Cross-protection between controlled acid-adaptation and thermal inactivation for 48 *Escherichia coli* strains

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Abstract

Given the importance of pH reduction and thermal treatment in food processing and food preservation strategies, the cross-protection between acid adaptation and subsequent thermal inactivation for 48 *Escherichia coli* strains was investigated. Those strains were selected among 188 *E. coli* strains according to their odds of growth under low pH conditions as determined by Haberbeck et al. (2015) [Haberbeck, L.U., Oliveira, R.C., Vivijs, B., Wenseleers, T., Aertsen, A., Michiels, C., Geeraerd, A.H., 2015. Variability in growth/no growth boundaries of 188 different *Escherichia coli* strains reveals that approximately 75% have a higher growth probability under low pH conditions than *E. coli* O157:H7 strain ATCC 43888. Food Microbiol. 45, 222–230]. First, the *E. coli* cells were acid and non acid-adapted during overnight growth in controlled acidic pH (5.5) and neutral pH (7.0), respectively, in buffered Lysogenic Broth (LB). After overnight growth, cells were washed and transferred to non-buffered LB adjusted to both pH 6.2 and 7.0. Next, they were immediately inactivated at 58°C. Thus, four conditions were tested in total by combining the different pH values during growth/thermal inactivation: 5.5/6.2, 5.5/7.0, 7.0/6.2 and 7.0/7.0. Acid adaptation in buffered LB at pH 5.5 increased the heat resistance of *E. coli* strains in comparison with non acid-adaptation at pH 7.0. For instance, the median D_{58}-value of strains inactivated at pH 7.0 was approximately 6 and 4 min for acid-adapted and non acid-adapted strains, respectively. For the non acid-adapted strains, the thermal inactivation at pH 6.2 and 7.0 was not significantly ($p > 0.05$) different, while for the acid-adapted strains, the thermal treatment at pH 6.2 showed a higher heat resistance than at pH 7.0. The correlation between the odds of growth under low pH previously determined and the heat resistance was significant ($p < 0.05$). Remarkably, a great variability in heat resistance among the strains was observed for all pH combinations, with D_{58}-values varying between 1.0 to 69.0 min. In addition, highly heat resistant strains were detected. Those strains exhibited D_{58}-values between 17.6 and 69.0 min, while *E. coli* O157:H7 (ATCC 43888) showed D_{58}-values between 1.2 and 3.1 min. In summary, results clearly showed that adaptation of *E. coli* cells to constant acidic pH results in cross-protection against thermal inactivation.

Keywords: Strain variability; Linear Mixed Model; *D*-value

1. Introduction

Bacteria are capable to adapt to different stress environments encountered in their natural habitat through highly coordinated cell mechanisms. When this adaptation occurs during exposure to a nonlethal stress factor, bacteria can further exhibit higher resistance to the same or to different stress factors, a

phenomenon known as cross-protection (Battesti et al., 2011). Cross-protection has important implications in food safety and in food processing optimizations. For instance, the use of hurdle technologies, where sub-lethal or mild stresses are applied, may induce multiple stress responses reducing the efficacy of subsequent treatments (Capozzi et al., 2009).

In literature, cross-protection between different stresses was studied for several foodborne pathogens such as *Salmonella* (Tassou et al., 2009; Xu et al., 2008), *Listeria monocytogenes* (Gabriel and Arellano, 2014; Koutsoumanis et al., 2003; Pittman et al., 2014) and *Escherichia coli* (Cheng et al., 2003; Stopforth et al., 2007; Usaga et al., 2014a; Velliou et al., 2011). Specifically, studies about cross-protection between low pH and other stress factors applied different acid adaptation methodologies, such as exposing the cells to gradual acidification as a result of glucose fermentation (Buchanan and Edelson, 1996; Sharma et al., 2005) or growing the cells in unbuffered media initially adjusted to low pH (Cheng et al., 2003; Gabriel and Nakano, 2011; Singh and Jiang, 2012; Usaga et al., 2014b; Velliou et al., 2011). In both methodologies the exact pH profile during adaptation is unknown. It has been observed that complex media initially adjusted to low or neutral pH showed a further pH reduction during growth due to the production of organic acids. For instance, during growth of *E. coli* O157:H7 in TSB (tryptone soya broth), the initial pH of 5.0 and 6.0 dropped to 4.5 and 5.0, respectively (Yuk and Marshall, 2004). Similarly, during *Salmonella enterica* ser. Enteritidis growth in TSB, the initial pH of 5.3 and 6.3 dropped to 4.9 and 5.2, respectively (Yang et al., 2014). Although considerable research has been devoted to acid adaptation of cells exposed to gradual acidification as a result of glucose fermentation on one hand or immediately exposed to media initially adjusted to low pH on the other hand, less attention has been paid to acid adaptation in a controlled pH environment, with Koutsoumanis and Sofos (2004) being a notable exception.

Phenotypic variability among strains of the same species has been studied for different aspects and for different bacterial species, like *E. coli* (Haberbeck et al., 2015; Oh et al., 2009; Saridakis et al., 2004; Whiting and Golden, 2002), *S. enterica* (Lianou and Koutsoumanis, 2013), *Listeria monocytogenes* (Aryani et al., 2015a, 2015b; Lianou et al., 2006) and *Lactobacillus plantarum* (Parente et al., 2010). The mechanisms causing phenotypic variability among strains of the same species can be attributed to genomic and epigenetic differences (Smits et al., 2006). How genetic differences in a population influences phenotypic variation and evolution is a major concern in modern biology (Bergström et al, 2014). Genetic variation can occur, for instance, by gene loss/genome reduction, genome rearrangement, expansion of functional capabilities through gene duplication, acquisition of functional capabilities through lateral gene transfer and gene expression differences (Carreto et al., 2011; Luo et al., 2010).

Moreover, many factors have been considered to contribute to the variability in thermal resistance of microorganisms, such as strain differences, physiological state of the cell, growth and experimental conditions (Aryani et al., 2015b). In the current study, variability is defined as in Aryani et al. (2015b): strain variability is the variability between strains from the same species, biological variability is the variability between biologically independent repetitions for each strain and experimental variability is the repeatability of parallel experimental replicates.

In the present work, we investigated three hypotheses. The first hypothesis is that growth of *E. coli* strains under low pH condition (acid-adaptation) induces cross-protection against subsequent thermal inactivation at 58 ˚C. Forty-eight *E. coli* strains were selected among 188 strains previously characterized according to their odds of growth under low pH (Haberbeck et al., 2015). Differently from previous studies, the strains were acid-adapted by growing the cells at pH 5.5 or pH 7.0 in buffered lysogenic broth (LB) which maintained the pH constant during the adaptation phase. This approach was chosen to evaluate the effectiveness of acid adaptation knowing the exact pH conditions during growth. Besides that, a constant pH in a buffered medium simulates more closely foods such as meat products, which contain naturally a range of potentially buffering molecules (Pösö and Puolanne, 2005). The second hypothesis is that the cells would be more heat resistant in heating medium of pH 6.2 than those in pH 7.0. It has been reported that the maximum heat resistance of *E. coli*, *Salmonella* Enteritidis and *Cronobacter* ssp. is obtained in slightly acidified media (Arroyo et al., 2009; Blackburn et al., 1997; Mañas et al., 2003). Four conditions were tested combining two pH values during growth (5.5 and 7.0) and two pH values for the heating menstruum (6.2 and 7.0). In summary, combining this two first hypotheses, we assume the following order of heat resistance: 5.5/6.2 > 5.5/7.0 > 7.0/6.2 > 7.0/7.0. The third hypothesis is that that strains with relatively high odds of growth under low pH (3.8 to 4.2), defined in Haberbeck et al. (2015), would show to be more heat resistant. Finally, we also had the objective to quantify the impact of strain variability on the thermal resistance (phenotypic response) of the *E. coli* strains tested.

2. Material and methods

2.1. Bacterial strains

The 48 *E. coli* strains used in this study are listed in Table 1. They were selected from the 188 strains in the previous study (Haberbeck et al., 2015) using a stratified sampling method. Since they have been isolated from a large number of diverse sources, most of these bacteria can be considered unique strains. However, it cannot be excluded that some isolates originating from the same source and having similar

odds of growth in acidic conditions (Table 1) are clonal. Stock cultures were maintained at -80 ˚C in LB with 25% v/v glycerol. Strains were activated by loop-streaking the stock cultures onto LB agar stock plates, which were then incubated for 24 h at 37 °C. Forty-seven strains plus the reference *E. coli* O157:H7 (ATCC 43888) were selected according to their odds of growth previously determined (Haberbeck et al., 2015). The selection was done in a stratified way as follows: firstly, 14 strains were randomly selected among the ones with higher odds of growth than the reference, secondly, 9 strains were randomly selected among the ones with lower odds of growth, and lastly 24 strains were randomly selected among the ones with higher and lower odds. By doing so, the stratification ensured to cover the different possibilities concerning lower and higher odds of growth.

2.2. Acid adaptation

Acid adaptation was achieved during pre-inoculum growth. LB was buffered with 0.1 M of 2-(N, morpholino) ethanesulfonic acid (MES) (AppliChem, Darmstadt, Germany) at pH 5.5 for the acid-adapted cells, and with 0.1 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, Bornem, Belgium) at pH 7.0 for the non acid-adapted cells. pH was adjusted with 1 M of NaOH and verified using a digital pH meter (Hanna, HI9125). Then, buffered LB was filter-sterilized with 0.22 µm cellulose acetate sterile filters (VWR International, Belgium) and kept at 4 ˚C until use. Prior to thermal treatments, single colonies were inoculated in microcentrifuge tubes with 400 µl buffered LB. The pre-inoculum was incubated at 37°C overnight under shaking at 200 rpm. After overnight growth and before proceeding with thermal treatment, pH stability was checked and confirmed for a random selection of pre-cultures. The working culture was obtained by centrifuging the pre-inoculum at 3600 g for 10 min (Centrifuge 5430, Eppendorf, Hamburg, Germany). The cell pellets were then washed once with 10 mM of potassium phosphate buffer (PPB) and finally resuspended in LB at pH 7.0 or 6.2 just before the thermal inactivation. The pH of LB for the thermal inactivation was adjusted with 1 M of NaOH or 37 % HCl followed by filter sterilization. In total, four conditions were tested combining two pH values during growth (5.5 and 7.0) and two during thermal inactivation (6.2 and 7.0). Along the text, the pH conditions are referred to as the combination of pH during growth/pH during thermal inactivation, i.e. 5.5/6.2, 5.5/7.0, 7.0/6.2 and 7.0/7.0.

2.3. Thermal inactivation

Thermal inactivation was accomplished at 58°C using a thermal cycler (Biometra®, Westburg, The Netherlands). The experiments were carried out using thin walled PCR tubes (Bioplastics, Landgraaf, The Netherlands) with 30 µL of the working culture. The initial cell concentration was on average 10^8 CFU/ml.

Samples were placed in the thermal cycler and to reduce and standardize the come-up time (time to reach the target temperature), temperature was initially set at 37°C for 30 s. Then the thermocycler block started to increase the temperature, and when the inactivation temperature of 58 ˚C was reached time zero samples were taken and immersed immediately in an ice-water bath. After 10 min, PCR tubes were removed and placed in the ice-water bath. Some strains were subjected to a second set of heat treatment experiments, limiting the duration at 58°C to 5 min; and some strains were subjected to a heat treatment increasing the time at 58 °C to 30 min. Previously to the 47 thermal inactivation, the inactivation kinetics was performed for *E. coli* O157:H7 (ATCC 43888) with samples taken at set time intervals, for all conditions. Survivors were counted by spot plating 20 µl of serial dilutions on tryptone soya agar (Oxoid, Basingstoke, England) supplemented with 0.6% (w/v) yeast extract (YE, Oxoid). Preliminary tests (data not shown) revealed that the number of colonies after acid adaptation followed by thermal inactivation was higher when plating on TSA supplemented with yeast extract. Plates were incubated at 37°C overnight. The thermal treatments were reproduced minimally two times on different days using two different single cell colonies from the activated cultures in LB agar plates. Exceptions, with more than two biological repetitions are detailed in Table 1.

2.4. Mathematical modelling of inactivation kinetics

The thermal inactivation curves of strain O157:H7 (ATCC 43888) were constructed by plotting $\log_{10}$ values of the surviving population against heating time. The inactivation kinetics modelling was performed by GInaFiT (Version 1.6), a freeware add-in for Microsoft® Excel (Geeraerd et al., 2005). The goodness-of-fit of the models was assessed by the root mean square error (RMSE). The RMSE has been proposed by David Ratkowsky (Ratkowsky, 2004) as a suitable indicator for the goodness-of-fit of a linear or a nonlinear model. Its magnitude should be comparable with the precision in the original data, an RMSE being much smaller indicates *overfitting*, while an RMSE much larger indicates a so-called *underfitting* regression model. Thermal inactivation curves with a log-linear behaviour were modelled with the 'log-linear' model (Eq. 1) (Bigelow and Esty, 1920),whereas, inactivation curves showing a shoulder prior to a log-linear region were modelled with the 'log-linear + shoulder' model (Eq. 2) (Geeraerd et al., 2000).

$$\log(N) = \log(N_0) - \frac{k_{max}.t}{Ln(10)} \quad (1)$$

$$\log(N) = \log(N_0) - \frac{k_{max}.t}{Ln(10)} + \log\left(\frac{e^{k_{max}S_l}}{1 + (e^{k_{max}S_l} - 1) \cdot e^{-k_{max}t}}\right) \quad (2)$$

where N is the microbial cell density (CFU/ml), N_0 is the initial population density (CFU/ml), k_{max} is the first order inactivation constant (1/min), S_l (min) represents the shoulder length and t is the time (min). D-values (decimal reduction time) were determined from the maximum inactivation rate (D-values = ln(10)/k_{max}). The time for 3 log-reductions (t_{3D}) was also obtained with GInaFiT.

For all other strains, two data points were obtained during inactivation, namely at time point zero (N_0) and after a determined inactivation time (N_t). This implies that there is no information on the inactivation curve shape, and we can only rely on a linear model description. Thus, the D-values for each condition, strain and biological replicate were estimated according to Eq. 3.

$$D = \frac{t}{\log(N_0) - \log(N_t)} \quad (3)$$

2.5. Statistical analysis

All statistical analyses were carried out in R (version 3.3.1, R Core Team, 2016). Distributions were fitted to the $LogD_{58}$ data and further evaluated with the "fitdistrplus" package (Delignette-Muller and Dutang, 2015; Pouillot and Delignette-Muller, 2010). The goodness-of-fit was evaluated through Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling statistical tests.

The relationship between *LogD* and the tested pH conditions (*Cond*) for all strains was described through a linear mixed model (LMM) (Eq. 4) with condition as the fixed effect and strain as the random effect.

$$LogD_{ij} = \alpha.Cond_i + \beta.Strain_{ij} \quad (4)$$

where *LogD* is the logarithm of the D-value (log min) for the i^{th} condition and the j^{th} strain, *Cond* is the fixed effect of condition with its correspondent parameter α, and *Strain* is the random effect accounting for the strains difference with its correspondent parameter β.

In a similar approach, the relationship between *LogD* and food related strains only was also investigated using LMM (Eq. 5) with strain as fixed effect and condition as random effect.

$$LogD_{ij} = \delta.Strain_i + \gamma.Cond_{ij} \quad (5)$$

where δ and γ are the parameters of the fixed and random effects. Both LMM were fitted to the data using the lmer function from package lme4. Afterwards a Tukey post hoc test ($p < 0.05$) was applied for multiple comparisons between the fixed effects. The *LogD* data is unbalanced due to the differences in the number of replicates per strains and pH condition, as detailed in Table 1. LMM is applied in fields where it is often difficult to ensure perfect balance of samples, such as in ecology and evolution (Schielzeth and Nakagawa, 2013; Van der Elst et al., 2013) and parasitology (Paterson and Lello, 2003).

The correlation between the odds of growth and the *LogD* was measured with the Spearman Rank-Order Correlation test (ρ) and its respective *p*-value, both obtained with the function "rankor" package "pvrank". The following arguments of the "rankor" function were used: "gaussian" as the type of approximation of approximation to the null distribution, "midrank" as the method for breaking ties, and "two-sided" as the type of alternative hypothesis.

3. Results

3.1. Inactivation kinetics of reference strain *E. coli* O157:H7 (ATCC 43888)

Prior to the inactivation of the 48 *E. coli* strains, the inactivation kinetics of the O157:H7 strain was performed in order to have an idea of the bacterial behaviour under the experimental setup used. This strain was chosen since it is used further as the reference strain. The kinetics during thermal inactivation at 58 ˚C for *E. coli* O157:H7 (ATCC 43888) was determined under the four pH combinations. Survival curves are depicted in Fig. 1. The survival curves obtained in this study showed two different profiles. Log-linear curves were observed for the pH 7.0 grown cells (non acid-adapted). By contrast, the inactivation kinetics of pH 5.5 grown cells (acid-adapted) were non-linear, showing an obvious shoulder prior to the initiation of linear inactivation. The log-linear model with shoulder was successful at fitting data, with *RMSE* values of 0.39 and 0.17 for conditions and 5.5/6.2 and 5.5/7.0, respectively (Table 2). For our experimental data, the RMSE value of each replicate equals from 0.08 to 0.48. The obtained pooled RMSE, ranging from 0.17 to 0.39, are of similar magnitude, and hence indicate an acceptable goodness-of-fit. A longer shoulder (or the presence of a shoulder) is related to increased thermal resistance in that region of the survivor curve. The magnitude of the shoulder was approximately equal to one log reduction time (D_{58}-value) for the acid adapted cells (Table 2). Due to the occurrence of shoulders in survival curves, D_{58}-values calculated from the straight line portion of the survival curves are not appropriate to use for

comparison and estimation of the thermal resistance of *E. coli* under different conditions. Thus, t_{3d}, including the shoulders, were used for comparisons instead of D_{58}-values. The t_{3d} values of acid-adapted cells were increased by 1.7 and 3.3 fold compared with non-adapted cells when thermal inactivation was accomplished at pH 7.0 and 6.2, respectively. For inactivation with pre-inoculum at pH 5.5, the cells showed on average a higher thermal resistance when inactivated at pH 6.2 than at pH 7.0, whereas for pre-inoculum at pH 7.0, the cells were on average more heat sensitive when inactivated at pH 6.2. However, the influence of the pH of the heating broth on the thermal resistance was not significant ($p > 0.05$) for the reference strain.

3.2. Variability in the thermal resistance of 48 strains

The descriptive statistics of the *D*-value of the 48 *E. coli* strains at 58 ˚C under four conditions are shown in Table 3. The median, 25th and 75th percentiles for 5.5/6.2 were greater than the corresponding values of other conditions (Table 3). The median for the D_{58}-values was almost doubled at 5.5/6.2 compared to 7.0/7.0. For thermal inactivation at pH 7.0, the median D_{58}-value for acid-adapted cells was 1.5 times higher than for non-adapted cells, and for thermal inactivation at pH 6.2 it was 1.4 times higher. The Median Absolute Deviation (MAD) was used to characterize these non-normally distributed data (Table 3). The variability increased at more stressful conditions, as the MAD value at 5.5/6.2 was 2.5 times higher than at 7.0/7.0.

A substantial variability of the thermal resistance was observed among the strains under the four pH conditions as illustrated in Figures S1-S4 in the Supplementary File, which depict graphics of the empirical and theoretical distributions in density and cumulative density function (CDF), Q-Q and P-P plot for each condition. The Logistic distribution best fitted the $LogD_{58}$ data for all conditions according to the performed goodness-of-fit statistical tests. Location and scale, parameters of the Logistic distribution fitted to $LogD_{58}$, are displayed in Table S1, Supplementary File.

Three highly heat resistant strains (strain EC57, EC73 and EC60) were observed among the 48 strains studied. When initially heat treated at the set time of 10 min at 58°C, no significant reduction in numbers was observed for those strains. Thus, they were subsequently treated at 58 ˚C up to 30 min. The *D*-values at 58°C of these three strains ranged from 17.6 to 69.0 min. The three strains were all isolated from the same source, the sewerage system in Antwerp (BE). Other strains from the same source, such as EC58, EC59, EC62, EC63 and EC74, did not show this extreme resistance. In addition to heat resistant strains, some very heat sensitive strains were also found, e. g., strain EC1623, EC1616, EC1607, all three

pathogenic strains (Table 1). For these strains, the survivor cell concentration was below the detection limit of 1.7 log CFU/ml after 10 min of thermal treatment. Thus, they were treated at 58 ˚C for 5 min only. The D_{58}-values of these three heat sensitive strains, calculated from 5-min thermal treatment, ranged from 1.4 to 4.5 min.

3.3. Cross-protection between acid adaptation and thermal inactivation for 48 *E. coli* strains

The LMM (Eq. 4) was fitted to the *LogD* with strain as a random effect, condition as a fixed effect and 7.0/7.0 as the reference condition. This condition was chosen as reference since we believe that it is the most optimal condition that results in less stressed cells during thermal inactivation. The D_{58}-values were log-transformed (*LogD*) to normalize the model residuals since the LMM model using the *D*-values did not present a normal distribution of the residuals (data not shown). An important feature of the LMM model is that it calculates the effect of *Con* in the *LogD* taking into account the strain variability due to the random effect. According to the LMM results (Table 4), the variability in the $LogD_{58}$ is explained mostly by the strain variability (0.07), followed by the residual variability (0.02). The residual random effect concern factors that influence the variability in the *LogD*, but were not measured or determined in this research.

The results for the fixed effects showed that the $LogD_{58}$ values were higher in all conditions compared to the reference 7.0/7.0. Further, Table 5 shows the post hoc Tukey results applied to the LMM comparing pairwise all conditions. The hypothesis of cross-protection between acid adaptation at pH 5.5 and thermal inactivation at 58 ˚C was accepted according to the post hoc Tukey test ($p < 0.05$) (Table 5). The average $LogD_{58}$ during thermal inactivation at pH 7.0 of acid adapted cells was 0.11 log min higher than that of non-adapted cells (comparison n˚ 2 in Table 5). Similarly, the average $LogD_{58}$ during thermal inactivation at pH 6.2 of acid adapted cells was on average 0.16 log min higher than that of non-adapted cells (n˚5 in Table 5). The major difference was between 5.5/6.2 and 7.0/7.0, where the average $LogD_{58}$ of the first condition was 0.21 log min higher than that of the second condition cells (n˚1 in Table 5). In general, the $LogD_{58}$ was significantly different ($p < 0.05$) among all conditions except between conditions 7.0/6.2 and 7.0/7.0 where this parameter was statistically similar ($p > 0.05$). Thus, the second hypothesis on increased resistance at thermal treatment at pH 6.2 was accepted ($p < 0.05$) for cells adapted at pH 5.5.

3.4. Result for food related strains

Among the 48 strains selected for this study, four strains are food related: EC1, EC3 and EC23 and *E. coli* O157:H7 (ATCC 43888). With the exception of *E. coli* O157:H7, abovementioned *E. coli* strains were

isolated from chicken and minced meat (Table 1). The D_{58}-values of each condition for those four food related strains are shown in Table S2 (Supplementary File). On average, higher D_{58}-values occurred at condition 5.5/6.2 followed by 7.0/7.0. *E. coli* O157:H7 (ATCC 43888) had the lowest D_{58}-values for all conditions, while EC23 had the highest. However, no significant difference in the $LogD_{58}$ between the conditions was found when applying the LMM (Eq. 4) using only the data from food related strains (data not shown). To investigate the differences in the *LogD* between the food related strains a LMM (Eq. 5) followed by a Tukey post hoc test ($p < 0.05$) was applied to the data (Table S3, Supplementary File). The $LogD_{58}$ for *E. coli* O157:H7 (ATCC 43888) was significantly ($p < 0.05$) different from the other 3 strains. The $LogD_{58}$ for EC1, EC23 and EC3 was on average 0.51, 0.80 and 0.54 log min higher than the *E. coli* O157:H7 strain, respectively.

3.5. Correlation between odds of growth under low pH and thermal inactivation

The correlation between the $LogD_{58}$ and the average odds of growth (Table 1) was analysed by the Spearman Rank-Order Correlation test for each condition (Fig. 2). The correlation was statistically significant ($p < 0.05$) for all conditions. The Spearman's correlation coefficients (ρ) indicate that there is a positive but weak correlation between the two variables. Acid-adapted strains have a higher correlation between $LogD_{58}$ and the average odds of growth than the non acid-adapted. Notably, the strain EC73 which had one of the highest $LogD_{58}$ values had an odds of growth 0.1 times lower than the reference (Table 1). The EC73 data points are located in the left upper corner of each subplot of Fig. 2. In contrast, EC1643 that had odds of growth 47 times higher than the reference was not among the most heat resistant strains showing an average $LogD_{58}$ of 0.5 log min in all conditions, as depicted in the data point located right of each subplot of Fig. 2.

4. Discussion

During food production and processing, foodborne microorganisms encounter a variety of stresses such as added chemicals (e.g. acids, salts and oxidants) and physical treatments (e.g. heat and pressure). Microorganisms adapted to these stresses may survive or even proliferate under conditions that could have ordinarily eliminated them, a phenomenon known as cross-protection (Rodriguez-Romo and Yousef, 2005). For example, the increase in acidity during sausage fermentation and the presence of salt in the formulation may induce an acid and osmotic adaptive response in pathogenic bacteria relevant for this product, such as *E. coli*. These bacteria, adapted to acid and osmotic stress during fermentation, may resist the further heating and smoking steps or persist during the product storage (Yousef and Courtney,

2003). The acidity of fruit juices may also induce bacterial acid adaptive responses, which could result in bacteria being more resistant to further pasteurization.

Our results showed that acid adaptation at controlled pH 5.5 significantly ($p < 0.05$) increased the heat resistance at 58 ˚C of the 48 *E. coli* strains. This result reflects the relevance of acid adaptation at a constant pH on the design of thermal treatments of food products in which both stresses are relevant, such as fruit juices. The heat treatments were performed at 58 ˚C aiming to observe an appreciable inactivation for all strains but not a total inactivation. However, to obtain a better view of the significance of our findings for the food industry, it would be necessary to extend the experiments to higher temperatures actually being applied in heat treatments, such as 70 to 75 ˚C for non-carbonated fruit juices (ICMSF, 2005), or as the usual pasteurization temperatures in the food industry, e.g. between 60 and 82 ˚C (Tewari and Juneja, 2007).

The acid adaptation at pH 5.5 and non-adaptation at pH 7.0 were performed in a buffered LB to ensure a constant pH during pre-culture growth. In the cross-protection literature between acid adaptation and thermal inactivation, acid adaptation is normally achieved either by growth in media with gradual acidification as a result of glucose fermentation, either by growth/survival in unbuffered media adjusted to mildly acidic or extremely acidic pH. Therefore, comparing our results with literature is not straightforward, as no reference using a buffered medium in the cross-protection between acid adaptation and thermal inactivation could be found. The hypothesis that *E. coli* cells adapted at pH 5.5 are more heat resistant than non-adapted cells was confirmed through the statistical analysis performed (Tables 4 and 5). Although direct comparisons cannot be made, overall, the finding that acid-adapted *E. coli* strains are more heat resistant than the non-adapted stains is in agreement with other studies (Cheng et al., 2003; Gabriel and Arellano, 2014; Mazzotta, 2001; Sharma et al., 2005; Singh and Jiang, 2012). For example, addition of different acids to Brain Heart Infusion (BHI) until pH 5.5 increased the heat resistance of *E. coli* K12 at 58 ˚C. The average k_{max} was 1.5 min^{-1} for unmodified BHI at pH 7.5, while it was 0.9, 1.1 and 0.6 min^{-1} for acid-adapted cells at pH 5.5 using acetic, lactic and hydrochloric acid, respectively (Velliou et al., 2011). *E. coli* O157:H7 (HCIPH 96055) pre-inoculated in slowly acidified nutrient broth with 1 % glucose (NBG) resulted in cells with thermal resistance almost 5 times greater than those grown in nutrient broth at pH 7.0 without glucose (Gabriel and Nakano, 2011). Usaga et. al (2014a) compared the heat resistance of *E. coli* O157:H7 (C7927 and ATCC 43895) and O111 for non-adapted, acid-adapted and acid-shocked cells in Tryptone Soya Broth (TSB). The authors define “acid-

shocked adaptation" when the TSB was adjusted to pH 5.0 with HCl, and "acid adaptation" when 1% of glucose was added to TSB. None of the acid adaptation/shocking methodologies resulted in more heat resistant O111 cells than the non-adapted cells. Contrarily, both O157:H7 strains subjected to acid shock adaptations exhibited greater heat resistance at 56°C in apple juice (pH 3.6). But, acid-adaptation with glucose only resulted in significantly more heat resistant cells for ATCC 43895 (Usaga et al., 2014a). These findings highlight that the thermal tolerance of *E. coli* strains can be significantly affected by the strain under study and the methodology for acid adaptation.

Blackburn et al. (1997) observed that *D*-values of *E. coli* O157:H7 at 64.5 ˚C and 3.5 % NaCl (w/w) were higher at pH 4.3 and 5.1 than at pH 7.0, besides that they suggested an optimal pH of 5.2 – 5.9 for *E. coli* survival during inactivation. However, other studies pointed a different direction, the lower the pH, the higher the sensitivity of the *E. coli* strains to heat (Dock et al., 2000; Juneja et al., 1999; Lee and Kang, 2009; Parry-Hanson et al., 2010; Riordan et al., 2000). In our experiments, the hypothesis of higher heat resistance when the inactivation media was adjusted to pH 6.2 compared to media adjusted to pH 7.0 was accepted ($p < 0.05$) for acid-adapted cells. When cells were adapted to the optimal pH 7.0, no significant ($p > 0.05$) difference in *LogD* between inactivation at pH 6.2 and 7.0 was observed. Thus, the order of heat resistance hypothesized initially was partially confirmed as 5.5/6.2 > 5.5/7.0 > 7.0/6.2 and 7.0/7.0.

The D_{58}-values, and consequently the $LogD_{58}$, showed a high variability among the 48 *E. coli* strains. According to the LMM results, the variability on the $LogD_{58}$ was explained mainly by the strain variability (0.07) followed by other factors (0.02) of data variability not measured in this study. Despite the different mathematical approach to quantify variability, Aryani et al. (2015b) showed that the strain variability was the main source of variability on the thermal resistance of 20 *L. monocytogenes* strains. The strain variability was ten times higher than the experimental variability and four times higher than the biological variability.

A wide range of *D*-values for thermal inactivation of pathogens can be found in the literature. The *D*-values of 17 *E. coli* O157:H7 was also significantly variable ranging from 2.6 to 21.5 min and 0.75 to 2.11 min at 55 and 60 ˚C, respectively (Whiting and Golden, 2002). In one of the most extensive studies to determine global thermal inactivation parameters for food pathogens, a large data set for *D*-values (n = 4066) of different pathogens at different conditions including *E. coli* (n = 382) was collected from literature to obtain average *D*-values (van Asselt and Zwietering, 2006). *E. coli* average log *D*-value (*LogD*)

361 at 70 ˚C was suggested to be -0.67 log min (D_{70}-value of 0.21 min), while at 58˚C was 0.46 log min for
362 *LogD* (D_{58}-value of 2.9 min). Our results show that the D_{58}-value varied for the 48 *E. coli* strains from 1 min
363 up to 69 min taking into account all conditions. The average D_{58}-value for all pH conditions was 5.2 min,
364 which was 2.3 min higher than the average D_{58}-value found by van Asselt and Zwietering (2006). When
365 considering only the four food related strains this parameter variation was between 1.22 and 15.5 min
366 and the average for all pH combination was 7.6 min, which was 2.44 min higher than the average D_{58}-
367 value of all 48 strains. The *D*-values obtained in the current study are limited by the lack of information on
368 the inactivation curve shape, as only a linear model was suitable to model the two data points used.
369 However, the authors believe that the two or more (in some cases) biological repetitions would allow to
370 calculate a valuable approximation to the *D*-values, even though it remains important to remember how
371 these *D*-values were calculated. Regarding the variability in the *D*-values found in the literature, ideally
372 the food processors may take a more "risk-based" approach, to characterize the heat resistance of a
373 range of strains instead of using a single very heat resistant or heat sensitive strain which in most cases
374 will lead to a uselessly conservative heat treatment or food safety issues (Condron et al., 2015).

375 Further analysis on the highly resistant strains found during our work would be of great importance. For
376 example, testing their behaviour under other temperatures or unveiling the genetic differences among
377 them and the other less resistant strains. Mercer et al. (2015) analysed the genomics of 29 *E. coli* strains
378 isolated from a beef processing plant showing variable heat resistance. Four non-pathogenic strains were
379 highly heat resistant compared to Shiga toxin-producing *E. coli* (STEC) strains, with a D_{60}-value of more
380 than 6 min. The genomic sequence identified a set of 6 unique genes for the highly resistant strains which
381 were termed as locus of heat resistance (LHR) coding for two small heat-shock proteins, a Clp protease,
382 several hypothetical proteins with predicted transmembrane domains, a putative sodium/hydrogen
383 exchanger and several peptidases (Mercer et al., 2015). This LHR is the same as found in heat resistant
384 strains of *Klebsiella pneumonia* (Bojer et al., 2010), *Cronobacter* spp. and other enterobacteria (Gajdosova
385 et al., 2011). Mercer et al. (2015) therefore postulated that this LHR was acquired by lateral gene transfer
386 between different members of the *Enterobacteriaceae.*

387 The Spearman's correlation test indicates a weak positive and statistically significant ($p < 0.05$) correlation
388 between $LogD_{58}$ and odds of growth (Fig. 2). This observation supports the hypothesis that strains with
389 higher odds of growth under the low pH tested in Haberbeck et al. (2015) would be better acid adapted
390 at pH 5.5 and consequently more heat resistant due to the cross-protection phenomenon. These results
391 must be interpreted with caution since the correlation coefficient was weak. It is possible, therefore, that

not all the strains with higher probability of growing in unbuffered media with pH between 3.8 and 4.6 (growth condition in the previous work) would have the same growth behaviour responses when growing under slightly higher constant pH 5.5. The resistance mechanisms underlying the growth under these different pH conditions are probably different. The different pH would trigger different adaptive responses and *E. coli* strains capable to adapt at pH 5.5 are not the same strains capable to grow better at lower pH. Different from our results, no correlation was observed between different food-related stresses in previous work. For instance, considering even lower pH values, not allowing growth, no correlation was observed between the resistance to acid inactivation at pH 3.0 and to thermal inactivation at 57 ˚C for 60 strains of *S. enterica* (Lianou and Koutsoumanis, 2013). None of the 12 *E. coli* strains, being 10 VTEC isolates (O157 and non-O157) and two laboratory non-pathogenic strains, showed consistently the highest resistance to acid, alkaline, heat and high hydrostatic pressure treatments. In addition, no correlation between the biofilm-forming ability and the resistance to those stresses was found (Alvarez-Ordóñez et al., 2013).

5. Conclusion

This study provided evidence that cross-protection between mild acid adaptation and thermal inactivation occurs. *E. coli* cells acid-adapted during growth at constant pH 5.5 were significantly more resistant to a subsequent thermal treatment at 58 ˚C compared to cells non acid-adapted during growth at pH 7.0. In addition, the acid-adapted *E. coli* cells were more heat resistant when the thermal inactivation medium was slightly acidic (pH 6.2) compared to neutral (pH 7.0). However, for non acid-adapted *E. coli* cells, no significant difference was observed between the inactivation medium pH of 6.2 and 7.0. A great variation in the heat resistance (*D*-values) at 58 ˚C among the 48 tested strains was observed in this study. Both effects of the cross-protection phenomenon as well as of the *D*-values variation should be carefully assessed within the context of the food processing from a food safety point of view. In addition, it is important that the design of food processes using low pH and thermal treatment should carefully include the consequences of those effects. This is especially important for nowadays food business operators, who are interested in introducing milder processing and preservation techniques.

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582

583 Table 1. Details of the 48 *E. coli* strains studied. The odds of growth were calculated by averaging the
584 odds of growth for all the conditions tested in Haberbeck et al. (2015). Reading key: strains with odds
585 ratio higher than one have a higher odds of growth than the reference, and strains with odds ratio lower
586 than one have a lower odds of growth than the reference. For most strains and pH conditions, two
587 biological repetitions were accomplished. The right column indicates the strain and pH condition in which
588 the number of biological repetitions was higher than two.

Strain	Average odd of growth	Details	N° of biological repetitions
ATCC 43888	1.0 (Ref)	O157:H7	
EC1643	45.7	Canine EPEC (Enteropathogenic *E. coli*), 11647-3 [a]	3 for 7.0/7.0
EC1642	25.3	Canine EPEC, 11646-1 [a]	3 for 5.5/7.0
ECOR[b] 26	24.9	O104:H21, Group B1 strain from a healthy infant in USA	
EC1	24.4	Isolated from minced meat in Leuven (BE) [c]	
EC63	23.6	Isolated from sewerage system in Antwerp (BE) [c]	
EC15	22.7	Isolated from chicken faeces in Belgium [c]	
BV17	22.3	Isolated from bird faeces in Belgium [c]	
EC16	22.1	Isolated from compost in Leuven (BE) [c]	
EC1633	20.8	ETEC (Enterotoxigenic *E. coli*), 288A [a]	3 for 7.0/7.0
EC3	18.3	Isolated from minced meat in Leuven (BE) [c]	
EC74	17.2	Isolated from sewerage system in Antwerp (BE) [c]	
APEC248	17.2	APEC (avian pathogen *E. coli*) [d]	4 for 7.0/7.0
BV26	17.2	Isolated from bird faeces in Belgium [c]	
BV45	15.3	Isolated from horse faeces in Belgium [c]	
EC13	14.2	Isolated from chicken faeces in Belgium [c]	
APEC124	13.9	APEC [d]	
BV13	13.1	Isolated from human faeces in Leuven (BE) [c]	3 for 7.0/7.0
EC59	12.8	Isolated from sewerage system in Antwerp (BE) [c]	
EC57	12.0	Isolated from sewerage system in Antwerp (BE) [c]	
EC23	11.8	Isolated from chicken meat in Leuven (BE) [c]	
EC1649	11.5	Feline EPEC, 43750 I [a]	
ATCC 43892	11.5	O29:NM	
BV50	11.5	Isolated from goose faeces in Belgium [c]	
BV8	11.4	Isolated from dog faeces in Leuven (BE) [c]	3 for 7.0/7.0
BV37	10.9	Isolated from goose faeces in Belgium [c]	
EC1640	10.9	Canine EPEC, 1004-1 [a]	
EC21	10.9	isolated from stream water in Belgium [c]	
ECOR[b] 58	9.9	O112:H8, Group B1 from a healthy lion in captivity in USA	
EC60	9.8	Isolated from sewerage system in Antwerp (BE) [c]	3 for 5.5/7.0 &

			7.0/7.0
EC62	9.8	Isolated from sewerage system in Antwerp (BE) [c]	
EC58	9.8	Isolated from sewerage system in Antwerp (BE) [c]	
ECOR[b] 30	9.5	O113:H2, 1 Group B1 strain from a healthy bison in Canada	
EC1645	8.4	Canine EPEC, B91-4 [a]	
ECOR[b] 01	6.7	Group A strain from a healthy person in USA	3 for 7.0/7.0
APEC15	5.8	APEC [d]	
EC1641	5.3	Canine EPEC, 1007-2 [a]	
ECOR[b] 65	5.3	O–:H10, Group B2 strain from a healthy ape in captivity in USA	3 for 7.0/7.0
APEC260	3.8	APEC [d]	
EC1607	0.3	CNF 1 (*E. coli* cytotoxic necrotizing factor-1), 559 [a]	
EC73	0.1	Isolated from sewerage system in Antwerp (BE) [c]	
EC1636	0.1	ETEC, 23A [a]	3 for 7.0/6.2 &7.0/7.0
EC1623	0.1	Bovine NTEC (Necrototoxic *E. coli*) II, B56 [a]	
BV23	0.1	Isolated from horse faeces in Belgium [c]	
EC28	0.1	Isolated from soil in Belgium [c]	3 for 7.0/7.0
ECOR[b] 08	0.1	O86, Group A strain from a healthy person in USA	
MG1655	0.1	K-12	
EC1616	0.1	CNF 2 (*E. coli* cytotoxic necrotizing factor-1), B177 [a]	

[a] Strains from Prof. J. Mainil, Laboratory of Bacteriology, Department of infectious and parasitic disease, Ulg, Liege, BE

[b] ECOR: *Escherichia coli* Reference Collection (http://www.shigatox.net/new/reference-strains/ecor.html). Group A, B1, B2, D and E are related to the phylogeny of *E. coli* (Clermont et al., 2000)

[c] Strains from the LMM (Laboratory of Food Microbiology), KU Leuven, Leuven, BE. Strains were isolated using first chromocult coliform agar. Then a PCR with the primers ECPAL-L and ECPAL-R was accomplished for identification. The primers amplify a segment of the pal gene encoding the peptidoglycan-associated lipoprotein which is conserved in *E. coli* and closely related bacteria. (Kuhnert et al., 1995)

[d] Strains from Prof. B. Goddeeris, Division Animal and Human Health Engineering, KU Leuven, Leuven, BE

597 Table 2. Thermal inactivation parameters of *E. coli* O157:H7 (ATCC 43888) at 58°C for different conditions
598 of pH during growth (acid and non acid adaptation) and thermal inactivation. Different letters in the same
599 column indicate significantly different parameters between conditions (Tukey, $p < 0.05$).

pH during growth	pH during thermal Inactivation	Shoulder length ± SE (min)	D_{58}-values ± SE (min)	t_{3d} (min)	RMSE
5.5	6.2	2.92 ± 1.24	3.08 ± 0.29 [c]	12.16	0.39
	7.0	2.88 ± 0.45	2.40 ± 0.12 [bc]	10.07	0.17
7.0	6.2	NA [a]	1.24 ± 0.07 [a]	3.72	0.36
	7.0	NA	2.02 ± 0.06 [ab]	6.05	0.23

600 [a] NA: Not applicable.

601 Table 3. Descriptive statistics of the D_{58}-values (min) of the 48 *E. coli* strains under different conditions of
602 pH during growth and thermal inactivation.

	pH during growth/thermal inactivation			
	5.5/6.2	5.5/7.0	7.0/6.2	7.0/7.0
Median	6.95	5.78	4.81	3.77
25th percentiles	4.63	3.82	3.03	2.94
75th percentiles	10.16	7.21	7.19	5.53
MAD [a]	2.44	1.53	1.91	0.99

603 [a] MAD: Median Absolute Deviation (obtained through mad () function in R, constant = 1).

604

605 Table 4. Linear mixed model (LMM) results for Eq. 4 describing the relationship between the *LogD* and
606 the different pH conditions (fixed effect) and strains (random effect). pH condition 7.0/7.0 is the
607 reference.

Random effects				Fixed effects			
Groups	Name	Variance	Std.Dev.	pH condition	Estimate	Std. Error	t value
Strain	Intercept	0.07	0.30		0.65	0.04	15.70
Residual		0.02	0.13	5.5/6.2	0.21	0.02	11.38
				5.5/7.0	0.11	0.02	6.06
				7.0/6.2	0.05	0.02	2.53

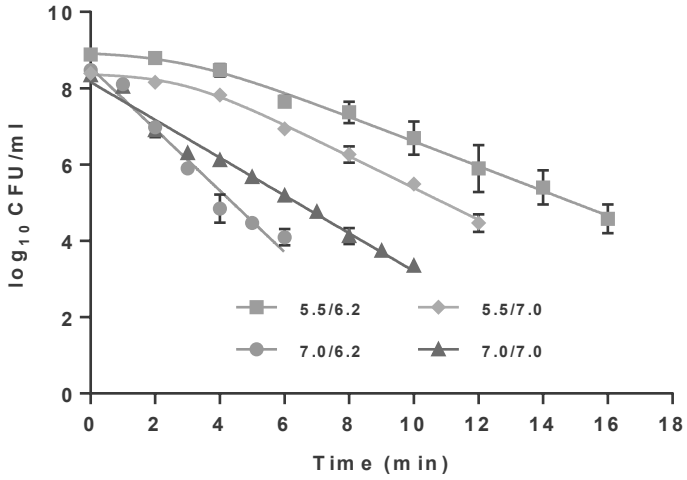
608

609

610 Table 5. Results from Tukey post hoc multiple comparisons ($p < 0.05$) test following the LMM (Eq. 4) that
611 describes the *LogD* according to the pH conditions for all 48 strains.

# comparison	Conditions comparison	Estimate	Std.Error	z value	Pr(>|z|)
1	5.5/6.2 - 7.0/7.0	0.21	0.02	11.38	<0.001
2	5.5/7.0 - 7.0/7.0	0.11	0.02	6.06	<0.001
3	7.0/6.2 - 7.0/7.0	0.05	0.02	2.53	0.06
4	5.5/7.0 - 5.5/6.2	-0.10	0.02	-5.24	<0.001
5	7.0/6.2 - 5.5/6.2	-0.16	0.02	-8.65	<0.001
6	7.0/6.2 - 5.5/7.0	-0.06	0.02	-3.44	0.003

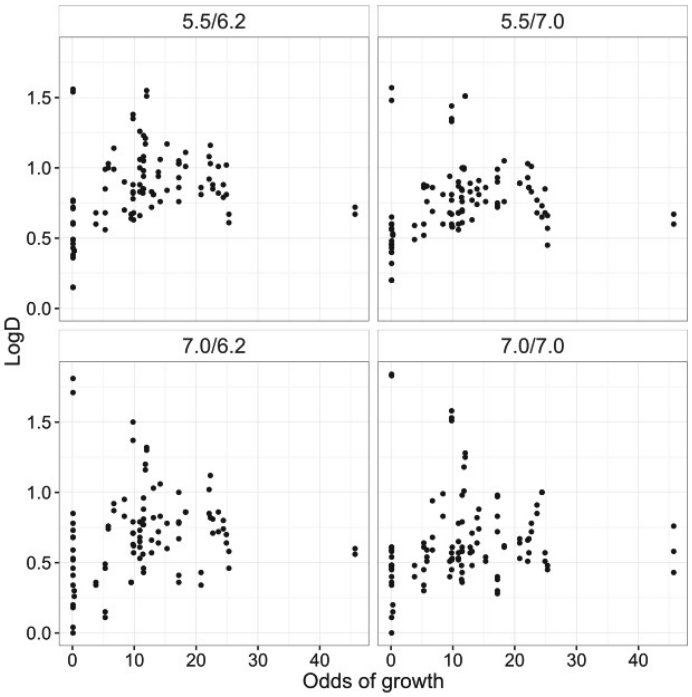
612



613

614 Fig. 1. Thermal inactivation curves of *E. coli* O157:H7 (ATCC 43888) at 58°C at the four combinations of pH
615 during growth in buffered LB adjusted to acidic (5.5) or neutral (7.0) pH conditions and subsequently
616 inactivated in LB adjusted to pH 6.2 or 7.0. Lines represent modelling results obtained with the software
617 GInaFiT (Geeraerd et al., 2005). The error bars represent the standard error.

618

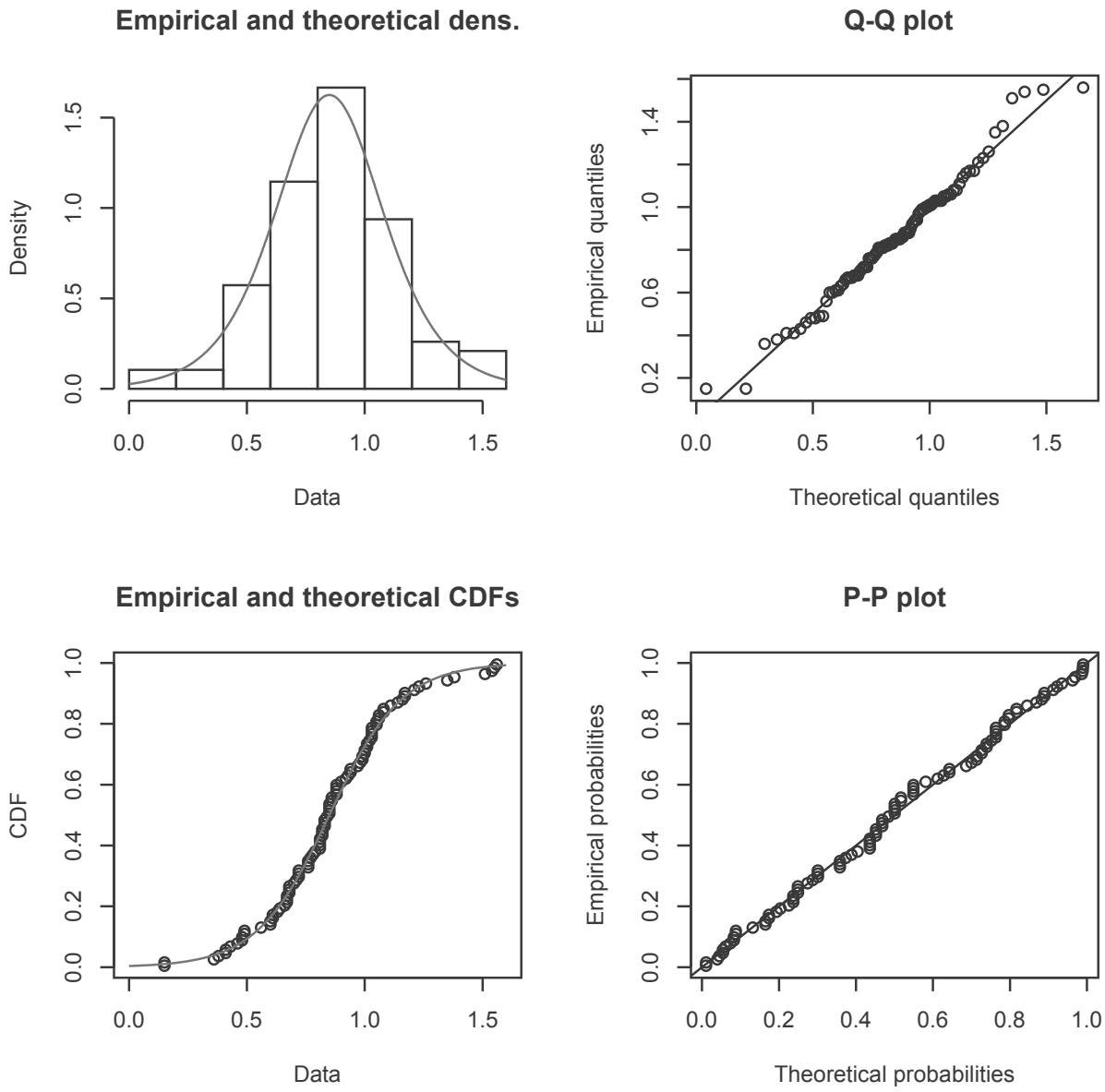


Condition	ρ	p value
5.5/6.2	0.40	<0.001
5.5/7.0	0.41	<0.001
7.0/6.2	0.32	0.002
7.0/7.0	0.31	0.001

ρ: Spearman's correlation coefficient

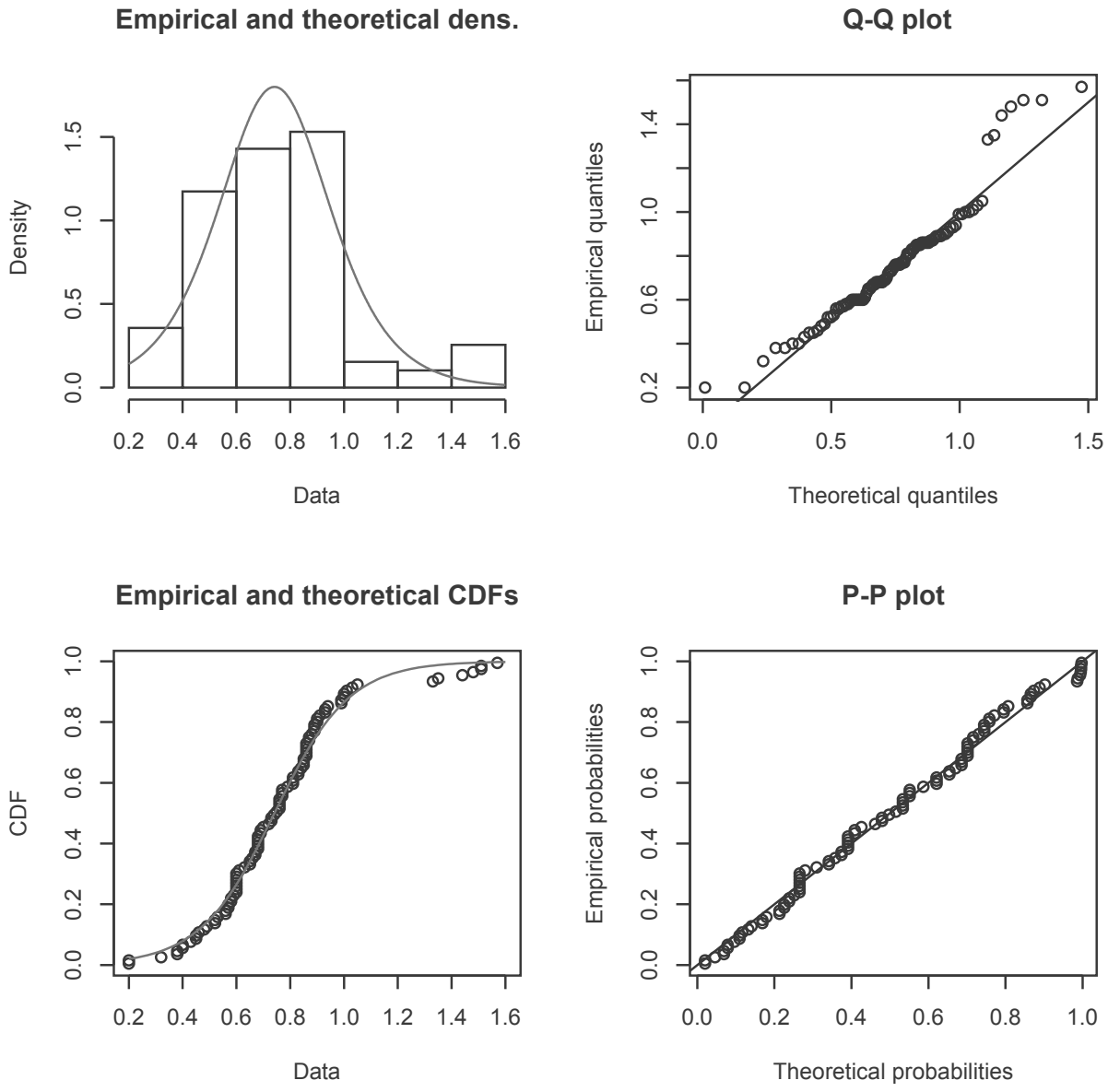
Fig. 2. $LogD_{58}$ for each condition in function of the odds of growth at low pH of the 48 strains. The table shows the results for the Spearman Rank-Order Correlation test (ρ) and the respective p-value for each condition.

625 Supplementary file



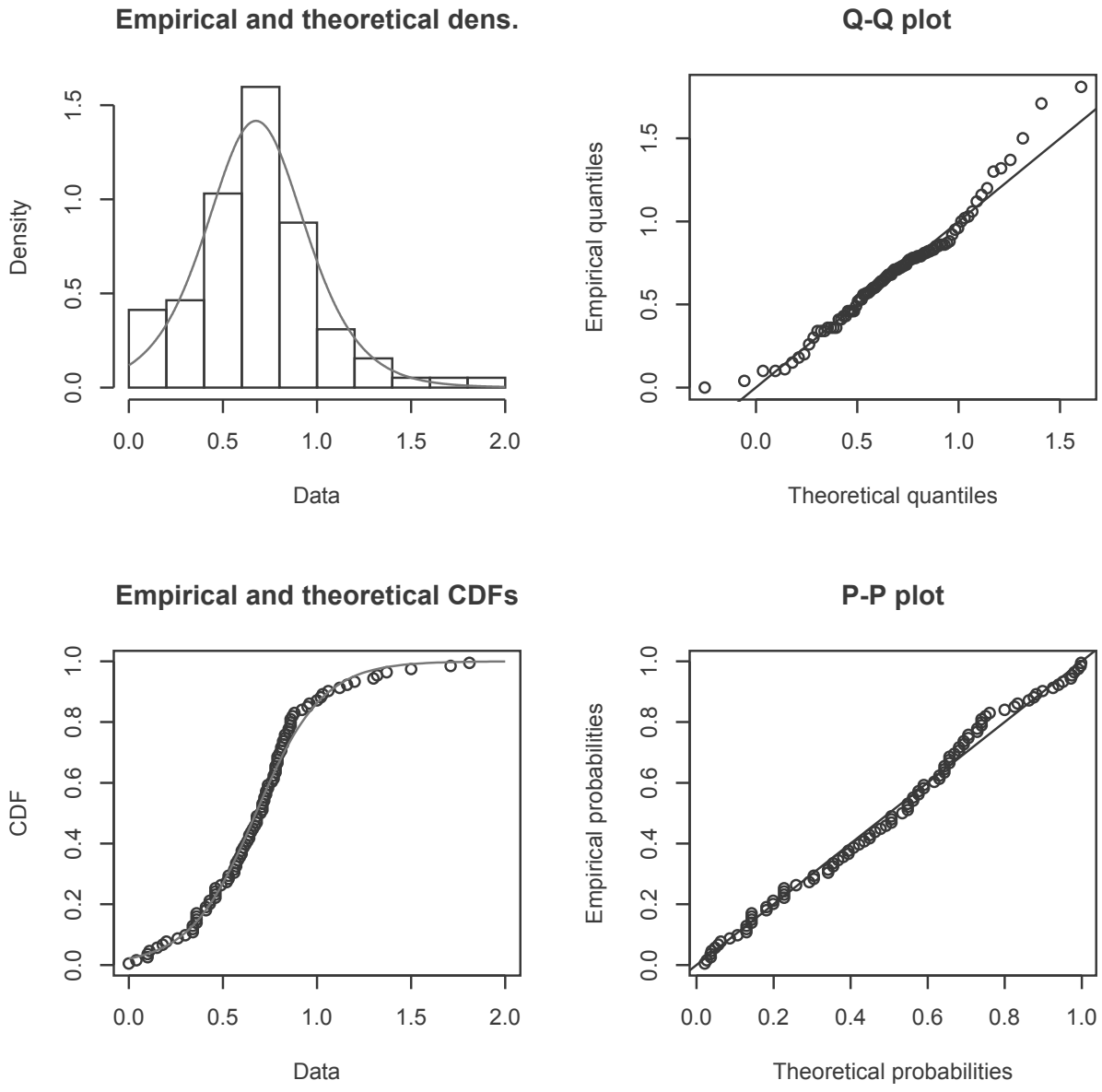
626

627 Fig. S1. Results of the fitting of a Logistic distribution to $LogD_{58}$ (Data) for condition 5.5/6.2. Clockwise: (a)
628 histogram of the empirical distribution overlaid with the density function (continuous line) of the
629 theoretical fitted distribution; (b) Q–Q plot; (c) P–P plot; (d) empirical density function of the data
630 overlaid with the cumulative density function (continuous line) of the theoretical fitted distribution.



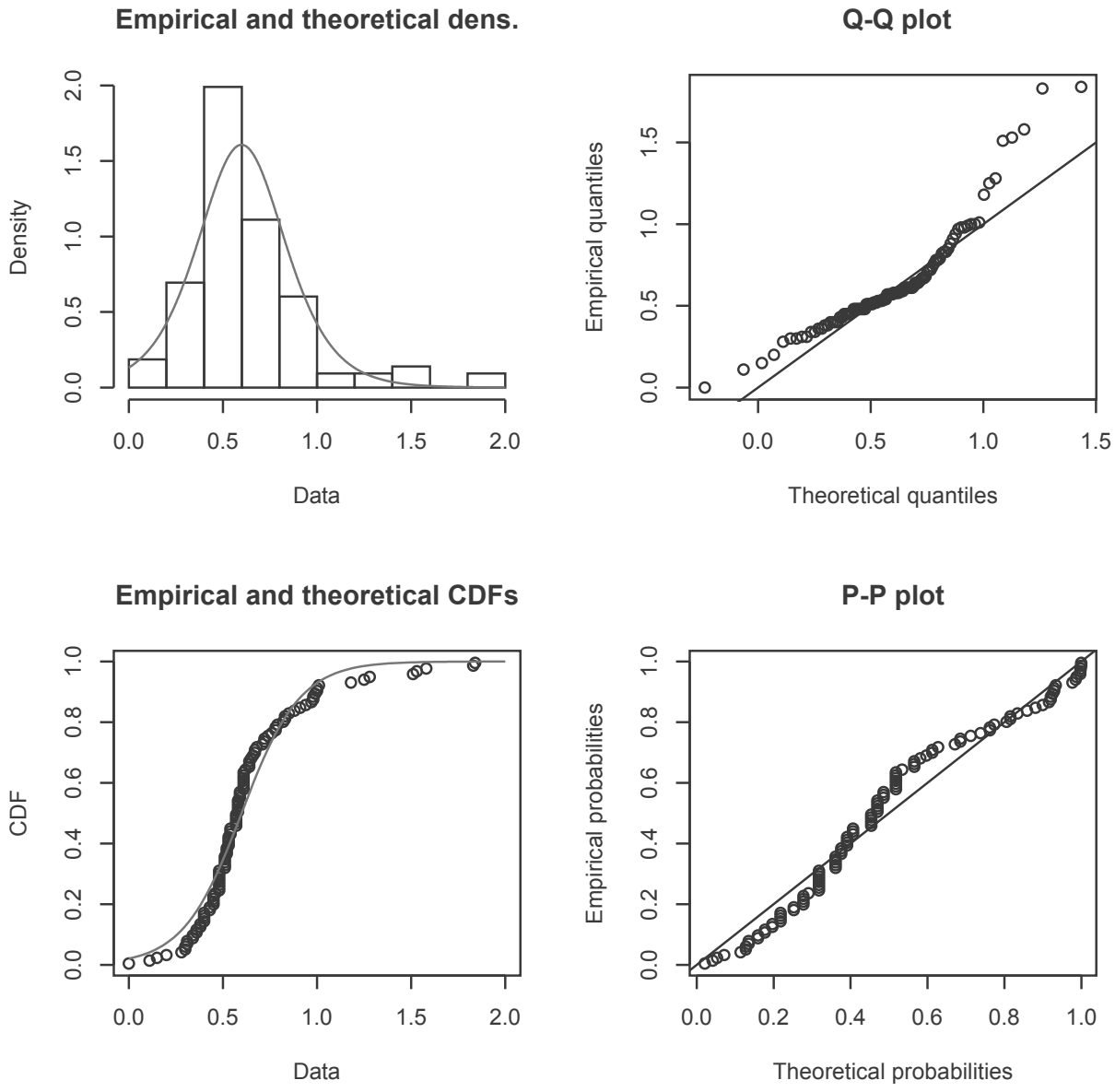
631

632 Fig. S2. Results of the fitting of a Logistic distribution to $LogD_{58}$ (Data) for condition 5.5/7.0. Clockwise: (a)
633 histogram of the empirical distribution overlaid with the density function (continuous line) of the
634 theoretical fitted distribution; (b) Q–Q plot; (c) P–P plot; (d) empirical density function of the data
635 overlaid with the cumulative density function (continuous line) of the theoretical fitted distribution.



636

637 Fig. S3. Results of the fitting of a Logistic distribution to $LogD_{58}$ (Data) for condition 7.0/6.2. Clockwise: (a)
638 histogram of the empirical distribution overlaid with the density function (continuous line) of the
639 theoretical fitted distribution; (b) Q–Q plot; (c) P–P plot; (d) empirical density function of the data
640 overlaid with the cumulative density function (continuous line) of the theoretical fitted distribution.



641

642 Fig. S4. Results of the fitting of a Logistic distribution to $LogD_{58}$ (Data) for condition 7.0/7.0. Clockwise: (a)
643 histogram of the empirical distribution overlaid with the density function (continuous line) of the
644 theoretical fitted distribution; (b) Q–Q plot; (c) P–P plot; (d) empirical density function of the data
645 overlaid with the cumulative density function (continuous line) of the theoretical fitted distribution.

646

647

Table S1. Logistic distribution parameters. SE: standard error

	pH during growth/thermal inactivation			
	5.5/6.2	5.5/7.0	7.0/6.2	7.0/7.0
Location ± SE	0.85±0.03	0.74±0.02	0.68±0.03	0.60±0.03
Scale ± SE	0.15±0.01	0.13±0.01	0.18±0.02	0.16±0.01

Table S2. Mean *D*-values ± standard deviation (min) for each food related strain according to the pH conditions and the median *D*-values for per condition (regarding only the food related strains).

Strain	5.5/6.2	5.5/7.0	7.0/6.2	7.0/7.0
EC1	6.85 ± 0.61	4.92 ± 0.47	5.90 ± 0.41	10.02 ± 0.11
EC23	15.50 ± 0.55	9.93 ± 0.21	15.15 ± 0.84	12.73 ± 2.48
EC3	11.55 ± 1.38	8.47 ± 2.72	7.22 ± 0.02	4.15 ± 0.01
O157:H7	3.03 ± 0.26	2.40 ± 0.09	1.24 ± 0.09	2.02 ± 0.06
Median	9.20	6.70	6.56	7.09

Table S3. Results from Tukey post hoc multiple comparisons ($p < 0.05$) test following LMM (Eq. 5) that describes the *LogD* according to the selected food related strains.

Strains comparison	Estimate	Std.Error	z value	Pr(>|z|)
EC1 - O157:H7	0.51	0.07	7.57	<0.001
EC23 - O157:H7	0.80	0.07	11.92	<0.001
EC3 - O157:H7	0.54	0.07	8.11	<0.001
EC23 - EC1	0.29	0.07	4.35	<0.001
EC3 - EC1	0.04	0.07	0.54	0.949
EC3 - EC23	-0.26	0.07	-3.81	<0.001