Impact of food environmental factors related to fermented sausages on *Salmonella* stress and virulence response

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Content

Preface

The present PhD thesis constitutes partial fulfilment of the requirements for obtaining a PhD degree at the Technical University of Denmark. The work was carried out in the period from August 2008 to December 2013 at the National Food Institute, Technical University of Denmark with research leader Søren Aabo as main supervisor (National Food Institute, DTU Food).

This PhD study is part of a cross-institutional project entitled: Control of foodborne infections from lightly preserved meat products through mathematical modelling and efficient HACCP-based control programmes – ConFood. ConFood was funded by the Danish Council for Strategic Research. The PhD study included a research stay at the Canadian Research Institute for Food Safety, University of Guelph. Apart from this all work was carried out at the division of Food Microbiology at the National Food Institute.

Sidsel Henriksen

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Janis Dyrby Nielsen for meticulously knitting her way through this manuscript

Implicitly, I am indebted to the everlasting love of family and friends

Summary

This thesis gives an introduction to *Salmonella*, its significance as a foodborne pathogen and clinical symptoms. An overview of fermented sausage production is provided, with special emphasis on the safety hurdles employed. The physiological response of *Salmonella* to the two main sausage processing stressors, acid and salt is covered, along with a description of genes responsible for the organisms ability to adapt and survive. A brief introduction to the pathogenicity of *Salmonella* is given, with focus on selected cognate regulators and environmental signals controlling *Salmonella* invasion. Also regulatory connections between stress response and virulence will be mentioned. The experimental work conducted during this PhD is documented in a published paper and three manuscripts;

To be able to follow the gene response of pathogens to food-related stress, we published a simple method for extracting stable and high quality and quantity prokaryotic RNA from contaminated meat. Using the RNA extraction method, it was possible to measure expression of bacterial stress genes in a food matrix by applying salt and heat stress. Most gene expression studies regarding stress-responses of pathogens have been performed in broth or broth-like food-models, not necessarily reflecting the situation in a real food-matrix. Summarizing conclusions were: Gene expression studies showed that heat and salt stress of *Escherichia coli* and *Salmonella* spp. in minced meat reproducibly induced > 5-fold induction of related stress gene expression. Thus, the method is applicable in studies of bacterial gene expression in a meat matrix.

With a notion of inherent growth-phase related differences in bacterial stress-responses and since sub-optimal conditions during storage and/or distribution of raw meat could support pathogen growth, we analysed the impact of exponential and stationary growth phase on survival of *Salmonella* during pre-processing freezing and during fermentation of sausage processing. Most inactivation studies of pathogens during sausage production have been conducted with stationary phase cells, presumably to reflect the worst case scenario. Summarizing conclusions were: *Salmonella* in exponential growth phase was more sensitive to freezing than stationary phase cells. This higher sensitivity was not apparent in the cells' response to the appertaining stresses encountered in the sausage environment during fermentation. We hypothesized that for the

exponential phase cells an acid tolerance response (ATR) was mounted at a higher pH and hence earlier in the fermentation process, than for the stationary phase cells, masking the inherent differences in stress response and ability to survive of the two physiological states.

With the aim of elucidating properties of food, or model food, components supportive of *Salmonella* survival during simulated gastric acid passage, we investigated the influence of meats and media with different buffer capacities on inactivation of *Salmonella enterica* serovar Typhimurium during dynamic pH lowering. Furthermore, we aimed at adding knowledge to the stationary phase acid tolerance response (ATR), possibly mounted during gastric passage, by investigating the relative gene expression pattern of *rpoS* and *ompR* encoding two major ATR regulators. To our knowledge, this is the first study analysing gene expression during dynamic pH changes in synthetic gastric fluid. Moreover, most acid challenge studies of pathogens have been conducted in a static pH environment. Summarizing conclusions were: Inactivation curves demonstrated that less buffered media provided higher protection of *Salmonella* from inactivation, compared to media with high buffer capacity. Results from gene expression studies displayed an induction of *ompR* and *rpoS* that were significantly higher in saline and buffered saline than in BHI. On account of these results we hypothesize that an ATR is induced instantaneously in saline, resulting in a higher acid tolerance and hence survival of *Salmonella* in media with low buffer capacity.

In identifying process parameters during sausages manufacturing, which could possibly diminish the risk of obtaining enteric infections from lightly preserved ready-to-eat meat products, we initiated investigations of targeting virulence genes as a novel means to control foodborne pathogens. We analysed the growth-medium of lactic acid bacteria and starter cultures used in sausage production for presence of factors affecting key regulatory virulence genes and hence infection potential of *Salmonella*. Previous studies have analysed the virulence inhibitory properties of probiotics, but to our knowledge no others have investigated this effect in strains with relevance for food-production. Summarizing conclusions were: The results obtained indicate that mixed and mono cultures of lactic acid bacteria used as starter cultures for production of fermented sausages secrete bioactive factors that reduce virulence of *Salmonella* through down-regulation of expression of virulence-associated genes.

Finally, a summarizing discussion and future perspectives is completing this thesis.

Resumé

Denne afhandling giver en introduktion til *Salmonella*, dens betydning som fødevarebåren patogen samt kliniske symptomer. Produktionen af fermenterede pølse bliver gennemgået med særlig vægt på de "hurdles", der benyttes for at øge fødevaresikkerheden. Det fysiologiske respons af *Salmonella* på to vigtige stressfaktorer under pølseforarbejdning, syre og salt, er dækket, sammen med en beskrivelse af gener, der er ansvarlige for organismernes evne til at tilpasse sig og overleve. En kort introduktion til patogeniciteten af *Salmonella* er givet, med fokus på udvalgte regulatorer og miljømæssige signaler, der kontrollerer dens invasion. Regulatoriske overlap mellem stressrespons og virulens vil blive også nævnt. Det eksperimentelle arbejde, udført i løbet af denne ph.d., er dokumenteret i en publiceret artikel og tre manuskripter;

For at kunne følge patogeners genrespons på fødevarerelateret stress publicerede vi en simpel metode til at udvinde stabilt prokaryot RNA af høj kvalitet og kvantitet fra kontamineret kød. Denne metode muliggør ekspressionsstudier af bakterielle stressgener i en fødevarematrix under påvirkning af salt- og varmestress. De fleste genekspressionsstudier vedrørende stressrespons af patogener er blevet udført i bouillon eller bouillonlignende fødevaremodeller, som ikke nødvendigvis afspejler vilkårene i en rigtig fødevare. De opsummerende konklusioner var: Reproducerbare genekspressionsstudier viste, at varme og salt stress medførte en > 5-fold induktion af relaterede stressresponsgener i *Escherichia coli* og *Salmonella* spp. i hakket kød. Metoden er anvendelig i studier af bakteriel genekspression i en kød matrix.

Med formodningen om at bakterielt stressrespons er relateret til vækstfase, og da sub-optimale betingelser under opbevaring og/eller distribution af råt kød kan understøtte vækst af patogener, undersøgte vi, om *Salmonella* i forskellige vækstfaser, henholdsvis eksponentiel og stationær, overlevede frysning og følgende fermentering af modelpølser forskelligt. De fleste inaktiveringsstudier af patogener under pølseproduktion er gennemført med celler i stationær fase, formentlig for at afspejle det værst tænkelige scenarie. De sammenfattende konklusioner var: *Salmonella* i eksponentiel vækstfase var mere følsom over for frysning end celler i stationær fase. Denne højere følsomhed gav sig ikke udslag i cellernes respons på stress i pølsemiljøet under fermentering. Vi antager, at cellerne i eksponentiel fase inducerer et syretolerancerespons ved en højere pH-værdi og dermed tidligere i fermenteringsprocessen end cellerne i stationær fase,

hvilket skjuler de iboende forskelle i stressrespons og evne til at overleve mellem de to fysiologiske tilstande.

Med henblik på at belyse egenskaber ved fødevarekomponenter, der understøtter overlevelse af *Salmonella* under simuleret passage gennem mavesyren, undersøgte vi indflydelsen af kødsaft og medier med forskellige bufferkapaciteter på inaktivering af *Salmonella* under dynamisk pH sænkning. Med sigtet at belyse det stationær-fase syretoleranceresponse, som muligvis bliver induceret under passage i mavesyren, analyserede vi den relative genekspression af *rpoS* og *ompR*, som koder for to centrale ATR regulatorer. Så vidt vides har der ikke tidligere været udført genekspressionsanalyser under dynamiske pH-ændringer i syntetisk mavesaft. Desuden er de fleste "syrechallenge" studier af patogener udført i et statisk pH-miljø. De sammenfattende konklusioner var: Inaktiveringskurver indikerede, at *Salmonella* var bedre beskyttet overfor inaktivering i medier med lav bufferkapacitet i forhold til i medier med høj bufferkapacitet. Resultater fra genekspressionsstudier viste, at induktionen *ompR* og *rpoS* var signifikant højere i fysiologisk saltvand og bufret saltvand end i BHI. På baggrund af disse resultater antager vi, at et ATR induceres umiddelbart i saltvand, hvilket resulterer i en højere syretolerance og dermed en øget overlevelse af *Salmonella* i medier med lav bufferkapacitet.

Under identificering af procesparametre, i produktionen af fermenterede pølser, som muligvis kan mindske risikoen for at få tarminfektioner fra letkonserverede kødprodukter, indledte vi undersøgelser af at sænke virulensgenekspression som middel til at kontrollere fødevarebårne patogener. Vi analyserede vækstmedier fra mælkesyrebakterier og starterkulturer, der anvendes i pølseproduktion, for tilstedeværelse af stoffer, der påvirker centrale regulatoriske virulensgener og dermed også infektionspotentialet af *Salmonella*. Tidligere undersøgelser har dokumenteret, at visse probiotiske bakterier har virulensgenhæmmende egenskaber, men så vidt vides har denne effekt ikke tidligere været undersøgt i stammer med relevans for fødevareproduktionen. Sammenfattende konklusioner var: Både blandede og renkulturer af mælkesyrebakterier, der anvendes som starterkulturer til fremstilling af fermenterede pølser, udskiller bioaktive stoffer, der reducerer virulensen af *Salmonella* gennem nedregulering af ekspressionen af virulens - associerede gener.

Endelig afslutter en sammenfattende diskussion og fremtidige perspektiver denne afhandling.

Chapter 1
Introduction

There is an increasing consumer demand for safe, minimally-processed and -preserved food (Gálvez et al. 2007). While a health and nutritional benefit will derive from lowering e.g. the salt and fat content, minimal preservation might give rise to products with intrinsic food safety problems. Fermented sausages consist of lightly preserved raw meat. Even under optimal hygiene conditions, fresh meat will be contaminated with fecal matter entailing the potential presence of foodborne pathogens (Nauta et al. 2013). Thus, the safety and shelf-life of minimally processed meat products have to rely on a high microbial quality of the raw material and on the inhibitory factors (hurdles) employed in product processing, controlling growth and survival of the given pathogens and spoilage bacteria. The main hurdles employed in production of fermented sausages are: high salt, low water activity, decrease of redox potential and pH, and growth of competitive microflora (Holck et al. 2011).

Traditionally, fermented sausages are considered safe due to the combined effect of hurdles which, in theory, should efficiently control pathogens (Barbuti & Parolari 2002). Despite this, foodborne outbreaks caused by consumption of fermented sausages contaminated with *Salmonella* and verocytotoxin producing *Escherichia coli* have been reported regularly, recently by: Gossner et al. (2012); Kuhn et al. (2011); Bone et al. (2010); Ethelberg et al. (2009); Schimmer et al. (2008) and Sartz et al. (2008).

A key to augment food safety is an understanding of the physiological response of foodborne pathogens to the stress they encounter in the food-processing chain along with during their infectious cycle. With the wish of adding to the knowledge of the molecular risk entailing the presence of *Salmonella* in food and with the overall aim of investigating how a food-environment influences the ability of *Salmonella* to survive and cause disease, the following studies were undertaken:

- Development of a method enabling gene expression studies of pathogens in a food matrix
- The impact of growth phases on the survival of *Salmonella* during fermentation of sausages
- The impact of food components on *Salmonella* inactivation during simulated gastric passage
- The impact of bioactive substances secreted by lactic acid bacteria employed for sausage fermentation on virulence determinants in *Salmonella*

Chapter 2

Salmonella – a zoonotic pathogen

2.1 Characteristics

Salmonella spp. are rod-shaped, predominantly motile, Gram negative, facultative anaerobe, invasive bacteria, closely related to the genus *Escherichia,* and as such, belonging to the *Enterobacteriaceae* family (Brenner et al. 2005). *Salmonella* is able to infect a wide range of unrelated host species and although being primarily intestinal, salmonellae are able to survive outside a host for extended periods (Giaccone et al.2012). The genus *Salmonella* is currently divided into two species: *S*. *enterica* and *S*. *bongori* (Tindall et al. 2005). *S*. *enterica* is further divided into six sub-species, with most zoonotic *Salmonella* belonging to the subspecies I (subsp. *enterica*) (EFSA 2012). Solely app. 50 of the more than 2600 known serotypes of *S*. *enterica* account for ~ 99% of all clinical isolates from humans and domestic mammals, and they belong to subspecies I (CDC 2008). The two most prevalent *Salmonella* serotypes *S*. Typhimurium and *S*. Enteritidis are broad host-ranged (un-restricted by the definition of Uzzau et al. (2000) – also differentiating between host-adapted and host-specific serotypes), whilst a few others are predominantly found in one particular host. Host-adaptation is seen in *S*. Dublin and *S*. Choleraesuis, which is strongly associated with cattle and pigs, respectively – still they can cause disease in other hosts and are highly pathogenic in humans. *S*. Typhi and *S*. Paratyphi A and C are host-restricted serotypes, with exclusive expression of systemic disease in human hosts (Coburn et al. 2007). Host-specific serotypes in animal hosts include *S.* Gallinarum and *S*. Abortusovis in poultry and sheep, respectively (Giaccone et al. 2012).

2.2 Clinical manifestations of *Salmonella*

The severity and nature of disease depends both on host susceptibility and the infectious *Salmonella* serotype, with manifestation of distinct syndromes in different hosts (Coburn et al. 2007). In humans four disease patterns can be recognized; enteric fever, gastroenteritis, bacteraemia, and chronic asymptomatic carriage (Pui et al. 2011).

The systemic disease, enteric fever, caused by *S*. Typhi and *S*. Paratyphi, is often associated with poor hygienic conditions, and infection typically occurs due to the ingestion of food and water contaminated with human waste (Parry et al. 2002). *S*. Typhi along with non-typhoid *Salmonella* (NTS) infections are endemic in many developing countries.

In developed countries the leading cause of human salmonellosis is non-typhoid *Salmonella*, which generally cause gastroenteritis and is transmitted through the ingestion of food or water contaminated with animal waste (Pui et al. 2011). Most *Salmonella* infections are self-limiting, yet severe complications such as bacteraemia can follow, especially in the elderly and young, pregnant women and immunocompromised individuals (Álvarez-Ordóñez et al. 2011). Systemic diseases require antimicrobial treatment, with the current drugs of choice being fluoroquinolones or expanded-spectrum cephalosporins e.g. ceftriaxone for children (Foley & Lynne 2007). Current measures to control *Salmonella* infections are being compromised by the emergence of multidrug-resistant strains worldwide, hereunder also by an increase in resistance to the aforementioned clinically important antimicrobial agents (reviewed by Hur et al. 2012; Mastroeni & Grant 2011). Recent figures from CDC show that 34.2% and 21.9 % of *S.* Typhimurium isolates are resistant to two and five antibiotic subclasses, respectively (CDC 2009). Moreover, it is still questionable whether antibiotics can penetrate the intracellular *Salmonella*-containing vacuoles (Lahiri et al. 2010). In developing countries mortality rates due to NTS is as high as 24% (reviewed by Fábrega & Vila 2013) and, despite antibiotic therapy, invasive NTS infections in immunocompromised adults in sub-Saharan African results in mortality rates above 20% (Mastroeni & Grant 2011).

The asymptomatic carrier of *Salmonella* may adversely infect others and thereby spread the disease. Non-typhoid *Salmonella* has been shown to persist in the gastrointestinal tract from 6 weeks to 3 months, depending on serotypes (Pui et al. 2011). Likewise, healthy hosts have been shown to shred both *S.* Typhimurium and *S*. Dublin, which remained viable outside the host for at least six months (Giaccone et al.2012).

Many factors are influencing the dose of *Salmonella* cells required to cause human infection including; host susceptibility, the matrix of delivery, and the virulence traits and physiological state of the bacterial cell (Humphrey 2004). Results from volunteer studies indicate that the infective dose for various serovars is 10^5-10^9 organisms (Kothary & Babu 2001) while epidemiological

evidence from several outbreaks reports that as few 1-10 cells can cause salmonellosis (Humphrey 2004; Teunis et al. 2010).

2.3 Burden of salmonellosis

Salmonella has long been recognised as an important zoonotic pathogen of economic significance in animals and humans. In 2011, 95,548 confirmed cases of human salmonellosis were reported from 27 European Union Member States. In foodstuffs, *Salmonella* was most often detected in meat and products thereof (EFSA 2013). A coordinated approach, probably especially in the poultry populations, has led to a reduction of human cases of salmonellosis in the EU by almost 40% compared to 2007(EFSA 2013). However, *Salmonella* remained the most frequently detected causative agent in food-borne outbreaks reported in the EU in 2011 (EFSA 2013). In Denmark the number of human *Salmonella* cases, in the same year, dropped to the lowest level since the 1980s (Anonymous 2012). Human salmonellosis was initially caused by the spread of *Salmonella* in broiler chickens and subsequently in swine and laying hens, whilst in present days almost half of the cases are acquired abroad (Anonymous 2013), due to national control programs (Wegener et al. 2003). Less progress has been made in the United States where, according to CDC estimates, foodborne diseases have reduced 25 % in the last 15 years, except *Salmonella* infections (CDC 2011). Moreover, non-typhoid *Salmonella* spp. remains to be the leading cause of hospitalizations and deaths, while the number of illnesses was exceeded only by norovirus (Scallan et al. 2011). On a global scale 93.8 million cases of gastroenteritis caused by *Salmonella* have been estimated to occur each year, with an estimated 80.3 million foodborne cases (Majowicz et al. 2010).

2.4 *Salmonella* in foods

The principal reservoir of *Salmonella* is the intestinal tract of a wide range of farmed and wild animals from where they spread and can prevail for a period of time in food, water, soil, insects, plants, surfaces, and even air-borne dust (Guard et al. 2011). Flies are a possible indirect vector as they may transmit the bacterium to foods (Ostrolenk & Welch 1942). Although the most common sources of salmonellosis are food of animal origin, in recent years, disease transmission routes involving fresh produce, as fruit and vegetables, have gained major concern in industrialized

countries (Pui et al. 2011). Hence a variety of foodstuffs of both animal and plant origin are at risk of becoming contaminated by faecal organisms either directly or indirectly (EFSA 2012). *Salmonella* transmission to food processing plants or food production equipment is also of great importance, whilst e.g. inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat (RTE) food may allow the organisms to multiply (EFSA 2012). *Salmonella* contamination in the food-chain can occur at any point from crop, farm, livestock feed, through food manufacturing, processing and retailing, as well as during food preparation at home, in restaurants and canteens (Gómez-Aldapa et al. 2012).

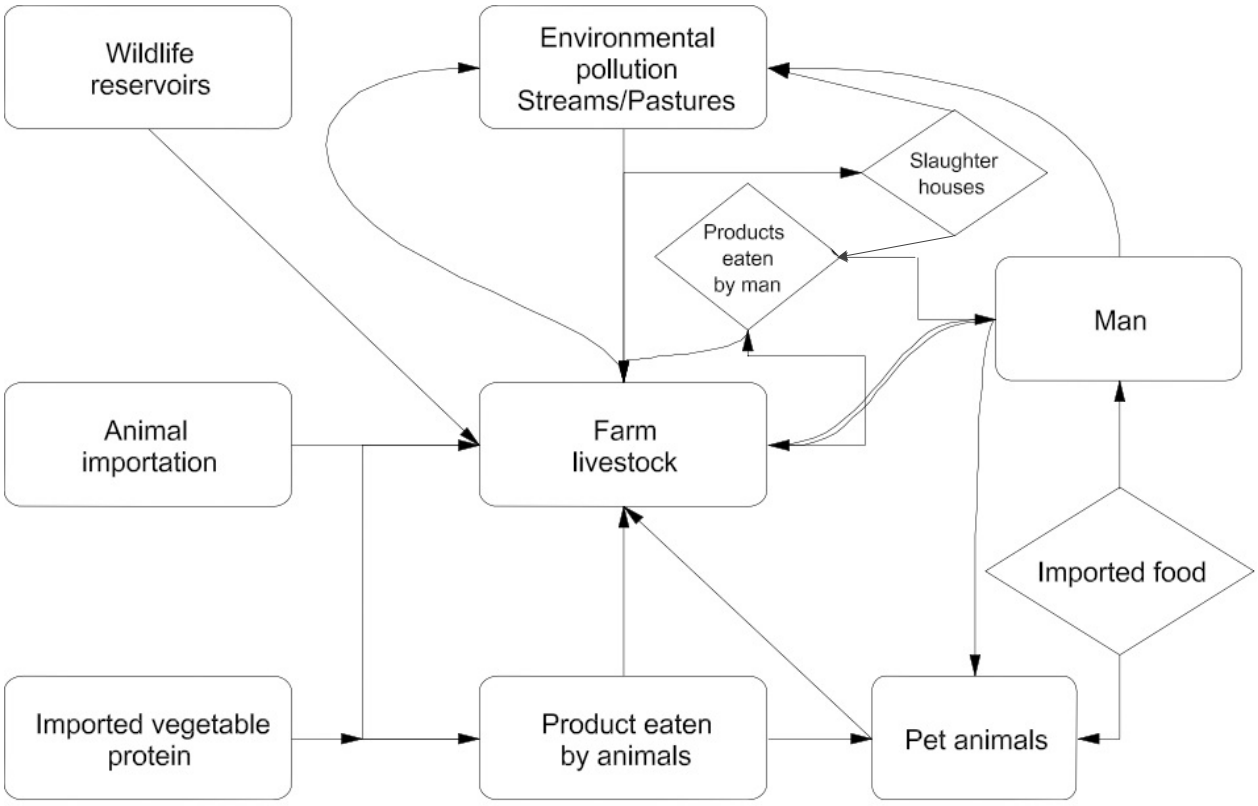

Figure 1. *Salmonella* transmissive cycle.
Source: Giaccone (2012).

Internationally, the serovars most frequently associated with human illness from contaminated foods are *S*. Enteritidis and *S*. Typhimurium. Human *S*. Enteritidis cases are mostly associated with the consumption of contaminated eggs and poultry meat while *S*. Typhimurium cases are most often linked to the consumption of contaminated pork, poultry and bovine meat (EFSA 2012). In

recent source attribution estimation, almost 57% of the human salmonellosis cases could be attributable to pigs in the EU (EFSA BIOHAZ Panel 2012).

2.5 Pathogens and outbreaks related to fermented sausages

Subclinical infections are common in animals (EFSA 2012), hence they can carry and excrete pathogens without any symptoms of disease, and contamination of the carcass during slaughter is a main entry for e.g. *Salmonella* in the production line (Giaccone et al. 2012). In order not to change the organoleptic properties of fermented sausages post-fermentation heating steps are rarely applied, hence the meat in these products is essentially raw. Due to this lack of thermal or other sterilizing treatments before consumption semi-dry and dry fermented sausages are considered ready-to-eat products.

According to EFSA (2012), 2.8% of the samples taken from minced meat and meat preparations from other species than poultry intended to be eaten cooked tested positive for *Salmonella* in the EU in 2010. Of even greater concern is the 1.8 % found in RTE foods, such as minced meat and meat preparations intended to be eaten raw. Occasional findings of up to 3-5 % of RTE meat products testing positive for *Salmonella* spp. have previously been reported (EFSA 2007).

Principally, raw meats from any animal species can be used for the production of fermented sausages, however, porcine meat is predominant – often in combination with e.g. beef (Barbuti & Parolari 2002). Due to the diversity of fermented sausages and since these products can be contaminated from many sources; raw meat, ingredients and/or processing equipment, and/or from post-processing contamination – the range of potentially associated pathogens could supposedly be wide. Epidemiological data show that i.a. *Salmonella* spp., enterohaemorrhagic *Escherichia coli* (EHEC) and *Staphylococcus aureus* have been implicated in foodborne outbreaks associated to the consumption of fermented sausages (Fernandes 2009). *Campylobacter jejuni*, *Yersinia enterocolitica, Listeria monocytogenes* and *Clostridium botulinum* may also be present in the products (Cabeza et al. 2009).

Foodborne gastrointestinal outbreaks especially by verocytotoxin producing *Escherichia coli* and *Salmonella* caused by consumption of fermented sausages have been reported regularly, recently by: Gossner et al., 2012; Kuhn et al., 2011; Bone et al., 2010; Ethelberg et al., 2009; Schimmer et

al., 2008 and Sartz et al., 2008. Sartz et al. (2008) identified that delayed start of fermentation, lack of heat-treatment, and a short curing period in cold temperature were the main factors enabling EHEC survival. Mainly, problems with *E.coli* and *Salmonella* have been reported in relation to shortly ripened semi-dry sausages (Leroy et al. 2006), also *Salmonella* has been related to food poisoning in sausages prepared with short fermentation periods and without starter cultures (Pontello et al. 1998).

Chapter 3
Principles of fermented sausage manufacture

Processed meat products may be preserved through fermentation, smoking, curing, heating etc. The production of fermented sausages constitutes a major part of the meat industry of continental Europe, with the strongest influence in Germany and Mediterranean countries (Ordóñez et al. 1999). Germans have the highest *per capita* consumption, around 4.5 kg per year (Lücke 2000a). In Spain alone the annual production has exceeded 190.000 tonnes (Cabeza et al. 2009), and in EU it is around 750.000 tonnes (Lücke 2000a). Fermented sausages are prepared from seasoned meat which is cured and stuffed into casings and left to ferment. Fermentation is carried out under controlled temperature and humidity conditions with subsequent ripening/drying (Ordóñez et al. 1999).

The manufacturing process of fermented sausages thus involves three main steps; formulation, fermentation and ripening/drying (Fig. 2). Variations in type and amount of raw materials and manufacturing conditions (e.g. temperature and duration of fermentation and ripening period) give rise to a wide variety of products with profound regional differences (Fernandes 2009).

Traditionally, sausages producers have relied on the indigenous flora of the meat or from the environment, so called “house flora”, to spontaneously ferment their products. Sequential inoculation of a new portion of sausage batter with material from a successfully fermented one, denoted back-slopping, was/is used to select for a desired bacterial population (Leroy et al. 2006). To standardize the production, improve consistency of quality and safety, and to shorten the ageing processes required for flavour formation, the sausage batter nowadays can be inoculated with commercial starter cultures (Hutkins 2006). Artisan products of a quality that might be superior to the uniform industrialized products continue to be produced by traditional means primarily by small manufactures (Leroy et al. 2006). While virtually all of the fermented sausages in northern Europe are produced with bacterial starter cultures, the percentage in southern Europe is commonly much lower – accounting for less than 30% of the production in Italy, Spain, Greece and Portugal when data was provided by Lücke et al. (1990). Seemingly, the rapid fermentation products using starter cultures do not differ in terms of food safety from the less acidic ones often produced without starters in southern Europe (Barbuti & Parolari 2002).

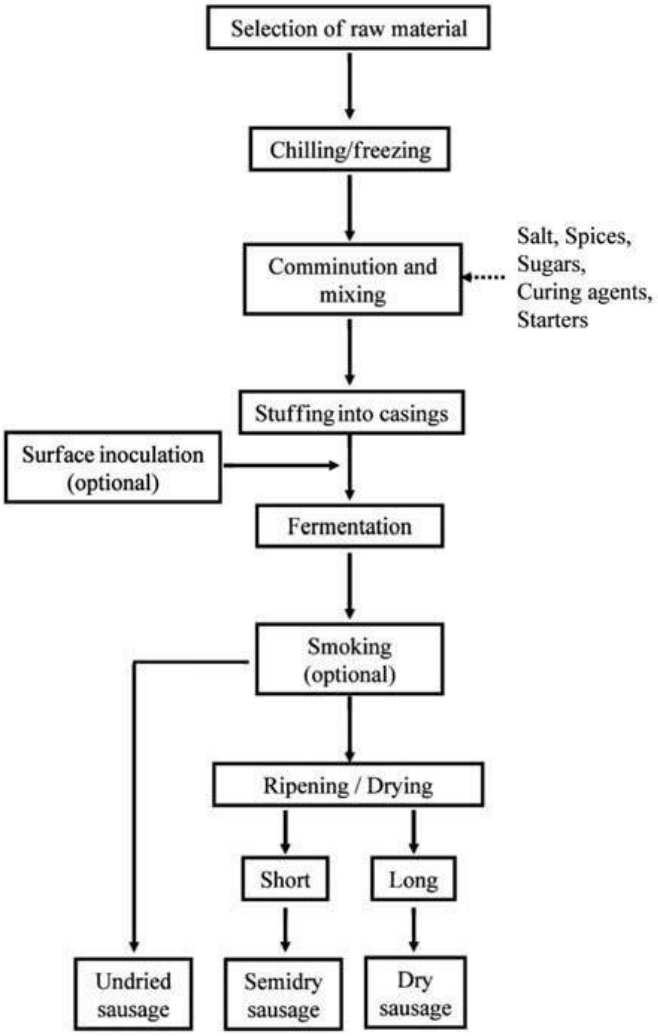

Figure 2. Manufacture of fermented sausages.
Source: Fernandes (2009).

There is an increasing consumer demand for safe, minimally-processed and -preserved food (Gálvez et al. 2007). While a health and nutritional benefit will derive from lowering e.g. the salt and fat content, minimal preservation might give rise to products with intrinsic food safety problems. The initial bacterial load is evidently of great importance for the microbial stability of the RTE products, and a reduction of the raw meat contamination will presumably lead to a safer product. This said, outbreaks related to post-processing contamination of RTE products with e.g. spices have also been reported (CDC 2010).

3.1 Hurdles in sausage production

Apart from ensuring a high quality of the raw material, safety and shelf-stability of RTE meat products is attained by controlling growth and survival of the given pathogens and spoilage during production by employing a combination of preservative factors (hurdles): high salt, low a_w, decrease of redox potential, growth of competitive microflora and a decrease in pH (Holck et al. 2011).

In theory, each hurdle is non-lethal - collectively they exert the inhibitory power to prevent microbial growth, but often hurdle technology does not lead to an inactivation of the microorganisms (Leistner 2000). Inadvertently this type of food preservation could give rise to sub-lethally stressed pathogens which, by synthesis of protective stress shock proteins, activate protective mechanisms and become more resistant or even more virulent. Entailing that the number of cells needed to initiate infection is decreased. Acid-induced resistance responses in *Salmonella* may lower the infectious dose from around 10^5 to 10 - 100 cells (Mashall 2003). A mere prevention of growth instead of inactivation of course also poses problems when the infectious dose of the pathogen is naturally low, as is true for the few cells needed to cause severe infection by verocytotoxic *E. coli* (Kothary & Babu 2001). Furthermore, adaptation to one type of stress can also result in an acquired cross-tolerance to other forms of stress of the surviving organisms (e.g., Leyer & Johnson 2003; Greenacre and Broclehurst 2006). This said, for pathogenic bacteria to cope with the concomitant inherent stresses in the fermented sausages, synthesis of several different stress shock proteins is possibly necessary, supposedly rendering the microorganisms metabolically exhausted (Leistner 2000). Moreover the hurdles might not merely result in an additive effect - a synergistic effect will arise when the hurdles hit different targets in the cell at the same time, thus disturbing homeostasis in more than one respect (Leistner 1995). The aspect of increased tolerance to stress by adaptation will be addressed in more detail. Traditionally, fermented sausages are considered safe due to the combination of hurdle factors which, in theory, should efficiently control pathogens (Barbuti & Parolari 2002). Although growth of pathogens, after the very early stage of fermentation before the pH is lowered (Nissen & Holck 1998), is unlikely to occur, they might still survive and be detected at levels higher than those established in the microbial criteria (absence of salmonellae in 25 g food). Prevalence data from the US reported by Levine et al. (2001) indicates that 1.4% of fermented sausages are contaminated with *Salmonella* spp.

As mentioned, fermented sausages have been implicated in recent outbreaks of foodborne illnesses due to gram-negative pathogens. Still they have a good safety record when compared to other foods (Fernandes 2009; Moore 2004).

A list of antimicrobial barriers dominating in fermented sausages can be seen in Table 1.

Table 1. Antimicrobial barriers in fermented sausages.

Property	
pH	4.5-5.8
acids	0.4-2.8%
a_w	0.7-0.95
salt	2-3%
Eh	Reduced
competition	Exclusion
casing	Exclusion
nitrite	60-200ppm*

* Dependent on regional legislation.
Source: Hutkins (2006), amended (data from Vignolo et al. 2010; Talon & Leroy 2006).

During the early stage of fermentation oxygen is depleted, the redox potential is lowered, and the growth of aerobic spoilage is restricted to the surface of the sausage (Lücke 2000a). Lactic acid starter cultures are known to scavenge sugars and other nutrients faster than their competitors (Hutkins 2006) although this kind of antagonism can seem unlikely in a nutrient-rich substrate as meat (Lücke 2000a). However, in solid-substrate fermentation of meat, the bacteria grow in micro-colonies in nests, rendering the competition for nutrients and the impairment by metabolic products appreciable in these defined areas. The microstructure is a significant hurdle in the stability of fermented sausages as it effects survival of pathogens as well as the desired ripening process (Leistner 1995). Moreover, lean meat contains considerable amounts of amino acids and peptides but only small amounts of sugar (Lücke 1994). For a rapid and amble pH reduction to occur, 0.3 – 0.8 % sugar (glucose and occasionally lactose or sucrose) is often added to the sausage mix (Fernandes 2009).

3.1.1 Water activity

Reducing the available water in food is a long-established method for controlling bacterial growth, as most spoilage and pathogenic species cannot grow under low water-activity conditions

(Fennema 1985). Based on water activity (a_w) fermented sausages can be denoted dry (a_w<0.90) or semi-dry (a_w between 0.90 and 0.95) (Lücke 2000a). Reduction of water activity is reliant on actual loss of water – 25-50% total weight loss in dry sausages or 10-15% in semi-dry sausages, and on the binding of water by NaCl and other solutes (Holck et al. 2011). The a_w of fresh meat is app. 0.99 and moisture is reduced almost exclusively from the muscle meat which has a water content around 80 %, while fat only holds 10 %. The fat content thereby influences the a_w indirectly by decreasing the water content (Roca & Incze 1990) – thus the leaner the sausages, the longer the drying time. Fermented sausages are either dried at a low rate for dry sausages (10-15°C for ≥4 weeks at a relative humidity of 65-90%), or at a fast rate for semi-dry sausages (5-16°C for 2-40 days). Semi-dry sausages are often smoked and slightly cooked by the heat used in the smokehouse, which occasionally can reach nearly 60°C for a strictly limited time (Vignolo et al. 2010). Dry sausages are, unlike their semi-dry counterparts, never exposed to pathogen inactivating temperatures and have attracted substantial interest regarding their safety. Due to a higher moisture-to-protein ratio, semi-dry sausages might require refrigeration, whilst dry sausages are considered shelf-stable (Vignolo 2010).

The minimum a_w for growth of *Salmonella* is approx. 0.94 (see Table 2), albeit lower values down to 0.90 have been reported depending on serotype, substrate, temperature and pH (Fernandes 2009). *Salmonella* is resistant to drying and has been found to survive in dry foods with a_w<0.2 for long periods (Pui et al. 2011).

Tolerance of *Salmonella* to different relevant factors are seen in Table 2

Table 2. Limits and optimum growth in relation to intrinsic and extrinsic factors for *Salmonell*a spp.

Conditions	Minimum	Optimum	Maximum
T °C	7.0 (5.2*)	35-43	46.2 (49.3 *)
pH	4.0 (3.8*)	7.0-7.5	9.5
Aw	0.94 (0.90*)	--	--
Tolerance to salt (%)	--	--	4

*Some serotypes.
Source: Giaccone (2012).

3.1.2 Curing

Sodium chloride is a main hurdle in processed meats. On average the quantity of salt added to fermented sausages is 2.0 – 3.0 % (Fernandes 2009). Whilst higher concentrations would augment food safety, it would also render the product unpalatable and inhibit growth of the starter

cultures. Evidently the actual salt concentration within the aqueous phase and in the final products will always be higher than in the initial mix as these products lose a substantial amount of water (Hutkins 2006). Apart from being an important hurdle and flavour enhancer, salt solubilizes and extracts myofibrillar proteins from the small lean meat particles after mincing (Ordóñez et al. 2010). These solubilized proteins form a gel between meat/meat and meat/fat particles in the matrix, giving rise to an increasingly firm structure with progressive ripening and drying of the products (Vignolo et al. 2010).

Curing with nitrite also serves as a preservation hurdle. The antimicrobial effect is highly pH dependent and experiments indicate that dependency on salt concentration and temperature might likewise be more important than the actual $NaNO_2$ concentration (Getty et al. 2000). Nitrite minimizes the outgrowth of *Clostridium botulinum* spores and development of botulinal toxin (Holck et al. 2011), but the inhibitory effect on other pathogens differs markedly (Lücke 2000a). Studies performed in pilot plant indicate that the inhibitory effect of $NaNO_2$ on *Salmonella* is minor if pH is under 5 while absence of $NaNO_2$ will result in growth of *Salmonella* in sausages with a pH > 5 (Gunvig et al. submitted 2013). Lücke (1994) also states that salt and nitrite play an important role in suppression of salmonellae early in the fermentation when the pH is still above 5.3. Apart from its growth inhibiting properties nitrate is added to sausages to obtain colour and cured flavour and to prevent lipid rancidity (Leroy & De Vuyst 1999).

Growth of *Salmonella* is generally inhibited in the presence of 3-4% NaCl although the salt tolerance increases with increasing temperature (D'Aoust 1989). An overview of the osmotic stress response activated by *Salmonella* in response to drying and augmented salt concentrations is given in 4.6.2

3.1.3 pH reduction

Lowering of pH by accumulation of lactic acid from the conversion of glycogen reserves in the muscle tissues and from the added sugar during product fermentation is essential for the safety of fermented sausages (Lücke 2000a). The lower external pH disturbs the homeostasis of different pathogens as well as spoilage bacteria e.g. pseudomonads (Leistner 2000). LAB produces lactic acid via glycolysis from hexoses while a smaller amount of acetic acid can be produced concomitantly from pentoses (Työppönen et al. 2003). The antimicrobial effect of acetic acid is

considerable, but it is only acceptable in low concentrations from a sensory point of view (Lücke 2000). Although *Salmonella* is more sensitive to acetic acid than to lactic acid, the former is also more prone to induce an acid tolerance response (ATR), rendering the cells more capable of surviving ensuing extreme acidic conditions (Álvarez-Ordóñez et al. 2008).

Fermentation conditions differ in terms of duration and temperature. In general, a higher fermentation temperature will lead to a more rapid acidulation to final pH values below 5, upon which the microbial safety of semi-dry sausages primarily relies (Fernandes 2009). Though, if acid formation for some reason is delayed or inhibited, the high fermentation temperature increases the risk of growth of mesophilic pathogens (Gunvig et al. submitted 2013). Starter cultures, as mentioned, are commonly used in semi-dry products, prepared in North America and in northern Europe, with accelerated pH drop, texture, and flavour development. Often the pH in dry sausages as manufactured in the Mediterranean is somewhat higher (pH 5 – 5. 8) and tends to rise during their long – up to several months – ripening (Vignolo et al. 2010). Addition of starter cultures is less common in these products, and their safety is primarily ensured by low a_w, which is the only hurdle that is strengthened over time. In general, dry sausages are fermented for 1-7 days at 12-24°C, while semi-dry sausages only ferment 1-2 days at 25-35°C (Vignolo et al. 2010). The lower pH of fermented sausages is important not only bacteriologically but also in enhancing drying speed since the water-holding capacity is reduced in the range near the isoelectric point of muscle protein (Roca & Incze 1990).

Salmonella has a wide pH growth range between 3.8 and 9.5 – see Table 2. While it is relatively resistant to low pH in the stationary growth phase (Lee et al. 1994), it can only survive moderately low pH (4-5) when exponentially growing (Rychlik & Barrow 2005). Adaptation can in both cases lead to increased acid resistance. Hence, these organisms may very well grow at the pH-values dominating in fermented sausages, but additional hurdles that render the growth conditions suboptimal most often lead to their inhibition.

3.1.4 Inhibitory substances produced by starter cultures

Addition of microorganisms for fermentation of meats may serve different purposes; improvement of food safety by antagonising pathogens, improvement of stability and extension of shelf-life, and changing of the sensory quality of the raw material (Lücke 2000b). The main groups

of bacteria important for meat fermentation are; lactic acid bacteria (LAB) and catalase-positive staphylococci (Talon & Leroy 2011). While the lactic acid bacteria generally serve all of the above purposes, the cocci induce and stabilize the desired sensory properties of the food (Lücke 2000b). Important properties of bacteria used in commercial starter cultures can be seen in Figure 3.

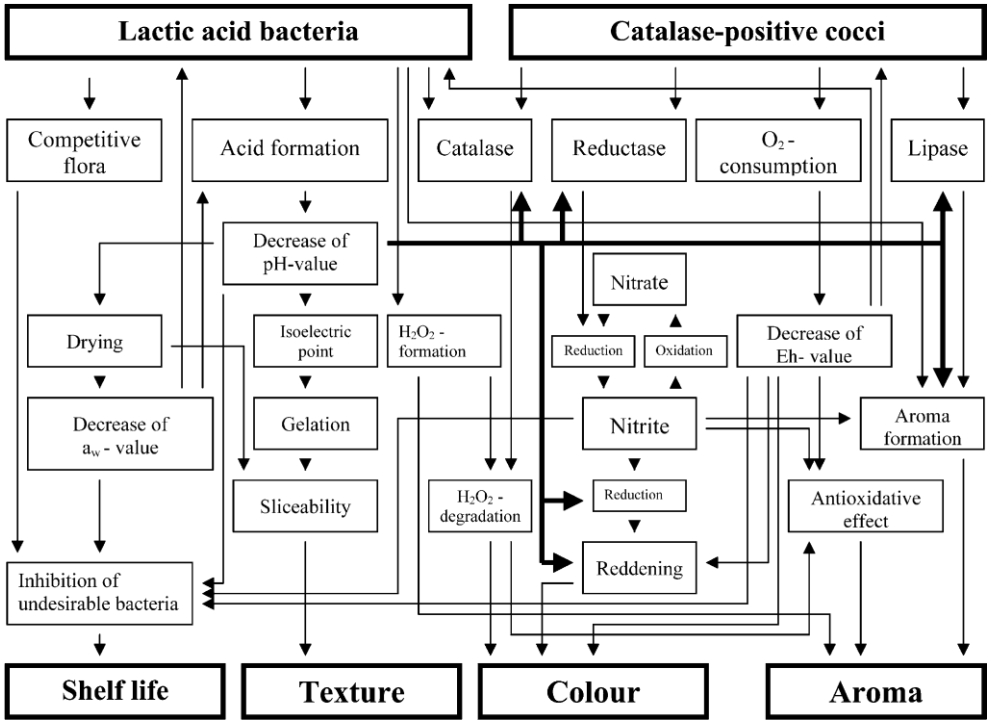

Figure 3. Interactions during the fermentation of sausages caused by the action of lactic acid bacteria and catalase-positive cocci
Source: Työppönen et al. 2003.

The LAB most commonly isolated from conventional dry fermented sausages or those used in commercial starter cultures are; *Lactobacillus sake*, *Lb. curvatus*, *Lb. plantarum*, *Pediococcus pentosaceus*, and *P. acidilacti* (Ordóñez et al. 1999). While the two latter generate lactic acid through a homofermentative pathway, the three lactobacilli are heterofermentative (Axelsson 2004). Commercially available catalase-positive and coagulase-negative strains are; *Staphylococcus xylosus, Staph. carnosus* and *Kocuria varians* (Fernandes 2009).

The formulation and fermentation conditions (high salt, low pH, low temperature) as well as an anaerobic environment strongly favour the desirable lactic acid bacteria.

It has been widely accepted that lactic acid starter cultures serve preservative functions beyond their acidifying and pH-lowering effects. Some produce antagonistic substances of which bacteriocins have attracted most attention as possible hurdles and food preservatives (Gálvez et al. 2007). Other metabolites secreted by LAB with inhibitory effect against Gram-negative bacteria *in vitro* have limited usage in food preservation as they might; be produced in insufficient amounts to be active e.g. reuterin, adversely affect the perception of the products e.g. diacetyl and hydrogen peroxide or raise regulatory concern e.g. benzoic acid (Lücke 2000b).

Bacteriocins produced by LAB are small ribosomally synthesized, antimicrobial peptides or proteins (De Vuyst & Leroy 2007). They are most active towards closely related bacteria, but frequently also towards spoilage organisms and food-borne pathogens, especially *Listeria monocytogenes* (De Vuyst & Leroy 2007). Several starter cultures for sausage production with anti-listerial activity are commercially available e.g. from the Danish manufacturer Chr. Hansen. Contrary to the initial belief that bacteriocins isolated from LAB were only active against other Gram-positive bacteria, several reports of bacteriocins from Gram-positive bacteria with inhibitory activity against Gram-negative bacteria have been presented (reviewed by Chalón et al. 2012). Bacteriocins primarily exert their inhibitory action by affecting the integrity of the cytoplasmic membrane of sensitive cells (Lücke 2000b). In Gram-negative bacteria the outer membrane (OM) serves as an efficient permeability barrier and a combined OM-disruptive treatment enabling the bacteriocin to reach its target of action will most often be needed for efficacy against e.g. *Salmonella* (Gálvez et al. 2007). Only a few bacteriocins – often times involving the approved food preservative nisin – have been studied in a food matrix as a control measure of *Salmonella* and then always in combination with OM disruptive methods (Chalón et al. 2012). As neither chemical treatments with e.g. chelating agents or detergents nor physical treatments as e.g. high hydrostatic pressure or ultrasonication may be acceptable for the industry (or the consumer) for destabilizing the OM – the somewhat narrow inhibitory spectrum of the bacteriocins known today is impeding their functionality since Gram-negative bacteria (and viruses), by far, are the main players regarding food borne gastrointestinal disease causing agents.

Recent research indicates that certain probiotic LAB strains secrete factors that have the ability to influence gene expression in specific pathogens. During the course of this PhD study we demonstrated that this ability is shared by commercial starter cultures and LAB isolates hereof

Regulation of the infection potential of foodborne pathogens by control of virulence genes may be a novel hurdle in food where a complete elimination cannot be guaranteed (Manuscript III).

3.2 Inactivation of pathogens in fermented sausages

Several studies have addressed the inhibition of pathogens in fermented sausages e.g., Hinkens et al. 1996; Ihnot et al. 1998; Nightingale et al. 2006; Ellajosyula et al. 1998; Chikthimmah & Knabel 2001; Nissen & Holck 1998; Heir et al. 2010; Porto-Fett et al. 2008; Hwang et al. 2009. Results obtained by e.g. Nightingale et al. (2006) suggest that *Salmonella* is capable of surviving dry and semi-dry sausage manufacturing processes. This might especially be true for acid adapted strains as also demonstrated by Smith et al. (1975). Challenge trials have been reviewed by Getty et al. (2000) and results confirm that *E.coli* O157:H7, if present in sufficiently high numbers, is able to survive the fermentation and drying of sausages. In general, fermentation and drying alone may not be sufficient to achieve the 6.5 $\log_{10}$ reductions of *Salmonella* in RTE meat products issued by USDA/FSIS in 2001 or the 5.0 $\log_{10}$ reduction of *E. coli* 0157:H7 in fermented products containing beef (Nightingale et al. 2006).

Porto-Fett et al. (2008) reported that fermentation and drying reduced numbers of *Salmonella* Typhimurium in soudjouck-style semi-dry sausage by 1.52 and 3.51 $\log_{10}$ CFU/g for sausages fermented to pH 5.3 and dried to a moisture-to-protein ratio (M:PR) of 1.09:1 or an pH of 4.8 and a M:PR of 1.16:1 respectively. An additional decrease of 0.88-3.17 $\log_{10}$ CFU/g was seen within 30 days of storage at 4, 10, or 21°C. Ellajosyula et al. (1998) reported a reduction of less than 2.0 $\log_{10}$ CFU/g of *S*. Typhimurium by a long fermentation (pH 4.7) in Lebanon bologna while a subsequent heating step (43.3°C for 20 hours, 46.1°C for 10 hours, or 48.9°C for 3 hours) reduced populations of *Salmonella* and *E. coli* 0157:H7 by > 7 $\log_{10}$ units. Ihnot et al. (1998) noted a 2.9 $\log_{10}$ CFU/g reduction of *S*.Typhimurium in pepperoni by fermentation (pH ≤ 4.8) and drying (M:PR 1.16:1). Storage of the pepperoni sticks for 56 days under vacuum at 4 or 21°C reduced the counts of the pathogen by 4.6 and 6.6 $\log_{10}$ respectively.

As seen in the before mentioned examples post-process (heat) treatment or longer fermentation and/or storage times may be necessary in achieving further reductions of surviving pathogens. For many types of sausages, though, thermal treatment will adversely change the sensory properties of the product.

Apparently, foods which are microbiologically stable become safer during storage, especially at ambient temperature (Leistner 2000). Salmonellae for instance which have survived fermentation and ripening of fermented sausages will disappear more rapidly if the products are stored at ambient temperature as opposed to under refrigeration where they survive longer and evidently pose a greater risk to the consumer (Leistner 1995). Results from inactivation studies of *E. coli*, *L. monocytogenes* and *Y. enterocolitica* in fermented sausages during maturation/storage also indicate that the practice of utilising a short maturation period and storage at refrigeration temperatures may result in unsatisfactory reductions of pathogens present (Lindqvist & Lindblad 2008). This was also seen in results reported by Nissen and Holck (1998), regarding survival of *E. coli* O157:H7, *L. monocytogenes* and *S. Kentucky* in Norwegian fermented, dry sausage. However, deterioration of the product due to a higher storage temperature (≥20°C) has been noted (Porto-Fett et al. 2008; Nissen & Holck 1998), and thus may not be applicable by the industry.

The reported pathogen reduction values based on pH/a_w/storage temperature and time vary from study to study, making it difficult to generalize the levels of reduction reached at the different steps in the production (Hwang et al. 2009). In a recent meta-analysis of 44 independent studies by McQuestin et al. (2009), the relative effect of temperature, pH and a_w on inactivation of *E. coli* in fermented meats was investigated. Data indicated that temperature (fermentation, maturation and storage) accounted for 61 % of the variability in the data while pH and a_w accounted for less than 8 %. This implies that under growth inhibitory conditions (e.g., low pH and a_w) the rate of inactivation of *E. coli* is dominated by temperature, even though temperatures typically used in fermented meat manufacture are not lethal to *E. coli.* Discrepancy probably also arises due to the marked diversity of fermented sausages in which pathogens are subjected to the combined effects of a wide variety of appertaining hurdles; leading to a complex and varying pattern of stress responses, cell injury and death. Recovery of injured cells will also differ markedly between direct counting and enrichment methods. In comparison, reductions reported by Nightingale et al. (2006) of *Salmonella* populations during fermentation and drying were ~ 1.0 to 2.0 log CFU/g lower when estimated with a solid repair medium (XLD-OVLY) rather than a selective media.

The safety of the manufacturing procedures could also be assessed using models describing the reduction of pathogens during production of fermented sausages (e.g., Hwang et al. 2009; Pond et al. 2001; Gunvig et al. submitted), with a notion of their inherent uncertainty in predictions of 0.5 to 1.0 $\log_{10}$ (Holck et al. 2011).

It is generally recognised that the lower the pH and/or a_w of the semi-dry or dry fermented sausage and/or the higher the storage temperature, the greater the reduction of pathogens (Hwang et al. 2009). In accordance to previous studies (Nissen & Holck 1998; Porto-Fett et al. 2008; Nightingale et al. 2006), Hwang et al. (2009) stated that *S.* Typhimurium was less viable than *E. coli* O157:H7 or *L. monocytogenes* following manufacture and storage of fermented sausages. The risk of heavy contamination of the sausage batter corresponding to the high inoculum levels (most often > 10^5 CFU/g) used in inhibition studies of pathogens in fermented sausages is probably small. Still, growth in temperature-abusive environments, delayed or inhibited acid formation during fermentation along with cross-contamination during processing may lead to elevated pathogen levels (Nightingale et al. 2006). A high level contamination was seen in a foodborne outbreak of salmonellosis where the incriminating Bologna sausage was demonstrated to hold 10^6 CFU/g (van Netten et al. 1986). Moreover *Salmonella* species, *E. coli* 0157:H7 and *L. monocytogenes* all have been reported to cause disease even when ingested in very low numbers (Teunis et al. 2010).

Chapter 4
Stress response of *Salmonella* and food-environmental adaptation

In the following sections the physiological response to the two main stressors (acid and salt) in fermented sausages, along with the genes responsible for the organisms' ability to adapt and survive are described.

Salmonella spp. are resilient and, as most foodborne pathogens, they can respond to a wide variety of external stresses and adapt rapidly to survive in a hostile environment. This is often associated with regulation of transcription of genes, encoding for stress proteins, which can enhance bacterial survival. Gene regulators respond to specific signals by inducing or inhibiting transcription, translation or altering stability of the gene product (Ramos et al. 2001). Transcriptional regulation of gene expression in bacteria commonly involves alternative sigma factors, two-component regulatory systems or transcriptional regulators (Spector & Kenyon 2011; Shen & Fang 2011).

4.1 Acid stress

4.1.1 Physiological response

In a solution, weak acids exist in equilibrium between an undissociated and a dissociated state. Undissociated fermentation acids, which are favoured by low pH, can penetrate the cell membrane and dissociate in the more alkaline interior. However, at a pH typical for dry sausage (4.8) only 10% of lactic acid is undissociated – the p*K*a of lactic acid is 3.86 – which should result in limited inhibitory effect (Työppönen et al. 2003).

Several explanations for the toxic effect of organic acids are reviewed by e.g. Capozzi et al. (2009) and Theron & Rykers Lues (2010): One relates directly to the detrimental effect of a lowered intracellular pH on cytoplasmic macromolecules e.g. RNA, DNA, proteins, protein synthesis, and biochemical pathways. A second explains that the dissociation of the acids inside the cells leads to an intracellular accumulation of anions, resulting in both end-product inhibition and a lowered a_w.

A third theory considers organic acids as uncouplers, transporting protons in a pH-driven process inside the cell, eventually leading to disruption of the proton motive force. Furthermore, the protein profiles of the outer membrane in Gram-negative bacteria are altered (Leyer & Johnson 1993).

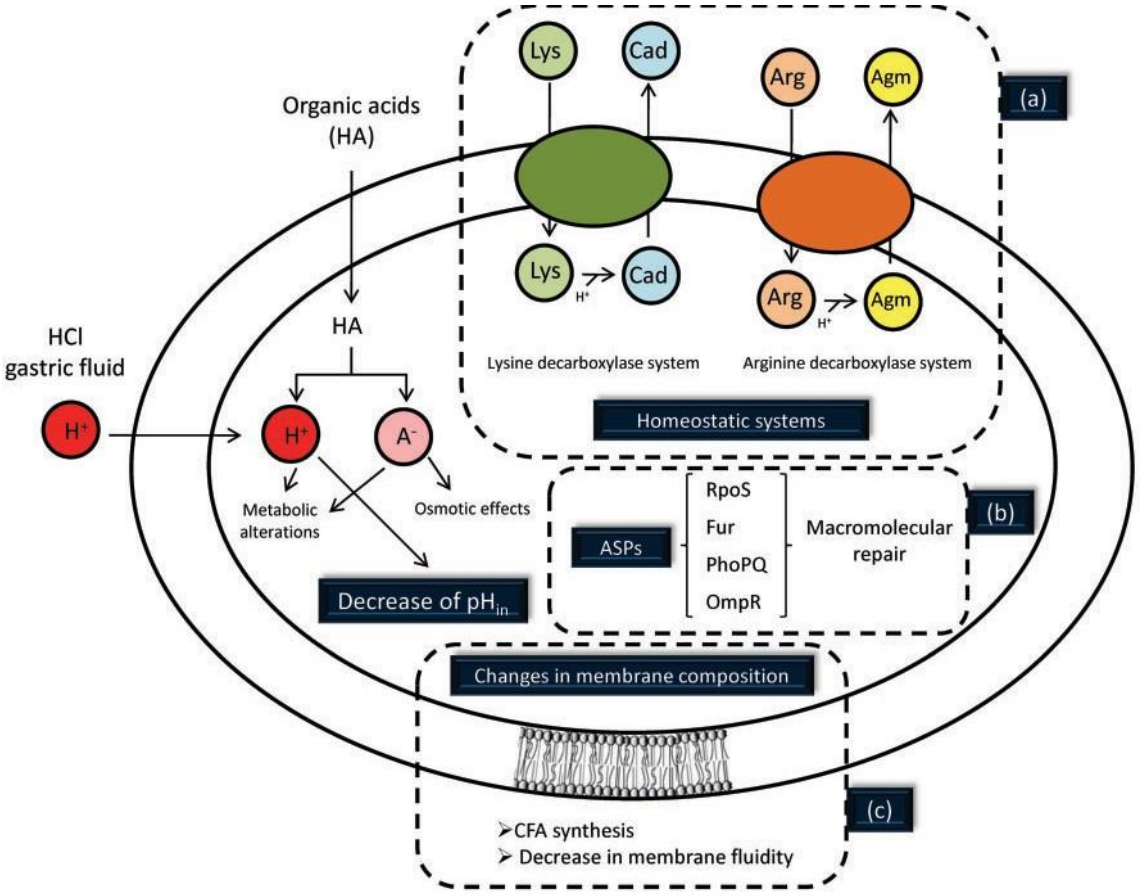

Figure 4. Schematic view of a *Salmonella* spp. cell containing all the known components of inducible acid-tolerance and acid- resistance systems. A low internal pH (pH_{in}) induces the expression of amino acid decarboxylase systems (a), the synthesis of ASPs (b), and modifications to membrane FA composition (c). Cad, cadaverine; Agm, agmatine. Source: Álvarez-Ordóñez et al. (2011).

4.1.2 pH homeostasis

As mentioned, the pH drop during fermentation disturbs the homeostasis of the bacteria present. Gram-negative bacteria try to keep a balanced intracellular pH of 7.6-7.8 over a wide range of external pH values (Foster 2000). Measurements of intracellular pH (pH_i) of cells exposed to pH 3.3

indicates that pH_i in unadapted cells is 0.5-0.9 pH units lower than in adapted cells (Foster & Hall 1991). The intracellular pH is maintained by pumps that expel protons from the cytoplasm (Foster 2000), along with the action of arginine and lysine decarboxylases (Álvarez-Ordóñez et al. 2010a). While these inducible systems seek to alkalinize pH_i, others involve complex regulatory networks that modulate the proteom and provide tolerance to acid stress (Foster 2000). Furthermore, the induction of alterations in the fatty acid composition of the membrane, for maintenance of membrane fluidity, is suggested to be of importance for stress tolerance in *Salmonella* (Álvarez-Ordóñez et al. 2008, 2010b). An overview of inducible acid-tolerance and acid-resistance systems can be seen in Figure 4.

4.2 Acid tolerance response

In *Salmonella* spp., adaptation at moderate pH can enhance resistance to otherwise lethal pH; a phenomenon intensively investigated and initiated by the pioneering work of Foster (1991; 1993; Foster & Hall 1990; Foster & Hall 1991). This phenomenon is designated Acid Tolerance Response (ATR) and has been shown to augment bacterial survival as much as 1000-fold over a 2-hour period at pH 3 (Foster & Hall 1990).

Acidic stress is frequently encountered by food borne pathogens in the food-processing system i.e.; during muscle to meat conversion – resulting in an ultimate pH of 5.4-5.7 in porcine muscle (Kylä-Puhju et al. 2004); by decontamination methods using organic acids – lactic acid was recently approved for bovine carcasses by the European Commision (COMMISSION REGULATION (EU) No 101/2013); as well as in acidic foods. While the ATR has direct implications for food safety enhancing the bacterial survival in acidic foods in e.g. fermented sausages (Smith et al. 1975), it also has the potential of increasing the survival of foodborne pathogens facing the acidic pH of the stomach before entering and colonizing the intestines. The infectious dose of pathogens in food thus correlates to their acid tolerance (Audia et al. 2000; Lin et al. 1995). Furthermore, ATR is known to induce cross-protection to other types of stress encountered by the pathogenic bacteria during its infectious cycle such as; osmotic, oxidative and short chain fatty acid stress (Leyer & Johnson 1993; Lee et al. 1995; Baik et al. 1996; Greenacre & Brocklehurst 2006).

The development of acid tolerance responses in *Salmonella* is complex i.e. with distinct responses occurring in log and stationary growth phases and with dependency on growth temperature,

stress level, exposure time, and on the properties of the acidulant (Spector & Kenyon 2012). *Salmonella* harbours several regulons which enable acid adaptation, most importantly those controlled by RpoS, Fur, PhoP, and OmpR (Rychlik & Barrow 2005). Regulatory connections to virulence encoding genes located on SPI-1 and SPI-2 will be mentioned in chapter 5.1.3.

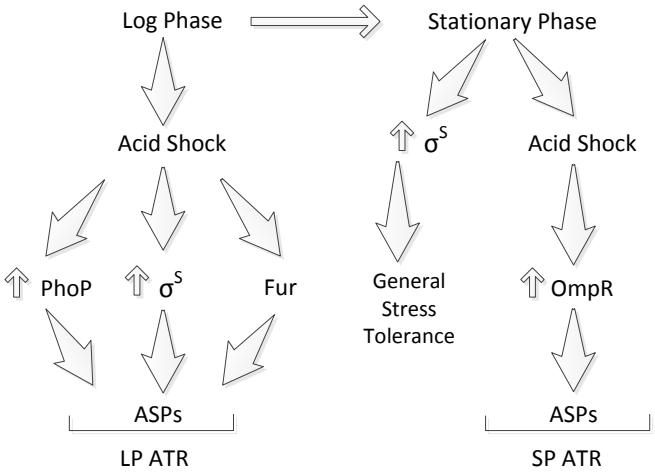

Figure 5. Overview of log phase and stationary phase acid tolerance responses of *S.* Typhimurium. Adated from Foster (2000).

To withstand the adverse effects of acid stress, the bacterial cell induces several groups of acid shock proteins (ASPs) during ATR (Audia el al. 2001). The function of these proteins is to prevent or repair the macromolecular damage caused by acid stress (Foster 1991; Foster 1993). In *Salmonella* Typhimurium proteomic analyses indicate that while acid shock induces 60 ASPs in log phase cells, 48 are induced in stationary phase cells with only 5 overlaps, supporting the idea of two separate systems (Audia et al. 2001). The log phase ATR (LP ATR) can be further divided into a transient system dependent on Fur, but not on RpoS, and a more efficient, sustained ATR which is RpoS-dependent (Lee et al. 1995). Likewise, *Salmonella* possesses two independently regulated stationary phase acid tolerance systems. Unadapted stationary phase cells are relatively resilient to pH 3 and 1,000-fold more acid tolerant than their log phase counterparts. RpoS is responsible for this acid tolerance which is pH-independent, but expressed as part of the general stress response upon entry into stationary phase (Lee et al. 1994). Though, upon exposure to a challenge of pH 3 for more than 4 hours, this resistance declines. If, however, the cells are preadapted at a

moderate pH of 4.5, they survive much longer (Lee et al. 1994). This phenomenon, referred to as the acid-induced stationary phase ATR (SP ATR), is OmpR-dependent but RpoS-, Fur and PhoPQ-independent, which differentiates it from the LP ATR (Lee et al. 1994).

The order of increasing tolerance could be denoted: log phase cells < acid-adapted log phase cells < stationary phase cells < acid-adapted stationary phase cells (Foster 2000).

4.3 Sigma factors

Sigma factors (σ^D, σ^E, σ^F, σ^H, σ^S, σ^N – also denoted RpoD, RpoE, etc.) are dissociable subunits of the prokaryotic RNA polymerase needed for promoter recognition and transcription initiation. While the vegetative RpoD is responsible for the expression of housekeeping genes during normal growth, the alternative sigma factors are activated and associates with the core RNA polymerase primarily during stress or changes in growth conditions (Shen & Fang 2011). By the selected binding to specific promoter sequences, the holoenzyme controls the expression of a subset of genes (regulon) which increases resistance to the given stressor(s).

4.3.1 RpoS

The alternative sigma factor RpoS, encoded by the gene *rpoS*, is essential for survival of *Salmonella* in stationary phase as well as under other various stress conditions including low pH (Fang et al. 1992). RpoS has also been shown to be involved in the acid-inducible log phase ATR, during which the level of RpoS increases dramatically (Lee et al. 1995). At least 10 of the 60 ASPs induced in log phase ATR are controlled by RpoS (Baik et al. 1996; Lee et al. 1995). The central role of *rpoS* in the stress response of *Salmonella* is reflected in the complexity of its regulation. Expression of *rpoS* and production of RpoS is regulated via transcriptional, translational, and protein stability controls (Lange & Hengge-Aronis, 1994).

In exponentially growing cells RpoS is rapidly degraded with reported half-lives of ~2 min. Upon entry into stationary phase or in response to certain stresses, the half-lives can increase ~10-fold (Zhou et al. 2001). RpoS proteolysis is regulated by the two-component response regulator MviA (known as RssB/SprE in *E. coli*) (Bearson et al. 1996) and the ClpXP protease (Schweder et al. 1996).

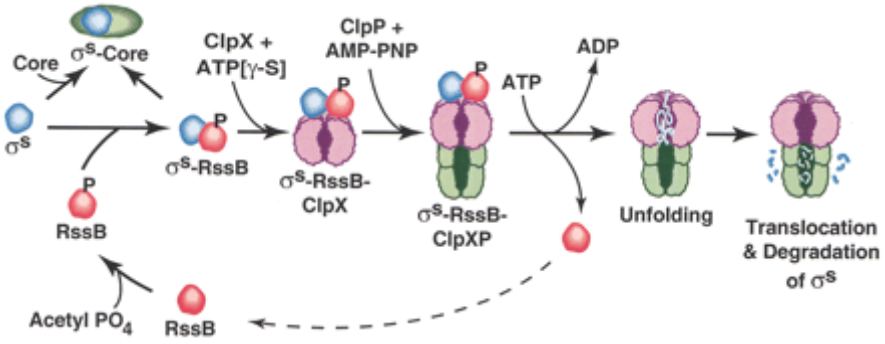

Figure 6. Model for the mechanism of action of RssB (MviA) in regulating σ^S (RpoS) degradation by ClpXP. Source: Zhou et al. (2001).

The phosphorylated form of MviA has high affinity for RpoS and makes it available for proteolytic degradation by ClpXP (see Figure 6). Hence, inactivation of *mviA* or *clpP* leads to an accumulation of RpoS, generally resulting in a more acid resistant phenotype (Bearson et al. 1996).

As opposed to the previous belief that the increased RpoS level induced by acid shock was mainly regulated on the level of proteolysis, Audia & Foster (2003) showed that *rpoS* translation is the major focal point. Stability and efficiency of translation of *rpoS* mRNA is in part controlled by small regulatory RNAs, but in acid shocked log phase cells, sequential formation of competing stem-loop structures in the 566 nt untranslated region (UTR) of the *rpoS* mRNA is believed to induce the increased translation (Audia & Foster 2003).

4.4 Fur

Fur is usually linked to the bacterial iron metabolism where it is a negative regulator of genes functioning in the assimilation or uptake of exogenous iron (Spector & Kenyon 2012). Surprisingly, *fur* mutants are acid sensitive and unable to mount a LP ATR (Foster and Hall 1992; Hall & Foster 1996). Furthermore, Foster and Hall showed that Fur positively regulates a subset of ASPs, indirectly or directly in an iron-independent manner. Proteins regulated by Fur in response to iron starvation or low pH form two distinct clusters with only seven proteins being influenced by both conditions (Rychlik & Barrow 2005). While both RpoS and Fur are related to the LP ATR, Fur is essential for the rapid but transient induction of a set of proteins at pH ~ 5 which enables

Salmonella to survive a subsequent challenge at pH 3. This induction is apparent 20-40 min after exposure, but disappears after 60 min where, in turn, the Rpos-dependent ATR is induced (Foster 1993; Lee et al. 1995). RpoS and Fur are essential for the response to low pH induced by organic acids (Rychlik & Barrow 2005).

4.5 Two-component signal transduction systems

Apart from alternative sigma factors, two-component signal transduction systems (TCSs) are commonly employed by bacteria to respond to environmental changes. Typically the TCSs are composed of a membrane-bound sensor kinase and a cytoplasmic transcriptional regulator. In response to certain environmental stimuli the sensor protein autophosphorylates and in turn transphosphorylates the transcriptional regulator. Activation of the response regulator by phosphorylation leads to conformational changes allowing the protein to bind to DNA (Beier & Gross 2006).

4.5.1 PhoP-PhoQ

The PhoP-PhoQ TCS modulates a large regulon controlling various cellular activities (~3 % of the genome) and is mainly activated by low Mg^{2+} concentrations (Groisman 2001; García Véscovi et al. 1996). The significance of PhoP in the ATR was discovered by the notion that a mutation in *phoP* eliminated adaptation to low pH (Foster & Hall 1990). The *phoPQ* operon is induced by moderate acid pH, even under high Mg^{2+} concentrations (Bearson et al. 1998). This suggests that PhoQ can sense pH by the influence of protons on the conformation of the Mg^{2+}-binding site. This signal activates the kinase activity of PhoQ, which phosphorylates the ASP PhoP. Phosphorylated PhoP induces the transcription of several genes, including the *phoPQ* operon itself (Audia et al. 2001). In *Salmonella* it has been shown that the main protecting role of the *phoPQ*-dependent acid tolerance response is against inorganic acid stress in log phase cells (Foster 2000).

4.5.2 OmpR-EnvZ

ompR insertion mutations have been shown to render stationary phase cells acid sensitive and unable to mount an inducible ATR (Bang et al. 2000). As observed on mRNA and protein levels,

ompR is induced by low pH (Bang et al. 2002). OmpR is the response regulator of a TCS in which EnvZ is the membrane-bound sensor kinase known to respond to changes in osmolarity (Pratt et al. 1996). During acid stress, however, OmpR is activated independently of EnvZ and phosphorylation has been shown to be mediated by acetyl phosphate (Bang et al. 2000). In a manner similar to the PhoPQ TCS, phosphorylated OmpR binds to DNA (including to its own low pH-specific promoter) and activates the transcription of target genes (Bang et al. 2002). Amongst these is *ompC*, which encodes the outer membrane porin, OmpC. Transcription of OmpC is increased by exposure to low pH, and the induction is dependent on OmpR (Foster et al. 1994). Thus, in addition to its osmoregulatory role, OmpR plays a central role in the acid-inducible stationary phase ATR (Lee et al. 1994).

Although, not in the scope of this thesis, it has to be mentioned that while the aforementioned acid tolerance in *Salmonella* functions well to protect cells down to pH 3, *E. coli* has additional acid resistance (AR) systems that protect cells to pH 2 or less (Foster 2000). While several studies have suggested that enterohemorrhagic *E. coli* is more acid resistant than commensal organisms, and correlations have been made to its low infectious dose, mutational analysis indicates that O157 and *E. coli* K-12 harbour the same AR systems (reviewed by Foster 2000).

4.6 Osmotic stress

4.6.1 Physiological response

A high amount of osmotically active substances i.e. salts and sugars (or desiccation) lower the water activity of food (Guiterrez et al. 1995). Also the lumen of the gastrointestinal tract has a relatively high salt concentration of 0.3 M NaCl (Álvarez-Ordóñez et al. 2011). A direct consequence for many bacteria of an osmotic upshift is a loss of water from the cytoplasm by osmosis, which causes the cell to shrink (plasmolysis). A ~50% reduction in the cytoplasmic volume in Gram-negative microorganisms has been reported at 0.3 M NaCl (Decad & Nikaido 1976). Removal of cell-bound water imposes both structural, physiological and biochemical stress, with implications for folding and assembly of proteins, regulation of gene expression and structure and function of membranes (Billi & Poots 2002). Dysfunctions in enzymes and/or electron transport chains will lead to oxidative stress and eventually to protein denaturation and mutation of DNA (Potts 1994). As the microbial membrane is permeable to water, Gram-negative bacteria

maintain the appropriate cell turgor by regulating the concentration of osmotic solutes in the cytoplasm and/or periplasm (Bremer & Krämer 2000). Turgor pressure is generally considered to be the mechanical force for cell extension, cell growth and cell division (reviewed by Sleator & Hill 2001). Thus, adaptation to changes in osmolarity is essential for the ability of salmonellae to survive and cause infection.

There is a clear distinction between dessication and hypertonicity - as the cells in the latter case are in an aqueous solution (albeit one with low water activity) - still, during the initial stages of slow air drying, water can also be balanced by osmotic adjustment (Billi & Potts 2002). While relatively little is known about the physiological and genetic response to desiccation in *Salmonella*, it is clear that there are regulatory overlaps to other stress response networks (Gruzdev et al. 2011).

An overview (see also Figure 7) of genetic loci responsible for adaptation to osmotic pressure will be given in the following:

4.6.2 Osmoadaptation

Osmoadaptation to low water activity environments will happen both at a genetic and a physiological level. The general belief is that *Salmonella* reacts to osmotic stress by means of an immediate uptake of potassium by the combined action of the inducible high-affinity system (Kdp) and the low-affinity system (Trk) (Bremer & Krämer 2000; Balaji et al. 2005). Then the ionic balance is reestablished by intracellular synthesis of the counter-ion of K^+, glutamate (Bremer & Krämer 2000).

While a high cytoplasmic concentration of K^+ is impairing cell functions, this initial response is followed by a more long-term countermeasure, involving highly elevated – either by uptake or *de novo* synthesis – cytoplasmic levels of osmoprotective compounds (Sleator & Hill 2001). These organic compounds, including; proline, glycine betaine, ectoine, and trehalose are often referred to as compatible, as they even in high internal concentration are tolerated by the cellular machinery. The ABC transporter complex *proU* (*proVWX*), and the H^+symporter *proP* are upregulated in response to hyperosmotic stress in *Salmonella* (Bremer & Krämer 2000; Balaji et al. 2005). Several studies have also found *proU* to be regulated by other environmental stresses e.g.

pH (reviewed by Foster & Spector 1995).These membrane systems function to transport various compatible compounds into the cell, such as glycine betaine, proline betaine, and proline (Bremer & Krämer 2000; Wood 2007).

While being an important osmoprotectant, the synthesis of prolin is not elevated under hyperosmotic conditions. Prolin is usually synthesized from glutamate via three enzymatic steps involving the action of ProB, ProA and ProC (Bremer & Krämer 2000).

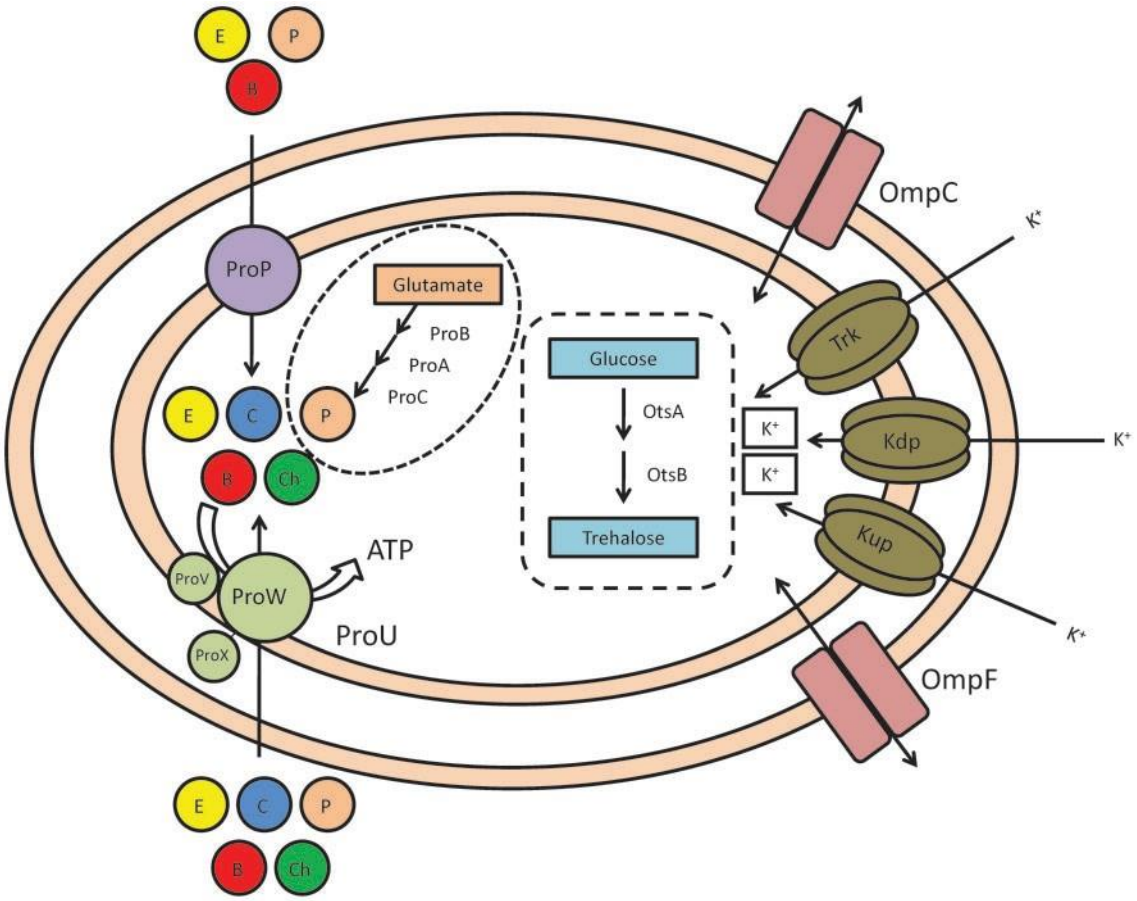

Figure 7. Schematic view of the systems for uptake and/or synthesis of potassium (K+), betaine (B), proline (P), choline (Ch), ectoine (E), carnitine (C) and trehalose in *Salmonella* spp.

Source: Álvarez-Ordóñez et al. (2011).

Trehalose, on the other hand, has to be synthesized internally – and becomes the main osmoprotectant in *Salmonella* if availability or supply of exogenous compatible compound is

inadequate (Spector & Kenyon 2012). Biosynthesis of trehalose involves the enzymes trehalose-6-phosphate phosphatase and trehalose-6-phosphate synthase encoded by *otsB* and *otsA*, respectively (Kaasen et al. 1992; Strøm & Kaasen 1993). The synthase OtsA catalyzes the enzymatic condensation of the precursors glucose-6-phosphate and UDP-glucose. Dephosphorylation of the intermediate via the *otsB*-encoded phosphatase generates free trehalose (Bremer & Krämer 2000). The *otsBA* operon is under regulatory control of RpoS and is thereby induced in stationary phase as well as by osmotic shock (Hengge 2008; Ibanez-Ruiz et al. 2000; Kaasen et al. 1992). Apart from being a compatible solute, it appears that trehalose can replace water and aid to maintain the structure and function of proteins and membrane lipids during desiccation (Potts 1994; Crowe et al. 1992). Furthermore, trehalose is associated to protection against thermal and oxidative stress (Howells et al. 2002).

Briefly, it should also be mentioned that, in response to elevated osmotic pressure, *Salmonella* can modify its outer membrane composition (Rychlik & Barrows 2005). As described in the previous chapter the two-component signal transduction system OmpR-EnvZ serves to detect osmolarity and to set the level of OmpF and OmpC synthesis. In response to high salt concentrations the OmpF porin is replaced by the OmpC porin, which forms a water-filled open channel with a smaller diameter, thus decreasing the influx of solutes into the periplasm (reviewed by Rychlik et al. 2005; Craeme-Cook et al. 1989).

Chapter 5
Pathogenicity

Although, *Salmonella* has evolved several overlapping stress response systems, and, as mentioned in previous chapters, may be subjected to several concomitant hurdles during food processing, acid resistance is increasingly important in determining the organisms infectious potential. During the infectious cycle of *Salmonella*, acid stress is the first major stress it encounters upon ingestion during gastric passage. Furthermore, acid adaptation confers cross-protection to a variety of other stresses i.a. also oxidative stress mounted by the phagocyte later in the infectious cycle (Leyer & Johnson 1993; Foster & Hall 1990; Kwon & Ricke 1998). The notion that e.g. oxygen or heat stress does not confer resistance to low pH adds to the central role of acid resistance (Rychlik & Barrow 2005). Apart from their important roles in controlling relevant stress responses both inside and outside the host the four major stress response regulators; RpoS, PhoP, Fur and OmpR have been shown to regulate different virulence determinants needed for colonization (Rychlik & Barrow 2005). While displaying increased susceptibility to acid pH *rpoS*, *phoP*, *fur* and *ompR* null mutants also show attenuated virulence in orally infected mice (Coynault et al. 1996; Wilmes-Riesenberg et al. 1996; Bearson et al. 2006b; Curtiss et al. 2009). Thus the ability of *Salmonella* to efficiently respond to environmental stresses is probably a prerequisite for survival in the food-chain and is also involved in determining the capacity of causing infection in multiple hosts.

5.1 Route of infection

Being enteric foodborne pathogens, *Salmonella* spp. typically re-enters the host upon ingestion of contaminated feed/food or water. Successful intestinal infection requires that a proportion of the organisms survive a succession of host defences; starting off with the acidic barrier of the stomach and while entering the intestines; the bactericidal activity of bile, antimicrobial peptides and weak acids produced by the commensal flora, decreasing oxygen tension and increasing osmolarity. Likewise, the pathogens must compete with the resident microbial flora for nutrients and space (Rychlik & Barrow 2005). Hereupon *Salmonella* typically colonizes the small intestine and invades the intestinal mucosa. *Salmonella* internalized in non-phagocytic or phagocytic cells is encased in a

nutrient deficient vacuole termed the *Salmonella*-containing vacuole (SCV) (reviwed by Ibarra & Steele-Mortimer 2009). Knodler et al. (2010) also suggest that a subpopulation of *Salmonella* hyperreplicates in the cytosol of epithelial cells, which are then extruded out of the monolayer, releasing *Salmonella* back into the lumen. Once released into the intestinal submucosa the pathogen can be phagocytized by resident macrophages and residing in phagosomes (or phagolysosomes) eludes the host's adaptive immune response (Parry et al. 2002). Localized infections of the intestine stimulate inflammatory responses that contribute to the diarrhoea and cramps of gastroenteritis, normally associated with self-limiting non-typhoidal *Salmonella* infections (Santos et al. 2009). Dissemination of *Salmonella* beyond the intestine and associated lymphoid tissue is host and/or host immune status dependent (Bäumler et al. 2000). *Salmonella* Typhimurium elicits inflammatory diarrhoea in calves and humans, whereas in infected mice a systemic disease characterized by bacterial multiplication in the liver and spleen is contracted, in lieu of gastroenteritis (Tsolis et al. 1999). The pathogenicity of systemic *Salmonella* infections, with some resemblance to human typhoid fever, has been extensively studied in the mouse model (Carter & Collins 1974).

Host cell internalization of *Salmonella* can, at least, occur via two distinct routes. Whereas one involves phagocytosis by professional phagocytes, such as macrophages, *Salmonella* can also actively invade phagocytic and non-phagocytic cells using a type III secretion system (T3SS) (reviewed by Ibarra & Steel-Mortimer 2009). For many Gram-negative pathogens, type III secretion systems, often likened to a molecular syringe, are important virulence determinants (Ibarra et al. 2010). The T3SS translocates effector proteins from the bacterial to the eukaryotic cytoplasm, which in turn alter host cell physiology (Santos et al. 2009). *Salmonella* possesses two distinct T3SSs, located on *Salmonella* pathogenicity island 1 (SPI-1) and 2 (SPI-2), respectively. Of the at least 21 SPIs known, SPI-1 and SPI-2 are the most studied (de Jong et al. 2012). The SPI-1 T3SS is involved in the invasion of epithelial cells and proinflammatory responses (Mills et al. 1995; Galán & Curtiss 1989; Galán 1996; Galán & Collmer 1999; Lostroh & Lee 2001). The coordinated action of the effector proteins SipA, SipC, SopB/SigD, SopD, SopE2 and SptP triggers rearrangement of the actin skeleton, promoting formation of localized ruffles in the host cell membrane and enabling internalization of the bacteria (reviewed in McGhie et al. 2009). SPI-2 T3SS, on the other hand is induced intracellularly, and is involved in the subsequent steps in the invasion – intracellular survival within macrophages and systemic spread (Shea et al. 1996; Hensel

et al. 1998). Intracellular replication and survival is mediated by SPI-2 i.e. by inhibiting various aspects of endocytic trafficking, including blocking the fusion between the SCV and the lysosome (Kuhle & Hensel 2004), and avoidance of NADPH oxidase-dependent killing by macrophages (Vazquez-Torres et al. 2000).

The complexity of regulation of SPI-1 and SPI-2, although being among the best-studied genetic systems, leads to its continued enigmatic nature. In the following a brief description of selected cognate regulators and, for this thesis interesting, global regulators and environmental signals that is believed to control *Salmonella* invasion will be presented.

Expression of the SPI-1 and SPI-2 T3SS is known to be tightly regulated by a large number of regulatory proteins that are responsive to a combination of intracellular and environmental signals (Ellermeier et al. 2005; Altier 2005; Ellermeier & Slauch 2008). These environmental cues for invasion, which are believed to direct the pathogen to the preferred site of entry in the host, include pH, osmolarity, oxygen tension, bile, divalent cation concentration, and the presence of short fatty acids (Bajaj 1996; Altier 2005). The preferred site of *Salmonella* invasion is the M-cells in the distal small intestine (ileum) (Jones et al. 2005). The environment in the ileum is characterized by being rich in nutrients, low in oxygen, high in osmolarity and divalent cations, and with a pH of 8 (Wisner et al. 2012). Hence, the expression of SPI-1 is repressed both by the presence of bile, characterising the proximal part of the small intestine, and by a high concentration of short chain fatty acids produced by the micro flora of the large intestine (Wisner et al. 2012). *In vitro*, SPI-1 genes are expressed in nutrient-rich broth (e.g. Luria-Bertani) at near neutral pH and with low oxygen concentration, but high osmolarity (Bajaj et al. 1996; Altier 2005; Ellermeier & Slauch 2007). The conditions in the SCV are markedly different, being nutrient-limited, low in osmolarity and divalent cation concentration, and with a pH between 4 and 5 (Wisner et al. 2012). SPI-2 genes have been shown to be expressed either during late stationary phase, when *Salmonella* grows in LB medium (Bustamante et al. 2008), or in acidic minimal medium with low concentrations of phosphate, calcium and magnesium (Deiwick et al. 1999).

5.1.1 HilA

HilA, encoded on SPI-1, is the central regulator of SPI-1 T3SS (Bajaj et al. 1995). The regulatory systems and environmental signals that alter the expression of SPI-1 T3SS genes also affect the

expression of *hilA*, and it is suggested that the level of expression of the SPI-1 locus is directly dependent on the level of HilA (Ellermeier & Slauch 2007). Ellermeier et al. (2005) showed in BALB/c mouse competition assays that a *hilA* deletion alone is phenotypically equivalent to a deletion of the entire SPI-1 locus. HilA activates the expression of the three operons encoding the primary effector proteins and structural genes of the T3SS, namely: *prg*/*org*, *inv*/*spa* and *sic*/*sip* (Lee et al. 1992; Bajaj et al. 1995; Bajaj et al. 1996; Eichelberg & Galán 1999). Apart of this direct activation, HilA also activates the expression of a second transcriptional activator InvF (encoded by the first gene in the *inv*/*spa* operon), involved in regulating the expression of SPI-1 secreted effector proteins and chaperones (Darwin & Miller 2000; Darwin & Miller 2001). *hilA* expression, it self, is directly controlled by three transcriptional activators with homologous DNA binding domains; HilC, HilD and RtsA (Schechter & Lee 2001; Olekhnovich & Kadner 2002; Ellermeier & Slauch 2003). These three transcription factors can independently activate their own, each other's and *hilA* expression – through complex feedforward and feedback mechanisms (Fig. 8).

5.1.2 HilD

HilD is dominant in this regulatory circuit, as in its absence there is limited *hilA* expression (Schechter et al. 1999). Still, HilD alone cannot induce *hilA* enough to stimulate invasion (Ellermeier et al. 2005). Additionally, many activating signals – both environmental and intracellular – have been shown to affect SPI-1 gene expression by controlling the level or modifying the activity of HilD (e.g. Ellermeier et al. 2005; Ellermeier & Slauch 2008; Baxter et al. 2003; Saini et al. 2010; Lopez-Garrido & Casadesus 2010; Martínez et al. 2011). In addition to positive regulation, SPI-1 gene expression is subjected to negative regulation e.g. by HilE (encoded outside SPI-1) that may repress *hilA* by direct protein-protein interaction with HilD, preventing HilD function (Baxter et al. 2003). Additionally, HilD serves to induce the expression of the only cognate regulator encoded on SPI-2, *ssrAB* (Ochman et al. 1996; Shea et al. 1996), hence mediating cross-talk between the two *Salmonella* pathogenicity islands (Bustamante et al. 2008). In response to an unidentified signal the phosphor kinase SsrA phosphorylates the DNA binding protein SsrB, activating the transcription of SPI-2 (Fass & Groisman 2009).

5.1.3 Global regulators

The genes of SPI-1 and SPI-2 are also under control of global regulators (Fig. 8), some of which have been described in previous chapters regarding the acid stress response in *Salmonella.*

Environmental signals, of which ion level is probably one, help determine when to turn SPI-1 on. Studies have shown that Fe^{2+} is abundant in the lumen of the anaerobic small intestine, but becomes limited upon invasion (Ellermeier & Slauch 2008). Fur, the primary ion regulatory protein in both *Salmonella* and *E. coli*, regulates HilD posttranslationally (presumably through an intermediate, ? in Fig. 8, being mechanistically a repressor), hence leading to an induction of SPI-1 in general, in high-iron conditions. Once in the iron limited SCV, the indirect activation of HilD is stopped (Ellermeier & Slauch 2008). The sensor potential of Fur seems to be broad, as the role of Fur in the LP ATR is probably independent of ion-levels (Hall & Foster 1996). In response to infection, reactive nitrogen species are expressed by macrophages and Fur-associated regulatory activity seems of importance for mounting a defence against nitrosative stress both in *E. coli* and in *S.* Typhimurium (Mukhopadhyay et al. 2004; Eriksson et al. 2003).

Osmolarity is probably another environmental cue for SPI-1 expression. As mentioned, apart from its role in the SP ATR, OmpR-EnvZ senses osmolarity. Although earlier studies suggested that OmpR-EnvZ modulates *hilA* expression by altering expression and/or activity of *hilC* (Lucas & Lee 2001), Ellermeier et al. (2005) have proposed that OmpR, somehow, regulates the function of HilD post-transcriptionally. While high osmolarity induces *hilA* expression it also causes changes in DNA supercoiling that affect gene transcription (Bajaj et al. 1996). Cameron & Dorman (2012) have shown that under conditions of relaxed supercoiling OmpR binds to *hilC* and *hilD* promoters, activating and repressing them, respectively. Likewise the supercoiling altering nucleoid associated proteins (NAPs), Hha and H-NS have been shown to repress transcription of *hilA* under conditions of low osmolarity (Olekhnovich & Kadner 2007). While, it has been proposed that the molecular role of HilC and HilD is to derepress *hilA* (Schechter et al. 1999, 2003; Schechter & Lee 2001) by counteracting the repression by H-NS, consensus on this notion has not been reached (Boddicker et a. 2003; Olekhnovich & Kadner 2004). In contrast, the NAPs HU and Fis are required for *hilA* expression (Schechter et al. 2003). Apart from the regulation of SPI-1 encoded genes, OmpR has been shown to bind directly to *ssrA* (originally *spiR*) and *ssrB* promoters under low osmolarity conditions, activating the SPI-2 locus (Feng et el. 2003; Lee et al. 2000).

Likewise, apart from participating in the resistance to acid pH (Foster and Hall 1990), the before mentioned two-component regulator PhoP-PhoQ, known to respond to environmental Mg^{2+} levels, plays a central role in controlling the expression of many virulence determinants in *Salmonella* – including invasion of epithelial cells (Groisman et al. 2001; Miller et al. 1989; Behlau and Miller 1993). A single amino acid substitution in PhoQ (*pho-24*) causes phosphorylated PhoP to accumulate, even when Mg^{2+} levels are high, and leads to hyperexpression of PhoP-activated genes as well as further repression of e.g. *hilA* (Bajaj et al., 1996; García Véscovi et al. 1996). The repression in expression of *hilA* renders the *pho-24* mutant unable to invade nonphagocytic cells while it is likewise unable to replicate within macrophages in vitro (Bajaj et al., 1996). PhoP-PhoQ has thereby also been demonstrated to be essential for the survival and replication of *Salmonella* within macrophages (Miller 1991; García Véscovi et al. 1994) where the regulon i.a. increases resistance to host defence antimicrobial peptides (Groisman et al. 1992). Thus, phosphorylated PhoP seems to play a dual role as a genetic switch in the inverse regulation of SPI-1 and SPI-2; suppressing expression of SPI-1 genes via HilE while activating expression of SPI-2 genes (via direct interaction with *ssrB* and post-transcriptionally with SsrA) under conditions of low cation concentration (Balaj et al. 1996; Behlau & Miller 1993; Deiwick et al. 1999; Fahlen et al. 2000). Yet, other studies have failed to link PhoP with SPI-2 expression (Kim & Falkow 2004), and at least some of the SPI-2 genes are expressed independently of PhoP-PhoQ (Beuzon et al. 2001). Lee et al. (2000), in contrast to the previously presented, found that acidic media (pH 4.5), rather than the magnesium concentration, affected SPI-2 expression, which underlines the complexity of the regulation.

Previous studies suggested that the observed repression of invasion genes in acidic pH conditions was mediated by PhoP-PhoQ (Miller 1991; Behlau & Miller 1993). However, Bajaj et al. (1996) measuring β-galactosidase levels of invasion gene fusions to *lacZY* saw no difference in pH regulation between a wild-type and a *phoP* mutant. Likewise, results presented by García Véscovi et al. (1996) suggest that pH is not the signal sensed by PhoP-PhoQ to modulate the expression of invasion genes. Still, studies suggest that acidic pH activates SPI-2 via the action of OmpR (Lee et al. 2000) while alkaline pH represses SPI-2 gene expression independently of PhoP-PhoQ (Miao et al. 2002).

Although much progress has been made to elucidate the complex regulation of SPI-1 and SPI-2, discrepancies in the interpretation of results and enigmas clearly still exist. The control of the,

presumably horizontally acquired (Hensel et al. 1997), pathogenicity islands has been incorporated in existing regulatory pathways, in part responsible for their coordinated spatiotemporal expression. While the functions of effectors of SPI-1 and SPI-2 have traditionally have been considered to be distinct, newer evidence suggests that there is a timely overlap of the two (reviewed by Hallstrom & McCormick 2011). Importantly for modern molecular microbiology, much still needs to be solved about the mechanisms by which a myriad of regulatory signals control invasion.

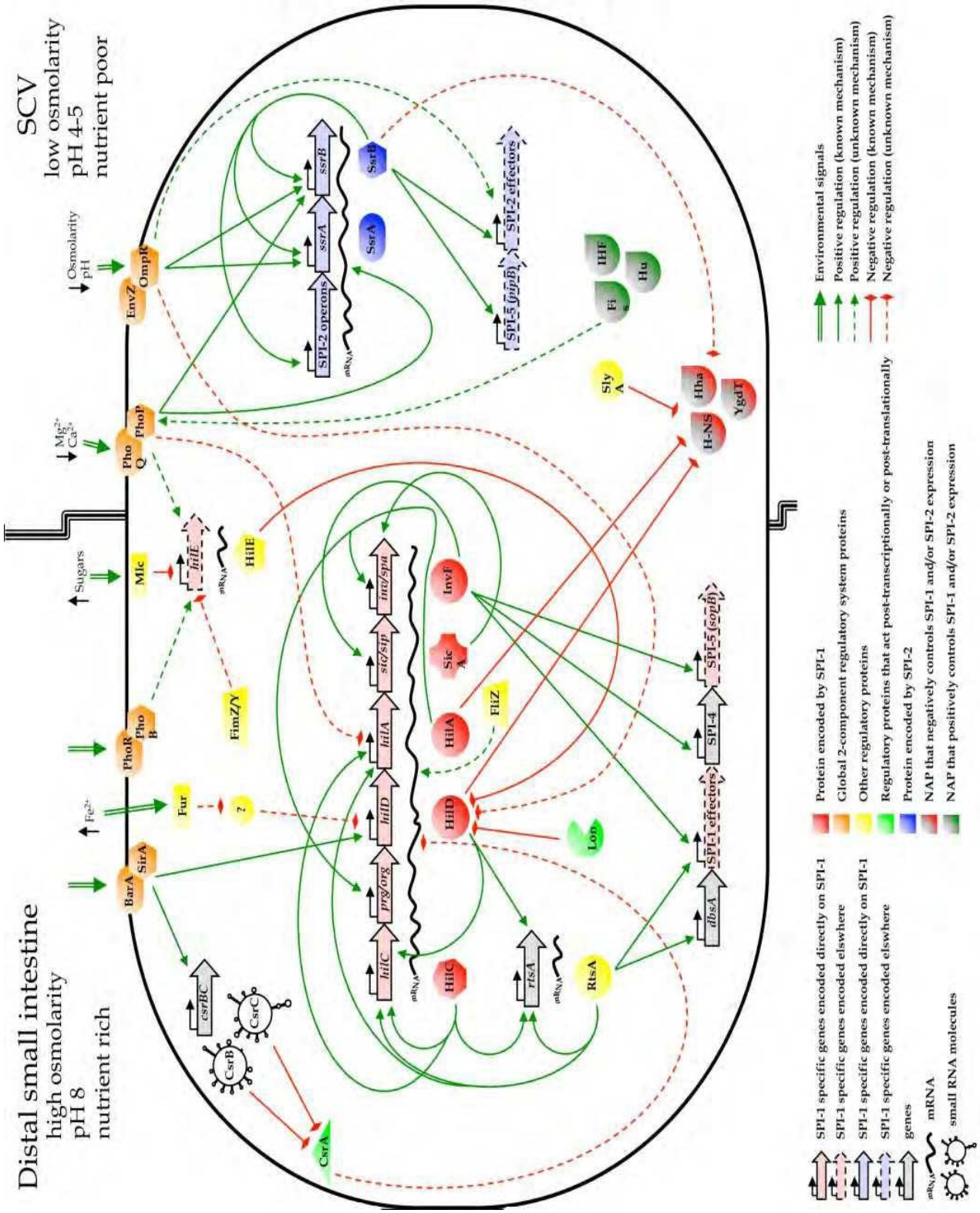

Figure 8. Model depicting the major modes of SPI-1 and SPI-2 regulation.

Source: Wisner et al. (2012).

Chapter 6

Paper I

Method Enabling Gene Expression Studies of Pathogens in a Complex Food Matrix

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Method Enabling Gene Expression Studies of Pathogens in a Complex Food Matrix▽

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We describe a simple method for stabilizing and extracting high-quality prokaryotic RNA from meat. Heat and salt stress of *Escherichia coli* and *Salmonella* spp. in minced meat reproducibly induced *dnaK* and *otsB* expression, respectively, as observed by quantitative reverse transcription-PCR (>5-fold relative changes). Thus, the method is applicable in studies of bacterial gene expression in a meat matrix.

A key component in understanding the ability of pathogens to survive in food products is to determine their response to food-related stresses. In recent years, advances in molecular techniques have enabled global studies of gene expression, using tools such as quantitative reverse transcription-PCR (qRT-PCR) and DNA microarrays. While these techniques have been employed in numerous studies examining the behavior of food-borne pathogens in broth or food-like broth models, only a few studies have investigated quantitative gene expression of pathogens in complex food matrices like dairy products (3, 7, 8, 11) or meat (4, 5, 9). Such investigations have been impeded by the complexity of food matrices, by inherent PCR-inhibiting compounds, and by low levels of target molecules (1). Diversity in foods necessitates methods applicable for different categories of food products. We wished to establish a method to investigate gene expression of bacteria directly from a complex food matrix that is often associated with transmission of pathogenic bacteria. As a model system we chose to study *Salmonella* spp. and verocytotoxic *Escherichia coli* in artificially contaminated minced meat.

Minced beef (8 to 15% fat) obtained in local Danish supermarkets was inoculated with a mix of strains of either *Escherichia coli* or *Salmonella enterica*. The *E. coli* mix consisted of *E. coli* serotype O26:H- (D3410), serotype O111:H- (D3411) obtained from Statens Serum Institut (SSI; Copenhagen, Denmark), and serotype O157:H7 from Danish beef meat (D3423) (2). The *Salmonella* mix consisted of *S. enterica* serovar Dublin (D3414), *S. enterica* serovar Typhimurium DT193 (D3415), and *S. enterica* serovar Derby (D3420) obtained from SSI. From −80°C frozen stocks, all strains were cultured at 37°C for 24 h on Luria-Bertani agar (LB; Oxoid, Greve, Denmark). A single colony from each strain was cultured individually and diluted 1:10 in prewarmed LB, and the *E. coli* mixed culture and the *Salmonella* mixed culture were prepared by pooling equal volumes of the three *E. coli* or the three *Salmonella* strain cultures. The mixed cultures were incubated until an absorbance at 600 nm (A_{600}) of 0.5 was reached, corresponding to approximately 5×10^8 CFU/ml as measured by plate counts. Samples of 5.0 g minced beef meat were weighed in stomacher filter bags and were stored at refrigeration temperature (5°C) or on ice during processing. All experiments with meat samples were performed in two technical replicates and were repeated in three biological replications. Ten-milliliter mixed cultures, or dilutions thereof, were pelleted by centrifugation (5,000 × *g* for 10 min) and resuspended in 0.5 ml LB to be used as inocula. Meat samples were inoculated and manually massaged for about a minute to distribute inocula. For heat stress conditions, meat samples were incubated at 45°C in a water bath for 30 min immediately after inoculation, and untreated controls were left at room temperature. For salt stress conditions, 1 ml of 17.5% NaCl solution was added to meat samples (5.0 g) to obtain 5% NaCl (in water phase) and were massaged manually before inoculation. Meat samples were stabilized with 10 ml RNAlater tissue collection medium (Ambion, Nærum, Denmark) and were manually massaged and left for 10 min in order to generate meat juice. The salt stress samples were stabilized with RNAlater 0 and 60 min after inoculation.

Meat juice (~9 ml) was collected and transferred to centrifuge tubes. Bacteria in the suspension (approximately 1×10^8 CFU per sample, determined by total plate counts) were harvested by centrifugation (5,000 × *g* for 10 min), and RNA was extracted from the pellets. Noninoculated meat samples, determined to contain a negligible level of bacteria ($<5 \times 10^4$ CFU/g), served as controls to examine the presence of RNA from the natural microflora as well as eukaryotic RNA from the meat and were processed as described above.

RNA extractions were performed with the RNeasy kit (Qiagen, Copenhagen, Denmark). Pellets from meat samples and broth cultures were treated with 0.2 ml Tris-EDTA buffer (Tris-EDTA, 10:1; pH 8) containing 3 mg lysozyme and 0.004 mg proteinase K and were vortexed for 10 s every second minute during a 10-min incubation period at room temperature. Cells were disrupted using Fastprep (MP Biomedicals, Illkirch, France) as recommended by the manufacturer, followed by a wash step with buffer RLT containing 2-mercaptoethanol (14.3 M; 10 μl per ml of buffer RLT). Samples were centrifuged at 14,000 × *g* for 2 min, supernatants were transferred to new tubes, and 500 μl of 96% ethanol was added. RNA extractions were continued according to Qiagen rec-

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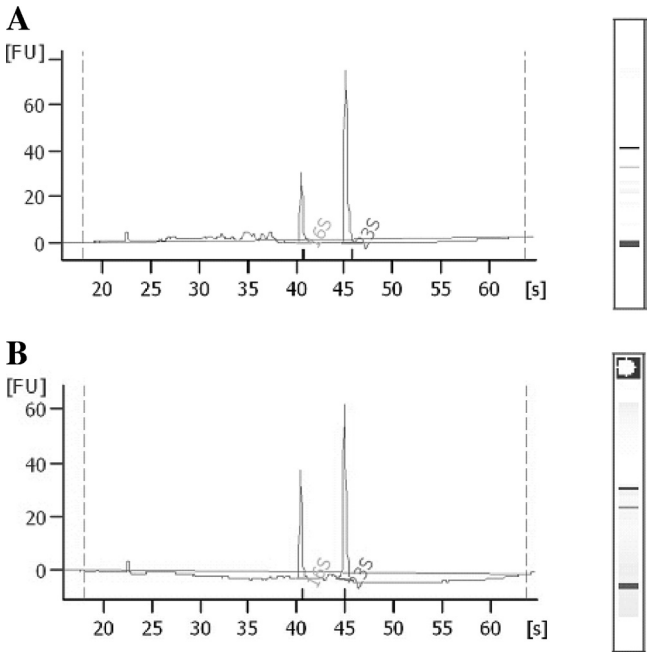

FIG. 1. Electropherograms (created using an Agilent 2100 Bioanalyzer) showing integrity of RNA samples extracted from an *E. coli* mixed culture inoculated into minced beef (A) and an *E. coli* mixed culture in LB broth (B). FU, fluorescence units.

ommendations, including an on-column DNase (Qiagen) treatment for 15 min. Purified RNA was quantified using a Nanodrop ND-1000 apparatus (ThermoScientific, Wilmington, DE), and the RNA quality was verified based on an optical density at 260 nm (OD_{260})/OD_{280} absorption ratio of >1.95 and an OD_{230}/OD_{260} absorption ratio of >2.0, and the integrity was further assessed either electrophoretically following ethidium bromide staining or with a 2100 Bioanalyzer (Agilent, Palo Alto, CA), resulting in clear patterns with prominent 16S and 23S ribosomal bands and RNA integrity number values of >7 (Fig. 1).

cDNA was synthesized using a SuperScript VILO cDNA synthesis kit (Invitrogen,Tåstrup, Denmark) with random hexamer primers and following the manufacturer's instruction, using 500 ng total RNA per sample (Invitrogen). Real-time PCR was completed on an ABI 7900HT Fast PCR system with SYBR green (Molecular Probes) as the fluorescent reporter. Primers (Table 1) were designed with Primer-BLAST (NCBI, Bethesda, MD). Amplification was carried out in triplicate in a 20-μl final volume containing 2.0 μl cDNA (100 ng), 10 μl Express SYBR greenER qPCR supermix with premixed ROX reference dye (Invitrogen), and each primer at a concentration of 0.2 μM. The cycling conditions were 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A subsequent melting curve determination between 60 and 95°C at a transition rate of 0.1°C/s confirmed the specificity of the PCR product. Each run included a negative control and a cDNA reaction without reverse transcriptase to rule out DNA contamination. Spectrophotometric measurements as well as quantitative PCR results showed none or very low contamination by RNA from eukaryotic tissue cells or background flora in noninoculated meat samples.

TABLE 1. Primer sequences of stress genes and reference genes used in this study

Gene (genus or species)	Primer orientation and sequence (5′–3′)
dnaK (*E. coli*)	Forward: CACCACGCCTTCTATCAT
	Reverse: GCCTTTAACTTCGACCCA
otsB (*E. coli*)	Forward: AGCAGGGAAAGTGTGTTGTC
	Reverse: ATCATCGCCCAGAAATACGG
gapA (*E. coli*)	Forward: CATCATCCCGTCCTCTACC
	Reverse: CGCCATACCAGTCAGTTT
dnaK (*Salmonella*)	Forward: CGATTATGGATGGAACGCAGG
	Reverse: GGCTGACCAACCAGAGTT
otsB (*Salmonella*)	Forward: GGTAGTCCGTGAGGTAGAGG
	Reverse: GGAGCCTGACGGTAGTGC
rpoD (*Salmonella*)	Forward: CTGAAAATACCACCAGCACC
	Reverse: CGGGTCAACAGTTCAACAGTG

To investigate potential bias due to inhibiting components in the meat, extractions of RNA from bacteria inoculated in meat and LB broth culture were concomitantly conducted using the same protocol. Threshold cycle (C_T) values of the investigated genes were consistently found at expected levels in the control samples over the range of experimental setups (data not shown). Amplification efficiencies for the primers (Table 1) recognizing *dnaK* (heat stress), *otsB* (salt stress), and *rpoD* and *gapA* (reference genes) were assessed based on the slopes of standard curves. The efficiencies were >90%, and the correlation coefficients were above 0.99 after optimization.

Measured mRNA levels of the target gene were normalized to the mRNA levels of the reference genes (*rpoD* and *gapA*), which were chosen for transcript stability under the given experimental conditions (data not shown). Normalized values were used to calculate ratios of expression levels (relative fold changes) in treated samples versus untreated control samples (Fig. 2) based on the $2^{-\Delta\Delta CT}$ method (6).

Using our RNA extraction method, it was possible to measure expression of bacterial stress genes in a food matrix by applying salt and heat stress. Following heat stress, an induction of *dnaK* expression was observed compared to untreated controls. Relative changes in expression of *dnaK* ranging from 24- to 41-fold were calculated for *E. coli* and *Salmonella* cultures (Fig. 2), although with some variation between biological replicates. Likewise, *E. coli* and *Salmonella* expression of *otsB* was increased >30- and 5-fold, respectively, after salt stress (data not shown). The variation in fold induction may have

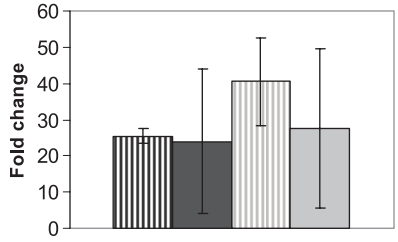

FIG. 2. Relative expression of *dnaK* after heat treatment (45°C, 30 min) of samples versus untreated samples and normalized to reference genes *gapA* (*E. coli* [dark gray]) and *rpoD* (*Salmonella* [light gray]). RNA was extracted from inoculated meat samples (vertically striped bars) and LB broth cultures (solid bars). Error bars show variations between two biological replicates, each measured in technical triplicate.

been related to small variations in timing of the sampling, which can influence the induction level (10). The extraction protocol was applied to meat samples inoculated with target bacteria levels ranging from 10^6 to 5×10^9 per gram of meat, which all yielded sufficient RNA (>50 ng/μl) and qPCR detection of reference genes (C_T values of <30).

The current study demonstrates that it is possible to extract bacterial RNA suitable for gene expression analyses directly from minced meat. The initial stabilization of the RNA without lengthy procedures or toxic chemicals as well as the easy and fast extraction procedure renders the method highly suitable for the study of gene expression related to changes in a minced meat environment.

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Chapter 7

Manuscript I

Growth and survival of exponential and stationary phase *Salmonella* during sausage fermentation

Growth and survival of exponential and stationary phase *Salmonella* during sausage fermentation

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Abstract

The raw meat used for fermented sausages will often contain leftovers from cutting up meat. During handling practices the microbiological food safety may be compromised if too high temperatures allow growth of pathogens such as *Salmonella.* High counts of *Salmonella* in exponential growth phase have been shown to be particular infectious compared to *Salmonella* in stationary phase. Also, adaptation can increase bacterial tolerance to environmental stress and inherent variability exists between exponential and stationary phase cells, with stationary phase cells generally being more resilient. To allow proper blending of ingredients during mincing, the meat has to be frozen and the stress encountered during freezing and thawing and exposure to ingredients as salt and nitrite could impose different bacterial survival depending on the growth phase. The aim of this study was to investigate the survival of exponential and stationary phase *Salmonella* during freezing and subsequent sausage fermentation. Minced meat was inoculated with exponential or stationary phase *Salmonella* Typhimurium and frozen at -18ºC for up to 35 days. The sausages were fermented at 25ºC, and survival of *Salmonella* was followed during fermentation for 3 days. During both short- and long-term freezing, no reduction of stationary phase cells was observed whereas exponential phase cells were reduced by more than 1.5 $\log_{10}$ units (30 fold). Without starter culture, growth of *Salmonella* in both growth phases was observed during the course of fermentation reaching comparable maximum population densities. This indicate that a disrupted or delayed fermentation may support growth of *Salmonella*, regardless of the growth phase of the organism, but also that in spite of a higher growth capacity of exponential cells their level of contamination in the raw material should be more than 30 fold higher than stationary cells to impose a higher risk. In contrast, when applying a commercial starter culture, a

rapid pH drop was achieved, which lead to a maximum reduction of approx. 1 $\log_{10}$ unit (10 fold) of *S.* Typhimurium DT12, independently of growth phases and other variable process parameters. Surprisingly, the growth phase of *Salmonella* seemed to have a minor influence on its tolerance to the stress encountered during the fermentation period of sausage production.

Introduction

Salmonella has long been recognized as an important zoonotic pathogen of economic significance in animals and humans and remained the most frequently detected causative agent in food-borne outbreaks reported in the EU in 2011 (EFSA, 2013). Fermented sausages have also been incriminated due to use of raw meat contaminated with e.g. *Salmonella.* According to EFSA (2012), 2.8 % of the samples taken from minced meat and meat preparations from other species than poultry, intended to be eaten cooked, tested positive for *Salmonella* in the EU in 2010, while 1.8 % of ready-to-eat (RTE) foods, such as minced meat and meat preparations intended to be eaten raw were positive for *Salmonella*. Occasional findings of up to 3 – 5 % of RTE meat products testing positive for *Salmonella*, have previously been reported (EFSA 2007). Quantitative data are rare, but more than 40 cfu/g *Salmonella* in pork cuttings has been reported (Hansen et al., 2010), however, the risk of heavy contamination of the sausage batter corresponding to the high inoculum levels (most often > 10^5 CFU/g) used in challenge studies in fermented sausages is probably small. Still, growth in temperature-abusive environments and delayed or inhibited acid formation during fermentation along with cross-contamination during processing may all lead to elevated pathogen levels (Nightingale et al., 2006). As an example, high-level contamination was seen in a foodborne outbreak of salmonellosis caused by Bologna sausage holding 10^6 cfu/g (van Netten et al., 1986). Several other *Salmonella* outbreaks have been linked to fermented sausages where epidemiological and molecular subtyping studies confirmed the etiology (Bremer et al., 2004; Nygård et al., 2007; Bone et al., 2010), and importantly, *Salmonella* species also have been reported to cause disease even when ingested in very low numbers (Teunis et al., 2010).

In order not to change the organoleptic properties of fermented sausages post-fermentation heating steps are rarely applied, so the meat in this type of products is essentially raw. The initial bacterial load is, therefore, of great importance for the microbial stability of a RTE product like fermented sausages. Lowering of pH is essential for reduction of pathogens during sausage

fermentation (Lücke, 2000), but lactic acid starter cultures are also known to produce factors with preservative functions beyond their acidifying effects such as bacteriocins. Bacteriocins may have possible hurdle and food preservative properties (Gálvez et al., 2007) but it is not known to which level these substances add to the control of pathogens during production of fermented sausages.

Even though foodborne outbreaks have been observed, fermented sausages are traditionally considered safe due to the combination of hurdle factors that, in theory, should efficiently control pathogens (Barbuti & Parolari, 2002). Nissen & Holck (1998) reported that growth of pathogens, after the very early stage of fermentation, before the pH is lowered, is unlikely to occur but in pilot plant studies using different starter cultures it has been shown that a delayed or inhibited acid formation in combination with the high fermentation temperature (24°C) increases the risk of mesophilic pathogen growth (Gunvig et al., submitted 2013).

The ability of *Salmonella* to initiate growth during fermentation may depend on the physiological state of the bacteria such as growth phase and ability to adapt to the environmental stress in the sausage. Stationary phase cells are naturally more resistant to most stresses, with e.g. unadapted stationary phase cells being 1,000-fold more acid tolerant than their exponential growth phase counterparts at pH 3 (Lee et al., 1994). *Salmonella* spp. are known to adapt rapidly to a variety of external stresses, which is often associated with transcription of genes encoding for stress proteins. Global regulators, of which the alternative sigma factor RpoS is of paramount importance in *Salmonella* (reviewed by Hengge-Aronis, 2002), mediate the complex regulatory control of the stress response. RpoS is responsible for the acid tolerance that is pH-independent, but expressed as part of the general stress response upon entry into stationary phase (Lee et al., 1994).

The growth phase of *Salmonella* in contaminated meat is unknown but suboptimal conditions during storage and/or distribution such as temperature abuse can potentially allow the organisms to multiply (EFSA, 2012). The freezing prior to mincing may cause *Salmonella* to reside in exponential growth phase and thus reduce the need for lag time prior to growth during fermentation.

It is likely that stationary and exponential phase cells will show variable tolerance/resistance to the succession of stresses encountered during the fermentation stage of sausage production. In this study we, therefore, aimed to: 1) investigate the survival of two phage types of *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) Definitive Types (DT) 12 and U292 in exponential

and stationary phase during freezing in minced meat and 2) examine the survival of the same *Salmonella* strains during fermentation in sausages prepared with this meat in the presence or absence of a commercial starter culture and $NaNO_2$. To our knowledge this is the first study analysing the effect of different growth phases on survival and growth of foodborne pathogens during freezing and subsequent process of sausage fermentation.

Material and Methods

Bacterial strains, media, and preparation of inocula. *S.* Typhimurium belonging to the phage types DT12 and DTU292, the latter isolated from a Danish outbreak in 2009, were used. Stationary cultures were prepared by inoculating a 1-µl-loopful of single colonies from calf blood agar into 20 ml Luria-Bertani (LB) broth and incubating with shaking for 19 h at 37ºC. The optical density (OD_{600}) of the inoculum was adjusted 0.1, corresponding to *approx*. 8.4 $\log_{10}$ cfu/ml and further diluted to 7.09 $\log_{10}$ cfu/ml to obtain the final inoculum used for the sausage batter. For preparation of the exponential phase cells, the 19 h culture was diluted to 4 $\log_{10}$ cfu/ml and, subsequently, cultured for about 4.5 h until an optical density of 0.05, likewise corresponding to a final inoculum of 7 $\log_{10}$ cfu/ml.

Simulating sausage fermentation. Minced beef meat with a fat content of 16–20 %, was purchased from a local shop. The mince was inoculated with *approx*. 4.5 $\log_{10}$ cfu/g of either exponential or stationary growth phase *Salmonella* cultures of the two phage types and thoroughly blended by hand massage. One portion was processed immediately while others were frozen at -18°C for 7 and >35 days, respectively.
For preparation of the sausage batters the inoculated meat portions were thawed overnight at 5°C before thoroughly mixing with standard sausage ingredients: NaCl (3 % wt/wt), dextrose (0.7 %) and $NaNO_2$ (either 0 or 100 ppm) and a starter culture commonly used in sausage production (Bactoferm F-1, Chr. Hansen; a mixture of *Staphylococcus xylosus* and *Pediococcus pentosaceus*) according to the manufacturer's recommendations. Batter prepared without addition of starter culture (spontaneous fermentation) served as controls. The sausage batter was stuffed into 60-ml sterile syringes with the tip end cut open, sealed, and incubated at 24 – 25ºC, in accordance to

Heir et al. (2010) and Gunvig et al. (submitted 2013). The pH decline was monitored throughout the fermentation by a pH logger (Lutron, WA-2015).

Microbial analysis and sampling. To analyse the effect of freezing on the survival of *Salmonella*, 5 g of meat thawed at 5 °C overnight was macerated by stomaching in 15 ml 0.9 % NaCl (wt/vol) at normal speed for 2 min. Cell counts were determined by spotting three 10 µl droplets from appropriate 10-fold dilutions made in 0.9 % NaCl on Xylose Lysine Deoxycholate (XLD) agar and incubated overnight at 37 ºC.

Cell counts of *Salmonella* during sausage fermentation were determined immediately after having mixed the batter (day 0) and twice a day for the following three days. At each sampling, a piece of sausage was withdrawn from the syringe by pressing the piston, with the first 0.5 cm being discarded. A sausage sample size of *approx*. 5 g was macerated by stomaching and prepared for bacterial analysis as described above for the freezing experiments.

Cell counts of indigenous lactic acid bacteria and starter culture were determined on DeMan, Rogosa and Sharpe (MRS) agar and incubated aerobically at 37 ºC.

Statistical analysis. The experiments were run in triplicate biological replicates. To approximate normal distribution all viable counts were $\log_{10}$-transformed. SAS Enterprise version 4.3 statistical software was used to perform statistical analysis of the data. The response variable in the statistical model was $\log_{10}$ (cfu/ml). Different conditions were compared: phage types (DT12 / DTU292), bacterial growth phase (exponential / stationary), addition of starter culture (+ / -) and $NaNO_2$ (0 ppm/100 ppm). To test the significance of differences under the above mentioned conditions, variance analyses were performed using the model PROC MIXED: $\log_{10}$ (cfu/ml) = time | type of condition, with phage type, bacterial growth phase, starter culture and $NaNO_2$ as class variables, respectively. The differences in the least-square means were used for pair-wise alignments. Alignments were considered statistically significantly different for *P* values < 0.05.

Results

Minced meat inoculated with either *S.* Typhimurium phage type DT12 or DTU292 was frozen for 7 or >35 days prior to preparation of sausage batter. Inoculated batches of non-frozen meat served

as controls. Furthermore, the effect of the following parameters on inactivation of *Salmonella* during the sausage fermentation was analysed: +/- starter culture, 0 ppm/100 ppm $NaNO_2$ and exponential/stationary growth phase cells. Tables 1 – 3 summarize the testing of combinations of the different parameters and the results of the statistical analyses.

Freezing effects. The effect of frozen storage at -18°C on survival of *S*. Typhimurium phage types, DT12 and DTU292 in minced beef meat is illustrated in Figure 1 showing exponential phase cells to be noticeably more sensitive than stationary phase cells. For both phage types in stationary phase viable counts were only marginally affected by short- and long-term freezing by *approx*. 0.1 log_{10} units of reduction. In contrast, a significant difference in the viable counts between the two phage types in exponential phase was observed after 7 days of freezing with average reductions of 0.6 log_{10} units and 0.9 log_{10} units of exponential phase DT12 and DTU292 cells, respectively. However, for both phage types significant reductions, around 1.2 log_{10} units, of the exponential phase cells were observed after >35 days of frozen storage.

Sausage fermentation without starter culture. The indigenous lactic acid bacteria (LAB) were enumerated, and the pH was monitored (Figure 3) in the sausages prepared without starter culture. The lactic acid bacteria grew 4 – 5 log_{10} units during the spontaneous fermentation, reaching levels of 8 log_{10} cfu/g after 25 h in the absence of $NaNO_2$ and after 48 h in the presence of $NaNO_2$ (Figure 2).
When no starter culture was applied, *Salmonella* DT12 and DTU292 grew during the fermentation with viable cell counts reaching maximum population density of 7 – 8 log_{10} cfu/g after *approx*. 48 h (Figure 4 and 5). No systematic differences in cell counts were observed between growth of exponential and stationary phase *Salmonella* after 20 h. Observations during the first 20 h were few and did not allow comparison between the two growth states in this period.

Sausage fermentation with starter culture. The sausages were inoculated with ~ 7 log_{10} cfu/g starter culture, which after *approx*. 20 h of fermentation had increased to 9 log_{10} cfu/g. At the tested concentration, $NaNO_2$ did not exert any antagonistic effect on the growth of this starter culture (Figure 2). The high starter culture inoculum resulted in a fast pH decline starting at around 3 h of fermentation (Figure 3). Presence of the commercial starter culture inhibited growth of both *S.* Typhimurium DT12 and DTU292. During fermentation of sausages inoculated with phage type

DT12, a maximum inactivation of *approx*. 1 $\log_{10}$ unit was attained for exponential and stationary phase cells alike, independently of the preceding freezing period of the meat (Figure 4). Difference in inactivation between the two phage types was observed for exponential phase cells, with DTU292 being most sensitive in sausages without added $NaNO_2$ ($P_{13, 15, 17} < 0.05$, Table 1). The combination of frozen storage (7 or 35 days), no $NaNO_2$ and low pH resulted in *approx*. 2.5 $\log_{10}$ units of inactivation of this phage type and the exponential phase cells were significantly more sensitive than the stationary phase cells ($P_{67, 69, 71} < 0.05$, Table 3) (Figure 5).

Comparison of survival of phage types DT12 and DTU292 during sausage fermentation. Comparative analyses of DT12 and DTU292 growth and survival were conducted under variable sausage processing conditions. No significant difference between the phage types in exponential phase was observed ($P_{1\text{-}6} > 0.28$, Table 1, Figures 4 and 5) in sausages produced with 100 ppm $NaNO_2$, regardless of the other variable parameters; +/- starter culture, +/- freezing. Likewise, in stationary phase, under the aforementioned conditions, no differences between the phage types could be observed ($P_{7\text{-}12} > 0.05$, Table 1, Figures 4 and 5). On the contrary, in sausages produced without $NaNO_2$, + starter culture and +/- freezing, significant differences in inactivation of the phage types were observed, both in exponential and stationary phase DT12 and DTU292 cells ($P_{13, 15, 17, 19, 23} < 0.05$, Table 1, Figures 4 and 5). One exception was seen in stationary phase cells in meat that had been frozen for 7 days ($P_{21} = 0.08$, Table 1, Figure 4D and 5D). While, in six out of seven statistical tests, DT12 was found to be significantly less sensitive to the sausage environment compared to DTU292 (Figure 4 and 5).

Effect of nitrite on *S.* Typhimurium phage types DT12 and DTU292. In exponential growth phase $NaNO_2$, at the concentration tested, did not have any influence on growth/inactivation of DT12, regardless of the other variable parameters; +/- starter culture, +/- freezing ($P_{25\text{-}30} > 0.05$, Table 2, Figure 4). Stationary growth phase DT12 cells, on the contrary, were significantly more sensitive in the presence of 100 ppm $NaNO_2$ under the following conditions; +/- starter culture, + (7 days)/- freezing ($P_{31, 33} < 0.05$, Table 2, Figure 4). A more complex growth/inactivation pattern was observed for phage type DTU292 (Figure 5). Exponential phase DTU292 cells were significantly more sensitive in the absence of $NaNO_2$ under the following conditions; +/- starter culture, + (35 days) freezing ($P_{41, 42} < 0.05$, Table 2, Figure 5F). No effect of $NaNO_2$ was observed on exponential phase DTU292 cells in sausages produced from fresh meat and +/- starter culture ($P_{37\text{-}38} > 0.37$,

Table 2, Figure 5B). Stationary phase DTU292 cells were significantly more sensitive in the presence of 100 ppm $NaNO_2$ under the following conditions; +/- starter culture, +/- freezing ($P_{43, 46, 47} < 0.05$, Table 2, Figure 5A, 5C and 5E).

Comparison of growth/inactivation of exponential and stationary phase *Salmonella* cells during sausage fermentation. For both phage types no significant differences in growth/inactivation between exponential and stationary phase cells was observed when 100 ppm nitrite was present during the sausage fermentations ($P_{49\text{-}54, 61\text{-}64, 66} > 0.05$, Table 3, Figure 4 and 5). One exception is DTU292 in sausages produced with starter culture from meat frozen for 35 days where stationary phase cells were more sensitive than their exponential counterparts ($P_{65} < 0.05$, Table 3, Figure 4E). In the absence of $NaNO_2$, there was a statistically significant difference between stationary and exponential phase cells, in two and four cases out of eight, for phage types DT12 and DTU292, respectively (Table 3). For DT12 it was when the meat had been frozen for >35 days independent on addition of starter culture ($P_{59, 60} < 0.05$, Table 3, Figure 4F). For DTU292, three cases of significant differences were observed when starter culture was added, *i.e.* independent on the meat being fresh or frozen, exponential phase cells inactivated faster than stationary phase cells during the fermentation ($P_{67, 69, 71} < 0.05$, Table 3, Figures 5B, 5D and 5F). The last case was when meat frozen for 7 days was spontaneously fermented in the absence of $NaNO_2$ ($P_{70} < 0.05$, Table 3, Figure 5D). Here, growth of exponential cells appeared to reach 7 $\log_{10}$ cfu/g faster than stationary cells despite initial count being significantly lower.

Discussion

Salmonella spp. have been linked to outbreaks having fermented sausages as incriminating source (Pontello et al., 1998; Bremer et al., 2004; Bone et al., 2010; Kuhn et al., 2011; Gossner et al., 2012), and several studies have, therefore, addressed the inhibition of this pathogen in fermented sausages (e.g. Ellajosyula et al., 1998; Hwang et al., 2009; Ihnot et al., 1998; Nightingale et al., 2006; Nissen & Holck, 1998; Porto-Fett et al., 2008). As also concluded for other pathogens, it is generally believed that control of *Salmonella* in fermented sausages depends on a combination of preservative factors, so-called hurdles, such as high salt, low a_w, decrease of redox potential, growth of competitive microbiota, and a decrease in pH (Holck et al., 2011). In particular, the

decrease in pH, by accumulation of lactic acid from the conversion of glycogen reserves in the muscle tissues, and from the added sugar during fermentation with starter cultures, has been highlighted as one of the most important hurdles with respect to safety of these products (Lücke, 2000; Moore, 2004; Gunvig et al., submitted 2013). Thus, if acid formation for some reason is delayed or inhibited, the high fermentation temperature increases the risk of growth of pathogens. Our study exemplifies this as fermentation with a commercial starter culture arrested growth of both *S.* Typhimurium phage types and in some cases reduced the number slightly with about 1 to 2 $\log_{10}$ units. In contrast, spontaneous fermentation by the natural lactic acid microbiota was not able to inhibit growth of *Salmonella* as viable cell counts increased with at least 2 $\log_{10}$ units during the course of spontaneous fermentation and final counts around 7 to 8 $\log_{10}$ units were obtained after 48 h (Figures 4 and 5).

Apart from the pathogen controlling effect of acidification during fermentation and the additional hurdles acting during maturation (Nightingale et al., 2006), the level of pathogen contamination of the raw meat also plays a role in ensuring safety of fermented sausages (Moore, 2004). Even that manufacturers purchase and use frozen raw meat during mincing of the batter, mainly in order to be able to blend ingredients properly, freezing will also prevent microbial growth. However, growth of pathogens might have occurred before freezing as a result of temperature abuse at different stages of the meat production chain as indicated for meat from cutting plants (Hansen et al., 2013). Consequently, this would potentially lead to a microbial community in different growth phases, and to our knowledge the present study is the first to analyse the possible effect of different growth phase on survival of a foodborne pathogen during freezing and subsequent sausage fermentation.

Our study found *Salmonella* in exponential growth phase to be significantly more sensitive to freezing in minced meat than stationary phase cells. The reduction during frozen storage of both *S.* Typhimurium phage types, DT12, and DTU292 in exponential growth phase was significant after 7 days at -18°C (Figure 1). In comparison, the reduction after >35 days of frozen storage of *Salmonella* in stationary phase was at about 0.12 log units for both phage types. These observations are in accordance with results obtained by Müller et al. (2012) where *S.* Typhimurium DT12 in stationary phase was reduced by less than 1 $\log_{10}$ units in minced pork during 336 days of freezing while cells in exponential growth phase were reduced 1.6 to 2.0 $\log_{10}$ units after 20 days without further reduction for up to 49 days. A freezing study on trimmed beef pieces found that

counts of stationary phase *Salmonella* cells were unchanged during long-term freezing (Dykes et al., 2001) while a reduction of *approx*. 0.5 $\log_{10}$ units during freezing of stationary phase *S.* Typhimurium in minced beef for 10 weeks was observed (Barrel, 1988). Although, beef may influence the inactivation of *Salmonella* differently from pork during frozen storage, *Salmonella* spp. are generally known for their tolerance to freezing (Archer, 2004).

Regarding growth capacity of *Salmonella* in meats, Müller et al. (2012) compared growth curves of exponential and stationary growth phase *Salmonella* cells during incubation at 25°C after frozen storage for up to 49 days. They found that exponential growth phase cells of *Salmonella* spp. initiated growth faster than stationary cells even though they were more sensitive to freezing. This led us to hypothesize that presence of *Salmonella* in exponential growth phase in meat for sausage production exhibited a higher risk of initiating growth during fermentation than stationary phase *Salmonella*.

It has been shown that the unset of acid adaptation/tolerance in *Salmonella* occurs at a higher pH, *approx*. 5.5, for exponentially growing cells compared to stationary phase cells where a pH of *approx*. 4.5 was required for maximum acid tolerance (Àlvarez-Ordóñez et al., 2012). Due to the relative high pH in the beginning of fermentation, it can thus be speculated that the exponential phase ATR will be induced earlier than the stationary phase ATR, which could lead to a higher acid tolerance of exponential phase *Salmonella*. Even if this was the case, our study found no evidence of systematic growth neither of exponential nor stationary phase cells, during sausages fermentation at 24 - 25°C when a commercial starter culture was used. However, a few examples of sporadic growth of both *Salmonella* phage types in exponential phase were observed in batters also added $NaNO_2$ (Figures 3A, 3C, 4A and 4B). In the batters added starter culture, the pH started to drop after 3 h from *approx*. 5.5 reaching pH 5.0 after 10 h and pH 4.6 to 4.8 after 20 h. At the applied fermentation temperature, the lag time of stationary phase *Salmonella* spp. in minced pork with a natural microbiota has been shown to be around 4 h (Møller et al., 2013). Also taking into account the presence of 4.5 % NaCl in the water phase and the use of $NaNO_2$, the lag time of *Salmonella* spp. is predicted to be prolonged even further (http://modelling.combase.cc/CombasePredictor.aspx). The observed sporadic growth of exponential phase *Salmonella* under these conditions indicates that these cells most likely rested in the exponential phase while kept frozen and, therefore, were able to react to the acidic environment immediately. It is interesting to note that this only took place in the presence of $NaNO_2$.

In the spontaneously fermented sausages, we observed that even though frozen storage for >35 days reduced the level of exponential phase *Salmonella* more than 1 $\log_{10}$ units, compared to stationary phase, the exponential inoculum managed to initiate growth and catch up with the stationary inoculum in 20 – 24 h (Figures 4E, 4F, 5E and 5F). In these sausages, the time of onset of the pH decline was delayed up to 4-fold compared to the sausages with commercial starter, leaving 10 - 12 h at pH 5.5 and *approx*. a total of 35 h with pH above 5.0 for *Salmonella* to initiate growth. This has been shown to be enough time for both exponential as well as stationary phase cells of both *S.* Typhimurium phage types to initiate growth after frozen storage when tested in meat juice (Müller et al., 2012). We observed growth of both phage types independent of preceding growth phases indicating no significant difference in risk of growth between exponential and stationary phase of *Salmonella* during erroneous sausage fermentation.

The inactivation during fermentation using commercial starter culture differed between the *S*. Typhimurium DT12 and DTU292. No statistically significant difference in inactivation was observed between exponential and stationary phase DT12 (Figure 4) while a significantly higher inactivation was observed for exponential phase cells of DTU292 in the absence of $NaNO_2$ (Figures 5B, 5D and 5F). It is unclear how adaptation takes place in *Salmonella* being arrested in exponential growth phase by freezing and has not yet entered active growth. It could be anticipated that exponential phase *Salmonella* was more sensitive to low pH stress compared to stationary phase cells. If the pH drop is too rapid it may avoid an adaptation to take place in the exponential phase cells but, on the other hand, allow it in stationary phase cells where the unset of acid adaptation may take place at the lower pH reached during the fermentation. This could only explain the behaviour of DTU292 and not DT12. The reason for the differences observed between these two phage types remains to be elucidated.

The observed growth and survival rates of exponential and stationary phase *Salmonella* were affected occasionally by presence of nitrite with eight out of 24 combinations showing statistically significant effects (Table 2). In general, phage type DT12 was minimally affected, whereas exponential DTU292 unexpectedly appeared most tolerant in sausages fermented with starter culture in the presence of $NaNO_2$ as compared to absence of $NaNO_2$ (Table 2). When effects were found for stationary phase cells, these cells were always most sensitive in the presence of $NaNO_2$. These observations indicate that some members of *Salmonella,* starting out fermentation in exponential phase, may have a higher potential to adapt to the hurdles, in this case nitrite, than

stationary phase cells. Although, nitrite is well-known to minimize the outgrowth of *Clostridium botulinum* spores and, thereby, the development of botulinal toxin (Holck et al., 2011), the inhibitory effect on gram-negative pathogens differs markedly (Lücke, 2000). Other studies indicate that the inhibitory effect of $NaNO_2$ on *Salmonella* is minor if pH is under 5 while absence of $NaNO_2$ may result in growth of *Salmonella* if pH stays above 5 during the fermentation stage (Gunvig et al, submitted 2013; Lücke, 1994). The European Directive 2006/52/EC allows the use of 150 ppm nitrite in fermented sausages (European Parliament, 2006), and a recent study showed that a concentration of 150 ppm reduced the number of *Enterobacteriaceae* with additionally 1 log_{10} units compared to a concentration of 75 ppm (Hospital et al., 2012). Growth of the starter culture applied in the present study appeared unaffected by the $NaNO_2$ concentration added whereas the indigenous lactic acid bacteria were sensitive, with the presence of $NaNO_2$ giving rise to significantly slower growth rate (Figure 2). Despite this observation, it did not result in any systematic difference in the growth of DT12 and DTU292, depending on whether $NaNO_2$ was added or not (Figures 4 and 5).

In conclusion, it was shown that *S.* Typhimurium in exponential growth phase was more sensitive to freezing in minced beef than stationary phase cells independent on phage types, and it appeared that the surviving exponential *S*. Typhimurium had rested in exponential phase during frozen storage. Surprisingly, this higher sensitivity of *S.* Typhimurium in exponential phase was not apparent in the cellular response to the appertaining stresses encountered in the sausage environment during fermentation. We hypothesize that for the exponential phase *S.* Typhimurium an acid tolerance response was mounted at a higher pH and, hence, earlier in the fermentation process than for the stationary phase, masking the inherent differences, in stress response and ability to survive, of the two physiological states. A different survival pattern was found for phage type DT12 and DTU292. No differences were observed between exponential and stationary phase for DT12 whereas exponential phase DTU292 clearly survived to a higher extent than stationary phase when both $NaNO_2$ as well as starter culture was applied. In case of spontaneous acidification by indigenous lactic acid bacteria, which mimicked erroneous fermentation, growth of *S.* Typhimurium was found independent on phage types, fresh or frozen meat and use of $NaNO_2$, underlining the huge importance of correct fermentation for controlling growth and reducing the risk of *Salmonella* infections from fermented sausages.

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Figures

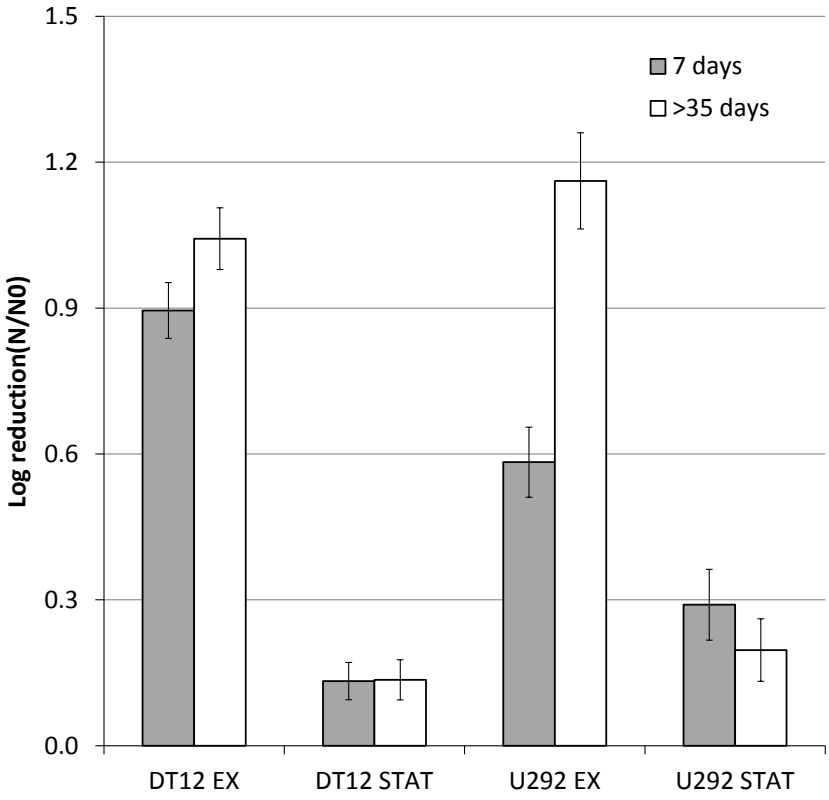

Figure 1. Survival of stationary (ST) and exponential (EX) growth phase *Salmonella* Typhimurium phage types DT12 and U292 in minced beef during frozen storage at -18°C for 7 (grey bars) and >35 days (white bars).

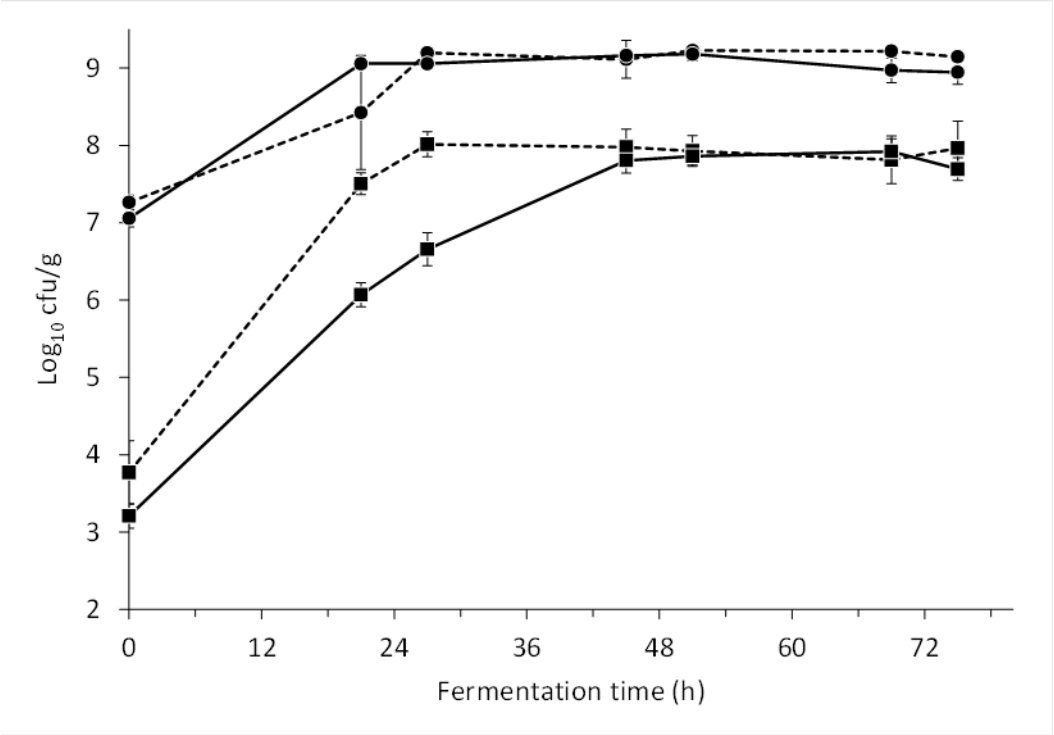

Figure 2. Counts of lactic acid bacteria (log_{10}cfu/g) in the sausage batters determined on MRS agar, aerobically incubated at 37°C 2-3 days. Sausage batters added starter culture are represented by ●, indigenous lactic acid bacteria by ■, whereas sausage batters added 100 ppm $NaNO_2$ are the full lines and the dotted lines represent batters without $NaNO_2$. As no statistically significant differences were found between fresh and frozen meat, average and S.E.M. of 0, 7 or >35 days of frozen storage are shown.

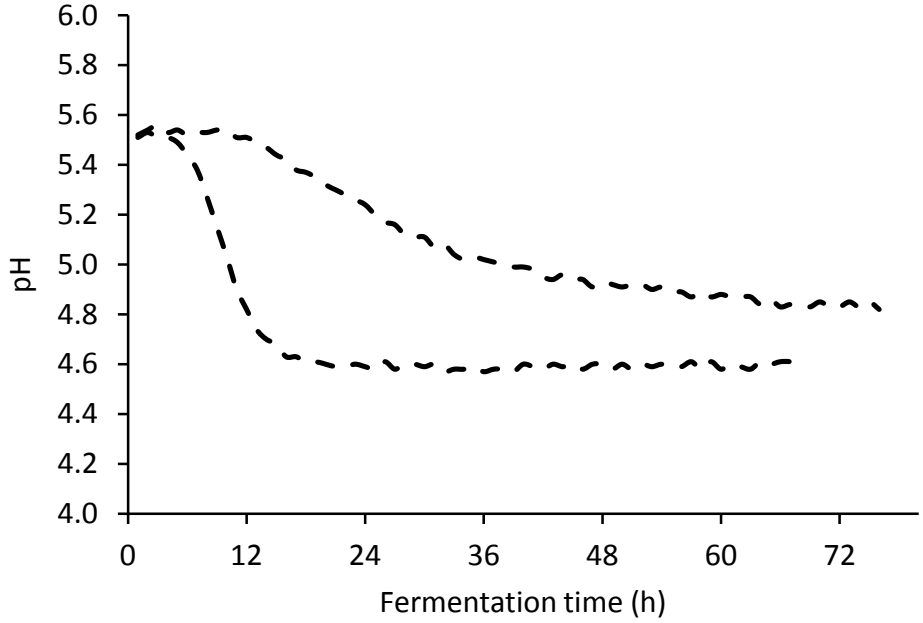

Figure 3. Dynamic pH profiles of sausage batters fermented spontaneously (dashed line) or with starter culture (full line) in the absence of $NaNO_2$.

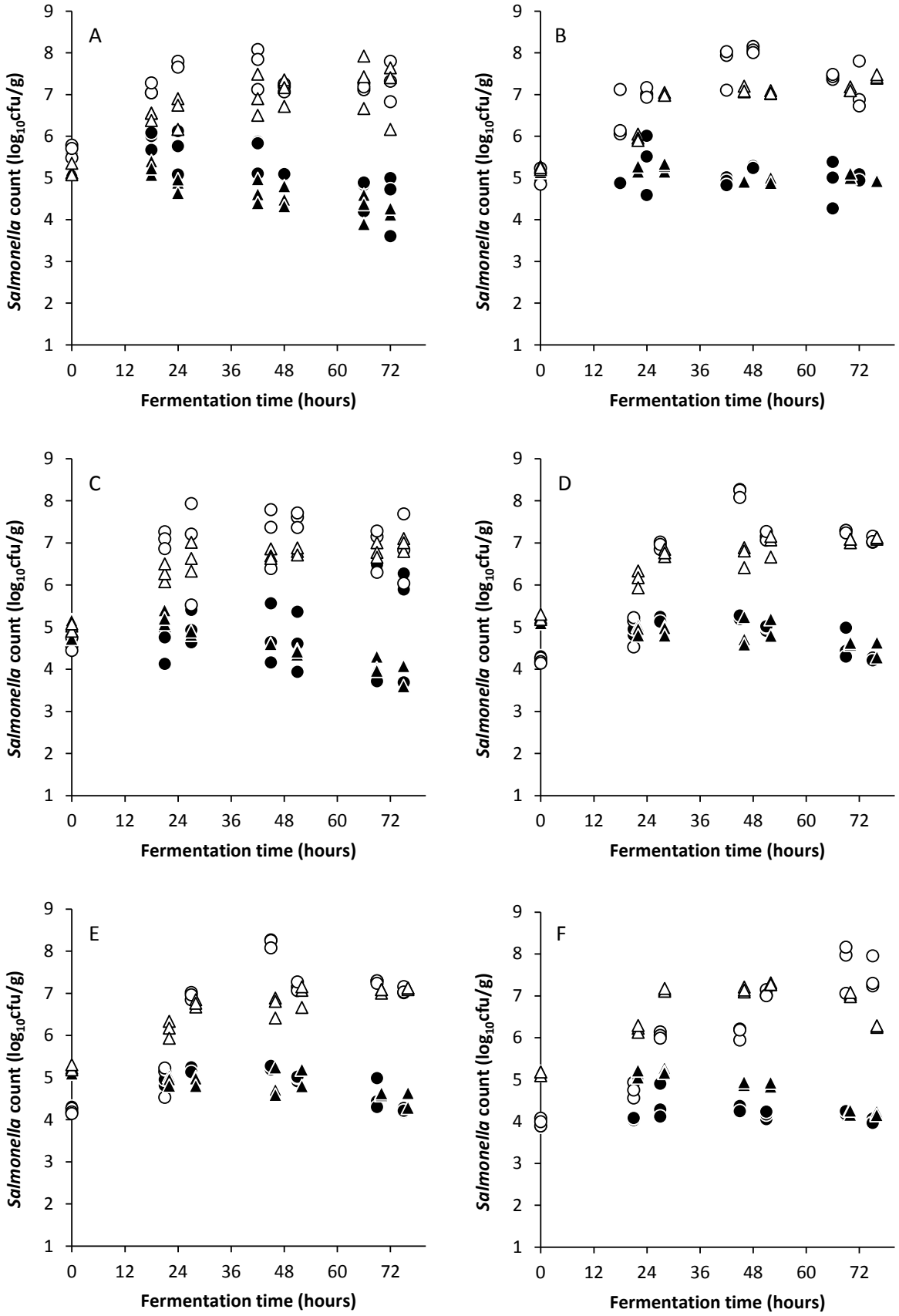
A
B
C
D
E
F
Salmonella count (log10cfu/g)
Fermentation time (hours)
0
12
24
36
48
60
72
1
2
3
4
5
6
7
8
9

Figure 4. Behaviour of stationary (▲△) and exponential (●○) growth phase *Salmonella* Typhimurium phage type DT12 in sausage batters prepared from fresh meat with (A) or without $NaNO_2$ (B), meat frozen at -18°C for 7 days with (C) and without $NaNO_2$ (D) and meat frozen for >35 days with (E) or without $NaNO_2$ (F). Closed symbols represent batters added starter culture and open symbols are the spontaneously fermented batters.

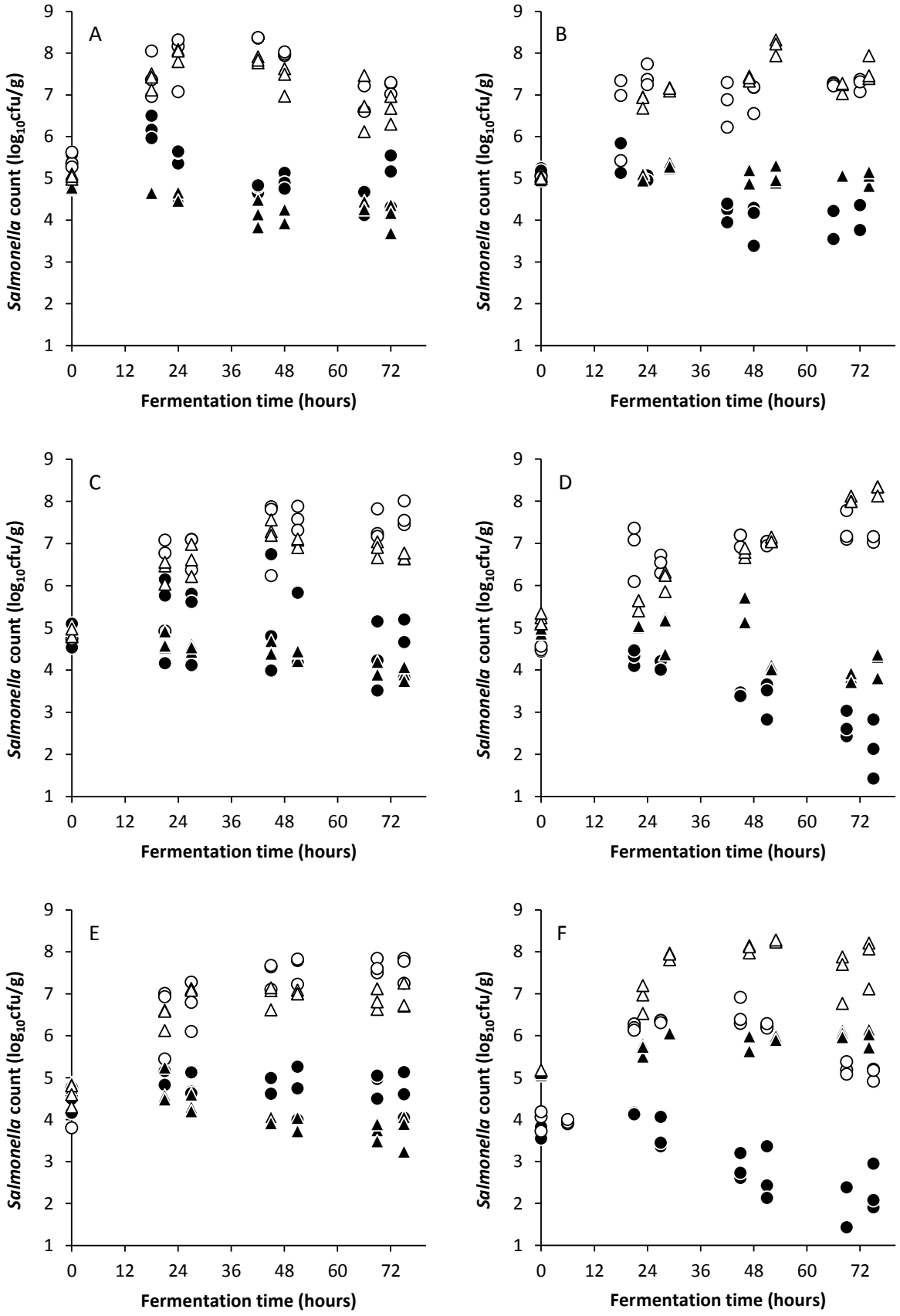
A
B
C
D
E
F
Salmonella count (log10cfu/g)
Fermentation time (hours)
0
12
24
36
48
60
72
1
2
3
4
5
6
7
8
9

Figure 5. Behaviour of stationary (▲△) and exponential (●○) growth phase *Salmonella* Typhimurium phage type DTU292 in sausage batters prepared from fresh meat with (A) or without $NaNO_2$ (B), meat frozen at -18°C for 7 days with (C) or without $NaNO_2$ (D) and meat frozen for >35 days with (E) or without $NaNO_2$ (F). Closed symbols represent batters added starter culture and open symbols are the spontaneously fermented batters.

Table 1. Pair-wise alignment of the two phage types, DT12 and DTU292, during different fermentation parameters.

Nitrite (ppm)		Bacterial growth phase		Starter culture		Freezing of meat batter (days)			P_n value
0	100	Ex	St	With	Without	0	7	>35	
	X	X		X		X			0.60_1
	X	X			X	X			0.92_2
	X	X		X			X		0.28_3
	X	X			X		X		0.75_4
	X	X		X				X	0.47_5
	X	X			X			X	0.70_6
	X		X	X		X			0.05_7
	X		X		X	X			0.19_8
	X		X	X			X		0.25_9
	X		X		X		X		0.93_{10}
	X		X	X				X	0.88_{11}
	X		X		X			X	0.57_{12}
X		X		X		X			$< 0.05_{13}$[a]
X		X			X	X			0.57_{14}
X		X		X			X		$< 0.05_{15}$[a]
X		X			X		X		0.47_{16}
X		X		X				X	$< 0.05_{17}$[a]
X		X			X			X	$< 0.05_{18}$[a]
X			X	X		X			$< 0.05_{19}$[b]
X			X		X	X			0.73_{20}
X			X	X			X		0.08_{21}
X			X		X		X		$< 0.05_{22}$[c]
X			X	X				X	$< 0.05_{23}$[b]
X			X		X			X	0.09_{24}

n: numeration of the specific test/parameter

a: phage type DTU292 most sensitive

b: phage type DT12 most sensitive

c: DTU292 grew to a higher level than DT12

Table 2. Pair-wise alignment of sausages fermented with (100 ppm) or without (0 ppm) nitrite.

Salmonella Phage type		Bacterial growth phase		Starter culture		Freezing of meat batter (days)			P_n value
DT12	DTU292	Ex	St	With	Without	0	7	>35	
X		X		X		X			$< 0.05_{25}$ [a]
X		X			X	X			0.11_{26}
X		X		X			X		0.59_{27}
X		X			X		X		0.47_{28}
X		X		X				X	0.18_{29}
X		X			X			X	0.11_{30}
X			X	X		X			$< 0.05_{31}$ [a]
X			X		X	X			0.72_{32}
X			X	X			X		$< 0.05_{33}$ [a]
X			X		X		X		0.79_{34}
X			X	X				X	0.57_{35}
X			X		X			X	0.21_{36}
	X	X		X		X			0.51_{37}
	X	X			X	X			0.37_{38}
	X	X		X			X		$< 0.05_{39}$ [b]
	X	X			X		X		0.67_{40}
	X	X		X				X	$< 0.05_{41}$ [b]
	X	X			X			X	$< 0.05_{42}$ [b]
	X		X	X		X			$< 0.05_{43}$ [a]
	X		X		X	X			0.05_{44}
	X		X	X			X		0.68_{45}
	X		X		X		X		$< 0.05_{46}$ [a]
	X		X	X				X	$< 0.05_{47}$ [a]
	X		X		X			X	0.62_{48}

n: numeration of the specific test/parameter

a: most sensitive with nitrite

b: most sensitive without nitrite

Table 3. Pair-wise alignment of exponential and stationary phase cells.

Phage type		Nitrite (ppm)		Starter culture		Freezing of meat batter (days)			P_n value
DT12	DTU292	0	100	With	Without	0	7	>35	
X			X	X		X			0.34_{49}
X			X		X	X			0.19_{50}
X			X	X			X		0.07_{51}
X			X		X		X		0.46_{52}
X			X	X				X	0.08_{53}
X			X		X			X	0.26_{54}
X		X		X		X			0.80_{55}
X		X			X	X			0.78_{56}
X		X		X			X		0.96_{57}
X		X			X		X		0.38_{58}
X		X		X				X	$< 0.05_{59}$ [a]
X		X			X			X	$< 0.05_{60}$ [c]
	X		X	X		X			0.13_{61}
	X		X		X	X			0.81_{62}
	X		X	X			X		0.38_{63}
	X		X		X		X		0.67_{64}
	X		X	X				X	$< 0.05_{65}$ [a]
	X		X		X			X	0.07_{66}
	X	X		X		X			$< 0.05_{67}$ [b]
	X	X			X	X			0.51_{68}
	X	X		X			X		$< 0.05_{69}$ [b]
	X	X			X		X		$< 0.05_{70}$ [c]
	X	X		X				X	$< 0.05_{71}$ [b]
	X	X			X			X	0.21_{72}

n: numeration of the specific test/parameter

a: stationary cells more sensitive than exponential cells

b: exponential cells more sensitive than stationary

c: stationary cells higher initial level than exponential cells

Chapter 8

Manuscript II

Buffer capacity of food components influences the acid tolerance response in *Salmonella* Typhimurium during simulated gastric passage

Buffer capacity of food components influences the acid tolerance response in *Salmonella* Typhimurium during simulated gastric passage

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Abstract

Enteric pathogens have evolved several mechanisms for handling acid stress which, along with other factors protecting them from inactivation during gastric acid passage, increase their likelihood of colonizing the intestine and causing infection (Watermann & Small 1998). The composition of the food ingested, hereunder especially buffer capacity, fat and protein content, has also been shown to effect the gastric acid survival of pathogens (Waterman & Small 1998). In this study, we used a computer-controlled fermentor to employ pH changes in synthetic gastric fluid, mimicking the dynamic pH during gastric passage. In order to minimise variation, *Salmonella enterica* serovar Typhimurium was contained in dialysis tubes, enabling simultaneous testing of biological triplicates under varying conditions. Meat and simple food-model substances with different buffer capacities were investigated for their ability to support survival of stationary phase *Salmonella* Typhimurium during simulated gastric acid passage. Surprisingly, we found that less buffered media provided higher protection of *Salmonella*, compared to media with high buffer capacity. By investigating the relative gene expression of *rpoS* and *ompR* encoding for two major stationary phase ATR regulators, we found an approx. four-fold increase in expression of *ompR* and an approx. three-fold increase of *rpoS* in saline and buffered saline, respectively, after 15 min of gastric acid challenge. The relative expression of these genes, were significantly lower in Brain Heart Infusion Broth having a higher buffer capacity. We suggest this to be associated with a varying ability of *Salmonella* Typhimurium to mount a stationary phase acid tolerance response (ATR) depending on the buffer capacity of the food vehicle.

Introduction

Being enteric foodborne pathogens, *Salmonella* spp. typically re-enter the host by ingestion of contaminated feed/food or water. Successful intestinal infection requires that a proportion of the organisms survive a succession of host defences, starting off with the acidic barrier of the stomach. Gastric survival is dependent on several factors related to the host, the ingested matrix and the enteric pathogen (Takumi et al. 2000): Kinetics of gastric pH and emptying vary between individuals, gender and age (Dressman et al. 1990; Russell et al. 1993). While characteristics of the food will affect gastric emptying time (reviewed by Smith 2003), and hence how long the pathogen is exposed to the gastric acid before entering the intestine, the food composition, hereunder especially buffer capacity, fat and protein content, has also been shown to alter the gastric acid survival of pathogens (Waterman & Small 1998). The physiological state of the pathogen and its previous history of exposure to sub-lethal stress are likewise important factors for determining their survival in a potentially lethal environment (Bearson et al. 1997). The pH of gastric acid in fastening state is 1-2, but transiently increases to as much as 5-7 after a meal (Koseki et al. 2011).

Enteric pathogens have evolved several mechanisms for handling acid stress and adaptation at moderate pH can enhance resistance to otherwise lethal pH (Foster & Hall 1990). The development of this so called acid tolerance response (ATR) is complex i.e. with distinct responses occurring in exponential and stationary growth phases (Spector & Kenyon 2012). *Salmonella* possesses two independently regulated stationary phase acid tolerance systems. Unadapted stationary phase cells are relatively resistant to low pH. The major stress response regulator RpoS is responsible for this acid tolerance which is pH-independent, but expressed as part of the general stress response upon entry into stationary phase. Also found is an acid-induced ATR system, of which OmpR is a key regulator (Lee et al. 1994).

Acidic stress is frequently encountered by food borne pathogens in the food-processing system i.e.; during muscle to meat conversion or by decontamination methods using organic acids. While the ATR has direct implications for food safety enhancing the bacterial survival in acidic foods, it also has the potential of increasing the survival of foodborne pathogens facing the acidic pH of the stomach before entering and colonizing the intestinal tract. The infectious dose of pathogens in food is thereby also strongly correlated to their acid tolerance (Audia et al. 2000; Lin et al. 1995). Furthermore, ATR is known to impart cross-protection to other types of stress including; osmotic,

oxidative and short chain fatty acids potentially encountered by the pathogen in the food environment or subsequently during its infectious cycle (Leyer & Johnson 1993; Lee et al. 1995; Baik et al. 1996; Greenacre & Brocklehurst 2006).

In this study we wanted to investigate the inactivation of stationary phase *Salmonella* Typhimurium during pH-conditions mimicking gastric acid passage hereunder; 1) the influence of meat and simple food-models and relation to buffer capacities. 2) the relative gene expression of *rpoS* and *ompR.* 3) the influence of RpoS. To our knowledge, this is the first study analyzing gene expression during dynamic pH changes in synthetic gastric fluid.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used are listed in Table 1. Cells from a stock culture stored at -80 °C in LB broth containing 15% glycerol were streaked on Xylose lysine deoxycholate (XLD) agar plates and incubated overnight at 37 °C. An 1 µl loopful gathered from several single colonies was inoculated in 10 ml brain heart infusion (BHI) broth and incubated overnight at 37 °C reaching a cell concentration of approx. 9 $\log_{10}$ CFU/ml. Overnight cultures were diluted ten-fold in BHI and centrifuged (5810R, Eppendorf AG) at 5000 x *g* for 15 min. The supernatants were discarded and pellets were resuspended in the relevant different media prepared.

Sample preparation

For the study of survival in differently buffered laboratory media, the following were selected: BHI (pH 7.4), saline 0.9% (w/v) NaCl, and saline buffered with disodium hydrogen phosphate (Na_2HPO_4) (2.5 g/l - as in BHI) and adjusted to pH 7.4. The pH was adjusted with 0.4 M HCl.
For testing of real food, meat-suspensions from either minced turkey breast and/or a mixture of pork 80% and beef 20% was produced. Minced meat was purchased from a local Danish supermarket, further comminuted by a regular kitchen blender for 60 seconds and stored at -21 °C. Prior to use samples were thawed at 5˚C overnight. The meat samples, raw or cooked by 2 min boiling, were macerated by stomaching in saline (Stomacher® 80, Seward Ltd.) for 1 min in filter stomacher bags. After stomaching, the filtrates were used for resuspension of bacterial pellets

obtaining meat concentrations of 33.3 g/l or 150g/l. For the survival in meat-suspensions, BHI, adjusted to pH 6, was used as a control medium.

When testing for importance of stress response genes, *S.* Typhimurium C5 Δ*clpP* and Δ*rpoS* and *S.* Typhimurium ST 4/74 Δ*rpoS* mutants were resuspended in saline adjusted to pH 5 with 0.4 M HCl.

Synthetic gastric fluid

Fresh synthetic gastric fluid (SGF) for each trial was prepared by dissolving analytical grade 4.1 g NaCl, 1.2 g KH_2PO_4, 0.2 g $CaCl_2$, 0.6 g KCl, 0.2 g lysozyme (Sigma-Aldrich L6876), 0.1 g bile extract (Sigma-Aldrich B8631), and 26.6 mg pepsin (Sigma-Aldrich P7000) in 2 l demineralized water, as described by Just & Daeschel (2003). When needed the pH of the SGF was adjusted to pH 5 with 0.4 M HCl. Prior to use the SGF was sterile filtered using a 0.22 µm membrane (Steritop™, Millipore™) and preheated to 37 °C on a thermo regulator.

Gastric acidification in pH-controlled fermentor

A pH computer-controlled fermentor (Biostat® B Benchtop fermentor, Braun Biotech International GmbH) provided programmed dynamic pH changes in SGF using 0.4 M HCl and 0.4 M NaOH for pH adjustment (Fig. 1a). The fermenter consisted of a 2 l reservoir containing SGF at 37 °C. For each test condition, a dialysis tube (Float-A-Lyzer®; cutoff 100 kD, Spectrum® Laboratories, Inc.) containing the inoculum mixed 1:10 with SGF with final cell concentrations of 7.04 $\log_{10}$ ± 0.11 CFU/ml, was submerged into the reservoir. Up to nine dialysis tubes could be tested simultaneously dependent on experimental set-up. This enabled biological triplicates and varying conditions to be run in the same trial, as described by Birk et al. (2012). Prior to use the tubes were washed twice with sterile water to remove the 0.1% sodium azide preservative and stored in sterile water at 5 °C, according to the recommendations of the manufacturer. The SGF in the reservoir was stirred at 150 rpm to ensure proton equilibrium between the dialysis tubes and the reservoir. pH meters were calibrated via two or three point calibrations using standard buffer solutions at pH 1.68, 4.01 and 7 prior to use. A secondary small-diameter electrode was employed for measurements inside the dialysis tubes (Micro pH electrode, Orion 8220BNWP, Orion ROSS®). The pH in the fermentor was adjusted automatically according to the following equation:

$$pH_t = (pH_{max} - pH_{min})e^{-kt} + pH_{min} \quad (1)$$

In this study we employ parameter estimates for pH in the stomach of young people having eaten a standard meal, according to Takumi et al. (2000). pH_t is the pH at time *t*, pH_{max} is the pH (5) at the start of acidification after a meal, whereas pH_{min} is the pH (2) at fastening state. The parameter *k* is the acidification rate ($1.6x10^{-2}min^{-1}$), which describes the pH decline from pH_{max} to pH_{min} (Fig. 1b).

Microbiological sampling and analysis

Prior to sampling the content of the dialysis tube was thoroughly mixed. At sampling times; 0, 30, 60, 90 min and hereafter every 15 min until 210 or 240 min, roughly 200 µl of inoculum was transferred from the dialysis tube. From appropriate 10-fold dilutions in buffered peptone water (Oxoid, Thermo Scientific), cell counts were determined by spotting three 10 µl droplets onto XLD agar followed by overnight incubation at 37 °C. To approximate normal distribution viable counts were log_{10} transformed. All results presented are means derived from biological duplicates or triplicates, as indicated in the results section. For each trial, the log transformed *Salmonella* counts were fitted to the Baranyi and Roberts non-linear survival model with no asymptote using the web edition of DMFit (http://modelling.combase.cc/DMFit.aspx). This procedure provided estimates of shoulder length, i.e. duration (min) before unset of inactivation, as well as maximum inactivation rate (IR, log_{10}CFU/ml/min). Shoulders and maximum inactivation rates were used as response variables in analyses of variance testing the differences between trials, media (BHI, saline, buffered saline), *Salmonella* wild types and mutants, and meat-suspensions (turkey, pork/beef). All analyses of variance were computed in Microsoft Office Excel 2007 using one- or two-factor ANOVA where appropriate with *P*-values < 0.05 being considered statistically significant. For pair-wise comparisons 95 % Least Significant Difference (LSD_{95}) values were used.

Determination of buffer capacities

Buffer capacities of media and food components were determined as described by Karow et al. (2013). By convention, buffer capacity is defined as the quantity of strong acid or base that must be added to one litre of buffer solution to change the pH one unit. Buffer capacity (ß) is defined as:

$$\beta = \frac{\Delta B}{\Delta pH} \qquad (2)$$

Where *Δ B* = concentration (in equivalents, Eq) of strong acid/base added. *ΔpH* = pH change caused by the addition of strong acid/base to the buffer system. The Eq for monovalent acids such as HCl is equal to the moles of titrant added (Karow et al. 2013). Titration curves were generated by adding 0.1 M HCl to 20 ml buffer system. For analysis, the pH was plotted versus the equivalents of acid added for each titration, normalized to the solution volume. Titration curves were subdivided in pH segments (ΔpH > 5, ΔpH 5-4, ΔpH 4-3, and ΔpH < 3), and the data points were fitted to a linear equation. In a given pH range the reciprocal of the slope of the linear curve gives the average buffer capacity β. Titration curve segments of ΔpH=1 were chosen when feasible, seldom segments of ΔpH= 0.5 had to be chosen in order to allow a linear fit. Titration curves and linear regressions were generated in Microsoft Office Excel 2007. The average buffer capacity was derived from means of two titration curves.

RNA extractions and qRT-PCR

Sampling during gastric acid challenge occurred in the first experiments at 30, 90 and 120 min and was subsequently changed to 0, 15, 30 min. Samples for qRT-PCR analysis were stabilized in 2 volumes of RNA*later* (Ambion) by incubation at room temperature for 10 min and subsequent storage at -20 °C until extraction. Samples were thawed on ice and centrifuged at 5000 x *g* for 15 min. RNA extractions were performed with the RNeasy kit (QIAGEN, Copenhagen, Denmark). Pellets were treated with 0.2 ml TE-buffer (Tris-EDTA 10:1, pH 8) containing 3 mg lysozyme and 0.004 mg proteinase K and further processed as recommended by the manufacturer, including an optional DNase treatment. Purified RNA was quantified using a Nanodrop ND-1000® (ThermoScientific, Wilmington, DE), and the RNA quality was verified by OD_{260}/OD_{280} nm absorption ratio > 1.95 and an OD_{230}/OD_{260} nm absorption ratio > 2.0. cDNA was synthesized using QuantiTect® Reverse Transcription Kit (Qiagen) with random hexamer primers following the manufacturers instruction, using 0.1 µg total RNA per sample.

Quantitative real-time PCR was performed on an ABI prism 7900HT from Applied Biosystems (Nærum, Denmark) with SYBR Green as the fluorescent reporter. Primers targeting the relevant stress response and reference genes (Table 2) were designed with Primer-BLAST (NCBI, Bethesda, MD). Amplification reactions were carried out in triplicate in transparent 384-well MicroAmp® Optical reaction plates (Applied Biosystems) in a total volume of a 12 µl containing 2.5 µl cDNA (~5 ng/µl), 6 µl Power SYBR® Green PCR Master Mix (Applied Biosystems) and each primer at a

concentration of 0.24 µM. Liquid handling was performed with an automated pipetting system (epMotion 5075 TMX, Eppendorf). The cycling conditions were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A subsequent melting curve determination between 60 and 95˚C at a transition rate of 0.1˚C/s confirmed the specificity of the PCR product. Each run included a negative control. Measured mRNA levels of the target gene were normalized to the mRNA levels of reference genes chosen for transcript stability under the given experimental conditions. Normalized values were used to calculate ratios of the expression levels in treated relative to untreated control samples using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Amplification efficiencies per amplicon group were determined to be 1.92, 1.93 and 1.94 for *rpoD*, *rpoS* and *ompR*, respectively using the LinRegPCR software (Ruijter et al. 2009). Single factor ANOVA (Microsoft Office Excel 2007) was used to assess the statistical significance of the differences between control and test groups. *P*-values < 0.05 were being considered statistically significant.

Results

A pH computer-controlled fermentor was employed to simulate the dynamic pH drop in the stomach after ingestion of a standard meal (Fig. 1). To be able to test various conditions and several biological replicates in the same experimental set-up, floating dialysis tubes containing different inocula were tested simultaneously in the fermentor. The pore size of the dialysis membrane allowed diffusion of protons, but not of proteins or bacterial cells across the membrane (Birk et al., 2012). In a validation study (data not shown), the performance of the system was tested in BHI to determine the inter- and intra-trial variance. No significant intra-trial variances ($P > 0.05$) were displayed amongst triplicates, and there were no significant differences on the two key parameters tested; duration until unset of inactivation (shoulder) and max. inactivation rate (IR) (shoulder; $P = 0.377$ and IR; $P = 0.162$) between two independent trials. Significant inter-trial variances, probably resulting from minor differences in the pH profile or in the inocula, were however encountered in subsequent trials (Fig 2).

Inactivation of *S*. Typhimurium C5 in presence of different media during modelled gastric acid challenge

As simple food models BHI, saline and buffered saline were applied to investigate the inactivation of *Salmonella* imposed by differences in pH and buffer capacity when added to the gastric acid model. From Figure 3 it appears that *Salmonella* challenged in BHI was inactivated faster than in either of the other media. While fitting the log transformed *Salmonella* counts to the non-linear survival model of Baranyi and Roberts (1994), it was observed that the unset of inactivation in saline occurred significantly later, at 90 min (pH 2.71), compared to 65 min and 69 min for BHI and buffered saline, respectively. The latter two media are displaying comparable buffer capacities and starting pH. The max. inactivation rates in the three media were significantly different, with saline and buffered saline displaying the highest and lowest rates, respectively. Measuring the pH inside the dialysis tubes, an approx. one hour delay in pH equilibrium across the membrane in buffered saline and BHI, was observed (data not shown).

The observed differences in survival could be a mere effect of pH exposure, but could also be related to buffer-induced differences in mounting of an acid tolerance response. To investigate this, expression of genes involved in the stationary phase ATR was measured by qRT-PCR. Relative gene expression analyses of *rpoS and ompR* were initially performed comparing the expression in the overnight culture to samples taken during acidification in the fermentor at 30, 60 and 120 min. The relative expression of *ompR* and *rpoS* was overall found to be upregulated at all time points and in all media during the simulated gatric acid challenge. At 30 min the expression of *ompR* was maximally induced in saline and comparable to the induction observed in BHI at 90 min. These results led us to hypothetize that the delayed start of inactivation in saline could be related to an earlier mouting of an ATR in this medium than in the buffered BHI. Subsequent studies with sampling at 0, 15 and 30 min were conducted to analyse if the gene expression pattern during the early drop in gastric pH and a potential acid adaption could account for the differences in inactivation curves. The results demonstrated significant differences in the relative expression of *rpoS* and *ompR* between BHI and saline (Fig 4). At 0 min, right after mixing of SGF and inoculum, an approx. 1 fold induction of *ompR* and *rpoS* in saline and of *rpoS* in buffered saline was observed. At 15 and 30 min the relative expression of *ompR* had increased more than 3.5 fold in saline and was significantly higher than in BHI. For *rpoS*, a maximum induction of approx. 3 fold was observed at 15 min in buffered saline, which decreased slightly at 30 minutes. The relative

expression of *ompR* and *rpoS* in BHI initially decreased while at 15 and 30 min an approx. 0.5 fold increase was observed.

Inactivation of *S*. Typhimurium C5 rpoS and clpP mutants during modelled gastric acid challenge

The gene expression analyses in different media indicated that *rpoS* was significantly upregulated during exposure to the dynamic pH-environment. This led us to investigate the survival of isogenic Δ*clpP* and Δ*rpoS* mutants of *S*. Typhimurium C5 (C5) in the gastric acid model.

Initial results with C5 indicated that survival in the gastric acid environment was independent of a functional *rpoS* gene as the inactivation of wild type and *rpoS* mutant did not differ significantly with shoulders ~ 99 and IRs = -0.11 for both strains. To avoid a bias in conclusions caused by a potential defect in *rpoS* in C5 as indicated by insufficient catalase activity (data not shown), an alternative strain, *S*. Typhimurium ST 4/74 (ST 4/74) (prototrophic parent of the SL1344 strain) was investigated for comparison. Two independent trials were performed, displaying a similar tendency in comparison between strains. Nevertheless, the duration until unset of inactivation (shoulder) varied significantly (P=0.003) between the trials while the rates of inactivation were not significantly different (P=0.768). The inactivation of C5 and ST 4/74 obtained from means of these two trials is presented in Figure 5. Similar to C5, the ST 4/74 WT and its isogenic *rpoS* mutant did not significantly differ, neither in duration until unset of inactivation nor in max. inactivation rate (Table 2). However, ST 4/74 differed significantly from C5 by displaying a longer duration before unset of inactivation. The max. inactivation rates of the wild type C5 strain and the ST 4/74 WT and *rpoS* mutant were not significantly different. The highest rate of inactivation was displayed by the C5 *rpoS* mutant. The unset of inactivation was delayed in the C5 *clpP* mutant compared to all the other strains while its rate of inactivation was not significantly different from the ST 4/74 WT and *rpoS* mutant strains. Gene expression analyses thus also indicated that the relative expression of *rpoS* was significantly up-regulated in the *clpP* mutant during gastric passage at 30-120 min compared to the wild type (data not shown).

Inactivation of *S*. Typhimurium in presence of meat during modelled gastric acid challenge

In order to investigate for a protective effect of food-components against gastric killing, proposed by several studies (Watermann & Small 1998; Koseki et al. 2010; Birk et al. 2012; Aviles et al. 2013), suspensions of different meat types were applied in the gastric acid model.

Initial studies run in duplicate, comparing meat-suspensions of raw mixed beef/pork (as a simple fermented sausage model) at a concentration of 33.3 g/l according to Birk et al (2012) to BHI, displayed corresponding inactivation curves for *Salmonella* (data not shown). As protection of *Salmonella* Dublin has been shown to depend on nature of the protein (Birk et al. 2012), the inactivation of *Salmonella* inoculated in BHI and suspensions of grinded and cooked turkey and mixed pork/beef meat, respectively, was compared. To obtain a meat concentration corresponding to a standard meal, 150 g/l was chosen according to Russell et al. (1993). No statistically significant differences in the shoulder duration between the control (BHI), turkey and pork/beef were observed (Fig 6). However, the rate of inactivation of *Salmonella* was significantly faster in BHI compared to meat suspensions. When applying the low meat-concentration (33.3 g/l), the inactivation curves were dropping log-linearly (data not shown). When a concentration of meat of 150 g/l was used an increase in viable counts occurred at approx. 150 min (pH 2.27), delaying the reduction of *Salmonella* below the detection limit. This increase in cell counts was reproducible and was observed for both the suspensions of beef/pork mixture and the turkey meat (Fig. 6).

Finally to establish if the observed differences in survival of Salmonella during modelled gastric acid exposure were related to buffer capacity of the simple media and meat-suspensions, the association between buffer capacity and the durations until unset of inactivation and the rates of inactivation, respectively was explored (Fig. 7). The results demonstrated that low buffer capacity media provided a longer duration before unset of inactivation compared to media and meat-juices with high buffer capacity. Nonetheless, once inactivation was induced, the max. inactivation rates were faster in the least buffered media.

Discussion

The low pH of the gastric acid in the stomach is considered the first major protective host barrier against foodborne pathogens (Smith 2003). Enteric pathogens have evolved several mechanisms for handling acid stress, and along with other factors protecting them from inactivation during gastric acid passage it increases their likelihood of colonizing the intestine and causing infection (Watermann & Small 1998). The composition of food has been suggested to influence bacterial survival. Thus, a high fat content has e.g. been epidemiologically linked to a low infectious dose

indicating a protective property during stomach passage (Kapperud et al. 1990). Also the buffer capacity of the food has been considered, with the rationale that a high buffer capacity confers a higher level of bacterial protection due to a delayed lowering of the pH (Takumi et al. 2000). The overall pH dynamics in the stomach thus depend on the meal but also on the ability of the host to produce acid, as well as on the emptying time of the stomach (Takumi et al. 2000; Koseki et al. 2011). While the pH of gastric acid in fastening state is 1-2, it transiently increases to as much as 5-7 after a meal (Koseki et al. 2011). A few studies have investigated pathogen inactivation in synthetic gastric fluid during exposure to dynamic pH conditions, including: (Takumi et al. 2000; de Jonge et al. 2003; Barmpalia-Davis et al. 2008; Koseki et al. 2011; Birk et al. 2012).

In the present study we employed a fermentor system, which could simulate the dynamic pH changes characteristic for gastric passage. Simultaneously it allowed investigation of different substances contained in dialysis tubes. The pH in the fermentor was computer-controlled adjusting on pH updates sent from the fermentor electrode every minute. Primarily doing comparisons and replicates within the same experiment was decided because even minor pH differences between trials had a significant impact on inactivation, particularly on the duration until unset of inactivation, leading to inconclusiveness. Despite this, the inactivation rates after unset of killing were not significantly affected, hence allowing comparison of this parameter between trials.

Takumi et al. (2000) estimated by a predictive modelling approach that 20-80% of ingested *E. coli* O157 would arrive in the intestine without being inactivated due to gastric passage. In the present study results showed that *Salmonella* was inactivated to undetectable levels (<30 CFU/ml), in all of the trials, at no later than 180 min. By taking the stomach emptying rate (Takumi et al. 2000) into account, within 120 min approx. 50 % of the meal would already be transported to the intestine. Our data indicate that at this time point and pH ~ 2.4 there will still be a substantial amount of viable bacteria left (at least 2 $\log_{10}$) thus supporting a substantial survival of *Salmonella*. We also expect some underestimation of the gastric survival due to the use of the selective media, XLD, for direct enumeration. This medium made it possible to discriminate between native microbiota of the meat and inoculated *Salmonella*. The selective medium, however, may leave more *Salmonella* unculturable, particular when the pH stress increases thus overestimating the level of inactivation as also argued by Aviles et al. (2013).

In *Salmonella* spp. adaptation at moderate pH can enhance resistance to otherwise lethal pH. This adaptation has been intensively investigated by the pioneering work of Foster (1991; 1993; Foster & Hall 1990; Foster & Hall 1991). The development of acid tolerance responses in *Salmonella* is complex i.e. with distinct responses occurring in exponential and stationary growth phases and with dependency on growth temperature, stress level, exposure time, and on the properties of the acidulant (Spector & Kenyon 2012). *Salmonella* harbours several regulons which enable acid adaptation, most importantly those controlled by RpoS, Fur, PhoP, and OmpR (Rychlik & Barrow 2005). In stationary phase, *Salmonella* possesses two independently regulated acid tolerance systems; one is RpoS-dependent but pH-independent, the other is pH- and OmpR-dependent but RpoS-independent. It is largely unclear to which extend these regulators are activated and which possible role they play during gastric acid exposure.

The alternative sigma factor RpoS, a global stress regulator encoded by the gene *rpoS*, is essential for survival of *Salmonella* in stationary phase as well as under other various stress conditions including low pH (Fang et al. 1992). Several studies have shown that *rpoS* deletion mutants display elevated susceptibility to acid pH (Fang et al. 1992; Lee et al. 1994; Lee et al. 1995; Bearson et al. 2006). Lee et al. (1994) for instance found that acid sensitivity increased at least 10-fold in an *rpoS* mutant. In contrast, in the present study, comparing inactivation of wild types and *rpoS* mutants of *S.* Typhimurium C5 and *S.* Typhimurium ST 4/74 during simulated gastric acid exposure, only minor differences between wild type and mutant were observed. During the study it was realised that C5 carries a deficient *rpoS* allele as suggested by Schwan et al. (2000). This could explain that the difference in inactivation between wild type and mutant for this strain was not statistically significant. However, a delayed inactivation of the C5 *clpP* mutant was observed with significantly slower max. inactivation rate compared to the C5 wild type. The increased acid resistance of the mutant probably stems from an augmented *rpoS* level, indicating that *rpoS* is not fully defective in C5. A serious *rpoS* defect in C5 is also somewhat contradicted as we did not find significant differences between ST 4/74 wild type and *rpoS* mutant either, although the inactivation rates were slower than observed for C5.

Unadapted stationary phase cells are relatively resilient to pH 3 and 1000-fold more acid tolerant than their log phase counterparts. RpoS is responsible for this acid tolerance, which is pH-independent, but expressed as part of the general stress response upon entry into stationary phase (Lee et al. 1994). If, however, the cells are preadapted at a moderate pH of 4.5, they survive

much longer (Lee et al. 1994). This phenomenon, referred to as the acid-induced stationary phase ATR, is OmpR-dependent but RpoS-independent and may in part explain why we did not observe any marked differences in survival between wild types and *rpoS* mutants. This led us to believe that the expression of *rpoS* would not increase in the stationary phase cells exposed to a dynamic acidic environment (below pH 5). However, qRT-PCR results showed that expression of *rpoS* was markedly induced, in saline and buffered saline, during simulated gastric acid challenge, not reflecting the seemingly insignificance of having a functional *rpoS* gene as shown with the mutants. Still, the induction of *rpoS* could also relate otherwise to stress experienced by the bacteria in these media and not to the SGF alone.

OmpR is the response regulator of a two-component regulatory system in which EnvZ is the membrane-bound sensor kinase known to respond to changes in osmolarity (Pratt et al. 1996). In addition to its osmoregulatory role, OmpR plays a central role in the acid-inducible stationary phase ATR (Lee et al. 1994). *ompR* insertion mutations have been shown to render stationary phase cells acid sensitive and unable to mount an inducible ATR (Bang et al. 2000). Observed on mRNA and protein levels, *ompR* is induced by low pH (Bang et al. 2002; Rychlik & Barrow 2005). During acid stress, however, OmpR is activated independently of EnvZ, and phosphorylation has been shown to be mediated by acetyl phosphate (Bang et al., 2000). Phosphorylated OmpR binds to DNA (including to its own low pH-specific promoter) and activates the transcription of target genes (Bang et al. 2002). Therefore, we expected up-regulation of expression of *ompR* in *S*. Typhimurium when exposed to the dynamic pH changes during simulated gastric passage. Gene expression analysis confirmed this notion, as an increased expression of *ompR* at all times and in all media was demonstrated, with the exception of BHI at the early (0 min) sampling point. Bang et al. (2000) found *ompR* transcription to be increased after 15 min in moderate pH reaching maximum levels after 30 min. In contrast, we observed that *ompR* transcription was induced immediately in media with low (saline) and medium (buffered saline) buffer capacities, and that a maximum level in these media was reached after 15 min, not changing significantly in the 30 min sample and still being comparably high at 120 min. In BHI a maximum 0.5-fold induction of *ompR* was observed. The up-regulation of *ompR* did not seem to be transiently expressed as it has been reported for other stress induced genes, such as *otsB* (Balaji et al. 2005).

To investigate the effect of buffer capacity on inactivation comparisons in saline, buffered saline (using the buffer system from BHI), BHI, and meat-suspensions were made. The buffered media

gave rise to a delayed pH drop, reaching pH 5 at approx. 30 min later than observed for saline. Several recent studies have proposed that the inherent high buffer capacities of some food components would prevent a rapid pH decline and then potentially protect enteropathogens during gastric acid passage (Birk et al. 2012; Koseki et al. 2011; Takumi et al., 2000). However, contradictory to this a significantly delay in inactivation of *Salmonella* was observed in the non-buffered saline compared to BHI. Moreover, it was observed that the fold change of expression of *ompR* was significantly higher for *S*. Typhimurium in saline compared to BHI at 0, 15 and 30 min. Along with the immediate induction of *ompR* in saline this could indicate that an acid tolerance response was mounted almost instantly in saline, which may support the increased survival of *Salmonella* in this medium, compared to the buffered BHI. Adaptation at pH ~ 4.5 thereby also seems to be a prerequisite for a maximum acid tolerance response in stationary phase cells (Lee et al. 1994; Àlvarez-Ordóñez et al. 2012). Furthermore, Lee et al. (1994) demonstrated that acid tolerance in *Salmonella* increased with exposure time to moderate acidity (pH 4.5). This together with our results support that an early ATR response is induced in the less buffered media, which in turn would lead to increased acid tolerance and survival of *Salmonella*. Relating buffer capacity to the duration until unset of inactivation (the shoulder of the curve) and maximum rate of inactivation, respectively, we saw that both decreased with increasing buffer capacity. While the novel observation of this study of an instantaneous ATR mounted under stressful conditions in saline could possibly explain the delay in inactivation, the slow inactivation rate in BHI could be explained by the observation of an enhanced ATR mounted in complex media, recently reported by Álvarez-Ordónez et al. (2012). Further studies is needed to elucidate how the buffer systems seem to deny *Salmonella* appropriate conditions for ATR development compared to the non-buffered saline, and why, on the other hand, inactivation occurs faster in saline once initiated.

In a recent study by Birk et al. (2012), they showed that pepsin, ovalbumin and a blended turkey meat-suspension, but not bovine serum albumin, protected *Salmonella* Dublin from inactivation in a simple gastric acid model. Our data did not support a protective effect from meat as we observed no significant difference in duration until unset of inactivation for meat-suspensions and control samples. Interestingly, Birk et al (2012) found no significant difference in inactivation between samples where pepsin and turkey meat were tested separately, and since we, on the contrary, applied pepsin to the SGF in the dialysis tubes, this could be the factor explaining the difference between the two studies.

Protection of bacteria during gastric passage may have other backgrounds than investigated in this study. It has been demonstrated that post-meal gastric fluid is less efficient in killing bacteria although the pH was adjusted to 1.5, thus avoiding the buffering effect from the food (Peterson et al. 1989). Others have suggested that entrapment of bacteria in food-particles or hydrophobic moieties may serve as a protective shelter, shielding the bacteria from the lethal pH of the stomach (d'Aoust 1985; Takumi et al. 2000; Aviles et al. 2013). Such factors may contribute to the unexplained increase in viable cell counts of *Salmonella* in the high-concentrated meat-suspensions at a growth restrictive pH of less than 2.5 seen as the second peak in the curves presented in Figure 6.

This study puts novel layers to the complexity of the acid tolerance response in *Salmonella,* which challenges conclusions. The data indicate that an efficient inactivation of *Salmonella* Typhimurium was reached after 3 hours in the gastric pH environment; still, significant numbers of bacteria will be able to pass to the intestine before that. The study also indicated a minor significance of RpoS for the survival of *Salmonella* during the acid challenge, although increased survival of a *clpP* mutant indicated some participation of *rpoS.* Accounting for the increased survival observed in non-buffered saline compared to the buffered, more complex media, BHI, we suggest that an instantaneous ATR is mounted under the pH stress encountered by the bacteria in saline, which is denied in the buffered system due to inappropriate pH dynamics in the moderate pH area. Supporting this hypothesis, *rpoS* and *ompR*, encoding for two major regulators of stationary phase ATR, were found to be highly induced in less buffered media. We also found that an inverse correlation seems to exist between buffer capacity and time before unset of inactivation and maximum inactivation rates. Lastly, in contrast to what has previously been reported (Birk et al. 2012) we found neither suspensions of turkey nor pork/beef meat to protect *Salmonella* from inactivation in the applied simulated gastric acid system, which could possibly relate to presence of pepsin.

Seemingly, ATR induction is more important for survival of *Salmonella* during gastric passage than the other factors investigated in this study.

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Tables and figures

Table 1. *Salmonella enterica* serovar Typhimurium strains used in this study

Strain	Genotype	Source
C5	Virulent wild type	Hormaeche (1979)
C5 ΔrpoS	*rpoS::amp*	Thomsen (2002)
C5 ΔclpP	Δ*clpP*	Thomsen (2002)
ST 4/74	Virulent wild type	Wray & Sojka (1978)
ST 4/74 ΔrpoS	*rpoS::kan*	Knudsen et al. (2012)

The strains were kindly donated by; J.E.Olsen, University of Copenhagen; L. E.Thomsen, University of Copenhagen; G. M. Knudsen, Technical University of Denmark.

Table 2. Oligo sequences of stress genes and reference genes used

Gene	Direction and sequence (5'-3')
ompR	Forward: TTCGTTTGCCTGACGACGTA
	Reverse: GCGAAGGGTGAAGAGGTTGA
rpoS	Forward: GTTGGACGCGACTCAGCTTT
	Reverse: TTTTACCACCAGACGCAGGTT
rpoD	Forward: CTGAAAATACCACCAGCACC
	Reverse: CGGGTCAACAGTTCAACAGTG
16SrRNA	Forward: ATTGACGTTACCCGCAGAAGA
	Reverse: GGGATTTCACATCCGACTTGA

Table 3. Mean maximum inactivation rate and shoulder for *Salmonella* Typhimurium C5 and ST 4/74, obtained from two independent gastric challenge trials.

	Shoulder (min)		Max. inactivation rate (log_{10}CFU/ml/min)	
strain	mean	SEM (n = 2)	mean	SEM (n = 2)
ST4/74	96 ^{A}	2.8	-0.073 ^{AC}	0.0111
ST4/74 ΔrpoS	94 ^{A}	3.9	-0.077 ^{AC}	0.0015
C5	106 ^{B}	6.6	-0.113 ^{AB}	0.0001
C5 ΔrpoS	105 ^{B}	4.7	-0.133 ^{B}	0.0204
C5 ΔclpP	110 ^{B}	7.0	-0.064 ^{C}	0.0003

$^{A, B, C}$ Different letters represent significant statistical differences evaluated by LSD_{95} values of 7.0 min for shoulder and 0.045 log_{10}CFU/ml/min for max. inactivation rate.

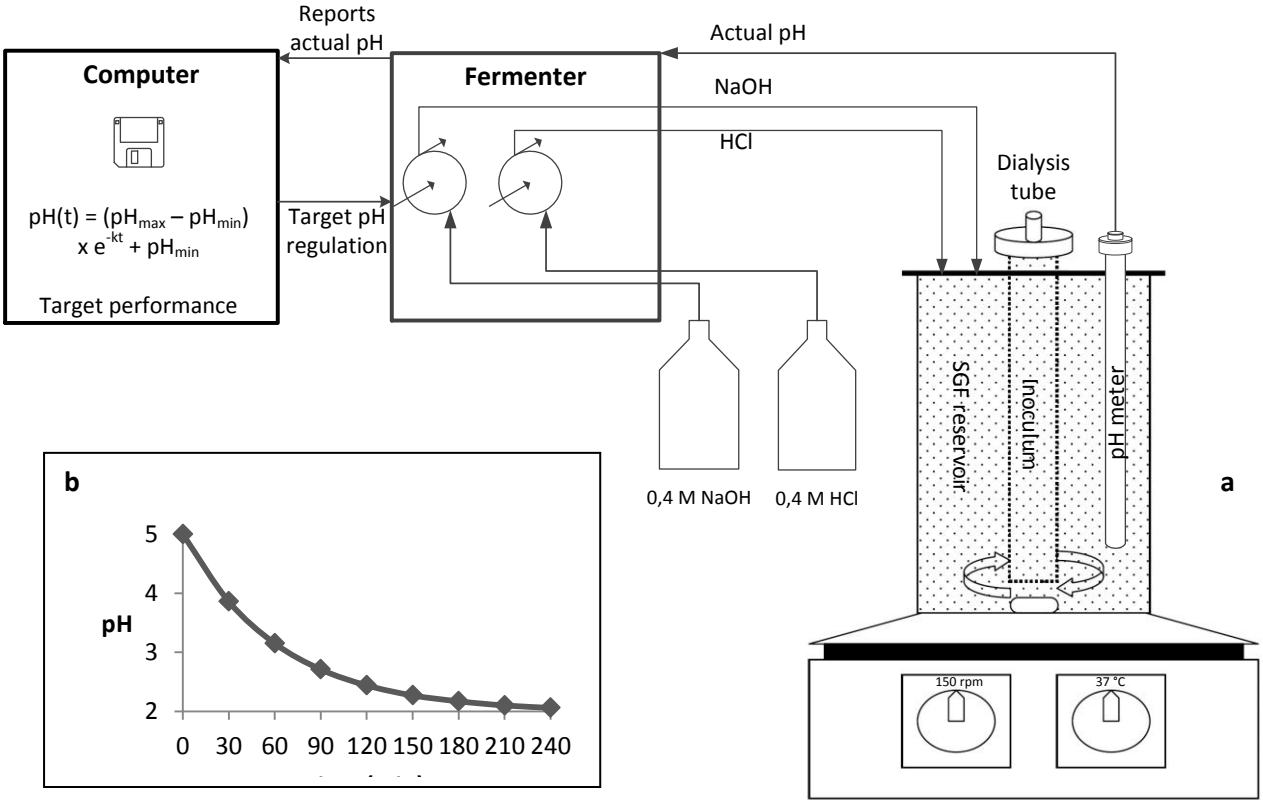


Figure 1. Schematic diagram of the simulated dynamic gastric acid system used in this study (a). pH-profile employed by the fermentor according to (1) (b).

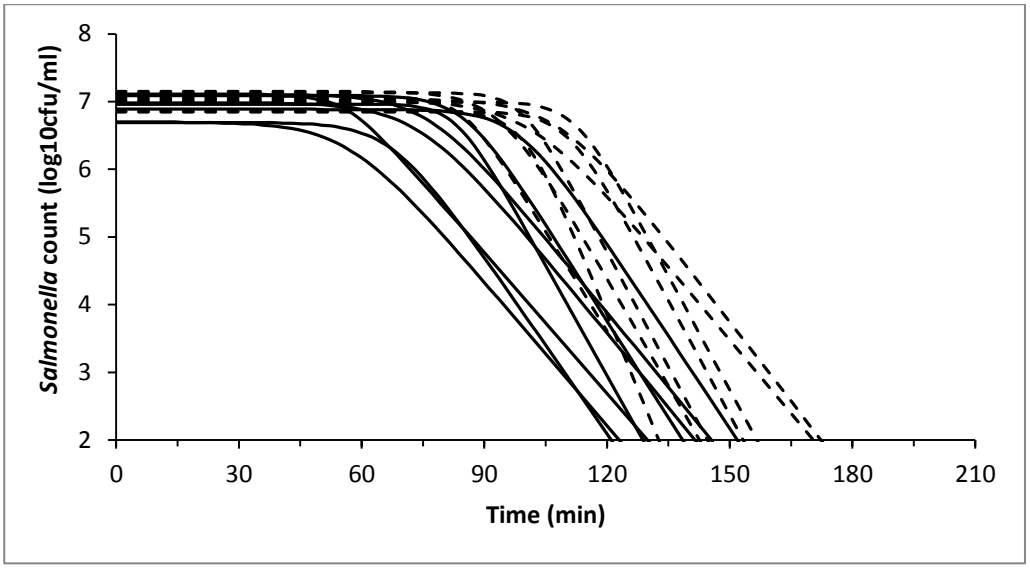


Figure 2. Display of inter-trial variance. Each fitted curve represents an independent trial. The solid lines are *Salmonella* in BHI while the dotted are in saline.

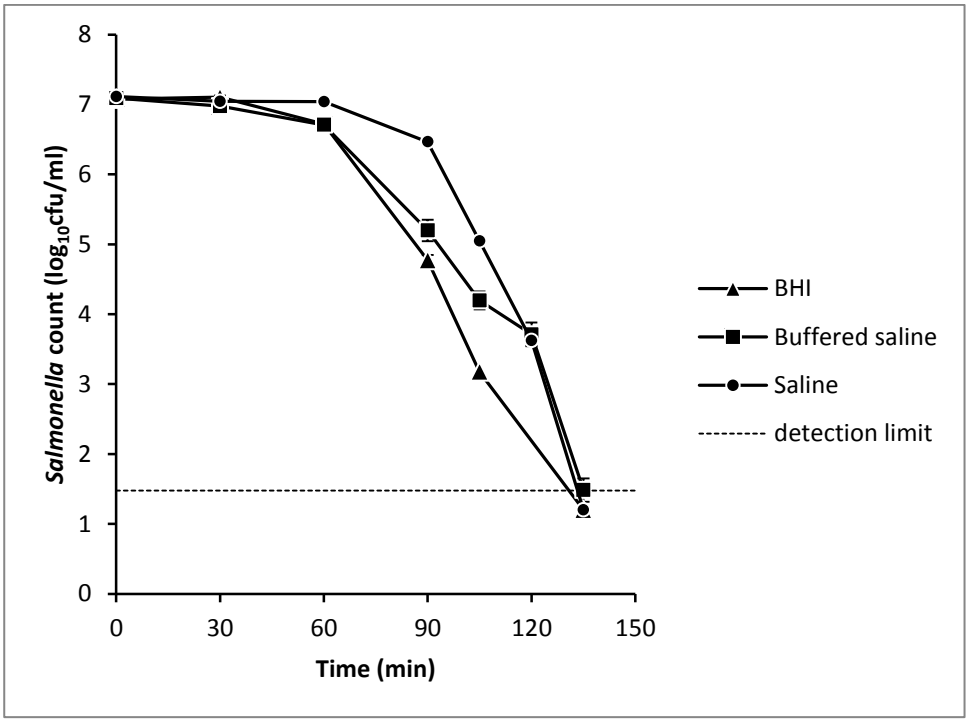
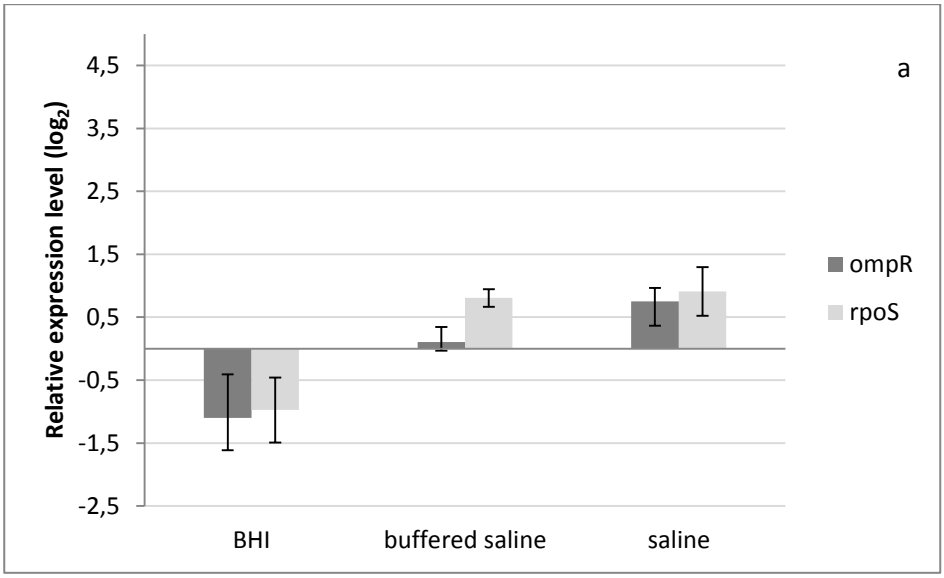
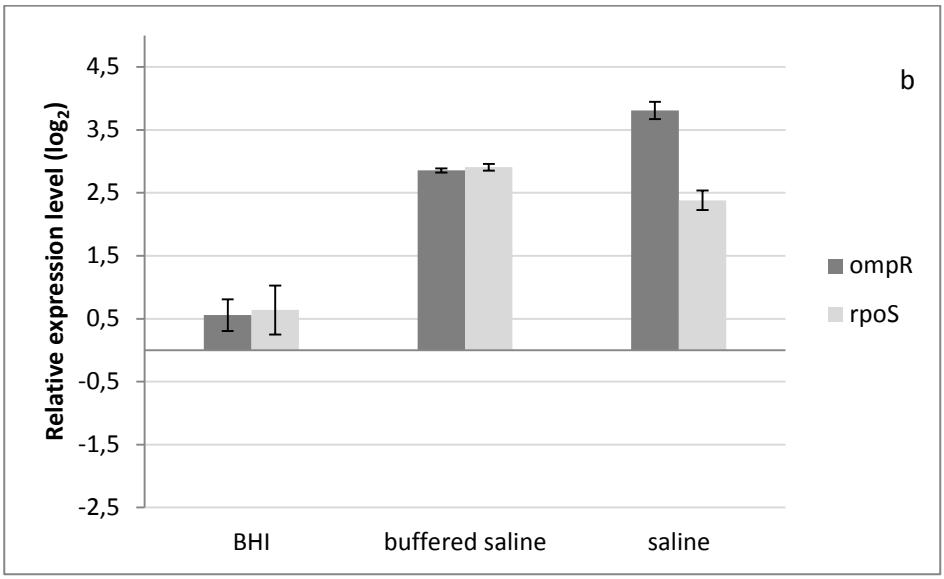


Figure 3. Inactivation of *S*. Typhimurium C5 in BHI, buffered saline and saline in a pH-controlled fermentor modelling gastric acid passage. Experiments were conducted in triplicate and data presented are means of results ± SEM (n=3).


a
Relative expression level (log2)
4,5
3,5
2,5
1,5
0,5
-0,5
-1,5
-2,5
BHI
buffered saline
saline
ompR
rpoS


b
Relative expression level (log2)
4,5
3,5
2,5
1,5
0,5
-0,5
-1,5
-2,5
BHI
buffered saline
saline
ompR
rpoS

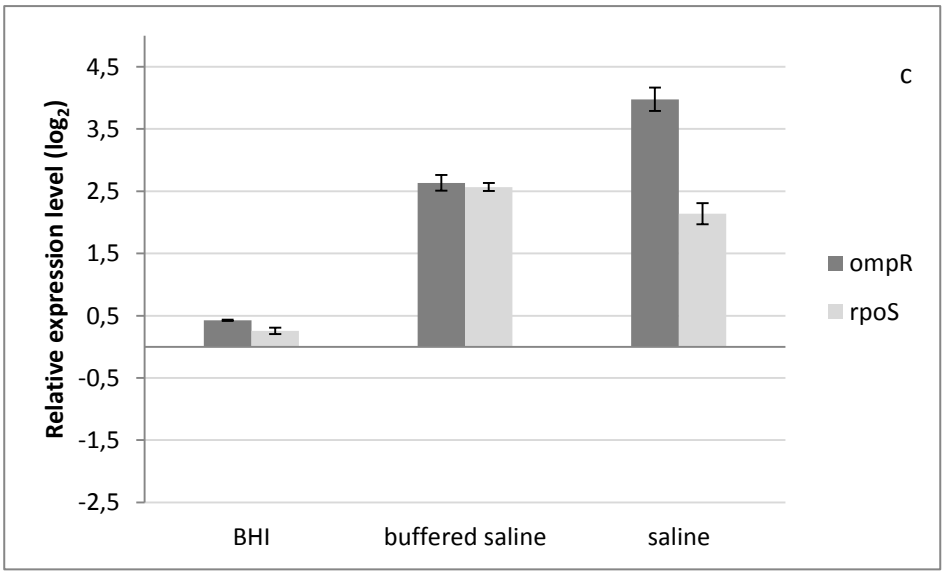

c
Relative expression level (log2)
4,5
3,5
2,5
1,5
0,5
-0,5
-1,5
-2,5
BHI
buffered saline
saline
ompR
rpoS

Figure 4. Relative expression (log_2) of *ompR* (dark grey) and *rpoS* (light grey) during gastric acidification with sampling at 0 min (a), 15 min (b) and 30 min (c) versus untreated samples and normalized to reference gene *rpoD*. The error bars show variation between three biological replicates, each measured in technical triplicates. At all time points expression in BHI is significantly different from either saline or buffered saline ($P < 0.001$).

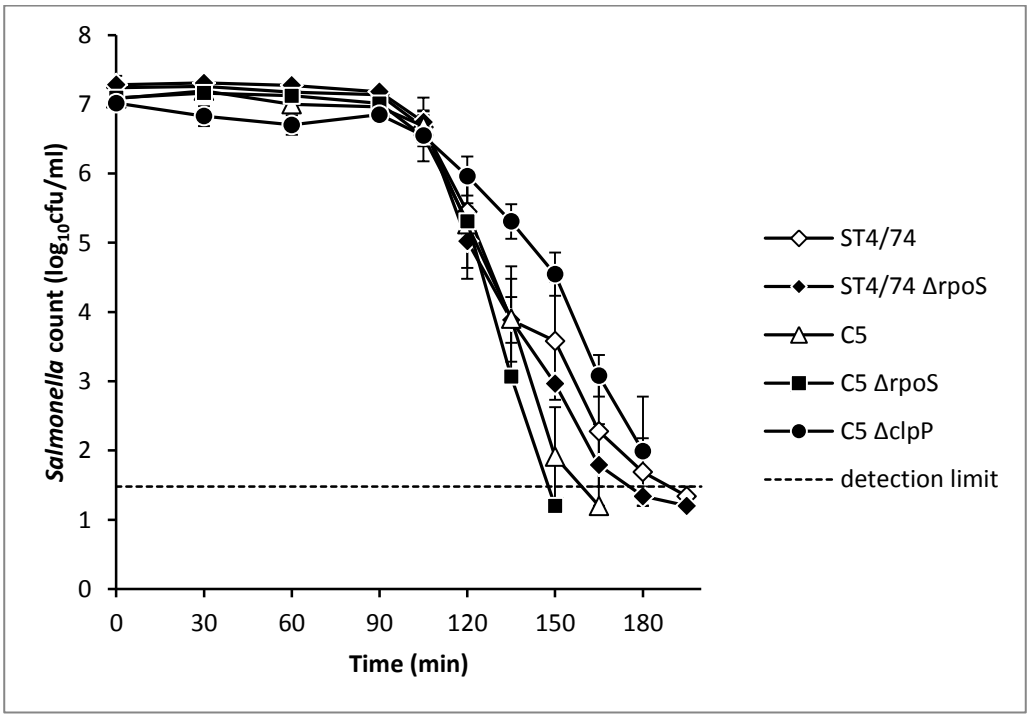


Figure 5. Inactivation during simulated gastric acid passage of *S*. Typhimurium strains C5 and 4/74 wild types and *rpoS* deletion mutants, plus an isogenic C5 *clpP* mutant. Results presented are means from duplicate trials ± SEM (n=2).

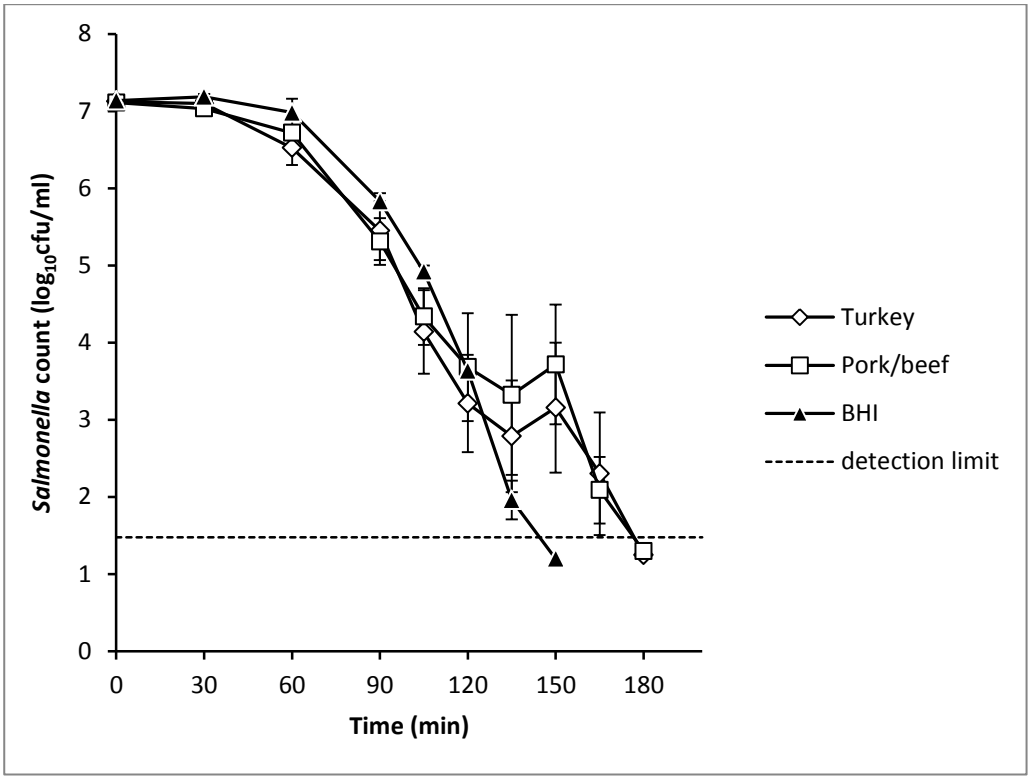


Figure 6. Influence of meat on the inactivation of *S.* Typhimurium during simulated gastric acid passage. Comparison of survival of *S.* Typhimurium challenged in BHI, turkey and pork/beef meat-suspensions, respectively. The data presented are means from two individual trials, each consisting of biological triplicates ± SEM (n=6).

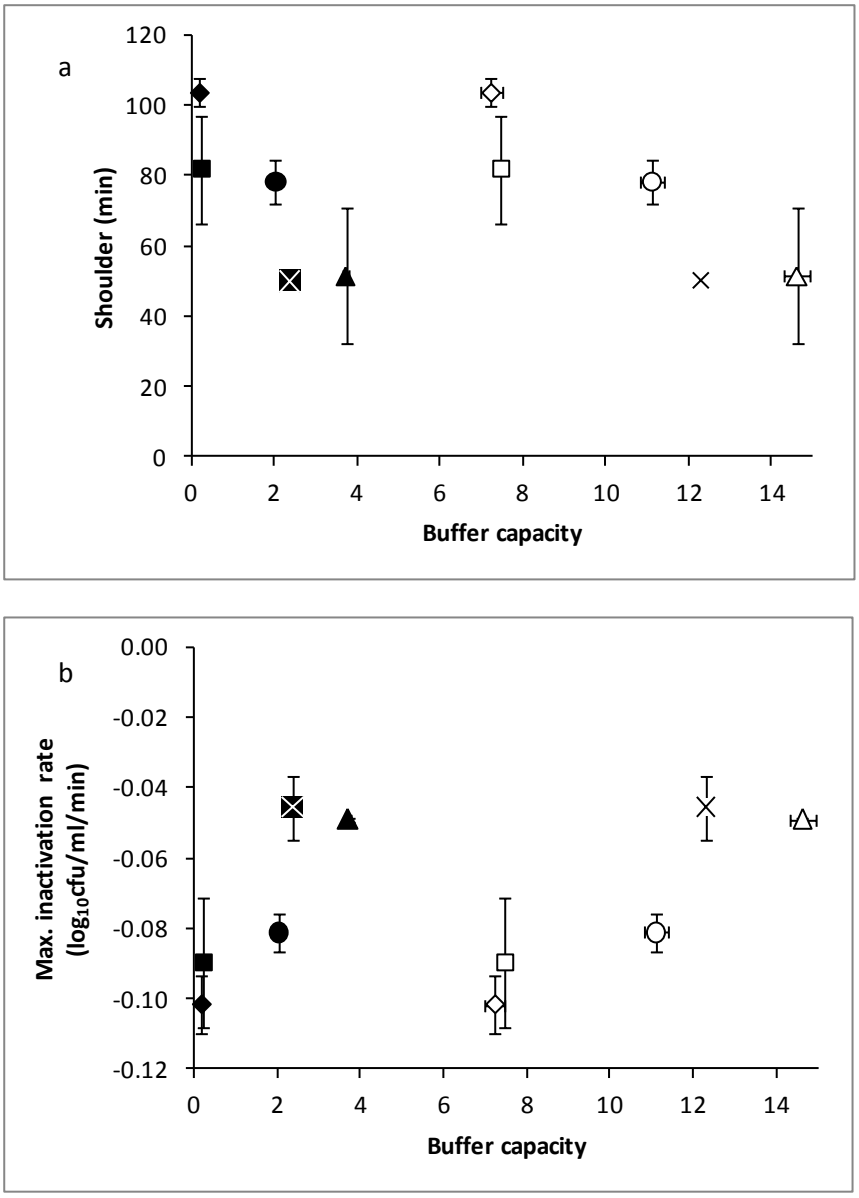


Figure 7. Effect of buffer capacity (closed symbols: ΔpH (5-4); open symbols: ΔpH (< 3)) on the inactivation kinetics ± SEM (n =2-9) (a: shoulder; b: max. inactivation rate) of *Salmonella* during dynamic acidification when inactivated in BHI (○), saline (◇), buffered saline (□), turkey meat suspension (△) or pork/beef meat suspension (×).

Chapter 9

Manuscript III

Inhibition of *Salmonella* Virulence gene expression by factors produced by Starter Cultures for fermentation of sausages

Inhibition of *Salmonella* Virulence gene expression by factors produced by Starter Cultures for fermentation of sausages

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Abstract

Lactic acid bacteria employed in sausage production serve different purposes; enhancement of food safety by antagonising pathogens, improvement of stability and extension of shelf-life by inhibition of spoilage, and changing of the sensory quality of the raw material (Lücke 2000b). Recent research imply that certain probiotic lactic acid bacteria strains can down-regulate virulence gene expression in specific pathogens (Ding *et al.*, 2005; Medellin-Peña *et al.*, 2007; Bayoumi & Griffiths, 2009; Tellez *et al*., 2011; Bayoumi & Griffiths, 2012), and we investigated if commercial starter cultures used for fermentation of sausages share this property. By use of promoter-reporter expression systems, we showed that the key regulator of *Salmonella* invasion, *hilA,* and of intracellular survival, *ssrB*, were down-regulated by ~ 99% and 86%, respectively when exposed to cell-free supernatants of strains belonging to species of *Lactobacillus* and *Pediococcus* grown in MRS broth. When investigating for *in vitro* effect of this down-regulation on *Salmonella* invasion in HT-29 tissue culture assays, both *Lactobacillus plantarum* and a commercial starter culture containing *Lactobaccillus curvatus* were able to attenuate invasion of *Salmonella*. The results obtained indicate that mixed and mono cultures of lactic acid bacteria used as starter cultures for production of fermented sausages secrete bioactive factors that reduce virulence of *Salmonella* through down-regulation of expression of virulence-associated genes. Methods to down-regulate infectivity of pathogens may be a novel hurdle in food where a complete elimination cannot be guaranteed.

Introduction

There is an increasing consumer demand for safe, minimally-processed and -preserved food (Gálvez *et al.*, 2007). While a health and nutritional benefit will derive from lowering e.g. the salt and fat content, minimal preservation may allow survival and growth of pathogens and thus give rise to products with intrinsic food safety problems. Lightly preserved ready-to-eat (RTE) meat products, such as fermented sausages, are basically still raw. Heat treatment is not commonly used as it would destroy the culinary quality of the products. Even under optimal hygiene conditions, fresh meat will be contaminated with fecal matter entailing the potential presence of food borne pathogens (Nauta *et al*., 2013). Thus, the safety and shelf-life of minimally-processed meat products has to rely on a high microbial quality of the raw material and on the inhibitory factors (hurdles) employed in product processing, controlling growth and survival of the given pathogens and spoilage bacteria. The main hurdles used in RTE meat products are: high salt, low water activity, decrease of redox potential and pH, and growth of competitive microflora (Holck *et al.*, 2011). Traditionally, fermented sausages are considered safe due to the combined effect of hurdles which, in theory, should efficiently control pathogens (Barbuti & Parolari, 2002). Despite this, food borne outbreaks caused by fermented sausages contaminated with *Salmonella* and verocytotoxin producing *Escherichia coli* have been reported regularly, recently by: Gossner *et al.*, (2012); Kuhn *et al.*, (2011); Bone *et al.*, (2010); Ethelberg *et al.*, (2009); Schimmer *et al.*, (2008) and Sartz *et al.*, (2008).

Lowering of pH is essential for reduction of pathogens during sausage fermentation (Lücke, 2000), and the specific pH lowering profile relies on the intrinsic characteristics of the starter culture. Consequently, choosing the right starter cultures is important for obtaining the appropriate level of pathogen reduction (Gunvig *et al.*, submitted), and notable differences amongst them have been demonstrated (Lahti *et al.*, 2001). Additionally, lactic acid starter cultures are known to serve preservative functions beyond their acidifying effects. Some produce antagonistic substances, i.e. bacteriocins, which exhibit antimicrobial effects particularly towards Gram-positive bacteria, such as *Listeria monocytogenes* (reviewed by Servin, 2004). Recent research indicates that certain lactic acid bacteria (LAB) affect expression of virulence-associated genes in specific Gram-negative pathogens as well (Ding *et al.*, 2005; Medellin-Peña *et al.*, 2007; Bayoumi & Griffiths, 2009). Moreover, probiotic LABs have been shown to affect attachment and invasion of *Salmonella in vitro* and *in vivo* through inhibition of the virulence genes *hilA and ssrB* (Tellez *et al.*, 2011;

Bayoumi & Griffiths, 2012) belonging to type III secretion systems (T3SS) being important virulence determinants (Ibarra *et al.*, 2010).

The T3SS' are instrumental for *Salmonella* pathogenicity and modulate the translocation of effector proteins from the bacterial to the eukaryotic cytoplasm, which in turn alter the eukaryotic host cell physiology (Santos *et al.*, 2009). Two major T3SS', located on *Salmonella* Pathogenicity Island 1 (SPI-1) and 2 (SPI-2), control invasion of epithelial cells and proinflammatory responses (Mills *et al.* 1995; Galán & Curtiss 1989; Galán 1996; Galán & Collmer 1999) plus intracellular survival within macrophages and systemic spread (Shea *et al.* 1996; Hensel *et al.* 1998), respectively. HilA is the key regulator of SPI-1 T3SS (Bajaj *et al.* 1995), and Ellermeier *et al.* (2005) have shown that a *hilA* deletion alone is phenotypically equivalent to a deletion of the entire SPI-1 locus in BALB/c mouse competition assays. The only cognate regulator encoded on SPI-2 is *ssrAB* (Ochman *et al.* 1996; Shea *et al.* 1996). Targeting key regulatory virulence-associated genes might be most efficient for virulence inhibition as inhibition of other genes in the regulon may only provide a partial attenuation as shown for e.g. *invH* (Aabo *et al.* 2000). Thus, an ability to inhibit *hilA* and *ssrB* in *Salmonella* could be an alternative means to control virulence in this clinically relevant foodborne pathogen.

The objective of this study was to investigate whether factors produced by lactic acid bacteria in starter cultures used for fermentation of sausages can inhibit the virulence regulatory genes *hilA* and *ssrB* in *Salmonella* as previously reported for probiotic lactic acid bacteria. If such factors are produced during fermentation or alternatively isolated and used as food additives, the perspective is a potential contribution to prevention of *Salmonella* infections from eating raw ready-to-eat products. We investigated cell-free supernatants from four different commercial starter cultures and from four LAB isolates for ability to inhibit virulence gene expression. This was done by analyzing luminescence activity of plasmid borne promoter-reporter fusions *hilA::luxCDABE* and *ssrB::luxCDABE* in *S. enterica* serovar Typhimurium. Further, to verify if virulence gene inhibition of *hilA* in *S.* Typhimurium also led to reduced invasion *in vitro,* testing in the human intestinal cell line, HT-29, was performed.

Methods

Bacterial strains and media

The bacterial strains used are listed in Table 1.

Lyophilized starter cultures and LAB were cultivated under anaerobic conditions at 37 °C in modified DeMan, Rogosa and Sharpe medium (mMRS; 10 g tryptone from casein, 8 g beef extract, 4 g yeast extract, 5 g sucrose, 1 ml tween 80, 2 g dipotassium hydrogen phosphate, 0.5 g L-cysteine HCL, 2 g diammonium hydrogen citrate, 5 g sodium acetate, 0.2 g magnesium sulphate, 0.04 g manganese sulphate in 1 liter distilled water) (Medellin-Peña *et al.*, 2007).

Salmonella Typhimurium LT2, stored at - 80 °C, was sub cultured aerobically in Luria–Bertani (LB) broth overnight at 37 °C with shaking. *S*. Typhimurium LT2 promoter-reporter *hilA::luxCDABE* and *ssrB::luxCDABE* constructs, a kind gift from Mohamed A. Bayoumi (Food Science Department, University of Guelph), were grown overnight at 37°C in LB broth supplemented with 50 microgram ml^{-1} of ampicillin (Amp).

Preparation of neutralized cell-free spent medium

Neutralized cell-free spent medium (nCFSM) of *Lactobacillus sakei*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, commercial starter cultures; BITEC LKB-5, BITEC LS-25, BITEC Advance LD-20, BITEC Advance RD-1 and *Lactobacillus acidophilus* La-5 was prepared as described by Medellin-Peña (2007) with minor modifications. Briefly, the LAB and starter cultures were grown anaerobically in mMRS medium for 16 h at 37°C and subsequently diluted 1:50 in fresh medium and re-incubated until an optical density of 2 (OD_{600}). After centrifugation at 6000 x *g* for 10 min at 4 °C, the supernatants, with a pH range of 4.82-5. 01, were harvested and adjusted to pH 7.2 (± 0.2) with 8 N NaOH in order to avoid effects caused merely by acidification. Sterilization of the supernatant was performed by filtering through a 0.2 µm-filter (Millipore). For 10 fold concentration, the nCFSM was freeze-dried (Unitop 600 SL, VirTis Co., Inc. Gardiner, NY, USA), stored at−20 °C, and reconstituted with sterile H_2O to 10 % of the original volume prior to use. As a control, unspent neutralized mMRS and LB were subjected to the same conditions as described above.

Screening of nCFSM for inhibition of growth and gene expression of *hilA* and *ssrB* in *S.* Typhimurium LT2

S. Typhimurium LT2 *hilA::luxCDABE* and *ssrB::lux-CDABE* constructs were grown at 37 °C for 19 hours in LB broth supplemented with Amp to an approximate cell density of 1 x 10^9 cells ml^{-1}. The culture was thereafter diluted 1:100 in fresh LB medium supplemented with 10 % of the 10 x nCFSM concentrate or neutralized mMRS or LB as controls. Two hundred microliters of each sample was distributed into triplicate wells of a sterile opaque Corning® 96-well plate (Sigma-Aldrich) and incubated at 37°C. Growth and luminescence were measured hourly for 24 h using a Victor multilabel counter (Wallac, PerkinElmer Life Sciences Canada, Woodbridge, ON, Canada). Growth was expressed by cell density (OD_{490}) and *hilA* and *ssrB* gene expression measured as luminescence was expressed as relative light units (RLU) - defined as luminescence counts min^{-1} and adjusted to OD_{490} (RLU/ OD_{490}).

Assessment of selected nCFSM effect on *in vitro* invasion of *S*. Typhimurium in cell culture

The human intestinal cell line HT-29 was obtained from the Canadian Research Institute for Food Safety (CRIFS) Culture Collection (Food Science, University of Guelph, ON, Canada). Cells were maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Invitrogen) and kept at 37°C in a CO_2 incubator (5 % CO_2). Confluent monolayers were prepared by seeding 5 x 10^5 cells in a 24-well tissue culture plate (Fisher Scientific) for 24 h. *S.* Typhimurium LT2 was statically grown at room temperature either with or without the addition of 10 % nCFSM for 16 h. Prior to infection, the bacterial cultures were adjusted to an OD_{600} corresponding to 1 x 10^7 cells ml^{-1} by adding fresh tissue culture medium. The adjusted bacterial cultures were added to each well at a multiplicity of infection of 10. The bacteria were allowed to invade for 1 h at 37°C in the presence of 5 % CO_2. The monolayers were washed twice with PBS after which they were incubated with 100 microgram gentamycin ml^{-1} in culture medium for 1 h to kill extracellular bacteria. After two subsequent washes with PBS the monolayers were lysed with 0.1% Triton X-100, and proper dilutions were plated on LB plates to enumerate the invading pathogens. Invasion was measured as the degree of recovery of intracellular *S*. Typhimurium treated for 16 hours with two selected nCFSMs (*Lactobacillus plantarum* and RD-1) in HT-29 cells compared to the control. Recovery was calculated using the following equation:

$$\% \text{ of Recovery} = \frac{(\text{Treatment group CFU ml}^{-1} \times 100)}{\text{Control group CFU ml}^{-1}}$$

Toxicity of the applied nCFSM on the HT-29 cell line was assessed by using a Scepter™ automated cell counter (MERCK MILLIPORE).

Statistics

All results presented are means derived from biological triplicates. Single factor ANOVA (Microsoft Office, Excel 2007) was used to assess the statistical significance of the differences between control and test groups. *P*-values <0.05 were being considered statistically significant.

Results

Effect of nCFSM on *hilA* and *ssrB* expression in *S.* Typhimurium LT2

Cell-free supernatants were screened for their ability to repress expression of *hilA* and *ssrB* in *Salmonella* Typhimurium LT2. The screening was performed by analysing luminescence activity of plasmid borne promoter-reporter fusions *hilA::luxCDABE* and *ssrB::luxCDABE* in *S*. Typhimurium LT2 (Bayoumi & Griffiths 2010). The nCFSMs from four starter cultures; BITEC LKB-5, BITEC LS-25, BITEC Advance LD-20, BITEC Advance RD-1 and four LAB isolates; *Lactobacillus sakei*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus* and the one probiotic strain; *Lactobacillus acidophilus* La-5 were tested for inhibition of virulence gene inhibition in both *S*. Typhimurium reporter strains. All but the probiotic *Lactobacillus acidophilus* (*P*=0.25) were shown to provide a significant down-regulation of the expression of *hilA* (*P*<0.05) (Fig. 1). This effect was evident after 7 h and was sustained during the 24 h course of the study (Fig. 2). The most pronounced effect was observed for nCFSMs from *Lactobacillus plantarum* and a mixed starter culture, RD-1, containing *Lactobacillus curvatus*. The latter exerted a significant ~ 99% inhibition of *hilA* expression (from over 80.000 RLU in the control medium compared to 600 in the sample containing 10% neutralized cell-free spent medium of RD-1) (*P*< 0.0002). MRS in itself has been reported to mask luminescence detection (Medellin-Peña *et al.*, 2007), but the modified MRS did

not interfere with light emission compared to LB (*P*=0.59) when comparing LB and mMRS groups depicted in Fig. 1), nor did modified MRS effect growth of the construct strains (data not shown). The significant reduction of *hilA* expression using the reporter construct was supported by qRT-PCR of mRNA in preliminary studies in which a 20-fold down-regulation was observed (data not shown).

The light emission from the *ssrB* construct was lower than for the *hilA* construct. Down-regulation was also observed for *ssrB*, again with the nCFSM from RD-1 showing the largest inhibition (from over 2.000 RLU in the control medium compared to 285 in the sample containing 10% neutralized cell-free spent medium of RD-1, *P*=0.00006) (Fig. 3).

Effects of nCFSM on growth rate and lag phase of *S.* Typhimurium LT2

No effects were observed in lag phase and growth rate of *S*. Typhimurium LT2 during 12 h incubation at 37°C in presence of 10 % neutralized CFSM from any of the starter cultures or LABs. An example of growth curves for *S*. Typhimurium LT2 with and without addition of *L. plantarum* and RD-1 can be seen in Figure 4. Similar results were obtained for the *hilA* and *ssrB* constructs (data not shown).

Influence of nCFSM on the invasion of *Salmonella* Typhimurium LT2 in cell culture

The ability of the nCFSMs to suppress intestinal epithelial invasion by *Salmonella* was assessed by a gentamicin protection cell culture assay using the colonic epithelial cell line, HT-29. A significant approx. 80% reduction in invasion (*P*<0.05) (Table 2) was observed when adding 10 % of nCFSMs from *Lactobacillus plantarum* and the starter culture RD-1 to the growth medium of *S.* Typhimurium LT2 16 h before infection. In contrast, when the nCFSMs were added just prior to infection, no significant effect on invasion was observed (data not shown).

No toxicity of the applied nCFSM on the HT-29 cell line could be shown, leaving out any bias from this on the invasion data.

Discussion

Inactivation of pathogens such as *E. coli* and *Salmonella* in fermented products can differ significantly while often reported being in the range of 0 - 3 $\log_{10}$ (Hwang *et al.*, 2009; Nightingale *et al.*, 2006; Holck *et al.*, 2011). The decline in pH during fermentation is essential for reducing pathogens (Lücke, 2000), and the choice of starter cultures, as well as recipe and process conditions, impacts the level of reduction significantly (Gunvig *et al.*, submitted; Lahti *et al.*, 2001; Heir *et al.*, 2010). In theory, each hurdle applied in the production of fermented sausages is non-lethal, albeit collectively they exert the inhibitory power to prevent microbial growth. However, often hurdle technology does not lead to an inactivation of the microorganisms (Leistner, 2000). Inadvertently, sub-lethal stress can lead to synthesis of protective stress response proteins and induction of higher stress tolerance or even higher virulence. As an example, acid-induced pH tolerance responses in *Salmonella* have been shown to lower the infectious dose from around 10^5 to 10 - 100 cells (Mashall, 2003). A mere bacteriostatic effect without inactivation also poses problems in cases where the infectious dose is naturally low, as shown for verocytotoxic *E. coli* (Kothary & Babu, 2001). Furthermore, adaptation to one type of stress can result in an acquired cross-tolerance to other forms of stress of the surviving organisms (e.g. Leyer & Johnson, 2003; Greenacre & Brocklehurst, 2006). The incomplete inactivation of pathogens and potential lowering of infectious doses underline the need to improve the control of pathogens, which are able to survive in the product.

It has been widely accepted that lactic acid starter cultures serve preservative functions beyond their pH-lowering effects of which production of bacteriocins has attracted most attention as possible hurdles and food preservatives (Gálvez *et al.*, 2007). Other LAB metabolites with inhibitory effect against Gram-negative bacteria *in vitro* have limited usage in food preservation due to constraints. Reuterin e.g. is produced in insufficient amounts to be active, substances as diacetyl and hydrogen peroxide adversely affect the sensory quality of the products, and benzoic acid raises regulatory concern (Lücke, 2000).

There is a need for development of new hurdle principles, and as a novel approach down-regulation of infectivity may be another possible means to control foodborne pathogens. Probiotic LABs have been shown to affect attachment and invasion of *Salmonella in vitro* and *in vivo* through inhibition of the virulence genes *hilA* and *ssrB* (Tellez *et al.*, 2011; Bayoumi & Griffiths, 2012). In

this study we investigated whether isolates of lactic acid bacteria and commercial starter cultures used for sausage production shared this property of being able to inhibit central virulence factors of *Salmonella*. The results obtained indicate that bioactive factors in the growth medium of both monocultures of LAB and mixed starter cultures noticeably down-regulate the expression of the virulence-associated genes *hilA* and *ssrB*. In *Salmonella* these two genes are essential for invasion of intestinal epithelial cells and for survival/multiplication inside macrophages and causing systemic infection, respectively. We chose to study *hilA* and *ssrB* encoding key regulators of T3SSs on SPI-1 and SPI-2, respectively. We thus expected suppression of *hilA* and *ssrB* to provide a stronger attenuation of *Salmonella* compared to selecting genes in the regulon being e.g. responsible for assembly or components of the T3SSs as *invH* (Aabo *et al*., 2000). We also expect the virulence inhibition to cover all *Salmonella* as these genes belong to the core genome (Wisner et al. 2012).

By use of promoter-reporter expression systems, we observed a > 99% inhibition of the expression of *hilA* and an 86% inhibition of *ssrB* expression in the presence of 10% of a neutralized cell-free supernatant of the commercial starter culture RD-1. However, all LAB isolates and starter cultures tested affected the virulence gene expression in *Salmonella*, although to a varying degree. Lactic acid bacteria are used in food processing because of their characteristic flavor formation but mainly because of their ability to lower the pH and to produce antimicrobial agents (Verluyten *et al.*, 2003). In this study we avoided the antagonistic effect of acidification by neutralizing the cell-free supernatants, in accordance with Bayoumi & Griffiths (2010). Thus the observed pattern in down-regulating expression of virulence genes is not associated to the capacity of LAB and starter cultures to lower pH.

Since invasion of the intestinal epithelium is believed to be mediated by SPI-1 and hence dependent on the transcription of *hilA*, it was tested whether the observed down-regulation of the gene expression would also prompt an attenuated invasion of *S*. Typhimurium in cell culture. The cell culture study showed that the down-regulation of virulence genes concomitantly leads to a reduction in *Salmonella* invasion, and that the 100 fold reduction in expression observed for *hilA* only converted into 5 fold reduction in invasion in cell culture. It is so far unknown if concentration of supernatants would provide a more efficient inhibition of invasion but may be expected as other studies have shown that the effects of bioactive substances can correlate to concentration (Bayoumi & Griffiths, 2010; Tellez *et al.*, 2011).

The results of this study are consistent with other reports showing that probiotic lactic acid bacteria exhibit an antagonistic effect on virulence determinants in enteropathogens. Thus, cell-free extracts from milk fermented by probiotic strains of *Lactobacillus* and *Bifidobacterium* have been shown to inhibit the expression of the virulence determinant *flaA* in *Campylobacter jejuni* (Ding *et al.*, 2005). Likewise, Medellin-Peña *et al.* (2007) found that cell-free supernatants of *Lactobacillus acidophilus* inhibited transcription of genes involved in quorum sensing and colonization in *E. coli* O157:H7 (EHEC). This work was extended by an *in vitro* cell culture assays and *in vivo* by a mouse model where attachment and colonization of *E. coli* O157:H7 were impeded by the *L. acidophilus* La-5 strain (Medellin-Peña & Griffiths, 2009). With respect to *Salmonella*, Bayoumi & Griffiths (2010) found that molecules secreted by a strain of *Bifidobacterium bifidum* down-regulated expression of the central virulence regulators *hilA* and *ssrB* in *Salmonella* Typhimurium, like in the present study, in a pH-independent manner. Further work demonstrated that chromatographically separated fractions from *B. bifidum* supernatants were able to inhibit expression of central virulence genes in *S*. Typhimurium and EHEC. The fractions were shown to reduce colonization and impede the ability of *S*. Typhimurium to survive and multiply in macrophages (Bayoumi & Griffiths, 2012). Recently, Tellez *et al.* (2011) observed a down-regulation of *ssrB* in *S*. Typhimurium by an extracted and purified a peptidic fraction from milk fermented with *Lactobacillus helveticus* (Tellez *et al*., 2010). In accordance with observations in the present study and by Bayoumi & Griffiths (2010) the neutralized fraction had no impact on *S*. Typhimurium growth. Furthermore, this peptidic fraction - either directly through its effect on virulence gene expression or indirectly through immunomodulation – was able to protect mice from infection when challenged with *Salmonella* (Tellez *et al.*, 2011). It is generally recognized that probiotic lactic acid bacteria can inhibit growth of enteropathogens, and this antimicrobial activity is mainly attributed to their lactic (and to a lesser extent acetic) acid production (reviewed by Servin, 2004). Likewise, it has been shown that probiotics can inhibit e.g. *Salmonella* associated diarrhea (Casey *et al.*, 2007). While the basis of their multifactorial mode of action remains largely un-clarified, the aforementioned studies indicate that modulation of virulence is probably an important feature in their protection against enteric infections.

In conclusion, this study clearly confirms that several monocultures of LAB as well as mixed consortia of bacteria in commercial starter cultures commonly used by the industry can secrete metabolites that affect the virulence of *Salmonella* without affecting its growth. Although the

nature and mode of action of the bioactive substances need to be elucidated, they have the potential of being a novel hurdle principle used in relevant food productions as a preventive measure against enteric infection. To substantiate the relevance of the findings, further studies will be needed in order to show to which degree the virulence gene inhibition is sustained when *Salmonella* leaves the environment of a fermented sausage and enters the gastrointestinal tract. Also it would be relevant to know if high concentrations of the inhibitory factors will exert any inhibition of virulence gene expression in the intestinal environment. The latter would open for use as a prophylactic, administered either as a pure substance or as a food additive.

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Table 1. *Bacterial strains, starter cultures and constructs used in this study.*

Strains or construct	Starter culture	Genotype/ phenotype	Source[a, b, c]
Salmonella enterica serovar Typhimurium LT2 SA 941256			CRIFS
S. Typhimurium *ssrB::lux*		P*ssrB*, *luxCDABE*, Amp^r	CRIFS[a]
S. Typhimurium *hilA::lux*		P*hilA*, *luxCDABE*, Amp^r	CRIFS[a]
Staphylococcus carnosus; *Kocuria varians*	BITEC LKB-5	Bacteriocin	[b]
Staphylococcus carnosus; *Lactobacillus sakei*	BITEC LS-25		[b]
Staphylococcus carnosus; *Lactobacillus sakei*	BITEC Advance LD-20		[b]
Staphylococcus carnosus; *Lactobacillus curvatus; Kocuria varians*	BITEC Advance RD-1		[b]
Lactobacillus sakei			[c]
Lactobacillus plantarum			[c]
Pediococcus acidilactici			[c]
Pediococcus pentosaceus			[c]
Lactobacillus acidophilus La-5		Probiotic LAB	CRIFS

CRIFS stock strains are deposited in the Canadian Research Institute for Food Safety (CRIFS) culture collection.

[a] Published by Bayoumi & Griffiths in 2009.

[b] Starter cultures were kindly donated by FRUTARUM (Germany)

[c] Lactic acid bacteria were kindly donated by Chr. Hansen (Denmark)

Table 2. *Invasion of* Salmonella *Typhimurium LT2 in HT-29 cell culture after exposure to two types of nCFSM for 16 hours prior to infection (% recovery).*

Strain & nCFSM	Intracellular *Salmonella* (CFU ml^{-1})	Invasion [a] (% recovery)
S. Typhimurium LT2 (control)	2.9×10^4	100[b]
S. Typhimurium LT2 + 10 % *L. plantarum* nCFSM	4.7×10^3	16[c]
S. Typhimurium LT2 + 10 % RD-1 nCFSM	5.8×10^3	20[c]

[a] The results are average values of three independent replicates

[b] Control CFU ml^{-1} was normalized to 100 % invasion ability

[c] The results are statistically significant ($P < 0.05$)

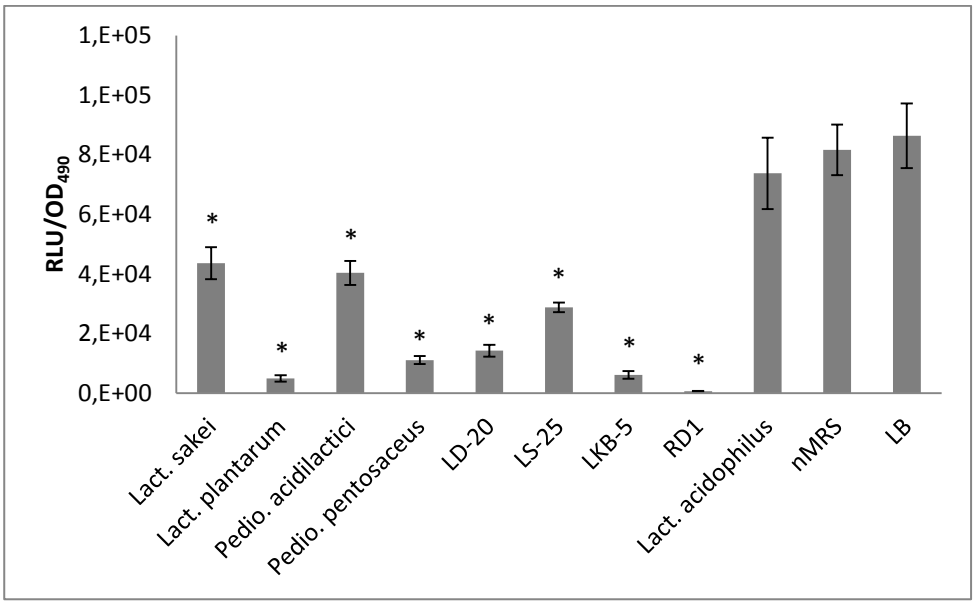

Figure 1. hilA *expression of* S*. Typhimurium LT2 grown for 12 h in LB with or without supplementation of 10 % nCFSM from different LABs and starters*. Data represents luminescence activity of *hilA::luxCDABE* construct and is shown by means ± standard deviations from three independent replicate trials and expressed as relative light units (RLU) - defined as luminescence counts min^{-1} and adjusted to OD_{490} (RLU/ OD_{490}). * Statistically significant difference $P < 0.05$.

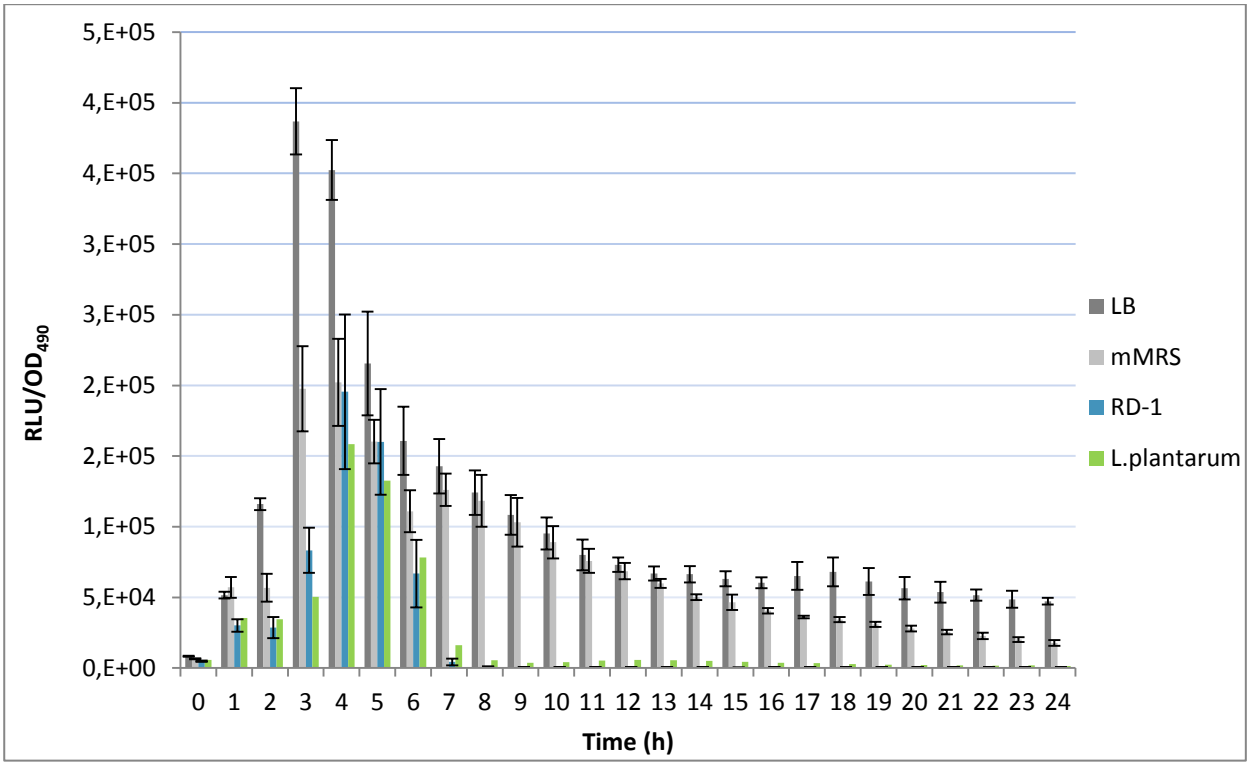

Figure 2. *Expression of* hilA *during 24-hour exposure to 10 % nCFSMs from RD-1 and Lactobacillus plantarum.* Expression was measured as luminescence activity of *hilA::luxCDABE* construct *in S.* Typhimurium LT2. Data are means ± standard deviations of results from three independent replicate trials and expressed as relative light units (RLU) - defined as luminescence counts min^{-1} and adjusted to OD_{490} (RLU/ OD_{490}).

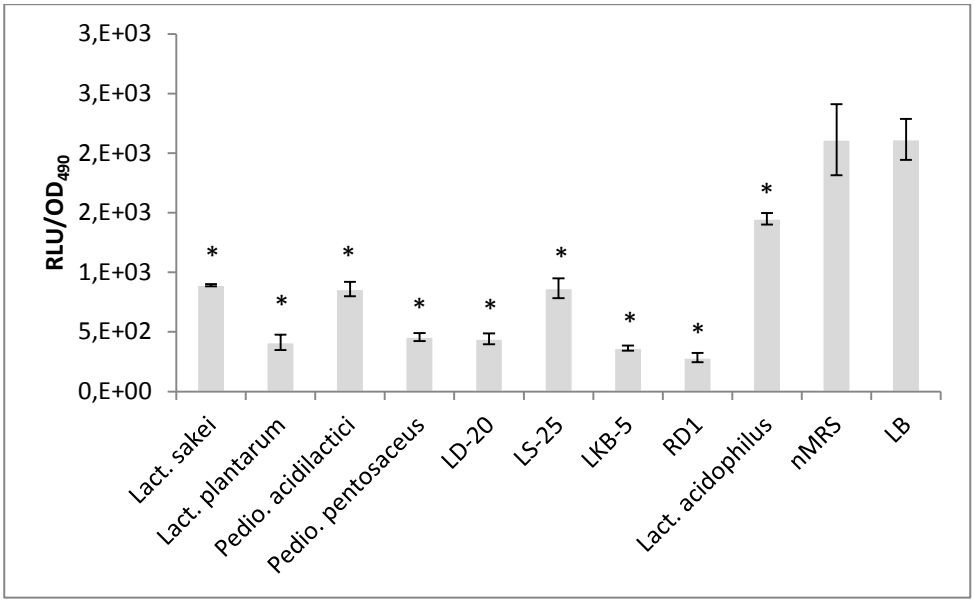

Figure 3. ssrB *expression in* S. *Typhimurium LT2 grown for 12 h in LB with or without supplementation of 10 % nCFSM from different LAB and starter cultures*. Expression is measured as luminescence activity of *ssrB::luxCDABE* construct with means ± standard deviations from three independent replicate trials and expressed as relative light units (RLU) - defined as luminescence counts min^{-1} and adjusted to OD_{490} (RLU/ OD_{490}).* Statistically significant difference $P < 0.05$.

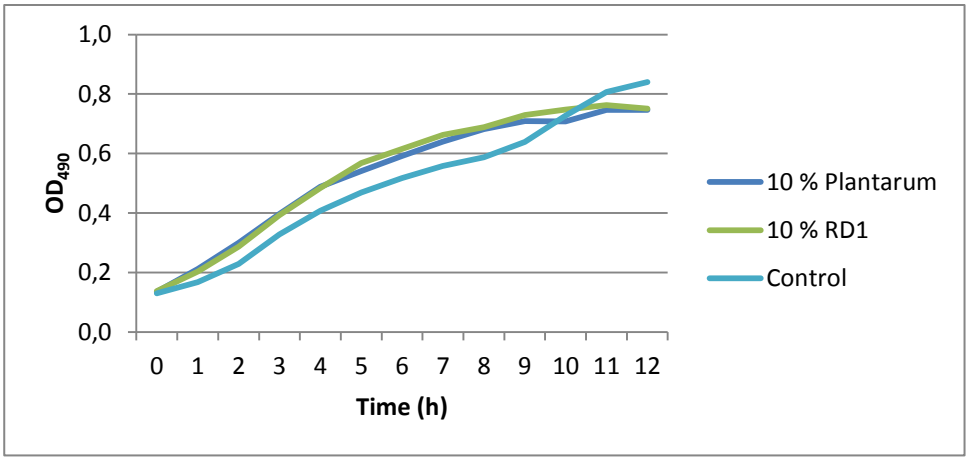

Figure 4. *Growth of* Salmonella *Typhimurium LT2 in presence of nCFSM from* L. plantarum *and RD-1 (expressed by* OD_{490}*)*.

Chapter 10
Discussion and future perspectives

The ambition starting this work was to be able to analyse the gene expression of *Salmonella* subjected to food-environmental stress related to fermented sausage processing. Results from such studies could have added to the knowledge on the molecular basis for bacterial survival, but more interestingly so, also of the infection potential of the stressed organisms - hence, refining the assessment of the molecular risk entailing the presence of *Salmonella* in fermented sausages. While molecular methods, such as quantitative real-time PCR (qPCR), are routinely applied for detection and quantification of microbial populations in food or during food processing, few studies analysing gene expression of bacteria in food by application of reverse-transcription qPCR (qRT-PCR) have been conducted (reviewed by Postollec et al. 2011). The extraction of RNA from complex or fatty food matrices remains a challenge, and we wanted establish a method enabling gene expression studies in a food matrix often associated with transmission of pathogenic bacteria. This is described in Paper I (Kjeldgaard et al. 2011). Biologically relevant transcriptomic studies are still impeded by the high concentration of RNA needed for reliable results. It has been shown that an inoculum level of around 8 log CFU/g *Salmonella* is ideal for optimal amount and purity of RNA for down-stream applications such as qRT-PCR (Sirsat et al. 2011; Kjeldgaard et al. 2011). Different sample preparation procedures have been reviewed (Brehm-Stecher et al. 2009), but their applicability would have to be tested for the specific food matrix. RNA is fragile and rapidly degraded, both unintendedly by ubiquitous RNases, but moreover the half-life of prokaryotic mRNA is only a few minutes (Levy 1975). In another complex food matrix, cheese, gene expression has been shown to vary with the applied RNA extraction method (Ulve et al. 2008; Monnet et al. 2008), underlining the importance of choosing an appropriate extraction protocol and stabilizing the RNA in the sample prior to extraction for reliable and reproducible results. Even so, when trying to apply the extraction method developed in the project (Paper I) - demonstrated to enable reproducible gene expression results from *Escherichia coli* and *Salmonella* spp. in minced meat - for RNA extractions from fermented sausage batter, the results were less reproducible. Different gene expression patterns of stress response genes were found depending on whether the bacteria were extracted from the food matrix by stomaching or hand massage

(data not shown). Furthermore, it became increasingly difficult to extract RNA of an appropriate concentration and purity during the course of fermentation, probably due to considerable changes in texture of the meat (data not shown). Compared to the raw material the complexity of a processed food matrix is increased, entailing also the presence of a higher amount of PCR-inhibiting substances, which could probably be released differently according to the extraction method employed. In the case of e.g. cheese or fermented sausages the high amounts of indigenous microflora will add further to the complexity and difficulty of analysing gene expression of pathogens in these matrices. Especially by diluting the abundance of target genes in the prokaryotic RNA extracted.

If key indicator genes were found the infectious potential of a pathogen exposed to process-related stress could be assessed in terms of the transcriptional expression pattern of this/these virulence markers. Applying qRT-PCR techniques would allow monitoring of gene expression at any time point during food processing and help isolating potential problematic steps, thereby ensuring microbiological safety of the products. Still, previously unknown genetic systems important for growth and survival of pathogens cannot be assessed by qRT-PCR as it is limited by only answering what you are asking for by the primers chosen. In which case microarray would be a better-suited alternative. Stress-response and virulence genes are expressed in response to environmental signals, enabling the pathogens to rapidly adapt to the environmental changes. The fast, and often so transient, changes in gene expression patterns underline the importance of determining the appropriate timeframe of sampling. Moreover, regulation will happen on different levels, not only by inducing or inhibiting transcription but also translation, or by altering stability of the gene product. In summary the aforementioned makes it challenging to relate gene expression to functionality and might perhaps impede the applicability of qRT-PCR in determining important processing steps or process variables in fermented sausage manufacturing, which inherently prompt a succession of different stresses.

Instead of trying to address the risk of a given product by looking at the genotypic traits of a potential contaminant expressed during food processing, a more direct way would be to examine it phenotypically by its survival capacity when exposed to relevant sausage processing stresses. In Manuscript I, we investigated the impact of growth phases on survival of two phage types of *Salmonella* during pre-processing freezing and subsequent fermentation with or without supplementation of $NaNO_2$ and starter culture, respectively. The growth phase of *Salmonella* in

contaminated meat is unknown, but suboptimal conditions during storage and/or distribution such as temperature abuse, can potentially allow the organisms to multiply. Whether growth of pathogens in the raw material of sausages would potentially compromise the microbial safety of the product has, to the best of our knowledge, not been addressed. Most inactivation and validation studies of pathogens during sausage production have been conducted with stationary phase cells, presumably to reflect the worst case scenario. It is generally accepted that rapidly dividing *Salmonella* in exponential growth phase is more sensitive to adverse conditions than their stationary phase counterparts, which have stopped dividing (Dodd et al. 2007). During sausage processing this could relate to the sub-lethal stress encountered by freezing or mild heating, osmotic and acid stress (Nyachuba & Donnelly 2005). In Manuscript I it was shown that *S.* Typhimurium in exponential growth phase was more sensitive to freezing in minced beef than stationary phase cells independent on phage types. Surprisingly, this higher sensitivity of *S.* Typhimurium in exponential phase was not reflected in the cellular response to the appertaining stresses encountered in the sausage environment during fermentation. We hypothesize that for the exponential phase *S.* Typhimurium an acid tolerance response was mounted at a higher pH and, hence, earlier in the fermentation process than for the stationary phase, masking the inherent differences in stress response and ability to survive of the two growth states. While no significant differences could be observed between exponential and stationary phase DT12, a different survival pattern was found for the outbreak-strain DTU292. For this phage type exponential phase cells survived to a higher extent than stationary phase cells in presence of both $NaNO_2$ and starter culture. It could be speculated that presence of $NaNO_2$ slightly delayed the pH-drop, providing an advantage for the presumably acid-adapted exponential phase DTU292. Also, Baik et al. (1996) found that exponential phase cells, in which acid tolerance had been induced, were better at tolerating weak acids than stationary phase cells. The reason for the difference observed between the two phage types remains to be elucidated and reminds us that even closely related organisms can differ in their physiology.

During spontaneous acidification by indigenous lactic acid bacteria, which mimicked erroneous fermentation with impeded drop in pH, growth of *S.* Typhimurium was observed independent on phage types and process variables, underlining the huge importance of proper fermentation for controlling growth and reducing the risk of contracting *Salmonella* infections from fermented sausages.

Recipe and process optimisation, along with choice of starter culture, has been shown to affect the level of pathogenic bacteria surviving the production processes of fermented sausages, thereby increasing the microbial safety of the products (Heir et al. 2010; Gunvig et al. submitted 2013). The consumer trend toward a demand for more "natural" products with low levels of salt and preservatives impede the utility of strengthening these classic, although efficient, hurdles in sausage production. While the combined effect of hurdles exerts the inhibitory power to prevent microbial growth, it seldom leads to inactivation of the microorganisms (Leistner 2000). Thus, food safety problems could be triggered in these products if the infectious dose of a potential pathogen is low, as recently demonstrated for i.a. *E. coli* and *Salmonella* by analysing outbreak data (Teunis et al. 2010). The simplest way of ensuring food safety of the products would be to apply a post-process heat treatment. For many types of sausages, though, thermal treatment will adversely change the sensory properties of the product. Methods to down-regulate infectivity of pathogens may be a novel hurdle in food where a complete elimination cannot be guaranteed. In Manuscript III we investigated the potential of lactic acid bacteria, employed for sausage fermentation, in attenuating virulence of *Salmonella*. We chose to study *hilA* and *ssrB* encoding key regulators of intestinal invasion and macrophage survival of *Salmonella* as we expected suppression of these genes to affect infection potential directly. The results obtained indicate that bioactive factors in the growth medium of both monocultures of lactic acid bacteria and mixed starter cultures noticeably down-regulate the expression of *hilA* and *ssrB*, but to a varying degree. Analysing gene expression by use of non-disruptive promoter-reporter expression systems enabled us to follow the expression over an extended time period, avoiding the sampling constraints of the more laborious qRT-PCR studies. An almost complete (>99 %) inhibition of *hilA* expression was exerted by a mixed starter culture, RD-1, containing *Lactobacillus curvatus*. It was further tested whether the observed down-regulation of the gene expression would also prompt an attenuated invasion of *S*. Typhimurium in cell culture. The cell culture study showed that the down-regulation of virulence genes concomitantly leads to a reduction in *Salmonella* invasion, and that the 100 fold reduction in expression observed for *hilA* converted into 5 fold reduction in invasion in cell culture. It is so far unknown if a higher concentration of supernatants would provide a more efficient inhibition of invasion, but it may be expected as other studies have shown that the effects of bioactive substances can correlate to concentration. Until recently it was generally accepted that *Salmonella* invasion was relying solely on the type III secretion system encoded by SPI-1 of which HilA is a key regulator. Newer evidence suggests, though, that *Salmonella* can enter non-

phagocytic cells by multiple T3SS-1-independent pathways (reviewed by Velge et al. 2012), which could explain why we did not see a more pronounced effect of the bioactive factors on invasion potential. Hence, currently it still needs to be established to what extent modulation of virulence gene expression affects the infection potential of pathogens. At this point, the nature of bioactive substances targeting T3SS in *Salmonella* is unknown, as is the mode of suppression of the infection mechanism. Future studies could aim at isolating the bioactive component(s). This probably by a sequential approach where supernatants would be fractionated and concomitantly tested for remnant bioactivity by the capacity to repress infection potential of *Salmonella* in cell culture virulence models. Animal models could substantiate the effects observed *in vitro*, and impact on the full infection cycle and ability to invade internal organs could be evaluated in mice challenge experiments. To substantiate the practical applicability of the bioactive compounds, the efficacy following incorporation into a food matrix (e.g. a fermented sausage) could be evaluated *in vivo* in a feeding experiment. This may show to which degree the virulence gene inhibition is sustained when *Salmonella* leaves the environment of a fermented sausage and enters the gastrointestinal tract. Also it would be relevant to know if high concentrations of the inhibitory factors will exert any inhibition of virulence gene expression in the intestinal environment. The latter would open for use as a prophylactic, administered either as a pure substance or as a food additive, as a preventive measure against enteric infection.

Acidification is a common preservation technique for controlling growth of pathogens in food and also a major barrier employed by the human host for defence against pathogens. As suggested in Manuscript I lowering of pH during fermentation can potentially induce an acid-tolerance response in surviving pathogenic bacteria, which may lower the infectious dose to as few as 10 - 100 cells (Marshall 2003). Such low infectious dose is also reported for out-breaks involving high-fat low-moisture matrices such as chocolate (Kapperud et al. 1990). Being enteric foodborne pathogens, *Salmonella* spp. typically re-enter the human host by ingestion of contaminated food or water. The pH of gastric acid is in fastening state 1-2, but transiently increases to as much as 5-7 after a meal (Koseki et al. 2011). Gastric survival is dependent on several factors related to the host, the ingested matrix and the enteric pathogen (Takumi et al. 2000). While characteristics of the food will affect gastric emptying time (reviewed by Smith 2003), and hence how long the pathogen is exposed to the gastric acid before entering the intestine, the food composition, hereunder especially buffer capacity, fat and protein content, has also been shown to effect the

gastric acid survival of pathogens (Waterman & Small 1998). Different food components could thereby play a critical role for the host defense against pathogens (Takumi et al. 2000). Aiming at elucidating properties of food, or model food, components enhancing *Salmonella* survival during simulated gastric challenge, we investigated the association between buffer capacity and inactivation of *Salmonella*. This by applying meat-suspensions and media with differing buffer capacities inoculated with *Salmonella* to a computer-controlled fermentor, which could simulate the dynamic pH changes characteristic for gastric passage after ingestion of a standard meal (Takumi et al. 2000). Surprisingly, low buffered media was demonstrated to provide higher protection of inactivation compared to media and meat-suspensions with high buffer capacity. We suggest that an acid tolerance response is likely to be induced during gastric passage, and that the less buffered media provides for a faster induction than the buffered. To substantiate this we analysed the relative gene expression pattern of *rpoS* and *ompR* encoding two major stationary phase acid tolerance response regulators. Consistent with our hypothesis a significantly faster and higher induction of expression of the response regulators was observed in the less buffered media compared to the buffered media.

Inactivation of *Salmonella* was initiated approx. half an hour later in saline compared to the complex media and meat-suspensions. Taking the gradual emptying of the stomach into account, this delay increases the risk of *Salmonella* reaching the intestine and causing infection considerably. Further studies are needed to elucidate how the buffer systems in the complex media seem to deny *Salmonella* appropriate conditions for induction of an ATR compared to the non-buffered saline and why, on the other hand, inactivation occurs faster in saline once initiated. While buffer capacity is clearly not the only property of food conferring protection of pathogens during gastric passage, it would have been interesting to investigate others such as high-fat, low-moisture and the degree of grinding of the food. More so, also the influence of the physiological state of the pathogen with pre-exposure to sub-lethal stress e.g. in a sausage environment would have been interesting to address in the gastric acid model.

The use of dialysis tubes enabled us to compare several properties or components in the same trial, leaving out bias from inter-trial variance and leading to higher conclusiveness. Still, although pH equilibrium across the membrane was suspected to be reached within minutes, this was not the case for BHI, and the pH drop was delayed in this medium compared to the intended. Testing inactivation in whole batch fermentations would have left out this discrepancy, but would have

markedly augmented the work-load and the inherent variance between biological triplicates. It is still unclear why the computer-controlled pH drop differed slightly and inconsistently in some trials, but decreased sensibility of the pH electrode in the lower pH area might hold some of the explanation.

In applying *rpoS* mutants to the gastric acid model, we saw only a minor effect on survival of having this key stress response regulator functioning. *Salmonella* possesses two independently regulated stationary phase acid tolerance systems, one of which is acid-induced but RpoS-independent. This may in part explain why we did not observe any marked differences in survival between wild types and *rpoS* mutants. It also seems reasonable to assume that the resilient bacterial cell possess several mechanisms functioning to protect against vital stresses such as acid. Furthermore, *rpoS* polymorphisms are common, not only in laboratory strains, but also in clinical and wild type isolates, which could suggest some phenotypic gain, and at least could entail that very closely related *Salmonella* serovars respond differently to stress (Jørgensen et al. 2000; Robbe-Saule et al. 2003). A relationship between stress response and virulence in *Salmonella* has been reported but seems to differ between animal species. It has been shown that while *rpoS* mutants are attenuated for mice (Coynault et al. 1996) it was not the case for chickens (Methner et al. 2004). In contrast, *hilA* mutants were largely cleared from the gut of infected chickens and provided significant protection against a virulent challenge strain (Bohez et al. 2007).

From a food safety point of view a sum up of the results obtained in this thesis suggests:

Growth of pathogens in raw material for sausage production as investigated in Manuscript I may not lead to a higher consumer risk.

Seemingly very important for survival both during food processing and gastric passage, the complexity of an acid tolerance response in *Salmonella* could be addressed further in future studies with the aim to identify factors that are critical in avoiding the pathogen to adapt and thus pose a greater risk to the host.

Inhibition of virulence gene expression using substances from lactic acid bacteria may be a promising control measure against pathogens in the future, but their potential use as food preservatives or prophylactics has to be investigated.

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