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Survival of *Listeria monocytogenes* during *in vitro* gastrointestinal digestion after exposure to 5 and 0.5 % sodium chloride

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ABSTRACT

The food industry is under pressure to reduce the NaCl content in food, but the consequences on the ability of *L. monocytogenes* to survive in the human host and cause listeriosis is not known. In this study, a recently developed internationally harmonized static *in vitro* digestion (IVD) model was used to investigate the survival of *L. monocytogenes* in the gastric and intestinal phases after exposure to 5 or 0.5% NaCl. Six isolates from three Scandinavian foodborne listeriosis outbreaks, all related to NaCl containing foods, the EGDe reference strain and an EGDe mutant, deleted for the major stress regulator gene, *sigB,* were included. A ten-fold reduction of NaCl in the cultivation media significantly reduced the survival fraction of the EGDe strain in the IVD model while one of the clinical outbreak isolates showed a significantly increased survival fraction. Finally, the EGDe strain was able to attach and invade cultured HT-29 cells after passage through the IVD model. Altogether, these results suggest that a reduction of the NaCl content from 5 to 0.5% prior to exposure to the IVD model has the potential to cause a change in the relative survival fraction and that the effect is strain dependent.

1. Introduction

Listeria monocytogenes is a food-borne pathogen that causes the serious disease listeriosis in humans (Schlech et al., 1983). Listeriosis has the highest case fatality rate among all foodborne zoonotic diseases surveilled under the EU system (EFSA, 2017). In particular, immunosuppressed individuals, elderly, pregnant woman, fetuses and infants are at higher risk for invasive listeriosis. Despite improved control measurements since the 1990s, which have greatly reduced the prevalence of *L. monocytogenes* in many food products (Buchanan et al., 2017) there is an increase in invasive listeriosis reported within the EU (EFSA, 2018).

NaCl is one of the most widely used food preservatives but, today, the human intake of NaCl is considered to be too high (Kloss et al., 2015). Excessive dietary intake of NaCl, often related to commercially processed food, may lead to vascular hypertension and subsequent cardiovascular disease, the leading cause of death worldwide (Strazzullo et al., 2009). The food industry is therefore under great pressure to reduce the NaCl content in ready-to-eat (RTE) foods (Anderson et al., 2010). Several studies have explored the inhibitory effect of NaCl on the growth of *L. monocytogenes* in food and found it tolerant to high levels of salt stress (Bergholz et al., 2010; Lorentzen et al., 2010; Schirmer et al., 2014). Some of the genes responsible for such osmotolerance are under regulation of Sigma B, which is a key regulator for the *L. monocytogenes* stressosome (NicAogáin and O'Byrne, 2016). Sigma B regulates transcription of several virulence and stress-associated genes including those that facilitate survival during passage through the gastrointestinal system (NicAogáin and O'Byrne, 2016). Previous studies have also identified Sigma B as a critical regulon for *L. monocytogenes* to survive adverse and fluctuating stressors present in the gastro-intestinal tract. However, this has not been simulated by exposure to both the gastric and intestinal environment and by including key digestive enzymes (Garner et al., 2006; Sue et al., 2004).

Survival in the gastro-intestinal tract is a biological key-event for dose-response models (Buchanan et al., 2009). It has been described that the environment *L. monocytogenes* encounters prior to infection may influence its survival fitness in the human host and its pathogenic potential (Gahan and Hill, 2014). Previous studies report that pre-exposure to elevated osmolarity (0.3 M NaCl for 1 h) increases the tolerance of *L. monocytogenes* to lethal concentrations of bile (Begley et al., 2002; Sleator et al., 2009). RTE foods can impose a number of environmental stressors on *L. monocytogenes*, such as osmotic stress (NaCl

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or sugars), organic acid stress (fermentation), acidic pH and cold stress (refrigeration). However, there is still limited knowledge about how these factors influence the survival and fitness of the pathogen *in vivo*.

In vitro digestion models are widely used within food science as *in vivo* models are costly and may raise ethical issues (Minekus et al., 2014). However, the use of different non-standardized models for simulating the human digestive system has proved to be inconsistent when comparing results across research groups. Therefore, an international consensus for static *in vitro* digestion models was published in 2014 (Minekus et al., 2014) and harmonized by several laboratories in 2015 (Egger et al., 2016). The standardized *in vitro* digestion model was documented to be physiologically comparable to *in vivo* porcine digestion of skim milk powder with regard to protein degradation and peptide formation (Egger et al., 2017).

Responses to stressors in the food matrix may facilitate the survival of *L. monocytogenes* through the human digestive system and increase the number of cells able to invade intestinal epithelial cells (NicAogain and O'Byrne, 2016). The current study explores how adaptation to different levels of NaCl stress influences *L. monocytogenes* survival in the digestion system by using an internationally harmonized static *in vitro* digestion (IVD) model (Minekus et al., 2014). To study the importance of the stressosome regulator Sigma B for survival through the digestive barriers, an EGDe Δ*sigB* mutant strain was included in the study. To our knowledge, this is the first time that survival of a foodborne pathogen has been tested under gastrointestinal conditions using the standardized *in vitro* digestion model (Minekus et al., 2014).

2. Materials and methods

2.1. Selection of L. monocytogenes isolates and strains

Scandinavian outbreak isolates used in the current study are shown in Table 1. In general, *L. monocytogenes* food isolates from outbreaks are often challenging to get hold of due to the long clinical incubation time and the relatively short shelf life of the food products. The Norwegian isolate was from an outbreak in 2007, involving 17 patients and causing three deaths (Johnsen et al., 2010). The Danish isolate was from RTE spicy meat roll and collected during an outbreak in 2013/2014, which involved 41 cases, including 17 fatalities (Kvistholm Jensen et al., 2016). The Swedish isolates include two food isolates and two clinical isolates from a Swedish outbreak in 2013/2014, which involved 48 patients. All the Swedish outbreak isolates belonged to the same sequence type (ST-7) (Dahl et al., 2017). Whole genome sequencing revealed that the Patient 2 isolate differed by eight single nucleotide polymorphisms (SNPs) compared to the Patient 1 isolate and the food isolates (Dahl et al., 2017). The eight SNPs were found in genes encoding 6-phospho-beta-galactosidase, 30S ribosomal protein, endoglucanase, tRNA-binding protein, peptidase, cell surface protein and glycerol dehydratase (Cecilia Jernberg, Public Health Agency, Sweden, personal communication). The EGDe reference strain and an EGDe Δ*sigB* mutant are previously described (O'Donoghue et al., 2016) (Table 1).

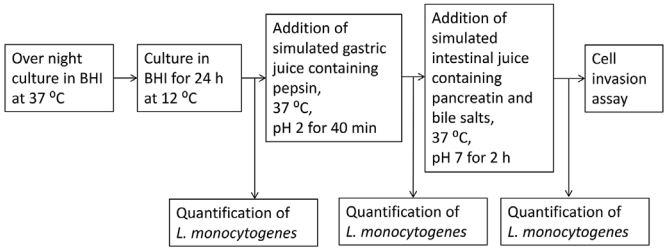

Fig. 1. Overview of the study design.

2.2. Exposure to NaCl

The experimental setup is outlined in Fig. 1. All isolates were grown in Brain Heart Infusion media (BHI, 237500, Bacto™ Becton, Dickinson and Company, Sparks, MID 21152 USA, 38800 Le Pont de Claix, France) to simulate the nutrient-rich environment of meat and other foods which are often associated with foodborne listeriosis (U.S Food and Drug Administration, 2003). The bacteria were first grown statically in a first pre-culture at 37 °C overnight and then transferred to a second pre-culture containing either 0.5% NaCl or 5% NaCl to simulate exposure to different salt concentrations in the water phase of food products. The second pre-culture was grown statically at 12 °C to simulate the growth conditions in solid food and temperature abuse (James et al., 2017). To reach a bacterial density of approximately 8 Log CFU/ml after 24 h of growth in pre-culture 2, different volumes of pre-culture 1 were transferred to pre-culture 2 to reach a total volume of 5 ml. Due to a lower growth rate at 5% NaCl than at 0.5% NaCl, 1 ml of pre-culture 1 was transferred to 4 ml of pre-culture 2 at 5% NaCl, and only 0.5 ml was transferred to 4.5 ml of pre-culture 2 at 0.5% NaCl. In pre-culture 2, *L. monocytogenes* was statically grown in 100 ml bottles with screw cap (not tightened) and wrapped in aluminum foil to ensure no exposure to light during the experiment.

2.3. Static in vitro digestion

A standardized *in vitro* digestion model was used to simulate the human gastrointestinal condition (Minekus et al., 2014). The digestive fate in the stomach and small intestine was investigated, while the oral phase was omitted since starch was not included in the substrate. After 24 h of growth in pre-culture 2, the bacteria were directly exposed to simulated gastric juice (Minekus et al., 2014). The time of gastric digestion for a liquid is reported to be between 5 and 45 min at 37 °C (Camilleri et al., 1989). The exposure time to pH 2 and porcine pepsin (2000U/ml, Sigma P7012) was therefore limited to 40 min and the incubation was performed at 37 °C with shaking (100 rpm). Subsequently, to simulate the intestinal phase, the pH was adjusted to 7 by adding 1 M NaOH, porcine pancreatin (trypsin 100U/ml, Sigma P7545) and porcine bile salts (10 mM, Sigma B8631) were added to the

Table 1
Isolates and strains used in the static *in vitro* digestion experiments.

Isolate	Reference	Isolated from	Provided by	Serogroup
1	EGDe wild type (O'Donoghue et al., 2016)	Rabbit	**	IIa (I/2a)
2	EGDe *ΔsigB* (O'Donoghue et al., 2016)	Laboratory	**	IIa (I/2a)
3	Outbreak, Norway (Johnsen et al., 2010)	Cheese brine	***	IIa (I/2a)
4	Outbreak, Denmark (Kvistholm Jensen et al., 2016)	Ready-to-eat spiced meat roll	****	IIb
5	Outbreak, Sweden* (Dahl et al., 2017)	Liver patê	*****	IIa
6	Outbreak, Sweden* (Dahl et al., 2017)	Boiled medwurst	*****	IIa
7	Outbreak, Sweden* (Dahl et al., 2017)	Patient 1	******	IIa
8	Outbreak, Sweden* (Dahl et al., 2017)	Patient 2	******	IIa

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bacterial cultures. The cultures were then incubated for an additional 120 min at 37 °C with shaking (100 rpm). *L. monocytogenes* was quantified at the start and after each digestive step by plating tenfold dilution in BPW (Buffered Peptone Water ISO, CM 1049, Oxoid) on bovine blood agar plates (CM 02071, Oxoid). The pH was measured in all 48 samples during the intestinal phase (PHM92 LAB pH meter, Radiometer Copenhagen) but due to practical considerations, detailed pH measurements were performed for only 20 samples from the gastric phase. However, the pH was measured in all samples using pH indicator strips at the start of each digestive step, immediately after addition of the respective gastrointestinal enzymes and chemicals (Merck, 1.09584.001). For all strains and conditions, three independent experiments were performed.

2.4. Cell culture assay

HT-29 cells were grown overnight to 80% confluency in 24 well culture plates, using 0.6 ml of McCoy's 5A Modified Medium supplied with 10% fetal bovine serum. The cells were incubated at 37 °C in a 5% (vol/vol) CO_2 atmosphere. Six ml of the EGDe strain suspension, collected after passage through the intestinal phase of the IVD model, was washed once in PBS, centrifuged (10000 rpm, room temperature) and thereafter re-suspended in 1100 µl McCoy's Modified Medium. A volume of 100 µl of the bacterial suspension was diluted and spread on blood agar plates for bacterial enumeration.

For invasion assays, six ml of the EGDe strain suspension collected after passage through the intestinal phase of the IVD model was washed, as described above, and added to cell monolayers. After incubation at 37 °C for 1.5 h, the cell culture plates were washed three times in McCoys Modified Medium. A volume of 500 µl McCoy's Modified Medium supplemented with 1 mg/ml gentamicin (Sigma) was then added to each well of the cell culture plates to kill non-invading bacteria, and the plates were incubated for an additional 1.5 h under the same conditions. The monolayers were then washed with PBS and lysed with 250 µl of ice-cold 0.1% (vol/vol) Triton X-100 (Sigma) in PBS and spread on blood agar plates. The invasion capacity was determined by dividing the number of invading bacterial cells with the total number of bacterial cells added, multiplied by 100. Overnight cultures of the *L. innocua* CCUG 15531 and the EGDe strain (not exposed to the IVD model) were included as negative and positive controls, respectively.

2.5. Statistics

Linear regression analysis, oneway ANOVA and pairwise comparison of means (the pwmean command) were performed using Stata, version 14. When the oneway ANOVA subsequent Bartlett's test for equal variances was significant, the Kruskal-Wallis equality-of-populations rank test was used. Residuals from the linear regression analysis were considered normally distributed by graphical histogram display. P-values equal to or below 0.05 were considered significant.

3. Results

3.1. The effect of different NaCl concentrations on L. monocytogenes survival during gastrointestinal digestion using the IVD model

The average bacterial concentration for all isolates before exposure to the gastric juice, independently of the NaCl concentration, was 8.2 Log CFU/ml (CI: 8.1–8.2, n = 48) (Fig. 2). After the gastric phase, an average bacterial concentration of 3.3 Log CFU/ml (CI 3.1–3.5) was observed (not including the *ΔsigB* mutant). After the intestinal phase the average bacterial concentration was 3.4 Log CFU/ml (CI 3.2–3.5) (not including the *ΔsigB* mutant). The concentration of each strain during the IVD model is shown in Fig. 2.

Oneway ANOVA analysis of the EGDe strain and the outbreak isolates altogether, did not reveal any significant difference in survival in the IVD model depending on exposure to different NaCl concentrations. However, when analyzing the effect of the ten-fold reduction in NaCl for individual strains, the survival fraction after passage through the whole IVD model was significantly ($P < 0.01$), but contrastingly altered for the EGDe strain and one of the Swedish patient isolates (nr 1) (Fig. 3). The EGDe strain demonstrated a significantly reduced survival fraction while the Swedish patient 1 isolate demonstrated a significantly increased survival fraction when the NaCl concentration was reduced. The EGDe strain showed a significantly higher survival fraction ($P = 0.05$) in the intestinal phase when it was exposed to 5% NaCl compared to 0.5% NaCl. In contrast, the Swedish patient 1 isolate did not show any significantly altered survival in individual digestive phases depending on exposure to different NaCl concentrations, but only when considering both the gastric and intestinal survival fractions together (Fig. 3).

A pairwise comparison of means of the total survival fraction through the IVD model, after exposure to 5% NaCl, revealed no significant difference between the EGDe strain and/or between the outbreak isolates. However, the *ΔsigB* mutant strain demonstrated a significantly lower total survival fraction compared to its EGDe background strain ($P = 0.01$). The reduced survival fraction of the *ΔsigB* strain, after exposure to 5% NaCl, could only be observed in the intestinal phase ($P < 0.01$). A pairwise comparison of means between the strains of the total bacterial survival fraction through the IVD model, after exposure to 0.5% NaCl, only revealed a significant difference between the EGDe strain and the Danish meat roll isolate ($P = 0.05$). The *ΔsigB* strain demonstrated a survival fraction similar to its EGDe background strain. Subsequent analysis of the survival fractions in the gastric and intestinal phase separately, after exposure to 0.5% NaCl, revealed no significant difference between EGDe and the *ΔsigB* mutant strain, or the Danish meat roll isolate. All the outbreak isolates and the EGDe strain demonstrated similar survival fractions in the gastric phase. However, in the intestinal phase, the Norwegian outbreak isolate and both Swedish clinical isolates demonstrated significantly increased survival ($P \leq 0.05$) compared to the EGDe strain.

3.2. Invasiveness in HT-29 cells after passage through the IVD model

To study the ability of *L. monocytogenes* to invade epithelial cells after passage through the IVD model, a cell culture assay with HT-29 cells was applied immediately after the intestinal phase. The experiments were limited to the *L. monocytogenes* EGDe strain, exposed to 5 or 0.5% NaCl and the IVD model. The observed invasion percentage in HT-29 cells ranged from 0.0 to 0.4% and 1.3–7.6% when the EGDe strain was exposed to 5 and 0.5% NaCl, respectively (Table 2). Oneway ANOVA analysis revealed a significant result according to Bartlett's test for equal variances (Prob > chi2 = 0.01) when analyzing the effect of the NaCl concentration upon cell invasiveness by the EGDe strain. Therefore, the Kruskal Wallis test was used. This test indicated a significantly increased ability of the EGDe strain to invade HT-29 cells after a tenfold reduction in the NaCl concentration ($X^2 = 3.9$, $P = 0.05$). Control experiments of the assay with overnight cultures of *L. innocua* CCUG 15531 and *L. monocytogenes* EGDe strain not pre-exposed to the IVD model confirmed that *L. innocua* CCUG 15531 were not able to invade HT-29 cells while the *L. monocytogenes* EGDe strain was (data not shown).

3.3. Strain dependent survival through the IVD model, regardless of NaCl concentration

In order to assess the isolate variability and simulation of complex RTE foods, which contain a range of different NaCl concentrations, the survival fraction for both NaCl concentrations were pooled for each isolate. Linear regression analysis resulted in significant impact of the isolate upon total digestive survival (Prob > F < 0.01, Adj. R-squared = 0.35 and F(7,40)) Only the *ΔsigB* mutant demonstrated

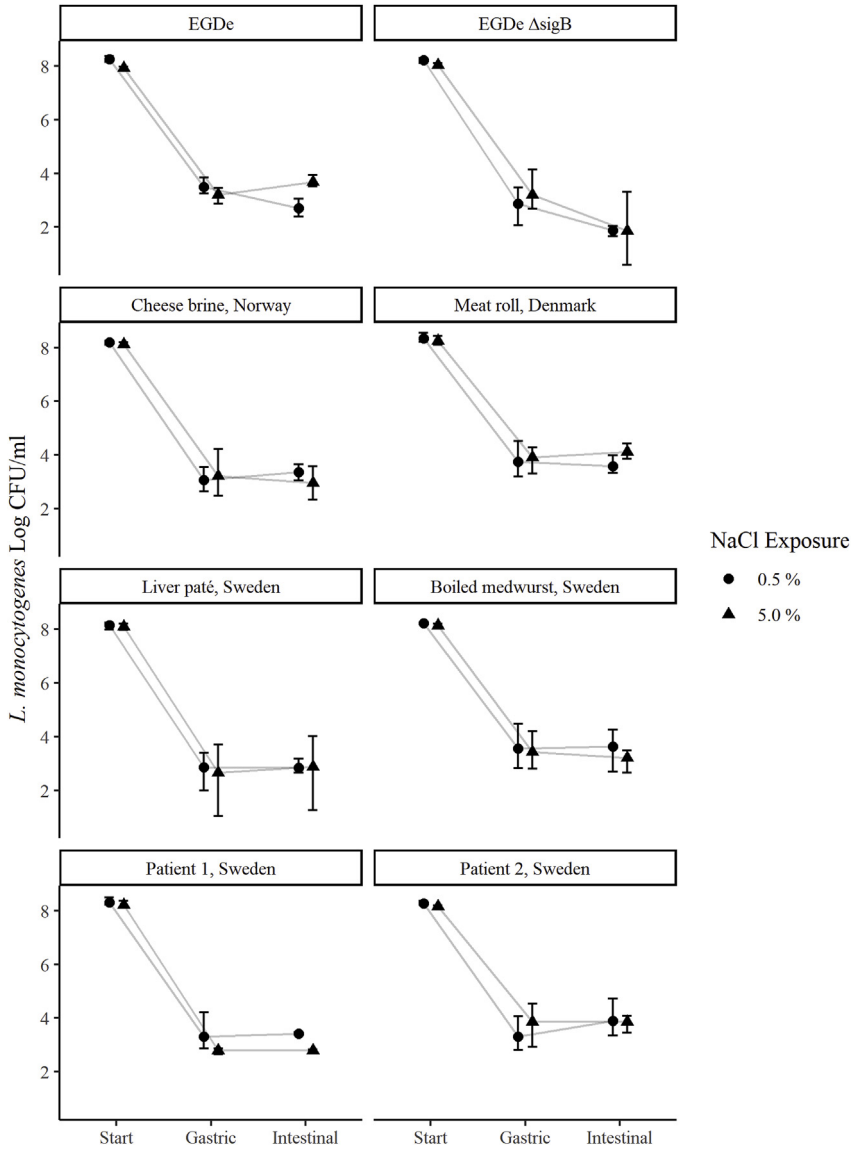

Fig. 2. Survival fraction of *L. monocytogenes* outbreak isolates and strains through the gastric and intestinal phases of the IVD model, divided by 0.5% NaCl (dot) and 5% NaCl (triangle) exposure (n = 3). The concentration (Log CFU/ml) is given at start, after the gastric phase (pH 2.1, 40 min) and after the intestinal phase (pH 7.0, 120 min). Data presented are mean values of three independent experiments and error bars representing the range.

significantly decreased survival ($P < 0.01$) in the IVD model compared to the EGDe wild type strain. The significant reduction in survival fraction occurred only after passing through the intestinal phase, and not in the gastric phase.

Among the outbreak isolates, the highest survival fraction mean was observed for the Swedish Patient 2 isolate (−4.3 Log CFU/ml, CI: −4.8 – (−3.8)), and the lowest survival fraction mean was observed for the Patient 1 isolate (−5.4 Log CFU/ml, CI: −5.5- (−4.4)), both originating from the Swedish outbreak.

4. Discussion

4.1. Exposure to different NaCl concentrations significantly affects the survival of L. monocytogenes in the IVD model

The aim of the current study was to explore how adaptation of *L. monocytogenes* to different levels of NaCl stress influences the survival fraction through the IVD model. Meat, meat products and dairy products are all NaCl-containing RTE food products often associated with *L. monocytogenes* contamination (U.S Food and Drug Administration, 2003). For public health reasons, many European countries have already started to reduce the NaCl content in RTE food. However, this may confer an adverse effect by resulting in faster growth of *L. monocytogenes* in the food products as the water activity increases. However, possible cross-protection against the digestive barriers after osmotic stress in the food products has been described (Sleator et al., 2009), which may lead to a higher survival fraction passing through the human digestive tract. Surprisingly, the present study detected no significant difference in survival fraction between isolates exposed to 5 and 0.5% NaCl when clustering the EGDe strain and the outbreak isolates altogether. The lower tolerance of the *ΔsigB* mutant was expected due to the likely reduced ability to cope with stress. However, when analyzing the isolates separately, exposure to different NaCl concentrations resulted in significant, but surprisingly contrasting survival fraction effects in the intestinal phase. The phenomena of cross-protection may explain why the EGDe strain demonstrated a significantly higher survival fraction in the intestinal phase when exposed to the higher NaCl concentration (Begley et al., 2002; Sleator et al., 2009). On the other hand, there are also studies reporting that exposure to osmotic stress may sensitize the bacteria to digestive stress (Barbosa et al., 2012; Garner

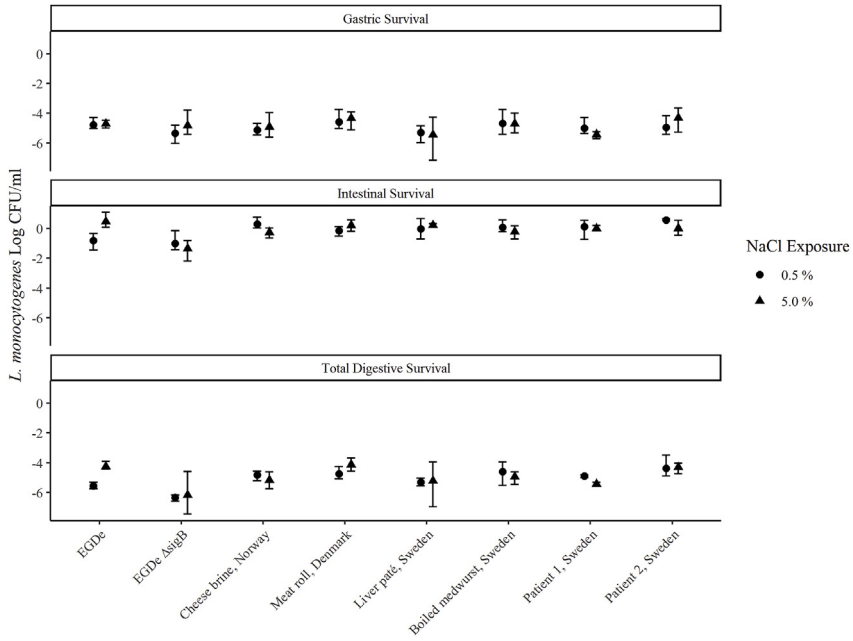

Fig. 3. Survival fraction of *L. monocytogenes* strains and isolates in the IVD model. The data represent the gastric step, the intestinal step and both steps together. Exposure to 0.5% NaCl (dot) and to 5% NaCl (triangle) is indicated by the mean values of three independent experiments and error bars representing the range.

Table 2
Invasivness of the EGDe strain in epithelial cells at 5 and 0.5% NaCl after *in vitro* digestion.

Experiment	NaCl (%)	Invasion (%)	Mean	Standard Error of the Mean
1	5.0	0.4	0.2	0.1
2	5.0	0.1		
3	5.0	0.0		
1	0.5	1.3	5.1	2
2	0.5	7.6		
3	0.5	6.5		

et al., 2006). This is in compliance with the significantly reduced survival fraction of the Swedish patient 1 isolate in the IVD model when the NaCl concentration was increased. Differences in survival fractions between the isolates were detected only in the intestinal phase, suggesting that stressors encountered in the intestine reveal more isolate dependent differences in stress tolerance than those encountered in the gastric phase. Our results suggest that exposure to NaCl may influence how strains and isolates of *L. monocytogenes* cope with stressors encountered in the digestive system and that there is a diversity among strains in how NaCl influence their stress resistance. After exposure to 0.5% NaCl, the Norwegian outbreak isolate, the Danish meat roll isolate and the two clinical Swedish outbreak isolates demonstrated significantly increased survival fractions in the IVD model compared to the EGDe strain. Such increased survival may have contributed to the severity of the respective listeriosis outbreaks. Surprisingly, such isolate-dependent differences in survival in the IVD model were not observed during exposure to 5% NaCl. In the current study, the initial concentration of *L. monocytogenes* was 8.2 Log CFU/g with a very low confidence interval, preventing potential bias in the results due to start concentration differences. By using a high inoculum concentration, it was still possible to quantify the bacteria after the intestinal step and to obtain enough cells to perform a cell culture assay, which is essential to document if the bacteria passing through the IVD model still have the potential to adhere, invade and cause disease. Barbosa et al. (2012) reported six *L. monocytogenes* isolates, isolated from cheese, to be more sensitive to acidic (pH 3.5) than osmotic stress (NaCl; 30–40%) prior to a static *in vitro* digestion model (Barbosa et al., 2012). After adding the bile salts to their IVD model, all strains decreased to a concentration below the level of detection, which is in contrast to the current study. However, Barbosa and colleagues exposed their strains to a higher NaCl concentration and used approximately a 6 Log in-put-concentration of *L. monocytogenes* and a longer digestion time than applied in the current study, which could explain the lower bacterial level after passage through the IVD model. Furthermore, it has been reported that *L. monocytogenes* isolates demonstrated different survival fractions after 90 min of exposure to the gastric environment (Barmpalia-Davis et al., 2008). Since the gastric phase lasted only 40 min in the present study, the short gastric exposure time may explain why no strain-dependent differences in survival were detected in the gastric phase.

The use of an in-put concentration of 8 Log CFU/g in the IVD model simulates a worst case scenario. However, given the low incidence of listeriosis in a population despite the ubiquitous nature of the causative bacterium, dose-response models presume illnesses results from exposure to high bacterial doses (Buchanan et al., 2017).

As expected, the *ΔsigB* mutant demonstrated a significantly lower survival fraction through the IVD model compared to its EGDe background strain, probably due to its inability to activate the stressosome. Surprisingly, deletion of *sigB* did not reduce the resistance to gastric pH, but rather sensitized the organism to the intestinal environment. Although Sigma B is regarded as the key stress regulator in *L. monocytogenes*, there are also other mechanisms responsible for coping with osmotic stress and low-pH stress, which do not belong to the Sigma B regulon, as reviewed by NicAogain and O'Byrne (2016). This may explain why deletion of *sigB* had a very limited effect on *L. monocytogenes* survival in the gastric phase (NicAogain and O'Byrne, 2016).

4.2. Invasion properties of the EGDe strain after passing through the IVD model

Key virulence genes in *L. monocytogenes* are important for attachment, invasion and the intracellular cycle. It has been reported that moderately increased salt stress could increase *L. monocytogenes* invasiveness in cell culture (Larsen and Jespersen, 2015; Lorentzen et al., 2011). However, the results in the current study demonstrated a significant lower invasion rate of HT-29 cells after the EGDe strain was exposed to 5% compared to when it was exposed 0.5% NaCl. Further

experiments with more strains will be needed to reveal if the salt concentration also has a similar effect on cell invasiveness of other *L. monocytogenes* strains and isolates.

4.3. *L. monocytogenes* survival fractions through the IVD model, after exposure to either 5 or 0.5% NaCl

All *L. monocytogenes* strains and isolates used in the current study showed surprisingly high survival fractions in the IVD model (Fig. 2), even though the bacteria were exposed to the simulated digestive fluids and enzymes in pure broth with limited protective solid matrix effects. The protective effect of solid food matrix is assumed to increase the survival of *L. monocytogenes* during digestion (Barmpalia-Davis et al., 2009). However, *in vitro* digestion with Lactic Acid Bacteria (LAB) and *L. monocytogenes* in Latin-style fresh cheese revealed that the food matrix only had a protective effect on the LAB, but not on *L. monocytogenes* cells (Silva et al., 2015).

When comparing the total digestive survival of the Swedish outbreak isolates, the Liver patê isolate and the other clinical isolates, the patient isolate nr 1 demonstrated a significantly decreased total survival fraction through the IVD model compared to the patient 2 isolate and the Boiled medwurst isolate. They all originated from the same outbreak and belonged to the same sequence type (Dahl et al., 2017). The only reported genotypic differences between the Swedish outbreak isolates were the presences of eight SNPs in the patient 2 isolate (Dahl et al., 2017). However, the Boiled medwurst isolate did not demonstrate a significantly reduced total survival fraction compared to the patient nr 2 isolate. Although whole genome sequencing is a precious and powerful tool for outbreak investigation and public health, there are still considerations to be made before predicting the physiological and pathogenic potential of a strain or an isolate (Adhikari and Curtis, 2016; Gill A., 2017).

Altogether, the results suggest that a reduction of the NaCl content from 5 to 0.5% in the BHI growth media has the potential to alter the relative fraction of viable bacteria, which reach the intestine and that the effect is isolate and strain dependent. A tenfold reduction in NaCl concentration also conferred a potentially increased epithelial cell invasion. However, further studies are needed, including adjustments of the IVD model to make it more representative of individuals belonging to the high-risk group for achieving invasive listeriosis. Individual physiological differences in gastric pH, pepsin content, gastric lipase levels, bile concentrations, level of proteolytic enzymes and transit time, should therefore be considered and implemented (Shani-Levi et al., 2017).

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.08.010.

References

Adhikari, S., Curtis, P.D., 2016. DNA methyltransferases and epigenetic regulation in bacteria. FEMS Microbiol. Rev. 40, 575–591.
Anderson, C.A., Appel, L.J., Okuda, N., Brown, I.J., Chan, Q., Zhao, L., Ueshima, H., Kesteloot, H., Miura, K., Curb, J.D., Yoshita, K., Elliott, P., Yamamoto, M.E., Stamler, J., 2010. Dietary sources of sodium in China, Japan, the United Kingdom, and the United States, women and men aged 40 to 59 years: the INTERMAP study. J. Am. Diet Assoc. 110, 736–745.
Barbosa, J., Borges, S., Magalhaes, R., Ferreira, V., Santos, I., Silva, J., Almeida, G., Gibbs, P., Teixeira, P., 2012. Behaviour of *Listeria monocytogenes* isolates through gastrointestinal tract passage simulation, before and after two sub-lethal stresses. Food Microbiol. 30, 24–28.
Barmpalia-Davis, I.M., Geornaras, I., Kendall, P.A., Sofos, J.N., 2008. Differences in survival among 13 *Listeria monocytogenes* strains in a dynamic model of the stomach and small intestine. Appl. Environ. Microbiol. 74, 5563–5567.
Barmpalia-Davis, I.M., Geornaras, I., Kendall, P.A., Sofos, J.N., 2009. Effect of fat content on survival of *Listeria monocytogenes* during simulated digestion of inoculated beef frankfurters stored at 7 degrees C. Food Microbiol. 26, 483–490.
Begley, M., Gahan, C.G., Hill, C., 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Appl. Environ. Microbiol. 68, 6005–6012.
Bergholz, T.M., den Bakker, H.C., Fortes, E.D., Boor, K.J., Wiedmann, M., 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. Foodborne Pathogens and Disease 7, 1537–1549.
Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., Whiting, R.C., 2017. A review of *Listeria monocytogenes*: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Contr. 75, 1–13.
Buchanan, R.L., Havelaar, A.H., Smith, M.A., Whiting, R.C., Julien, E., 2009. The key events dose-response framework: its potential for application to foodborne pathogenic microorganisms. Crit. Rev. Food Sci. Nutr. 49, 718–728.
Camilleri, M., Colemont, L.J., Phillips, S.F., Brown, M.L., Thomforde, G.M., Chapman, N., Zinsmeister, A.R., 1989. Human gastric emptying and colonic filling of solids characterized by a new method. Am. J. Physiol. 257, G284–G290.
Dahl, V., Sundqvist, L., Hedenström, I., Löfdahl, M., Alm, E., Ringberg, H., Lindblad, M., Wallensten, A., Thisted Lambertz, S., Jernberg, C., 2017. A nationwide outbreak of listeriosis associated with cold-cuts, Sweden 2013-2014. Infect. Ecol. Epidemiol. 7, 1324232.
Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, M.J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A.R., Martins, C., Meynier, A., Miralles, B., Murray, B.S., Pihlanto, A., Picariello, G., Santos, C.N., Simsek, S., Recio, I., Rigby, N., Rioux, L.-E., Stoffers, H., Tavares, A., Tavares, L., Turgeon, S., Ulleberg, E.K., Vegarud, G.E., Vergères, G., Portmann, R., 2016. The harmonized INFOGEST in vitro digestion method: from knowledge to action. Food Res. Int. 88, 217–225.
Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brugger, C., Durr, D., Stoll, P., Vergeres, G., Portmann, R., 2017. Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. Food Res. Int. 102, 567–574.
EFSA, European Centre for Disease Prevention and Control, 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA Journal 15 e05077-n/a.
EFSA Panel on Biological Hazards (BIOHAZ), 2018. *Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU. EFSA Journal 16 e05134-n/a.
Gahan, C.G., Hill, C., 2014. *Listeria monocytogenes*: survival and adaptation in the gastrointestinal tract. Front Cell Infect Microbiol 4, 9.
Garner, M.R., James, K.E., Callahan, M.C., Wiedmann, M., Boor, K.J., 2006. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. Appl. Environ. Microbiol. 72, 5384–5395.
Gill, A., 2017. The importance of bacterial culture to food microbiology in the age of genomics. Front. Microbiol. 8, 777.
James, C., Onarinde, B.A., James, S.J., 2017. The use and performance of household refrigerators: a review. Compr. Rev. Food Sci. Food Saf. 16, 160–179.
Johnsen, B.O., Lingaas, E., Torfoss, D., Strom, E.H., Nordoy, I., 2010. A large outbreak of *Listeria monocytogenes* infection with short incubation period in a tertiary care hospital. J. Infect. 61, 465–470.
Kloss, L., Meyer, J.D., Graeve, L., Vetter, W., 2015. Sodium intake and its reduction by food reformulation in the European Union — a review. NFS Journal 1, 9–19.
Kvistholm Jensen, A., Nielsen, E.M., Bjorkman, J.T., Jensen, T., Muller, L., Persson, S., Bjerager, G., Perge, A., Krause, T.G., Kiil, K., Sorensen, G., Andersen, J.K., Molbak, K., Ethelberg, S., 2016. Whole-genome sequencing used to investigate a nationwide outbreak of listeriosis caused by ready-to-eat delicatessen meat, Denmark, 2014. Clin. Infect. Dis. 63, 64–70.
Larsen, N., Jespersen, L., 2015. Expression of virulence-related genes in *Listeria monocytogenes* grown on Danish hard cheese as affected by NaCl content. Foodb. Pathog. Dis. 12, 536–544.
Lorentzen, G., Mennen, S., Olsen, R.L., Skjerdal, T., 2011. Invasiveness of *Listeria monocytogenes* strains of Caco-2 cells in response to a period of extreme salt stress reflecting salt-curing and rehydration of cod (Gadus morhua L.). Food Contr. 22, 1040–1045.
Lorentzen, G., Olsen, R.L., Bjørkevoll, I., Mikkelsen, H., Skjerdal, T., 2010. Survival of *Listeria innocua* and *Listeria monocytogenes* in muscle of cod (Gadus morhua L.) during salt-curing and growth during chilled storage of rehydrated product. Food Contr. 21,

292–297.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Menard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food - an international consensus. Food & Function 5, 1113–1124.
NicAogain, K., O'Byrne, C.P., 2016. The role of stress and stress adaptations in determining the fate of the bacterial pathogen *Listeria monocytogenes* in the food chain. Front. Microbiol. 7, 1865.
O'Donoghue, B., NicAogain, K., Bennett, C., Conneely, A., Tiensuu, T., Johansson, J., O'Byrne, C., 2016. Inhibition of *Listeria monocytogenes* growth by blue light is mediated by reactive oxygen species: a role for sigma B and the blue light sensor, Lmo0799. Appl. Environ. Microbiol. 82, 4017–4027.
Schirmer, B.C., Heir, E., Lindstedt, B.A., Moretro, T., Langsrud, S., 2014. Use of used vs. fresh cheese brines and the effect of pH and salt concentration on the survival of *Listeria monocytogenes*. J. Dairy Res. 81, 113–119.
Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C.V., 1983. Epidemic listeriosis — evidence for transmission by food. N. Engl. J. Med. 308, 203–206.
Shani-Levi, C., Alvito, P., Andrés, A., Assunção, R., Barberá, R., Blanquet-Diot, S., Bourlieu, C., Brodkorb, A., Cilla, A., Deglaire, A., Denis, S., Dupont, D., Heredia, A., Karakaya, S., Giosafatto, C.V.L., Mariniello, L., Martins, C., Ménard, O., El, S.N., Vegarud, G.E., Ulleberg, E., Lesmes, U., 2017. Extending in vitro digestion models to specific human populations: perspectives, practical tools and bio-relevant information. Trends Food Sci. Technol. 60, 52–63.
Silva, C.C., Domingos-Lopes, M.F., Magalhaes, V.A., Freitas, D.A., Coelho, M.C., Rosa, H.J., Dapkevicius, M.L., 2015. Short communication: Latin-style fresh cheese enhances lactic acid bacteria survival but not *Listeria monocytogenes* resistance under in vitro simulated gastrointestinal conditions. J. Dairy Sci. 98, 4377–4383.
Sleator, R.D., Watson, D., Hill, C., Gahan, C.G., 2009. The interaction between *Listeria monocytogenes* and the host gastrointestinal tract. Microbiology 155, 2463–2475.
Strazzullo, P., D'Elia, L., Kandala, N.B., Cappuccio, F.P., 2009. Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies. Br. Med. J. 339, b4567.
Sue, D., Fink, D., Wiedmann, M., Boor, K.J., 2004. sigmaB-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. Microbiology 150, 3843–3855.
U.S. Department of Health and Human Services, U.S. Department of Agriculture, 2003. Quantitative Assessment of Relative Risk to Public Health from Foodborne *Listeria Monocytogenes* Among Selected Categories of Ready-to-eat Foods. U.S. Food and Drug Administration.