New insights on *Listeria monocytogenes* growth in pressurised cooked ham: a piezo-stimulation effect enhanced by organic acids during storage

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Abstract

The aim of the present study was to understand growth and survival responses of *Listeria monocytogenes* during the storage of high pressure processed (HPP) cooked ham formulated with organic acids to inhibit growth of the pathogen. Cooked ham batches were manufactured without organic acids (control), with potassium lactate (2.8% or 4%) or with potassium lactate and sodium diacetate (2.0% + 0.11% or 2.0% + 0.45%). Products were aseptically sliced and inoculated with 10^7 cfu/g or 10^2 cfu/g of either *L. monocytogenes* CTC1034 (a meat isolate) or a cocktail of three isolates (12MOB045Lm, 12MOB089Lm and Scott A). Vacuum-packed samples with 10^7 cfu/g were HPP at 600 MPa for 3 min, whereas samples with 10^2 cfu/g were not HPP. Growth or survival of *L. monocytogenes* was determined during subsequent storage at 8, 12 and 20 ºC. Growth or survival was characterized by fitting the experimental data using the primary logistic model and the log-linear with shoulder model, respectively. Secondary models were fitted to characterize the effect of temperature on growth kinetic parameters without or with HPP. For cooked ham without organic acids, growth rates of *L. monocytogenes* were slightly increased by HPP and lag times were longer. Interestingly, for cooked ham with organic acids, the HPP had a significant stimulating effect on subsequent growth of *L. monocytogenes* (piezo-stimulation). At 20 ºC, the growth rates of *L. monocytogenes* in cooked ham with lactate were up to 4-fold higher than those of the same product without HPP. The observed enhancement of the piezo-stimulating effect of organic acids on growth rates during storage of HPP cooked ham represents a challenge for the use of organic acids as antimicrobials in these products. A predictive model available as part of the Food Spoilage and Safety Predictor (FSSP) software seemed useful to predict growth and growth boundary of *L. monocytogenes* in non-pressurised cooked ham. This model was calibrated to take into account the observed piezo-stimulating effect and to predict growth of *L. monocytogenes* in HPP cooked ham with organic acids.

Keywords

High pressure processing; Food safety; Deli meat products; Post-lethality treatments; Safe shelf-life

1 Introduction

High pressure processing (HPP) is a non-thermal technology with increasing application in the food industry. HPP can extend the shelf-life of perishable food while ensuring food safety (Rendueles et al., 2011). HPP is particularly interesting as a post-lethality treatment (PLT) for ready-to-eat (RTE) foods that are exposed to microbial contamination after thermal treatments such as cooked meat products commercialised in convenience format (i.e. diced, sliced and packaged). HPP causes microbial inactivation not only of spoilage microorganisms but also of pathogens like *Listeria monocytogenes*, the most relevant foodborne pathogen for this type of products (Buchanan et al., 2017).

The *Listeria* zero tolerance followed by countries such as USA forces food manufacturers to design specific risk mitigation strategies. In this framework, the Listeria rule (FSIS, 2014) rate the RTE food manufacturers according to the RTE product risk. The safest operating procedures are those validated as Alternative 1, in which PLT aiming to reduce pathogen loads are combined with antimicrobial agents (AMA) to inhibit the pathogen growth during the product shelf-life. The so called Alternative 2 consists in either the application of a PLT (Alternative 2a) or an AMA (Alternative 2b, considered as a higher risk than alternative 2a). While, the highest risk occurs when operating procedures rely exclusively on sanitation and good manufacturing practices (i.e. Alternative 3). Among AMA, organic acids and their salts (lactate, acetate, diacetate) are food additives frequently used as *L. monocytogenes* growth inhibitors in cooked meat products (Pérez-Rodríguez et al., 2017).

The effectiveness of the specific strategies needs to be validated (FSIS, 2013, 2014). In case of HPP, the microbial inactivation during processing is of primary importance. This inactivation is influenced by processing parameters such as pressure, time and temperature as well as by product characteristics that may favour lethality or protect microorganisms during HPP (Hereu, Dalgaard, et al., 2012; Rendueles et al., 2011). Therefore, the validation should be carried out through a product-oriented approach (Hereu et al., 2014). Moreover, the potential occurrence of resistant cells after HPP makes it necessary to take into consideration the behaviour of surviving bacteria, as for example cooked ham and refrigerated storage may offer conditions enabling the recovery

and subsequent growth of *L. monocytogenes* during the product shelf-life (Jofré and Serra, 2016). The effect of organic acids and their salts have been extensively studied and several predictive tools can be used to design products not supporting the growth of the pathogen (Mejlholm et al., 2010). However, scarce information is available about the possible interaction between HPP and antimicrobials, particularly organic acids and their salts. Based on the antimicrobial hurdle concept (Leistner, 2007), an additive or a synergistic effect may be expected. However, in previous studies with cooked ham an increased HPP resistance of *L. monocytogenes* was observed by the presence of lactate in the product formulation but the subsequent growth or survival of the pathogen during the product shelf-life was not studied (Bover-Cid et al., 2016).

The present work was carried out to study the behaviour of *L. monocytogenes* during storage of HPP cooked ham formulated without or with natural antimicrobials often used by the meat industry, i.e. potassium lactate (E-326) and sodium diacetate (E-262). The *L. monocytogenes* growth and growth-boundary model included in the Food Spoilage and Safety Predictor (FSSP, v4.0) was used to design experiments where some formulations were close to the growth boundary of the pathogen. This allowed the combined effect of HPP and organic acids to be studied close the growth boundary, which is important as products stabilized against growth of *L. monocytogenes* are desirable.

2 Material and methods

2.1 Cooked ham manufacture and characterization

Cooked ham was manufactured *ad-hoc* using pork meat and the following ingredients (g/kg): water, 120; salt, 20.7; sodium tripolyphosphate, 5.8; dextrose, 5.8; carragenate, 2.3; sodium ascorbate, 0.6; and sodium nitrite 0.1. For the 34 combinations of conditions studied (see 2.3) five different batches were manufactured, one without organic acids as control product, two with potassium lactate (HiPure Corbion®, Montmeló, Spain) at 2.8% and 4.0% and two with potassium lactate and sodium diacetate (Grama Aliment SL, Les Preses, Spain) at 2% + 0.11% or 2% + 0.45%. The concentrations of potassium lactate and sodium diacetate were selected close

to the growth/no growth boundary for *L. monocytogenes* at 8 ºC and 12 ºC, respectively, according to the predictive model "Growth of *Listeria monocytogenes* in chilled seafood and meat products" available in the Food Spoilage and Safety Predictor (FSSP v4.0) software (http://fssp.food.dtu.dk). Meat was minced in a cutter to a particle size of 6 mm. Ingredients were homogenized in a mixer for 30 min, stuffed into an impermeable plastic film, and cooked in an oven at 68 °C for 5 h resulting in a product core temperature of 65 °C. For each formulation up to five blocks of *ca*. 3 kg each were manufactured.

Product a_w was measured with an AquaLab™ instrument (Series 3; Decagon Devices Inc., Pullman, WA, USA). pH was measured by direct measurement with a penetration probe (52-32; Crison Instruments SA, Alella, Spain) connected to a portable pH-metre (PH 25; Crison Instruments). Concentrations of organic acids were determined from an acid extract of a cooked ham sample by HPLC, using an ion exclusion column (Transgenomic ICSepICE-ORH-801, Chrom Tech. Inc., MN, USA) with a refractive index (RI) detector. Nitrites were determined by spectrofluorometry and sodium by flame atomic absorption spectroscopy according to the Spanish official methods (Anonymous, 1979). The fat, protein and water contents were determined according to the AOAC official method 2007.04 (Anderson, 2007) with a FoodScan™ device (FOSS, Hillerød, Denmark).

2.2 *L. monocytogenes* strains and pre-culture conditions

Strains of *L. monocytogenes* used in the present study included: the meat isolate CTC1034 (serotype 4b) from the IRTA culture collection and previously used in our studies dealing with HPP meat products (Bover-Cid et al., 2015; Bover-Cid et al., 2011; Hereu, Bover-Cid, et al., 2012; Hereu, Dalgaard, et al., 2012; Hereu et al., 2014); the reference strains 12MOB045LM (genoserotype II) and 12MOB089LM (genoserotype IV) from the European Reference Laboratory for *L. monocytogenes*, both recommended for challenge tests with meat products (EURL Lm, 2014); and Scott A (4b) a clinical isolate frequently included in HPP inactivation studies (van Boeijen et al., 2008).

Strains were kept at 80 °C in Brain Heart Infusion (BHI) broth (Beckon Dickinson, Sparks, Md., USA) with 20% glycerol until used. These conditions provided slightly more pressure resistant cells (conservative approach) than pre-culturing at refrigeration temperatures, though without modifying the growth rate of the pathogen during the subsequent growth (Hereu et al., 2014). Thawed cultures of the strain CTC1034 were directly used to inoculate cooked ham slices at *ca*. 10^7 cfu/g or were diluted to 10^5 cfu/g with physiological saline (0.85% NaCl and 0.1% Bacto Peptone) to inoculate cooked ham slices at *ca*. 10^2 cfu/g (see section 2.3). A cocktail including 12MOB045LM, 12MOB089LM and Scott A (Lm-mix) was prepared by mixing the respective thawed cultures at equal concentrations before being directly inoculated (1% v/w) to the products or diluted as described for the CTC1034 strain.

2.3 Challenge tests, HPP and storage conditions

Cooked hams with the five different formulations (see section 2.1) were sliced in the laboratory under aseptic conditions. Slices of each type of cooked ham were surface spiked with either the *L. monocytogenes* CTC1034 strain or with the cocktail of three strains (i.e. 12MOB045LM, 12MOB089LM and Scott A). This inoculation was performed by using a laminar flow cabinet to avoid contamination with other microorganisms. The inoculum level for either the single strain or the mix of strains was 1% (v/w) to reach a final concentration of *ca*. 10^7 cfu/g for products to be HPP and *ca*. 10^2 cfu/g for non-pressurised products. These different inoculum levels between HPP and non-pressurised products were necessary to enable quantitative characterization of the growth curve. The inoculated volume was spread on the whole surface of the ham slices with a single-use sterile Digralsky spreader and then let to be adsorbed for 2 min. under a laminar flow of sterile air. Inoculated slices of each product were vacuum packaged (EV-15-2-CD; Tecnotrip, Terrassa, Spain) in PET/PE bags (oxygen permeability < 50 cm^3/m^2/24 h and low water vapour permeability < 15 mg/m^2/24 h; Sacoliva S.L., Barcelona, Spain). Samples were pressurised at 600 MPa for 3 min using commercial high pressure processing equipment (Wave 6000; Hiperbaric, Burgos, Spain) at an initial water temperature of 15 °C. The come-up rate was on average 220 MPa/min and the pressure release almost instantaneous (< 6 s). Samples inoculated with the lower

inoculum were not pressure treated and used as controls. Pressurised and non-pressurised samples were stored at 8 and 12 °C for 16 to 90 days. These temperatures are recommended by the European Reference Laboratory of *L. monocytogenes* Guidelines to conduct challenge test to study the safe-shelf life of ready-to-eat food (EURL Lm, 2014). Furthermore, for products inoculated with *L. monocytogenes* strain CTC1034 storage at 20 ºC during 10 to 58 days was also studied to better characterize the effect of HPP and organic acids on growth of the pathogen. A total of 34 experimental conditions combining product formulation, *L. monocytogenes* strains, storage temperatures and HPP were studied (see Tables 1 and 2).

2.4 Microbiological analysis

To monitor *L. monocytogenes* growth behaviour, samples from all 34 experimental conditions in the study were periodically analysed with a total of 30 to 44 data points distributed all along the storage period. Each sample was homogenized 1/10 in a bag Blender Smasher® (bioMérieux, Marcy-l'Étoile, France) and 10-fold serially diluted in physiological saline (0.85% NaCl and 0.1% Bacto Peptone). Enumeration of *L. monocytogenes* was performed on the CHROMagar™ *Listeria* chromogenic media (CHROMagar, Paris, France) incubated at 37 °C for 24 h. To achieve a quantification limit of 2 cfu/g, 5 ml of the 1/10 diluted homogenate was pour plated into plates with a diameter of 14 cm. For samples with expected concentration of *L. monocytogenes* below this quantification limit, the presence/absence of the pathogen was investigated by enrichment of 25 g-samples in 225 ml tryptic soy broth (Becton Dickinson) supplemented with 0.6% yeast extract (TSBYE) and incubated for 48 h at 37 °C. After enrichment, the presence of *L. monocytogenes* was detected by plating on CHROMagar™ *Listeria*. For modelling purposes, absence in 25 g was computed as -1 Log cfu/g, presence below the quantification was computed as -0.3 Log cfu/g.

Additionally, the potential contamination by lactic acid bacteria (LAB) in cooked ham samples (both pressurised and non-pressurised) was checked along the experiments by plating the homogenized 1/10 dilution into MRS (de Man Rogosa and Shape) agar plates (Merck), which were incubated at 30 ºC for 72h under anaerobiosis. For the experiments carried out with the

control ham at 8 ºC, a high sampling frequency was carried out (i.e. on days 0, 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 14, 15 and 17). No LAB were detected and this is likely related to the aseptic conditions applied during cooked ham manipulation (slicing and packaging). For the other trials the absence of LAB (<10 cfu/g) was verified occasionally with 3 to 4 sampling times along the storage time.

2.5 Primary growth modelling

To estimate the kinetic growth parameters for each growth curve, the primary Logistic growth models with delay ($\lambda > 0$) and without delay ($\lambda = 0$) (Eq. (1), (Rosso et al., 1996)) were fitted to the log-transformed counts using the nls2 and nls function form the respective nls2 and nls packages of R (R Core Team, 2013).

$$\text{If } t < \lambda \qquad \text{Log}(N_t) = \text{Log}(N_0)$$

$$\text{If } t \geq \lambda \qquad \text{Log}(N_t) = \text{Log}\left(\frac{N_{max}}{1+\left(\frac{N_{max}}{N_0}-1\right)\left(\exp\left(-\mu_{max}\ (t-\lambda)\right)\right)}\right) \qquad \text{Eq. (1)}$$

Where t is time (d); N_0 is the bacterial concentration (cfu/g) at time zero; N_t is the bacterial concentration (cfu/g) at time t, N_{max} is the maximum bacterial concentration (cfu/g), λ is the lag time (d) and μ_{max} is the maximum specific growth rate (d^{-1}).

The *F*-test was applied to determine the statistical significance of the estimated lag time for each growth curve (Dalgaard, 1995).

For the combination of conditions not supporting growth and compromising the viability of the pathogen a log-linear with shoulder primary model (Eq. (2), (Geeraerd et al., 2000)) was fitted to the data.

If $t \leq S$;

$$\text{Log}(N) = \text{Log}(N_0)$$

If $t > S$;

$$\mathrm{Log}(N) = \mathrm{Log}(N_0) \quad \left(\frac{k_{max}\ t}{\mathrm{Ln}(10)}\right) + \mathrm{Log}\left(\frac{\exp^{(k_{max}\ S)}}{1+\left[\exp^{(k_{max}\ S)}-1\right]\ \exp^{(-k_{max}\ t)}}\right) \qquad \text{Eq. (2)}$$

Where t is time (d); N_0 is the bacterial concentration (cfu/g) at time zero, k_{max} is the maximum specific inactivation rate (d ^{1}) and S is the shoulder (d). The F-test was applied to determine the statistical significance of the shoulder for each growth curve.

2.6 Comparison of observed and predicted growth rates

The growth rates observed at different combination of experimental conditions were compared with those predicted by the model of Mejlholm and Dalgaard (2009) available in the Food Spoilage and Safety Predictor (FSSP v4.0) as "Growth of *Listeria monocytogenes* in chilled seafood and meat products". This model was previously found suitable to predict the growth behaviour of *L. monocytogenes* in cooked ham (Mejlholm et al., 2010). The comparison was performed to facilitate a quantitative evaluation of effects by experimental condition rather than as an evaluation of the specific predictive model. Growth was predicted by taking into account storage temperature and product characteristics for each experimental condition (see Table 3). Observed and predicted growth was compared by calculation of bias- (B_f) and accuracy (A_f) factors for the μ_{max}–values (Dalgaard and Jorgensen, 1998). The bias factor values were calculated so that numbers lower than 1 always indicated that predicted growth was slower than observed growth. As an example, a B_f-value of 0.75 indicates predicted growth rates to be 25% slower than observed growth rates (Mejlholm et al., 2010; Ross, 1996). A_f-values > 1.5 have previously been shown to indicate incomplete models or systematic deviation between observed and predicted μ_{max}-values (Mejlholm and Dalgaard, 2013).

2.7 Secondary growth modelling

Secondary modelling was applied to assess the effect of the storage temperature on the primary growth parameters (μ_{max}, λ and N_{max}) of *L. monocytogenes* in cooked ham without added organic

acids. The modified Ratkowsky square root model (Eq. (3); (Ross and Dalgaard, 2004)) was used to fit the growth rate (μ_{max}, d ^{1}) values determined at different storage temperatures.

$$\sqrt{\mu} = \sqrt{\mu_{ref}} \cdot \left(\frac{T - T_{min}}{T_{ref} - T_{min}}\right) \quad \text{Eq.(3)}$$

Where μ_{ref} is the estimated growth rate (d^{-1}) at a reference temperature, T_{ref} is the temperature of reference fixed at 25 ºC (Mejlholm and Dalgaard, 2009), and T_{min} is the estimated theoretical minimum temperature for *L. monocytogenes* growth. The relative lag time (RLT) concept, defined as the ratio of the lag time to the generation time (GT = Ln(2)/μ_{max}) was used to develop a secondary lag time (λ) model (Eq. (4), (Ross and Dalgaard, 2004)). Where a potential effect of storage temperature on RLT was modelled as previously described (Hereu et al., 2014) with the parameters k_0 and k_1 characterizing a potential temperature dependance of RLT.

$$\lambda = RLT \cdot \frac{Ln(2)}{\mu_{max}} = k_0 + \frac{k_1}{T^2} \cdot \frac{Ln(2)}{\mu_{max}} \quad \text{Eq. (4)}$$

The effect of storage temperature on log(N_{max}) was described by using a simple linear equation (Eq. (5)) where a is log(N_{max}) at 0°C and b a slope parameter.

$$Log(N_{max}) = a + b \cdot T \quad \text{and} \quad N_{max} = 10^{(a + b\,T)} \quad \text{Eq. (5)}$$

Following the two-step modelling approach, a one-step or global regression procedure was applied. A global model (Eq. (6)) integrating the primary model (Eq. (1)) and the secondary models for λ, μ_{max} and N_{max} was fitted to the data set with 350 Log cfu/g values for cooked ham without added organic acids. The *F*-test was applied to assess the need of two different models for non-HPP and HPP products. The goodness of fit of the developed models was assessed by means of residual sum of square (RSS), root mean square error (RMSE) and determination coefficients (R^2 and R^2_{adj}).

If $t < \lambda$

$$\mathrm{Log}(N_t) = \mathrm{Log}(N_0) \qquad \text{Eq. (6)}$$

If $t \geq \lambda$

$$\mathrm{Log}(N_t) = \mathrm{Log}\left(\frac{10^{(a+b\,T)}}{1+\left(\frac{10^{(a+b\,T)}}{N_0}-1\right)\cdot \exp\left(\mu_{ref}\cdot\left(\frac{T-T_{min}}{T_{ref}-T_{min}}\right)^2\right)\cdot t\left(k_0+\frac{k_1}{T^2}\cdot\frac{\mathrm{Ln}(2)}{\mu_{ref}\cdot\left(\frac{T-T_{min}}{T_{ref}-T_{min}}\right)^2}\right)}\right)$$

3 Results and discussion

3.1 *Listeria monocytogenes* behaviour in non-pressurised cooked ham

Growth and survival responses of *L. monocytogenes* in 17 challenge tests for cooked ham without HPP are shown in Fig. 1 (empty symbols) with fitted kinetic parameters from primary models shown in Table 1. As expected, growth of the pathogen was observed for non-pressurised cooked ham formulated without organic acids (Fig. 1a, b and c). Similar growth curves were found for *L. monocytogenes* CTC1034 and for the mix including the reference strains 12MOB045LM, 12MOB089LM and Scott A (Fig. 1). The expected prevention of growth due to added organic acids was found for products stored at 8, 12 and 20 ºC (Fig. 1h, i and k, empty symbols). Under these conditions, the viability of *L. monocytogenes* was compromised. A log-linear decreasing trend was observed, with k_{max} as maximum specific inactivation rate, after surviving for some time with the shoulder parameter being statistically significant ($p < 0.05$) in most survival curves (survival parameter estimates are also included in Table 1). The addition of 2.8% lactate in non-pressurised cooked ham (Fig. 1d, empty symbols) extended the lag time in comparison with the control without lactate, but it did not prevent growth of *L. monocytogenes*. In this case, the wide dispersion of the observed levels of *L. monocytogenes* along the storage made the estimation of growth kinetic parameters more uncertain than in the control products, as indicated by the goodness of fit parameters (Table 1). At 20 ºC, *L. monocytogenes* was able to grow in the presence of 4% lactate and with a combination of 2% lactate plus 0.11% diacetate (Fig. 1g and j), although

these conditions prevented growth at 8 °C (Fig. 1h) and 12 °C (Fig. 1e). As expected, lag times were longer and growth rates lower compared to control product without organic acids (Table 1). With 2% lactate plus 0.45% diacetate inactivation of *L. monocytogenes* was observed for non-pressurised cooked ham at 12 °C and inactivation was faster at 20 °C (Fig. 1k; Table 1). The faster inactivation at a higher temperature under growth-preventing conditions (Fig. 1i and k) is in agreement with previous studies of both *L. monocytogenes* and *E. coli* (Ross et al., 2008; Zhang et al., 2010). LAB were not detected (i.e. < 10 cfu/g) in any of the samples analysed along the experiments, therefore *L. monocytogenes* behaviour was not determined by the interaction with endogenous LAB.

For cooked ham non-HPP growth responses were in accordance with those predicted by the FSSP model without LAB interaction, as shown by the B_f-value of 0.89 indicating that growth rates on average were predicted to be 11% slower that observed. Without or with added organic acids the B_f-values were, respectively, 0.95 and 0.84 (Table 3). Of the 17 experimental conditions for non-pressurised products, growth or no-growth responses were correctly predicted for 15 trials, whereas for cooked ham with 4% lactate the prediction of growth with μ_{max} of 0.25 d^{-1} was fail-safe as slight inactivation was observed for both CTC1034 and Lm-mix (Fig. 1e, Table 1). This difference between observed and predicted growth may be due to minor deviations between actual and measured product characteristics. If for example the product pH actually were 6.09 rather than the measured 6.15 (Table 3) then the applied model would correctly predict no-growth for this product formulation with high lactate concentration.

3.2 *Listeria monocytogenes* behaviour in HPP cooked ham

The applied HPP (600 MPa for 3 min) caused a significant inactivation on *L. monocytogenes* of about 7 log units (Results not shown). Just after the HPP, *L. monocytogenes* was detected in all samples though at levels below the quantification limit in most of the samples, hampering a more precise quantification of the log reductions.

In the HPP control cooked ham (Fig. 1a, b, c, full symbols) the surviving *L. monocytogenes* cells were able to initiate growth after a relatively short time post-HPP. Without added organic acids

the lag times and growth rates in HPP cooked ham (Table 2) were slightly higher than those observed for non-HPP products (Table 1). Some works have dealt with the behaviour of piezo-tolerant isolates of *L. monocytogenes* ScottA and LO28 in comparison with the wild type counterpart (Joerger et al., 2006; Karatzas and Bennik, 2002; Van Boeijen et al., 2010). In these works, *L. monocytogenes* mutants exhibited identical or slightly lower growth rate in comparison with the wild-type strain. These studies applied lower pressures (150 MPa to 500 MPa) than those used in the present work and by the meat industry nowadays. Besides, they were performed in simple laboratory media such as brain heart infusion (BHI). Under these conditions the effect of food matrix components was omitted and thus results may not be comparable with the findings of the present study carried out with meat products.

Surprisingly, *L. monocytogenes* was able to grow in HPP products formulated with organic acids at concentrations that prevented growth in non-pressurised cooked ham (Fig 1h, e, i). This was observed both for *L. monocytogenes* CTC1034 and for the mix of strains (Fig. 1). In these challenge tests, the estimated growth parameters were less accurate due to the occurrence of results below the quantification limit. The unexpected growth could result from HPP-resistant cells indicating the occurrence of a heterogeneous *L. monocytogenes* population including piezo-sensitive and piezo-resistant fractions (Hereu et al., 2014; Van Boeijen et al., 2010). Thus, the unexpected growth could represent the behaviour of the cells that were able to resist, recover from potential sub-lethal damage and grow during the subsequent refrigerated storage of the product. However, growth rates of *L. monocytogenes* in HPP products with organic acids were markedly higher than for non-pressurised products with the same concentrations of organic acids. This was most pronounced at 20 ºC where *L. monocytogenes* was able to grow up to 4-fold faster in comparison with the corresponding non-HPP conditions (Fig. 1f, g, j; Tables 1 and 2). Thus, in the presence of organic acids a pronounced piezo-stimulation by HPP was observed. The comparison of observed and predicted growth contributed to the quantification of this piezo-stimulating effect as the applied growth and growth boundary model included in the FSSP software did not take this effect into account. With HPP and without added organic acids the B_f value of 0.71 showed predicted growth rates to be 29% slower that observed, whereas with both

HPP and added organic acids B_f was 0.29 and predicted growth rates were 71% slower that observed (Table 3). Of the 17 experimental conditions with HPP, growth or no-growth responses were correctly predicted for 12 (Fig.1, Table 1) and fail-dangerous predictions were obtained with 2% lactate plus 0.11% diacetate at 8°C and with 2% lactate plus 0.45% diacetate at 12°C and 20°C (Fig. 1). With 2% lactate plus 0.45% diacetate at 12 °C and at 20 ºC just a few samples showed concentrations higher than those measured immediately after HPP, suggesting these conditions to be close to the growth boundary (Fig. 1i, 1k). However, the piezo-stimulating effect due to HPP and organic acids moved the growth boundary conditions.

If organic acids are used to control *L. monocytogenes* growth in HPP cooked ham, it is very important that concentrations of these antimicrobials are sufficient to efficiently prevent growth of the pathogen. Therefore, the piezo-stimulating effect needs to be taken into account. A mathematical model and software to predict the required concentrations of organic acids or their salts depending on product characteristics, storage conditions and HPP would be most useful but to our knowledge is not available. However, for a specific HPP of 600 MPa for 3 min at 15 °C the *L. monocytogenes* growth and growth boundary model from the FSSP software can be calibrated to cooked ham with added organic acids. This is obtained by multiplying the μ_{ref}-value in the Mejlholm and Dalgaard (2009) model with a value of 3.4 corresponding to $1/B_f$ for HPP cooked ham with organic acids (Table 3) as previously reported for other cardinal parameter models (Østergaard et al., 2014; Pin et al., 1999). The calibrated model is product specific and it can be used to predict the inhibiting effect of lactate and diacetate on growth rates of *L. monocytogenes* in HPP cooked ham with added organic acids. Importantly, this model calibration does not influence the predicted growth boundary.

The observed piezo-stimulation of *L. monocytogenes* growth is unlikely to be due to differences or changes on the major physico-chemical characteristics (such as the pH, a_w, etc.) of the products as the same batch of cooked ham was used with or without HPP and no change in pH of samples was recorded after HPP. The possible effect of the amount of glycerol (0.2%) added on the matrix as a results of the inoculation with a *L. monocytogenes* culture was also considered negligible

according to previous findings published in Hereu et al. (2014), where the growth of *L. monocytogenes* inoculated on cooked ham adding no glycerol, 0.0002 and 0.2% glycerol was studied in parallel. However, it cannot be excluded that HPP cause organic acids to react with components in cooked ham and that this may reduce their antimicrobial activity. If this was the case it becomes important to test the piezo-stimulating effect in other foods. For some bacteria, the recovery after HPP is favoured under less oxidative conditions (Kimura et al., 2017). Besides the removal of oxygen by vacuum packaging, the addition of lactate and diacetate, with recognised antioxidant potential (FAO/WHO, 1995), could contribute to a better recovery, but this hardly explains the piezo-stimulation observed in the present study. To better understand the piezo-stimulating effect it seems important to determine if fast growing *L. monocytogenes* in HPP cooked with organic acids retain this growth potential after isolation from the product. Furthermore, it would be interesting to compare at genomic and transcriptomic level wild and fast growing *L. monocytogenes* isolates from HPP cooked ham with and without added organic acids. The influence of different initial fluid temperatures for HPP has been scarcely studied. In dry-cured ham, the initial fluid temperature within the rage from 7.6 to 24.4 ºC had no impact on *L. monocytogenes* inactivation by HPP (Bover-Cid et al., 2011). However, the impact of this processing parameter on the subsequent growth of the pathogen, particularly in the presence of organic acids with a piezo-stimulating effect observed in the present study remains to be elucidated, and this is another point for potential future studies.

3.3 Secondary modelling

The secondary and global modelling was used to more precisely describe the quantitative effect of storage temperature on *L. monocytogenes* growth in cooked ham without organic acids and both without and with HPP. Fig. 2 shows the effect of storage temperature on the observed growth kinetic parameters and the fit of the secondary models for μ_{max} and λ. No significant differences ($p > 0.05$) were observed between *L. monocytogenes* CTC1034 and the mix of strains, thus data was considered together.

One step fitting of the global model showed growth of *L. monocytogenes* to be statically different for products without or with HPP ($p = 0.008$). Growth of *L. monocytogenes* was faster in products submitted to HPP, confirming the piezo-stimulation effect in cooked ham without added organic acids. Therefore, two different secondary models were used to describe the effect of storage temperature on growth rates (Fig. 2a, Table 4).

In a previous study dealing with *L. monocytogenes* CTC1034 in cooked meat products without organic acids, HPP at 400 MPa (5 min) did not cause a significant difference on the μ_{max} in comparison with non-pressurised products (Hereu et al., 2014). In fact, the model obtained in the present work describes a very similar *L. monocytogenes* behaviour to that of the previous model build with non-pressurised and 400 MPa-treated products (Hereu et al., 2014) as well as to the behaviour predicted by the FSSP model (results not shown). This finding could suggest that higher pressure levels (i.e. 600 MPa, as applied in the present study) may be necessary to cause a detectable increased growth rate. In this line, (Jofré et al., 2008) carried out challenge tests with *L. monocytogenes* inoculated at 10^4 cfu/g in cooked ham with 1.8% potassium lactate in comparison with cooked ham (without lactate) and during the subsequent chill storage after HPP at 600 MPa (for 5 min at 10 °C), more positive samples were recorded in cooked ham with lactate compared to the control cooked ham. However, the effect of HPP on the subsequent growth rate of piezo-resistant bacteria has been scarcely studied from a quantitative perspective and the present study provides new information.

A substantial variability of lag times at the same storage temperature were observed particularly for HPP products (Fig. 2b). The observed data is in line with the previous work (Hereu et al., 2014), in which lag time of *L. monocytogenes* (previously frozen as in the present study) was extended when HPP was applied in comparison with non-HPP products (Fig. 2b, Table 4). Opposed to Hereu et al. (2014) fitting of the global model and *F*-testing showed RLT-values to be independent of the storage temperature (i.e. $K_1 = 0$, Table 4). Lag time extension due to HPP can be related to the time taken by *L. monocytogenes* cells to recover from the sub-lethal damage

caused by HPP before growth is initiated i.e. physiological lag. However, HPP may also create fractions of growing and non-growing cells that contribute to the observed population lag time (Hereu et al., 2014; Koutsoumanis, 2008).

From a practical point of view, it has been reported that HPP caused a reduction of the invasiveness of wild type *L. monocytogenes* isolates (Stollewerk et al., 2017) and piezo-tolerant mutants of *L. monocytogenes* seemed less virulent, and thus appear of lesser concern to human health than the wild type (Joerger et al., 2006; Karatzas et al., 2003). However, current detection and enumeration methods in food are not able to distinguish between these mutants and wild type cells. These issues are neither taken into account by the microbiological criteria regulations for *Listeria monocytogenes* nor by the guidelines to assess the safe shelf-life of RTE foods such as cooked ham (EURL Lm, 2014; European Commission, 2005). The assumption of equal growth potential of *L. monocytogenes* in both non-pressurised and pressurised meat products, stated in some risk assessments dealing with HPP products (Lerasle et al., 2014) is not supported by the results of the present study. Organic acids not only increase the growth rate of *L. monocytogenes* cells surviving HPP (Fig. 1), but they also protect the pathogen from the lethal effects of HPP (Bover-Cid et al., 2016). Consequently, the risk of non-compliance with microbiological criteria regulation could be higher than expected if these findings are not taken into account when designing and validating HPP for cooked meat products.

4. Conclusions

Besides a piezo-protective effect during processing, salts of organic acids exert a piezo-stimulating effect on surviving cells that can increase growth rate of *L. monocytogenes* in cooked ham as much as 4-fold. The mechanisms underlying this important piezo-stimulating effect remain to be elucidated. However, the present study emphasises the need of a product-oriented approach to design, evaluate and implement high pressure processing, taking into account the specific formulation used for product manufacture.

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Table 1 Estimated parameter values resulting from fitting the primary kinetic models to the *L. monocytogenes* counts on cooked ham not pressurised.

Experimental conditions				Kinetic parameters					Goodness of fit[c]			
Temperature (ºC)	**Added lactate (%)**	**Added diacetate (%)**	**Strain**	**G[a] I[b]**	**Log N_0 (Log cfu/g)[a,b]**	**λ (d)[a] s (d)[b]**	**μ_{max} (d^{-1})[a] $-k_{max}$ (d^{-1})[b]**	**Log N_{max} (Log cfu/g)[a]**	**n**	**RSS**	**RMSE**	**R^2_{adj}**
8	-	-	CTC1034	G	2.5	0.5	1.010	8.4	38	1.77	0.23	0.988
8	-	-	Mix	G	2.6	1.6	1.020	8.0	38	1.88	0.24	0.986
8	2.8	-	CTC1034	G	2.2	43.9	0.460	5.9	38	30.02	0.94	0.743
8	2.8	-	Mix	G	2.4	50.4	0.300	5.8	38	23.50	0.83	0.694
8	2.0	0.11	CTC1034	I	2.4	0.0	-0.001	-	38	4.71	0.37	0.253
8	2.0	0.11	Mix	I	2.3	63.1	-0.069	-	38	0.82	0.15	0.750
12	-	-	CTC1034	G	2.7	0.6	2.046	8.6	33	1.14	0.20	0.993
12	-	-	Mix	G	2.4	0.7	1.842	8.3	33	0.95	0.18	0.994
12	4.0	-	CTC1034	I	2.8	19.0	-0.145	-	35	6.61	0.46	0.904
12	4.0	-	Mix	I	2.6	7.0	-0.038	-	35	6.57	0.45	0.455
12	2.0	0.45	CTC1034	I	2.6	21.4	-0.143	-	31	11.62	0.64	0.820
12	2.0	0.45	Mix	I	2.4	28.1	-0.133	-	31	6.45	0.48	0.846
20	-	-	CTC1034	G	2.7	0.1	4.692	8.8	32	0.94	0.18	0.993
20	2.8	-	CTC1034	G	2.5	1.8	1.503	7.1	32	0.48	0.13	0.996
20	4.0	-	CTC1034	G	2.6	4.7	0.571	6.9	42	1.64	0.21	0.984
20	2.0	0.11	CTC1034	G	2.6	2.2	0.712	7.0	39	2.15	0.25	0.982
20	2.0	0.45	CTC1034	I	2.5	7.7	-0.190	-	44	9.99	0.49	0.854

[a] G, for conditions supporting growth the logistic with delay model was fitted to the data (Eq. (1)) to estimate the kinetic parameters Log N_0: initial bacterial concentration; λ: lag time; μ_{max}: maximum specific growth rate; Log N_{max}: maximum bacterial concentration.

[b] I, when conditions not supporting growth caused a loss of *L. monocytogenes* viability (i.e. inactivation), the log-linear with shoulder model was fitted to the data (Eq. (2)) to estimate the kinetic parameters Log N_0: initial bacterial concentration; S: shoulder; k_{max}: inactivation rate; Log N_{max}: maximum bacterial concentration.

[c] n: number of data (cell concentrations, Log cfu/g) included for fitting; RSS: residual sum of squares; R^2_{adj}: adjusted coefficient of determination. Values obtained for experiments at each combination of conditions.

Table 2 Estimated parameter values resulting from fitting the primary kinetic models to the *L. monocytogenes* counts on cooked ham pressurised (at 600 MPa/3 min/15 ºC).

Experimental conditions				Kinetic parameters					Goodness of fit[c]			
Temperature (ºC)	**Added lactate (%)**	**Added diacetate (%)**	**Strain**	**G[a] I[b]**	**Log N_0 (Log cfu/g)[a,b]**	**λ (d)[a] s (d)[b]**	**μ_{max} (d^{-1})[a] $-k_{max}$ (d^{-1})[b]**	**Log N_{max} (Log cfu/g)[a]**	**n**	**RSS**	**RMSE**	**R^2_{adj}**
8	-	-	1034	G	-0.1	1.4	1.350	7.8	38	17.66	0.72	0.942
8	-	-	Mix	G	-0.2	3.2	1.700	7.3	37	26.04	0.89	0.920
8	2.8	-	1034	G	1.1	38.2	1.080	6.6	21[e]	13.60	0.89	0.899
8	2.8	-	Mix	G	1.1	26.3	0.522	7.5	26[e]	11.84	0.73	0.942
8	2.0	0.11	1034	G	0.8	38.2	1.070	5.5	16[e]	3.10	0.51	0.944
8	2.0	0.11	Mix	G	0.6	34.0	1.360	5.2	19[e]	2.27	0.39	0.949
12	-	-	1034	G	-0.7	1.5	2.236	7.8	37	13.07	0.63	0.966
12	-	-	Mix	G	-0.7	2.9	2.709	7.8	36	9.42	0.54	0.976
12	4.0	-	1034	G/NG[d]	-0.9	1.3	1.475	0.2	16[e]	10.31	0.89	0.144
12	4.0	-	Mix	G/NG[d]	-1.0	0.3	0.279	1.1	22[e]	15.71	0.91	0.400
12	2.0	0.45	1034	NG[d]	-	-	-	-	36	-	-	-
12	2.0	0.45	Mix	NG[d]	-	-	-	-	36	-	-	-
20	-	-	1034	G	-0.3	0.0	5.517	8.6	29	10.94	0.66	0.952
20	2.8	-	1034	G	-0.5	1.7	6.169	7.1	29	7.01	0.53	0.976
20	4.0	-	1034	G	0.6	3.9	2.520	6.3	35	10.23	0.57	0.953
20	2.0	0.11	1034	G	-0.5	2.9	3.300	7.0	30	5.97	0.48	0.982
20	2.0	0.45	1034	I	-0.1	36.3	-0.183	-	40	19.13	0.89	0.048

[a] For conditions supporting growth the logistic with delay model was fitted to the data (Eq. (1)) to estimate the kinetic parameters Log N_0: initial bacterial concentration after the HP treatment; λ: lag time; μ_{max}: maximum specific growth rate; Log N_{max}: maximum bacterial concentration.

[b] When conditions not supporting growth caused a loss of *L. monocytogenes* viability, the loglinear with shoulder model was fitted to the data (Eq. (2)) to estimate the kinetic parameters Log N_0: initial bacterial concentration after the HP treatment; S: shoulder; k_{max}: inactivation rate; Log N_{max}: maximum bacterial concentration.

[c] n: number of data (cell concentrations, Log cfu/g) included for fitting; RSS: residual sum of squares; R^2_{adj}: adjusted coefficient of determination. Values obtained for experiments of each combination of conditions.

[d] no clear growth (NG) or inactivation was observed.

[e] data indicating no growth (i.e. below the quantification limit) were excluded for the primary growth model fitting. Growth parameters correspond to the worse case scenario represented by recovered cells that were able to initiate growth.

Table 3 Comparison of observed and predicted growth rates.

	n	**Bias factor (B_f)**	**Accuracy factor (A_f)**
non-HPP			
Without added acids[a]	5	0.95	1.08
With added acids[b]	5	0.84	2.05
All data[a,b]	10	0.89	1.49
HPP			
Without added acids[a]	5	0.71	1.40
With added acids[b]	7	0.29	3.42
All data[a,b]	12	0.42	2.36
Both non-HPP and HPP	22	0.58	1.97

[a] FSSP input parameters: pH = 6.07; water phase salt = 2.71%; water phase lactate (endogenous) = 7,034 ppm

[b] FSSP input parameters for cooked ham with 2.8% K-lactate: pH=6.11; water phase salt = 2.72%; water phase lactate (endogenous+added) = 34,369 ppm. For cooked ham with 4% K-lactate: pH= 6.15; water phase salt = 2.82%; water phase lactate (endogenous+added) = 45,171 ppm. For cooked ham with 2% K-lactate plus 0.11% Na-diacetate: pH 5.88; water phase salt = 2.88%; water phase lactate (endogenous+added) = 26,717 ppm; water phase diacetate = 1,247 ppm.

Table 4. Parameter estimates of global regression model (Eq.(6)) for the growth of *L. monocytogenes* in cooked ham formulated without organic acid salts, obtained for two data sets (from non-pressurised and pressurised products).

	Growth rate model parameters		**Lag time model parameter**	**Maximum population density parameter**		**Goodness of fit**			
	μ_{ref} (d^{-1})	T_{min} (°C)	k_0[a]	a[b]	b	**RSS**	**RMSE**	R^2	R^2_{adj}
Non-HPP	7.958	-0.644	1.49	7.88	0.046	129.1	0.38	0.957	0.956
HPP	8.719	-1.656	2.76	6.37	0.121				
Common model	8.649	-1.334	2.51	7.23	0.076	142.5	0.41	0.953	0.952

[a] k_1 in Eq. (6) was not statistically significant and in this case k_0 corresponds to the relative lag time (RLT).

[b] The parameter a corresponds to Log(N_{max}) at 0 °C.

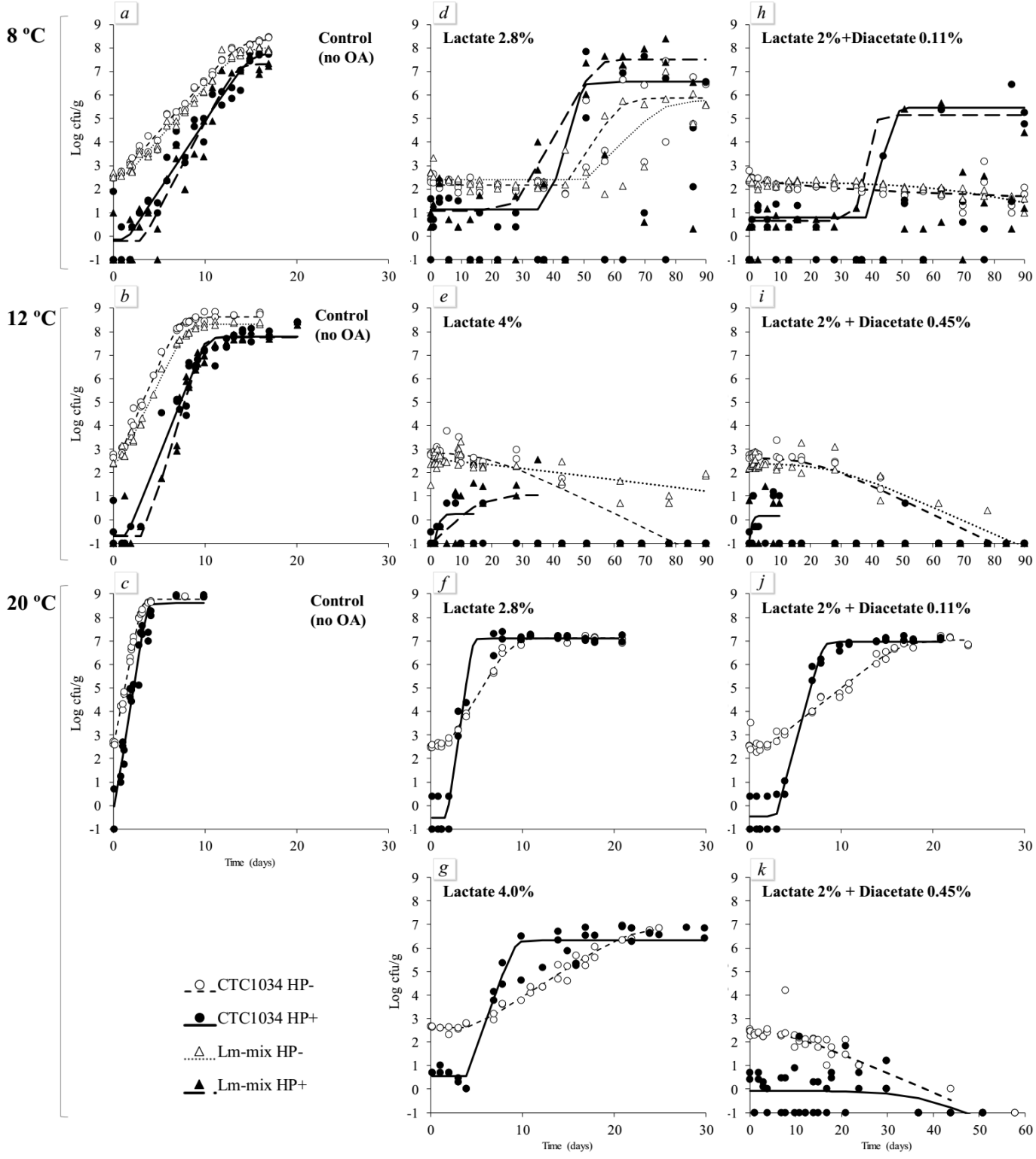

Fig 1. Growth of *L. monocytogenes* in cooked ham formulated without (control) or with organic acids and stored at 8, 12 or 20 ºC. Symbols represent cell concentration (Log cfu/g) and lines the fitted data. Non-pressurised (HP-) and pressurised (HP+, 600 MPa/ 3min/15 ºC) samples are represented with empty and solid symbols, respectively.

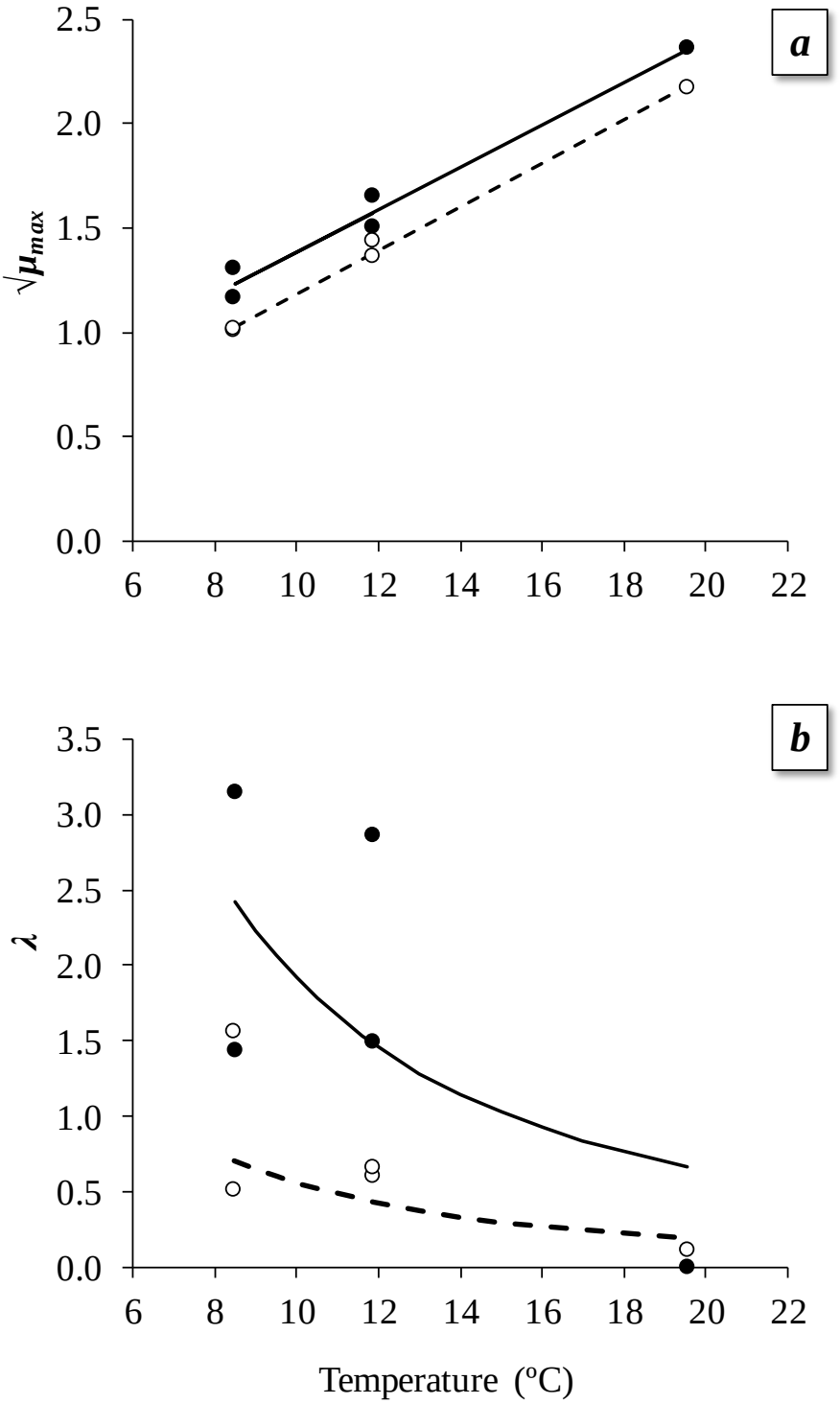

Fig 2. Effect of storage temperature on square root transformed growth rate (μ_{max}, plot *a*) and lag time (λ, *plot b*). Data and model fit for non-pressurised samples are shown with empty symbols and dashed lines, respectively. Data and model fit for pressurised samples (600 MPa/3 min/15 ºC) are shown with solid symbols and continuous line, respectively. Estimated parameters values obtained with the global one-step regression are shown in Table 4.