Food Research International 134 (2020) 109253

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Variability in lag-duration of *Campylobacter* spp. during enrichment after cold and oxidative stress and its impact on growth kinetics and reliable detection

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ARTICLE INFO

Keywords:
Baranyi-model
ISO 10272-1:2017
Bolton broth
Campylobacter jejuni
Campylobacter coli
Sub-lethal injury
Bacterial growth curve

ABSTRACT

Campylobacter jejuni and *Campylobacter coli* continue to be the leading cause of zoonotic gastroenteritis in the European Union, making reliable detection in food important. Low storage temperatures and atmospheric oxygen concentrations during food production can cause sub-lethal damage or transient non-culturability which is why ISO 10272-1:2017 includes an enrichment step to repair cell damage and increase cell concentrations, thereby supporting detection of campylobacters from foods.

The aim of this study was to assess the variability in lag-duration of *C. jejuni* and *C. coli* during enrichment after different food-relevant stress treatments and evaluate its impact on growth kinetics and reliability of detection outcomes. Therefore, 13 *C. jejuni* and 10 *C. coli* strains were subjected to cold stress during refrigerated and frozen storage. Refrigerated storage did not significantly reduce culturability, but frozen storage reduced cell concentrations by 1.6 ± 0.1 $\log_{10}$cfu/ml for both species.

Subsequently, cells were enriched following ISO 10272-1:2017-A and cell concentrations were determined over time and lag-duration and growth rate were determined by fitting the Baranyi-model. Without prior stress treatment, mean lag-duration for *C. jejuni* and *C. coli* was 2.5 ± 0.2 h and 2.2 ± 0.3 h, respectively. Refrigerated storage increased lag-duration for *C. jejuni* to 4.6 ± 0.4 h and for *C. coli* to 5.0 ± 0.4 h and frozen storage increased lag-duration to 5.0 ± 0.3 h and 6.1 ± 0.4 h for *C. jejuni* and *C. coli*, respectively.

Comparison of strain- and biological variability showed that differences in recovery after cold stress can be attributed mainly to strain variability since strain variability after refrigeration and freeze stress increased respectively 3-fold and 4-fold while biological variability remained constant.

A subset of strains was also subjected to oxidative stress that reduced cell concentrations by 0.7 ± 0.2 $\log_{10}$ cfu/ml and comparison of recovery patterns after oxidative and freeze stress indicated that recovery behaviour was also dependent on the stress applied. A scenario analysis was conducted to evaluate the impact of heterogeneity in outgrowth kinetics of single cells on the reliability of detection outcomes following ISO protocol 10272-1:2017. This revealed that a 'worst-case'-scenario for successful detection by a combination of the longest lag-duration of 7.6 h and lowest growth rate of 0.47 h^{-1} still resulted in positive detection outcomes since the detection limit was reached within 32.5 h. This suggests that other factors such as competitive microbiota can act as a causative factor in false-negative outcomes of tested food samples.

1. Introduction

Campylobacter spp. are Gram-negative, microaerophilic, spiral-shaped rods that live as commensals in the intestinal tract of predominantly birds, but also sheep, cattle and pigs, cats and dogs and they can survive in environmental waters and even sand (Jones, 2001; Ogden et al., 2009; Vandamme & De Ley, 1991). *Campylobacter* spp. continues to be the leading cause of registered zoonotic gastroenteritis in the European Union (EU) for more than 10 years, with approximately 84% and 10% of campylobacteriosis cases caused by *Campylobacter jejuni* and *Campylobacter coli*, respectively (European Food Safety Authority, 2019).

Abbreviations: BB, Bolton broth; CAB, Columbia Blood Agar; ESBL, Extended-spectrum beta-lactamase; EU, European Union; FS, Freeze stress; HI, Heart Infusion broth; ISO, International Organization for Standardization; RC, Reference condition; RS, Refrigeration stress; ST, Sequence type

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https://doi.org/10.1016/j.foodres.2020.109253
Received 27 November 2019; Received in revised form 26 March 2020; Accepted 16 April 2020
Available online 21 April 2020

Thermotolerant campylobacters need at least a temperature as high as 30 °C to grow but grow optimally at a temperature range of 37–42 °C and require an atmosphere with reduced oxygen levels of 3–5% (Adams & Moss, 2014; Mead, 2004). This often leads to the conclusion that campylobacters seem to be fragile, also since they appear to be much more sensitive to environmental conditions compared to other food-borne pathogens (Jasson, Uyttendaele, Rajkovic, & Debevere, 2007; Mihaljevic et al., 2007). Indeed, it has been shown that *Campylobacter* spp. are highly susceptible to desiccation, low pH and heat stress even below 60 °C (Chaveerach, Ter Huurne, Lipman, & Van Knapen, 2003; Nguyen, Corry, & Miles, 2006; Oosterom, De Wilde, De Boer, De Blaauw, & Karman, 1983). During food production, *Campylobacter* spp. can be exposed to increased atmospheric oxygen concentrations, which can lead to the production of reactive oxygen species (ROS), which can cause growth arrest and damage of the cell membrane and important proteins within the cell (Gundogdu et al., 2016; Imlay, 2003; Klančnik et al., 2009). Removal of ROS is mediated by several enzymes including superoxide dismutase (SOD), Catalase (KatA), cytochrome *c* peroxidase and alkyl hydroperoxide reductase (Ahp) (van Vliet, Ketley, Park, & Penn, 2002). It has been shown that *Campylobacter* spp. cannot grow below 30 °C, partly due to a lack of cold shock proteins (Bhaduri & Cottrell, 2004; Hazeleger, Wouters, Rombouts, & Abee, 1998; Parkhill et al., 2000). Studies on the effect of freeze stress and survival of *Campylobacter* spp. during storage at temperatures of −20 °C and lower showed that decrease in cell concentration was highest at the begin of frozen storage and remained relatively stable for several weeks, which probably can be attributed to the formation of ice crystals during the initial stage of freezing (Bhaduri & Cottrell, 2004; Georgsson, Thornorkelsson, Geirsdottir, Reiersen, & Stern, 2006; Lee, Smith, & Coloe, 1998; Maziero & De Oliveira, 2010).

Although *Campylobacter* spp. are generally unable to multiply outside the animal host, it has been shown that cells are able to survive long-term under adverse environmental conditions (Lee et al., 1998; Park, 2002; Sopwith et al., 2008; Wilson et al., 2008). So, even though campylobacters have to endure oxidative stress and unfavourably low temperatures during transport and storage of food products, cells have shown to be able to survive the hostile environment (Sampers, Habib, De Zutter, Dumoulin, & Uyttendaele, 2010) and possibly cause disease. This, together with the high potential to cause illness (Black, Levine, Clements, Hughes, & Blaser, 1988) increases the importance of a sensitive, yet selective detection method to verify food safety measures.

Currently, the protocol of the International Organization for Standardization (ISO), ISO 10272-1:2017 is applied in the European Union for the detection of *Campylobacter* spp. from foods. It consists of three procedures, two of which contain selective enrichment to amplify cells to a detectable level (procedures A and B). Procedure A uses Bolton Broth and is applied when injured campylobacters are expected in the food product whereas procedure B uses Preston Broth and is applied when high amounts of background microflora are expected (International Organization for Standardization, 2017). Regardless of the procedure chosen, growth initiation might be preceded with a lag-phase (Zwietering, Jongenburger, Rombouts, & Van 't Riet, 1990). The lag-duration is not only dependent on the severity of stress a cell endured prior to enrichment, but can also be affected by strain-dependent differences in robustness and ability to recover from stress (Booth, 2002; Jasson et al., 2007). Strain variability has been previously described by Whiting and Golden (2002) as an inherent property of microorganisms which cannot be reduced when strains undergo identical treatments under the same conditions. Next to strain variability, also biological variability has to be considered. Aryani, Den Besten, Hazeleger, and Zwietering (2015) define biological or reproduction variability as the difference between independently reproduced experiments of the same strain performed on different experimental days from new pre-cultures and newly prepared media (Aryani et al., 2015). Both strain variability and biological variability can have an impact on reliable detection of *Campylobacter* spp. when following ISO protocols, since increased recovery duration in enrichment might lead to false-negative outcomes.

In this study, the effect of different food-relevant stresses on the lag-duration of 23 *Campylobacter* isolates was assessed. Biological and strain variabilities in the obtained lag-duration λ were quantified and compared.

Furthermore, a scenario analysis and Monte Carlo simulations on outgrowth kinetics of sub-lethally injured *Campylobacter* spp. during enrichment were conducted to evaluate the impact of strain variability in recovery and growth kinetics on the reliability of detection outcomes following ISO protocol 10272-1:2017.

2. Materials and methods

2.1. Bacterial strains and preparation of stationary phase cultures

A selection of 13 *Campylobacter jejuni* and 10 *Campylobacter coli* strains of different origin (human, food and environmental isolates) and sequence type (ST) was collected (details can be found in Table S1 of the supplementary materials). Whenever possible, clonal complexes or STs frequently associated with disease were selected (Colles & Maiden, 2012; Dearlove et al., 2016). *Campylobacter* stock cultures were grown in Heart Infusion broth (HI, Bacto HI, Becton, Dickinson and Company) for 24 h at 41.5 °C, then supplemented with 15% glycerol (Fluka) and stored at −80 °C. To obtain pre-cultures for stress and enrichment experiments, *C. jejuni* and *C. coli* were plated from the −80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, Netherlands) and 0.5% agar (Bacteriological agar No.1, Oxoid)) and grown microaerobically for 24 h at 41.5 °C. Subsequently, single colonies were re-suspended in HI and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterwards, a 1:500 dilution was made in unselective Bolton broth (BB, Oxoid, supplemented with 5% (v/v) sterile lysed horse blood (BioTrading Benelux B.V., Mijdrecht, Netherlands without the addition of selective supplements) and cultured for 24 h at 41.5 °C to reach the stationary phase. For the application of oxidative stress, cultures were prepared by diluting the culture grown in HI in a ratio of 1:500 in Bolton broth (BB, Oxoid) without supplementation of horse blood and antibiotics and this culture was subsequently grown for 24 h at 41.5 °C. Cell concentrations were determined by plating appropriate dilutions on CAB. All cells were cultured under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) in flushed jars (Anoxomat WS9000, Mart Microbiology, Drachten, Netherlands) unless stated otherwise.

2.2. Application of stress treatments

2.2.1. Refrigeration stress and freeze stress

All 23 strains cultured to the stationary phase in unselective Bolton broth with the addition of 5% sterile lysed horse blood were decimally diluted in peptone physiological salt solution (PPS, Tritium Microbiologie) to a cell concentration of approximately 10^4 cfu/ml for application of freeze stress and 10^3 cfu/ml for application of refrigeration stress (considering the difference in stress severity and consequent difference in reduction in cell viability, aiming at a cell concentration of approx. 10^3 cfu/ml after application of stress) and inoculated 1:6 in 5 ml of Bolton broth with addition of 5% (v/v) sterile lysed horse blood and without addition of selective supplements in 15 ml plastic tubes (Greiner centrifuge tubes, Merck). Tubes were placed standing upright at −20 °C or 4 °C for 64 ± 1 h for frozen and refrigerated storage, respectively. Afterwards, refrigeration-stressed cultures were transferred to room temperature and allowed to warm up for 10 min. Freeze-stressed cultures were transferred to room temperature and allowed to stand until defrosted. Cell concentrations after both stresses were determined by plating appropriate dilutions on CAB and stress-induced reduction in cell numbers was calculated.

2.2.2. Combined mild refrigeration and atmospheric oxygen stress

A selection of strains, namely *C. jejuni* strains WDCM 00005 and 81-176 and *C. coli* strains Ca 2800 and WDCM 00004 were exposed to atmospheric oxygen. Cultures grown to the stationary phase in unselective Bolton broth (without the addition of sterile lysed horse blood) were diluted to 10^4 cfu/ml and inoculated 1:10 in 27 ml of unselective Bolton broth without supplementation of sterile lysed horse blood in sterile 250 ml Erlenmeyer flasks with cotton stopper. Samples were incubated at 12 °C for 64 ± 1 h at 160 rpm shaking conditions to induce atmospheric oxygen and mild refrigeration stress. Cell concentrations after atmospheric oxygen stress were determined by plating appropriate dilutions on CAB and stress-induced reduction in cell numbers was calculated.

2.3. Quantification of growth parameters during enrichment

Infusion bottles were filled with 42 ml of Bolton Broth, closed with a rubber stopper and aluminium cap and sterilized. Subsequently, bottles were supplemented with 5% sterile horse blood and 450 µl of the selective enrichment supplement (Oxoid SR0208E). Bottles were filled with 5 ml of either reference or stressed cultures, resulting in a starting cell concentration in the enrichment broth of approximately 2 $\log_{10}$ cfu/ml. Additions of fluids to sterilized infusion bottles was achieved using syringes to puncture the rubber stopper of the bottles. The head space of infusion bottles was flushed for 2 min with a gas-mixture of 5% O_2, 10% CO_2 and 85% N_2 by a home-made gas flushing device using syringes to puncture the rubber stopper. Inoculated infusion bottles were incubated in water baths at 37 °C for the first 5 h and subsequently transferred to 41.5 °C for the remaining 43 h following ISO 10272-1:2017. At regular time intervals, 1–2 ml samples were taken from the bottles using a syringe and after each second sample, bottles were flushed again with the appropriate gas mixture. Samples were immediately decimally diluted in PPS, plated onto CAB and incubated for 48 h at 41.5 °C. Two biologically independent reproductions per strain and stress treatment were performed on different days.

2.4. Model fitting to estimate growth parameters during enrichment

Plate counts were transformed to $\log_{10}$ cfu/ml and growth curves were constructed using Microsoft Excel 2010. Growth curves were fitted with the modified Gompertz model (Zwietering et al., 1990), the three-phase model (Buchanan, Whiting, & Damert, 1997) and Baranyi-model (Baranyi & Roberts, 1994) using the Solver add-in of Excel. The models were ranked based on the mean square error of the model (MSE_{model}) previously described by (den Besten, Mataragas, Moezelaar, Abee, & Zwietering, 2006).

Baranyi-model:

$$\log_{10} N(t) = \log_{10} N(0) + \frac{\mu}{\ln(10)} \cdot A(t) - \frac{1}{\ln(10)} \cdot \ln\left[1 + \frac{\exp[\mu \cdot A(t)] - 1}{10^{[\log_{10} N(\max) - \log_{10} N(0)]}}\right] \quad (1)$$

$$A(t) = t + \frac{1}{\mu} \cdot \ln[\exp(-\mu \cdot t) + \exp(-\mu \cdot t_{lag}) - \exp(-\mu \cdot t - \mu \cdot t_{lag})] \quad (2)$$

t =	elapsed time during enrichment (h)
$\log_{10} N(t)$ =	population at time t ($\log_{10}$ cfu/ml)
$\log_{10} N(0)$ =	initial cell population ($\log_{10}$ cfu/ml)
μ =	maximum specific growth rate (h^{-1})
$\log_{10} N(\max)$ =	final population ($\log_{10}$ cfu/ml)
t_{lag} =	lag-duration of the growth curve (h)

Modified Gompertz model:

$$\log_{10} N(t) = \log_{10} N(0) + (\log_{10} N(\max) - \log_{10} N(0)) * \exp\left\{-\exp\left[\frac{\frac{\mu}{\ln(10)} * \exp(1)}{\log_{10} N(\max) - \log_{10} N(0)} * (\lambda - t) + 1\right]\right\} \quad (3)$$

t =	elapsed time during enrichment (h)
$\log_{10} N(t)$ =	population at time t ($\log_{10}$ cfu/ml)
$\log_{10} N(0)$ =	initial cell population ($\log_{10}$ cfu/ml)
μ =	maximum specific growth rate (h^{-1})
$\log_{10} N(\max)$ =	final population ($\log_{10}$ cfu/ml)

Three-phase linear model:

Lag Phase: For $t \le t_{lag}$, $\log_{10} N(t) = \log_{10} N(0)$ (4)

Exponential Growth Phase: For $t_{lag} < t < t_{\max}$, $\log_{10} N(t) = \log_{10} N(0) + \frac{\mu}{\ln(10)}(t - t_{lag})$ (5)

Stationary Phase: For $t \ge t_{\max}$, $\log_{10} N(t) = \log_{10} N(\max)$ (6)

t =	elapsed time during enrichment (h)
t_{lag} =	time when lag-duration ends (h)
t_{max} =	time until maximum population density is reached (h)
$\log_{10} N(t)$ =	population at time t ($\log_{10}$ cfu/ml)
$\log_{10} N(0)$ =	initial cell population ($\log_{10}$ cfu/ml)
μ =	maximum specific growth rate (h^{-1})
$N(max)$ =	final population ($\log_{10}$ cfu/ml)

The two-tailed *t*-test was used to evaluate the statistical significance of differences in the reduction after stress treatments between species as well as differences in lag-duration of cultures during enrichment at different conditions ($p < 0.05$).

To evaluate the increase in lag-duration due to stress pre-treatment, the mean lag-duration derived from enrichments of stressed cells was subtracted from the mean lag-durations derived from enrichments of the reference condition following equation (7).

$$\Delta_{lag} = \left(\frac{\lambda_{stressrep.1} + \lambda_{stressrep.2}}{2}\right) - \left(\frac{\lambda_{ref.conditionrep.1} + \lambda_{ref.conditionrep.2}}{2}\right) \quad (7)$$

$\lambda_{\text{stress rep.1}}$ = lag-duration (h) after stress of reproduction 1
$\lambda_{\text{stress rep.2}}$ = lag-duration (h) after stress of reproduction 2
$\lambda_{\text{ref.condition rep.1}}$ = lag-duration (h) in reference condition of reproduction 1
$\lambda_{\text{ref.condition rep.2}}$ = lag-duration (h) in reference condition of reproduction 2

2.5. Quantifying biological and strain variability

To quantify variability, methods previously described by Aryani et al. (2015) were applied. Biological and strain variability were calculated for all strains and histories according to equations (8) and (9).

Biological variability:

$$MSE_{Biological} = \frac{RSS}{df} = \frac{\sum_{S=1}^{23} \sum_{R=1}^{2} (\lambda_{RS} - \lambda_S)^2}{n - p} \quad (8)$$

MSE =	mean square error
λ_{RS} =	lag-duration (h) of each biological reproduction "*R*" and strain "*S*"
λ_S =	average lag-duration (h) of λ_{RS} from two biological reproductions for strain "*S*"
df =	no. of data points (n = 2*23) minus the number of parameters (p = 1*23)

Strain variability:

$$MSE_{Strain} = \frac{RSS}{df} = \frac{\sum_{S=1}^{23} (\lambda_S - \bar{\lambda})^2}{n - p} \quad (9)$$

λ_S =	average lag-duration (h) of λ_{RS} from two biological reproductions for strain "*S*"
$\bar{\lambda}$ =	average lag-duration (h) of all 23 strains
df =	no. of data points (n = 23) minus the number of parameters (p = 1)

The *F*-test was used to compare biological and strain variability in lag-duration during enrichment of the reference condition, as well as variability in lag-duration during enrichment after refrigeration stress

and freeze stress. Data was considered significantly different at a p-value of 0.05 or lower.

2.6. Predictive modelling for scenario analysis

A three-phase linear model (Buchanan et al., 1997) was used to predict the bacterial growth curve during enrichment and to identify factors which could lead to false-negative detection outcomes. Equations (4) until 6 were used.

By varying different biological parameters such as the initial cell concentration ($\log_{10} N_0$), lag-duration (λ) and bacterial growth rate (μ) a scenario analysis was conducted to assess when/if the detection level set to 3 $\log_{10}$ cfu/ml was reached. The initial cell concentration was set to -2 $\log_{10}$ cfu/ml at the beginning of enrichment to mimic a scenario wherein 10 g of food product containing 1 cell is mixed with 90 ml of enrichment broth. The maximum cell concentration ($\log_{10} N_{max}$) was set to 9 $\log_{10}$ cfu/ml. By varying the duration of recovery λ (h) and growth rate μ (h^{-1}), predictions on growth after freeze stress and worst-case scenario analyses were conducted. Variations in lag duration λ were simulated by using the mean lag-duration derived after model fitting of growth kinetics during enrichment after freeze-stress of 23 strains (5.4 h) as well as the mean value with subtraction and addition of two standard deviations (± 2.2 h). For variation of growth rate μ, the mean values (0.93 h^{-1}) was used as well as the mean value with subtraction and addition of two standard deviations (± 0.47 h^{-1}).

The analysis was conducted using Microsoft Excel 2010 using the Solver add-in. Furthermore, the risk of false-negative detection outcomes after enrichment following ISO 10272-1:2017 procedure A was determined by means of a Monte Carlo simulation using the @RISK version 7.5 (Palisade Corporation) add-in in Microsoft Excel 2010. The impact of changes in μ and λ on the ability to reach the detection limit of 3 $\log_{10}$ cfu/ml was assessed. The parameters μ and λ were estimated to be normal-distributed with the calculated standard deviations from all reproductions for refrigerated and freeze-stressed strains. To determine the risk of false-negative detection outcomes of freeze-stressed cells after 48 h of enrichment in Bolton broth, simulations were done with 100.000 iterations using Latin Hypercube sampling in combination with a Mersenne twister random number generator.

3. Results

3.1. Reduction in cell concentration of C. jejuni and C. coli after cold stress

The reduction in cell concentrations of 13 *C. jejuni* and 10 *C. coli* strains after 3 days of refrigerated and frozen storage at +4 °C and −20 °C, respectively, were determined and are presented in Fig. 1. The reductions of the *C. jejuni* strains after refrigerated storage ranged from none to 0.2 $\log_{10}$ cfu/ml with an average for the 13 strains of 0.1 ± 0.02 $\log_{10}$ cfu/ml. *C. coli* showed a similar reduction ranging from none to 0.3 $\log_{10}$ cfu/ml with an average of the 10 strains of 0.1 ± 0.03 $\log_{10}$ cfu/ml. A two-tailed *t*-test showed no significant differences in reduction between the two species after refrigeration ($p = 0.61$).

Storage at −20 °C resulted in a mean reduction of 1.5 ± 0.05 $\log_{10}$ cfu/ml with reduction ranging from 0.9 to 1.7 $\log_{10}$ cfu/ml for *C. jejuni*. For *C. coli* reductions ranged from 1.1 to 2.1 $\log_{10}$ cfu/ml with an average of 1.7 ± 0.09 $\log_{10}$ cfu/ml. A two-tailed *t*-test showed a rather similar, but just significantly different reduction after frozen storage for both species ($p = 0.04$).

3.2. Lag-duration of C. jejuni and C. coli during enrichment after different treatments

Lag-duration during enrichment of 13 *C. jejuni* and 10 *C. coli* strains without prior stress treatment as well as after refrigerated and frozen storage were determined by fitting the growth curves with the Baranyi-model. Initially, data were fitted with three different growth models; the Baranyi-model (Baranyi & Roberts, 1994), three-phase model (Buchanan et al., 1997) and modified Gompertz model (Zwietering et al., 1990). The reason for this was to assess and select the model which showed the best fit overall for the experimental data collected. Overall, the three-phase model showed the worst fit (highest MSE for 55% of the fittings), followed by the modified Gompertz model (highest

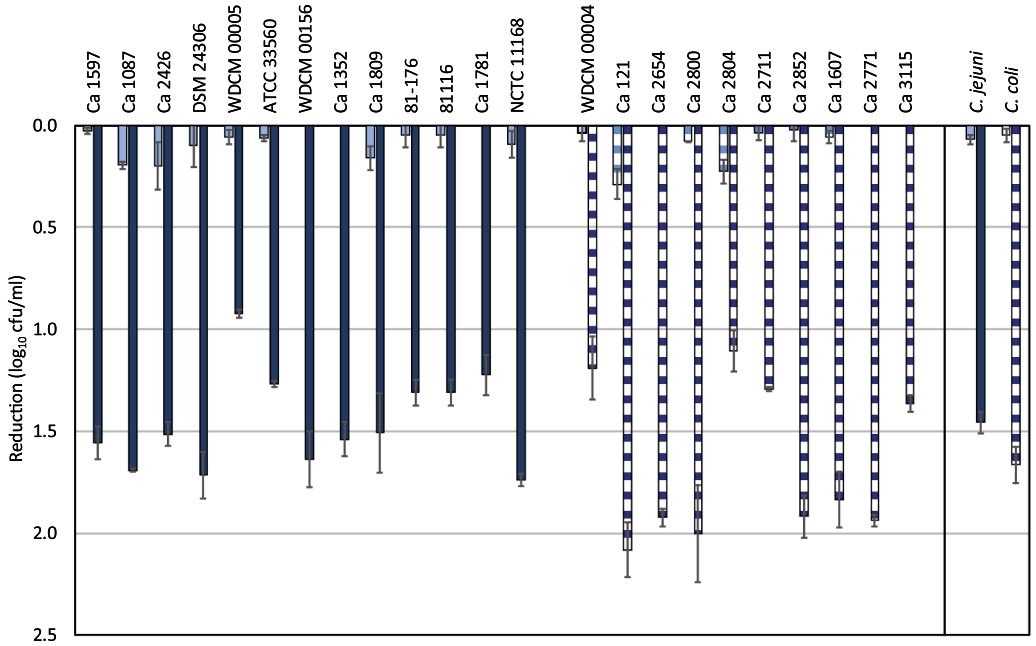

Fig. 1. Reduction in cell concentration of *C. jejuni* (filled bars) and *C. coli* (striped bars) after refrigerated storage (light blue coloured bars) and frozen storage (dark blue coloured bars). Error bars depict the standard error of the biological reproductions (n = 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MSE_{model} for 25% of the fittings), and the Baranyi-model (highest MSE_{model} for 20% of the fittings). Because the fitting performances of the Baranyi and the modified Gompertz models were rather comparable, all data sets were fitted with both models and lag-duration estimates were compared. Outcomes of this analysis revealed that the model choice did not have a significant influence on the estimated lag-duration of any group ($p = 0.06$ for lag of reference cells, $p = 0.36$ for lag of refrigeration-stressed cells and $p = 0.13$ for lag of freeze-stressed cells). Since the Baranyi model gave the best fit overall, this model was chosen for data representation. Mean lag-duration for each strain and after each history is depicted in Fig. 2. Without prior stress treatment, mean lag-duration for *C. jejuni* and *C. coli* was 2.5 ± 0.2 h and 2.2 ± 0.3 h, respectively. Refrigerated storage increased mean lag-durations for *C. jejuni* to 4.6 ± 0.4 h and for *C. coli* to 5.0 ± 0.4 h. Frozen storage led to a longer lag-duration for both species, with a mean lag-duration of 5.0 ± 0.3 h and 6.1 ± 0.4 h for *C. jejuni* and *C. coli*, respectively. A trend for interspecies differences in lag-duration could only be seen after frozen storage ($p = 0.02$).

3.3. Quantifying variability

Fig. 3 shows the calculated biological and strain variability using the lag-duration determined for 23 strains and two reproductions per strain after three different experimental treatments; without prior stress (reference condition), after refrigeration stress and after freeze stress. Comparison of biological variability between the reference condition and refrigeration stress (just significant, $p = 0.03$), reference condition and freeze stress ($p = 0.11$) as well as comparison between the biological variability of both stress treatments ($p = 0.76$) indicated that biological variability remained relatively constant for all three treatments.

Strain variability increased as the severity of stress increased, namely approximately three times higher compared to the reference condition after refrigerated storage and approximately four times higher after frozen storage. Comparison of strain variability between the reference condition and stress treatments showed significant differences ($p \ll 0.001$, for both, refrigeration and freeze stress). Biological and strain variability were almost equal during enrichment without prior stress ($p = 0.03$). After refrigeration and freeze stress, strain variability was significantly higher than reproduction variability ($p \ll 0.001$, in both cases). Notably, similar conclusions could be made when the lag durations were estimated using the modified Gompertz model (see Fig. S1 of the supplementary materials).

3.4. Effect of stress history on recovery during enrichment

After determination of lag-duration after freeze stress, one fast- and one slow-recovering strain of each species were selected to investigate whether trends in lag-duration are similar after application of combined mild refrigeration and atmospheric oxygen stress. Strain selection was made after comparing the lag-duration of reference cells with the lag-duration of freeze-stressed cells. For *C. jejuni*, strain WDCM 00005 was chosen as a fast-recovering strain and strain 81-176 was selected as a slow-recovering strain. For *C. coli*, strain Ca 2800 showed the fastest recovery after freeze-stress, while strain WDCM 00004 was chosen as a slow-recovering strain. Fig. 4 depicts the described difference in lag-durations in enrichment of the four selected strains after oxidative and freeze stress. Oxidative stress reduced cell concentrations by 0.7 ± 0.2 log_{10} cfu/ml on average, while freezing resulted in a mean decrease of 1.4 ± 0.5 log_{10} cfu/ml (corresponding data is displayed in sFig. S3 of the supplementary material). Visualization of lag-duration after the different stresses allowed comparison of stress-dependent recovery

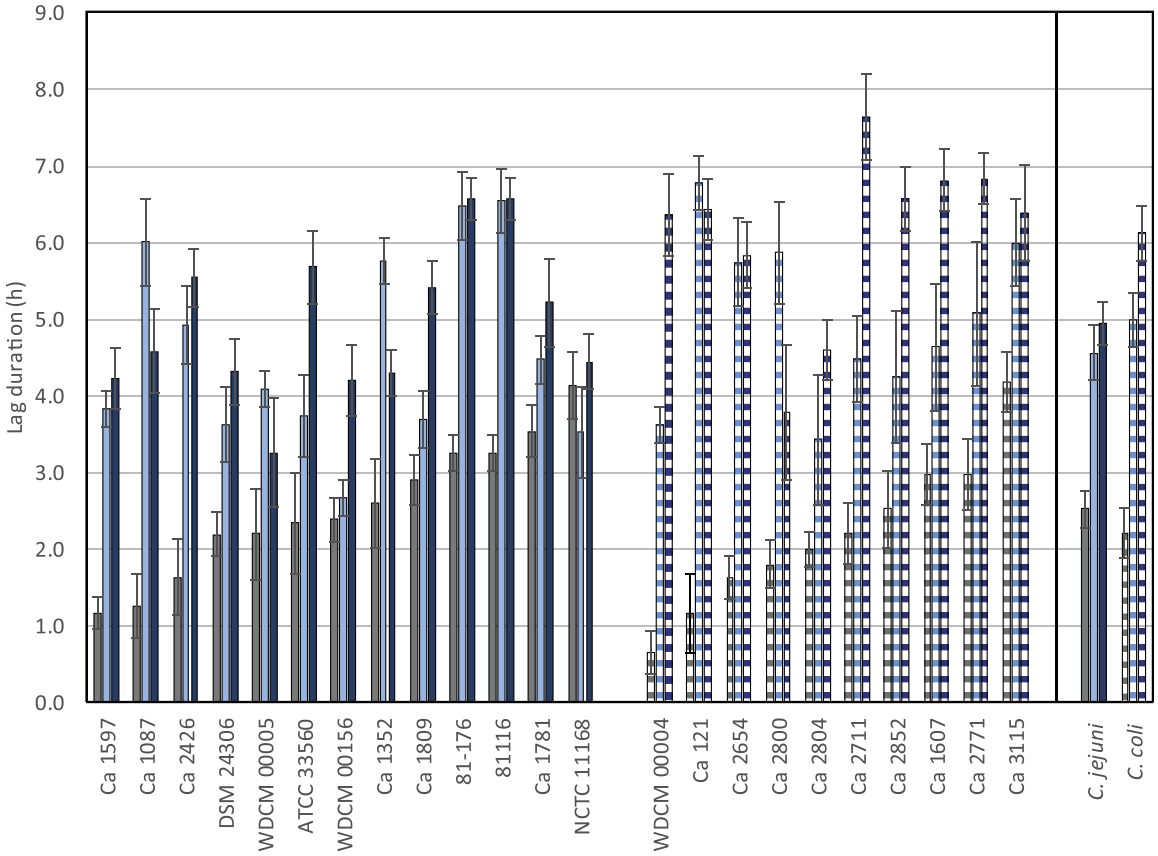

Fig. 2. Recovery duration of *C. jejuni* (filled bars) and *C. coli* (striped bars) with(out) prior stress treatment. Mean lag-duration without prior stress treatment (grey coloured bars), after refrigerated storage (light blue coloured bars) and frozen storage (dark blue coloured bars). Error bars depict the standard error of the biological reproductions (n = 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

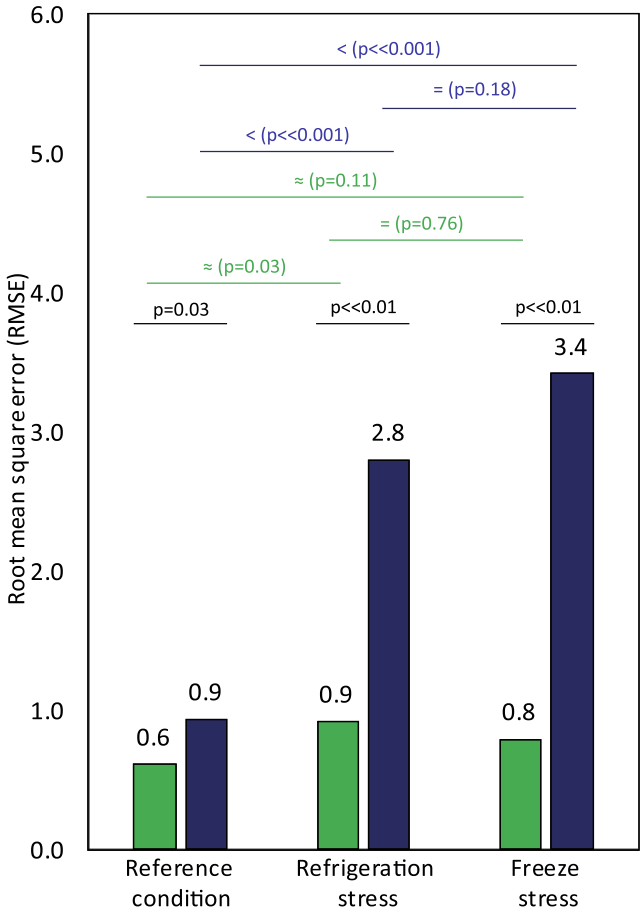

Fig. 3. Comparison of biological variability (green coloured bars) and strain variability (blue coloured bars) of the lag-duration after three experimental treatments. Significance testing showed significant differences between biological and strain variability in growth experiments conducted after all three treatments. Comparison of biological variability between the sets of experiments showed no significant difference, except between reference condition (RC) and refrigeration stress (RS) ($p = 0.03$). Comparison of strain variability between RC and RS as well as RC and freeze stress (FS) showed significant differences ($p < 0.05$ in both cases). No significant difference could be seen after comparing biological variability in growth experiments conducted after both stress treatments ($p = 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

behaviour of each strain. *C. jejuni* isolate WDCM 00005 and *C. coli* isolate Ca 2800 recovered relatively fast from freeze stress (Δ_{lag} of 1.1 h and 2.0 h, respectively). Exposure to atmospheric oxygen concentrations led to an increase in Δ_{lag} for strain WDCM 00005 by approximately a factor 3 (2.8x) and a factor 2.0 for strain Ca 2800. Recovery of *C. jejuni* strain 81-176 was comparable after both stresses (Δ_{lag} of 3.3 h and 2.9 h after freeze and oxidative stress, respectively) and lag-duration of *C. coli* strain WDCM 00004 was similar after both stresses as well. These stress effects indicate that after similar reduction, recovery behaviour during enrichment following ISO 10272-1:2017 was not only strain-dependent but also affected by the type of stress the population encountered prior to enrichment.

3.5. Scenario analysis

The data obtained for the 23 *Campylobacter* isolates from enrichments conducted after cold stress treatments was used to predict growth kinetics during enrichment following ISO 10272-1:2017, procedure A. According to this procedure, one *Campylobacter* cell present on/in 10 g or ml of food sample is enriched in 90 ml of Bolton Broth for 48 h,

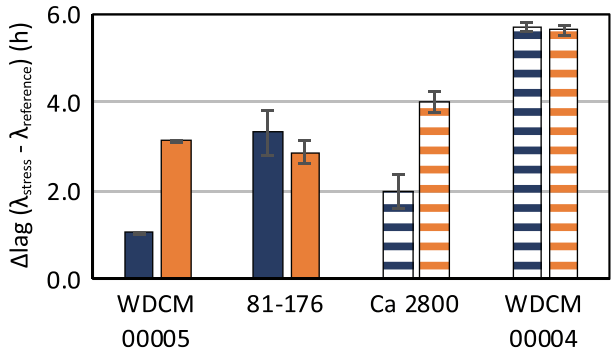

Fig. 4. Comparison of absolute lag-durations of four strains after freeze-stress (dark blue coloured bars) and oxidative stress (orange coloured bars). Two fast- and slow-recovering strains after freeze stress of *C. jejuni* (filled bars) and *C. coli* (striped bars) were subjected to oxidative stress and subsequently enriched following ISO 10272-1:2017, procedure A. The bars depict the increase in lag-durations to compare relative values for lag-duration. Error bars depict the standard error of the biological reproductions ($n = 2$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resulting in an initial concentration of -2 $\log_{10}$ cfu/ml. Subsequently, 10 µl of the enriched broth is streaked onto selective solid media and *Campylobacter*-typical colonies are confirmed. Therefore, cell concentrations after enrichment have to be at least 2 $\log_{10}$ cfu/ml in order to transfer on average one cell onto selective solid media. In this study the detection limit was set to 3 $\log_{10}$ cfu/ml to minimize the risk of false-negative outcomes after enrichment. Fig. 5 shows the outcomes of the scenario analysis. To simulate variations in lag-duration λ, initially the mean lag derived after model fitting of the growth kinetics during enrichment of the 23 freeze-stressed isolates was used (5.4 h) (Fig. 5, B). Particularly short but especially long recovery durations were also simulated by usage of the mean value with subtraction (Fig. 5, A) and addition (Fig. 5, C) of two standard deviations (± 2.2 h). For variation of growth rate µ, the mean values (0.93 h^{-1}) was used as well as the mean value with subtraction (dotted line) and addition (striped line) of two standard deviations (± 0.47 h^{-1}). A worst-case scenario analysis was conducted to simulate the growth kinetics of a single cell in 100 ml Bolton broth with a maximum lag duration of 7.6 h and a minimal growth rate of 0.47 h^{-1}. Results show that the detection limit was reached within 32.5 h (marked by a black arrow in Fig. 5, C). This indicates, that false-negative detection outcomes are probably not due to slow growth and long lag-duration. The outcomes of the scenario analysis can be underpinned by the Monte Carlo simulation that was conducted to determine the risk of false-negative detection outcomes of freeze-stressed cells after 48 h of enrichment in Bolton broth. For that, 100.000 iterations were used and for the majority of simulations (94.3%) the detection limit of 3 $\log_{10}$ cfu/ml was reached already within 24 h while the probability of reaching the detection limit at the end of enrichment (after 48 h) was 100%. Outcomes of the Monte Carlo simulation are displayed in Fig. S4 of the supplementary materials.

4. Discussion

4.1. Reduction in cell concentration of C. jejuni and C. coli after cold stress

In this study, cold stress treatments were applied for 64 h to cells suspended in Bolton broth base supplemented with 5% sterile lysed horse blood. Trials were done in a food-based fluid (chicken rinse) following methods described by Birk and colleagues (Birk, Ingmer, Andersen, Jørgensen, & Brøndsted, 2004) to assess reduction over time after refrigeration and freeze stress and results indicated that cells of the same inoculum showed comparable reduction in both media (data not shown). Also, reduction in cell concentration over time during

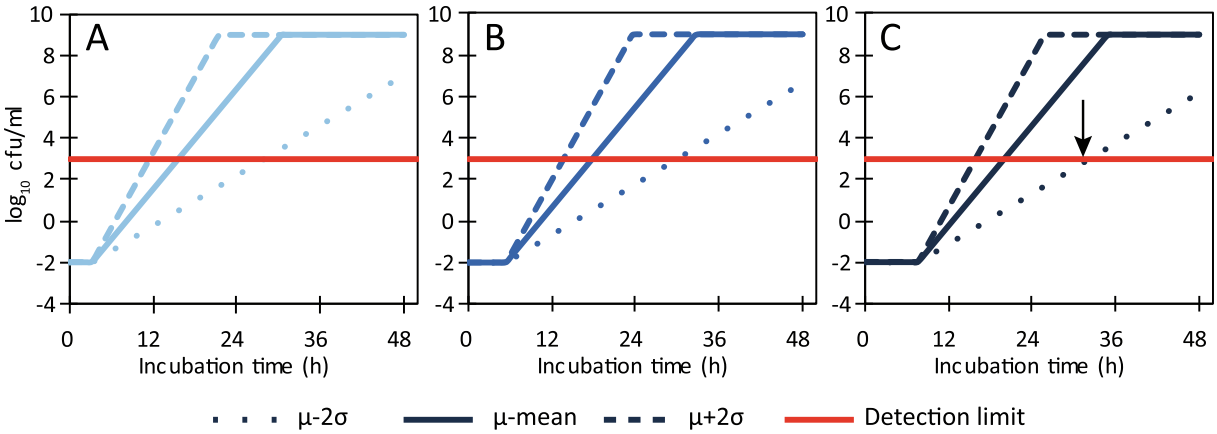

Fig. 5. Scenario analysis of growth kinetics of freeze-stressed *Campylobacter* spp. in monoculture during enrichment in Bolton Broth following ISO 10272-1:2017. Initial inoculation level (log N_0) was set to one *Campylobacter* cell/100 ml of Bolton broth. Simulation of differences in lag-duration were achieved by plotting (A) the mean lag-duration derived from all enrichments conducted after freeze stress treatments −2 standard deviations (3.2 h), (B) mean lag-duration (5.4 h) and (C) mean lag-duration +2 standard deviations (7.6 h). For all three scenarios, growth rates were estimated by plotting the mean growth rate (0.93 h^{-1}) as well as low (0.47 h^{-1}) and high (1.39 h^{-1}) growth rate. Low and high growth rates were estimated by plotting mean growth rate ± 2 standard deviations. Worst case scenario is depicted in the dotted line of graph (C). The black arrow indicates the time point during enrichment, when the detection limit of 3 log_{10} cfu/ml is reached (t = 32.5 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

short-term refrigerated and frozen storage was monitored in trial experiments to set the cold storage time. Results showed, that freezing at −20 °C resulted in an initial sharp decrease in cell concentration which is probably due to the formation of ice crystals during freezing but remained relatively constant until the end of the measurement (5 days, data not shown). Refrigerated storage at +4 °C in BB base resulted in minor reduction of cell concentrations. This might be attributed to decrease in metabolic rate of cells cultured at refrigeration temperatures in BHI previously described by Hazeleger and colleagues (Hazeleger et al., 1995). Overall, after refrigerated and frozen storage in BB, a reduction of less than 0.5 log_{10} cfu/ml and approximately 1.5 log_{10} cfu/ml was reached in the current study, respectively. Those results are in line with other studies conducted on the survival after exposure to cold temperatures (Bhaduri & Cottrell, 2004; Georgsson et al., 2006; Haddad et al., 2009; Klančnik, Zorman, & Smole Možina, 2008; Maziero & De Oliveira, 2010; Sampers et al., 2010). Throughout this study, the unselective solid medium CAB was used for the determination of cell concentrations after the application of stress in order to increase the chance to detect sub-lethally injured cells, however it cannot be excluded that the tested stresses might trigger the viable but nonculturable (VBNC) state (Portner, Leuschner, & Murray, 2007; Rollins & Colwell, 1986).

4.2. Lag-duration of C. jejuni and C. coli during enrichment after cold stress

Results of this study showed, that refrigerated storage did not result in a major decrease in cell concentration (approximately 0.1 log_{10} cfu/ml for both species). Nonetheless, lag-duration after refrigerated storage increased significantly by a factor of approximately 2.0 compared to the lag-duration of strains without prior stress treatment. Although refrigeration stress resulted in a relatively low reduction in culturability compared to freeze stress, strain variability in subsequent lag-duration after both stresses was comparable ($p = 0.18$) and significantly higher than for reference cells. Interestingly, no correlation could be found for both cold-stress treatments between reduction and subsequent lag-duration for the tested *C. jejuni* and *C. coli* strains (corresponding data can be found in Fig. S2 of the supplementary materials). It has been shown, that *Campylobacter* spp. do not express cold-shock proteins, which are often associated with cell division at low temperatures (Hazeleger et al., 1998; Phadtare, Alsina, & Inouye, 1999). However, cells have demonstrated to be able to show respiration, chemotaxis and protein synthesis at temperatures as low as 4 °C, although at rates much lower than at 37 °C (Hazeleger et al., 1998). Metabolism appears to be affected by low temperatures, as the production of succinate, an amino acid which is secreted during growth was decreased at low temperatures (Hofreuter, 2014; Höller, Witthuhn, & Janzen-Blunck, 1998). It might be possible, that even though cells did not incur severe damage to their cell membrane during refrigeration, cells still need to adapt to the rapid change of temperature from refrigeration to enrichment temperatures of 37 °C. It has been shown, that changes in temperature even within the range of growth lead to transcriptional changes over time with gene up- and downregulation lasting for at least 50 min (Stintzi, 2003).

The impact on cell viability of storage at temperatures comparable to those of conventional household freezers has been studied in detail (Georgsson et al., 2006; Jasson et al., 2007; Maziero & De Oliveira, 2010; Sampers et al., 2010). However, relatively little is known about the recovery behaviour of freeze-stressed *Campylobacter* cells during enrichment. In this study, frozen storage significantly increased the lag-duration of both *Campylobacter* species compared to reference cells by a factor of approximately 2.3. It has been recognized that freezing mainly results in damage to the membrane resulting in cell leakage as well as to DNA or DNA synthesis due to ice crystal formation during the freezing process (Wesche, Gurtler, Marks, & Ryser, 2009). Humphrey and Cruickshank described that *Campylobacter* cells showed to be more sensitive to antibiotic agents after exposure to freeze stress (Humphrey & Cruickshank, 1985). The observed increase in lag-duration might be attributed to a combination of these factors. Next to a general period which is needed to adapt to the enrichment environment, cells need to initiate and carry out repair processes to deal with structural damages. This might be further impeded by the presence of the cocktail of antibiotic compounds in Bolton broth (International Organization for Standardization, 2017).

4.3. Effect of oxidative stress history on recovery during enrichment

In this study, four strains were selected to be subjected to oxidative stress based on their recovery behaviour during enrichment after freeze-stress. The strains selected were two fast-recovering and two slow-recovering strains of both, *C. jejuni* and *C. coli*. This was done to investigate, whether those strains showed a similar recovery pattern after exposure to oxidative stress. Results showed that the recovery trend was not consistent. This might be due to the lack of a general stress response system as it can be found in other bacteria and therefore

differences in cellular stress response (Parkhill et al., 2000). On average, reduction after the oxidative stress treatment applied in this study was relatively limited (0.7 ± 0.2 $\log_{10}$ cfu/ml). Under aerobic conditions, more reactive oxygen species accumulate potentially leading to damage of cellular components such as nucleic acids and proteins (Gundogdu et al., 2016; Kaakoush et al., 2009; Oh, McMullen, & Jeon, 2015). Generally, *C. jejuni* has developed specific adaptation mechanisms for survival under atmospheric oxygen which deal with the removal of ROS from the cytoplasm (Kim, Oh, Kim, & Jeon, 2015). It has also been recognized, that *C. jejuni* is less susceptible to oxidative stress at low temperatures than at 42 °C, suggesting that temperature can affect oxidative stress resistance (Garénaux et al., 2008). Indeed, results of preliminary experiments showed that oxidative stress treatments conducted for 64 h in Bolton broth at 20 °C led to a significantly higher reduction than at 12 °C (data not shown). These experiments also showed, that reduction was less when cells were suspended in Bolton broth base without the addition of sterile horse blood or selective supplements than when stress treatments were conducted in sterile water (data not shown). It has been suggested that pyruvate itself and in a combination with sodium metabisulfite and ferrous sulphate can have a protective effect against oxidative stress in aerobic conditions (Chou, Dular, & Kasatiya, 1983; Verhoeff-Bakkenes, Arends, Snoep, Zwietering, & De Jonge, 2008). The former two can be found in Bolton broth base. Consequently, in this study, cells grown to stationary phase were transferred into Bolton broth base and subsequently exposed to atmospheric oxygen by shaking incubation at 12 °C for 64 h. It is possible, that the limited reduction observed might be attributed to the combination of low temperature and choice of Bolton broth as a medium with protective components for coping with oxidative stress.

4.4. Scenario analysis

In this study, the detection limit was set to 3 $\log_{10}$ cfu/ml, which equals on average to the transfer of 10 cells onto selective agar after enrichment, in order to reduce the risk of false-negative detection outcomes. Also, an initial contamination of 1 cell per 10 g or ml of food product was applied to mimic the growth kinetics of single cells during enrichment. In reality, contamination on broiler meat in Europe is often higher and in the range of 10^2-10^3 cfu/10 g of food sample (Guyard-Nicodeme et al., 2015), but here the aim was to provide growth predictions based on the lowest initial cell concentration since this will decrease the chance of overestimating positive detection outcomes. In this study, the average μ_{max} was set to 0.93 h^{-1} as well as the 2.5 and 97.5 percentile (0.47 and 1.39 h^{-1}) to include strain specific slow and fast growth rate. Comparison with literature showed, that μ_{max} of *Campylobacter* in different growth media is often between 0.7 and 0.9 h^{-1}. Battersby and colleagues determined a mean μ_{max} of *Campylobacter* spp. in Bolton broth of 0.7 h^{-1} (Battersby, Walsh, Whyte, & Bolton, 2016) and Hazeleger and colleagues calculated the μ_{max} of *Campylobacter* spp. during growth at approximately 40 °C in Brain Heart Infusion broth to 0.7–0.9 h^{-1} (Hazeleger et al., 1998). However, this scenario analysis is based on the outcomes of experiments conducted with isolates from various isolation sources in monoculture in culture media after undergoing a single cold-stress treatment. In reality, cells are often confronted with a multitude of processing steps which can induce sub-lethal damage (Keener, Bashor, Curtis, Sheldon, & Kathariou, 2004). The combination of stressful factors could lead to increased lag-duration. A worst-scenario analysis showed, that a single cell at the beginning of enrichment with a maximum growth specific rate as low as 0.47 h^{-1} could reach the detection limit still within 48 h even with lag duration as long as 23.5 h (data displayed in Fig. S5). However, with the stress conditions applied in this study, no lag-duration above 8 h was found. The scenario of presence of competitive microbiota has not been considered in this study. In practice, meat, especially broilers can also be contaminated with Extended-spectrum beta-lactamase (ESBL-) producing Enterobacteriaceae. In the United Kingdom and Belgium, ESBL-producing bacteria were found on approximately 65% and 60% of tested broilers, respectively (Depoorter et al., 2012; Randall et al., 2017) and in a study from the Netherlands, 94% of all chicken breasts tested were positive for ESBL-producing bacteria (Stuart et al., 2012). Therefore, there is a possibility of co-culture scenarios during enrichment wherein the growth of *Campylobacter* cells is suppressed by a more dominant strain following the principle of the Jameson effect, that is often attributed to production of specific inhibitors of growth by one species against another (Hazeleger, Jacobs-Reitsma, & den Besten, 2016; Mellefont, McMeekin, & Ross, 2008; Overdevest et al., 2011). ESBL-producing *Enterobacteriaceae* have been recognized as a challenge for reliable detection of campylobacters in food (Hazeleger et al., 2016). Further research will focus on the impact of competitive microbiota on the growth kinetics and detection outcomes of *Campylobacter* spp. during enrichment following ISO 10272-1:2017.

5. Conclusion

Refrigerated and frozen storage led to an increase in lag-duration of 13 *C. jejuni* and 10 *C. coli* strains in Bolton broth. Variability in lag-duration could be mainly attributed to strain variability, since biological variability was constant for all cold stress treatments and rather comparable to the reference condition. Exposure of cells to oxidative stress before enrichment showed that lag duration was not only strain dependent but also influenced by the type of stress applied. A scenario analysis on the growth kinetics of *Campylobacter* spp. during enrichment in monoculture highlighted that even in a worst-case scenario starting from one cell the limit for further successful detection was reached within 32.5 h and the probability of reaching the detection limit within 48 h was 100%. Based on these data, it seems that failures in reliable detection outcomes are not due to prolonged lag-duration and/or a reduction in specific maximum growth rate even to values as low as 0.47 h^{-1}. The outcomes of this research narrow down the reasons for false-negative detection outcomes as they regularly occur in practice. As competitive microbiota challenge the success of enrichment-based detection, the effect of competitive microbiota on reliable detection will be subject for further research.

CRediT authorship contribution statement

M.I. Lanzl: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **M.H. Zwietering:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision. **W.C. Hazeleger:** Methodology, Formal analysis, Writing - review & editing, Supervision. **T. Abee:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Supervision. **H.M.W. den Besten:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no conflict of interest to declare.

Acknowledgments

The authors thank Ourania Misiou for excellent lab work, Dr. Marc Wösten and Dr. Nancy Bleumink-Pluym from the Department of Infectious Diseases & Immunology, Infection Biology of Utrecht University and Dr. Menno van der Voort from Wageningen Food Safety Research for providing strains used in this research. Furthermore, the authors thank all project partners (Biomérieux, DSM, Innosieve Diagnostics, Merck Darmstadt, NWO and Wageningen Food Safety

Research) for their financial and in-kind contributions. This work is part of the research programme “Rapid and reliable enrichment-based detection of foodborne pathogens” with project number 15002, which is partly financed by the Dutch Research Council (NWO).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109253.

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