Enhanced high hydrostatic pressure lethality in acidulated raw pet food formulations was pathogen species and strain dependent

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ABSTRACT (max 200 words)

Feeding dogs and cats with raw meat-based pet food is taking relevance in the recent years. The high a_w of these products together with the no cooking before its consumption by the animal pose a risk due to the potential occurrence and growth of foodborne pathogens. High pressure processing (HPP) is a non-thermal emerging technology that can be used as a lethality treatment to inactivate microorganisms with a minimum impact on the sensory and nutritional traits of the product. The purpose of the present study was to evaluate the variability in pressure resistance of different strains of the relevant foodborne pathogens *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes* in raw pet food formulated without and with lactic acid. In general, *Salmonella* and *L. monocytogenes* strains showed a higher resistance to HPP than *E. coli* strains. In lactic acid acidulated formulations, the susceptibility to HPP of *L. monocytogenes* was markedly enhanced. The resistance to HPP was not only dependent on the microorganism but also on the strain. Thus, the selection of the proper strains should be taken into account when designing and validating the application of HPP as a control measure within the HACCP plan.

Keywords (max 6): high hydrostatic pressure, mathematical modelling, inactivation kinetics, pet food, pathogenic bacteria, piezo-resistance.

1. Introduction

Health benefit claims have been boosting pet owners to shift from traditional dry and canned pet foods to raw pet food diets (Davies et al., 2019). These type of diets are perceived as more nutritious and natural as the components are not heated and mantain the thermosensitive components, which are associated with a series of potential benefits including improved behaviour, shinier coat, better palatability and prevention of disorders affecting body systems (Davies et al., 2019; Freeman et al., 2013). In this respect, for instance, a recent observational study found significantly lower allergy/atopy skin signs after the age of 1 year in dogs eating more than 20% of diet as raw (Hemida et al., 2021).

However, the lack of heat treatments as a microbial kill step in the manufacturing process of raw pet food may pose a health risk as raw materials may harbour pathogenic bacteria (Jones et al., 2019; Nüesch-Inderbinen et al., 2019). The prevalence of bacterial pathogens has been investigated in raw pet foods. In a study conducted with 196 frozen raw pet food samples ordered online in the USA, 16.3 %, 7.6% and 4.1 % were positive for *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli*, respectively (Nemser et al., 2014). In investigations of foodborne illnesses associated with these three pathogens in cats and dogs, raw pet food was confirmed as the incriminated food by whole genome sequencing (Jones et al., 2019). Moreover, FDA has been recalling raw pet foods contaminated with *Salmonella, L. monocytogenes* and *E. coli* (FDA, 2021). Due to low infectious dose, a "zero tolerance" policy for *Salmonella* in raw pet foods is implemented in many countries, e.g. in the European Union through Regulation (EC) No 142/2011 (Commission, 2011) and in the USA Compliance Policy Guide Sec 690.800 *Salmonella* in Food for Animals (FDA, 2013).

To ensure the compliance of regulatory requirements and guarantee the microbiological safety of raw pet food non-thermal preservation strategies can be applied to kill pathogenic bacteria while maintaining the nutritional and freshness traits. In this respect, High Pressure Processing (HPP) has been increasingly adopted by food and pet food producers worldwide as a killing step (Anonymous, 2019). The efficacy of HPP as microbial lethal treatment depends on the type

of microorgansim and the process parameters, mainly pressure and holding time (Bover-Cid et al., 2012, 2011). The physico-chemical characteritics of the food matrix have also a very strong influence on the microbial inactivation associated with HPP. Therefore, the industrial application of HPP technology needs to be validated and, whenever possible, optimised taking into account the specific pet food formulation. Moreover, pet food acidification by means of organic acids such as lactic acid has shown to be effective for inactivating *Salmonella* in rendered chicken used for raw pet food manufacture (Dhakal et al., 2019). To date, the effects of the combination of HPP technology application followed by freezing storage, currently recommended by manufacturers, with other hurdles such as acidification, on pet food microbiological safety have not been evaluated.

Studies to investigate the pressure-resistance of different strains of *Salmonella* (Sherry et al., 2004; Tamber, 2018; Whitney et al., 2007), *E. coli* (Liu et al., 2015; Whitney et al., 2007) and *L. monocytogenes* (Van Boeijen et al., 2008) in liquid culture media or phosphate buffer solution have indicated that microbial responses to HPP are diverse. Screening tests are necessary to establish the levels of pressure-resistance of different microorganisms and within the same species of a microorganism (Tamber, 2018; Van Boeijen et al., 2008). Moreover, since the pressure-resistance of a microorganism also depends on the matrix characteristics including pH and fat content (Bover-Cid et al., 2017; Li and Farid, 2016; Possas et al., 2017), the characterization of bacterial pressure-resistance in the matrix in which the implementation of HPP must be optimized or evaluated is highly recommended. Strains with the greatest resistance at different conditionsshould be used in challenge tests for simulating the worst-case scenarios in risk assessments (Tamber, 2018; Serra-Castelló et al., 2021).

In this framework, this work aimed at i) determining the pressure-resistance of different *Salmonella*, *E. coli* and *Listeria monocytogenes* strains in both non-acidulated and acidulated raw pet food and ii) to study the inactivation kinetics of the most pressure-resistant *Salmonella* strains in raw pet food.

2. *Material & Methods*

2.1. Bacterial strain and culture preparation

The high-pressure resistance of 10 *Salmonella* strains, 5 *E. coli* strains and 10 *L. monocytogenes* strains from the strain collection of the Food Safety and Functionality Programme of IRTA with different serotype and origin (Table 1) were characterized. As shown in Table 1, strains isolated from both food matrixes used as ingredients of raw pet food (raw meat) and clinical isolates were included.

Individual pure cultures of the selected strains were prepared by growing a loopful of the frozen stock culture (- 80 ºC) on Plate Count Agar (PCA, Merck, Darmstadt, Germany) at 37 ºC overnight (18 h). A colony was picked and grown in a new plate of PCA at 37 ºC for a second overnight. Bacterial biomass was collected and resuspended with a cryoprotectant solution (0.3% of beef extract (Difco Laboratories, Detroit, MI, USA), 0.5% of Tryptone (Oxoid Ltd., Basingtok, Hampshire, UK) and 20% of glycerol) and properly distributed in aliquots. Culture was frozen at -80 ºC until being used to obtain freeze-stressed cells. The frozen culture is representative of the status of the strain in raw materials usually stored frozen to produce the raw pet food.

2.2. Raw pet food preparation

The composition of a food in terms of ingredients and additives, and particularly the physico-chemical characteristics, is known to influence the efficacy of HPP. To overcome this point, the study was performed through a product-oriented approach, using the real food matrix. The raw ingredients for pet food manufacture were provided by Affinity Petcare SA (L'Hospitalet de Llobregat, Spain). The formulation of the pet food was as follows (% w/w on wet basis): chicken (80%), vegetables (18%), antioxidants (1%) and vitamins and minerals (1%). Prepared raw pet food was kept frozen at -20 ºC until use.

Immediately before the experiments, the necessary aliquots of raw pet food were thawed at room temperature for 1h . For the acidulated samples, 5 ml of lactic acid based acidulant provided by Corbion® (Amsterdam, The Netherlands) (71 % v/v of lactic acid) per kg of

product, was added to samples (with an initial pH of *ca*. 6.8) 24 hours before the pressurization in order to lower the pH to reach a stable pH of *ca*. 5.70. The addition of the acidulant did not significantly affect ($p > 0.05$) the a_w of the acidulated samples (a_w=0.991 ± 0.001) with respect to samples without acidulant (a_w=0.992 ± 0.001). Just before the pressurization, samples were independently inoculated with *Salmonella, E. coli* or *L. monocytogenes* strains at *ca*. 7.5 log cfu/g (1% v/w). Samples of 25 g were vacuum-packed in PA/PE plastic bags (oxygen permeability of 50 cm^3/m^2/24 h and a low water vapor permeability of 2.8 g/m^2/24 h; Sistemvac, Estudi Graf S.A., Girona, Spain). Samples were kept at 8 ± 1ºC until being pressurized. The a_w and pH of samples were measured before and after HPP treatments with an Aqualab™ equipment (Series 3, Decagon Devices Inc., Pullman, WA, USA) and a pH meter PH25 (Crison Instruments S.A., Alella, Spain), respectively.

2.3. High pressure processing

In order to be able to quantitatively screen the pressure-resistance of different strains of *Salmonella, E. coli* and *L. monocytogenes* strains, a the lower pressure levels within the range of HPP usually applied at industrial level was selected. Thus, vacuum-packed raw pet food samples were pressurised at 400 MPa for a holding time of 5 min in a 120-liter Wave 6000 industrial equipment (Hiperbaric, Burgos, Spain). The pressurization fluid was water and was set up with an initial temperature of 9 ºC. Compression heating was expected to be about 3ºC / 100 MPa (Patazca et al., 2007). The average pressure come up rate was 200 MPa/min, while the release was almost immediate (< 6s).

2.4. Inactivation kinetics

For three *Salmonella* strains (CTC1022, GN0082 and GN0085) the kinetics of inactivation was assessed at 600 MPa, being a pressure level widely used at industrial level to increase food safety of meat products. Holding times of 0, 1, 2, 3, 5, 7 and 10 min were evaluated using the same procedures and equipment described in sections 2.1-2.3. Before microbiological analysis, pressurized samples were kept at 4 ºC for 1 h. In addition, samples were microbiologically

147 analysed after a storage of 24 hours at 4ºC in order to evaluate the potential recovery of
148 pressure-injured cells.

149 *2.5. Microbiological determinations*

150 Raw pet food samples were ten-fold diluted in 0.1 % Bacto Peptone (Difco Laboratories,
151 Detroit, MI, USA) with 0.85 % NaCl (Merck, Darmstadt, Germany) and homogenized for 60
152 seconds in a Smasher blender (bioMérieux, Marcy-l'Étoile, France). The homogenates were
153 serially diluted and plated onto chromogenic media: CHROMagar™ *Salmonella* Plus (SPCM,
154 CHROMagar, Paris, France) incubated at 37 °C for 2-5 days for the enumeration of *Salmonella*,
155 CHROMagar Listeria (CHROMagar) incubated at 37 °C for 2-5 days for the enumeration of *L.*
156 *monocytogenes* and REBECCA® EB agar (bioMérieux, Marcy-l'Étoile, France) incubated at
157 37 ºC for 24 hours for the enumeration of *E. coli*. For samples with expected concentration of
158 *Salmonella* or *L. monocytogenes* below the limit of detection by plate counting (4 cfu/g,
159 resulting from plating 4 ml of homogenate in a 14 cm diameter plate), the presence of the
160 pathogen was investigated by enrichment of 25 g-samples 1/10 diluted and homogenized in
161 peptone water. The presence of *Salmonella* was determined after an enrichment of the
162 homogenate in Rappaport-Vassiliadis (RV) broth (Oxoid Ltd., Basingstoke, Hampshire, UK)
163 for 48 h at 41.5 °C. The presence of the pathogens in the enriched homogenates was confirmed
164 by PCR using the PrepSEQ™ Rapid Spin Sample Preparation Kit (Applied Biosystems) and
165 MicroSEQ™ *Salmonella* spp. Detection Kit and MicroSEQ® *Listeria monocytogenes* Detection
166 Kit (Applied Biosystems) following the instructions of the manufacturer. Microbiological
167 determinations were conducted in pressurized and non-pressured samples in triplicate.
168 Inactivation of the pathogens *Salmonella* spp., *L. monocytogenes* and *E. coli* in pet food samples
169 was expressed in terms of logarithmic reductions as the difference between counts before HPP
170 treatments (N_0) and after treatments (N), *i.e.*, log (N_0/N).

171 *2.6. Data analysis and curve fitting*

172 The Log-linear with tail (Eq. 1, (Geeraerd et al., 2000)) model was fitted to the *Salmonella* spp.
173 concentration versus pressure holding time (min) data for both acidulated and non-acidulated

pet food products. Data obtained immediately after HPP treatments and 24 hours after treatments were used for model fitting by using the nls2 and nls R packages (R Core Team, 2019). The root mean square error (RMSE) was calculated as measure for goodness-of-fit.

If $t \leq t_{shift}$

$$log\ (N) = log\ (N)_i - \frac{k_{max} \cdot t}{Ln(10)}$$

If $t \geq t_{shift}$

$$log\ (\mathrm{N}) = \log(N_{res})$$

Where: $log\ (N)$ bacterial concentration (log cfu/g) at a specific time (t); $log(N)_i$ is the initial bacterial concentration (log cfu/g); k_{max} is the inactivation rate (ln/min); t_{shift} is the time (min) for the appearance of resistance tail and $log\ (N_{res})$ is the residual bacterial concentration (log cfu/g).

3. Results and Discussion

3.1. HPP resistance of Salmonella spp., E. coli and L. monocytogenes in raw pet food

The results of the log reduction of the *Salmonella* spp., *E. coli* and *L. monocytogenes* strains due to HPP in products without the addition of acidulant (non-acidulated) and acidulated are shown in Figure 1.

In non-acidulated products little variability was found either between replicates within the same trial and between results from different trials and strains of *Salmonella*, *E. coli* and *L. monocytogenes*, (Coefficients of variation from 0.27 to 3.53 %). For the three pathogens studied, the strain specific resistance to HPP varied significantly ($p < 0.05$). For *Salmonella* spp., the CECT34136^T (type strain) was the most sensitive to HPP achieving 3.90 log reductions (Figure 1a). In contrast, *Salmonella* strains CTC1022, GN0085, GN0082, CTC1003 and CCUG21272 showed greater resistance (Figure 1a), with an average of logarithmic reductions of less than 0.5 log, which would not be considered microbiologically relevant considering the

accuracy of the plate count determination (CAC/GL 61, 2007). Results of the present work showed that both the most sensitive strain of *Salmonella* (CECT34136^T) and one of the most resistant strains (GN0082) to HPP belonged to the Enteritidis serotype, pointing out the wide variability that can be present not only between serotypes but also between strains from the same serotype.

In case of *L. monocytogenes*, the inactivation of the evaluated strains when HPP was applied in non-acidulated product was similar to that of *Salmonella* spp., being the 12MOB045LM the strain with a greatest HPP sensitivity (3.35-Log inactivation) and 12MOB049LM, CTC1769 and the clinical isolate Scott A (CCUG32843), the strains with a greatest resistance to HPP (inactivation <0.5 log) (Figure 1b). As observed for *Salmonella*, different susceptibility to HPP was found for *L. monocytogenes* strains with the same serotype (e.g. CTC1011 and EUR045LM), confirming that inactivation was more dependent on the *L. monocytogenes* strain rather than on the serotype. Comparing the three evaluated species, *E. coli* was the most sensitive to HPP (mean inactivation of 2.50 log), showing the largest variability in its inactivation response compared to *Salmonella* and *L. monocytogenes* (Figure 2), being *E. coli* CTC1029 and LMG2092^T the most-pressure resistant strains (1.27-1.14 log reductions) and CTC1028 the most susceptible strain (5.15 log reductions) (Figure 1c).

Generally, Gram-positive bacteria have been described as being more resistant to pressure than Gram-negative bacteria (Arroyo et al., 1997; Fonberg-Broczek et al., 2005; Moreirinha et al., 2016; Wuytack et al., 2002). However, some studies have shown that Gram-negative bacteria (especially *E. coli*) are more resistant to pressure than Gram-positive bacteria in raw poultry meats (Kruk et al., 2011; 2014; Yuste et al., 2006). The discrepancy among the studies can be explained by the fact thatthe ability of microorganisms to withstand environmental stresses (not only pressure but also other food processing treatments) is much related to each specific strain rather than the characterisitics of the cell envelop of Gram-positive or Gram-negative bacteria(Bartlett, 2002; den Besten et al., 2018; Considine et al., 2011Jofré et al., 2010;). In this line, in the present study, no significant differences ($p < 0.05$) in the HPP-inactivation were found between *Salmonella* and *L. monocytogenes* (Figure 2)).Moreover, although *E. coli*

showed the greatest susceptibility to HPP (Figure 2), the *E. coli* strains CTC1029 and LMG2092^T showed to be more HPP-resistant than some strains of *Salmonella* (CECT34136^T) and *L. monocytogenes* (12MOB045LM and CECT4031^T) (Figure 1).

In acidulated raw pet food, and as observed in non-acidulated products, the magnitude of the HPP-inactivation of the pathogens was species and strain-dependent (Figure 1 and 2). While in *Salmonella* and *E. coli* the effect of the acidulation only resulted in a slight increase (up to *ca*. 1 log unit) of the reduction produced by HPP, the impact of acidulation was more remarkable for *L. monocytogenes*, resulting in a larger enhancement of both the lethality (up to *ca*. 3 log units more than in non-acidulated pet food) and the variability (Figure 2). It is worth mentioning that among the Gram-negative species the HPP-lethality enhancement due to lactic acid addition was not observed in a higher proportion of the strains (40 and 60% for *Salmonella* and *E. coli*, respectively), compared to *L. monocytogenes* (20%).

It is well reported that one of the main consequences of HPP application on microbial cells is the membrane damage (Bowman et al., 2008). The level of the membrane damage depends on the pressure applied, being estimated that pressures at or above 400 MPa result in membrane disruption and cell leakage (Tauscher, 1995). It also depends on the membrane properties such as membrane fluidity and fatty acid composition (Casadei et al., 2002; Serra-Castelló et al., 2021). Within this context, some studies have reported that for some strains, a higher HPP-resistance of the cells is related with a larger proportion of cyclopropane fatty acids in the membrane (Charoenwong et al., 2011; Tamber, 2018). Additionally, a synergistic protective effect of cyclopropane fatty acids was reported by (Chen and Gänzle, 2016), showing that the disruption of the cyclopropane fatty acid synthase not only increased the *E. coli* lethality of the HPP treatment but also increased the *E. coli* susceptibility to lactic acid, demonstrating that this enzyme contributes to the resistance of both stresses.

Interestingly, results of the present work showed that some strains with higher sensitivity to HPP (e.g. *Salmonella* strain CECT34136T, and *L. monocytogenes* strains 12MOB045LM and CECT4031) were also more susceptible to the lactic acid addition. The same was seen for some of the most HPP-resistant or piezo-resistant strains in which the effect of lactic acid on pathogen

inactivation was less pronounced (< 0.5 log reduction difference), indicating that the presence of lactic acid practically did not modify their resistance to HPP. However, this trend was not observed for all the strains, indicating that in addition to the bacterial membrane composition, other factors may be related to the microbial resistance to HPP and acidity, such as proteins and energy-dependent cofactors. Within this framework, many proteins involved in pressure-resistance were reported to be stress proteins that their expression was governed by stress-responsive alternative sigma factors, such as σ^S and therefore, by the RpoS gene (Gayán et al., 2019, 2017; Landini et al., 2014). Additionally, (Tamber, 2018) found that differences between *Salmonella* strains resistance and their catalase activity when exposed to citric acid, suggesting a role for RpoS in coordinating the acid-resistance response and indicating that RpoS could be an important factor not only for the resistance to HPP but also for the acidity and the synergistic effect of both stresses.

Despite the intrinsic characteristics of the strains described above, the conditions in which the strains were stored before being applied to the raw food and the composition of the pet food matrix could also have had an impact on the HPP-resistance of the pathogens. As frozen raw materials are usually used for the manufacturing of raw pet food, frozen bacterial cultures were used in the present study in order to reproduce the conditions to which the pathogens could have been submitted if they were present in the raw materials. Accordingly, the results of the present study integrated the possible effect of the mechanisms developed by cells as a response to freeze stress on the resistance to subsequent stresses, e.g. HPP and acidification (Hereu et al., 2014), whose resistance will be in turn affected by the nature of the raw pet food components, as it may contain substances that affect the susceptibility of the pathogen to HPP.

3.2. Inactivation kinetics of Salmonella spp. in raw pet food by HPP and lactic acid

Since the *Salmonella* strain CTC1022 was the most pressure-resistant during screening conducted with inoculated raw pet food without lactic acid and *Salmonella* GN0082 and GN0085 showed to be the most pressure-resistant strains in raw pet food with lactic acid, their inactivation kinetics during HPP were quantitatively assessed in order to quantify the impact of

283 HPP technological parameters (pressure level and holding time) on *Salmonella* inactivation.
284 Data and inactivation kinetics of the 3 piezo-resistant strains of *Salmonella* (CTC1022, GN0082
285 and GN0085) in raw pet food treated by HPP, formulated without and with lactic acid (non-
286 acidulated and acidulated) and enumerated immediately and 24 h after application of HPP are
287 shown in Figure 3, and the fitted kinetic parameters of the Log-linear with tail model (Eq. 1) are
288 summarized in Table 2.
289 Results of the enumeration performed 24 hours post-HPP resulted in a lower inactivation of
290 *Salmonella* (between 1 to 2 Log) in products without lactic acid (non-acidulated) and in a *ca*. 1
291 Log in acidulated products. These results indicated that *Salmonella* could recover from sublethal
292 injury during the storage of samples for 24 hours under refrigeration at 4 ºC and be quantified
293 by plate count in the selective chromogenic media. The differences cannot be related to growth
294 as the minimum growth temperature of *Salmonella* is 5 - 8 ºC (FSAI, 2019; (ICMSF, 1996).
295 Recovery of the leakage of ions (Na^+, K^+, Mg^{2+} and Ca^{2+}) induced by HPP at 400 MPa has been
296 reported in *E. coli* during subsequent storage at 20 – 37 ºC for up to 24 hours (Ma et al., 2019).
297 Although in the present study the storage temperature was lower, similar physiological
298 mechanisms could be employed by *Salmonella*. Several studies have reported the occurrence of
299 sublethal damage in foodborne pathogens after HPP (Schottroff et al., 2018). From the practical
300 point of view, these findings indicate that when the efficacy of HPP is assessed by measuring
301 the pathogen counts immediately after the treatment, it can be overestimated.
302 The Log-linear with tail model clearly fitted the shape of the inactivation curve of *Salmonella*
303 (Figure 3), indicating the presence of subpopulations with different resistance to pressure (tail
304 effect), phenomenon that is usually reported in bacterial HPP-inactivation kinetics (Bover-Cid
305 et al., 2012, 2011; Patterson, 2005; Patterson et al., 1995). In this context, (Ma et al., 2019)
306 indicated that the *E. coli* cell death increased with increasing pressure (100 - 500 MPa) although
307 the lethal effect was inconsistent with the injury effect, results that could be possibly related
308 with the resistance tail effect.
309 In non-acidulated products, the inactivation rate (k_{max}) estimated immediately after treatments
310 was higher than the inactivation rate estimated from the results of the determinations 24 hours

311 post-HPP. In addition, the $logN_{res}$ value, corresponding to the concentration of the resistant tail
312 was lower with the fit of the model to data immediately after HPP. About 5-log reducion were
313 recorded after 5 min of holding time at 600 MPa when measured immediately after HPP.
314 However, a maximum lethality of 3.5 log reduction was recorded when *Salmonella* cells were
315 allowed to recover 24h post-HPP.
316 The enhancement of the lethality of HPP due to the addition of lactic acid was already seen
317 during the pressure increase phase of the treatment (come-up), resulting in an earlier start of the
318 inactivation curve from lower initial values compared to the non-acidulated product (although
319 the initial inoculum level of *Salmonella* before HPP was equivalent in both products). The
320 inactivation rate (k_{max}) of *Salmonella* in acidulated products was considerably higher and a
321 greater inactivation of the pathogen was observed before the appearance of the resistance tail.
322 Moreover, the addition of lactic acid contributed to reduce the level at which the resistance tail
323 (residual *Salmonella* concentration) appeared, thus enhancing HPP efficacy.
324 Interestingly, the differences between the inactivation rate (k_{max}) obtained with *Salmonella*
325 counts immediately after HPP and 24 hours post-HPP were minimized with the addition of
326 lactic acid (Table 2). These results could be associated with the fact that in a more acidic
327 environment, the pressurized *Salmonella* cells could not repair the sublethal damage caused by
328 the HPP treatment during 24 hours in refrigeration (4 ºC). From the practical perspective, 5 log
329 reductions could be achieved in the acidulated raw pet food after 3 min at 600 MPa.

330 ## *4. Conclusions*

331 The study provides scientific data on the HPP-response of *Salmonella*, *E. coli* and *L.*
332 *monocytogenes*, increasing the knowledge on the variability of the HPP lethal effect. The wide
333 species and strain variability in bacterial HPP inactivation should be considered in risk
334 assessments evaluating the effect of HPP and specifically when validating its efficacy to be used
335 as a control measure within the HACCP plan. The present study has identified some HPP-
336 resistant strains of the pathogens that can be used in challenge tests to assess the efficacy of
337 HPP in raw pet food products. The acidulated formulation enhances the HPP lethality with a

variable extent depending on the species and strain. The potential relevance of sublethal injury in the overestimation of the immediate effect of HPP has also been pointed out, which needs to be considered when interpreting the results of validation studies.

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6. Declaration of conflict of interests

Authors declare no conflict of interest. The funders provided the raw materials for preparing the raw pet food product used in the study. They had no responsability on the desing of experiments, data collection and analysis or decision to publish.

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FIGURE CAPTIONS

Figure 1. Mean logarithmic reductions for each strain of *Salmonella* (a*), L. monocytogenes* (b) and *E. coli* (c) strains in HPP treated (400 MPa, 5 min) raw pet food without (white bars) and with lactic acid (black bars). Standard deviation is shown with error bars.

Figure 2. Boxplot of the mean log reductions of *Salmonella, L. monocytogenes* and *E. coli* strains in HPP treated (400 MPa for 5 min) raw pet food without (white boxes) and with lactic acid (grey boxes). Standard deviation is shown with error bars. Outliers are shown as empty circles

Figure 3. Inactivation kinetics of the *Salmonella* strains CTC1022 (a), GN0082 (b) and GN0085 (c) HPP treated at 600 MPa in raw pet food without (circles) and with lactic acid (triangles). Symbols represent the observed *Salmonella* counts and lines the fit of the Log-linear with tail model to data. Empty symbols and dashed lines correpond to determinations immediately post-HPP and full symbols and continuous lines correspond to determinations from 24 hours post-HPP.

Table 1. Bacterial strains used in the present study.

Pathogen	Strain	Serotype	Origin
Salmonella enterica	CECT702	Panama (9,12:1,v:1,5)	Sewage, Albufera Lake
	CECT4565	Senftenberg (1,3,19:g,s,t)	Clinical
	CECT705	Agona (1,4,12:f,g,s:-)	Eggs
	CTC1003	London (3, 10 : l, v: 1, 6)	Pork meat
	CTC1022	Derby (1, 4, 12: f, g: -)	Pork meat
	CECT34136^{T}	Enteritidis (1, 9, 12:g, m:-)	Clinical
	CCUG21272	Mbandaka	Clinical
	GN0085	Typhimurium (1,4,5,12:i:1,2)	Chicken meat
	GN0082	Enteritidis (9,12:g,m:-)	Chicken meat
	CTC1756 (monophasic)	Derby (4:g,f:-)	Pork meat sausage
Escherichia coli	CTC1028	O6	Pork meat
	CTC1029	O2	Pork meat
	CTC1030	O78	Pork meat
	LMG2092^{T}	O1:K1:H7	Urine
	CECT5947	O157:H7 (non toxigenic; stx2-)	Human
Listeria monocytogenes	12MOB045LM	1/2c	Pork meat
	12MOB089LM	4b	Bacon
	CTC1011	1/2c	Meat
	Scott A (CCUG32843)	4b	Clinical
	CECT4031^{T}	1a	Meat
	CTC1034	4b	Cured ham
	12MOB102LM	4b	Salmon
	CTC1769	1/2a	Salmon
	12MOB049LM	1/2b	Industrial environment
	12MOB050LM	4b	Industrial environment

Table 2. Estimated kinetic inactivation parameters and goodness-of-fit resulting from fitting the Log-linear with tail model to *Salmonella* inactivation data on raw pet food pressurized at 600 MPa for up to 10 min.

Strain	Product and determination time		Kinetic parameters[a]			$log\ N_{res}$ (log cfu/g)	*RMSE*
			$log(N)_i$ (log cfu/g)	k_{max} (1/min)	t_{shift} (min)		
CTC1022	Control	Immediately post-HPP	7.06 ± 0.15	2.42 ± 0.14	5.82 ± 0.29	0.94	0.440
		24 hours post-HPP	6.86 ± 0.04	1.19 ± 0.03	7.33 ± 0.18	3.06	0.124
	With lactic acid	Immediately post-HPP	5.36 ± 0.24	3.74 ± 0.33	3.37 ± 0.24	0.86	0.602
		24 hours post-HPP	5.75 ± 0.26	3.14 ± 0.35	4.03 ± 0.36	0.19	0.644
GN0082	Control	Immediately post-HPP	6.94 ± 0.15	2.00 ± 0.14	5.49 ± 0.34	2.19	0.444
		24 hours post-HPP	6.71 ± 0.07	0.98 ± 0.05	7.82 ± 0.39	3.38	0.220
	With lactic acid	Immediately post-HPP	5.55 ± 0.28	2.15 ± 0.25	5.69 ± 0.60	0.50	0.807
		24 hours post-HPP	6.06 ± 0.21	3.22 ± 0.29	3.92 ± 0.29	-0.44	0.535
GN0085	Control	Immediately post-HPP	7.24 ± 0.15	1.73 ± 0.13	6.40 ± 0.43	2.45	0.425
		24 hours post-HPP	7.09 ± 0.07	0.85 ± 0.05	8.54 ± 0.49	3.95	0.223
	With lactic acid	Immediately post-HPP	6.42 ± 0.32	3.35 ± 0.44	4.32 ± 0.46	0.14	0.813
		24 hours post-HPP	5.77 ± 0.19	2.67 ± 0.26	3.93 ± 0.31	1.21	0.482

$log(N_0/N)_i$: initial bacterial concentration; k_{max}: inactivation rate; *t*: time; t_{shift}: time for the appearance of resistance tail, $log(N_{res})$: residual bacterial concentration and *RMSE*: root mean square error.

[a]: Parameter estimate ± standard error

Figure 1

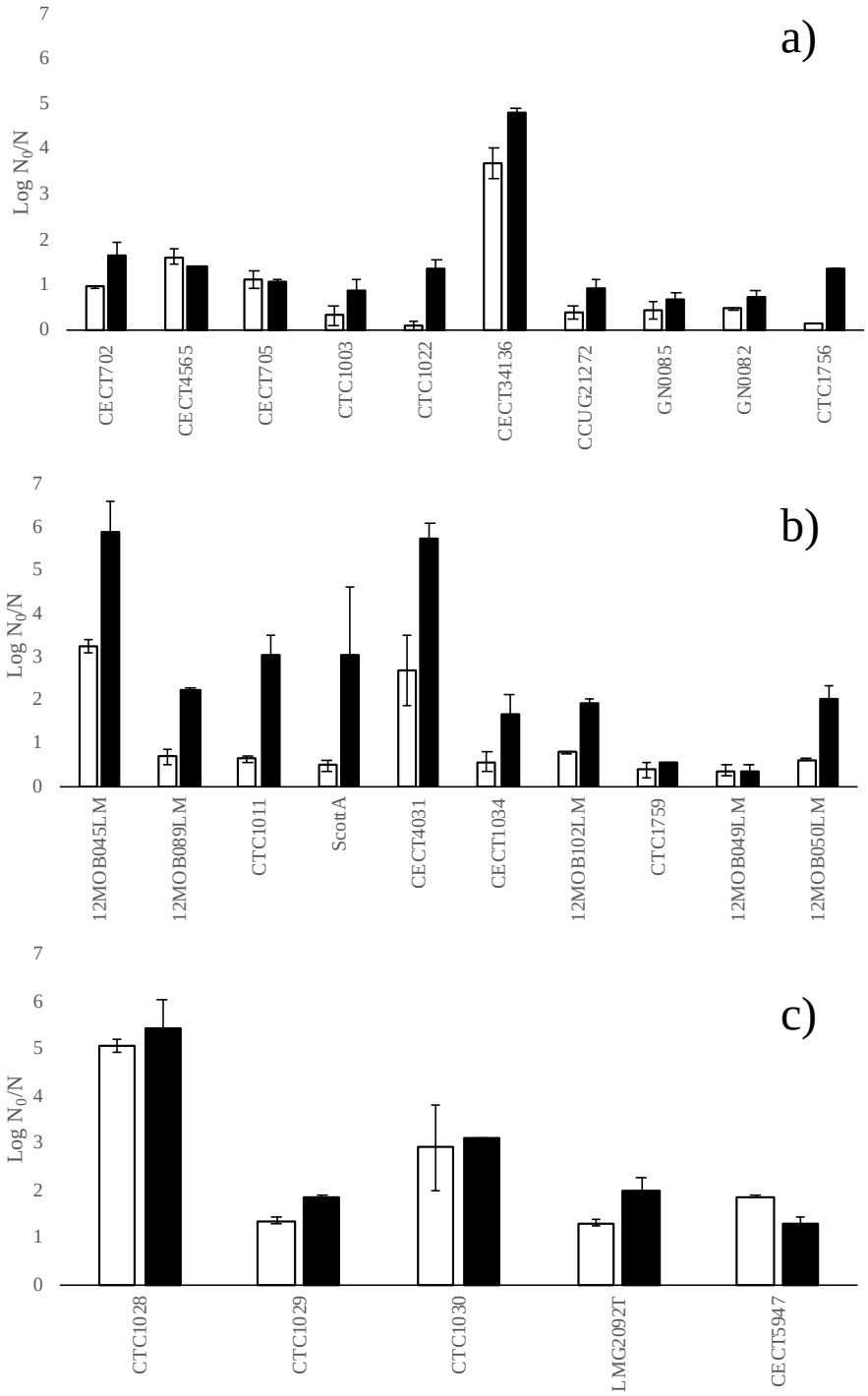

a)
Log N_0/N
0
1
2
3
4
5
6
7
CECT702
CECT4565
CECT705
CTC1003
CTC1022
CECT34136
CCUG21272
GN0085
GN0082
CTC1756
b)
Log N_0/N
0
1
2
3
4
5
6
7
12MOB045LM
12MOB089LM
CTC1011
ScottA
CECT4031
CECT1034
12MOB102LM
CTC1759
12MOB049LM
12MOB050LM
c)
Log N_0/N
0
1
2
3
4
5
6
7
CTC1028
CTC1029
CTC1030
LMG2092T
CECT5947

Figure 2

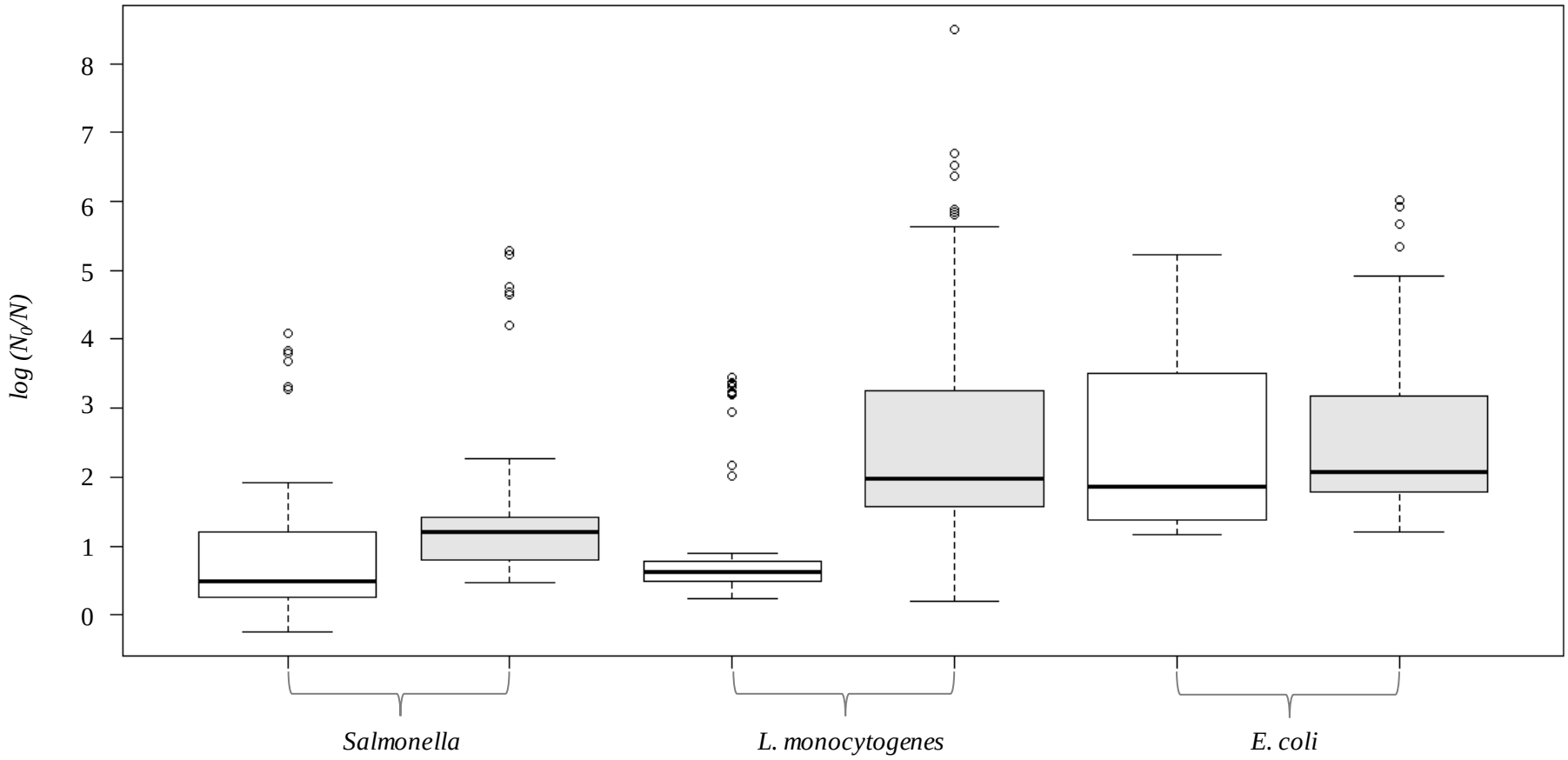
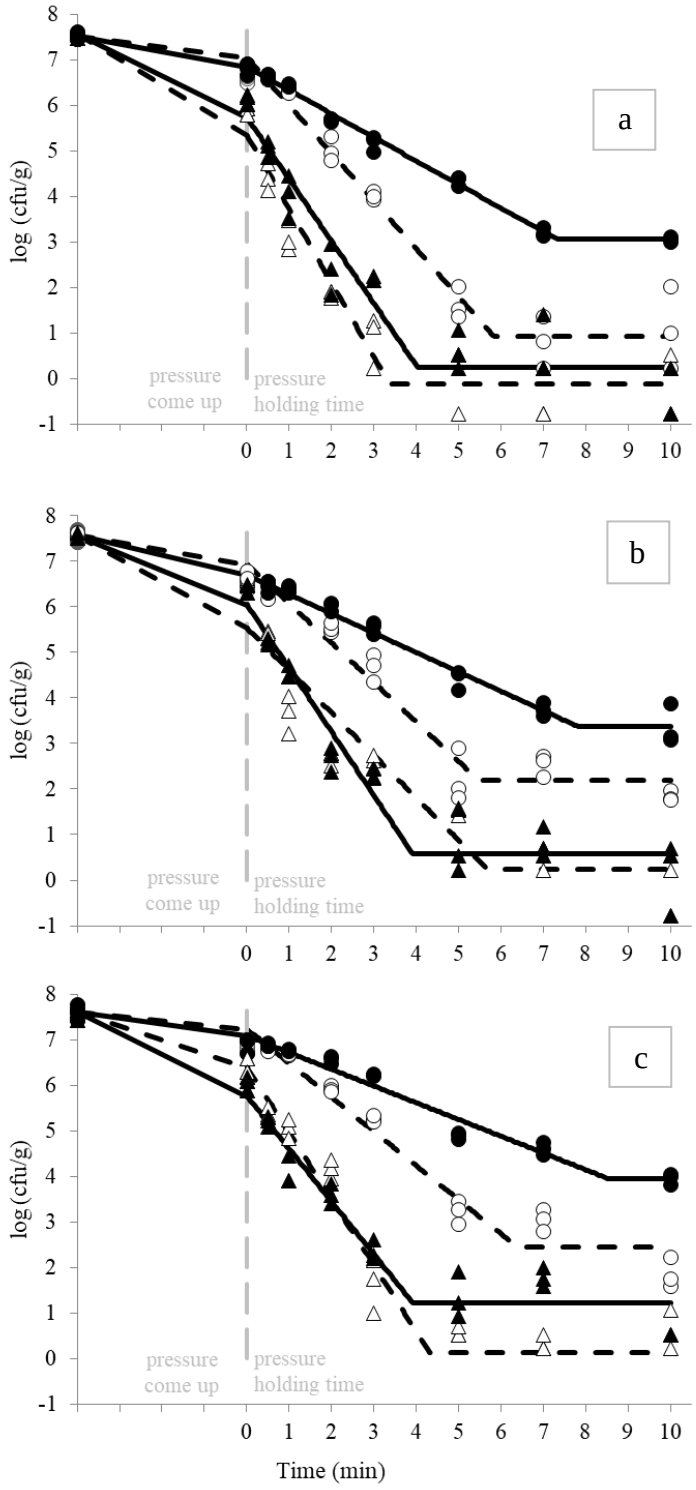


Figure 3


a
b
c
log (cfu/g)
pressure come up
pressure holding time
Time (min)

Highlights

- HPP-resistance variability among pathogenic strains was assessed in raw pet food
- *Salmonella* and *L. monocytogenes* strains showed higher resistance than *E.coli*
- Enhanced lethality in acidulated formulations was species and strain dependent
- Sublethal injury can let to an overestimation of HPP lethality