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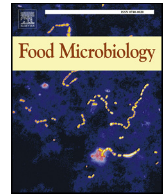

Survival of *Listeria monocytogenes* during *in vitro* gastrointestinal digestion after exposure to 5 and 0.5 % sodium chloride

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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).

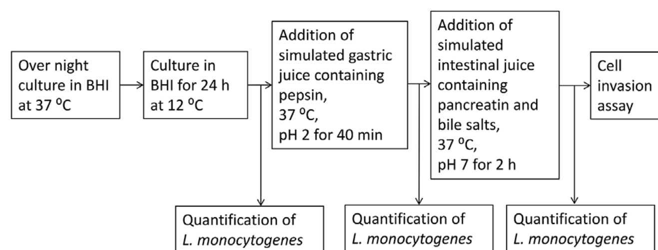

Fig. 1. Overview of the study design.

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 Δ*sigB* (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

*Belong to the same outbreak. ** Department of Molecular Biology, Umeå University. *** Norwegian Veterinary Institute. ****National Food Institute, Technical University of Denmark. ***** National food agency, Sweden. ****** The public Health Agency of Sweden.

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