, UK). The moisture content was calculated at each sampling time for each medium to generate a moisture loss curve. The density of each medium was also measured by using a density meter (DMA35, Anton Paar GmbH, Graz, Austria). The percentage of solid content was provided by media manufacturer or calculated based on the ingredients. The entire experiment was replicated twice for each medium. Therefore, six observations were obtained at each sampling point for each medium. The calculation of the moisture (M) content (%, wet basis (wb)) at each sampling time (t) is as follows:

Mt (%) = the moisture content at each sampling time = ((Wt - Wp - Ws) / (Wt - Wp)) × 100

Where,

V = medium volume (cm^3),

ρ = density of medium (g/cm^3),

Ws = solid content (g) = V × ρ × solid content (%).

Wt = the weight of the pan with gradually drier medium (g) at the time, t

Wp = the weight of the pan (g)

t = time (hours)

3.6 RNA Isolation

Total RNA was isolated from Lm568 and its Δ*sigB* mutant exposed to 0.5% (Low) and 5.0% (High) salt (1 hour at 15 °C) in order to analyse transcription of *gbuA*, *betL* and *opuCA*. The two strains were grown aerobically in MM at 15 °C for 3 days. Then, 250 µl of this culture was added to 5 ml of fresh MM for both strains and incubated at 15 °C for 24 hours, where according to the previously obtained growth data, the cell density of Lm568 and its Δ*sigB* mutant would have reached approximately 8 Log(CFU/ml). Cells were harvested by centrifugation at 2767 × *g* and 15 °C for 10 minutes, followed by addition of 2.5 ml of MM with 0.5 and 5.0% NaCl to the pellets. Following the ± salt stress treatment (1 hour at 15 °C), total RNA was extracted using the Ultraclean Microbial RNA isolation kit (MoBio Laboratories Inc.). To eliminate the contamination

with DNA, the extracted RNA was treated with 1 µl of DNase I (Ambion, Austin, TX, USA) for 1 hour at 37 °C. Following to the manufacturer's instructions, 2 units (1 µl) of DNase I was used to remove potentially up to 1 µg of DNA from RNA in a 25-100 µl reaction volume. The concentration and purity of extracted total RNA was determined using an Ultrospec™ 1100 *pro* UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, NJ, USA) to measure Abs_{260nm} and Abs_{260nm} / Abs_{280nm}. A ratio of Abs_{260nm} / Abs_{280nm} ranging from 1.9 to 2.1 indicates presence of pure RNA.

3.7 Reverse-Transcriptase PCR and Analysis of Gene Transcription

Extracted total RNA was immediately transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen Inc., Mississauga, ON) following the manufacturer's instructions. Based on the concentration, a variable amount of sample RNA and RNase-free water was mixed with 2 µl of gDNA wipeout buffer to a total volume of 14 µl. The mixture was incubated at 42 °C for 2 minutes to remove any contaminating DNA. The reverse transcription reaction consisted of 1 µl of Quantitech reverse transcriptase, 4 µl of RT buffer, 1 µl of random RT primers and 14 µl of RNA template mixture including 2 µl of gDNA wipeout buffer. The reaction was incubated for 15 minutes in a 42 °C water bath followed by 3 minutes at 95 °C to inactivate the reverse transcriptase. All cDNA was stored at -20 °C until further use.

For the transcription analysis, the following PCR reaction was used: 0.2 µl of ID Tag™ DNA polymerase (5 U/µl), 2.5 µl of 10X reaction buffer (with 20 mM $MgCl_2$), 1.0 µl of 10 mM dNTPs (2.5 mM of each), 1.0 µl of template, 19.3 µl of DEPC water (MoBio Laboratories Inc.), and 0.5 µl of each of the forward and reverse primer pair (10 mM). PCR kits were purchased from ID labs™ Inc. (London, ON, Canada). The oligonucleotide primers used in this study are shown in Table 6. Primers were designed according to guidelines found in the StrataClone PCR Cloning kit booklet by using Primer 3 software (available online: http://primer3.sourceforge.net/e), and checked using the NCBI Blast program to confirm the total gene specificity of the nucleotide sequences. Nucleotide sequences were synthesized by Sigma Genosys (Sigma-Genosys, Canada). PCR products were detected by electrophoresis on a 1% agarose gel (TAE buffer) (MoBio Laboratories Inc.) containing 2 µl of 10 mg/ml EB and visualized with UV

illumination. Duplicate experiments with independently prepared cultures were performed for each treatment.

Table 6. PCR primers used in RT-PCR

Primers	Sequence (5' to 3')	Target gene	Annealing temperature (°C)	Product size (bp)
sigB-F (internal)	AAAAACCTAGTAGAGTCCATCG	*sigB*	55	234
sigB-R (internal)	TCGGACCTAACTCTTTGATTC	*sigB*	55	234
sigB-F (whole)	ATGAAAGCAGGTGGAGGAG	*sigB*	58	1000
sigB-R (whole)	AAATTATTTGATTCAACTGCCT	*sigB*	58	1000
betL-F	AGTCCGATTGGCTCGATTCGAC	*betL*	60	238
betL-R	CCAAGCAGAAATTCCCCAGTGG	*betL*	60	238
gbuA-F	GCTTCCAAAGCATCTTCTTTAC	*gbuA*	55	232
gbuA-R	GACTGGATAGTTCTTTTCCGTC	*gbuA*	55	232
opuCA-F	GTTTGTTTTATCGGTCCAAGTG	*opuCA*	57	238
opuCA-R	CTCTTCGGACCATTTAAGTAA	*opuCA*	57	238
rRNA-F	GTGCCTAATACATGCAAGTCGA	16S*rRNA*	65	235
rRNA-R	TAGGCCATTACCCTACCAACTA	16S*rRNA*	65	235

*Primers sequences designed based on the sequenced *L. monocytogenes* EGD-e strain, and genebank accession number AL591824

3.8 High Performance Liquid Chromatography (HPLC) Determination of Osmolyte Contents in Media

HPLC was used to detect the presence and quantify the amount of glycine betaine, carnitine and proline present in BHI, TSB-glu and PPS. HPLC-grade water and acetonitrile (ACN) (ACROS, New Jersey, USA) were used as mobile phase solvents. A Reverse Phase (RP) HPLC method was used. Samples were chromatographed on a Perkin Elmer series 200 HPLC system equipped with an Agilent ZORBAX 300SB-C18 column (150 × 4.6 mm, I.D. 5 µm; Chromatographic Specialties Inc., Brockville, Ontario, Canada), a Perkin Elmer series 200 pump and a Perkin Elmer series 200 UV/VIS Detector (Norwalk, CT, USA). Before passing through the C18 column, mobile phase solvents were degassed and filtered through an organic filter (0.2 µm pore size, Whatman, Inc., NJ. USA). Osmolyte standard solutions and samples were also filtered through the 0.2 µm filters. The chromatographic conditions were as follows: column at room temperature (20-21 °C, flow rate 0.5 ml/min, an ion pairing agent Trifluoroacetic acid (TFA) (Sigma-Aldrich, Oakville, ON, Canada) was added to the water and ACN at a concentration of 0.1% (v/v), the sample injection volume was 10 µl, and the UV detector was set at 195 nm. The gradient profiles of the solvent system consisting of A (water containing 0.1% TFA) and B (ACN containing 0.1% TFA) were optimized as follows (Table 7):

Table 7. The optimized gradient profile of mobile phase solvent used in HPLC analysis of the osmolyte glycine betaine, carnitine and proline content.

Step	Time (min)	Flow rate (ml/min)	%A (water with 0.1% TFA)	%B (ACN with 0.1% TFA)
0	0.5	0.5	98.5	1.5
1	2	0.5	98.5	1.5
2	4	0.5	70	30
3	5	0.5	70	30
4	14	0.5	98.5	1.5

External standard solutions covering the desired concentration range were chromatographed and a standard curve was obtained by plotting the peak area versus concentration. Then, an identical volume of sample was chromatographed, and the peak area of a compound of interest was used to determine the compound concentration as calculated from the standard curve. Each of the osmolyte standard solutions was also added as internal standards to confirm the peak identity for the compound of interest in the samples. The internal standard solution was used to minimize errors due to sample preparation, apparatus, and operator technique (Nielsen, 2003). The HPLC analysis of samples was performed in triplicates.

3.9 Statistical Analyses

Counts of Lm568 and its Δ*sigB* mutant obtained in growth and desiccation survival experiments were log transformed (Log(CFU/ml)) and expressed as means ± standard deviation of the six samples from two independent trials with triplicate samples for each experimental treatment (n=6). As described in sections 3.4.2 and 3.5.4 above, the modified Gompertz and Weibull equations were fitted to each of the six observations to obtain estimates for the model parameters. To compare the effect of the experimental parameters (strains, growth media, growth temperature, desiccation media, osmoadaptation, initial desiccation salt level, pre-culture with or without osmolytes, and desiccation with or without osmolytes), an analysis of variance (ANOVA) was performed on the critical model parameters for growth and desiccation survival using the SYSTAT®11.0 software package (Systat Software Inc., San Jose, CA, USA). The Tukey post hoc test was used to compare the means of the model parameters at the 5% significance level. To ensure the suitability of the models, four indices (r^2, MSE_{model}, A_f and *F*-test) as described by den Besten et al. (2006) were calculated to statistically evaluate the model fit. These indices were calculated by fitting the modified Gompertz and Weibull models to the data from the two independent trials with triplicate experiments at once. For r^2, the values range from 0 to 1 and the closer the values are to the maximum value the better the fit of the model. For MSE_{model}, the smaller the value for a given model is, the better the fitness of model to describe the data is. For the accuracy

factor A_f, when the value is closer to 1, it indicates perfect agreement between observed and fitted data (Vogel et al., 2010). The *F*-test is used to decide whether or not the fitting performance of the models was statistically acceptable. When appropriate, one-way ANOVA with the Tukey post hoc test, (5% significance level), was also performed to compare the Log transformed counts of the two strains obtained at individual time points during the different treatments in the growth and desiccation survival experiments.

CHAPTER 4. RESULTS

4.1 Confirmation of the Deletion of the *sigB* Gene in the Mutant by DNA Sequencing

Sequencing of the *sigB* gene in *L. monocytogenes* 568 with M13-F and M13-R primers yielded 831 base pair (bp) and 808 bp sequences from each end, respectively, to cover the entire gene (780 bp). In the *L. monocytogenes* 568 Δ*sigB* mutant, sequencing of the *sigB* gene with M13-F and M13-R primers generated sequences of 471 bp and 476 bp, respectively. Those four sequences were analyzed using the BLAST program maintained at the NCBI web site (http://blast.ncbi.nlm.nih.gov/Blast). The *sigB* sequence in *L. monocytogenes* 568 showed significant homology (identities, 99%; E value, 0.0) with the *lmo0895* gene in *L. monocytogenes* strain EGD-e (GeneBank ID NC_003210), which encodes the RNA polymerase sigma factor SigB (Glaser et al., 2001). The successful deletion of the *sigB* gene in the mutant was confirmed by DNA sequencing, which showed 100% identity with a 99 bp and 124 bp sequence in the front and end part, respectively, of the *sigB* gene sequence in *L. monocytogenes* strain EGD-e. The deleted part in the *L. monocytogenes* 568 Δ*sigB* mutant was found between nucleotides 99 to 657 leading to the removal of the middle part of the *sigB* gene to yield a shortened gene fragment of 223 bp. The details of the DNA sequencing results and gene identities for the *sigB* gene in *L. monocytogenes* 568 and its Δ*sigB* mutant are given in Table 8. The photograph of the electrophoresis gel containing PCR products from DNA sequencing can be found in the appendix (Figure 16).

Table 8. DNA sequencing results for the *sigB* gene in *L. monocytogenes* 568 and its isogenic *L. monocytogenes* Δ*sigB* mutant

Gene	Primer	Length of sequencing product (bp)	Similar gene in Genebank Entry for *L. monocytogenes* strain EGD-e (GeneBank ID NC_003210)	Query range coverage (Query start to end position)	Score	E value	Identities
sigB	M13-F	831	*sigB lmo0895* Query (930671-931450) - RNA polymerase sigma factor SigB	930671-931445	1413 bits (765)	0.0	99%
sigB	M13-R	808	*sigB lmo0895* Query (930671-931450) - RNA polymerase sigma factor SigB	930700-931450	1369 bits (741)	0.0	99%
Δ*sigB*	M13-F	471	*sigB lmo0895* Query (930671-931450) - RNA polymerase sigma factor SigB	930671-930769	183 bits (99)	4e-50	100%
Δ*sigB*	M13-F	471	*sigB lmo0895* Query (930671-931450) - RNA polymerase sigma factor SigB	931328-931450	228 bits (124)	2e-63	100%
Δ*sigB*	M13-R	476	*sigB lmo0895* Query (930671-931450) - RNA polymerase sigma factor SigB	931328-931450	228 bits (124)	2e-63	100%
Δ*sigB*	M13-R	476	*sigB lmo0895* Query (930671-931450) - RNA polymerase sigma factor SigB	930671-930769	183 bits (99)	4e-50	100%

4.2 Phenotypic Comparison of the Wild Type Lm568 and Its Δ*sigB* Mutant

4.2.1 Gram Staining

Microscopy of Gram stained Lm568 Δ*sigB* mutant cells showed these to morphologically be identical to the wild type Lm568 cells (Figure 4). Cells from both strains could be described as being dark purple and rod-shaped, as expected for Gram-positive *Listeria* spp.

Lm568 Δ*sigB*

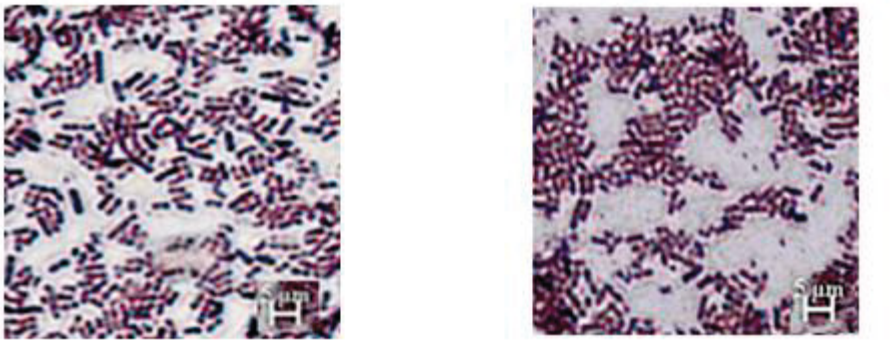


Figure 4. Gram stained *L. monocytogenes* 568 and its Δ*sigB* mutant cells were observed using a Nikon Eclipse 50i light microscope under a 100 x oil immersion objective. Scale bars, 5.0 μm.

4.2.2 KOH Gram Reaction and Catalase Test

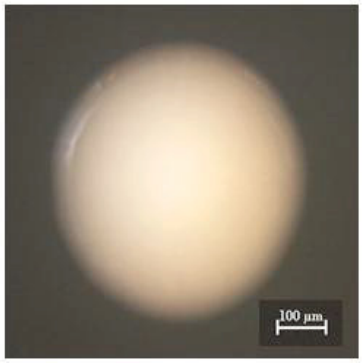
Other biochemical tests were also performed showing that the Δ*sigB* mutant was not different from the wild type Lm568 in terms of observed physiological properties. For the KOH Gram reaction, both strains remained "watery" on glass slides, as expected for Gram-positive bacteria. For catalase test, both strains produced gas bubbles on glass slides, as is expected for catalase positive bacteria.

4.2.3 Colony and Cell Morphology Assay

In order to study the influence of sodium chloride on colony and cell morphology of Lm568 and its Δ*sigB* mutant in solid and liquid media, growth of both strains was observed on BHIA with 0.5 and 5.0% NaCl and in BHI with 0.5 and 5.0% NaCl at 15 °C for 72 hours. None of the strains formed visble colonies on BHIA with 10 and 20% NaCl after incubation at 15 °C for 4 and 5 days, respectively. For both strains, the colonies on BHIA ± 5.0% NaCl were found to be white, hemispheric, and smooth-surfaced with regular outlines, however, the size of the colonies tended to be smaller on BHIA with 5.0% NaCl than on BHIA with 0.5% NaCl (Figure 5). Since the incubation conditions were the same (15 °C for 72 hours), the presence of 5.0% NaCl in BHIA delayed the growth of the colonies as indicated in results from the growth experiment (Table 9), where cells in 5.0% NaCl medium exhibited slower growth rates ($p<0.05$) than in BHI with 0.5% NaCl. The change in NaCl concentration affected cellular morphological characteristics similarly for both strains. Increasing the level of NaCl to 5.0% resulted in the formation of chains consisting of 3 to 5 cells, fewer single rods, and an increase in cell aggregation for both strains (Figure 6). The size of individual Lm568 and Δ*sigB* mutant cells (average length of 2.23 $\pm$ 0.06 μm) grown in 5.0% NaCl was not different from the average length of single cells (2.24 $\pm$ 0.07 μm) found in BHI with 0.5% NaCl.

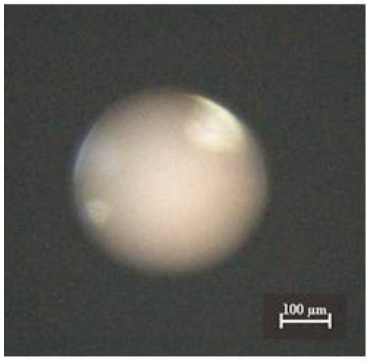
BHIA with 0.5% NaCl

Lm568 (a)

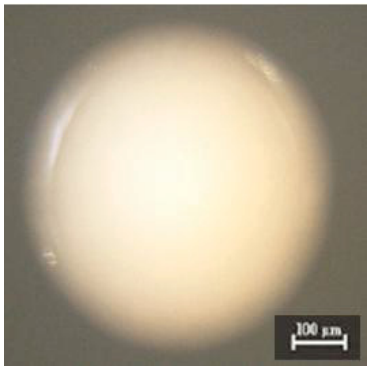


BHIA with 5.0% NaCl

Lm568 (b)



Δ*sigB* (a)



Δ*sigB* (b)

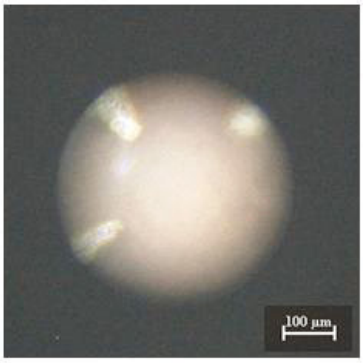


Figure 5. Microscopic images of colonies of *L. monocytogenes* 568 and its Δ*sigB* mutant on plates of BHIA with 0.5 (a) and 5.0% (b) NaCl after 72 hours at 15 °C were obtained using a Nikon Eclipse 50i light microscope equipped with a 4 x objective. Scale bars, 100 µm.

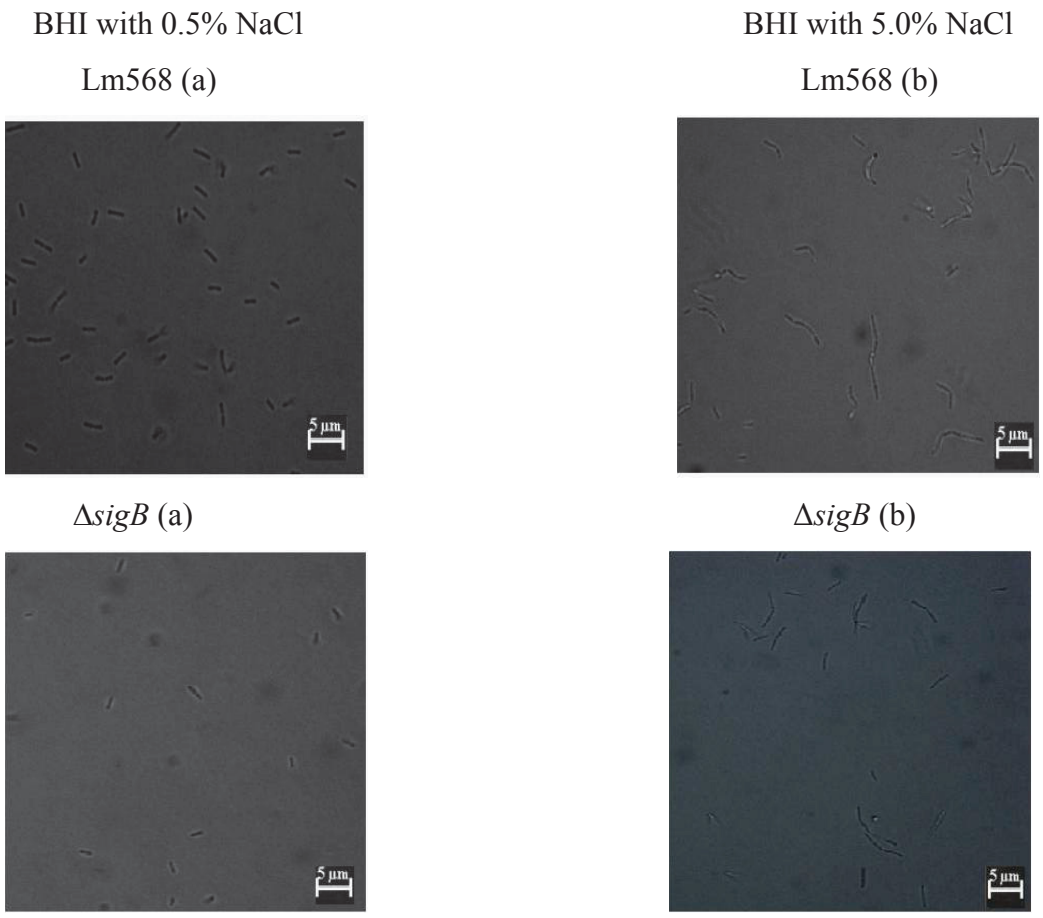


Figure 6. Phase contrast microscopic images of *L. monocytogenes* 568 and its Δ*sigB* mutant cells grown in BHI with 0.5 (a) and 5.0% (b) NaCl at 15 °C for 72 hours were obtained using a Nikon Eclipse 50i light microscope equipped with a 100 x oil immersion objective. Scale bars, 5.0 µm.

4.3 Comparative Study of the Growth Kinetics of the Wild Type Lm568 and Its Δ*sigB* Mutant

The growth kinetics of Lm568 and its Δ*sigB* mutant was studied to compare the growth of the strains in different growth media (BHI, TSB-glu, PPS and MM) with low and high salt levels at 15 °C over 96 hours (see section 3.4 in the M & M). In the second series of experiments, an MM containing five essential amino acids (Premaratne et al., 1991) was used to determine the influence of exogenous osmoprotectants on the growth of Lm568 and its Δ*sigB* mutant. Specifically the growth of both strains was measured in MM ± 5.0% NaCl and 1 mM of the osmolytes; glycine betaine, carnitine, or proline.

All the growth curves were modelled using the modified Gompertz equation and the three calculated model fitness indices (MSE_{model}, R^2, A_f) (Tables 9 and 10) indicated that the model was a good fit for the observed growth curves obtained for both strains. Also, the *f* values (Tables 9 and 10), which were consistently lower than 1.48, indicated that the fit of the modified Gompertz equation to the observed data is suitable ($p<0.05$).

Results presented in Table 9 show that Lm568 and its Δ*sigB* mutant were able to grow in BHI, TSB-glu and PPS as well as in MM supplemented with 0.5% and 5.0% NaCl when incubated at 15 °C for 96 hours. The fact that both strains grew in MM indicated that the essential growth factors required for the growth of both strains at low and high osmotic strengths were present in MM. The average initial populations for both strains in all treatments were 5.0 Log_{10} CFU/ml as also reflected in the model's estimate for the initial population (A parameter, Table 9).

Pairwise comparison of the model parameters in Table 9 shows that there were no significant differences ($p>0.05$) between the growth kinetics exhibited by wild type and mutant strains in any of the tested media (BHI, TSB-glu, PPS and MM ± 5.0% NaCl).

However, as expected, the salt level significantly affected ($p<0.05$) the B, D, and M parameters for both strains in BHI, TSB-glu, PPS, and MM. For each strain in each growth medium, the value of the B parameter which represents the growth rate, was significantly lower ($p<0.05$) in the presence of 5.0% NaCl than in 0.5% NaCl. The same significant ($p<0.05$) observation could be made for the value of the D parameter (maximum minus initial populations in Log_{10} CFU/ml), as the size of the maximum

population decreased with the increase in NaCl concentration in all growth substrates. Increasing NaCl concentrations also significantly ($p<0.05$) delayed the on-set of exponential growth, as indicated by the M parameter value estimates of the time when the exponential growth rate is maximal.

The nutritionally richer growth media BHI and TSB-glu tended to result in faster growth rates (B), higher maximum populations (D) and earlier onset of the exponential phase (M) than the less nutritional growth media; PPS and MM. For both strains, the values of the B parameter in BHI and TSB-glu were regardless of the NaCl concentrations significantly higher ($p<0.05$) than in PPS and MM. In low NaCl concentration media, there were no significant differences between the B parameters for BHI and TSB-glu or PPS and MM. However, the growth rate (B parameter) was significantly higher ($p<0.05$) in PPS with 5.0% NaCl compared to in MM with 5.0% NaCl. The value of the D parameter was highest ($p<0.05$) in BHI with 0.5% and lowest ($p<0.05$) in PPS with 5.0% NaCl for both strains. The largest final populations of both strains in the stationary phase were observed in BHI with 0.5% and 5.0% NaCl. The time to the maximum exponential growth (M parameter) was found to be significantly ($p<0.05$) shorter in BHI and TSB-glu compared to in MM with 0.5% and 5.0% NaCl for both strains, indicating MM as a minimal nutrient medium delayed the onset of exponential growth of both strains. No significant ($p>0.05$) differences were observed for the M parameters obtained during growth of both strains in BHI and TSB-glu with 0.5% and 5.0% NaCl, respectively.

Lm 568 in MM with 5.0% NaCl in the presence of 1 mM of glycine betaine or carnitine exhibited faster growth rates (B), higher maximum populations (D) and earlier onset of exponential phase (M) than the Δ*sigB* mutant (Table 10 and Figure 7 for glycine betaine). The average initial populations for both strains in all treatments were 5.0 Log_{10} CFU/ml as also reflected in the model's estimate for the initial population (A parameter, Table 10).

The salt level significantly affected ($p<0.05$) the B, D, and M parameters for both strains in MM in the presence of osmolytes. For each strain in each growth media, the values of the growth rate (B) and difference between maximum and initial populations (D) were significantly higher ($p<0.05$) in the presence of 0.5% NaCl than in 5.0% NaCl.

Similarly, an increase ($p<0.05$) for the M parameter was observed when the NaCl concentration increased, indicating increasing NaCl concentrations significantly ($p<0.05$) delayed the onset of exponential growth.

The addition of osmolytes except proline significantly enhanced ($p<0.05$) the growth of both strains in MM regardless of the salt levels, resulting in the faster growth rates (B), the higher maximum populations (D), and earlier onset of exponential phase (M) than in the absence of osmolytes in MM. For both strains, the addition of 1 mM proline had no significant effect ($p>0.05$) for the B, D, and M parameters in MM ± 5.0% NaCl.

Glycine betaine was the more effective osmolyte ($p<0.05$) compared to carnitine in terms of improving the growth of the Δ*sigB* mutant in MM with 5.0% NaCl, resulting in the faster growth rates (B), the higher maximum populations (D), and earlier onset of exponential phase (M). However, glycine betaine and carnitine worked equally well ($p>0.05$) to improve growth of Lm568 in MM ± 5.0% NaCl and the Δ*sigB* mutant in MM with 0.5% NaCl, as indicated by the B, D, and M parameters.

The individual growth curves of Lm568 and its Δ*sigB* mutant in MM ± 5.0% NaCl, and MM ± 5.0% NaCl and glycine betaine are shown in Figures 7 and 8, respectively. The individual growth curves of Lm568 and its Δ*sigB* mutant in BHI ± 5.0% NaCl, TSB-glu ± 5.0% NaCl, PPS ± 5.0% NaCl, MM ± 5.0% NaCl and carnitine, and MM ± 5.0% NaCl and proline are shown in Figures 17, 18, 19, 20, and 21 in the appendix, respectively.

Table 9. Modelling the growth of *L. monocytogenes* 568 and its Δ*sigB* mutant using the modified Gompertz equation: The growth kinetics were determined in BHI with 0.5% and 5.0% NaCl, TSB-glu with 0.5% and 5.0% NaCl, PPS with 0.85% and 5.0% NaCl, and MM with 0.5% and 5.0% NaCl during incubation at 15 °C for 96 hours.

Growth media	Salt level (%)	Strain	Parameter estimates				Statistical indices of fit of model			
			A (Log_{10} CFU/ml)	D (Log_{10} CFU/ml)	B (Log_{10} CFU/ml x time)	M (hours)	MSE_{model}	R^2	A_f	f^*
BHI	0.5	Lm568	5.01[a**]±0.02***	4.47[a]±0.02	0.08[a]±0.00	17.83[a]±0.26	0.030	0.988	1.00	1.05
		Δ*sigB* mutant	5.01[a]±0.02	4.47[a]±0.02	0.08[a]±0.00	17.73[a]±0.38	0.031	0.988	1.00	1.06
	5.0	Lm568	4.98[a]±0.02	4.33[b]±0.01	0.07[b]±0.00	20.13[b]±0.11	0.026	0.989	1.00	1.02
		Δ*sigB* mutant	4.98[a]±0.01	4.33[b]±0.01	0.07[b]±0.00	20.18[b]±0.02	0.026	0.989	1.00	1.03
TSB-glu	0.5	Lm568	5.01[a]±0.02	4.32[b]±0.02	0.08[a]±0.00	17.52[a]±0.41	0.035	0.985	1.00	1.01
		Δ*sigB* mutant	4.99[a]±0.04	4.34[b]±0.03	0.08[a]±0.00	17.48[a]±0.14	0.035	0.986	1.00	1.06
	5.0	Lm568	4.99[a]±0.00	4.23[c]±0.01	0.07[b]±0.00	20.15[b]±0.04	0.026	0.989	1.00	1.05
		Δ*sigB* mutant	4.99[a]±0.00	4.23[c]±0.00	0.07[b]±0.00	20.16[b]±0.01	0.026	0.990	1.00	1.04
PPS	0.85	Lm568	5.00[a]±0.04	2.43[d]±0.03	0.06[c]±0.00	20.48[c]±0.42	0.013	0.984	1.00	1.12
		Δ*sigB* mutant	4.95[a]±0.02	2.42[d]±0.05	0.06[c]±0.00	20.69[c]±0.09	0.012	0.984	1.00	1.11
	5.0	Lm568	4.99[a]±0.00	1.62[e]±0.00	0.05[d]±0.00	31.85[d]±0.21	0.011	0.995	1.00	1.02
		Δ*sigB* mutant	4.99[a]±0.01	1.64[e]±0.02	0.05[d]±0.00	31.69[d]±0.43	0.011	0.998	1.00	1.04
MM	0.5	Lm568	4.97[a]±0.02	3.39[f]±0.02	0.06[c]±0.00	22.09[e]±0.10	0.007	0.996	1.00	1.17
		Δ*sigB* mutant	4.99[a]±0.00	3.39[f]±0.02	0.06[c]±0.00	22.74[e]±0.51	0.007	0.995	1.00	1.11
	5.0	Lm568	5.00[a]±0.03	2.57[g]±0.02	0.02[e]±0.00	57.86[f]±2.36	0.007	0.991	1.00	1.12
		Δ*sigB* mutant	4.97[a]±0.02	2.58[g]±0.04	0.02[e]±0.00	59.58[f]±3.66	0.013	0.984	1.00	1.13

* *f* value lower than1.48 indicate that the fit of model is suitable ($p<0.05$).

**values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

*** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 10. Modelling the growth of *L. monocytogenes* 568 and its Δ*sigB* mutant using the modified Gompertz equation: The growth kinetics were determined in MM with 0.5% and 5.0% NaCl, MM with 0.5% and 5.0% NaCl with glycine betaine (B, 1 mM), MM with 0.5% and 5.0% NaCl with carnitine (C, 1 mM) and MM with 0.5% and 5.0% NaCl with proline (P, 1 mM) during incubation at 15 °C for 96 hours.

Growth media	Salt level (%)	Strain	Parameter estimates				Statistical indices of fit of model			
			A (Log_{10} CFU/ml)	D (Log_{10} CFU/ml)	B (Log_{10} CFU/ml x time)	M (hours)	MSE_{model}	R^2	A_f	f^*
MM	0.5	Lm568	$4.97^{a**}\pm0.02^{***}$	$3.39^{a}\pm0.02$	$0.06^{a}\pm0.00$	$22.09^{a}\pm0.10$	0.007	0.996	1.00	1.17
		Δ*sigB* mutant	$4.99^{a}\pm0.01$	$3.39^{a}\pm0.02$	$0.06^{a}\pm0.00$	$22.74^{a}\pm0.51$	0.007	0.995	1.00	1.11
	5.0	Lm568	$5.00^{a}\pm0.03$	$2.57^{b}\pm0.02$	$0.02^{b}\pm0.00$	$57.86^{b}\pm2.36$	0.007	0.991	1.00	1.12
		Δ*sigB* mutant	$4.97^{a}\pm0.02$	$2.58^{b}\pm0.04$	$0.02^{b}\pm0.00$	$59.58^{b}\pm3.66$	0.013	0.984	1.00	1.13
MM (B)	0.5	Lm568	$4.99^{a}\pm0.02$	$4.52^{c}\pm0.02$	$0.07^{c}\pm0.00$	$17.71^{c}\pm0.14$	0.009	0.996	1.00	1.06
		Δ*sigB* mutant	$4.98^{a}\pm0.03$	$4.53^{c}\pm0.04$	$0.07^{c}\pm0.00$	$17.68^{c}\pm0.17$	0.007	0.997	1.00	1.06
	5.0	Lm568	$5.01^{a}\pm0.03$	$3.85^{d}\pm0.02$	$0.05^{d}\pm0.00$	$27.27^{d}\pm0.08$	0.004	0.998	1.00	1.04
		Δ*sigB* mutant	$5.00^{a}\pm0.02$	$3.27^{e}\pm0.04$	$0.04^{e}\pm0.00$	$35.15^{e}\pm0.04$	0.017	0.994	1.00	1.04
MM (C)	0.5	Lm568	$4.99^{a}\pm0.01$	$4.50^{c}\pm0.01$	$0.07^{c}\pm0.00$	$17.69^{c}\pm0.06$	0.013	0.991	1.00	1.02
		Δ*sigB* mutant	$4.96^{a}\pm0.03$	$4.50^{c}\pm0.00$	$0.07^{c}\pm0.00$	$17.59^{c}\pm0.34$	0.022	0.987	1.00	1.03
	5.0	Lm568	$4.99^{a}\pm0.01$	$3.84^{d}\pm0.02$	$0.05^{d}\pm0.00$	$27.31^{d}\pm0.12$	0.006	0.996	1.00	1.11
		Δ*sigB* mutant	$4.95^{a}\pm0.06$	$3.01^{f}\pm0.04$	$0.03^{f}\pm0.00$	$40.43^{f}\pm0.02$	0.013	0.989	1.00	1.05
MM (P)	0.5	Lm568	$4.99^{a}\pm0.01$	$3.39^{a}\pm0.00$	$0.06^{a}\pm0.00$	$22.17^{a}\pm0.02$	0.024	0.985	1.00	1.05
		Δ*sigB* mutant	$4.99^{a}\pm0.01$	$3.39^{a}\pm0.00$	$0.06^{a}\pm0.00$	$22.16^{a}\pm0.01$	0.004	0.988	1.00	1.05
	5.0	Lm568	$5.00^{a}\pm0.02$	$2.53^{b}\pm0.04$	$0.02^{b}\pm0.00$	$57.51^{b}\pm0.15$	0.026	0.988	1.00	1.16
		Δ*sigB* mutant	$5.00^{a}\pm0.02$	$2.55^{b}\pm0.01$	$0.02^{b}\pm0.00$	$59.39^{b}\pm3.47$	0.015	0.981	1.00	1.11

* *f* value lower than1.48 indicate that the fit of model is suitable ($p<0.05$).

**values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

*** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

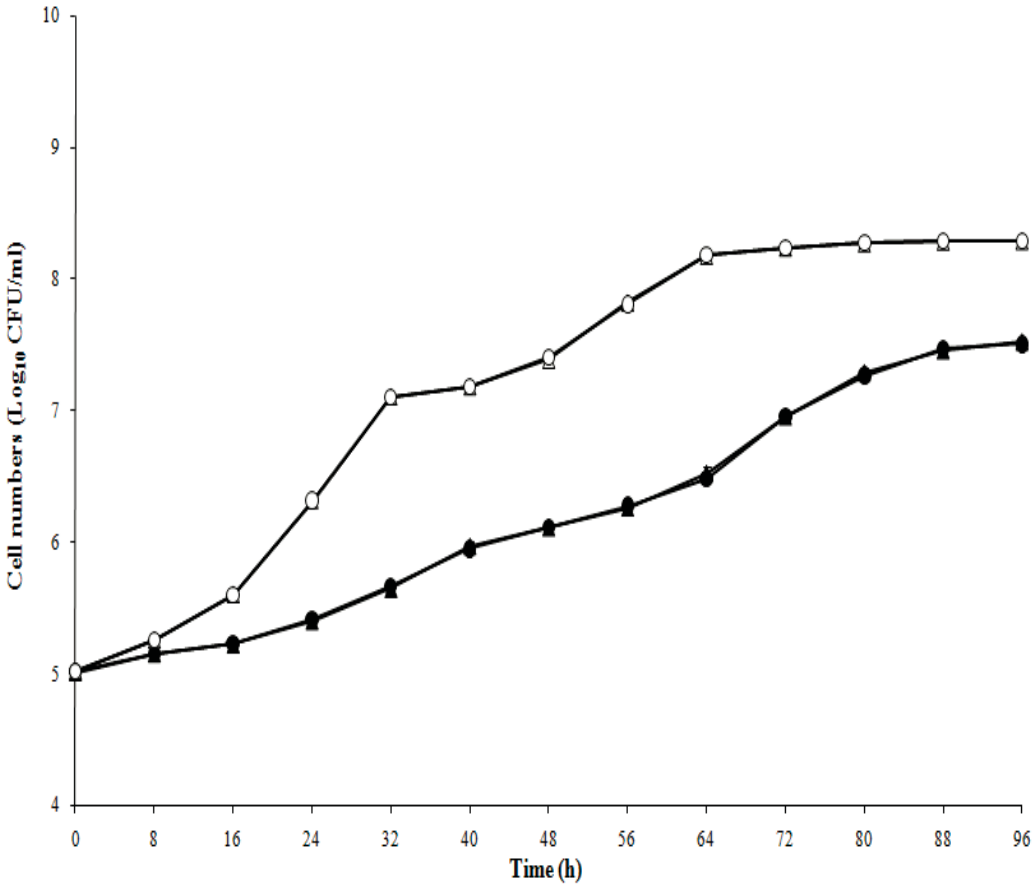


Figure 7. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM with 0.5% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

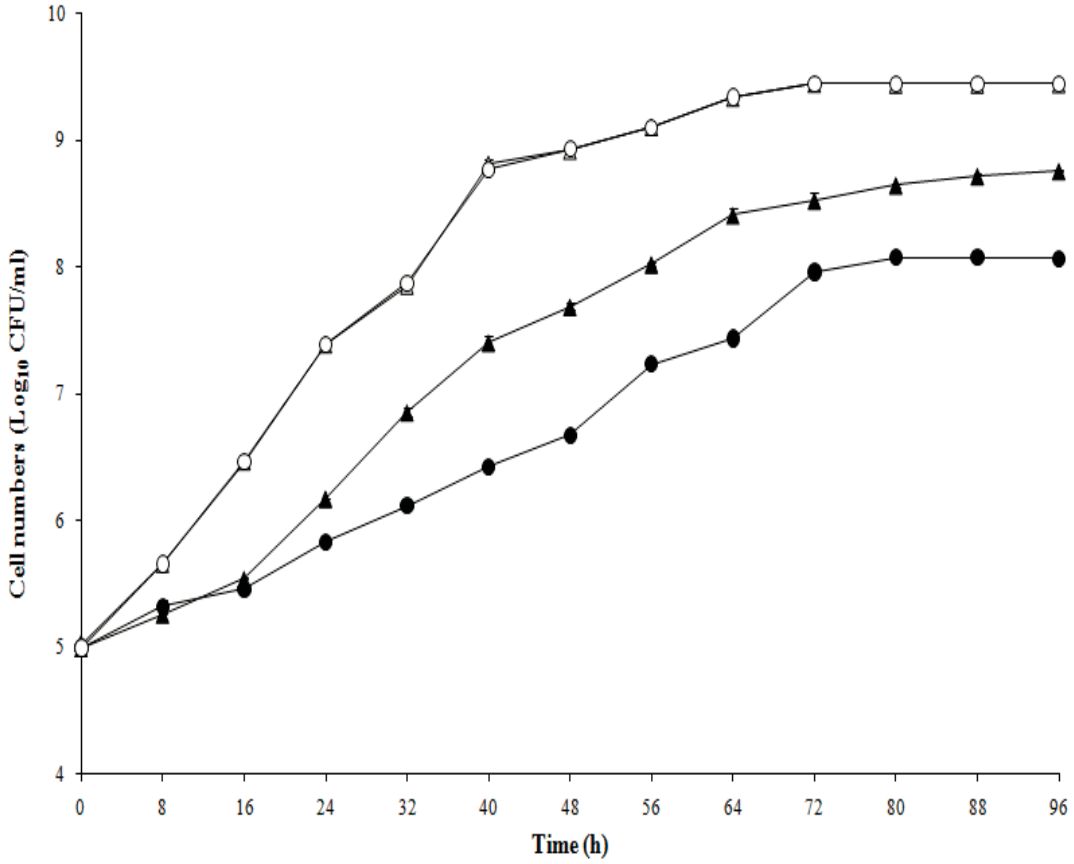


Figure 8. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM in the presence of glycine betaine (1 mM) with 0.5% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

4.4 Comparative Study of the Desiccation Inactivation of the Wild Type Lm568 and Its Δ*sigB* Mutant

4.4.1 Influence of Different Positions in a Mini Desiccation Chamber on the Desiccation Inactivation of the Wild Type Lm568 and Its Δ*sigB* Mutant

For both treatments prior to desiccation, the initial populations of both strains were approximately 10^8 CFU/cm^2. The four calculated indices (MSE_{model}, R^2, A_f, and f) indicated the Weibull model (Mafart et al., 2001) fitted the non-linear inactivation kinetics well for both strains (Table 11). For example, the f values were consistently lower than 1.61, indicating the fit of the Weibull model to all data is statistically suitable ($p<0.05$). The inactivation curves for both strains desiccated in the four different positions are shown in Figure 20 in the appendix.

The results (Table 11) showed the position in the desiccation chamber had no significant ($p>0.05$) effect on survival of both strains, as indicated by the lack of differences in the delta (time to the first log reduction) and p (shape parameter) parameters estimated using the Weibull model (Mafart et al., 2001), as well as in the total reductions in cell numbers after desiccation for 21 days at 43% RH. For both strains, the numbers of survivors in the four different positions reached final levels of 10^5 CFU/cm^2 after 21 days (Figure 22). Only the p parameter was significantly different ($p<0.05$) between the desiccation curves of Lm568 and its Δ*sigB* mutant (Table 11).

Similarly when the *Listeria* strains were desiccated in TSB-glu with 5.0% NaCl, the position appeared to have no significant effect ($p>0.05$) on the delta, p parameters and total reductions in cell numbers after desiccation for 21 days (Table 12). At all positions, the two Weibull model parameters (delta and p) indicated that Lm568 and its Δ*sigB* mutant behaved similarly ($p>0.05$) under the desiccation challenge (15 °C and 43% RH for 21 days). Also, the numbers of survivors for both strains only decreased slightly to reach 10^7 CFU/cm^2 after 21 days.

In summary, these results (Tables 11 and 12) showed that inactivation of both strains was not influenced significantly ($p>0.05$) in relation to one of the four different positions (Figure 3, Left front, Right front, Left back, and Right back) in the mini

desiccator. In the following desiccation experiments, each strain was still placed at a fixed position in the desiccator in order to be able to accurately investigate whether differences in the desiccation inactivatin of Lm568 and its Δ*sigB* mutant was caused by the experimental factors (± *sigB*, desiccation substrates, ± 5.0% NaCl, ± osmolytes during pre-culture or desiccation).

Table 11. Impact of position in the desiccation chamber on desiccation inactivation kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant: Cells were cultured in TSB-glu with 0.5% NaCl at 15 °C for 3 days, and then desiccated (15 °C and 43% RH) in TSB-glu with 0.5% NaCl for 21 days. Petri dishes containing the inoculated stainless steel coupons were placed in four different positions in a mini desiccator.

Strains	Position	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
			Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f^*
Lm568	**Left front**	**2.78^{a**} ±0.03*****	**0.45^{a} ±0.03******	**0.287^{a} ±0.005**	**0.015**	**0.981**	**1.00**	**1.04**
Lm568	**Right front**	**2.78^{a} ±0.03**	**0.45^{a} ±0.03**	**0.290^{a} ±0.002**	**0.016**	**0.989**	**1.00**	**0.94**
Lm568	**Left back**	**2.78^{a} ±0.03**	**0.44^{a} ±0.05**	**0.287^{a} ±0.007**	**0.012**	**0.985**	**1.00**	**0.96**
Lm568	**Right back**	**2.78^{a} ±0.04**	**0.44^{a} ±0.04**	**0.286^{a} ±0.006**	**0.010**	**0.980**	**1.00**	**1.06**
Δ*sigB* mutant	**Left front**	**2.78^{a} ±0.04**	**0.42^{a} ±0.03**	**0.277^{b} ±0.007**	**0.009**	**0.988**	**1.00**	**0.88**
Δ*sigB* mutant	**Right front**	**2.78^{a} ±0.04**	**0.42^{a} ±0.03**	**0.275^{b} ±0.004**	**0.010**	**0.990**	**1.00**	**1.03**
Δ*sigB* mutant	**Left back**	**2.78^{a} ±0.03**	**0.43^{a} ±0.02**	**0.275^{b} ±0.004**	**0.006**	**0.992**	**1.00**	**0.99**
Δ*sigB* mutant	**Right back**	**2.78^{a} ±0.04**	**0.43^{a} ±0.02**	**0.279^{b} ±0.001**	**0.009**	**0.989**	**1.00**	**1.10**

*f value lower than1.61indicate that the fit of model is suitable ($p<0.05$).

**values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 12. Impact of position in the desiccation chamber on desiccation inactivation kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant: Cells were cultured in TSB-glu with 5.0% NaCl at 15 °C for 3 days, and then desiccated (15 °C and 43% RH) in TSB-glu with 5.0% NaCl for 21 days. Petri dishes containing the inoculated stainless steel coupons were placed in four different positions in a mini desiccator.

Strains	Position	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
			Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f^*
Lm568	**Left front**	**1.14^{a**}±0.04*****	**15.93^{a}±0.14******	**0.503^{a}±0.010**	**0.001**	**0.998**	**1.00**	**1.11**
Lm568	**Right front**	**1.14^{a}±0.04**	**15.94^{a}±0.14**	**0.503^{a}±0.009**	**0.002**	**0.994**	**1.00**	**1.19**
Lm568	**Left back**	**1.14^{a}±0.03**	**15.74^{a}±0.20**	**0.505^{a}±0.006**	**0.001**	**0.999**	**1.00**	**1.26**
Lm568	**Right back**	**1.15^{a}±0.04**	**15.74^{a}±0.20**	**0.505^{a}±0.006**	**0.001**	**0.999**	**1.00**	**1.26**
Δ*sigB* mutant	**Left front**	**1.14^{a}±0.04**	**15.83^{a}±0.19**	**0.496^{a}±0.009**	**0.001**	**0.999**	**1.00**	**1.05**
Δ*sigB* mutant	**Right front**	**1.14^{a}±0.05**	**15.83^{a}±0.19**	**0.496^{a}±0.009**	**0.001**	**0.998**	**1.00**	**1.04**
Δ*sigB* mutant	**Left back**	**1.14^{a}±0.03**	**15.81^{a}±0.03**	**0.498^{a}±0.014**	**0.002**	**0.998**	**1.00**	**1.24**
Δ*sigB* mutant	**Right back**	**1.14^{a}±0.03**	**15.81^{a}±0.03**	**0.498^{a}±0.014**	**0.002**	**0.998**	**1.00**	**1.24**

*f value lower than1.61indicate that the fit of model is suitable ($p<0.05$).

**values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

4.4.2 Effect of Different Desiccation Substrates on the Survival of Lm568 and Its Δ*sigB* Mutant During Desiccation on Stainless Steel Coupons at 15 °C and 43% Relative Humidity

To simulate the food production environments with both relatively low and high nutrient contents and with low and high salt levels, the desiccation survival of Lm568 and its Δ*sigB* mutant was determined on SS surfaces with cells initially suspended in PPS, TSB-glu, and BHI with low (0.5%) and high (5.0%) NaCl concentrations and incubated at 15 °C and 43% RH in the desiccation chamber for 21 days. Prior to desiccation, for all treatments the initial populations of both strains on the SS coupons were approximately 10^8 CFU/cm^2. The resulting non-linear inactivation kinetics were suitably modelled using the Weibull model, as indicated by the four statistical indices (MSE_{model}, R^2, A_f, and f) (Table 13). The low values of MSE_{model}, and the values of R^2 and accuracy factor (A_f) being close to 1 indicated the fitness of the model to describe the data. Moreover, the fact that the calculated values of f were lower than 1.61 indicate the fit of the model is statistically suitable ($p<0.05$). The desiccation survivor curves for Lm568 in BHI, TSB-glu, and PPS with low and high NaCl levels at 15 °C and 43% RH are shown in Figure 9. And the desiccation survivor curves for its Δ*sigB* mutant in BHI, TSB-glu, and PPS with low and high NaCl levels at 15 °C and 43% RH are shown in Figure 23 in the appendix.

The modelling results (Table 13) showed that the non-osmoadapted cells of the Δ*sigB* mutant in PPS followed by desiccation in PPS with low salt was significantly ($p<0.05$) more sensitive to the desiccation stress compared with Lm568, as indicated by the difference in the delta parameters (time to the first log reduction). However, eventually the survivors for both strains reached similar ($p>0.05$) levels with initial levels being reduced by 2.9 Log (CFU/cm^2) after 21 days (Table 13). Beside this treatment, the desiccation survival kinetics of Lm568 and its Δ*sigB* mutant were not different ($p>0.05$) from each other in any of the other treatments during the test period (Table 13).

The cells of both strains were most desiccation resistant ($p<0.05$) in BHI indicating that a nutritionally richer desiccation substrate in all treatments scenarios resulted in the longest time ($p<0.05$) to obtain the first log reduction (delta) and the highest survivor levels ($p<0.05$) after 21 days (Table 13). In contrast, the cells of both strains suspended in PPS, which is not a nutrient-rich substrate, were the least resistant ($p<0.05$) to the desiccation shock with the shortest times

($p<0.05$) to obtain the first log reduction (delta) and the lowest survivor levels ($p<0.05$) after 21 days (Table 13). It is important to point out that even in PPS, the residual populations of both strains stabilised at levels of 10^5 - 10^7 CFU/cm^2 depending on the different treatments after 14 to 21 days, indicating the survival and persistence of Lm568 subjected to desiccation conditions could increase the risk for cross-contamination during food processing.

Osmoadaptation prior to desiccation significantly improved ($p<0.05$) the survival of both strains in TSB-glu and PPS, respectively (Table 13), as indicated by the larger ($p<0.05$) values of the delta parameters and the lower ($p<0.05$) reduction levels in survivors after 21 days for osmoadaptated cells compared with non-osmoadaptated cells during desiccation regardless of the initial salt levels (Table 13).

The protective effect of the presence of high initial salt levels during the desiccation period was observed in TSB-glu and PPS for both strains (Table 13). It can be seen clearly that irrespectively of osmoadaptation, the reduction levels were significantly lower ($p<0.05$) for cells desiccated with high initial salt levels than for cells desiccated with low initial salt levels. Also, the values of the delta parameters were significantly ($p<0.05$) greater for cells desiccated with high initial salt levels compared to cells desiccated in low initial salt levels (Table 13). Among the four treatments in TSB-glu and PPS, it was desiccation with high initial salt levels of osmoadapted cells from both strains which achieved significantly better ($p<0.05$) survival compared with the other three treatments, as indicated by the largest ($p<0.05$) values of the delta parameters and the smallest ($p<0.05$) reduction levels after 21 days (Table 13). Moreover, in TSB-glu and PPS substrates, the survivors of both strains in each treatment reached stable population levels after 14 to 21 days (Figures 9 and 23).

Surprisingly, osmoadaptation during pre-culture and desiccation with the high initial salt levels had no significant protective effect ($p>0.05$) on the desiccation survival of both strains in BHI, as indicated by the modelling results (delta) (Table 13). And in this desiccation substrate, the reduction patterns of both strains in all treatments behaved similarly with delta values of ~31 days leading to final reductions in survivors of only 0.85 Log (CFU/cm^2) after 21 days (Table 13).

Table 13. Modelling of desiccation inactivation kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant in complex media: Cells were pre-cultured in BHI, TSB-glu, and PPS with 0.5% (Low) or 5.0% (High) NaCl level at 15 °C for 3 days, and then desiccated (15 °C and 43% RH) in each media with low or high NaCl level for 21 days on stainless steel coupons.

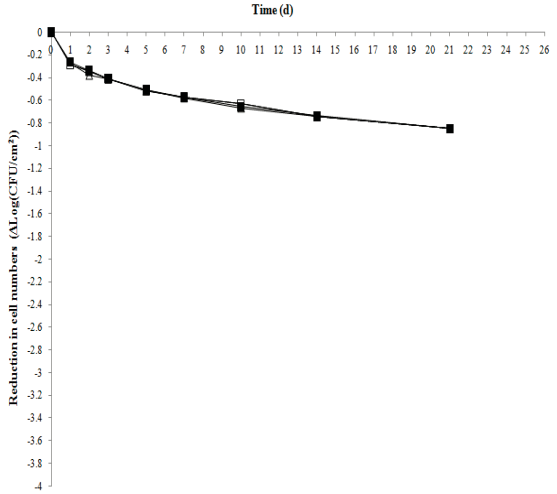
Desiccation substrate	Strains	Osmoadaptation	Desiccation salt	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
					Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f*
BHI	Lm568	No	Low	0.85^{a**}±0.04***	31.88^a±0.53****	0.365^a±0.010	0.001	0.997	1.00	1.45
BHI	Lm568	No	High	0.85^a±0.03	32.53^a±0.65	0.370^a±0.005	0.001	0.998	1.00	1.38
BHI	Lm568	Yes	Low	0.85^a±0.04	32.41^a±0.27	0.371^a±0.004	0.001	0.998	1.00	1.06
BHI	Lm568	Yes	High	0.85^a±0.05	31.57^a±0.91	0.374^a±0.005	0.001	0.998	1.00	1.26
BHI	Δ*sigB* mutant	No	Low	0.85^a±0.04	32.10^a±0.83	0.365^a±0.009	0.001	0.998	1.00	1.34
BHI	Δ*sigB* mutant	No	High	0.85^a±0.04	32.06^a±0.16	0.373^a±0.001	0.001	0.997	1.00	1.42
BHI	Δ*sigB* mutant	Yes	Low	0.85^a±0.04	32.36^a±0.19	0.370^a±0.004	0.001	0.997	1.00	1.06
BHI	Δ*sigB* mutant	Yes	High	0.85^a±0.04	31.78^a±0.52	0.365^a±0.009	0.001	0.997	1.00	1.17
TSB-glu	Lm568	No	Low	2.78^b±0.05	0.44^b±0.05	0.286^b±0.008	0.014	0.982	1.00	0.95
TSB-glu	Lm568	No	High	1.37^c±0.04	6.38^c±0.06	0.368^a±0.013	0.008	0.964	1.00	1.20
TSB-glu	Lm568	Yes	Low	1.52^d±0.04	1.54^d±0.04	0.170^c±0.002	0.001	0.995	1.00	1.13
TSB-glu	Lm568	Yes	High	1.14^e±0.04	15.79^e±0.19	0.504^d±0.009	0.001	0.998	1.00	1.06
TSB-glu	Δ*sigB* mutant	No	Low	2.79^b±0.07	0.43^b±0.04	0.281^b±0.006	0.010	0.987	1.00	1.08
TSB-glu	Δ*sigB* mutant	No	High	1.38^c±0.04	6.38^c±0.05	0.365^a±0.013	0.006	0.970	1.00	1.38
TSB-glu	Δ*sigB* mutant	Yes	Low	1.51^d±0.05	1.50^d±0.04	0.169^c±0.003	0.001	0.993	1.00	1.14
TSB-glu	Δ*sigB* mutant	Yes	High	1.14^e±0.05	15.59^e±0.26	0.499^d±0.013	0.001	0.998	1.00	1.30
PPS	Lm568	No	Low	2.90^f±0.04	0.30^f±0.01	0.261^e±0.003	0.005	0.993	1.00	1.28
PPS	Lm568	No	High	1.52^d±0.04	4.70^g±0.11	0.303^f±0.007	0.002	0.991	1.00	1.04
PPS	Lm568	Yes	Low	1.99^g±0.04	0.84^h±0.03	0.227^g±0.002	0.002	0.994	1.00	1.29
PPS	Lm568	Yes	High	1.44^h±0.03	7.53^i±0.20	0.428^h±0.008	0.003	0.984	1.00	1.23
PPS	Δ*sigB* mutant	No	Low	2.89^f±0.05	0.04^j±0.00	0.182^i±0.003	0.009	0.989	1.00	1.48
PPS	Δ*sigB* mutant	No	High	1.51^d±0.04	4.61^g±0.12	0.306^f±0.007	0.002	0.989	1.00	1.11
PPS	Δ*sigB* mutant	Yes	Low	1.99^g±0.04	0.81^h±0.03	0.224^g±0.005	0.002	0.995	1.00	1.23
PPS	Δ*sigB* mutant	Yes	High	1.44^h±0.03	7.41^i±0.27	0.421^h±0.013	0.004	0.982	1.00	1.11

*f value lower than1.61indicate that the fit of model is suitable ($p<0.05$). **values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

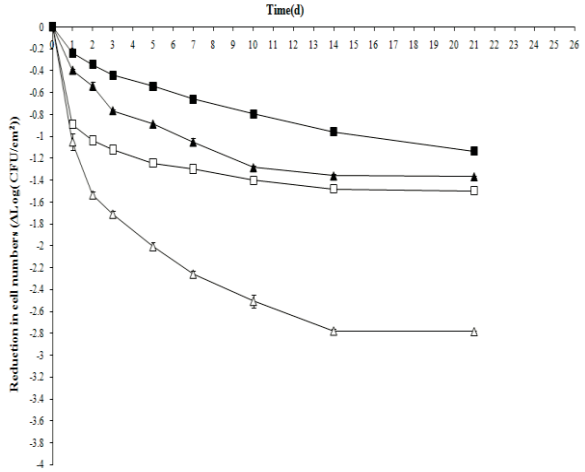
***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

a)



b)



c)

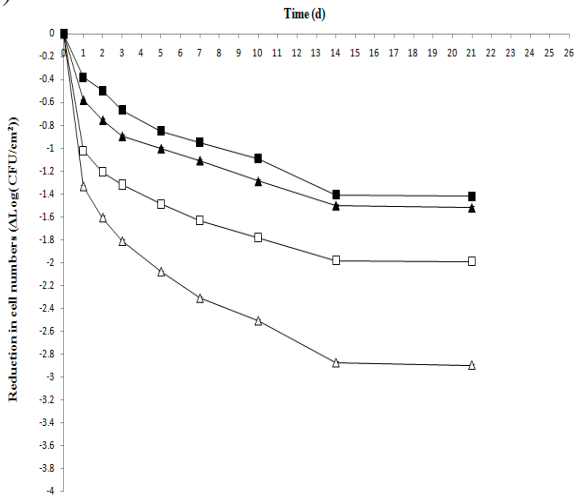


Figure 9. Effect of salt during pre-culture and desiccation on the survival of *L. monocytogenes* 568 cells on stainless steel coupons incubated at 15 °C and 43% RH for 21 days in the desiccator. Lm568 were pre-cultured in a) BHI, b) TSB-glu, and c) PPS with 0.5% NaCl at 15 °C for 3 days and desiccated in each substrate with 0.5% NaCl (Δ) and 5.0% NaCl (▲), respectively. Osmoadapted cells pre-cultured in a) BHI, b) TSB-glu, and c) PPS with 5.0% NaCl (15 °C for 3 days) were desiccated in each substrate with 0.5% NaCl (□) and 5.0% NaCl (■), respectively. Reduction in cell numbers ($\Delta Log(CFU/cm^2)$) at each sampling point is expressed as $Log(N_t/N_0)$). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

4.4.3 Effect of Osmoadaptation, Osmolytes, and Desiccation Salt on Survival of Lm568 and Its Δ*sigB* Mutant Suspended in Minimal Media During Desiccation on Stainless Steel Coupons at 15 °C and 43% Relative Humidity

To determine whether pre-culture with each of osmolytes (1 mM, glycine betaine, carnitine, and proline) in MM without and with simultaneous osmoadaptation would improve the desiccation survival of Lm568 and its Δ*sigB* mutant when desiccated at 15 °C and 43% RH for 21 days in the same MM medium with no osmolytes, the survivor curves were constructed experimentally and modelled using the Weibull model. Prior to desiccation, the initial populations for both strains in all treatments were approximately 10^8 CFU/cm^2. The four indices of the model fit (the smaller values of the MSE_{model}, the values of R^2 and A_f near to 1, and the f values lower than 1.61) indicated the suitability of the model to describe the data (Tables 14, 15, 16, 17, and 18). The survivor curves from this experiment are shown in Figures 24, 25, 26, and 27 in the appendix, respectively.

The results showed that pre-culture of Lm568 in MM containing one of the osmoytes (glycine betaine, carnitine or proline) consistently enhanced ($p<0.05$) its survival during desiccation in both low and high NaCl level media, as indicated by the increase in the Weibull delta parameter for osmolyte treatments (Table 14 and Figure 10 for glycine betaine). However, the protective effect of pre-culturing with osmolytes only persisted for the non-osmoadapted and osmolyte treated cells desiccated with a low initial NaCl level, which contained higher survivor counts ($p<0.05$) after 21 days (Figure 10 for glycine betaine).

Pre-culturing with glycine betaine prior to desiccation was more effective ($p<0.05$) for improving the survival of Lm568 during desiccation in comparison with the pre-culturing with carnitine and proline (Table 14). The values of the delta parameter were significantly ($p<0.05$) greater for cells pre-cultured with glycine betaine than with carnitine and proline regardless of the osmoadaptation and desiccation salt treatments. There were no significant difference ($p>0.05$) between the delta parameters obtained for carnitine and proline. However, after 21 days, the difference in survivor counts for the three osmolyte treatments was no longer significant ($p>0.05$).

For the Δ*sigB* mutant (Table 15), prior to desiccation, pre-culturing with glycine betaine or carnitine in MM with 0.5% NaCl significantly improved ($p<0.05$) its survival during desiccation with a low initial salt level, as indicated by the higher ($p<0.05$) Weibull delta

parameter and the lower ($p<0.05$) reduction level in survivors after 21 days. Pre-culturing in glycine betaine resulted in a significantly higher ($p<0.05$) delta parameter than pre-culturing with carnitine, but final survivor levels after 21 days were similar ($p>0.05$) for the two osmolytes. The presence of proline in MM with 0.5% NaCl prior to desiccation had no significant effect ($p>0.05$) on the survival of the Δ*sigB* mutant during desiccation with a low initial NaCl level.

Growth of the Δ*sigB* mutant in 5.0% NaCl prior to desiccation and any one of the osmolytes improved ($p<0.05$) its initial survival (delta parameter) during desiccation with a high initial NaCl level, however, there were no significant differences ($p>0.05$) for the reduction in survivors after 21 days compared to osmoadaptated cells pre-cultured in the absence of the osmolytes prior to desiccation in MM with 5.0% NaCl. Osmoadaptated cells pre-cultured with glycine betaine were more resistant to the desiccation stress in the presence of a high initial NaCl level during the initial inactivation period compared to osmoadaptated cells grown with carnitine and proline, resulting in higher values ($p<0.05$) of delta parameter for the glycine betaine than in carnitine and proline. Cells pre-cultured with carnitine and proline prior to desiccation behaved similarly ($p>0.05$) in the first log reduction period (delta parameter). The final reduction in survivors was not significantly different ($p>0.05$) among the three osmolytes.

For both strains (Tables 14 and 15), osmoadaptated cells desiccated with high initial NaCl levels were significantly ($p<0.05$) more resistant to the desiccation stress (larger delta parameter values and lower reduction levels in survivors after 21 days) compared with the non-osmoadaptated cells exposured to low initial NaCl levels in the desiccation period, indicating both osmoadaptation during pre-culture and desiccation with a high initial NaCl level enhanced significantly the survival of both strains at 15 °C and 43% RH for 21 days.

The modelling results (Table 16) showed the non-osmoadaptated Δ*sigB* mutant cells during desiccation with a low initial NaCl level were more sensitive ($p<0.05$) to the desiccation shock compared with its parent strain (Lm568), but the survivor counts for both strains reached the same reduction levels of 3 Log (CFU/cm^2) after 14 to 21 days (Figure 24). Also, relative to Lm568, the osmoadaptated Δ*sigB* mutant cells when desiccated in a high initial NaCl level underwent its first log loss in viability faster ($p<0.05$) and exhibited an increased ($p<0.05$) reduction in survivors after 21 days. For non-osmoadaptated cells desiccated in MM with 0.5% NaCl after pre-culture with osmolytes (Table 17), the delta parameters were significantly greater ($p<0.05$) for Lm568 than for the Δ*sigB* mutant. The reduction in survivors after 21 days was

similar ($p>0.05$) for both strains, with the exception of cells pre-cultured with proline where the loss of viability after 21 days for the Δ*sigB* mutant was significantly greater ($p<0.05$) than for Lm568. Similarly, when desiccating osmoadaptated cells pre-cultured with osmolytes in MM with 5.0% NaCl (Table 18), survival of Lm568 was significantly ($p<0.05$) better than its Δ*sigB* mutant as observed by the Weibull delta parameters and the reduction levels after 21 days. The results indicated that even in the presence of osmolytes in MM prior to desiccation, the Δ*sigB* mutant was more sensitive to the desiccation conditions in the minimal defined media (MM) compared with Lm568.

Table 14. Desiccation survival kinetics of *L. monocytogenes* 568 following pre-culture with glycine betaine, carnitine or proline: Cells were pre-cultured in low (0.5%) or high (5.0%) salt minimal medium (MM) with 0 (control) or 1 mM of each osmolyte at 15 °C for 3 days followed by desiccation (15 °C and 43% RH) in MM with low or high NaCl for 21 days on stainless steel coupons.

Osmoadaptation	Pre-culture with osmolyte	Desiccation salt	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
				Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f^*
No	No	Low	2.82^{a**}±0.02***	0.32^{a}±0.01****	0.257^{a}±0.003	0.005	0.992	1.00	1.22
Yes	No	High	1.70^{b}±0.02	5.01^{b}±0.05	0.490^{b}±0.003	0.025	0.979	1.00	1.39
No	Yes(B)	Low	2.67^{c}±0.02	1.07^{c}±0.02	0.331^{c}±0.003	0.002	0.999	1.00	1.44
No	Yes(C)	Low	2.67^{c}±0.02	0.86^{d}±0.01	0.308^{d}±0.001	0.001	0.999	1.00	1.27
No	Yes(P)	Low	2.68^{c}±0.02	0.87^{d}±0.01	0.310^{d}±0.001	0.001	0.999	1.00	1.21
Yes	Yes(B)	High	1.71^{b}±0.03	6.51^{e}±0.02	0.528^{e}±0.003	0.005	0.985	1.00	1.42
Yes	Yes(C)	High	1.70^{b}±0.03	6.00^{f}±0.07	0.445^{f}±0.006	0.001	0.995	1.00	1.16
Yes	Yes(P)	High	1.71^{b}±0.04	6.01^{f}±0.02	0.442^{f}±0.002	0.003	0.988	1.00	0.74

*f value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 15. Desiccation survival kinetics of *L. monocytogenes* 568 Δ*sigB* following pre-culture with glycine betaine, carnitine or proline: Cells were pre-cultured in low (0.5%) or high (5.0%) salt MM with 0 (control) or 1 mM of each osmolyte at 15 °C for 3 days followed by desiccation (15 °C and 43% RH) in MM with low or high NaCl for 21 days on stainless steel coupons.

Osmoadaptation	Pre-culture with osmolyte	Desiccation salt	Reduction in cell numbers after desiccation for 21 days $\Delta Log(CFU/cm^2)$	Weibull parameter estimates		Statistical indices of fit of model			
				Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f*
No	No	Low	2.83[a**] ± 0.02***	0.13[a] ± 0.01****	0.215[a] ± 0.002	0.007	0.991	1.00	0.72
Yes	No	High	2.02[b] ± 0.03	2.27[b] ± 0.04	0.358[b] ± 0.003	0.009	0.979	1.00	0.93
No	Yes(B)	Low	2.67[c] ± 0.02	0.69[c] ± 0.00	0.286[c] ± 0.001	0.001	0.999	1.00	1.26
No	Yes(C)	Low	2.68[c] ± 0.02	0.48[d] ± 0.01	0.259[d] ± 0.002	0.003	0.999	1.00	1.17
No	Yes(P)	Low	2.82[a] ± 0.02	0.12[a] ± 0.00	0.191[e] ± 0.003	0.001	0.999	1.00	1.29
Yes	Yes(B)	High	2.01[b] ± 0.04	3.45[e] ± 0.02	0.429[f] ± 0.001	0.005	0.987	1.00	1.37
Yes	Yes(C)	High	2.02[b] ± 0.05	2.98[f] ± 0.07	0.413[g] ± 0.006	0.014	0.970	1.00	1.01
Yes	Yes(P)	High	2.02[b] ± 0.05	2.98[f] ± 0.03	0.415[g] ± 0.001	0.015	0.967	1.00	0.95

**f* value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 16. A comparative study of desiccation tolerance of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM: Cells were cultured in MM with 0.5% (Low) or 5.0% (High) NaCl at 15 °C for 3 days, and desiccated (15 °C and 43% RH) in MM with low or high NaCl for 21 days on stainless steel coupons.

Strains	Osmoadaptation	Pre-culture with osmolyte	Desiccation salt	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
					Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f^*
Lm568	**No**	**No**	**Low**	**2.82^{a**}±0.02*****	**0.32^{a}±0.01******	**0.257^{a}±0.003**	**0.005**	**0.992**	**1.00**	**1.22**
Lm568	**Yes**	**No**	**High**	**1.70^{b}±0.02**	**5.01^{b}±0.05**	**0.490^{b}±0.003**	**0.025**	**0.979**	**1.00**	**1.39**
Δ*sigB* mutant	**No**	**No**	**Low**	**2.83^{a}±0.02**	**0.13^{c}±0.01**	**0.215^{c}±0.002**	**0.007**	**0.991**	**1.00**	**0.72**
Δ*sigB* mutant	**Yes**	**No**	**High**	**2.02^{c}±0.03**	**2.27^{d}±0.04**	**0.358^{d}±0.003**	**0.009**	**0.979**	**1.00**	**0.93**

*f value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 17. Desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant in low salt MM following pre-culture with glycine betaine, carnitine or proline (1 mM): Cells were cultured in 0.5% (Low) NaCl MM with 1 mM of each osmolyte for 3 days at 15 °C, and desiccated (15 °C and 43% RH) in MM with low NaCl for 21 days on stainless steel coupons.

Strains	Osmoadaptation	Pre-culture with osmolyte	Desiccation salt	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
					Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	*f**
Lm568	**No**	**Yes(B)**	**Low**	**2.67^a** ±0.02*****	**1.07^a±0.02******	**0.331^a±0.003**	**0.002**	**0.999**	**1.00**	**1.44**
Δ*sigB* mutant	**No**	**Yes(B)**	**Low**	**2.67^a±0.02**	**0.69^b±0.00**	**0.286^b±0.001**	**0.001**	**0.999**	**1.00**	**1.26**
Lm568	**No**	**Yes(C)**	**Low**	**2.67^a±0.02**	**0.86^c±0.01**	**0.308^c±0.001**	**0.001**	**0.999**	**1.00**	**1.27**
Δ*sigB* mutant	**No**	**Yes(C)**	**Low**	**2.68^a±0.02**	**0.48^d±0.01**	**0.259^d±0.002**	**0.003**	**0.999**	**1.00**	**1.17**
Lm568	**No**	**Yes(P)**	**Low**	**2.68^a±0.02**	**0.87^c±0.01**	**0.310^c±0.001**	**0.001**	**0.999**	**1.00**	**1.21**
Δ*sigB* mutant	**No**	**Yes(P)**	**Low**	**2.82^b±0.02**	**0.12^e±0.00**	**0.191^e±0.003**	**0.001**	**0.999**	**1.00**	**1.29**

**f* value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 18. Desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant in high salt MM following pre-culture with glycine betaine, carnitine or proline (1 mM): Cells were cultured in 5.0% (High) NaCl MM with 1 mM of one of the osmolytes for 3 days at 15 °C, and desiccated (15 °C and 43% RH) in MM with high NaCl for 21 days on stainless steel coupons.

Strains	Osmoadaptation	Pre-culture with osmolyte	Desiccation salt	Reduction in cell numbers after desiccation for 21 days $\Delta Log(CFU/cm^2)$	Weibull parameter estimates		Statistical indices of fit of model			
					Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f^*
Lm568	**Yes**	**Yes(B)**	**High**	**1.71^{a}** ±0.03*****	**6.51^{a}±0.02******	**0.528^{a}±0.003**	**0.005**	**0.985**	**1.00**	**1.42**
Δ*sigB* mutant	**Yes**	**Yes(B)**	**High**	**2.01^{b}±0.04**	**3.45^{b}±0.02**	**0.429^{b}±0.001**	**0.005**	**0.987**	**1.00**	**1.37**
Lm568	**Yes**	**Yes(C)**	**High**	**1.70^{a}±0.03**	**6.00^{c}±0.07**	**0.445^{c}±0.006**	**0.001**	**0.995**	**1.00**	**1.16**
Δ*sigB* mutant	**Yes**	**Yes(C)**	**High**	**2.02^{b}±0.05**	**2.98^{d}±0.07**	**0.413^{d}±0.006**	**0.014**	**0.970**	**1.00**	**1.01**
Lm568	**Yes**	**Yes(P)**	**High**	**1.71^{a}±0.04**	**6.01^{c}±0.02**	**0.442^{c}±0.002**	**0.003**	**0.988**	**1.00**	**0.74**
Δ*sigB* mutant	**Yes**	**Yes(P)**	**High**	**2.02^{b}±0.05**	**2.98^{d}±0.03**	**0.415^{d}±0.001**	**0.015**	**0.967**	**1.00**	**0.95**

**f* value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

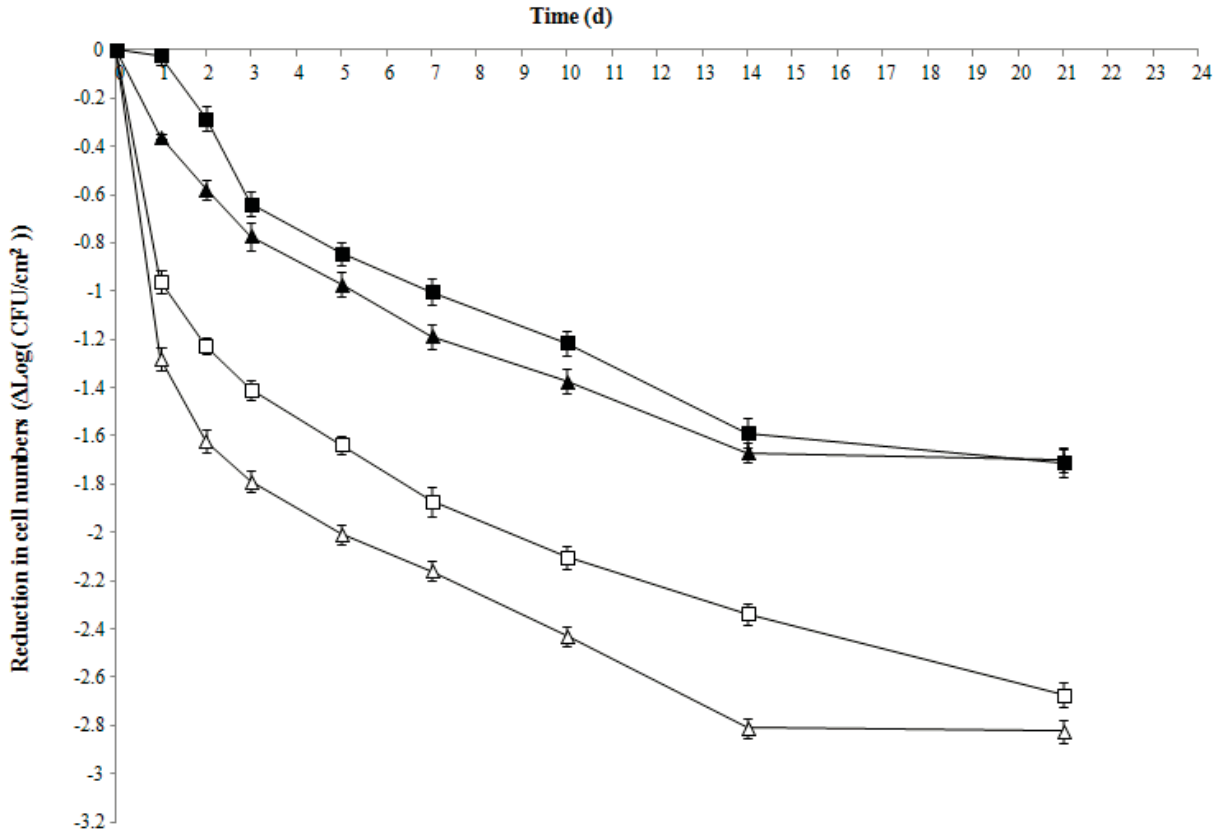


Figure 10. Protective effect of pre-culture with glycine betaine on desiccation survival kinetics of *L. monocytogenes* 568. The cells of Lm568 were pre-cultured in MM with 0.5% NaCl and glycine betaine (1 mM) (□) and without glycine betaine (Δ) at 15 °C for 3 days and desiccated in MM with 0.5% NaCl on stainless steel at 15 °C and 43% RH for 21 days. The cells of Lm568 were osmoadapted in MM with 5.0% NaCl and glycine betaine (1 mM) (■) and without glycine betaine (▲) at 15 °C for 3 days and desiccated in MM with 5.0% NaCl, respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

To test whether the presence of exogenous osmolytes would improve the desiccation survival of Lm568 and its Δ*sigB* mutant on desiccation substrates, desiccation survival experiments were carried out in a MM substrate with a low (0.5%) or high (5.0%) NaCl level in the presence of glycine betaine, carnitine, or proline to an initial concentration of 1 mM. Prior to desiccation, starting populations of 10^8 CFU/cm^2 for both strains were evenly spotted on stainless steel coupons to investigate the inactivation kinetics at 15 °C and 43% RH for 21 days. The non-linear inactivation kinetics were suitably modelled using the Weibull model, as indicated by the four indices of the model fits (MSE_{model}, R^2, A_f, and f) (Tables 19, 20, 21 and 22). The inactivation kinetics of Lm568 and its Δ*sigB* mutant in each of the desiccation substrates is shown in Figures 28, 29, 30 in the appendix.

For Lm568 (Table 19 and Figure 11 for glycine betaine), when desiccated as non-osmoadapted cells in a low initial NaCl MM, exogenous glycine betaine, carnitine and proline significantly improved its survival during air-drying. The values of the delta parameter were significantly greater ($p<0.05$) for cells desiccated with each exogenous osmolyte than for cells desiccated without exogenous osmolytes, however, there were no significant differences ($p<0.05$) in survivor counts (2.83 Log (CFU/cm^2)) observed after 21 days (Figure 11 for glycine betaine). The cells desiccated with exogenous glycine betaine were significantly more resistant ($p<0.05$) to the initial desiccation stress (delta parameter) compared with the cells desiccated with the other osmolytes. The presence of carnitine and proline in the desiccation substrates affected initial survival equally ($p>0.05$).

When the difference in survival on each day was calculated between non-osmoadapted Lm568 cells desiccated in a low initial NaCl MM with or without one of osmolytes, it was shown that the difference reached maximal levels after 2 days. Subsequently, the difference decreased gradually and reached close to zero difference after 21 days for glycine betaine and 14 to 21 days for carnitine and proline. Also, glycine betaine had more of an impact (0.17 ΔLog(cfu/cm^2)) than carnitine and proline (0.10 ΔLog(cfu/cm^2)) (Figures 31 a), b), and c)).

When desiccating osmoadapted Lm568 cells in MM with a high initial NaCl level, only exogenous glycine betaine significantly improved ($p<0.05$) its survival during the first log reduction period (delta parameter), but after 21 days, the survivors plateaued at the same level ($p>0.05$) obtained after desiccation in MM without exogenous osmolytes (Figure 11 for glycine betaine).

When osmoadapted Lm568 cells in MM were desiccated in a high initial NaCl level, the difference in survival between osmoadapted Lm568 cells with and without glycine betaine increased gradually over the initial 14 days and reached the maximal level after 14 days, after which it sharply decreased to close to zero level after 21 days (Figure 31 d), e), and f)).

For the Δ*sigB* mutant (Table 20), the Weibull delta values were regardless of the salt treatments during pre-culture and desiccation significantly greater ($p<0.05$) for cells desiccated with one of the exogenous osmolytes than for cells desiccated without osmolytes. However, no difference ($p>0.05$) in the reduction in survivors after 21 days was observed between the treatments. Exogenous glycine betaine, again, exerted significantly greater ($p<0.05$) protection during the initial desiccation period (delta parameter) compared with exogenous carnitine and proline. Exogenous carnitine and proline equally ($p>0.05$) contributed to the increased survival of the Δ*sigB* mutant cells within the first log reduction period. However, the survivors among all three osmolytes treatments dropped to the similar levels ($p>0.05$) after 21 days.

For the Δ*sigB* mutant, the difference in survival between non-osmoadapted cells with glycine betaine, carnitine, or proline and without osmolytes reached maximal levels after 2 days. Then, it decreased gradually until no difference was present after 21 days for glycine betaine and after 14 to 21 days for carnitine and proline. Also, glycine betaine was more protective (0.10 ΔLog(cfu/cm^2)) than carnitine and proline (0.08 ΔLog(cfu/cm^2)) (Figures 31 a), b), and c)).

When the osmoadapted Δ*sigB* mutant cells were desiccated in a high initial NaCl MM, the calculated difference in survival between cells with and without osmolytes increased to maximal levels after 14 days, after which it essentially decreased to zero after 21 days. Again, glycine betaine caused the highest level of protection (0.18 ΔLog(cfu/cm^2)) relative to carnitine and proline (0.12 ΔLog(cfu/cm^2)) (Figures 31 d), e), and f)).

Lm568 consistently survived significantly better than the Δ*sigB* mutant in both the desiccation treatments with non-osmoadapted cells being desiccated with low initial NaCl levels and exogenous osmolytes, and osmoadaptated cells being desiccated with high initial NaCl levels and exogenous osmolytes (Tables 21 and 22). For the desiccation with low NaCl and osmolytes treatment (Table 21), the Weibull delta values were significantly higher ($p<0.05$) for non-osmoadapted cells of Lm568 than for those of the Δ*sigB* mutant, however, both strains were reduced to similar survivor levels ($p>0.05$) after 21 days. This indicated Lm568 survived the initial desiccation period significantly better ($p<0.05$) than its Δ*sigB* mutant. The Weibull

modelling parameters (Table 22) showed that the time to the first log reduction (delta parameter) was significantly greater ($p<0.05$) for the osmoadapted cells of Lm568 than for the Δ*sigB* mutant when desiccated with a high initial NaCl level in the presence of osmolytes. Also, the number of survivors of Lm568 after 21 days were greater ($p<0.05$) than those of the Δ*sigB* mutant. (Figures 28, 29, and 30, and Table 22), showing the survival of osmoadapted Lm568 was significantly better than the survival of the osmoadapted Δ*sigB* mutant throughout the desiccation period in MM with a high initial NaCl level and presence of exogenous osmolytes.

Table 19. Desiccation survival kinetics of *L. monocytogenes* 568 suspended in MM with 0 (control) or 1 mM of exogenous glycine betaine, carnitine or proline: Cells were cultured in MM with 0.5% (Low) or 5.0% (High) NaCl at 15 °C for 3 days, and desiccated (15 °C and 43% RH) in MM with 0 and 1 mM of each exogenous osmolyte and low or high NaCl for 21 days on stainless steel coupons.

Osmoadaptation	Desiccation salt	Osmolyte in the desiccation medium	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
				Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f^*
No	Low	No	2.82^{a**}±0.02***	0.32^{a}±0.01****	0.257^{a}±0.003	0.005	0.992	1.00	1.22
Yes	High	No	1.70^{b}±0.02	5.01^{b}±0.05	0.490^{b}±0.003	0.025	0.979	1.00	1.39
No	Low	Yes (B)	2.83^{a}±0.04	0.50^{c}±0.01	0.286^{c}±0.002	0.002	0.998	1.00	1.03
No	Low	Yes (C)	2.83^{a}±0.03	0.39^{d}±0.01	0.267^{d}±0.001	0.004	0.994	1.00	1.05
No	Low	Yes (P)	2.83^{a}±0.03	0.40^{d}±0.01	0.270^{d}±0.002	0.005	0.993	1.00	1.06
Yes	High	Yes (B)	1.71^{b}±0.03	6.55^{e}±0.04	0.483^{e}±0.004	0.001	0.998	1.00	0.97
Yes	High	Yes (C)	1.71^{b}±0.03	4.97^{b}±0.04	0.473^{f}±0.005	0.018	0.952	1.00	1.18
Yes	High	Yes (P)	1.71^{b}±0.04	4.97^{b}±0.03	0.476^{f}±0.002	0.020	0.948	1.00	1.53

*f value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 20. Desiccation survival kinetics of *L. monocytogenes* 568 Δ*sigB* suspended in MM with 0 (control) or 1 mM of exogenous glycine betaine, carnitine or proline: Cells were cultured in MM with 0.5% (Low) or 5.0% (High) NaCl at 15 °C for 3 days, and desiccated (15 °C and 43% RH) in MM with 0 and 1 mM of each exogenous osmolyte and low or high NaCl for 21 days on stainless steel coupons.

Osmoadaptation	Desiccation salt	Osmolyte in the desiccation medium	Reduction in cell numbers after desiccation for 21 days ΔLog(CFU/cm^2)	Weibull parameter estimates		Statistical indices of fit of model			
				Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f*
No	Low	No	2.83^{a**}±0.02***	0.13^a±0.01****	0.215^a±0.002	0.007	0.991	1.00	0.72
Yes	High	No	2.02^b±0.03	2.27^b±0.04	0.358^b±0.003	0.009	0.979	1.00	0.93
No	Low	Yes (B)	2.83^a±0.02	0.23^c±0.00	0.235^c±0.001	0.008	0.990	1.00	1.21
No	Low	Yes (C)	2.83^a±0.03	0.17^d±0.01	0.222^d±0.002	0.006	0.992	1.00	1.08
No	Low	Yes (P)	2.83^a±0.04	0.17^d±0.00	0.224^d±0.001	0.004	0.994	1.00	1.34
Yes	High	Yes (B)	2.02^b±0.03	2.65^e±0.05	0.358^b±0.004	0.002	0.995	1.00	1.10
Yes	High	Yes (C)	2.02^b±0.04	2.53^f±0.03	0.343^e±0.001	0.001	0.996	1.00	1.21
Yes	High	Yes (P)	2.02^b±0.04	2.51^f±0.03	0.344^e±0.002	0.001	0.997	1.00	1.30

*f value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 21. Comparison of desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM with 1 mM of exogenous glycine betaine, carnitine or proline: Cells were cultured in MM with 0.5% (Low) NaCl at 15 °C for 3 days, and desiccated (15 °C and 43% RH) in MM with 1 mM of each exogenous osmolyte with low NaCl for 21 days on stainless steel coupons.

Strains	Osmoadaptation	Desiccation salt	Osmolyte in the desiccation medium	Reduction in cell numbers after desiccation for 21 days $\Delta Log(CFU/cm^2)$	Weibull parameter estimates		Statistical indices of fit of model			
					Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f*
Lm568	**No**	**Low**	**Yes (B)**	**2.83^{a}**$\pm$0.04*****	**$0.50^{a}\pm0.01$******	**$0.286^{a}\pm0.002$**	**0.002**	**0.998**	**1.00**	**1.03**
Δ*sigB* mutant	**No**	**Low**	**Yes (B)**	**$2.83^{a}\pm0.02$**	**$0.23^{b}\pm0.00$**	**$0.235^{b}\pm0.001$**	**0.008**	**0.990**	**1.00**	**1.21**
Lm568	**No**	**Low**	**Yes (C)**	**$2.83^{a}\pm0.03$**	**$0.39^{c}\pm0.01$**	**$0.267^{c}\pm0.001$**	**0.004**	**0.994**	**1.00**	**1.05**
Δ*sigB* mutant	**No**	**Low**	**Yes (C)**	**$2.83^{a}\pm0.03$**	**$0.17^{d}\pm0.01$**	**$0.222^{d}\pm0.002$**	**0.006**	**0.992**	**1.00**	**1.08**
Lm568	**No**	**Low**	**Yes (P)**	**$2.83^{a}\pm0.03$**	**$0.40^{c}\pm0.01$**	**$0.270^{c}\pm0.002$**	**0.005**	**0.993**	**1.00**	**1.06**
Δ*sigB* mutant	**No**	**Low**	**Yes (P)**	**$2.83^{a}\pm0.04$**	**$0.17^{d}\pm0.00$**	**$0.224^{d}\pm0.001$**	**0.004**	**0.994**	**1.00**	**1.34**

*f value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

Table 22. Comparison of desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM with 1 mM of exogenous glycine betaine, carnitine or proline: Cells were cultured in MM with 5.0% (High) NaCl at 15 °C for 3 days, and desiccated (15 °C and 43% RH) in MM with 1 mM of each exogenous osmolyte with high NaCl for 21 days on stainless steel coupons.

Strains	Osmoadaptation	Desiccation salt	Osmolyte in the desiccation medium	Reduction in cell numbers after desiccation for 21 days $\Delta Log(CFU/cm^2)$	Weibull parameter estimates		Statistical indices of fit of model			
					Delta (days to 1st log reduction)	P (days)^p	MSE_{model}	R^2	A_f	f*
Lm568	**Yes**	**High**	**Yes (B)**	**1.71^{a**}±0.03*****	**6.55^{a}±0.04******	**0.483^{a}±0.004**	**0.001**	**0.998**	**1.00**	**0.97**
Δ*sigB* mutant	**Yes**	**High**	**Yes (B)**	**2.02^{b}±0.03**	**2.65^{b}±0.05**	**0.358^{b}±0.004**	**0.002**	**0.995**	**1.00**	**1.10**
Lm568	**Yes**	**High**	**Yes (C)**	**1.71^{a}±0.03**	**4.97^{c}±0.04**	**0.473^{c}±0.005**	**0.018**	**0.952**	**1.00**	**1.18**
Δ*sigB* mutant	**Yes**	**High**	**Yes (C)**	**2.02^{b}±0.04**	**2.53^{d}±0.03**	**0.343^{d}±0.001**	**0.001**	**0.996**	**1.00**	**1.21**
Lm568	**Yes**	**High**	**Yes (P)**	**1.71^{a}±0.04**	**4.97^{c}±0.03**	**0.476^{c}±0.002**	**0.020**	**0.948**	**1.00**	**1.53**
Δ*sigB* mutant	**Yes**	**High**	**Yes (P)**	**2.02^{b}±0.04**	**2.51^{d}±0.03**	**0.344^{d}±0.002**	**0.001**	**0.997**	**1.00**	**1.30**

*f value lower than 1.61 indicated that the fit of model is suitable ($p<0.05$).

** values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.

***the mean of calculated values ($Log_{Day0} - Log_{Survivors\ after\ 21\ days}$) from the two independent trials with triplicates (n=6) ± standard deviation.

**** the mean of parameter values from the two independent trials with triplicates (n=6) ± standard deviation.

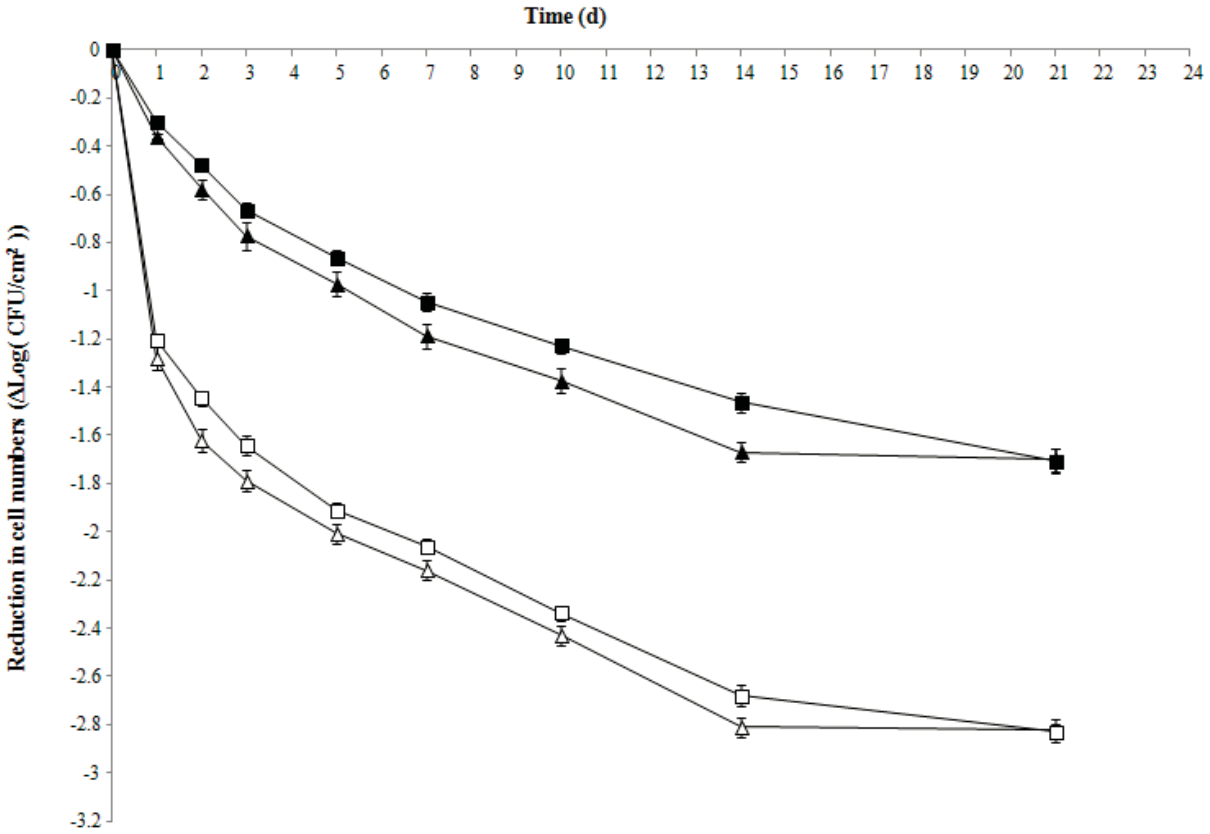


Figure 11. Protective effect of glycine betaine in the desiccation substrate (MM ± 0.5% NaCl) on the survival kinetics of *L. monocytogenes* 568 on stainless steel coupons incubated at 15 °C and 43% RH for 21 days. Cells of Lm568 were pre-cultured in MM with 0.5% NaCl at 15 °C for 3 days and desiccated in MM with 0.5% NaCl and glycine betaine (1 mM) (□) and without glycine betaine (Δ), respectively. Cells of Lm568 osmoadapted in MM with 5.0% NaCl (15 °C, 3 days) were desiccated in MM with 5.0% NaCl and glycine betaine (1 mM) (■) and without glycine betaine (▲), respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

4.4.4 Moisture Loss Measurements

During the whole dehydration process, the desiccation happened more quickly in BHI, TSB-glu, PPS, and MM with 0.5% NaCl than in the same substrate with 5.0% NaCl (Figures 12, 13, 14, and 15). Also, the desiccation in the front position of a mini desiccator tended to be faster than in the back position of a mini desiccator for each substrate regardless of the salt levels. In the equilibrium state, the moisture contents in BHI and TSB-glu with 0.5% NaCl (Figures 12 and 13) in the front and back positions were approximately 10% (wb), while the moisture contents in the two substrates with 5.0% NaCl in the front and back positions reached similar levels of 5.0% (wb). However, in PPS and MM (Figures 14 and 15), the moisture contents in the substrates with 0.5% and 5.0% NaCl in the front and back positions were reduced to levels close to 0% in the equilibrium state. In this experiment, the data logger recorded stable levels of 48% RH in front and back positions, and not the expected 43% RH as were used in the *Listeria* desiccation experiments (Sections 4.4.1, 4.4.2, and 4.4.3). The deviation may have been caused by the poor sensitivity of the data logger at 15 °C. Since the water activity of the statured K_2CO_3 solution was measured as 0.43 at 15 °C, the RH in the desiccation chamber was still considered to be 43% RH during this experiment as well as in the desiccation treatments. Also, differences in the surface-area-to-volume ratio between 2 ml of broth (the surface-area-to-volume ratio is 9.82 cm^2/ml) in this measurement and the 10 µl of cell suspensions (the surface-area-to-volume ratio is 25 cm^2/ml) used in the desiccation treatments may have resulted in different evaporation rates at 15 °C and 43% RH. The 10 µl of cell suspensions on SS coupons were visibly dry after 1 to 2 days at 15 °C and 43% RH. Also, it only took 1 to 2 hours to re-equilibrate the desiccator to 43% RH after removing the SS coupon samples on each sampling day during the *Listeria* desiccation treatments. Therefore, the measurements of moisture content in this experiment can only be used to roughly predict the pattern of the drying process happening in the different desiccation substrates during the *Listeria* challenge experiments.

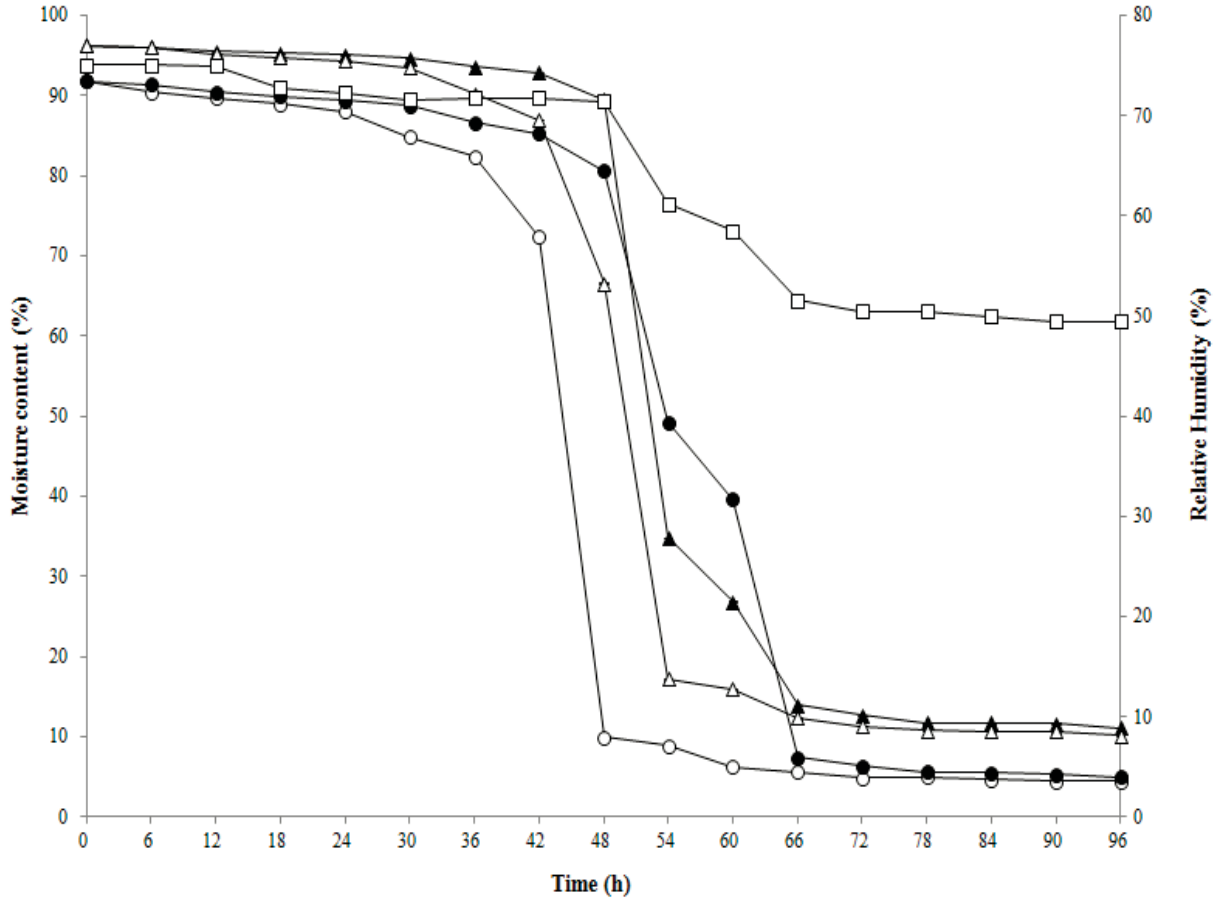


Figure 12. Measurements of moisture content in BHI with 0.5% NaCl in the front (Δ) and back(▲) positions, respectively, and with 5.0% NaCl in the front (○) and back (●) positions, respectively, during desiccation at 15 °C for 4 days. The value of relative humidity (□) was recorded by the data logger. The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

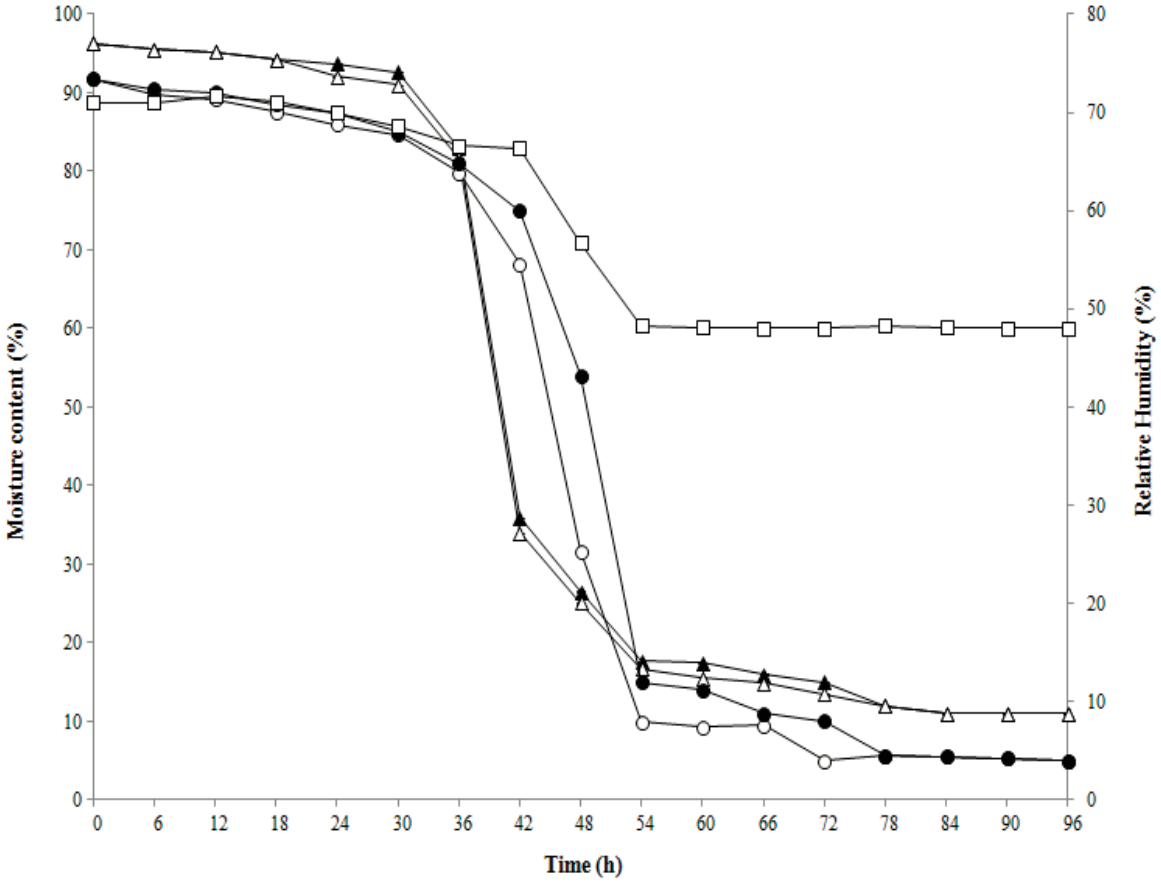


Figure 13. Measurements of moisture content in TSB-glu with 0.5% NaCl in the front (Δ) and back (▲) positions, respectively, and with 5.0% NaCl in the front (○) and back (●) positions, respectively, during desiccation at 15 °C for 4 days. The value of relative humidity (□) was recorded by the data logger. The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

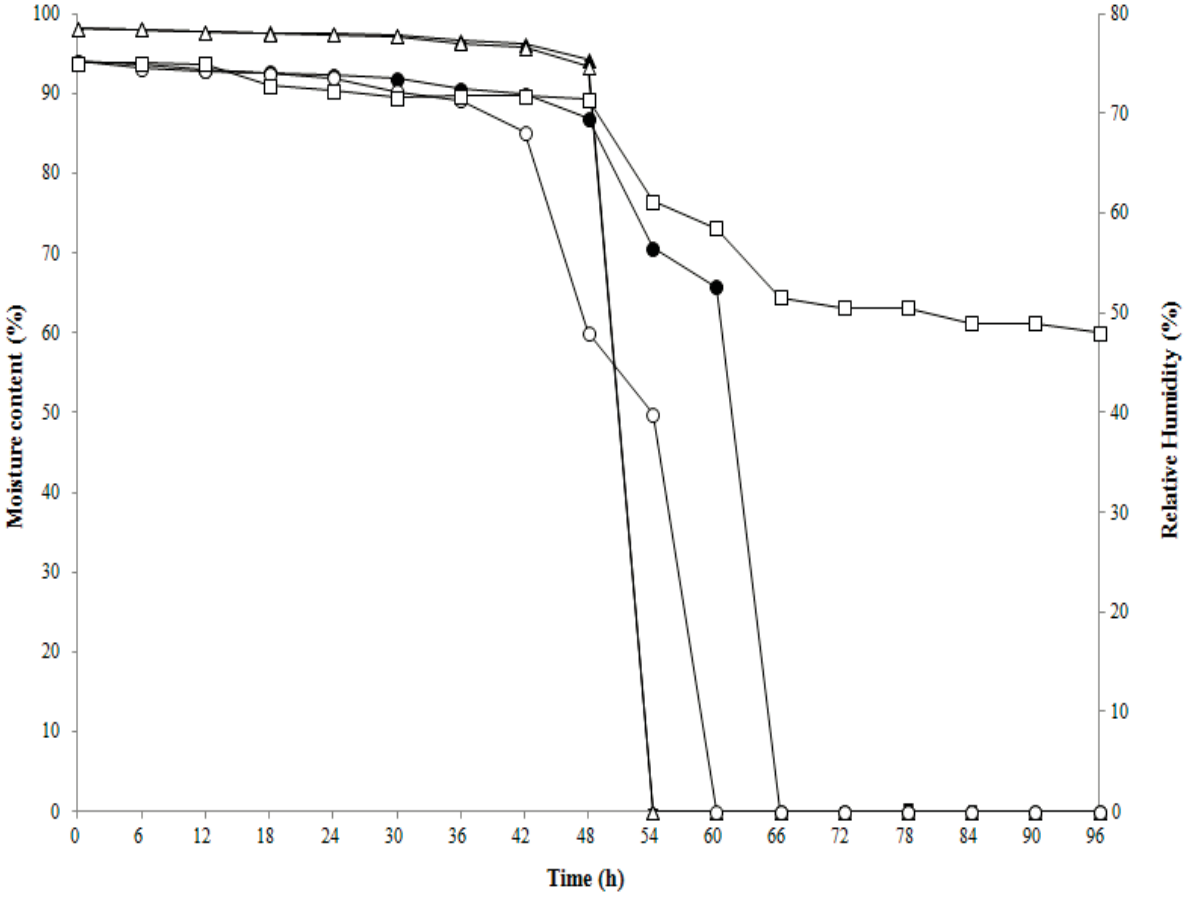


Figure 14. Measurements of moisture content in PPS with 0.85% NaCl in the front (Δ) and back (▲) positions, respectively, and with 5.0% NaCl in the front (○) and back (●) positions, respectively, during desiccation at 15 °C for 4 days. The value of relative humidity (□) was recorded by the data logger. The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

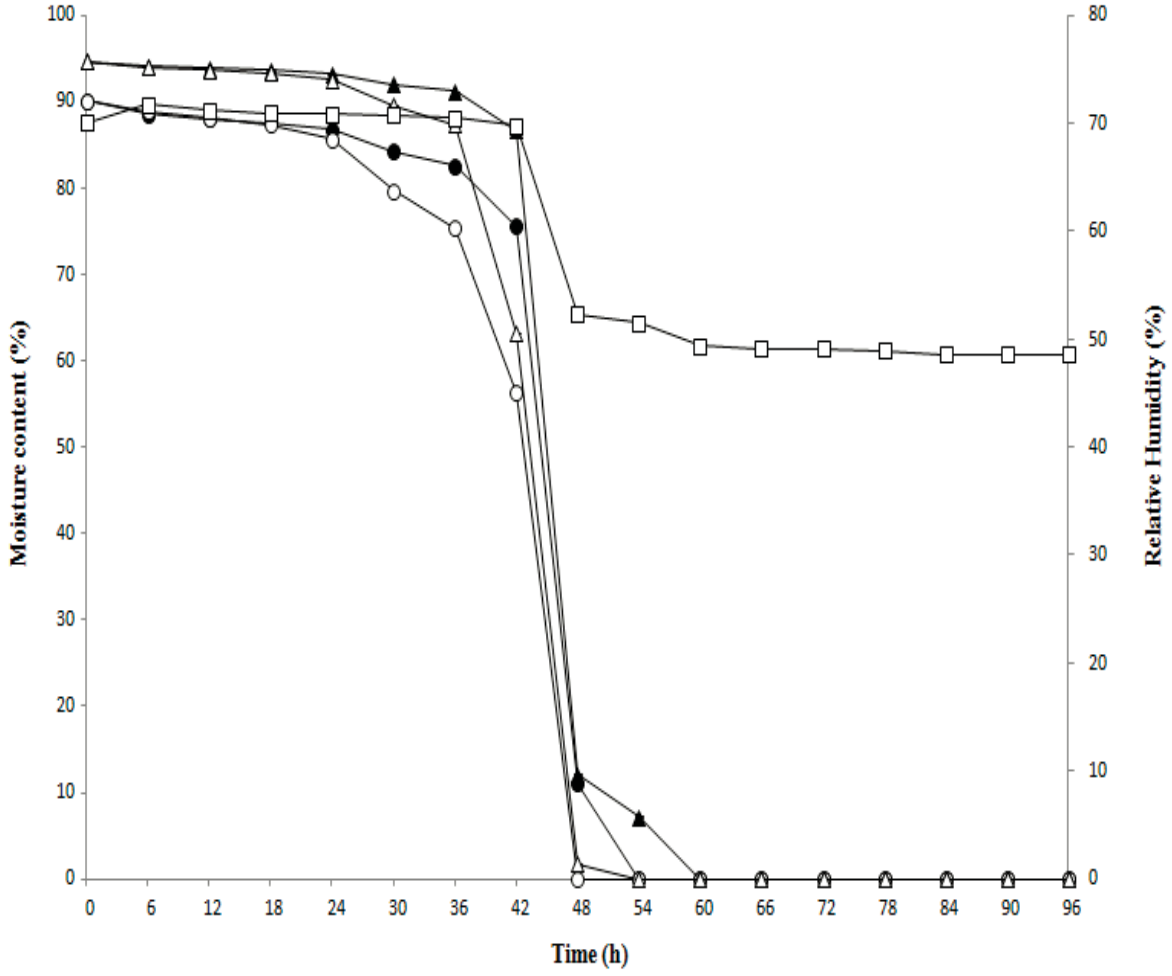


Figure 15. Measurements of moisture content in MM with 0.5% NaCl in the front (Δ) and back (▲) positions, respectively, and with 5.0% NaCl in the front (○) and back (●) positions, respectively, during desiccation at 15 °C for 4 days. The value of relative humidity (□) was recorded by the data logger. The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

4.5 Transcriptional Analysis

The transcription of all three osmolyte transporter genes (*betL*, *gbuA*, and *opuCA*) were detected in Lm568 during growth in both low osmolarity (MM with 0.5% NaCl) and high osmolarity (MM with 5.0% NaCl) medium (Table 23). The transcription of all three genes was also indicated in the Δ*sigB* mutant grown in MM with low osmolarity (Table 23). However, under the high osmolarity condition, only transcripts of *betL* and *gbuA* were found in the Δ*sigB* mutant, indicating the deletion of *sigB* affected transcription of *opuCA* (Table 23). The result demonstrated that σ^B plays a positive role in regulating the transcription of *opuCA*, which encodes the main carnitine transport system, under the osmotic stress conditions. A pair of *rRNA* primers was used as a positive control to ensure that RNA had been extracted and properly converted to DNA. The *sigB* whole and internal primers were used to ensure that the Lm568 Δ*sigB* mutant expressed a truncated version of the *sigB* gene. Straight PCR with no addition of reverse transcriptase (RT) to DNAse treated RNA was carried out with the *betL* primers as a negative control to confirm the absence of DNA contamination in the RNA samples used in the transcriptional analysis. Images of the agarose electrophoresis gels containing the PCR products from experiments carried out in MM with 0.5% and 5.0% NaCl are shown in Figure 32 a) and b), respectively, in the appendix.

Table 23. RT-PCR analysis of transcription of genes encoding osmolyte transporters in *L. monocytogenes* 568 and its Δ*sigB* mutant during exposure to MM ± 5.0% NaCl at 15 °C.

Primer	Lm568		Lm568 Δ*sigB* mutant	
	0.5 % NaCl	5.0% NaCl	0.5 % NaCl	5.0% NaCl
***rRNA* (+RT, *Listeria* positive control)**	+	+	+	+
RNA (-RT, negative control, *betL* primers)	-	-	-	-
***sigB* whole**	+	+	+a	+a
***sigB* internal**	+	+	-	-
gbuA	+	+	+	+
betL	+	+	+	+
opuCA	+	+	+	-

a. Short fragment of *sigB* expressed

4.6 HPLC Determination of Osmolyte Contents in the Growth and Desiccation Substrates

Presence of glycine betaine, carnitine, and proline was detected in BHI and TSB-glu, respectively (Figures 33 and 34), whereas, PPS only contains glycine betaine (Figure 35) or contains carnitine and proline at levels lower than the detection limit of 0.005 mg/ml. Using the reverse phase HPLC, the retention time for glycine betaine was 5.5 minutes, and the retention times were 6.5 and 6.7 minutes for carnitine and proline, respectively. The results for determination of osmolyte contents (Table 24) showed that the largest ($p<0.05$) and smallest ($p<0.05$) amounts of glycine betaine were present in TSB-glu and PPS, respectively. BHI contains a higher level ($p<0.05$) of carnitine than TSB-glu. The contents of proline in BHI and TSB-glu were provided by manufacturer (Difco). The HPLC results of trehalose were provided by Dr. Tim Ells (Personal communication, Agriculture and Agri-Food Canada, Kentville, Nova Scotia). The amount of trehalose, which is a known carbohydrate osmolyte, was 4 times higher in BHI than in TSB-glu.

Table 24. Osmolyte contents in the growth and desiccation substrates: BHI, TSB-glu, and PPS.

	Osmolytes			
Substrate	**Glycine betaine (mg/ml)**	**Carnitine (mg/ml)**	**Proline (mg/ml)**	**Trehalose (mg/ml)**
BHI	**0.24[a]****±0.01*****	**0.07[a]±0.00**	**2.00**	**0.31**
TSB-glu	**0.29[b]±0.02**	**0.06[b]±0.00**	**2.00**	**0.072**
PPS	**0.14[c]±0.01**	**ND***	**ND**	**ND**

*Not detected.
**values in the same column followed by different letters indicate significant differences ($p<0.05$) from each other as determined by the Tukey post hoc test.
***The mean of values from the triplicate samples (n=3) ± standard deviation.

CHAPTER 5. DISCUSSION

5.1 Phenotypic Comparison of the Wild Type Lm568 and Its Δ*sigB* Mutant

Maintenance of cell wall integrity was in one study shown to be influenced by σ^B in *L. monocytogenes* (Abram, Starr, Karatzas, Matlawska et al., 2008). In this study, an unusual pattern of Gram staining was observed for the *L. monocytogenes* 10403S (Lm10403S, serotype 1/2a, streptomycin resistant) Δ*sigB* mutant cells which stained red (i.e., Gram negative) after growth on BHIA for 24 hours at 37 °C. In the meanwhile, they conducted another study to investigate the σ^B regulon's involvement in the utilization of glycerol and found σ^B controls the expression of DapE. DapE contributes to biosynthesis of diaminopimelate which is required for assembly of the peptidoglycan cell wall (Abram et al., 2008). Thus, the cell wall defect observed in the mutant and led to the unusual Gram-staining pattern might have been due to the lack of DapE expression. In our study, however, cells of Lm568 and its Δ*sigB* mutant obtained from BHIA incubated at 15 °C for 3 days stained purple, as expected for Gram-positive bacteria, suggesting σ^B does not impact the integrity of the cell wall in our strain. The use of different strains, protocols to create the mutant and incubation temperatures may have yielded different results and conclusions. Both strains behaved as expected when subjected to the rapid Gram identification with KOH and were catalase positive. Also, both strains resembled each other in terms of cell and colony morphologies observed in BHI and BHIA with 0.5% or 5.0% NaCl incubated for 3 days at 15 °C. Those results indicated the deletion of *sigB* in a *L. monocytogenes* 568 background (serotype 1/2a) had no influence on major morphological characteristics under our experimental conditions.

5.2 The Effect of Sodium Chloride on Cell and Colony Morphology of Lm568 and Its Δ*sigB* Mutant

Researchers have suggested that the cellular elongation of *L. monocytogenes* cells grown in medium with high NaCl concentrations is an adaptive survival mechanism (Isom, Khambatta, Moluf, Akers, & Martin, 1995). In their study, as NaCl concentrations increased at 37 °C, the length of filaments or elongated cells also increased. The temperature was shown to influence the length of filaments. According to Brzin (1973), in order to produce the longest filaments, the optimal growth temperature must be combined with the highest NaCl concentration. He pointed out the longest filaments were observed after 24 hours at 37 °C on human serum agar containing 8 to 9% NaCl, while elongation was slower and less distinct at 22 °C. At 22 °C, another research group observed that cell elongation was only observed in the combination of 10% NaCl and pH 5.0 (HCl) rather than in the presence of NaCl alone (Bereksi, Gavini, Benezech, & Faille, 2002). In our study, the cells of Lm568 and its Δ*sigB* mutant grown in BHI with 5.0% NaCl (initial pH 6.7) at 15 °C for 3 days aggregated and formed long chains with no change in the length of individual cells (2.23 μm) compared with the length of single cells (2.24 μm) found in BHI with 0.5% NaCl incubated under the same conditions. The use of the sub-optimal growth temperature of 15 °C may explain why no cellular elongation was observed. The formation of the long chains, filaments and cell aggregation may be involved in the cellular adaptation to the osmotic stress. Both of our strains developed colonies with smooth outlines on BHIA at 15 °C for 3 days, regardless of the NaCl concentration (0.5% or 5.0%). According to Brzin (1975) in their further study of growth of *L. monocytogenes* on salt containing agars, the colonies showed regular outlines after incubation for 2 days at 10 °C; however, the outlines of colonies became irregular with growth of some individual bacilli at the border after 24 hours at 37 °C. Moreover, long twisted or straight filaments were formed beyond the border of the colonies at 37 °C. This result and the previous result in 1973 demonstrated that growth temperature influences the formation of filaments when cells were cultured in high NaCl concentrations. Scanning electron microscopy would be needed to future investigate whether Lm568 and its Δ*sigB* mutant form filaments under the conditions employed in our study (BHI with 5.0% NaCl and 15 °C).

5.3 Growth Kinetics of the Wild Type Lm568 and Its Δ*sigB* Mutant

In *L. monocytogenes*, σ^B has been shown to play a positive role for growth under conditions of high osmolarity which is commonly applied in the preservation of minimally processed foods (Abram, Starr, Karatzas, Matlawska, Boyd, & Wiedmann et al., 2008; Becker, Cetin, Hutkins, & Benson, 1998; Chaturongakul & Boor, 2006; Fraser et al., 2003). *L. monocytogenes* is detected in numbers above the legal safety level in ready-to-eat fish and cheese products, which are characterized by containing elevated salt levels (Caly, Takilt, Lebret, & Tresse, 2009). This prompted us to investigate the role of the σ^B-controlled stress response mechanism in this pathogen's persistence, survival and growth under elevated salt conditions.

The results from the first part of the growth kinetics study (Table 9) showed that σ^B is not involved in regulation of growth in BHI, TSB-glu, PPS, and MM with 0.5% or 5.0% NaCl. Similar results were reported in previous studies (Abram et al., 2008; Fraser et al., 2003). According to Abram et al. (2008), the Δ*sigB* mutant exhibited similar growth rates compared with its isogenic wild type strain (Lm10403S) in BHI in the presence of additional NaCl, with the exception of BHI with 10% NaCl where the growth of the Δ*sigB* mutant was extremely impaired compared to that of its isogenic parent. Another study (Fraser et al., 2003) indicated that the wild type strain (Lm10403S) and its Δ*sigB* mutant grew with identical growth rates and lag times in BHI with 0.5% or 3.0% NaCl and in MM in the presence or absence of 5.0% NaCl, respectively. The results in our study showed that the growth rates of both strains in BHI, TSB-glu, and PPS with a high osmolarity level were greater than rates in MM with a high osmolarity level, which is in agreement with observations made by Amezaga and his colleagues (1995). They reported that growth of *L. monocytogenes* ATCC 23074 was slow in MM with high osmolarity compared with the growth in BHI under the same osmotic shock. They found out the availability of osmolytes including amino acids and peptides in BHI contributed to this difference observed. When *L. monocytogenes* grew in peptone (peptide)-containing media, such as BHI, the cells can accumulate peptides from the medium to become adapted to the osmotic stress. Thus, the peptone was found to play two important roles. On one hand, it serves as a nutritional supplement for protein synthesis while on the other hand, it is a source of osmo-active amino acids and peptides functioning to maintain turgor (Amezaga et al., 1995). Due to the presence of various peptone, amino acids, and nutrients in BHI, TSB-glu, and PPS used in our

study, we hypothesized that the presence of higher nutrient levels as well as osmolytes in BHI, TSB-glu, and PPS enhances the growth of both strains in 5.0% NaCl in comparison to their growth in MM with 5.0% NaCl. *L. monocytogenes* is known to be able to accumulate glycine betaine, carnitine and proline in response to osmotic stress (Beumer, Giffel, Cox, Rombouts, & Abee, 1994). HPLC results confirmed the presence of glycine betaine, carnitine, and proline in BHI, TSB-glu, and PPS. The HPLC results showed the BHI broth contains 0.24 mg/ml (2 mM) of glycine betaine which is in agreement with previous findings (Sleator, Gahan, & Hill, 2001; Smith, 1996). Several studies showed *L. monocytogenes* is capable of accumulating carnitine by importing it from BHI when grown in high salt levels (Angelidis & Smith, 2003b; Cetin et al., 2004; Smith, 1996). Two studies indicated that BHI broth contains carnitine (Gardan, Duche, Leroy, & Labadie, 2003; Raimann et al., 2009). From the HPLC analysis, the presence of carnitine in the BHI broth used in our study was detected at levels of 0.07 mg/ml (0.434 mM or 0.43 nmol/mg on wet basis). Smith (1996) used ^{13}C nuclear magnetic resonance (NMR) technique to determine the amount of carnitine in meat ranged from 0.23 to 0.95 nmol/mg (fresh weight) depended on different types of meat. Because the formula of the BHI broth used in our study contains brain and heart tissue from animals (Difco, BD Canada, Oakville, ON), the level of carnitine in BHI as determined by HPLC analysis is comparable to the results reported by Smith (1996). HPLC analysis confirmed the presence of proline in BHI which is in agreement with the amino acid analysis providing by the manufacturer. Due to the co-elution of proline and carnitine, carnitine concentrations were calculated by subtraction of the proline content obtained from the manufacturer of the bacteriological substrates. With that in mind, the HPLC protocol developed in our study is adequate to identify and quantify the presence of three osmolytes; glycine betaine, carnitine and proline. However, further refinement of the HPLC method and mass spectrometry for further confirmation of peak identity is recommended for future studies. The HPLC results showed that the content of glycine betaine is higher in TSB-glu than in BHI, whereas BHI contains more carnitine than TSB-glu. Soybean is a main component in TSB-glu (Difco, BD Canada, Oakville, ON). The fact that glycine betaine is a predominant osmolyte in plant tissues, whereas carnitine is a main osmolyte in animal tissues (Beumer et al., 1994) explains the observed difference in osmolyte contents. PPS contains glycine betaine at a significantly lower level ($p<0.05$) compared to BHI and TSB-glu (Table 24). PPS may contain carnitine or proline at levels below the detection limit of 0.005 mg/ml. This and the lower

nutrient levels may explain why the growth rates of both strains in PPS with 5.0% NaCl were significantly lower ($p<0.05$) than those in BHI and TSB-glu.

The reasons for the lack of significant differences ($p>0.05$) being observed between Lm568 and its Δ*sigB* mutant for all growth parameters in BHI, TSB-glu, PPS, and MM with 5.0% NaCl (Table 9), will be discussed in the following section.

Firstly, although energy stress response is known to be positively related to σ^B (Chaturongakul & Boor, 2004), the energy sources in BHI, TSB-glu, PPS, and MM were probably adequate and cells were not starved. Also, the presence of high levels of nutrients and osmolytes in BHI and TSB-glu may have masked any minor differences in growth kinetics. According to HPLC results by Dr. Tim Ells (Personal communication, Agriculture and Agri-Food Canada, Kentville, Nova Scotia), trehalose, a disaccharide osmoprotectant, is present in BHI in concentrations of 0.31 mg/ml. Ells and Truelstrup Hansen (2011) observed that a Lm568 with a mutated *treA*, which encodes a phospho-α-(1, 1)-glucosidase required for the hydrolysis of trehalose-6-phosphate, was able to accumulate exogenously-added trehalose during growth. This Δ*treA* mutant was found to be more resistant than Lm568 when incubated in BHI with 20% NaCl, presumptively due to high levels of intracellular trehalose. The study also pointed out that a secondary but less efficient transport system may exist for the accumulation of trehalose. Also, to date, no mechanism has been identified for listerial synthesis of trehalose. Also, BHI which as a rich growth media potentially contains an undefined mixture of different osmolytes, including peptides and amino acids, may cause the cells to experience stress levels which are not severe enough to activate the transcription of σ^B (Abram et al., 2008). Abram and colleagues (2008) found that only exposure to very high salt concentrations (10% NaCl) in BHI induced the activation of σ^B. In our study, the final population levels of both strains were significantly higher ($p<0.05$) (Table 9) in BHI with 5.0% NaCl than in TSB-glu and PPS with 5.0% NaCl. It is likely that BHI contains more nutrients and osmolytes to support growth of both strains in an environment with 5.0% NaCl. For the other nutrient-rich media, TSB-glu, trehalose was detected in levels of 0.072 mg/ml. The higher levels of glucose in TSB-glu (Table 24) serve as an energy source rather than source of osmolytes for *L. monocytogenes* (Smith, 1996). Due to the complexity of this media, it is possible that different types of osmo-active amino acids and peptides are present to create a less stressful growth environment for both strains even in the

presence of 5.0% NaCl. Similarly, the nutrient compositions of PPS and MM with 5.0% NaCl may not stress the cells enough to activate σ^B.

Secondly, it is known that the general stress proteins not under σ^B control contribute to the survival of *L. monocytogenes* at high salt concentrations (Gardan et al., 2003; Cotter, Emerson, Gahan, & Hill, 1999). During the study, Gardan and other researchers (2003) indicated that *clpP* encoding a protein belonging to the Clp family played a positive role in the osmotic tolerance of *L. monocytogenes*. The in-frame *clpP* deletion mutant exhibited a stress sensitive phenotype compared with its isogenic parent strain when submitted to salt stress conditions. A previous study of ClpP had indicated that the transcription of *clpP* is under the control of σ^A (Gaillot, Pellegrini, Bregenholt, Nair, & Berche, 2000). LisRK encodes a two-component regulatory system which allows target gene expression to be adapted to the changes in environments. This regulatory system is able to ensure optimal growth under the stress conditions, including osmotic stress shown to activate LisRK (Cotter et al., 1999). Thus, in BHI, TSB-glu, PPS, and MM with 5.0% NaCl, respectively, those σ^B independent stress proteins may be activated equally in Lm568 and the Δ*sigB* mutant cells to allow for similar growth rates and patterns.

Another explanation may be found in the fact that σ^B is not the only sigma factor involved in the osmoregulation of *L. monocytogenes*. Recently, another alternative sigma factor σ^L was found to facilitate the stress resistance of *L. monocytogene* when exposure to elevated NaCl concentrations (Raimann et al., 2009). A recent study of sigma factors in *L. monocytogenes* including σ^B, σ^C, σ^H, and σ^L applied transcriptomic and phenotypic analyses to identify regulons for each of these four transcriptional regulators (Chaturongakul et al., 2011). The results showed regulons for these four sigma factors exhibited a considerable overlap. Thirty one genes were regulated by both σ^B and σ^L, and 10 of these genes were co-regulated by σ^B, σ^L, and at least one additional regulator (Chaturongakul et al., 2011). According to this finding, regulons under σ^B control during the osmotic stress conditions may possibly also be controlled by σ^L, thus, these regulons during the osmotic shock may be still transcribed and subsequently translated to stress proteins to protect the cells even in the absence of σ^B. Further research is needed to investigate this hypothesis.

Lastly, growth in substrates with 5.0% NaCl, may rely on mechanisms of transporting or synthesising osmolytes in a σ^B-independent or only partly σ^B-dependent manner. Verheul and other researchers (1995) indicated the existence of a proton motive force-dependent di- and tri-

peptide transporter system in *L. monocytogenes* Scott A. This system was shown to have a broad substrate spectrum, especially with high affinity for various proline-containing peptides, which functioned as osmolytes to maintain turgor under the osmotic stress (Amezaga et al., 1995). Therefore, this transporter system plays an important role in osmoregulation during growth of *L. monocytogenes* at high osmolarity (Verheul et al., 1995). The regulation of the transporter system has recently been found to be also under the control of PrfA (Chaturongakul et al., 2011). For proline, one of the known osmolytes in *L. monocytogenes* (Beumer et al., 1994), sequence analysis of *proBA*, which encodes the proline biosynthesis system, has shown this operon has a single σ^A consensus promoter which is proximal to *proB* (Sleator et al., 2001). To date, no transport system for proline has been described in *L. monocytogenes* (Sleator et al., 2001). For glycine betaine and carnitine in *L. monocytogenes*, the intracellular accumulation of both osmolytes is achieved by uptake rather than by synthesis in *L. monocytogenes* (Sleator et al., 2001). The uptake of the two osmolytes is mediated via three transporters. BetL and Gbu are glycine betaine transporters, and OpuC is the main carnitine transporter (Cetin et al., 2004; Fraser et al., 2003; Wemekamp et al., 2002). σ^B of *L. monocytogenes* was shown to play an important role in regulating the expression of glycine betaine and carnitine transporters in osmotic stress conditions, since the Δ*sigB* mutant exhibited a reduced ability to use glycine betaine and carnitine compared with its isogenic wild-type strain (Becker, Cetin, Hutkins, & Benson, 1998). For *L. monocytogenes*, the study of the role of σ^B in osmoregulation by Cetin and other researchers (2004) showed that transcription of *betL* was regulated in a σ^B-independent manner, while *gbuA* was transcribed from dual promoters, where one is σ^B dependent and the other is σ^A dependent. However, the transcription of *opuC* was exclusively under the control of σ^B. Therefore, it is possible that the Lm568 Δ*sigB* mutant cells accumulated glycine betaine via the gbuA system from BHI, TSB-glu, and PPS in 5.0% NaCl to combat the osmotic stress, and thus grew as well as Lm568. Also, the nutrient- and osmolyte-rich properties of these media may mask the salt-stress sensitive phenotypes associated with inactivation of σ^B. Thus, such medium background makes it difficult to study the utilization of each osmolyte (glycine betaine, carnitine, and proline) by Lm568 and its Δ*sigB* mutant strains under high NaCl conditions.

The second growth experiment and transcriptional analysis using RT-PCR were conducted to (1) determine whether the presence or absence of 1 mM concentrations of glycine betaine, carnitine or proline had any impact on the growth patterns of both strains in a minimal

medium with 0.5% or 5.0% NaCl levels and to (2) study the role of σ^B in the protective effect of exogenous glycine betaine and carnitine. The concentration level of 1 mM osmolytes used in our study is comparable to the 1 to 10 mM osmolyte concentration range found to be normally present in muscle tissue (Beumer et al., 1994). RT-PCR results showed the *opuCA* transcription depended on σ^B in Lm568 indicating that the uptake of carnitine as an osmolyte may be impaired in the mutant strain when grown in MM in the presence of 5.0% NaCl. This result from the transcriptional analysis is consistent with the observation of the impaired growth of the Δ*sigB* mutant which exhibited significantly slower growth rates ($p<0.05$) and lower final population levels ($p<0.05$) upon entering into the stationary phase compared with the growth of Lm568 (Table 10). This finding is consistent with early reports which demonstrated the transcription of the carnitine transporter, OpuC, primarily depended on σ^B after an osmotic upshift (Cetin et al., 2004; Fraser et al., 2003; Chaturongakul & Boor, 2004; Chaturongakul et al., 2011). However, the addition of carnitine (1 mM) into MM with 5.0% NaCl enhanced ($p<0.05$) the growth of the Δ*sigB* mutant compared with its growth in MM with 5.0% NaCl and no carnitine. Our mutant still expressed *betL* and *gbuA* which would allow the Δ*sigB* mutant to uptake carnitine from the environment. Or the presence of exogenous carnitine may in itself exert an osmoprotective effect as was shown for glycine betaine (Dreux, Albagnac, Sleator, Hill, & Carlin, 2008). In their study, exogenous glycine betaine was found to increase survival during desiccation of a triple deletion mutant *L. monocytogenes* LO28 ΔBCG (Δ*betL* Δ*opuC* Δ*gbu*) which is incapable of importing glycine betaine. Exogenous or extracellular glycine betaine may therefore protect cells against desiccation.

In *Bacillus subtilis*, a majority of genes associated with stress response are usually under control of dual promoters in order to mediate flexible gene expression in response to rapidly changing environmental conditions. The transcriptions of only a few genes were solely σ^B dependent (Cetin et al., 2004). For *L. monocytogenes*, a recent study demonstrated many regulons under seven regulator proteins (σ^B, σ^C, σ^H, σ^L, PrfA) and the heat shock-related negative regulators (CtsR and HrcA) were co-regulated by more than one regulator (Chaturongakul et al., 2011). Fraser et al. (2003) observed a residual pool of carnitine in Lm10403S Δ*sigB* mutant cells cultured in MM in the presence of carnitine (1 mM) and 3% NaCl, although it was also shown that σ^B played an important role in the uptake of carnitine. Wemekamp and other researchers (2002) found a mutant with multiple deletions of *betL*, *gbu*, and *opuC* in a *L. monocytogenes*

LO28 background was capable of accumulating carnitine in BHI with 6.0% NaCl, indicating the presence of a possible fourth osmolyte transporter for carnitine. In the meanwhile, Angelidis and Smith (2002) indicated both Gbu and BetL can mediate carnitine uptake in response to osmotic stress. Gbu can transport carnitine regardless of the type of salt stress. However, BetL can mediate weak carnitine uptake in response to NaCl stress but not KCl stress. The RT-PCR results in our study showed expression of *gbu* and *betL* is observed in both strains when grown in MM with 0.5 and 5.0% NaCl, which is in agreement with the finding by Fraser et al. (2003). It is therefore possible that Gbu and BetL mediate the uptake of carnitine to improve the growth of the cells even in the absence of σ^B.

It is also seen that the growth of Δ*sigB* mutant was slower than that of Lm568 in MM in the presence of glycine betaine (1 mM) and 5.0% NaCl, although *gbu* and *betL* were transcribed in the Δ*sigB* mutant cells. Since the end-point RT-PCR method used in our study only qualitatively identified the transcription of the three genes in Lm568 and the Δ*sigB* mutant when grown in MM with 0.5% or 5.0% NaCl, real-time PCR is needed to further analyse to what degree the genes are inducible in the Δ*sigB* mutant compared with Lm568. As the expression of *gbu* was partially σ^B dependent (Cetin et al., 2004), loss of the sigma factor may lead the Δ*sigB* mutant cells to accumulate less glycine betaine from environments compared with Lm568. Also Fraser et al. (2003) observed glycine betaine accumulation was partly σ^B dependent in their study of the Gbu transporter alone when BetL was inactivated. During the growth of Lm10403S and its Δ*sigB* mutant in MM with glycine betaine (1 mM) and 3.0% NaCl, the wild type cells were found to take up glycine betaine faster ($p<0.05$) than the Δ*sigB* mutant cells. The results from our study also showed the exogenous glycine betaine significantly improved ($p<0.05$) the growth of the Δ*sigB* mutant cells in the high osmolarity condition (Table 10), indicating the uptake and/or presence of glycine betaine as an osmolyte can enhance the growth of *L. monocytogenes* in the absence of σ^B at high osmolarities.

For the Δ*sigB* mutant cells grown a low osmolarity, all three genes (*gbuA*, *betL*, and *opuCA*) were transcribed independently of the σ^B-deletion. Use of a quantitative real-time PCR technique to compare the transcription levels of each gene in Lm568 and its Δ*sigB* mutant would determine, if the 30% reduction in the Lm10403S Δ*sigB*'s *opuCA* transcription observed by Fraser and other researchers (2003) at low osmolarity conditions compared to the wild type level, would also be seen for the Lm568 Δ*sigB* mutant. Lm568 expressed *gbuA*, *betL*, and *opuCA*

during growth at both low and high osmolarities, and addition of exogenous glycine betaine and carnitine improved the growth of Lm568 in MM regardless of the salt content. Moreover, in MM with 0.5% NaCl, addition of glycine betaine and carnitine enhanced the growth of Lm568 and its Δ*sigB* mutant to the same extent with the same growth rate and maximum density level, indicating the deletion of *sigB* has no effect on the uptake of glycine betaine and carnitine in MM with low osmolarity. Glycine betaine and carnitine were equally effective in improving Lm568 growth under low or high osmotic stress conditions, which is consistent with the report by Beumer et al. (1994). However, for the Δ*sigB* mutant, although it seems glycine betaine and carnitine both stimulate growth in low osmolarity MM, glycine betaine was more effective than carnitine at improving its growth in the same substrate with high osmotic-strength conditions. This difference is probably due to the absence of *opuC* (main carnitine transporter) expression in MM with 5.0% NaCl.

In the present study, it is also found that 1 mM exogenous proline did not function as an osmolyte for any of the strains during growth in MM ± 5.0% NaCl. Another study also indicated that proline at the same concentration had no protective effect on *L. monocytogenes* (Patchett et al., 1992). Patchett et al. (1992) hypothesised a low-affinity uptake system for proline may exist in *L. monocytogenes*. Much higher proline concentrations (10 mM) were found by Beumer et al. (1994) to significantly improve the growth of *L. monocytogenes* in high osmotic-strength conditions in MM. Also, 10 mM proline was required to complement the *L. monocytogenes* mutant wherein the proline biosynthesis system had been deleted (Sleator et al., 2001). The two observations may be attributed to the presence of a low-affinity uptake system for proline in *L. monocytogenes*. The other possibility for those two observations is that the osmolyte effect on a per molar basis is less efficient for proline than for the other osmolytes or some of the proline gets diverted and used for protein synthesis. In food, proline plays a minor role as an osmoprotectant since free proline is available only in small amounts and the majority of proline is present in proteins (Beumer et al., 1994). In addition, our study showed that the deletion of *sigB* had no impact on the growth of *L. monocytogenes* in MM with proline at low or high NaCl concentrations.

5.4 Kinetics of the Desiccation Inactivation of the Wild Type Lm568 and Its Δ*sigB* Mutant

5.4.1 Effect of Different Desiccation Substrates on Survival During Desiccation on Stainless Steel coupons at 15 °C and 43% Relative Humidity

Recently, it was reported that *L. monocytogenes* is capable of surviving on a stainless steel food grade contact surface in a dry environment for extended periods of time (up to three months) (Vogel et al., 2010). This potentially presents a risk of foods becoming cross-contaminated, especially ready-to-eat foods, when coming in contact with contaminated surfaces. The role of σ^B as a stress response regulator during desiccation or matric stress has not previously been studied in *L. monocytogenes*, but a recent report indicated σ^B is involved in the desiccation tolerance of *S. aureus* (Chaibenjawong & Foster, 2011). To investigate whether the σ^B is involved in the persistence of *L. monocytogenes* on SS surfaces under low RH conditions, the desiccation kinetics of Lm568 and its Δ*sigB* mutant was compared in our study. In order to simulate realistic food production conditions, 15 °C and 43% RH, and organic material rich or poor desiccation substrates were chosen as the experimental desiccation conditions to probe the kinetics of desiccation inactivation of both strains over a 21 day period. Stationary-phase cells from both strains were used to study the role of σ^B in the desiccation tolerance of *L. monocytogenes*, since stationary-phase cells strongly express σ^B in response to various stresses (Hecker et al., 2007). In our study, the expression of σ^B- dependent phenotypes depended on the desiccation substrate. It was only in PPS that the non-osmoadapted Δ*sigB* mutant cells, which were desiccated with a low initial NaCl level, were found to be more sensitive to desiccation compared with Lm568. HPLC results showed the osmolytes content was lower in PPS compared to BHI and TSB-glu. Thus the combination of osmolyte- and nutritent-limited conditions in PPS probably meant that mutant cells became impaired in their response to the desiccation stress, which typically involves osmolyte transporters and osmotic stress proteins to protect cells (Potts, 1994). Since glycine betaine accumulation is partially σ^B-dependent under high osmolarities (Fraser et al., 2003), the Lm568 Δ*sigB* mutant may accumulate less glycine betaine in PPS compared with its wild-type strain, thus resulting in rapid loss in viability during the initial desiccation period. Also, the σ^B-dependent osmotic stress proteins RelA and Kpd may be absent in the desiccated Δ*sigB* mutant cells in PPS. Therefore, the desiccation-stress sensitive

phenotypes of the Δ*sigB* mutant cells were observed in the initial phase of the experiment. In the study of desiccation tolerance in *S. aureus*, the Δ*sigB* mutant displayed a significantly ($p<0.05$) higher sensitivity to desiccation in phosphate-buffered saline (PBS) relative to its wild-type strain after air-dying for 4 and 31 days at 25 °C (Chaibenjawong & Foster, 2011). However, in our study, the final survivor levels after 21 days in PPS were not significantly different ($p>0.05$) between Lm568 and the Δ*sigB* mutant (Table 13), and survivors for both strains stayed at stable levels from 14 to 21 days (Figures 9 c), and 23 c)). This may indicate σ^B is involved in the initial desiccation period, while a σ^B-independent or only partly σ^B-dependent mechanism may contribute to the long-term viability of *L. monocytogenes*. Exposure to desiccation stress for 21 days in PPS may also have evoked starvation or energy stress due to loss of water and lack of carbon sources in PPS. The long-term viability of *L. monocytogenes* L61 (serotype 1/2a) during exposure to energy stress was reported to be σ^B-independent by Moorhead et al. (2003). Moreover, large changes in cell metabolism probably occurred during desiccation, thus leading to oxidative stress within the cell (Chaibenjawong & Foster, 2011). As discussed above σ^B is known to be partly involved in the oxidative stress response for *L. monocytogenes* (Section 2.3.1).

The desiccation kinetics of the Δ*sigB* mutant cells followed a pattern similar to that of Lm568 in both BHI and TSB-glu over 21 days. The presence of high levels of nutrients and osmolytes in both desiccation substrates may have masked the desiccation-sensitive phenotypes associated with the deletion of *sigB*. In BHI and TSB-glu, the high levels of proline (2 mg/ml or 20 mM) may compensate the absence of *sigB*, since proline can function as an osmolyte to enhance the osmotic tolerance of *L. monocytogenes* at concentrations above 10 mM (Sleator et al., 2001; Beumer et al., 1994). The σ^B-independent glycine betaine transporter, BetL, appeared to be responsible for the maximum uptake of glycine betaine in the initial period during the increased osmotic shock (Mendum, 2002). The uptake of glycine betaine in the Δ*sigB* mutant via BetL may protect the cells in order to reduce the loss of viability during the initial desiccation period and thus exhibit a similar death rate comparable to that of Lm568 in the first log reduction period (Table 13).

Trehalose, as a nonreducing sugar, has been reported to function as osmolyte in many studies (Breeuwer, Lardeau, Peterz, & Joosten, 2003; Chaibenjawong & Foster, 2011; Stroem, 1993; Welsh, 1999). Chaibenjawong and Foster (2011) indicated exogenous trehalose improved

the desiccation tolerance of *S. aureus* in PBS following air-drying at 25 °C for one month. Breeuwer and co-workers (2003) found the stationary-phase cells of *Ent. sakazakii* (now *Cronobacter sakazakii*) subjected to desiccation were more resistant than exponential-phase cells. They determined that significantly higher amounts of intracellular trehalose in the stationary-phase cells partly contributed to this difference. In *E. coli*, intracellular accumulation of trehalose, which was induced by osmotic stress, promotes its desiccation tolerance (Stroem, 1993; Welsh, 1999). Very few studies of trehalose in *L. monocytogenes* have been reported. The bacteria does not normally accumulate trehalose intracellularly as the disaccharide is imported as trehalose-6-phosphate by a PTS system (*treB*), and then split into glucose and glucose-6-phosphate by phosphotrehalase, the product of *treA* (Ells & Truelstrup Hansen, 2011). Although these authors observed that intracellular accumulation of trehalose in a Δ*treA* mutant significantly improved the desiccation tolerance and Potts (1994) described how extracellular trehalose also protected bacteria under desiccation, in our study the trehalose present in BHI and TSB-glu would be expected to serve as a food source with no effect on desiccation sensitivity.

The σ^B-independent di- and tri-peptide transporter systems, which contribute to enhanced growth of *L. monocytogenes* at high osmolarities (discussed in 5.3), may cause the Δ*sigB* mutant cells to exhibit the same inactivation kinetics as Lm568 during desiccation for 21 days. Due to the higher levels of solid content in BHI and TSB-glu versus the lower level of solid content in PPS, the drying time may be prolonged as the higher solid contents hold on to the water. Experimentally in the equilibrated state between substrates and air when conditioned at 43% RH, the absolute moisture contents of the BHI and TSB-glu substrates were observed to be higher than in PPS (Figures 12, 13, and 14). Together the combination of a slower drying rate and higher final moisture content in BHI and TSB-glu compared with PPS may have contributed to erasing any differences in inactivation between Lm568 and its Δ*sigB* mutant during desiccation for three weeks in the nutrient rich substrates.

Air-drying caused Lm568 and its Δ*sigB* mutant cells to lose viability, but the size of the reduction was highly dependent on desiccation substrates. BHI appeared to protect the strains the most during desiccation, followed by TSB-glu and then PPS (Table 13). The high levels of nutrients and mixture of known and unknown osmolytes in BHI may have provided the cells with the most effective protection against desiccation. In BHI, proteins from the animal tissue may have contributed to the desiccation resistance. In the study of desiccation tolerance of *S.*

aureus by Chaibenjawong and Foster (2011), they observed addition of 3% (w/v) Bovine serum albumin (BSA) into PBS resulted in 15-fold protection compared with the cells suspended in PBS only. Studies of survival of *Acinetobacter* spp. on dry surfaces showed the survival in BSA was prolonged compared to in distilled water (Jawad, Heritage, Snelling, Gascoyne, & Hawkey, 1996). In addition, the four times higher concentration of trehalose in BHI than in TSB-glu (Table 24) may partly contribute to the higher desiccation protective effect observed in BHI than in TSB-glu. Chaibenjawong and Foster (2011) observed the presence of 100 mM trehalose in PBS has more effective protection effect than the presence of both 10 mM and 1 mM trehalose in PBS for the desiccation tolerance of *S. aureus*. The higher levels of proline-containing peptides in BHI (Amezaga et al., 1995) may also be partly involved in the desiccation protective effect observed. *L. monocytogenes* contains a peptide transporter system with high affinity for various proline-containing peptides (Verheul et al., 1995). We also found both strains survived better in TSB-glu during desiccation for 21 days than in PPS. This is consistent with the previous finding for *L. monocytogenes* N53-1 (Vogel et al., 2010). Similarly, *Salmonella enteritis* was reported to be more desiccation resistant in TSB than in PBS (De Cesare, Sheldon, Smith, & Jaykus, 2003) . In the TSB-glu substrate, the presence of glucose (dextrose) and other nutrients as energy sources, and osmolytes such as carnitine, proline, and trehalose, which were absent or present at undetectable levels in PPS, may explain the enhanced survival of both strains in TSB-glu compared with PPS.

A protective effect of osmoadaptation was observed for both strains regardless of the initial desiccation salt levels applied during desiccation for 21 days in TSB-glu and PPS. This is consistent with a previous report demonstrating that prior to desiccation, osmoadaptation improved the survival of *L. monocytogenes* during long-term desiccation period (Truelstrup Hansen & Vogel, 2011; Vogel et al., 2010). When cells of both strains were exposed to 5.0% NaCl before desiccation, the σ^B-independent or only partly σ^B-dependent mechanisms associated with osmotic stress may have become activated, including accumulation of osmolytes via transporters or *de novo* synthesis systems for the synthesis of osmotic stress related proteins. Thus, the pre-osmoadapted cells were more resistant to another form of osmotic stress, namely desiccation or matric stress. Welsh and Herbert (1999) observed that osmoadaptation prior to desiccation improved the desiccation tolerance of *E. coli* as the previous osmotic shock induced intracellular accumulation of trehalose. This process is most likely an example of cross-

resistance. Several studies have demonstrated that a bacterium, which is exposed to one stress, may become more resistant to subsequent applications of different or identical stresses (Herbert & Foster, 2001; Hill, Cotter, Sleator, & Gahan, 2002; Welsh, 1999). Previous exposure of *L. monocytogenes* to nutrient-depleted conditions was found to increase its resistance to heat shock (Herbert & Foster, 2001). Moreover, the pre-adaptation of *L. monocytogenes* cells to mild acid stress (pH 5.5) improved their survival during subsequent exposure to severe acid stress at pH 3.5 (Hill et al., 2002).

Desiccation with a high initial NaCl level increased the desiccation tolerance of Lm568 and its Δ*sigB* mutant. This observation is in agreement with recent reports (Truelstrup Hansen & Vogel, 2011; Vogel et al., 2010), where *L. monocytogenes* N53-1 (persistent serotype 1/2a strain, environmental food plant isolate) cells desiccated with a high initial NaCl level (5.0%) were more resistant to the desiccation stress. According to Vogel and co-workers (2010), *L. monocytogenes* suspended in substrates with 5.0% NaCl was able to auto-aggregate and strongly attach to the plastic surface, thus, this characteristic in combination with the salt stress response may explain the protective effect of the high initial NaCl level (5.0%) during desiccation. Our microscopic results also showed both Lm568 and the Δ*sigB* mutant cells aggregated and formed long chains during growth in 5.0% NaCl (Figure 6). Also, we found that the addition of 5.0% NaCl to desiccation substrates may have reduced the rate of air-drying, thus providing bacteria with more time to adjust their metabolic process to adapt to the desiccation stress (Figures 12, 13, and 14). Fuster et al. (2007) observed slow air-drying reduced the survival level of *Enterobacter cloacae* to below the detection level within 24 hours, however, under rapid air-drying conditions, the survival of *Ent. cloacae* decreased rapidly to undetectable levels within 2 hours. They suggested that loss of water from cells over a short time may damage cell membranes in an irreversible way until death. Among the four treatments (± osmoadaptation and ± desiccation with a high initial NaCl level) at 15 °C and 43% RH for 21 days, the combination of osmoadaptation and desiccation with a high initial NaCl level resulted in the highest number of survivors of Lm568 and its Δ*sigB* mutant, whereas non-osmoadapted cells desiccated with the a low initial NaCl level were the most vulnerable to exposure to the desiccation stress for 21 days.

Interestingly, BHI as a desiccation substrate improved the survival of both strains so much as to mask the effect of osmoadaptation, desiccation with a high initial NaCl level, and the combination of the two factors. This is probably due to the abundance of nutrients and osmolytes

in BHI. The importance of nutrient richness was similarly reported by Kim et al. (2008). According to their studying, desiccation survival of *Ent. sakazakii* on SS coupons at 23-85% RH and 25 °C for 42 days, was more influenced by the nutrient-richness of the desiccation substrate (infant formula broth) than by biofilm formation, which has been previously shown to enhance desiccation tolerance of *L. monocytogenes* (Truelstrup Hansen & Vogel, 2011), *Rhizobium leguminosarum* (Vanderlinde et al., 2010), and *Pseudomonas putida* (Chang et al., 2007).

Since osmoadaptation and desiccation with a high initial NaCl level were demonstrated to protect cells during desiccation in TSB-glu and PPS, this appears to be an example of cross-resistance caused by the application of the hurdle technology which is commonly applied in the food industry as a preservative method. Therefore, when designing a hurdle technology process which combines the application of multiple sub-lethal stresses to the microflora in foods, the built-up of cross-resistance should always be avoided to ensure processed foods are safe for human consumption.

5.4.2 Effect of Osmoadaptation, Osmolytes, and Desiccation Salt on Survival of Lm568 and Its Δ*sigB* Mutant During Desiccation on Stainless Steel Coupons in MM at 15 °C and 43% Relative Humidity

To determine whether pre-culture with osmolytes would improve the desiccation survival of Lm568 and its Δ*sigB* on SS coupons at 15 °C and 43% RH for 21 days, MM with 0.5% or 5.0% NaCl and the addition of one of the osmolytes (1 mM, glycine betaine, carnitine or proline) was used as the growth substrate, while MM with 0.5% (Low) or 5.0% NaCl (High) was used as a desiccation substrate. The results from the control treatments, where previously non-osmoadaptated and osmoadaptated cells of both strains were desiccated with a low or high initial NaCl level, showed the osmoadaptation and desiccation with a high initial NaCl level increased desiccation tolerance of both strains. This result is in accordance with the results observed in BHI, TSB-glu, and PPS and discussed in section 5.4.1. Dreux et al. (2008) also demonstrated osmoadaptation and desiccation with a high initial NaCl level improved the desiccation survival of *L. monocytogenes* on parsley leaves. Moreover, they observed pre-osmoadapted cells when desiccated in MM with 3.0% NaCl were more resistant to the desiccation stress than non-osmoadapted cells during exposure to desiccation in MM at 48% RH for 4 days.

For Lm568, modelling of its desiccation kinetics indicated pre-culture with glycine betaine, carnitine, or proline enhanced desiccation survival over the 21 days, irrespectively of osmoadapatation and initial desiccation salt levels. RT-PCR results showed the three osmolyte transporters were activated in Lm568 during the growth at both 0.5% and 5.0% NaCl in MM. Thus, the cells were able to accumulate glycine betaine or carnitine when it was available in MM during growth, and subsequently become better adapted to the desiccation stress. Also, Dreux et al. (2008) observed the protective effect of glycine betaine against desiccation stress is independent of intracellular glycine betaine accumulation by the known uptake systems. Kets et al. (1994) reported that lactic acid bacteria (LAB) with intracellular accumulation of osmolytes (glycine betaine or carnitine) were more resistant to drying. The uptake of proline by a low-affinity proline transporter, which was hypothesised by Patchett et al. (1992) and Sleator et al. (2001), may contribute to the enhanced survival during desiccation. Under desiccation conditions, since large changes in cell metabolism may occur, it is possible that small amounts of proline accumulated via its low-affinity transporter during growth may play a protective role during desiccation stress, although proline at 1 mM did not improve the growth of Lm568 in MM with 0.5% or 5.0% NaCl. The osmolytes' protective effect was only observed during the first log reduction period for osmoadaptated cells desiccated with a high initial NaCl level, since the final survivor counts after 21 days stayed at the same level as the survivors in the control group (Table 14). This indicated the osmoadaptation and desiccation with a high initial NaCl level alone contributed to the long-term survival during desiccation conditions. During the initial desiccation period, pre-culture with glycine betaine played a larger role for desiccation survival of Lm568, regardless of the osmoadaptation and desiccation salt treatment, than carnitine or proline, which were equally protective. Glycine betaine has previously been reported to be the preferred and most effective osmolyte for *L. monocytogenes* (Sleator, Francis, O'Beirne, Gahan, & Hill, 2003; Smith, 1996; Wemekamp et al., 2002) during exposure to solutions with elevated osmotic pressure, however, its importance during desiccation survival has not previously been shown. The preference of *L. monocytogenes* for glycine betaine in osmotic adaptation has not been fully explained. According to Smith (1996), during a study of the kinetics of osmolyte transport in *L. monocytogenes*, the uptake rate for glycine betaine was determined to be significantly higher than that for carnitine at high osmolarity. Glycine betaine has also been found to be a more effective osmolyte than carnitine in *E. coli* (Peddie, Lever, Hayman, Randall, & Chambers,

1994). These workers indicated that glycine betaine is a more effective stabilizer of protein structure compared with carnitine and the longer carbon chain of carnitine decreases its osmoprotective function.

The presence of two, glycine betaine and carnitine, of the three osmolytes in the pre-culture substrate similarly protected the Δ*sigB* mutant during the subsequent desiccation trial while pre-culture with proline had no impact on its survival during desiccation with a low initial NaCl level. The ability of mutant to accumulate glycine betaine or carnitine (discussed in section 5.3) during the pre-culture phase may have contributed to their enhanced survival during desiccation. Based on studies of σ^B in *B. subtilis*, it is hypothesized that under low osmolarity, the transcription of the low-affinity proline transporter may be under the control of σ^B, thus explaining why proline pre-culture did not protect the mutant. In *B. subtilis*, where the regulatory components of the σ^B system are very similar to those found in *L. monocytogenes* (Hecker & Volker, 2001), the σ^B-dependent *opuE* gene in *B. subtilis* was found to encode the proline transporter (Hecker et al., 2007). However, in *L. monocytogenes* a protective effect of pre-culture with proline was observed for Δ*sigB* cells desiccated with a high initial NaCl level after prior osmoadaptation. Higher osmolarity may have activated one of the other regulator systems, such as σ^L which has been shown to be induced during osmotic shock (Raimann et al., 2009). It is possible that the transcription of the proline transporter may also be controlled by one of the other sigma factors, which are also activated under the osmotic stress. Identification of overlaps among the regulons for the alternative sigma factors and other regulatory systems in *L. monocytogenes* have made it clear that a complex regulator network exists to control the organism's gene expression in response to changing environmental conditions. In *B. subtilis*, the σ^B-dependent proline transporter is also controlled by σ^A in response to osmotic stress (Hecker et al., 2007).

The application of glycine betaine during the pre-culture provided both the parent and Δ*sigB* mutant strains with the most protection against desiccation under all treatments. Beside the faster uptake rate of glycine betaine observed by Smith (1996) in *L. monocytogenes*, the absence of σ^B may have led to higher levels of intracellular glycine betaine in the Δ*sigB* mutant compared to intracellular levels of carnitine where import may have been impaired due to the σ^B-dependence of the OpuC carnitine transporter under the osmotic shock.

The protective effect of pre-culture with the osmolytes were only observed during the initial desiccation period for osmoadapted cells desiccated with a high initial NaCl level, since after 21 days, the survivors from all three osmolyte treatments attained the same levels as the survivors from the control group (Table 15). This indicates the osmoadaptation and desiccation with a high initial NaCl level also in the Δ*sigB* mutant contributed to desiccation resistance.

5.4.3 Protective Effect of Osmolytes in the Desiccation Substrate During Desiccation of Lm568 and Its Δ*sigB* Mutant on Stainless Steel Coupons in MM at 15 °C and 43% Relative Humidity

During desiccation for 21 days, the addition of exogenous glycine betaine, carnitine, or proline to the desiccation substrate enhanced the survival of both strains during the initial desiccation period for non-osmoadaptated cells which were desiccated with a low initial NaCl level. Each osmolyte protected cells most during the first 2 days, after which its protection slowly decreased to zero after 21 days. It indicates that during the beginning of the desiccating period, the cells from both strains might have been able to take up glycine betaine, carnitine, or proline in order to combat the desiccation shock, however, after 21 days, survivor counts for both strains reached the same levels regardless of whether they had been treated with osmolytes or not. Dreux et al. (2008) indicated exogenous glycine betaine during desiccation at 60% RH improved the survival of *L. monocytogenes* on parsley leaves in a concentration-dependent manner. But, after 4 days, the difference in survival between the exogenous osmolyte treated and controls was no longer significant. Additional survival mechanisms may be involved during the long-term desiccation survival of both strains, such as the two-component regulatory system LisRK, which is involved in the osmosensing and osmoregulation of *L. monocytogenes*, and *htrA*, which is associated with osmotolerance and under the transcriptional control of LisRK (Sleator & Hill, 2005). Or, the osmolytes may have become degraded in the desiccation substrate after 21 days.

Addition of exogenous glycine betaine, carnitine, or proline to the desiccation substrate with a high initial NaCl level also enhanced the initial desiccation tolerance of osmoadapted Δ*sigB* mutant cells. The protective effect of each osmolyte on Δ*sigB* mutant cells lasted for 14 days, however, for the wild-type it was only glycine betaine that enhanced survival under the same conditions. The rate of uptake for glycine betaine has previously been reported to be seven-

fold faster than the uptake rate for carnitine for *L. monocytogenes* exposed to high osmolarity (8% NaCl) (Smith, 1996). In our study, where the combination of desiccation or matric stress (43% RH) and high osmolarity (5.0% NaCl) is likely to be a more severe form of osmotic stress, it may be hypothesized that the difference in uptake rates may have caused carnitine to be accumulated in insufficient amounts to improve the survival of Lm568 during the initial desiccation period. A similar explanation may be applied to proline under the same conditions. Glycine betaine protected Lm568 cells in the first 14 days, and showed negligible protection after 21 days.

As discussed above, desiccation survival of osmoadapted Lm568 Δ*sigB* mutant cells in MM with 5.0% NaCl were initially enhanced by all three osmolytes, and other σ^B-independent carnitine or proline transporter may have transported carnitine or proline from the desiccation substrate into the Δ*sigB* mutant cells. Based on the observation that an *E. coli* Δ*sigS* mutant grew faster than the parent strain in the nutrient limiting conditions (King, Seeto, & Ferenci, 2005; Notley, King, & Ferenci, 2002), Nystrom (2004) hypothesized that competition exists between sigma factors in *E. coli*. In *E. coli*, the σ^{70}, which is the housekeeping sigma factor, is involved in the control of growth-related functions, whereas σ^S as a general stress response sigma factor responsible for the stress response (Nystrom, 2004). Under conditions of nutrient-limitations, the σ^S functions as the main stress regulator to inhibit σ^{70} in the *E. coli* wild-type strain. However, in the absence of σ^S, σ^{70} responds to the nutrient-limitation stress by transcribing housekeeping genes linked to growth functions which in turn allows the Δ*sigS* mutant cells to grow faster than the wild type (Nystrom, 2004). Similarly, faster growth rates were observed for *L. monocytogenes* Δ*sigB* mutants compared with the growth rates of the parent strain during growth in both a complex medium at 3 °C and chemically defined medium with 3% NaCl at 37 °C (Abram et al., 2008; Brondsted, Kallipolitis, Ingmer, & Knochel, 2003). In *B. subtilis*, cells lacking *sigB* also grew faster than the wild-type cells under glucose-limited conditions (Schweder, Kolyschkow, Volker, & Hecker, 1999). In our study, the difference between the wild type and mutant strain may be related to the balance between the sigma factors being interrupted.

At the end of the 21 day desiccation period, the survivors of each strain in all three osmolytes treatments stayed at the same levels as the survivors in the control group, indicating additional survival mechanisms unrelated to osmolytes may be involved in the long-term desiccation survival of both strains.

SigB improves the desiccation survival of Lm568 in MM regardless of the desiccation conditions. Modeling of the inactivation kinetics (Tables 17, 18, 21 and 22) consistently showed that the Δ*sigB* mutant was more sensitive to the desiccation stress treatments compared to its parent strain. As MM only contains the basic nutrients required for listerial growth (Premaratne et al., 1991), cells would be exposed to both starvation and desiccation stress. The combination of these stresses provides a more adverse condition, which may fully activate the σ^B regulon to better combat the adverse conditions. Hecker and Völker (2001) indicated under standard laboratory conditions, neither energy depletion nor physical stress alone can trigger complete activation of the σ^B regulon in *B. subtilis*, since a full induction of the σ^B regulon can be achieved when the combination of a mild physical stress and energy depletion is applied. If this observation is extended to the activation of σ^B regulon in *L. monocytogenes*, it may explain the desiccation-sensitive phenotypes of the Lm568 Δ*sigB* mutant in PPS (Section 5.4.1) and MM, but not in BHI-glu and TSB which are nutrient-rich medium (Section 5.4.1). The results in MM showed that the role of σ^B is more pronounced for osmoadaptated cells which are subsequently desiccated with a high initial NaCl level in combination with osmolytes during pre-culture or in the desiccation substrate. Under those treatments, the initial death rates of the Δ*sigB* mutant cells were greater than those of Lm568 cells, and after 21 days, the survivors of the Δ*sigB* mutant cells were lower than those of Lm568. Although σ^B-independent regulatory systems may become activated to aid in the survival of the Δ*sigB* mutant cells during those treatments, they are unable to restore the survival of the Δ*sigB* mutant to the same level as that of the wild-type strain. However, for cells pre-cultured and desiccated in MM with 0.5% NaCl with either pre-culture with glycine betaine or carnitine or desiccation with each of the three osmolytes, σ^B is mainly involved in the initial desiccation period, since the survivors of the Δ*sigB* mutant cells reached the same levels as the parent Lm568 after 21 days. It illustrates that a σ^B-independent regulatory mechanisms may be involved in the long-term survival of both strains in response to desiccation stress.

Generally, the number of survivors for both strains remained at stable levels between day 14 and 21, with the lowest survivor level of approximately 10^5 CFU/cm^2 being attained by the Δ*sigB* mutant. It leaves an important question as to what factors besides σ^B enable *L. monocytogenes* to survive desiccation for three weeks (and longer) at such high levels? Since bacteria may change their normal morphology and physiological activity during drying, a study

of *Salmonella enterica* susp. *enterica* ser. Enteritidis at reduced water activity indicated that exposure to reduced water activity induced filamentation of the cells. The formation of filamentation may contribute to their elevated tolerance to drying stress, since filamentous cells maintained their membrane integrity after exposure to drying (Kieboom et al., 2006). At this point it is not known whether *L. monocytogenes* forms filaments when desiccated, however, future studies of the morphology using for example SEM may shed light on this and provide us with more clues to understanding desiccation tolerance in *L. monocytogenes*.

The high initial population levels of 10^8 CFU/cm^2, which was inoculated on SS coupons to investigate the desiccation kinetics of both strains, could possibly lead to the release of nutrients from death or injured cells to sustain the remaining survivors, thus maintaining of the long-term viability of the remaining cells (Herbert & Foster, 2001). Different inoculation levels should be used in a future study to investigate whether the high level of inoculation contributes to the high amount of survivors after 3 weeks. In a recent study of desiccation tolerance of *L. monocytogenes* by Hingston and colleagues (2011), they found that the initial cell density level of *L. monocytogenes* has no significant impact on desiccation survival kinetics, however, biofilm cells, which reached a maximum cell density levels after 48 hours at 100% RH, appeared to harbour an increased resistance to desiccation. This is contrary to the study of desiccation tolerance in *S. aureus* where higher initial cell densities were found to provide greater survival during desiccation (Chaibenjawong & Foster, 2011). Another study of the desiccation survival of *S.* Enteritidis and *S. aureus* on SS coupons similarly showed the death rates were slower for higher inoculation levels than for lower inoculation levels when air-drying at room temperature (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). These apparent discrepancies may be due to real differences between prokaryotes from different genera, variations between the experimental conditions or methods by which the desiccation survival kinetics were analysed.

CHAPTER 6. CONCLUSION

The persistence of *L. monocytogenes* on food contact surfaces potentially increases the risk of cross-contamination of food products with this pathogen, especially ready-to-eat foods. In the food industry, this risk has been considered to be reduced by keeping the surface of food processing equipment dry, as it was assumed that the survival of bacteria would be reduced. However, our study showed Lm568 is able to withstand desiccation stress on SS coupons for an extended period (≥21 days), thus increasing the risk of cross-contamination of foods coming into contact with dry contaminated surfaces in the food industry.

Deletion of *sigB* reduced *L. monocytogenes*' survival during desiccation in the osmolyte- and nutrient-limited substrates (PPS and MM) but not in the osmolyte- and nutrient-rich substrates (BHI and TSB-glu). This finding led us to conclude that additional σ^B-independent survival mechanisms may be involved in the long-term survival of Lm568 and its Δ*sigB* mutant. Also, osmoadaptation during growth or desiccation with a high initial NaCl level, which reflects salt concentrations commonly used in food preservation, consistently increased the desiccation survival of both strains. The combination of osmoadaptation during growth and desiccation with a high initial NaCl level further enhanced both strains' survival on SS coupons. Protective mechanisms evoked by osmoadaptation are therefore likely to be independent of the SigB regulon.

When designing a cleaning and sanitation protocol for food contact surfaces, cells recently introduced and deposited on surfaces can be easily killed by the recommended concentration of disinfectants. However, cells not removed during the cleaning and sanitation process have the potential to grow and/or persist when exposed to stress conditions, such as low relative humidity and nutrient contents. Our study found that the growth kinetics of the wild type and the Δ*sigB* mutant did not differ from each other over a wide range of nutritional growth conditions at 15 °C. The quantity of the bacterium in the food processing environment can be seen as a balance between new introduction, growth, and survival on one hand, and removal, inhibition, and death on the other hand. Consequently, rational design of cleaning and sanitation programs should include considerations of the frequency and efficiency of activities to eliminate *Listeria* on food contact surfaces in light of its ability to grow and survive once introduced to the food plant.

Our results showed osmolytes such as glycine betaine, carnitine, and to a lesser extent, proline, play important protective roles during growth and survival of Lm568 and its Δ*sigB* mutant when subjected to osmotic and matric stresses. The RT-PCR results suggested that σ^B is involved in the activation of one of the carnitine transport systems under the high osmotic stress conditions. Pre-culturing with osmolytes prior to desiccation protected both strains. Similarly, the presence of osmolytes during desiccation increased both strains' survival. The addition of glycine betaine during pre-culturing or desiccation provided the most effective desiccation protection of both strains. Thus, foods with a high content of osmolytes, when preserved with NaCl and/or processed by air-drying, may inherently increase the survival and possible persistence of *L. monocytogenes*.

In conclusion, σ^B is partly involved in the desiccation survival of Lm568 on SS coupons for 21 days; however, additional σ^B-independent mechanisms may play important roles for the long-term (more than 21 days) desiccation survival.

Future studies should be designed to study the changes in morphology of *L. monocytogenes* during desiccation to help us understand more about the mechanisms of desiccation survival for this pathogen. Thus, the morphological changes during desiccation should be investigated by SEM and other advanced microspectroscopic methods (e.g., attenuated total reflection FTIR and confocal Raman microspectroscopy). Genetic mechanisms of importance for survival other than SigB should also be investigated to elucidate how far the similarities between osmotic and matric stress reach. The application of the chemical agents and physical forces as part of the cleaning protocol for the food contact surfaces are relatively effective methods to remove bacteria which persist on surfaces. However, in this context, an understanding of the kinetics of biofilm formation and desiccation survival would be helpful for the development of effective sanitation strategies including the frequency with which it is necessary to take food processing equipment apart for "deep" cleaning. Finally while salting or drying are effective food preservation methods to reduce the survival and/or to inhibit the growth of bacteria in foods, it is important to understand the impact of endogenous osmolytes in foods. In the future, perhaps strategies to prevent intracellular accumulation of osmolytes in bacteria can be pursued to extend shelf-life and enhance food safety.

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APPENDIX

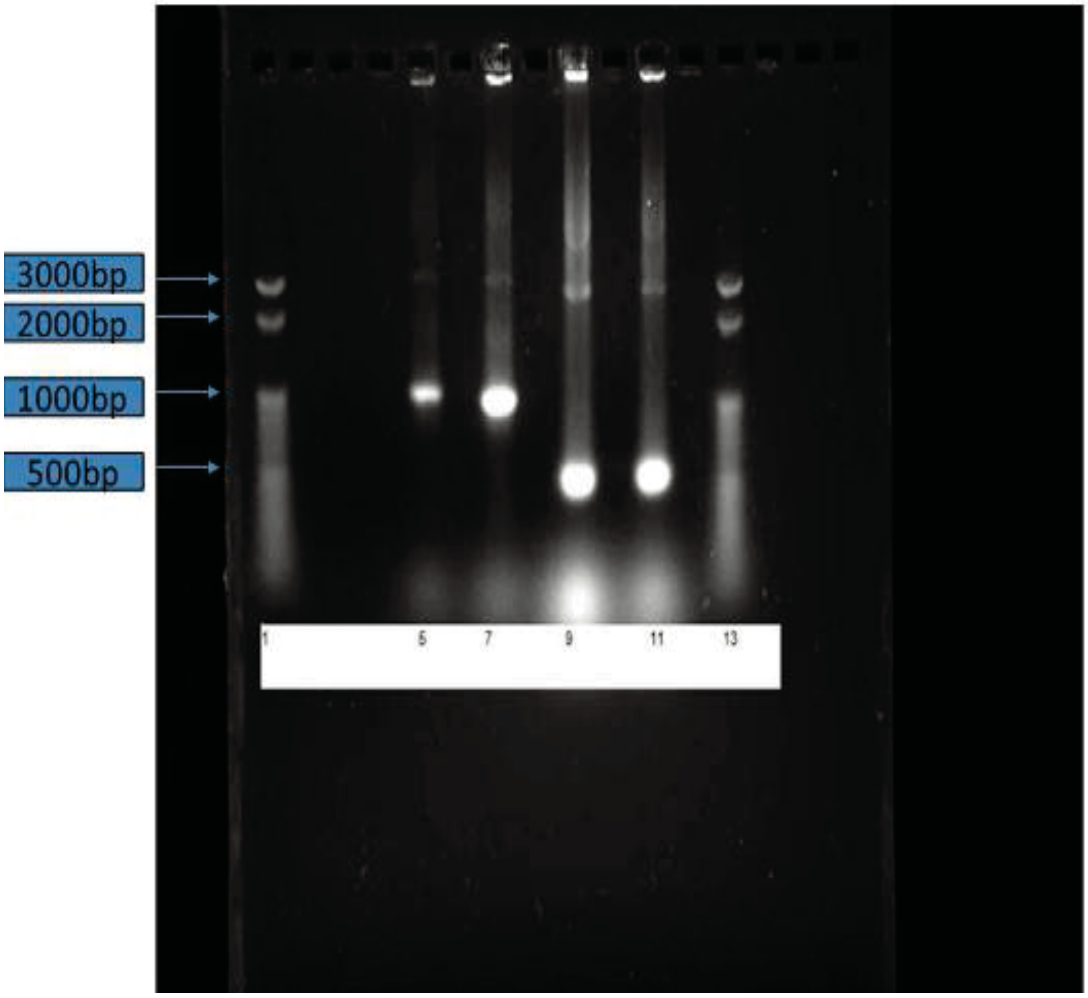


Figure 16. Photograph of agarose gel containing PCR products used for DNA sequencing of the *sigB* gene from *L. monocytogenes* 568 and its Δ*sigB* mutant. Lane 1 and 13 is a DNA ladder, lane 5 and 7 is the pSC-A cloned *sigB* gene from Lm568 amplified with the M13-R and M13-F primers, and lane 9 and 11 contains the pSC-A cloned *sigB* from the Δ*sigB* mutant amplified with M13-R and M13-F primers. The results shown are representative of two independent experiments.

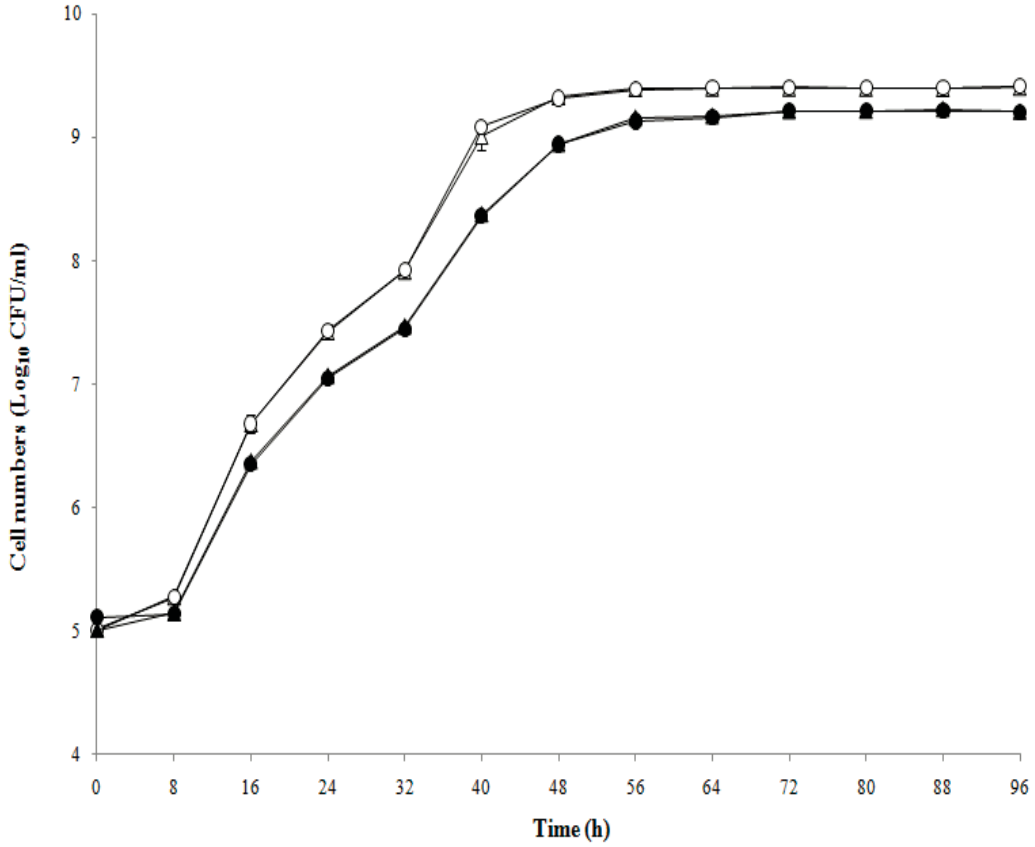


Figure 17. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in BHI with 0.5% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

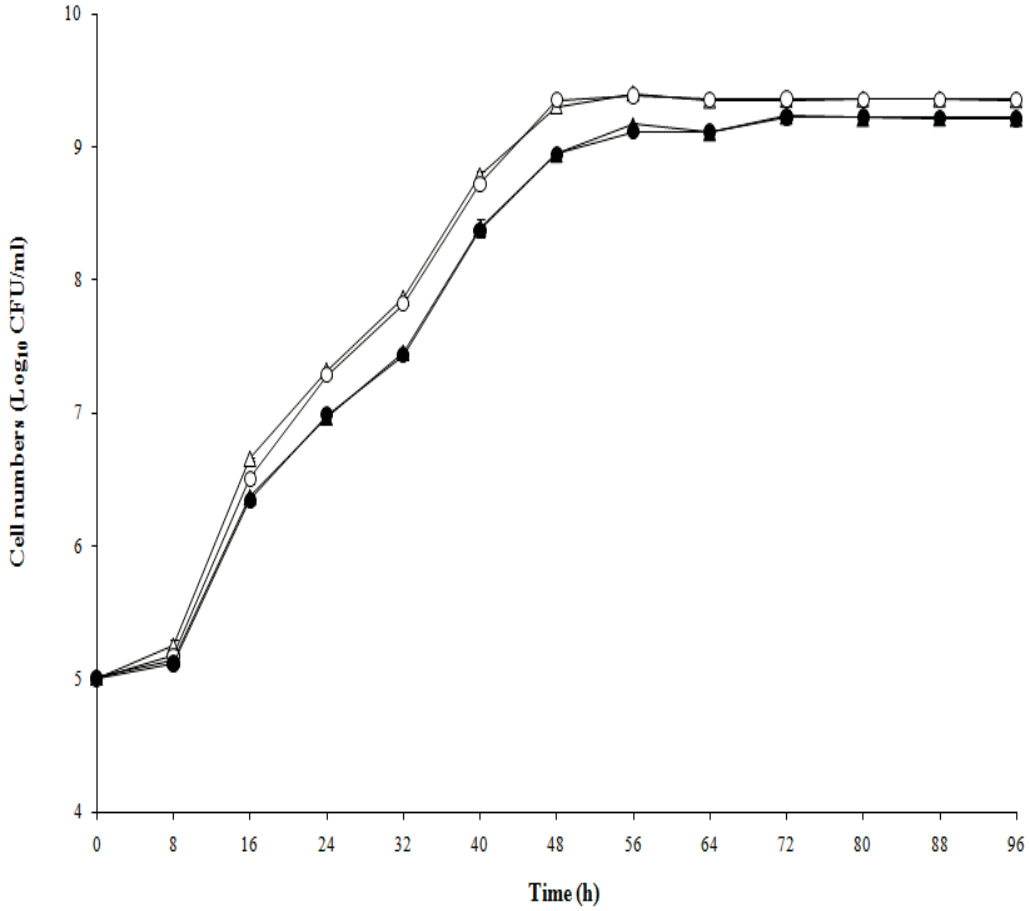


Figure 18. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in TSB-glu with 0.5% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

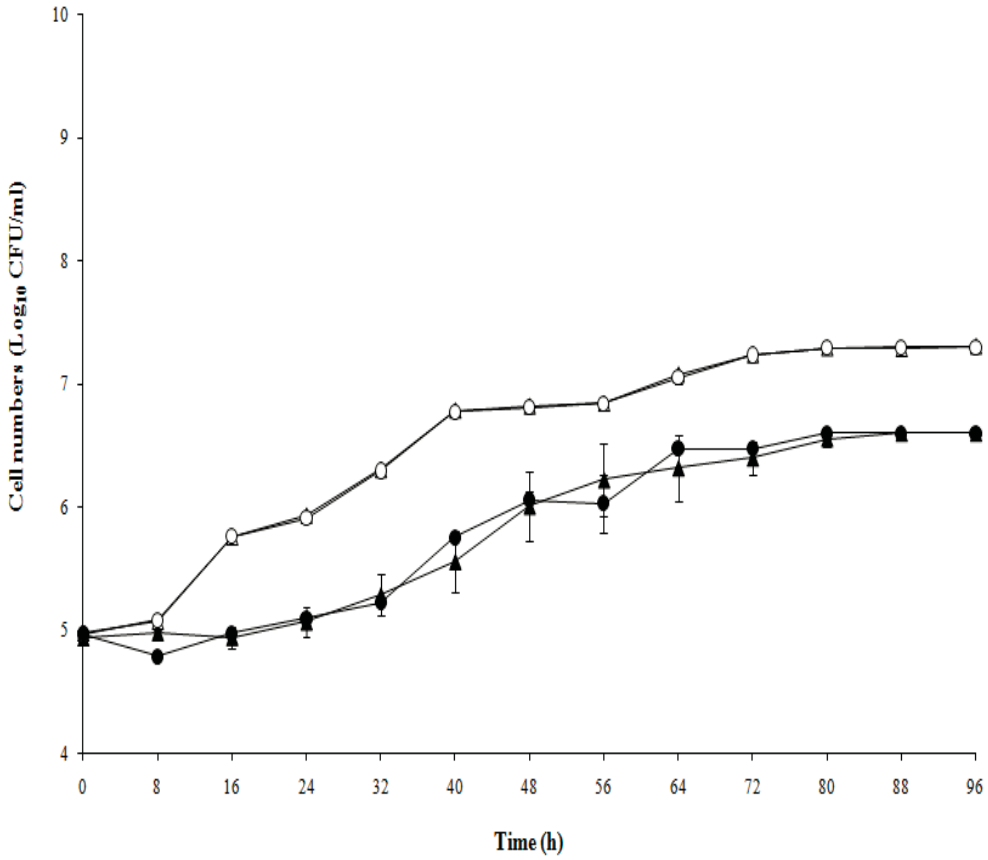


Figure 19. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in PPS with 0.85% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

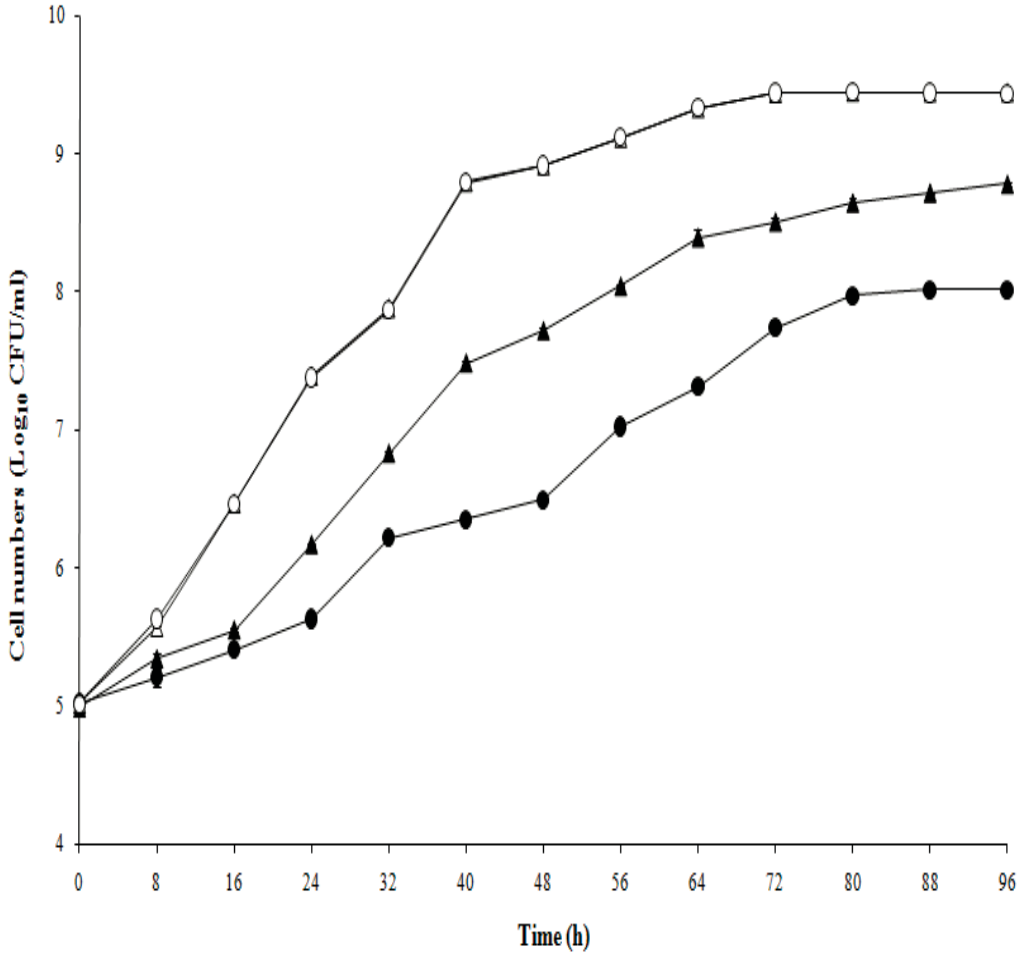


Figure 20. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM in the presence of carnitine (1 mM) with 0.5% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

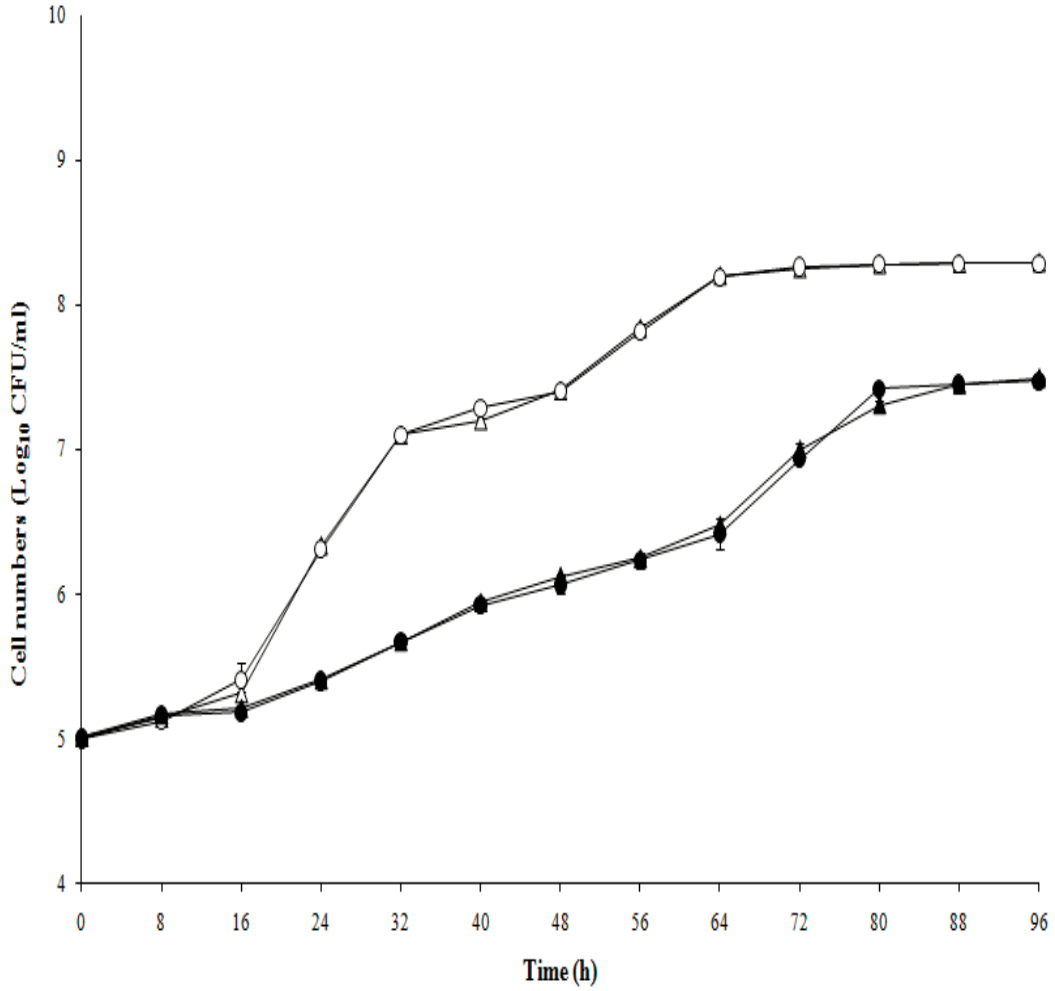


Figure 21. Growth of *L. monocytogenes* 568 and its Δ*sigB* mutant in MM in the presence of proline (1 mM) with 0.5% NaCl (Δ,○) and 5.0% NaCl (▲,●), respectively, at 15 °C for 4 days. The values (Log_{10} CFU/ml) at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements. Triangle symbols signify the wild type *L. monocytogenes* while circle symbols denote the Δ*sigB* mutant.

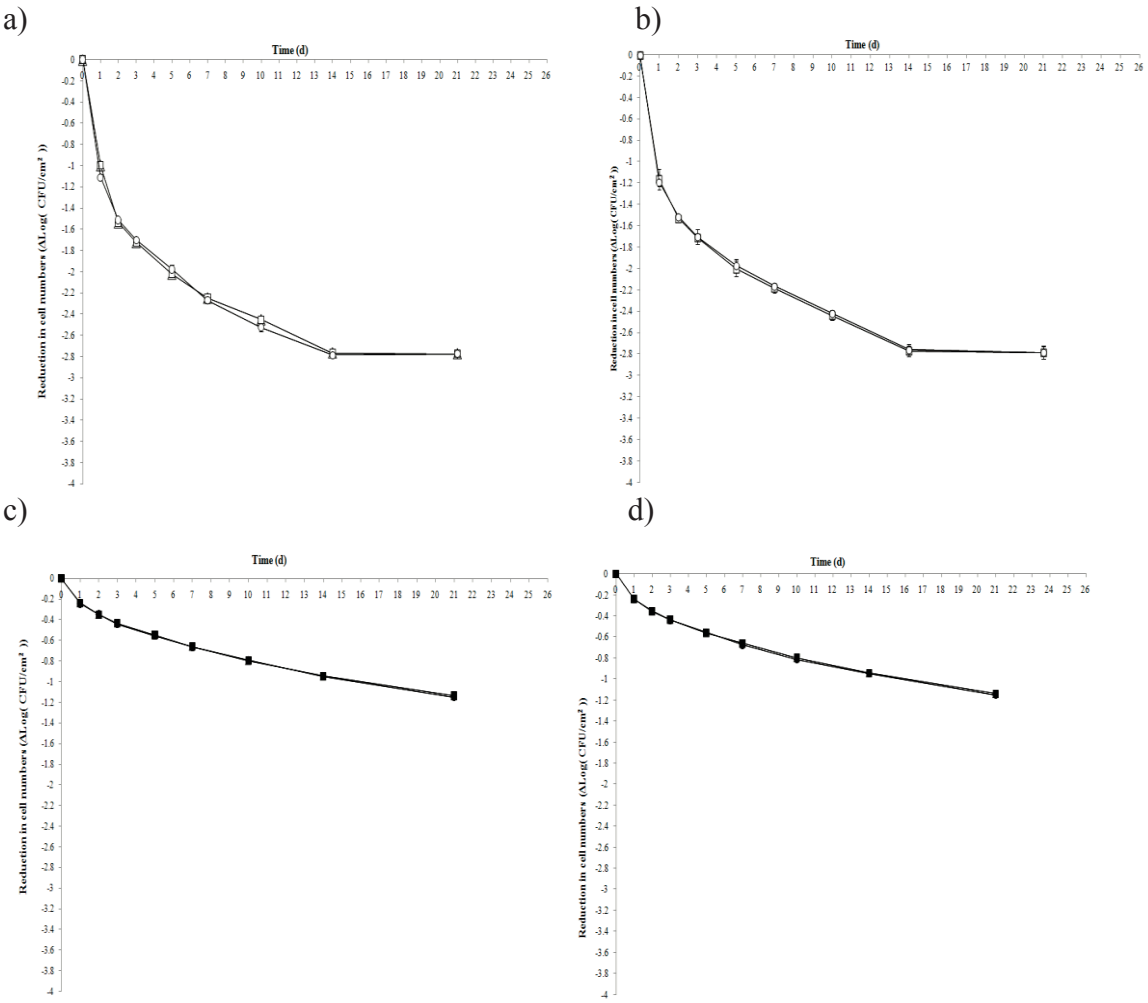


Figure 22. Desiccation survival of *L. monocytogenes* 568 and its Δ*sigB* mutant on stainless steel coupons (SS) placed in four different positions in the desiccator for 21 days at 15 °C and 43% RH. The cells of Lm568 a) and its Δ*sigB* mutant b) were pre-cultured in TSB-glu with 0.5% NaCl at 15 °C for 3 days and desiccated in TSB-glu with 0.5% NaCl on SS coupons loaded into Petri dish bottoms placed in different four positions (Left front Δ, Right front □, Left back ◊, and Right back ○). The same experiment was conducted with cells of Lm568 c) and its Δ*sigB* mutant d) which had been pre-cultured in TSB-glu with 5.0% NaCl at 15 °C for 3 days followed by desiccation in TSB-glu with 5.0% NaCl on SS coupon placed in the four positions (Left front ▲, Right front ■, Left back ♦, and Right back ●). Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

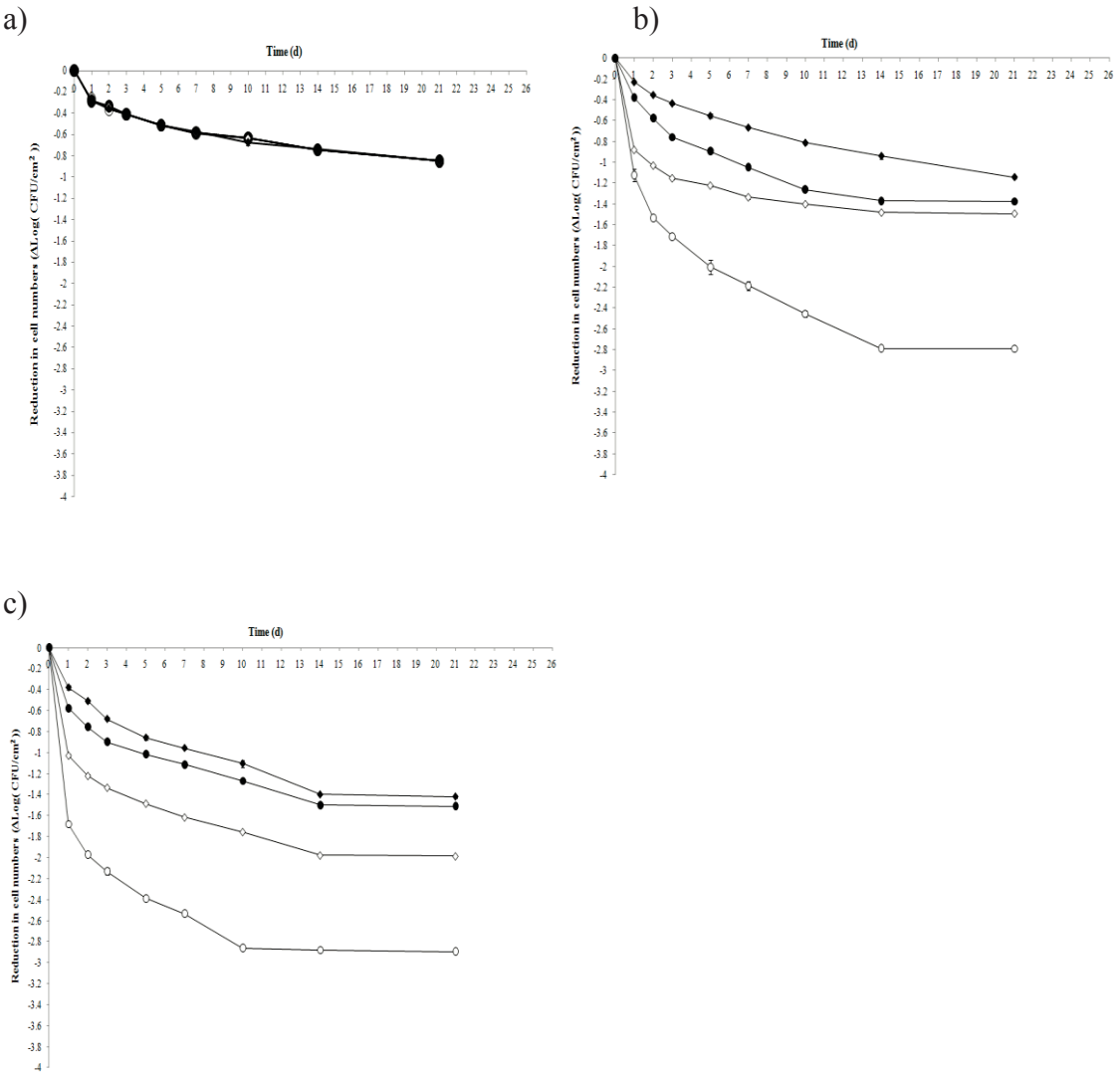


Figure 23. Effect of salt during pre-culture and desiccation on the survival of *L. monocytogenes* 568 Δ*sigB* mutant cells on stainless steel coupons incubated at 15 °C and 43% RH for 21 days in the desiccator. Lm568 Δ*sigB* mutant cells were pre-cultured in a) BHI, b) TSB-glu, and c) PPS with 0.5% NaCl at 15 °C for 3 days and desiccated in each substrate with 0.5% NaCl (○) and 5.0% NaCl (●), respectively. Osmoadapted cells in a) BHI, b) TSB-glu, and c) PPS with 5.0% NaCl (15 °C for 3 days) were desiccated in each substrate with 0.5% NaCl (◊) and 5.0% NaCl (♦), respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

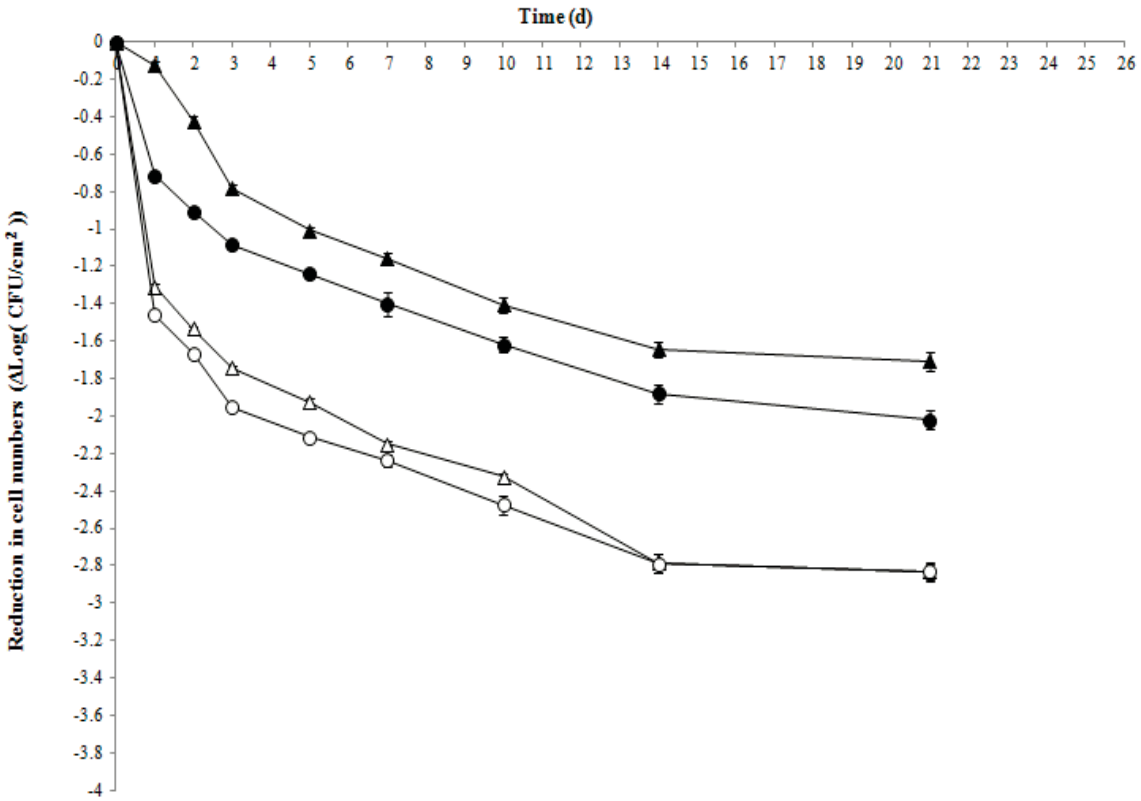


Figure 24. Survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant during desiccation in a defined minimal media (MM) on stainless steel at 15 °C and 43% RH for 21 days. Cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl (15 °C for 3 days) and desiccated in MM with 0.5% NaCl. The osmoadapted (MM with 5.0% NaCl at 15 °C for 3 days) cells of Lm568 (▲) and its Δ*sigB* mutant (●) were desiccated in MM with 5.0% NaCl. Reduction in cell numbers ($\Delta Log(CFU/cm^2)$ at each sampling point is expressed as $Log(N_t/N_0)$). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

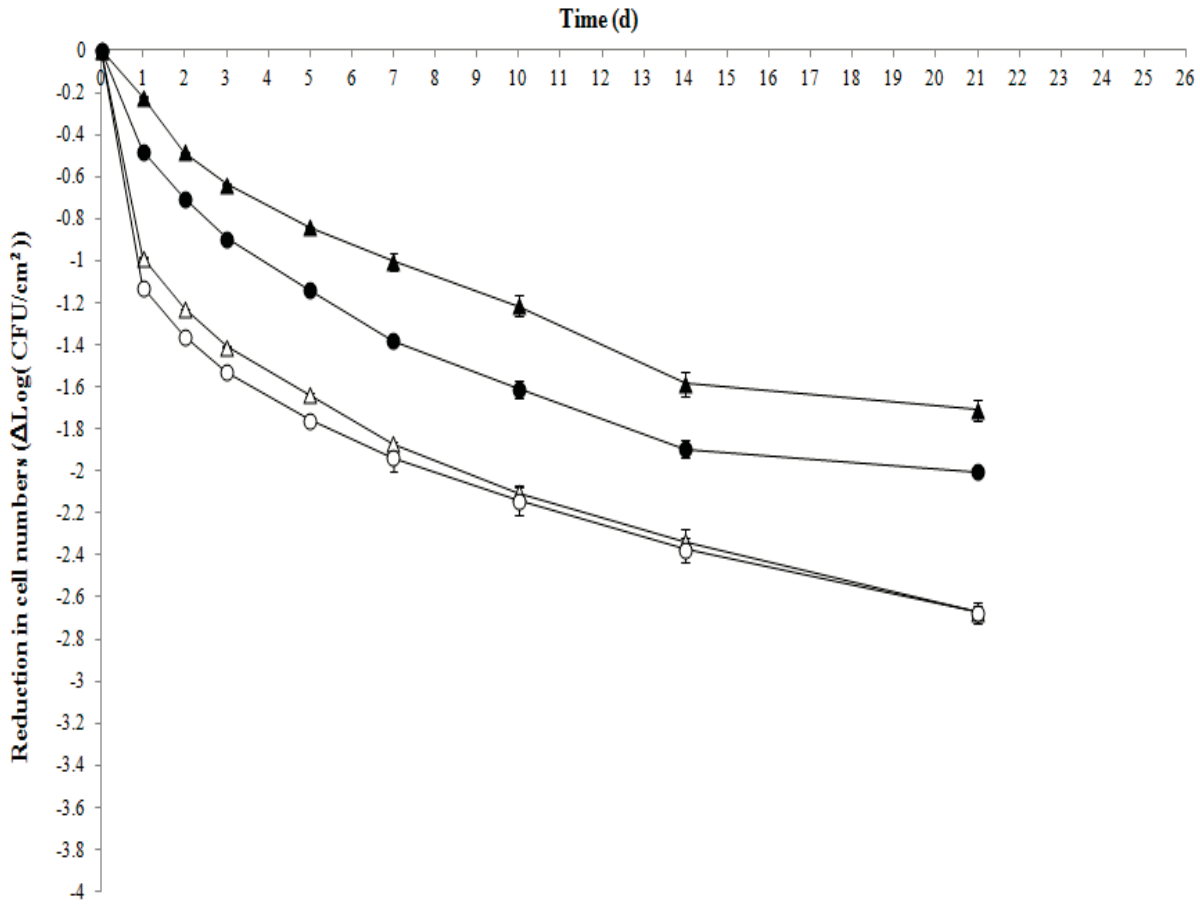


Figure 25. Effect of pre-culture with glycine betaine on desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant. The cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl and glycine betaine (1 mM) at 15 °C for 3 days and desiccated in MM with 0.5% NaCl on stainless steel at 15 °C and 43% RH for 21 days. Lm568 (▲) and its Δ*sigB* mutant (●) were osmoadapted in MM with 5.0% NaCl and glycine betaine (1 mM) at 15 °C for 3 days and desiccated in MM with 5.0% NaCl, respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

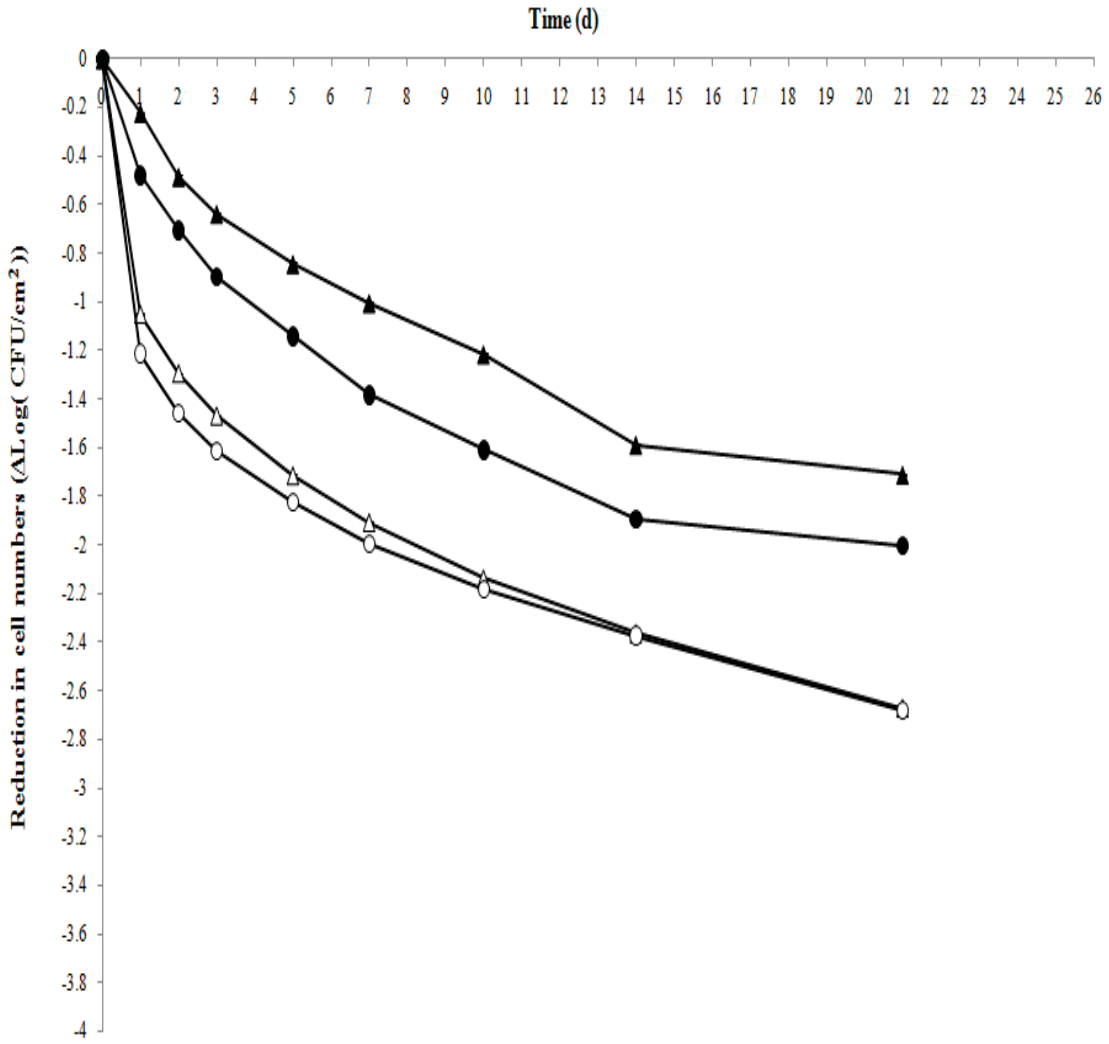


Figure 26. Effect of pre-culture with carnitine on desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant. The cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl and carnitine (1 mM) at 15 °C for 3 days and desiccated in MM with 0.5% NaCl on stainless steel at 15 °C and 43% RH for 21 days. Lm568 (▲) and its Δ*sigB* mutant (●) were osmoadapted in MM with 5.0% NaCl and carnitine (1 mM) at 15 °C for 3 days and desiccated in MM with 5.0% NaCl, respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

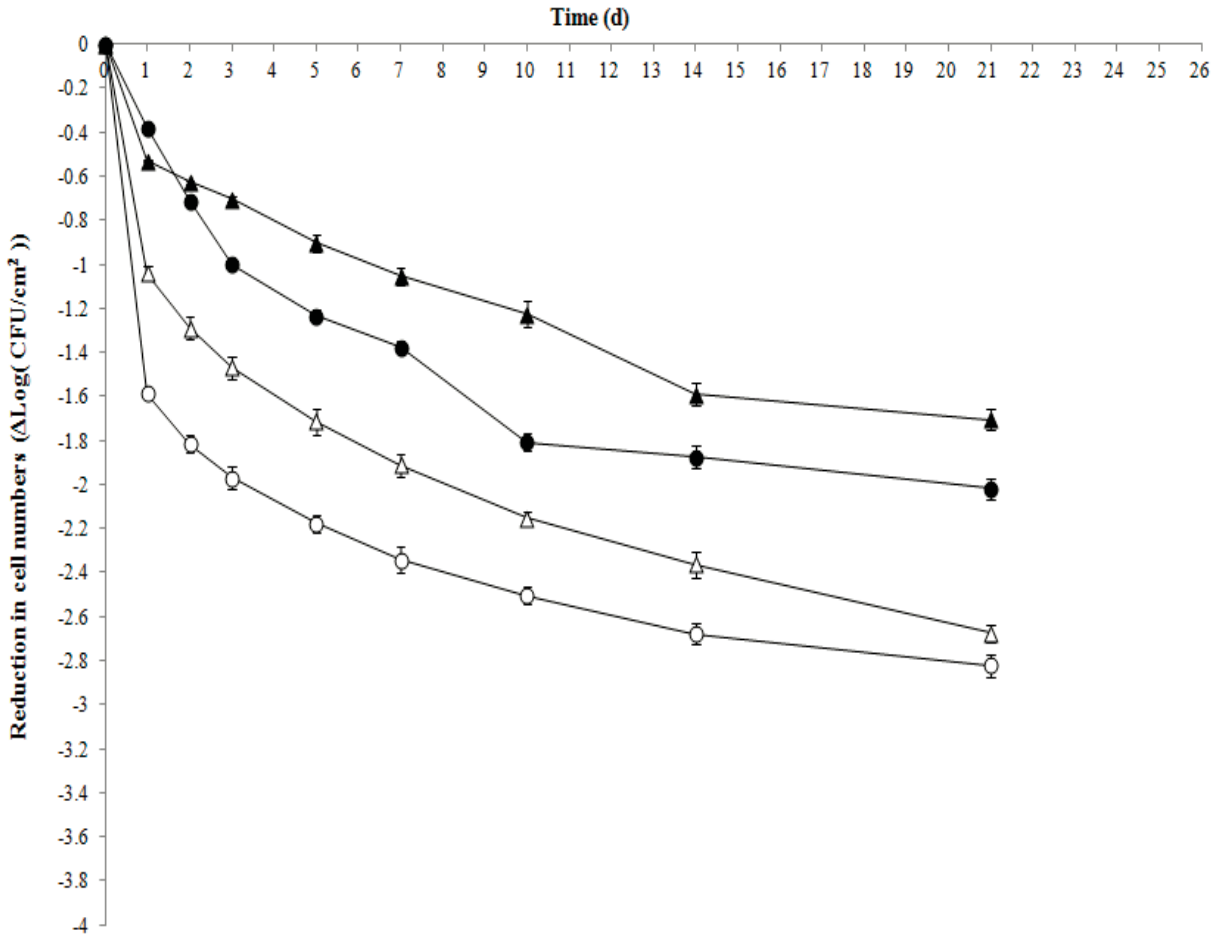


Figure 27. Effect of pre-culture with proline on desiccation survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant. The cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl and proline (P, 1 mM) at 15 °C for 3 days and desiccated in MM with 0.5% NaCl on stainless steel at 15 °C and 43% RH for 21 days. Lm568 (▲) and its Δ*sigB* mutant (●) were osmoadapted in MM with 5.0% NaCl and proline (1 mM) at 15 °C for 3 days and desiccated in MM with 5.0% NaCl, respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

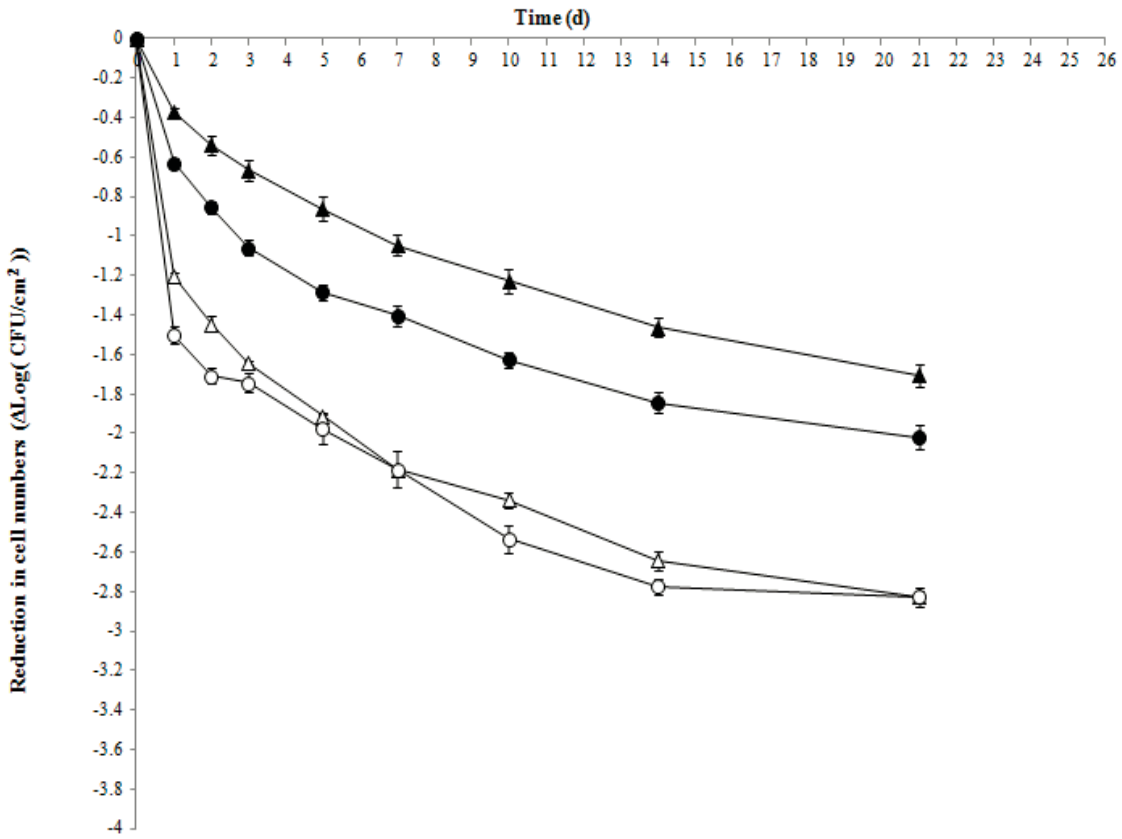


Figure 28. Effect of glycine betaine in the desiccation substrate (MM ± 0.5% NaCl) on the survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant on stainless steel coupons incubated at 15 °C and 43% RH for 21 days. Cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl at 15 °C for 3 days and desiccated in MM with 0.5% NaCl and glycine betaine (1 mM). Cells of Lm568 (▲) and its Δ*sigB* mutant (●) osmoadapted in MM with 5.0% NaCl (15 °C, 3 days) were desiccated in MM with 5.0% NaCl and glycine betaine (1 mM), respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

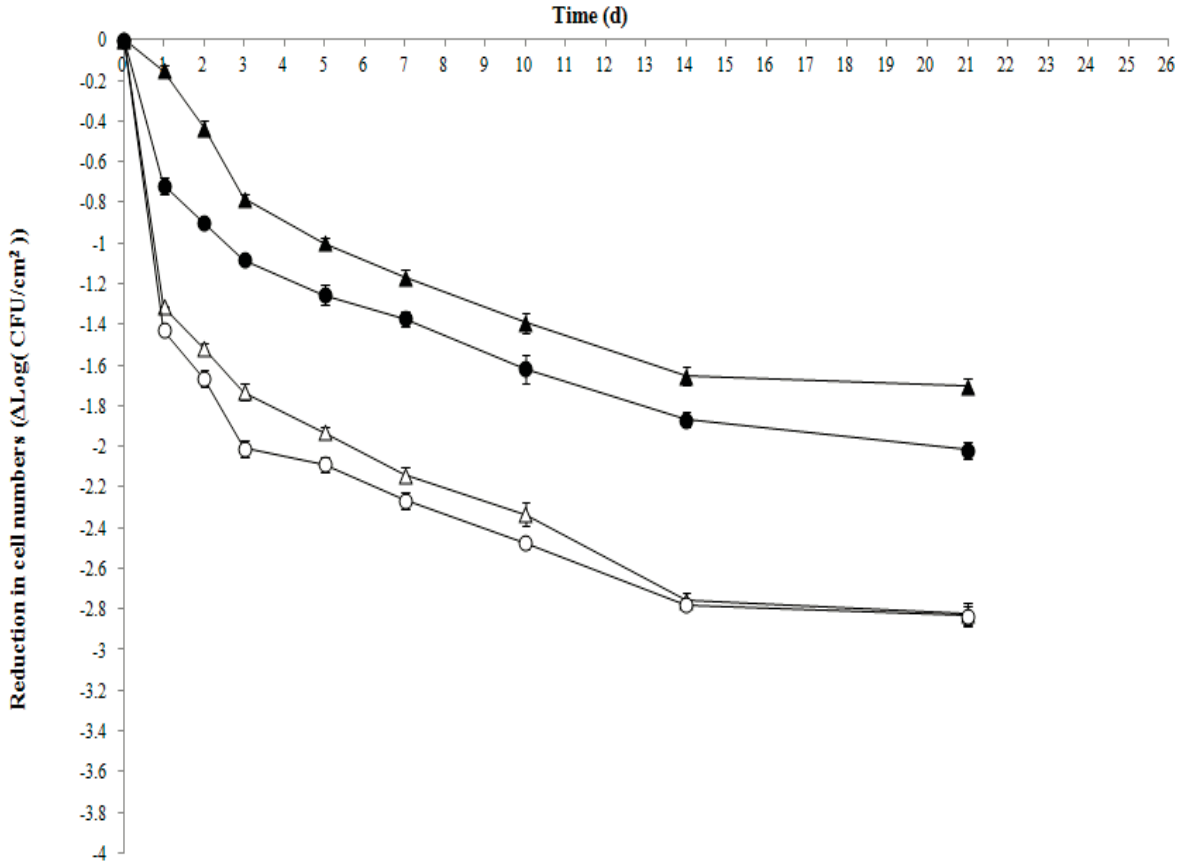


Figure 29. Effect of carnitine in the desiccation substrate (MM ± 0.5% NaCl) on the survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant on stainless steel coupons incubated at 15 °C and 43% RH for 21 days. Cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl at 15 °C for 3 days and desiccated in MM with 0.5% NaCl and carnitine (1 mM). Cells of Lm568 (▲) and its Δ*sigB* mutant (●) osmoadapted in MM with 5.0% NaCl (15 °C, 3 days) were desiccated in MM with 5.0% NaCl and carnitine (1 mM), respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

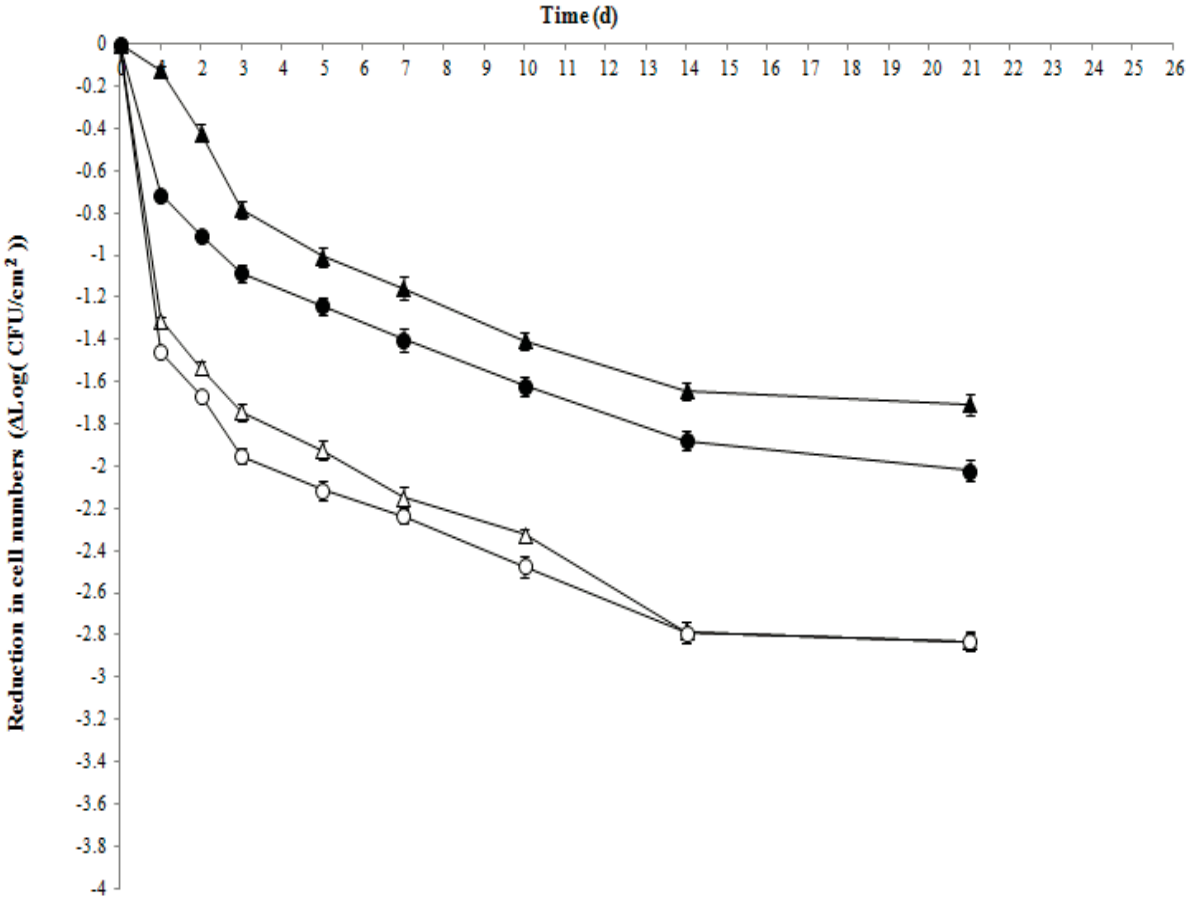


Figure 30. Effect of proline in the desiccation substrate (MM ± 0.5% NaCl) on the survival kinetics of *L. monocytogenes* 568 and its Δ*sigB* mutant on stainless steel coupons incubated at 15 °C and 43% RH for 21 days. Cells of Lm568 (Δ) and its Δ*sigB* mutant (○) were pre-cultured in MM with 0.5% NaCl at 15 °C for 3 days and desiccated in MM with 0.5% NaCl and proline (1 mM). Cells of Lm568 (▲) and its Δ*sigB* mutant (●) osmoadapted in MM with 5.0% NaCl (15 °C, 3 days) were desiccated in MM with 5.0% NaCl and proline (1 mM), respectively. Reduction in cell numbers (ΔLog(CFU/cm^2) at each sampling point is expressed as Log(N_t/N_0)). The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) and the error bars indicate the standard deviation of six measurements.

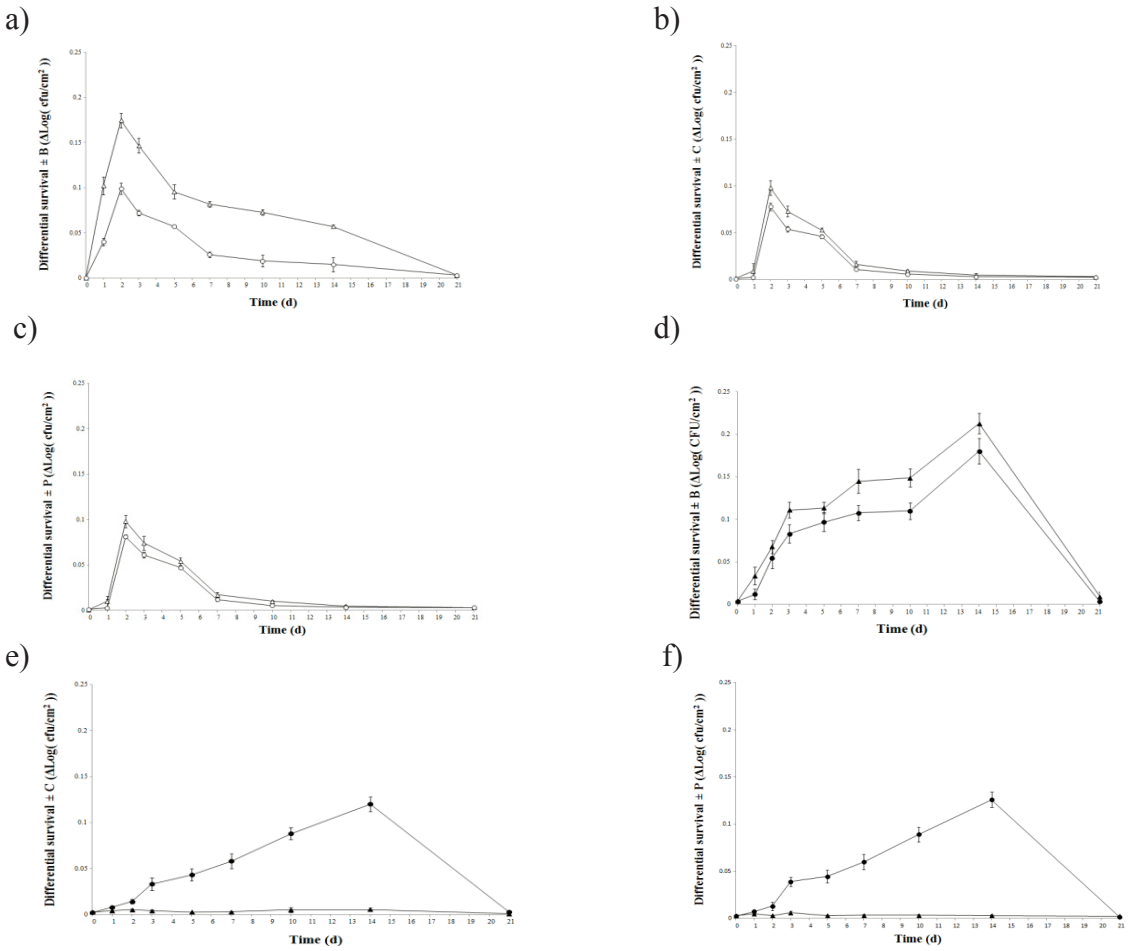
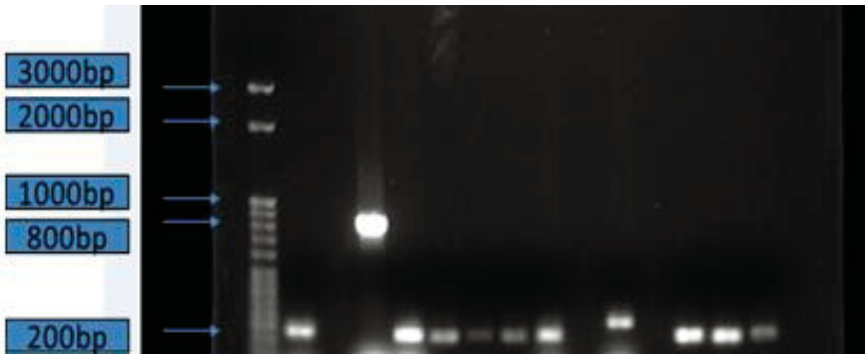


Figure 31. Difference in survival between non-osmoadapted *L. monocytogenes* 568 (Δ) and its Δ*sigB* mutant (○) desiccated in MM with 0.5% NaCl with glycine betaine (B, 1 mM) and without glycine betaine a), with carnitine (C, 1mM) and without carnitine b), and with proline (P, 1mM) and without proline c) at 15 °C and 43% RH for 21 days. Difference in survival between osmoadapted *L. monocytogenes* 568 (▲) and its Δ*sigB* mutant (●) desiccated in MM with 5.0% NaCl with glycine betaine (B, 1 mM) and without glycine betaine d), with carnitine (C, 1mM) and without carnitine e), and with proline (P, 1mM) and without proline f) at 15 °C and 43% RH for 21 days. Differential survival in cell numbers ($\Delta Log(CFU/cm^2)$ at each sampling point is expressed as $Log(N_{day\ with\ osmolyte}) - Log(N_{day\ without\ osmolyte})$. The values at each sampling point represent the mean of the six samples (triplicate samples from the two independent trials) from the subtraction of the two measurements and the error bars indicate the standard deviation of six samples from the subtraction of the two measurements.

a)



b)

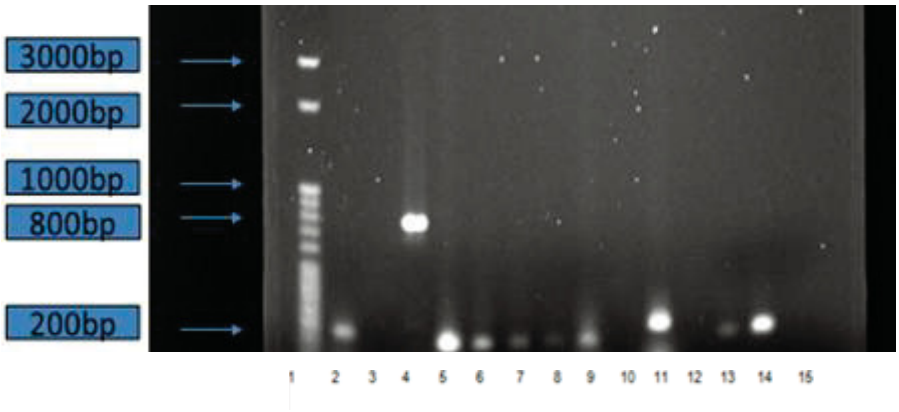


Figure 32. Transcriptional analysis by reverse transcriptase PCR of osmolyte transporters (*betL, gbuA* and *opuCA*) in *L. monocytogenes* 568 and its Δ*sigB* mutant exposed to MM with 0.5% NaCl at 15 °C for 1 hour a) and to MM with 5.0% NaCl at 15 °C for 1 hour b). Lane 1, DNA ladder; Lane 2, *rRNA* amplicon from Lm568 cDNA (positive control); Lane 3, *betL* amplicon from Lm568 RNA with no RT added to the reaction mixture (negative control); Lane 4, Lm568 amplicon of *sigB* after PCR of cDNA with the *sigB* whole gene primers; Lane 5, Lm568 amplicon of *sigB* after PCR of cDNA with the *sigB* internal primers; Lane 6, Lm568 amplicon of *gbuA* from cDNA; Lane 7, Lm568 amplicon of *betL* from cDNA; Lane 8, Lm568 amplicon of *opuCA* from cDNA; Lanes 9-15 contain amplicons from the same reactions (*rRNA*, *betL* amplicon from RNA and no RT, *sigB* whole, *sigB* internal, *gbuA*, *betL* and *opuCA*) obtained with cDNA derived from Δ*sigB* mutant RNA. The results shown are representative of those from two independent experiments, each performed in duplicate.

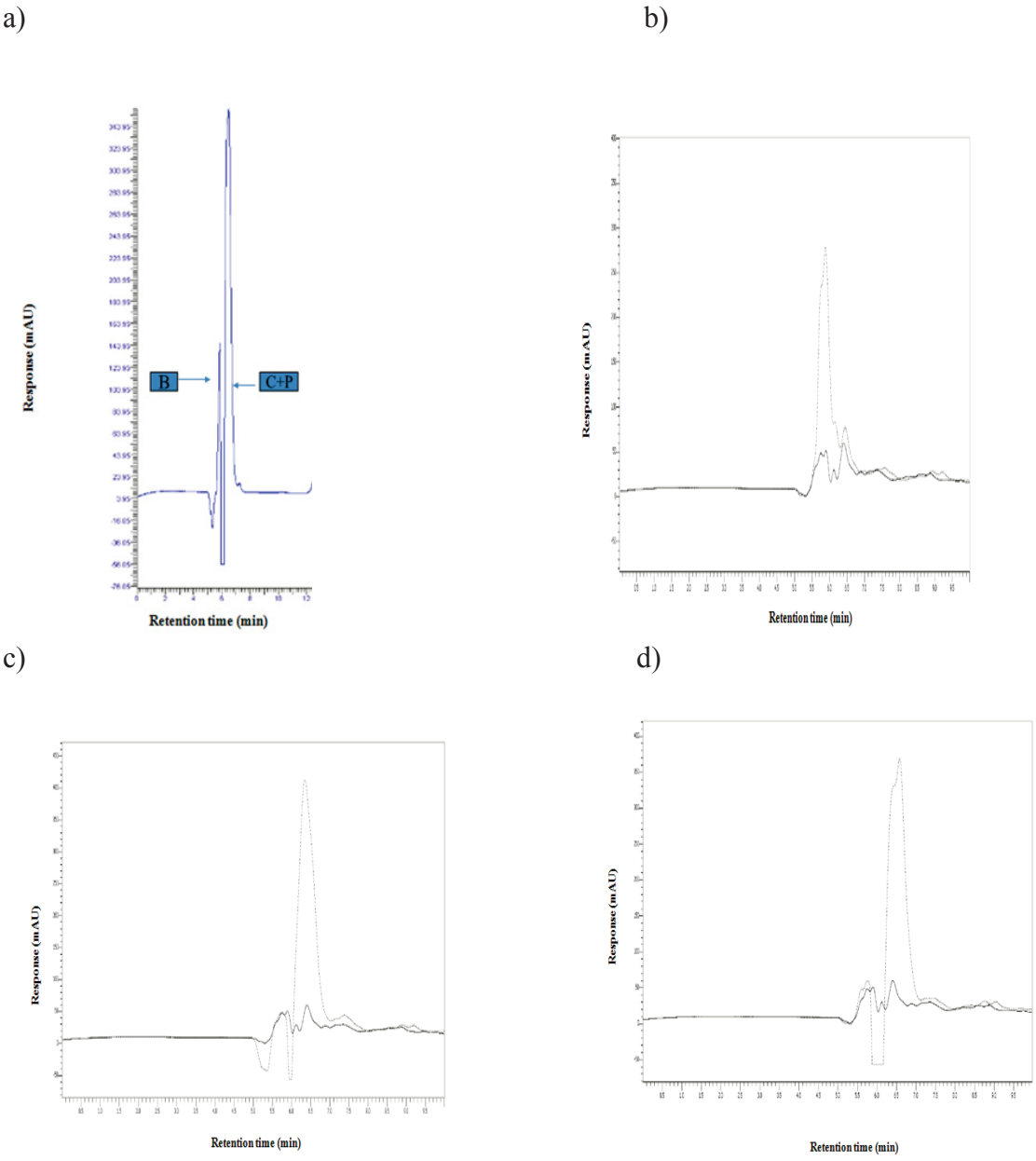


Figure 33. HPLC analyses of osmolytes in the growth and desiccation substrates (BHI). a) Chromatogram of mix standards of glycine betaine (B), carnitine (C) and proline (P). b) Chromatograms of 10-fold diluted BHI sample (solid line) and this sample with internal standard of B (1 mg/ml, dotted line). c) Chromatograms of 10-fold diluted BHI sample (solid line) and this sample with internal standard of C (1 mg/ml, dotted line). d) Chromatograms of 10-fold diluted BHI sample (solid line) and this sample with internal standard of P (1 mg/ml, dotted line).

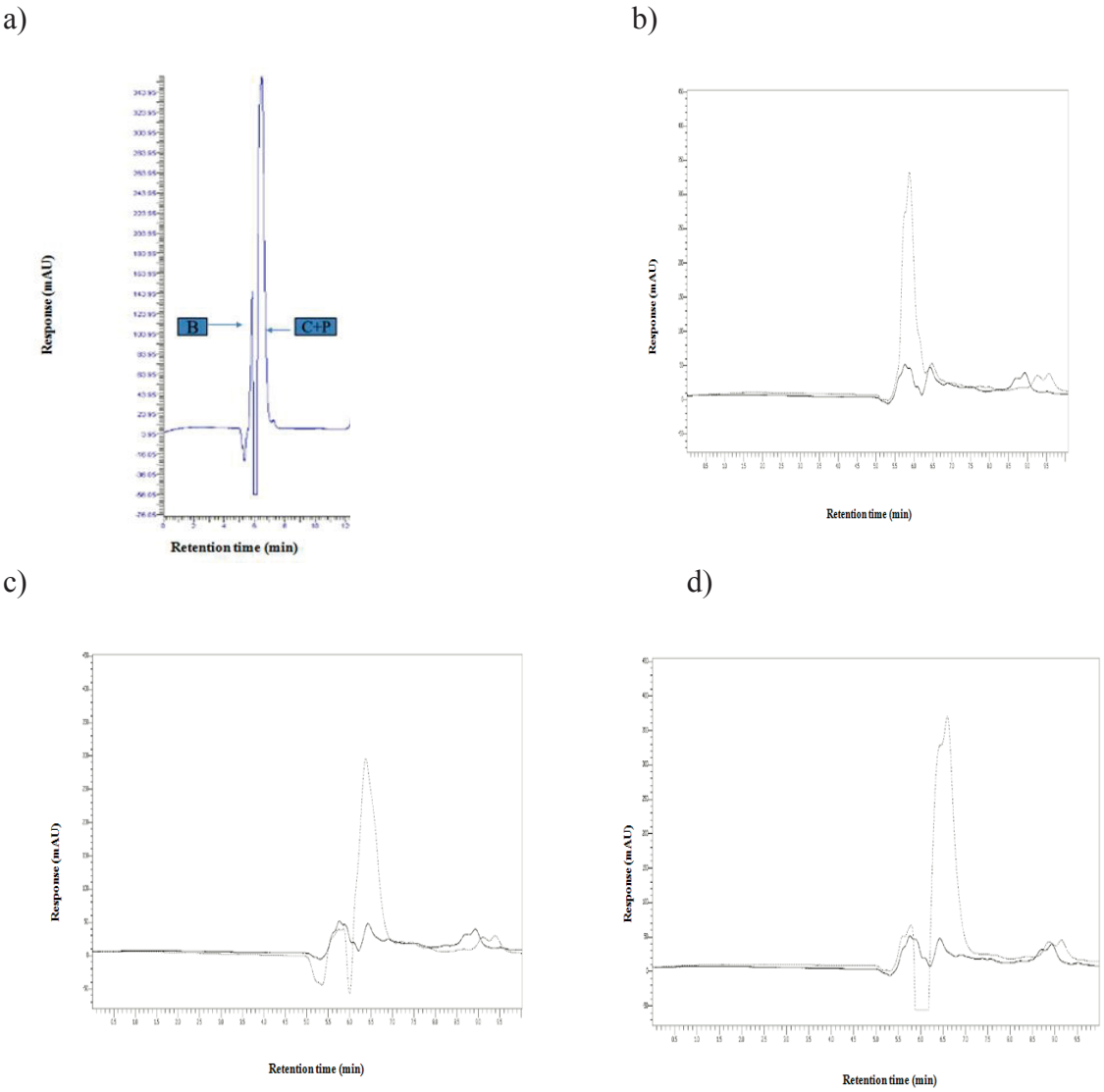
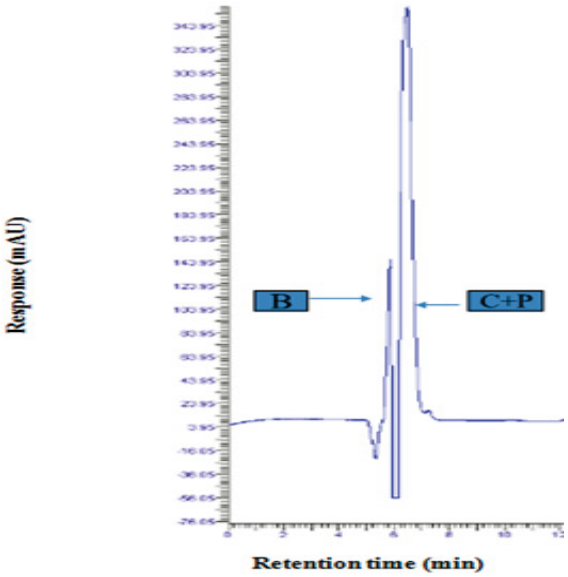


Figure 34. HPLC analyses of osmolytes in the growth and desiccation substrates (TSB-glu). a) Chromatogram of mix standards of glycine betaine (B), carnitine (C) and proline (P). b) Chromatograms of 10-fold diluted TSB-glu sample (solid line) and this sample with internal standard of B (1 mg/ml, dotted line). C) Chromatograms of 10-fold diluted TSB-glu sample (solid line) and this sample with internal standard of C (1 mg/ml, dotted line). d) Chromatograms of 10-fold diluted TSB-glu sample (solid line) and this sample with internal standard of P (1 mg/ml, dotted line).

a)



b)

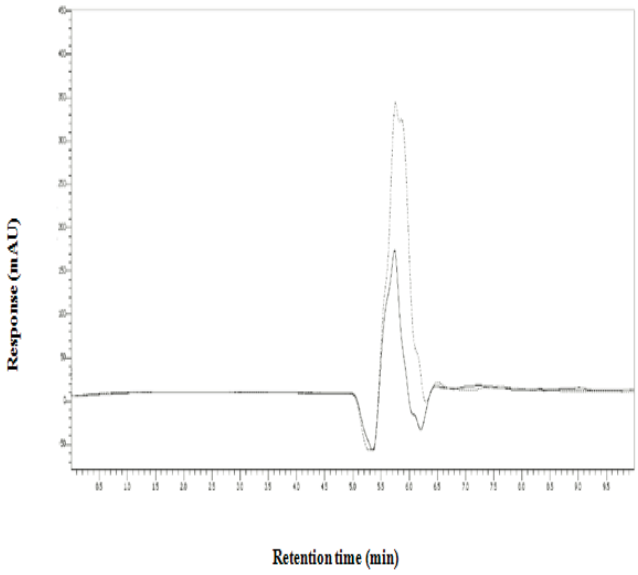


Figure 35. HPLC analyses of osmolytes in the growth and desiccation substrates (PPS). a) Chromatogram of mix standards of glycine betaine (B), carnitine (C) and proline (P). b) Chromatograms of half-fold diluted PPS sample (solid line) and this sample with internal standard of B (1 mg/ml, dotted line).

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