The impact of disinfection stressors on *Listeria monocytogenes* in challenge testing of foods

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Introduction

Listeria monocytogenes is a foodborne pathogen causing listeriosis (Schlech *et al.*, 1983; Cartwright *et al.*, 2013). The mortality rate can exceed 25% and listeriosis has the highest proportion of hospitalisation cases of all zoonotic diseases under EU surveillance (EFSA, 2017). *L. monocytogenes* infections are most commonly reported in people over 64 years old. The trend for an increase in foodborne listeriosis has been attributed to the rising proportion of older people, along with the higher consumption of ready-to-eat (RTE) food products (Cartwright *et al.*, 2013; EFSA, 2017; Rossi *et al.*, 2008).

According to the European Food Law (European Commission, 2005), the required sampling and analysis regimes are different for food products that support the growth of *L. monocytogenes* than for products that do not. Accordingly, European guidelines for conducting *L. monocytogenes* challenge tests were developed to determine whether a specific food product supports the growth of the bacterium (Beaufort *et al.*, 2014). Under these guidelines, *L. monocytogenes* should be acclimated to the temperature at which the experiment will be undertaken prior to inoculation of the food to shorten the lag phase during the challenge testing and to obtain maximum growth potential during the food's shelf life. These guidelines do not account for factors other than temperature influencing the physiological state of the inoculated strains.

L. monocytogenes contamination of RTE foods, heat-treated or not, usually occurs during food production, when the food is handled and/or is in contact with the production environment. Some *L. monocytogenes* strains can persist in food production facilities for years (Ferreira *et al.*, 2014) despite thorough cleaning and disinfection, conferring a continuous risk for contaminating production environments and food products. Persistent in-house strains are likely to be frequently exposed to washing agents and chemical disinfectants, particularly alkali treatments, which are frequently used to disinfect hard surfaces (Giotis *et al.*, 2010; Taormina and Beuchat, 2002b). The sub-lethal stress imposed by alkaline disinfectants may alter the resistance to subsequent stressors present in food products and in the human host (Giotis *et al.*, 2008; Giotis *et al.*, 2010; Segal *et al.*, 1981; Taormina and Beuchat, 2002a), resulting in altered growth potential.

Food production companies and researchers have used challenge testing to estimate the storage time up to a 100-fold increase in concentration of *L. monocytogenes*, and to assess the storage time before *L. monocytogenes* represents a food safety risk (De Cesare *et al.* 2018; Mejlholm *et al.* 2010; Pal *et al* 2008; Skjerdal *et al* 2010; Skjerdal *et al* 2014). According to the guidelines, the inoculum should be prepared to obtain immediate growth and maximum growth rate in the food products. However, an environmental contamination route, including stress imposed by a disinfectant, will alter the physiological state of the bacterial inoculum, compared with an inoculum grown under traditional laboratory conditions (Eom *et al.*, 2009). This may result in a prolonged lag phase for stressed bacteria compared with unstressed bacteria (Guillier *et al.*, 2005). If *L. monocytogenes* contamination arises from an environmental contamination route, the cells may require an extended time to recover, showing reduced growth potential in food products compared with an inoculum in good physiological condition, even if cold-adapted. The presence of environmental stressors may thus cause a discrepancy between naturally contaminated samples and challenge test data, consequently affecting food safety, food economy and food waste.

L. monocytogenes is characterised as a hardy agent, able to grow under anaerobic conditions, at high salt concentrations and under refrigeration temperatures (Chan and Wiedmann, 2009; Liu, 2008; Lorentzen *et al.*, 2010; Schirmer *et al.*, 2014). The bacterial growth rate in food can be decreased with stressors such as low pH, organic acids, nitrite, low water activity, background flora, modified atmospheres and cold temperatures (Mejlholm *et al.*, 2010; Mejlholm and Dalgaard, 2015b). These stress factors function as hurdles and are used separately

or in combination to reduce the growth rate of pathogens in food products.

Underestimations of pathogen growth in food products may lead to unacceptable high risks for consumers, but overestimation of growth may lead to unnecessary food waste. Considering the effect of relevant environmental stressors on the inoculum can lead to a more realistic *Listeria* shelf life without compromising food safety. In their review, Álvarez-Ordóñez *et al.* (2015) also pointed out that comparing the European guidelines with any alternative methodology is worthwhile.

The aim of this study was to evaluate how the inoculation procedure involving pre-exposure to a commercial chlorinated alkaline disinfectant influences the growth potential of *L. monocytogenes* in RTE food products. RTE chicken and RTE sliced deli meat were chosen as food model matrices because both products are widely used, have relatively long shelf lives and have been reported as food vehicles for *L. monocytogenes* transmission (Cartwright *et al.*, 2013).

Materials and Methods

■ *L. monocytogenes* strains and food products

Eight strains of *L. monocytogenes*, previously used in challenge tests to study growth in similar meat products, were used for inoculation of RTE food products (Skjerdal *et al.*, 2010). All strains are listed in Table 1. All these strains demonstrated rapid growth at 4°C in a previous study (Skjerdal *et al.*, 2010). Two laboratory strains, whereas Scott A was used as a reference strain, were included in the cocktail for both RTE chicken and RTE sliced deli meat. The respective cocktails further contained four isolates from similar food products and from food production facilities (Table 1). Because *L. monocytogenes* strains were selectively chosen in accordance with the specific product, statistical comparison of bacterial growth between these products was not performed.

TABLE 1 / Selected in-house *Listeria monocytogenes* strains for challenge testing.

ID number	Source	Used for inoculation of	Serotype*
VI 59788	Unknown product	RTE chicken	II c
VI 59789	RTE chicken	RTE chicken	II a
VI 59790	Sliced deli meat	RTE sliced deli meat	II a
VI 59791	Sliced deli meat	RTE sliced deli meat	II a
VI 59792	Meat balls	RTE chicken and sliced deli meat	II c
VI 59793	Wiener sausage	RTE chicken and sliced deli meat	II c
NVH-FMN	Laboratory	RTE chicken and sliced deli meat	IV b
Scott A	Laboratory	RTE chicken and sliced deli meat	IV b

*Serotype analysed by PCR (ANSES 2013, ANSES 2014)

RTE chicken and sliced deli meats were used as food matrices; they are heat-treated products with a commercial shelf life of 18 and 35 days, respectively. The package size was 200 g for the RTE chicken and 100 g for the RTE sliced deli meat. Food characteristics given on the product packages from the manufacturer are described in Table 2. In total, 369 packages of RTE chicken and 189 packages of RTE sliced deli meat from three different batches were included in the challenge test studies. All food packages were kindly provided by a private

food business company. Challenge tests with BHI broth (BactoTM brain heart infusion, Becton, Dickinson and Company, Sparks, MD, USA) were performed in parallel with the challenge tests using food matrices to confirm the ability of the *L. monocytogenes* strains to grow. A potential effect of stress was expected to be more readily detectable in RTE chicken than in the RTE sliced deli meat, because previous challenge tests of the latter product have documented only limited growth of *L. monocytogenes*.

TABLE 2 / Food characteristics provided by the manufacturer on the product package.

	RTE chicken	RTE sliced deli meat
Shelf life	18 days	35 days
Ingredients in 100 g	109 g chicken[a] Water Salt (1.5%) Spices (including paprika) Glucose Garlic Rapeseed and sunflower oil	58% beef and pork Water Salt Spices Glucose Onion Smoke flavouring Starch
Preservation	E262 (sodium acetate) E325 (sodium lactate)	Anti-oxidant E315 E325 (sodium lactate) E261 (potassium acetate) E326 (potassium lactate)
Nutritional content in 100 g	980 KJ 25 g protein 0 g carbohydrate 15 g fat	998 KJ 12 g protein 6.5 g total carbohydrate, including 0.2 g sugars 18.6 g fat, including 7.3 g unsaturated fat, 8.5 g monounsaturated fat and 2.4 g polyunsaturated fat 1.7 g salt
Modified atmosphere	70% CO_2 and 30% N2	Yes, but not specified
Recommended storage temperature	0-4°C	0-4°C

[a] 109 g of raw chicken was needed for 100 g of the final chicken product

■ Preparation and characterisation of inoculum

The standard inoculation culture was prepared according to EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in RTE foods, Version 3 – 6 June 2014 (Beaufort *et al.*, 2014). In brief, strains were inoculated from frozen stocks into BHI broth and cultured separately at 37°C for 24 h. For pre-adaptation to cold, in accordance with the food storage temperature, 100 µl of each pre-culture was transferred into 9 ml of BHI broth and incubated separately for 7 days at 4°C. Following this adaptation, equal volumes of all *L. monocytogenes* cultures were pooled into a mixed culture and enumerated in accordance with part 2 of the horizontal method for the detection and enumeration of *L. monocytogenes* (Anonymous *et al.*, 2004). The mixed culture was immediately diluted in BHI and subsequently in physiological saline water (sodium chloride, Merck, Darmstadt, Germany) aiming for a *L. monocytogenes* concentration in the food of 100 colony forming units (CFU)/g when inoculating with 100 µl (Beaufort *et al.*, 2014). The dilutions in physiological saline water were applied to ensure equal and minimal carry-over concentrations of BHI between standard ino-

culum and the chlorinated alkaline-stressed inoculum.

For the challenge tests with disinfectant-stressed inoculum, standard cultivation methods were used, except that the bacterial suspension was not diluted because the subsequent exposure to stress was expected to reduce the level of viable bacteria. The chlorinated alkaline disinfectant Titan Hypo (Lilleborg AS, Oslo, Norway) was diluted in physiological saline water to a concentration of 0.5 or 1% after addition of 1 ml of the BHI containing bacteria. After 5 min exposure to disinfectant, 1 ml of the bacterial suspension was transferred to 9 ml of physiological saline water for dilution as suggested by Eom *et al.* (2009). Both the concentration and the exposure time were in accordance with the producers' recommendations for disinfection of environmental surfaces in food processing plants. A preliminary experiment revealed that physiological saline water and the solutions containing 0.5 and 1% of Titan Hypo had pH values (AOAC 981.12, 1982) of 6.9, 10.6 and 11.1 respectively. After adding 1 ml of BHI containing the inoculum, the pH changed to 7.2, 7.8 and 8.3, respectively. The final pH values in the inoculum samples were 7.2 for the control, 7.5 for the 0.5% solution Titan Hypo and 8.2 for the 1% solution of Titan Hypo.

■ Inoculation and storage of food samples

The inoculation was performed on the production date or the day after the production date, except for the last batch of RTE sliced deli meat, which due to logistical reasons were inoculated 5 days after the production date.

The product packages from three different batches were inoculated with 100 µl of the mixed cultures through a septum (ø 15 mm white, hard, PBI Dansensor A/S, Ringsted, Denmark) using a needle (0.6*25 mm) and syringe. The needle was used to spread the bacteria on the surface. The inoculated area of the food package was marked to facilitate later quantification of 25 g food matrix (De Cesare *et al.*, 2018). After inoculation, the inoculated food packages and control broth were constantly stored at 4°C until eight days after the expiry date.

The concentration of *L. monocytogenes* in the challenge test food packages and corresponding BHI tubes was assessed three times during the storage time, except for three biological replicates of RTE chicken and respective BHI tubes, which were sampled nine times during the storage period. A preliminary experiment revealed more rapid growth in RTE chicken than in RTE sliced deli meat, and the RTE chicken was therefore prioritised for more frequent sampling. Quantification of *L. monocytogenes* was performed according to a modified version of ISO 11290-2 using buffered peptone water (BPW, Oxoid, Hampshire, England) as diluent and agar *Listeria* according to Ottaviani Agosti (ALOA) and ALOA® supplement (Biomérieux, Marcy L'Etoile, France) as the agar medium (Anonymous *et al.*, 2004). BPW was used for optimal recovery of stressed cells. In each challenge test, the level of *L. monocytogenes* was determined from at least three biological and technical replicate samples. To ensure a minimal effect of the inoculation in modified atmosphere (MAP) conditions, the MAP was measured prior to inoculation and at three days post-inoculation (DanSensors MAP analyser, Ringsted, Denmark).

■ Characterisation of RTE chicken and sliced deli meat

Control samples from all test batches were initially analysed for natural contamination of *L. monocytogenes* using the ISO 11290-1 standard method for *L. monocytogenes* detection. The total aerobic count of the challenge test batches was analysed at the beginning and at the end of the storage period. Briefly, after blending, samples were diluted in unbuffered peptone water (UPW) (Becton, Dickinson and Company), plated on plate count agar (PCA) (Becton, Dickinson and Company) and the plates were incubated at 20°C for three days before counting colonies. The water activity (NMKL No. 168) and the pH (AOAC 981.12; 1982) were also analysed both at the start and at the end of the storage period. Additional single food packages from three different batches (n = 3) of RTE chicken and sliced deli meat were analysed for both extrinsic (MAP) and intrinsic (pH, water activity, dry matter, organic acids, NaCl and lactic acid bacteria) properties (Table 3). The analyses of pH, water activity, dry matter (NMKL No 23, 1991), organic acids (internal method of the commercial laboratory) and NaCl concen-

tration (internal method of the commercial laboratory using the chloride concentration and silver nitrate titration) were subcontracted to a commercial laboratory. The headspace MAP analysis in the food packages was performed using the PBI DanSensors CheckMate 9900 MAP analyser according to the manufacturer's instructions. The concentrations of organic acid in the water phase was calculated from the average dry matter weight and percentage of organic acid, using Food Spoilage and Safety Predictor (FSSP) software (Mejlholm, Gunvig *et al.* 2010, Mejlholm and Dalgaard 2013).

■ Statistical analysis

The food characteristic parameters were described with the mean and standard deviation (SD). Growth potentials were calculated from the log CFU/g difference of the median at the end and the median at the start (Beaufort *et al.*, 2014). The main aim of the study was to evaluate the effect of stress, not to categorise the food products in their respective growth category set by the regulations, and growth potentials below 0.5 log CFU/g were included in the regression analysis. The growth potentials were determined at the expiry dates and eight days after the expiry dates, because the sensory shelf life of the products were normally longer than the shelf life set by the food producer. The highest estimated growth potential among batches was used for final growth potential estimation of the specific product.

Statistical analyses were performed using Microsoft Excel 2010 and STATA, version 14. Multiple linear regression analysis was used to predict the growth potential, accounting for batch, level of stress exposure of the inoculum and inoculum concentration. The multiple regression model included predictors, which reached a p-value of < 0.2 in univariate regressions. The final model was determined by backward selection to obtain variables with p-values of < 0.05. Residuals were predicted and normality tested by using the Shapiro-Wilk test. Potential outliers were evaluated by their residual and leverage values.

Results

■ Growth potential of stressed and non-stressed *L. monocytogenes* in RTE chicken and sliced deli meat

L. monocytogenes strain mixtures were pre-exposed to either 0, 0.5 or 1% chlorinated alkaline disinfectant before inoculation of RTE chicken or sliced deli meat. The inoculum concentrations and the growth potentials of the *L. monocytogenes* strain mixtures in RTE chicken and sliced deli meat are given in Tables 4 and 5 and Figure 1. Growth of *L. monocytogenes* greater than 0.5 log CFU/g was observed for at least one of the challenge test repetitions within each category, except for the 0.5% stressed inoculum in RTE sliced deli meat.

■ Growth potential of *L. monocytogenes* in RTE chicken

Disinfectant exposure resulted in a concentration-dependent reduction in the growth potential of *L. monocytogenes* in chicken. A linear regression model, which included concentration of disinfectant and inoculum concentration as predictors for the growth potential estimations, revealed a significant effect of both inoculum concentration and the level of stress after 18 days of storage. The p-values were < 0.01 and 0.02, respectively, explaining 76% of the growth potentials (adj. R-squared). Potential batch variation had no significant effect on the growth potential for either of the products. A negligible batch effect is supported by the limited inter-batch variation observed for the food characteristics (Table 3). The inoculum concentration ($p = 0.02$) and the level of stress ($p < 0.01$) both had a significant effect on the final day of storage, explaining 70% of the growth potential estimates. There was an increased difference in growth potential in the RTE chicken between the stressed and non-stressed inoculum from the expiry date to eight days post-expiry.

TABLE 3 / Means and standard deviation (SD) of product characteristics for RTE chicken and RTE sliced deli meat, n=3.

Food characteristics	Batch	RTE chicken		RTE sliced deli meat	
		Mean	SD	Mean	SD
Total aerobic count, day 0 (Log CFU/g)	1, 2 and 3	1.75	0.23	1.91	1.08
pH, day 0		6.41	0.02	5.93	0.06
Water activity, day 0		0.98	0.00	0.97	0.01
Detection of *L. monocytogenes* in 25 g, day 0		ND[a]	ND	ND	ND
Total aerobic count, last day of storage (log CFU/g)	1, 2 and 3	6.86	0.37	7.07	0.66
pH, last day of storage		6.47	0.07	5.70	0.17
Water activity, last day of storage		0.98	0.00	0.97	0.00
Detection of *L. monocytogenes* in 25 g, last day of storage		ND	ND	ND	ND
Initial concentration of lactic acid bacteria (log CFU/g)	4, 5 and 6	1.70	0.19	1.90	0.44
NaCl (%)[b]		1.0	0.02	2.50	0.04
Dry matter (g/100 g)		34.9	0.67	37.2	0.59
pH		6.40	0.06	5.90	0.00
CO2 % in headspace gas at equilibrium		55.0	0.91	2.30	0.26
N2 % in headspace gas at equilibrium		45.0	0.92	97.7	0.28
O2 % in headspace gas at equilibrium		0.1	0.01	0.1	0.01
Nitrite (ppm)		<0.16	-	4.50	1.85
Acetic acid (ppm)[b]		307	17	981	40
Citric acid (ppm)[b,c]		56	6	-	-
Lactic acid (ppm)[b]		12096	82	17224	159

a Not detected (ND).
b In the aqueous phase of the product
c Values below the level of quantification (LOQ = 20) increased due to matrix effects and were <59, <99 and <63 for the three tested samples of sliced deli meat

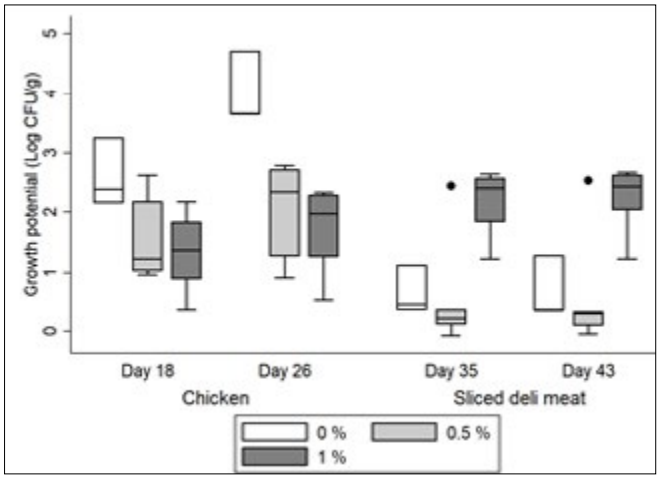

FIGURE 1 / Growth potentials of *L. monocytogenes* (log CFU/g) in RTE chicken and sliced deli meat at the expiry date (18 and 35 days, respectively) and eight days past the expiry date after exposure of the inoculum to 0, 0.5 and 1% chlorinated alkaline disinfection stress (n=3-5). Each box indicates the median (middle line in the box), the 25th (lower line of the box) and 75th (upper line of the box) percentiles, lower and upper adjacent value (single line) and outliers (dots)

Due to a potential compartment effect in solid foods, we compared the impact of disinfection on the growth parameters using BHI broth, representing more homogenous growth conditions than RTE chicken. The 1% stressed inoculum clearly demonstrated larger variance in *L. monocytogenes* concentration throughout the storage period, compared with the 0.5% stressed cells and the control (Figure 2).

■ Growth potentials of *L. monocytogenes* in RTE sliced deli meat

According to the linear regression model, disinfectant exposure resulted in a concentration-dependent increase in the growth potential in sliced deli meat (Table 4, 5 and Figure 1). In RTE sliced deli meat, stress and inoculum concentration had significant effects, with p-values of < 0.01 at both the expiry date and eight days past the expiry date and explained 89 and 86% of the observed growth potentials, respectively. One observation in the 0.5% stress exposed group for sliced deli meat (Figure 1) was defined as an outlier, because its residual value was 4.2 with a leverage of 0.8; it was therefore excluded from the regression model.

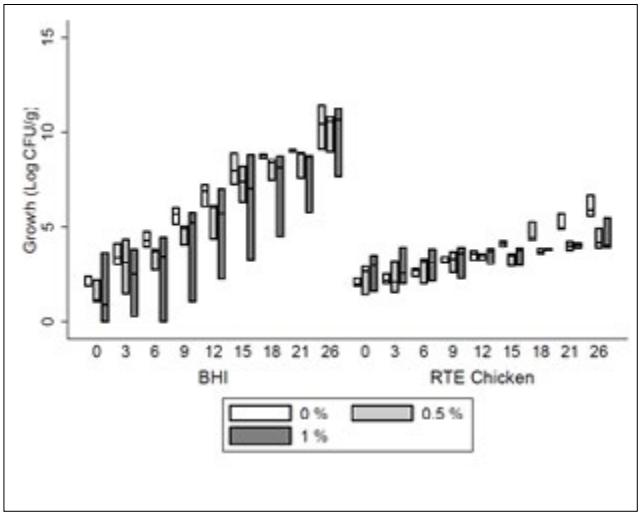

FIGURE 2 / Growth of a *L. monocytogenes* cocktail consisting of six strains (log CFU/g) in BHI broth and in RTE chicken from day 0 to day 26 (eight days post-expiry) after exposure of the inoculum to 0, 0.5 and 1% chlorinated alkaline disinfection stress (n=3-5). Each box indicates the median (middle line in the box) and the 25th (lower line of the box) and 75th (upper line of the box) percentiles. The high concentration of *L. monocytogenes* in BHI at day 26 may be due to sedimentation of cells.

TABLE 4 / Challenge test data on RTE chicken.

			Expiry date		Eight days past expiry date	
Batch[a]	Desinf.[b]	Day 0[c]	Day 18[c]	δ[d]	Day 26[c]	δ[e]
1	0	2.3	4.5	2.2	6.0	3.7
2	0	1.9	4.3	2.4	5.6	3.7
3	0	2.0	5.3	3.3	6.7	4.7
1	0.5	1.5	3.7	2.2	4.2	2.7
2	0.5	2.7	3.7	1.0	3.9	1.3
3	0.5	2.9	3.9	1.0	4.9	2.0
3	0.5	2.8	3.9	1.1	5.4	2.6
3	0.5	1.0	3.6	2.6	4.6	0.9
3	0.5	3.3	4.7	1.3	6.1	2.8
1	1	1.7	3.8	2.2	4.0	2.3
2	1	3.5	3.9	0.4	4.1	0.5
3	1	3.0	3.9	0.9	5.4	1.3
3	1	2.0	3.8	1.8	4.2	2.2
3	1	2.0	3.8	1.7	4.3	2.3
3	1	3.6	4.5	1.0	5.4	1.8

[a] The original batch
[b] Level of chlorinated alkaline stress (%)
[c] Concentration on day 0, 18 and 26 (log CFU/g)
[d] Growth potential at expiry date (δ, log CFU/g)
[e] Growth potential eight days past expiry date (δ, log CFU/g)

TABLE 5 / Challenge test data on RTE sliced deli meat.

			Expiry date		Eight days past expiry date	
Batch[a]	Desinf.[b]	Day 0[c]	Day 35[c]	δ[d]	Day43[c]	δ[e]
1	0	2.2	2.5	0.4	2.5	0.4
2	0	2.4	3.5	1.1	3.7	1.3
3	0	1.9	2.4	0.4	2.3	0.4
1	0.5	3.3	3.6	0.4	3.6	0.3
2	0.5	3.8	4.1	0.2	4.2	0.3
2	0.5	4.0	4.1	0.1	4.1	0.1
2	0.5	1.5	3.9	2.5	4.0	2.6
3	0.5	3.8	3.7	-0.1	3.8	0.0
1	1	2.5	3.7	1.2	3.7	1.2
2	1	1.6	4.0	2.4	4.1	2.4
2	1	1.3	3.9	2.6	4.0	2.7
2	1	1.3	4.0	2.7	3.9	2.6
3	1	1.8	3.6	1.8	3.8	2.0

[a] The original batch
[b] Level of chlorinated alkaline stress (%)
[c] Concentration on day 0, 35 and 43 (log CFU/g)
[d] Growth potential at expiry date (δ, log CFU/g)
[e] Growth potential eight days past expiry date (δ, log CFU/g)

Characteristics of the food

Prior to inoculation, *L. monocytogenes* was not detected in any of the challenge test batches used. Regarding potential alteration of the MAP during inoculation, the inoculation procedure was evaluated as satisfactory, as shown in Table 6. The aerobic viable count increased in the challenge test batches of RTE chicken from an average ± standard error of the mean (SEM) of 1.8 ± 0.1 log CFU/g on the day of inoculation to 6.9 ± 0.2 log CFU/g at eight days after the expiry date (Table 3). In RTE sliced deli meat, the aerobic viable counts increased from 1.9 ± 0.3 log CFU/g on the day of inoculation to 7.1 ± 0.2 log CFU/g at eight days after the expiry date (Table 3). The water activity was consistent in both product types during storage and was considered to have a stable impact on *L. monocytogenes* growth during storage: 0.98 in the RTE chicken and 0.97 in the RTE sliced deli meat (Table 3). The pH was also stable in both products: 6.4 ± 0.0 and 5.9 ± 0.0 on the day of inoculation and 6.5 ± 0.0 and 5.7 ± 0 eight days after the expiry date in RTE chicken and sliced deli meat, respectively (Table 3). Additional characteristics of one sample, from three different batches of RTE sliced deli meat and RTE chicken, are also shown in Table 3. The level of dry matter was 34.9 ± 0.4% in the RTE chicken and 37.2 ± 0.3% (average ± SEM) in the RTE sliced deli meat (Table 3), and was used as input for the FSSP to calculate organic acid concentrations in the water phase of the products.

TABLE 6 / Control measurements of modified atmosphere packaging (MAP) 3 days after inoculation, means and standard deviation (SD), n =3, for CO_2, N_2 and O_2.

	CO_2 (%)		N_2 (%)		O_2 (%)	
	Mean	SD	Mean	SD	Mean	SD
RTE chicken, inoculated	56.1	0.5	43.9	0.5	0.0	0.0
RTE chicken, non-inoculated	53.8	0.6	46.2	0.6	0.1	0.1
RTE sliced deli meat, inoculated	2.6	0.4	97.3	0.4	0.1	0.0
RTE sliced deli meat, non-inoculated	2.4	0.1	97.5	0.1	0.1	0.0

Discussion

General considerations of the growth potential estimations

Maximising food safety and product shelf life and determining criteria for the withdrawal of foods require a correct estimation of the growth potential of *L. monocytogenes* in RTE foods at realistic production conditions. This study compared *L. monocytogenes* growth in RTE foods with a traditional inoculum preparation method and an alternative method including an environmental stressor, frequently used in food processing facilities. This new protocol for inoculum preparation was assumed to simulate a more realistic contamination route than the standard prepared inoculum for challenge testing. The "*Listeria* shelf life" of specific foods is often set as the time needed for a 2 log CFU/g increase in the *L. monocytogenes* level, based on the assumption that the concentration of *L. monocytogenes* is 1 CFU/g immediately after contamination. According to the guidelines for challenge tests (Beaufort *et al.*, 2014), the most important factors affecting *Listeria* shelf life and bacterial growth potential in food products are the properties of the inoculated strain(s), the inoculation level, the physiological state of the inoculated bacterial cell(s), the intrinsic properties of the food (*e.g.* pH, NaCl content, aw, associated microflora and antimicrobial constituents) and the extrinsic properties (*e.g.* time-temperature profile, gas atmosphere and moisture). The possible impact of these properties is assessed below.

All strains in the current study were either strains isolated from similar food products, or laboratory strains. Due to the food-matrix-specific composition of strains, to prepare a representative inoculum, only four of the strains were represented in both inoculums. Due to the different strains in the two products, the observed growth kinetic data for the two products cannot be compared and must be assessed separately.

As expected, the inoculation concentration significantly influenced the estimation of growth, and the inoculation concentration thereby had to be included in the linear regression analysis when analysing factors affecting growth potential. In contrast to naturally contaminated samples, where starting inoculum concentrations usually are below 1 log CFU/g (Beaufort *et al.*, 2007; Mejlholm *et al.*, 2015a; Pouillot *et al.*, 2007; Skjerdal *et al.*, 2014), the current study used starting concentrations between 1 and 4.0 log CFU/g. The European guidelines recommend a contamination level of 100 CFU/g in the food. However, one study claimed that the bacterial cell-to-cell variability has serious consequences for the challenge test design and that the inoculum concentration should be 3 log CFU/g to reach an acceptable level of variability and a consistent estimation of pathogen behaviour (Francois *et al.*, 2006). A low initial concentration of *L. monocytogenes* may also reduce growth potential due to lower ability to compete with the background flora and to the Jameson effect (Mellefont *et al.*, 2008). On the other hand, high inoculum concentrations may reduce the duration of the exponential growth phase, which may also lead to underestimation of growth potential (Francois *et al.*, 2006; Lardeux *et al.*, 2015). In the present study, the stressed inoculum concentration was challenging to standardise, despite preliminary studies and efforts to standardise the inoculum procedure. The protocol used in the current study may therefore introduce a bias to the growth potential estimates, as shown by the current results and by others (*e.g.* McManamon *et al.*, 2017). The stress exposure through the addition of a disinfectant agent in the current study likely introduced other caveats. However, removing any residual disinfectant agent by centrifugation prior to inoculation would not be representative of an environmental contamination route. Another alternative would be to use a reagent that chemically neutralises the stressor effect, but this is unrealistic for a natural contamination route and residuals from the neutralisers, which are not naturally present in the food industry plants, may potentially interact with the pathogen and the food matrix. Thus, it is difficult to make a stressed inoculum that contains a predictable concentration of bacteria and that is also representative of a realistic contamination route.

In addition to imposing increased stress on the inoculum, the disinfectants may have raised the pH of the food matrices, at least locally, which may have influenced the bacterial growth potential in the foods. If so, this pH effect can explain the contrasting effects of disinfectants in the chicken meat and in the sliced deli meat. Despite a 10-fold dilution of the disinfectant, disinfectant residues may interfere differently with the intrinsic qualities of the RTE chicken matrix compared with the sliced deli meat matrix. For example, the pH ranged by one unit between the stressed and non-stressed inoculum; thus the pH may change in the food matrix due to a carry-over effect of the alkaline disinfectant. The pH of chicken meat is closer to the optimum pH for growth of *L. monocytogenes* than the pH of sliced deli meat. A slightly increased pH due to the carry-over of disinfectants from the stressed inoculum could therefore lower the growth potential in chicken meat due to stress imposed on the bacteria, but give more favourable growth conditions in sliced deli meat due to a more favourable pH. However, higher pH in microenvironments in the inoculated samples due to carry-over of disinfectant was not possible to measure, because larger sample sizes are needed for pH measurements.

The decreasing growth potential in the RTE chicken may be due to a reduced physiological state of the bacterial cells after chlorinated alkaline stress (Eom *et al.*, 2009). Eom *et al.* (2009) reported that exposure to sodium hypochlorite affects both the lag-phase and the specific growth rate of *L. monocytogenes* strains. At 4°C, 75 ppm of sodium hypochlorite results in a higher specific growth rate than pre-exposure to 25 ppm, in broth and in a food matrix simulating crab meat (Eom *et al.*, 2009).

The food-specific intrinsic qualities also affect the behaviour of *L. monocytogenes* (Mejlholm *et al.*, 2015). An important difference between the RTE chicken and the sliced deli meat matrices is sodium nitrite, which is present in the sliced deli meat, but not in the chicken. Castellani and Niven (1955) showed that the bacteriostatic effect of nitrite was inversely proportional to pH. The chlorinated alkaline disinfectant may confer a local pH increase in the sliced deli meat during the inoculation procedure and thereby reduce the negative effect of nitrite and organic acids on the growth of *L. monocytogenes*.

The water activity and pH values were also consistent in both product types during storage. The variations in food characteristics in the current study are therefore not likely to provide any significant bias to the results.

■ Growth kinetics in RTE chicken versus BHI broth after stressing the inoculum

Due to the matrix effect in complex food products such as RTE chicken, it is also useful to analyse the effect of disinfection upon the growth in BHI broth, which is a more homogenous substrate. Therefore, RTE chicken and BHI broth were inoculated in parallel to compare the effect of disinfectant on growth of *L. monocytogenes*. The 1% stressed cells in broth clearly demonstrated larger variance in the concentration of *L. monocytogenes* than the 0.5% stressed cells and the controls throughout the storage period. The challenge of standardising the inoculum concentration when stressing the cells with disinfection was thereby further confirmed throughout the storage period (Figure 2).

■ Impact of sub-lethal stress on product categorisation according to EU microbiological criteria

According to the results from the current study, the level of stress imposed by the chlorinated alkaline disinfectant would not affect how the regulations categorise RTE chicken depending on growth potential. Except for one outlier in the 0.5% category of RTE sliced deli meat, all inoculated batches demonstrated *L. monocytogenes* growth levels of 0.5 log CFU/g or more, and are thereby categorised as “Ready to eat foods, able to support the growth of *L. monocytogenes* other than those intended for infants and special medical purposes” (Category 1.2) (European Commission, 2005). Nevertheless, the effect of disinfectant stress on the inoculum would affect how the food business operator would define a proper shelf life for this product.

The effect of chlorinated alkaline stress prior to contamination may have the potential to significantly alter the estimated concentration of *L. monocytogenes* in food products, thereby affecting food safety, food waste and the economy of the food industry. Further studies should be performed using single strains to determine the effect of stress imposed by disinfectants upon lag-phases and maximum growth rates of *L. monocytogenes* in food products.

Conclusion

The current study revealed significant changes in the growth potential of *L. monocytogenes* when the inoculum was pre-exposed to a commercial alkaline disinfection agent, consequently affecting the shelf life in terms of food safety. However, it is not clear whether the effect of the disinfectant was related to increased pH in the food matrix due to carry-over into the food matrix or to a change in the physiological condition of the inoculated bacteria. Furthermore, the significant impact of the inoculum concentration on growth potential and the challenge standardising the day 0 contamination levels indicate that the alternative method for inoculum preparation including stress is not recommended when performing traditional challenge tests with *L. monocytogenes* in RTE chicken and sliced deli meat.

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