High-pressure processing inactivation of *Salmonella* in raw pet food for dog is enhanced by acidulation with lactic acid

Cristina Serra-Castelló[a], Aricia Possas[a1], Anna Jofré, Margarita Garriga, Sara Bover-Cid*

IRTA, Food Safety and Functionality Programme - Finca Camps i Armet, E-17121 Monells, Spain

[a]These authors contributed equally to this work

***Corresponding:** Sara Bover-Cid, IRTA, Food Safety and Functionality Programme - Finca Camps i Armet, E-17121 Monells, Spain. sara.bovercid@irta.cat

[1]Present address: Aricia Possas, Department of Food Science and Technology, Faculty of Veterinary, Agrifood Campus of International Excellence (ceiA3), University of Cordoba, 14014, Córdoba, Spain.

Abstract

Raw pet food market is growing at rapid rate due to the raising perception as a natural option and the potential health benefits. However, raw pet food also may pose health concerns due to the occurrence of pathogenic bacteria such as *Salmonella* spp. High-pressure processing (HPP) is known as a non-thermal technology to inactivate microorganisms in food, preserving the nutritional characteristics with minimal impact on organoleptic traits. In this framework, the effects of pressure intensity (450-750 MPa), pressure-holding time (0-7 min) and lactic acid concentration (0-7.2 g/kg) on the inactivation of *Salmonella* spp. by HPP in chicken-based raw pet food intended for dogs was evaluated though a central composite design. *Salmonella* reduction ranged from 0.76 to >9 log units depending on the combination of factors, which were all linearly correlated with inactivation. The rate of inactivation slowed down after an initial rapid drop of *Salmonella* levels during treatments, which was reflected as a quadratic term of holding time. The interaction between factors and the quadratic terms of pressure and lactic acid concentration were not statistically significant and therefore not included in the final model. According to the stochastic assessment, after treatments at 500 MPa for 4 min, the probability of a non-acidulated product being contaminated with *Salmonella* decreased to 0.03 %. For these products, an increase in holding-time duration from 4 to 6 min at 500 MPa, decreased the probability of non-conforming products by approximately 50-fold. Remarkably, for products acidulated with 3.6 g/kg of acid lactic, the same increase in treatment duration reduced the probability of non-conforming products in approximately 475-fold. The results highlight the relevant influence of processing parameters and intrinsic factors associated with the product formulation (i.e. lactic acid causing a slight pH decrease) on the lethality of *Salmonella* in pressurized

raw pet food. The polynomial model provided constitutes a useful decision-support tool for optimizing HPP of raw pet food, considering matrix acidulation by lactic acid as a strategy to enhance *Salmonella* lethality to comply with current regulations concerning pet food microbiological safety.

Keywords: HPP, modelling, predictive microbiology, pet food, salmonellosis**.**

1. Introduction

Raw pet food is composed of pieces of uncooked meat together with animal by-products and vegetables not subjected to thermal treatments, prepared at domestic environments or supplied commercially as fresh, frozen or freeze-dried products (Freeman et al., 2013; Davies et al., 2019). Feeding dogs with products containing raw meat has become a popular practice in recent years, since these products are considered as a more "natural" option in comparison with conventionally processed pet food (Davies et al., 2019; Hellgren et al., 2019). Improvements on pet behaviour, immune function, skin and dental health are among the claimed benefits of raw pet food diets (Joffe & Schlesinger, 2002; Finley et al., 2008).

Regulations of different countries apply zero tolerance regarding the occurrence of *Salmonella* in pet food (European Parliament and Council, 2009; European Commission, 2011; FDA, 2013). Therefore, manufacturers should ensure that raw pet food placed in the market is not contaminated with this pathogen. *Salmonella* prevalence is higher in raw pet food than in conventional processed pet food because raw food does not undergo a lethality process to inactivate bacteria (Hellgren et al., 2019). In Italy, a survey conducted with chicken raw material available for pet food manufacture resulted in the detection of *Salmonella* in 12% of the evaluated samples (Bacci et al., 2019). Van Bree et al. (2018) reported 20% out of 35 commercial samples of raw pet food contaminated with *Salmonella* in the Netherlands. Domesle et al. (2021) reported a turkey-based raw pet food contaminated with three different serovars of *Salmonella.* The occurrence of outbreaks or sporadic cases of animal salmonellosis associated with contaminated dog foods provides evidence of the risk of feeding-*Salmonella* contaminated products to pets (Schotte et al., 2007; Behravesh et al., 2010; Imanishi et al., 2014; Jones et al., 2019).

To limit the health risk for animals due to contaminated raw pet food, high-pressure processing (HPP) is proposed as a non-thermal process to inactivate pathogenic bacteria in

this type of products, with minimal impact on nutritional and organoleptic characteristics. It has been demonstrated that the efficacy of HPP to promote bacterial inactivation depends on a series of factors, including processing parameters and matrix related intrinsic factors, e.g. fat, protein, pH and a_w (Hereu et al., 2012; Bover-Cid et al., 2015; Possas et al., 2017; Bover-Cid et al., 2019; Serra-Castelló et al., 2021). However, studies on *Salmonella* inactivation on raw meat-based pet food by HPP are scarce.

Predictive microbiology models are practical tools to understand and quantify the impact of factors that affect microbial behaviour in foods and to optimize the application of technological interventions such as HPP. The survival kinetics of *Salmonella* have been modelled in dry pet food during heat treatment (Rachon et al., 2016) and during long term storage (Lambertini et al., 2016), but to date no modelling approach has been conducted to describe the inactivation of *Salmonella* due to the application of HPP in a raw pet food intended for dog.

In this context, the purpose of the present study was to build and to evaluate a mathematical model describing the inactivation of *Salmonella* in chicken-based raw pet food intended for dogs by HPP as a function of processing parameters, i.e., pressure intensity and holding time, as well as lactic acid concentration as a key parameter of product formulation. The lactic acid was added to lower the pH of raw pet food in order to evaluate to which extent acidulation enhanced pressure-inactivation of *Salmonella*.

2. Material & Methods

2.1. Experimental design

A Central Composite Design (CCD) was performed in order to evaluate the influence of the three variables: pressure intensity (450-750 MPa), pressure-holding time (0-7 min) and lactic acid concentration (0-7.2 g/kg) on the efficacy of HPP treatments to inactivate *Salmonella*

spp. in chicken-based raw pet food samples. Twenty-one trials were randomly performed in triplicate in accordance with the CCD, consisting of i) eight trials on factorial points, ii) six trials on axial points, iii) seven trials on the central point to enable the evaluation of the experimental error and the lack-of-fit of the model. The experimental layout regarding variables and levels is shown in Table 1 and the specific combination of conditions for the twenty-one trials performed are depicted in Table 2.

The ranges set for the technological factors (Table 1), *i.e.* pressure intensities and pressure-holding times, were set based on previous studies, which demonstrated the effectiveness of HPP treatments at 450-750 MPa for up to 7 min to inactivate pathogenic bacteria in foods, including pet food (Jofré et al., 2009; Bover-Cid et al., 2017; Serra-Castelló et al., 2021).

2.2. Bacterial strain and culture preparation

A three-strain cocktail mixture of *Salmonella* Derby CTC1022, *Salmonella* Typhimurium GN0085 and *Salmonella* Enteritidis GN0082, isolated from pork and chicken meat, was used for samples inoculation. These strains were selected based on their higher pressure-resistance in comparison with other 7 *Salmonella enterica* strains tested in a previous screening in which inoculated pet food samples were pressurized at 400 MPa for 5 minutes (Serra-Castelló, et al., 2021). Each strain was grown on Plate Count Agar (PCA, Merck, Darmstadt, Germany) at 37 ºC for 18 h. A colony was picked and confluently grown in a new PCA plate at 37 ºC for 18 h. Bacterial biomass was collected and resuspended with a cryoprotectant solution consisting of 0.3% of beef extract (Difco Laboratories, Detroit, MI, USA), 0.5% of Tryptone (Oxoid Ltd., Basingtok, Hampshire, UK) and 20% of glycerol and frozen at -80 ºC until being used. Cultures were thawed at room temperature before being used. The freeze culture is representative of the status of *Salmonella* in raw materials used to produce the raw pet food, which are usually stored frozen. Moreover, frozen cultures are known to be more resistant to

HPP than freshly growth cultures, thus this procedure allow to account for the worse-case scenario (Hereu et al., 2014).

2.3. Raw pet food preparation/formulation

The raw ingredients for pet food manufacture were provided by Affinity Petcare SA and prepared according to a commercial formulation as described in Serra-Castelló et al. (2021). Briefly, raw pet food included chicken (as the main component), vegetables, antioxidants and vitamins and minerals. *Salmonella* was not detected in non-inoculated samples (25 g) of raw pet food. Pet food was prepared in a block format of ca. 10 cm diameter and stored frozen as 1.5 cm-thick slices. Before the experiments, the necessary number of slices were thawed, and lactic acid was incorporated to the samples according to the concentrations set in the CCD (Table 1) by adding the appropriate amount of a lactic acid solution (71 % v/v) kindly provided by CORBION® and kept at 4 ± 1 ºC during 24 h before pressurization Samples were inoculated with the *Salmonella* cocktail at a concentration of 10^8-10^9 cfu/g and vacuum-packed in PA/PE 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) 1h before HPP. 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 with a penetration 52–32 probe connected to a PH 25 portable pH-meter (Crison Instruments S.A., Alella, Spain), respectively.

2.4. High-pressure processing

Vacuum-packed raw pet food samples were pressurised at the target time-pressure combinations corresponding to the CCD (Table 1). For pressures up to 600 MPa, the equipment used was a Wave 6000 Hiperbaric (Burgos, Spain), while a pilot equipment (Thiot ingenierie, Bretenoux, France – Hiperbaric, Burgos, Spain) was used for pressures above 600

147 MPa. The come up of pressure was on average 200 MPa/min, while the release was almost
148 immediate. The initial temperature of pressurization fluid (water) was set at 9ºC. Compression
149 heating was expected to be about 3 ºC/100 MPa (Patazca et al., 2007).

150 **2.5. Microbiological determinations**

151 Raw pet food samples were 10-fold diluted in 0.1 % Bacto Peptone (Difco Laboratories,
152 Detroit, MI, USA) with 0.85 % NaCl (Merck, Darmstadt, Germany) and homogenized for 1
153 min in a Blender Smasher (bioMérieux, Marcy-l'Étoile, France). The homogenates were
154 serially diluted and plated onto *Salmonella* Plus chromogenic medium (SPCM, CHROMagar™
155 *Salmonella* Plus; CHROMagar, Paris, France). Colonies were enumerated after incubation at 37
156 °C for 2 to 5 days (in case of pressurized samples). For expected counts below the detection
157 limit by plate counting (4 cfu/g, resulting from plating 4 ml of homogenate in a 14 cm-
158 diameter plate), the presence of *Salmonella* spp. was investigated in 25 g of sample after
159 selective enrichment of the homogenate in Rappaport-Vassiliadis (RV) broth (Oxoid Ltd.,
160 Basingstoke, Hampshire, UK) for 48 h at 41.5 °C. The presence of *Salmonella* in the enriched
161 homogenates was confirmed by PCR using the PrepSEQ™ Rapid Spin Sample Preparation
162 Kit (Applied Biosystems) and MicroSEQ™ *Salmonella* spp. Detection Kit (Applied
163 Biosystems). For modelling purposes, detection of *Salmonella* below the plate detection level
164 was considered -1.0 log cfu/g. Microbiological determinations were conducted in vacuum-
165 packaged samples, pressurized (HPP) or non-pressurized (non-HPP) and either acidulated or
166 non-acidulated in triplicate for each combination of factors considered in the CCD. Vacuum-
167 packaged non-acidulated or acidulated samples that were not pressurized were defined as
168 controls. Inactivation of *Salmonella* spp. in vacuum-packaged pet food samples was
169 expressed in terms of logarithmic reductions as the difference between counts in non-
170 acidulated or acidulated pressurized-samples (N) and controls, i.e., their respective non-
171 acidulated or acidulated non-pressurized samples (N_0), i.e., log (N/N_0).

2.6. Data analysis and statistical modelling

The statistical significance of the differences in the pH of raw pet foods before and after HPP was tested through a t-test. The effects of pressure intensity, pressure holding time and acid lactic concentration on the inactivation of *Salmonella* spp. in raw pet food was investigated by using the Response Surface Methodology. The "rsm" package for R software (R Core Team, 2019) was used to fit quadratic model for each response shown in Equation 1.

$$\log(N/N_0) = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^{\,2} + \sum_{i=j=1}^{n} \beta_{ij} x_i x_j \qquad \text{Equation 1}$$

Where log (N/N_0) is the logarithmic reduction of *Salmonella*; β_0 is a constant; β_i- β_n are model coefficients and x_i-x_n are the independent variables (i.e., pressure intensity, pressure holding time and lactic acid).

To obtain the polynomial equation that best fitted to the experimental data without compromising parsimony, only the significant terms ($p \leq 0.05$) derived from each factor were kept in the final model as indicated by a backward stepwise regression approach. The goodness of fit and the statistical significance of the model were evaluated by means of the root mean square error (RMSE) and the significance of the regression model and the estimated parameters as well as the lack-of-fit test. Response surface graphs were drawn with the value of the independent variable not shown but kept at the central point of the CCD.

2.7. Model performance evaluation

Observed inactivation data (i.e. log reduction) obtained in additional independent experiments were compared with model predictions in order to evaluate its performance. Treatments with foreseeable conditions to be applied at industrial level (EFSA BIOHAZ Panel et al., 2022), i.e. 500 MPa for 4 and 6 min, were applied in products formulated with 3.6 g/kg of lactic acid and products not acidulated. The observed experimental data was compared with model

predictions, taking into consideration the 95 % prediction interval of the model. The model was considered acceptable when inactivation observed data were within the 95 % prediction interval of the model.

3. Results

3.1. Reductions of *Salmonella* spp. in raw pet food due to HPP

The addition of lactic acid in raw pet food at concentrations ranging from 0 to 7.2 g/kg yielded samples with pH varying from 6.97 to 5.72, respectively (Table 2). Differences in *Salmonella* counts between non-acidulated and acidulated samples before HPP were not microbiologically relevant (<0.5 log units). No significant differences were detected between the pH of samples before and after HPP treatments ($p > 0.05$). The a_w of samples was neither affected by HPP application nor the addition of lactic acid and was ≥ 0.99 in all cases.

Inactivation of *Salmonella* by HPP expressed as log (N/N_0) for each combination of factors of the CCD is shown in Table 2. By increasing both pressure intensity and pressure-holding time, an increase in *Salmonella* inactivation was observed. The maximum reduction achieved in the present experiments was 9.33 log units, when a treatment at the highest pressure level evaluated was applied (i.e. 750 MPa, Trial 21). During this treatment, levels of *Salmonella* decreased to values below plate count detection, although its presence was detected after enrichment of 25 g of the sample. The increase in pressure intensity from 450 to 750 MPa while keeping time and lactic acid concentrations at the central point of the CCD (i.e. 3.5 min and 3.6 g/kg, respectively), increased the inactivation by 7.3 additional log units. Moreover, for treatments at 600 MPa in products containing 3.6 g/kg of lactic acid, an increase in holding time from 0 to 7 minutes resulted in a 6 log reduction (Trials 6 and 16). Considering the addition of lactic acid, an increase from 1.5 to 5.7 g/kg of raw pet food, led to an increase of the HPP inactivation by 1.4 additional log units of reduction in treatments at 511 MPa/1.4

219 min (Trials 2 and 3). The same increase in lactic acid concentration at 689 MPa/1.4 min
220 resulted in an acid-related reduction of *Salmonella* of 2.5 log (Trials 17 and 18). In these
221 experiments, the increase in lactic acid concentrations reduced the pH of raw pet food
222 samples from 6.5 to 5.8 (Table 2).

223 **3.2. Modelling the inactivation of *Salmonella* spp. in raw pet food by HPP**

224 The coefficients of the empirical model (Equation 1) quantifying the relationship between the
225 *Salmonella* inactivation in raw pet food and the independent factors evaluated, i.e. pressure,
226 pressure-holding time and lactic acid concentration, are shown in Table 3. The model is
227 statistically significant as indicated by the F-value = 268.1 ($p \leq 0.00001$) and the non-
228 significant lack-of-fit test (F-value = 5.2; $p > 0.05$). Moreover, the low RMSE value of 0.677
229 indicated a satisfactory goodness of fit.

230 The response surface graphs generated based in the obtained model are shown in Figure 1.
231 The three factors evaluated were positively correlated with the inactivation of *Salmonella* spp.
232 in raw pet food and are present in the model as linear terms ($p \leq 0.05$). Effect estimates
233 indicated that pressure intensity was the quantitatively most important factor influencing
234 inactivation, followed by pressure-holding time. Interactions between the factors were not
235 significant ($p > 0.05$) and thus not included in the final model.

236 A non-linear relationship between *Salmonella* inactivation and pressure-holding time was
237 marked and reflected by the presence of a quadratic term in the model. It means that by
238 increasing the duration of pressure treatments, there is a slowing down on reductions, with
239 higher inactivation rates at the beginning of pressurization (Figure 1a and 1b). The results of
240 model performance evaluation are shown in Table 4. The model could be successfully applied
241 to predict the inactivation of *Salmonella* in raw pet food containing 0 or 3.6 g/kg of lactic acid

242 treated at 500 MPa for 4 and 6 min, as independent data obtained in additional experiments
243 carried out at these conditions fall within the 95 % prediction interval of the model.

244 The contour plot showing the combination of pressure intensity and holding time that allow to
245 accomplish a target isoreduction level in raw pet food containing 4 g/kg of lactic acid is
246 shown in Figure 2a. It can be deduced by checking the plot that by applying treatments at 500
247 MPa for 6 min, a 4 log reduction in *Salmonella* levels would be achieved. Additionally, to
248 achieve a 6 log reduction at 600 MPa, treatment duration might be at least of 4 min.

249 **4. Discussion**

250 **4.1. *Salmonella* spp. inactivation in raw pet food by HPP**

251 The results of the present study highlighted the role of processing parameters on the lethality
252 of HPP, as reported in previous investigations in foods other than raw pet food (Bover-Cid et
253 al., 2017; Possas et al., 2017). Moreover, they revealed that the HPP-resistance of *Salmonella*
254 in chicken-based raw pet food was lower in comparison with dry-cured meat products and
255 comparable to the inactivation levels achieved with the pressurization of the pathogen in
256 liquid matrices or culture broth. *Salmonella* reductions in the range of 4-8 log were reported
257 after pressurization of culture broth at 350-550 MPa up to 10 min (Lee & Kaletunç, 2010;
258 Maitland et al., 2011), while notably lower reductions, within the range 2-4 log, were reported
259 in dry-cured ham (with a a_w of 0.88) subjected to 450-750 MPa for 5 min. These differences
260 would be associated with the protective effect of the low a_w of the matrix on the lethality of
261 HPP on *Salmonella*, since higher microbial reductions have been quantified in matrices with
262 higher a_w, such as the raw pet food under study ($a_w > 0.99$) (Bover-Cid et al., 2015; Georget et
263 al., 2015). Besides the effect of a_w, additional reductions in raw pet food in comparison with
264 other meat products can be associated with the pH decrease through the addition of lactic acid

265 which could be explained by the lower resistance of pathogens to HPP in more acidulated
266 conditions (Alpas et al., 2000).

267 Due to the lack of studies dealing with the pressure-induced inactivation of *Salmonella* in raw
268 pet food, comparison of results with data obtained during raw poultry pressurization seems
269 reasonable, since chicken meat is the main ingredient of the raw pet food under study (80 %
270 w/w). Reductions of 3.35 and 3.5 log in *Salmonella* levels were achieved after the
271 pressurization at 450 MPa for 5 min of inoculated ground chicken (Sheen et al., 2015) and
272 chicken fillets (Kruk et al., 2011), respectively. In line with these investigations, in the present
273 study the application of 450 MPa for a slightly shorter time yielded a slightly lower log
274 reduction (2 log, Trial 1).

275 In the present study, acidulation by adding acid lactic was effective in increasing *Salmonella*
276 inactivation. Besides acidulation, additional control measures can be applied together with
277 HPP to promote the inactivation of *Salmonella* and to avoid the growth of pressure-injured
278 cells during storage of raw pet food, including refrigeration of pressurized products (Jofré et
279 al., 2010; Lerasle et al., 2014). For instance, Morales et al. (2009) found no recovery of
280 pressure-injured cells of *Salmonella* in chicken fillets subjected to treatments at 300 and 400
281 MPa for up to 20 min during the subsequent storage at 4 °C for 72 hours. Therefore, the
282 storage of pressurized raw pet food under refrigeration according to manufacture
283 recommendations would assist the compliance with current regulations for *Salmonella*.

284 The non-linear relationship between *Salmonella* and pressure-holding time found in the
285 present article is compatible with the occurrence of a tail of resistant cells which may indicate
286 the presence of subpopulations of *Salmonella* with different susceptibilities to pressure
287 (Tamber, 2018). The same non-linear trend was observed in other studies modelling the
288 microbial pressure-induced inactivation in foods (Hereu et al., 2012; Tananuwong et al.,

289 2012; Lerasle et al., 2014). From the technological point of view, the occurrence of a tail
290 during microbial inactivation has remarkable implications. Since the inactivation rate in the
291 tail part is drastically reduced, no significant additional *Salmonella* reductions would be
292 achieved by increasing processing times, which means that additional operational costs
293 derived from increased pressure-holding times could be avoided. Based on capital costs, an
294 economically reasonable holding time to be applied at industrial level was estimated in a
295 maximum of 6 min (Garriga et al., 2004). On the other hand, regarding food safety, the
296 occurrence of a tail of resistant cells is a concern during the subsequent storage and handling
297 practices. Even if resistant cells may be sublethally damaged, they can recover and initiate
298 growth if the intrinsic and storage conditions are favourable (Hereu et al., 2014).

299 Recommendations regarding the required lethality of HPP treatments to eliminate *Salmonella*
300 in raw pet food have not been established. However, the application of technologies
301 alternative to the thermal treatment such as HPP must ensure the reduction of the loads of
302 pathogenic microorganisms in foods in about 4 to 6 log reductions (IFT, 2002). Considering
303 that a HPP treatment should assure those reductions of *Salmonella* in raw pet food, the model
304 developed in this study can be applied, for instance, to set the appropriate processing
305 parameters, assuming the addition of a fixed lactic acid concentration.

306 On the other hand, according to the requirements established in the US for the production of
307 fully cooked poultry products, a lethality process which must include a cooking step may
308 assure a 7-log reduction of *Salmonella* (CFR, 2018). Simulations using the developed model
309 indicate that this target inactivation would only be achieved in raw pet food formulated with
310 lactic acid. For example, a 7-log reduction would be achieved when applying a treatment at
311 600 MPa for at least 4.2 min in raw pet food containing 7 g/kg of lactic acid (Figure 2b). By
312 reducing the lactic acid concentration to 6 g/kg, the minimum holding time of a HPP
313 treatment at 600 MPa required to achieve the target inactivation would increase to 5 min

(Figure 2b). Therefore, the model developed in the present study can be applied to define HPP parameters and lactic acid concentrations required to achieve desired levels of *Salmonella* inactivation, being an important tool for process assessment and optimization in view of food safety assurance.

4.2. Validation of HPP as a killing step in raw pet food using the FSO concept

The validation of a control measure provides evidence that a specific process will result in products that meet microbiological and quality requirements (Zwietering et al., 2010). Considering that there is no specification of the number of *Salmonella* reductions that may be reached during HPP treatments applied to pet food, the management of the food safety of this product can be approached through Food Safety Objective (FSO) concept (ICMSF, 2002). In the present study a stochastic approach (Zwietering et al., 2010) was used to evaluate the probability that HPP treatments would result in products that comply with current regulations concerning *Salmonella* in pet food. The FSO is the maximum level of the pathogen that are tolerated at the moment of consumption and can be calculated by means of Equation 2.

$$H_0 - \sum R + \sum I \leq FSO \qquad \text{Equation 2}$$

where H_0 is the initial level of *Salmonella* contamination in raw pet food; ΣR is the total reduction of *Salmonella* during processing, e.g. by HPP application; and ΣI is the total *Salmonella* increase (growth and/or recontamination) during the whole process.

To determine whether a food batch meets an FSO, the distribution of initial levels of the pathogen (H_0) within a food must be understood (van Schothorst et al., 2009). The initial *Salmonella* concentration in chicken-based raw pet food was estimated by applying the probabilistic approach published by Valero et al., 2014 based on presence/absence data

provided by the pet food producer and was described by a normal distribution with mean -1.55 log cfu/g and standard deviation 0.51 log cfu/g.

Growth of *Salmonella* and recontamination after HPP treatments were deemed negligible (i.e., $\Sigma I = 0$) since products were pressurized in their package and after HPP they stored frozen or under refrigeration temperatures not supporting the growth of *Salmonella* (ICFMH, 1996). *Salmonella* reduction observed in HPP treatments were expressed as normal distributions (ΣR, Table 5). The *FSO* was set at < -1.41 log cfu/g, which corresponds to the logarithm of 1 cfu in 25 g of product, the maximum level of *Salmonella* in accordance with regulations that require no detection in 25 g of product. It is assumed that 95% of the distribution of concentration must satisfy the test limit so that the FSO is met.

The stochastic assessment indicated that a high number of contaminated product units could be present in a lot, i.e., up to ca. 38 %. The percentage of non-conforming products regarding the *FSO* and the overall distribution of *Salmonella* in acidulated and non-acidulated products subjected to pressurization are shown in Table 5. After treatments at 500 MPa/4 min, the probability of a non-acidulated product being contaminated with *Salmonella* decreased to 0.03 %. For these products, an increase in holding-time duration from 4 to 6 min at 500 MPa, decreased the probability of non-conforming products in approximately 50-fold (Table 5). Remarkably, for products acidulated with 3.6 g/kg of acid lactic, the same increase in treatment duration reduced the probability of non-conforming products in approximately 475-fold.

By increasing the acid lactic concentration from 0 to 3.6 g/kg and applying 500 MPa for 4 min, the probability of non-conforming units was reduced by approximately 30-fold, while the same increase in lactic acid concentration in parallel with the increase in pressure-holding time from 4 to 6 minutes would reduce the prevalence of *Salmonella* expressed as percentage

of contaminated units per batch to approximately 0. The impact of acidulation and HPP treatments in the distribution of *Salmonella* in raw pet food can be seen in Figure 3, where it can be noted that the distribution of *Salmonella* in acidulated products is shifted to the left of the graph, representing lower concentrations.

5. Conclusions

The inactivation of *Salmonella* spp. by HPP in chicken-based raw pet food intended for dogs was dependent of the pressure intensity and holding time and could be notably enhanced by the lactic acid addition in the product formulation. By increasing the values of the three factors, higher inactivation is quantified, although the inactivation rate significantly decreases at holding times of 4-6 min due to the occurrence of a tail of pressure-resistant cells, which should be considered not only from the food safety point of view but from the operational and economic perspective. The model developed in the present study is suitable to assess and optimize the impact of HPP conditions. The model constitutes a useful decision support tool to assist pet food producers on setting appropriate combinations of processing parameters and lactic acid concentrations on raw chicken-based pet food formulations to achieve desired levels of *Salmonella* inactivation to assure the compliance with the microbiological criteria regulation.

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7. 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 responsibility on the design of experiments, data collection and analysis or decision to publish.

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576 **Figure captions**

577

578 **Figure 1.** Response surface graphs of high-pressure processing (HPP)-induced
579 inactivation of *Salmonella* spp. in raw pet food according to the developed model. (a)
580 Pressure intensity and lactic acid concentration effects; (b) Holding time and lactic acid
581 concentration effects. The factors not included in each graph are maintained at the
582 central value of the central composite design; time = 3.5 min in graph (a) and pressure =
583 600 MPa in graph (b).

584

585 **Figure 2.** Contour plots describing the inactivation effect of high-pressure processing
586 (HPP) in raw pet food at different combinations of (a) pressure intensity and pressure-
587 holding time at a lactic acid concentration = 4 g/kg of raw pet food and (b) lactic acid
588 and pressure-holding time at 600 MPa. Numbers in each line indicate the inactivation
589 value, i.e. log (N/N_0).

590

591 **Figure 3.** Probability distribution of the initial level of contamination of *Salmonella*
592 (log cfu/g) in chicken-based raw pet food (H_0, - - -) and after pressurization at 500
593 MPa for 4 min of products acidulated with 3.6 g/kg (——) and non-acidulated
594 products (·····). The vertical dashed line indicates the FSO < -1.4 log cfu/g.

595

Table 1. Selected variables (factors) and the corresponding five levels used in the Central Composite Design (CCD).

Levels[a]	Factors		
	Pressure intensity (MPa)	**Holding time (min)**	**Lactic acid (g/kg)**
-1.68	450	0.0	0.0
-1.0	511	1.4	1.5
0	600	3.5	3.6
+1.0	689	5.6	5.7
+1.68	750	7.0	7.2

[a] Considering the circumscribed central composite experimental design for three factors, the scaled value for α relative to the coded values ± 1 was 1.68 ($2^{3/4}$) in order to maintain rotatability and orthogonality.

Table 2. *Salmonella* inactivation on raw pet food samples after high pressure processing treatments at each combination of the Central Composite Design (CCD).

Trial	Pressure (MPa)	Time (min)	Lactic acid (g/kg)[a]	Inactivation (log N/N_0)[b]
1	450	3.5	3.6 (6.08 ± 0.07)	-2.01 ± 0.15
2	511	1.4	1.5 (6.50 ± 0.03)	-0.84 ± 0.07
3	511	1.4	5.7 (5.77 ± 0.02)	-2.21 ± 0.04
4	511	5.6	1.5 (6.50 ± 0.03)	-3.05 ± 0.14
5	511	5.6	5.7 (5.77 ± 0.02)	-4.66 ± 0.08
6	600	0.0	3.6 (6.16 ± 0.04)	-0.76 ± 0.07
7	600	3.5	0.0 (6.97 ± 0.05)	-3.67 ± 0.14
8	600	3.5	3.6 (6.09 ± 0.06)	-5.32 ± 0.25
9	600	3.5	3.6 (6.09 ± 0.07)	-5.59 ± 0.20
10	600	3.5	3.6 (6.22 ± 0.05)	-5.38 ± 0.14
11	600	3.5	3.6 (6.22 ± 0.05)	-5.31 ± 0.20
12	600	3.5	3.6 (6.22 ± 0.05)	-5.49 ± 0.14
13	600	3.5	3.6 (6.22 ± 0.05)	-5.27 ± 0.27
14	600	3.5	3.6 (6.22 ± 0.05)	-5.24 ± 0.51
15	600	3.5	7.2 (5.72 ± 0.08)	-6.80 ± 0.31
16	600	7.0	3.6 (6.08 ± 0.07)	-6.84 ± 0.03
17	689	1.4	1.5 (6.55 ± 0.05)	-4.92 ± 0.29
18	689	1.4	5.7 (5.78 ± 0.10)	-7.42 ± 0.30
19	689	5.6	1.5 (6.55 ± 0.05)	-8.40 ± 1.60
20	689	5.6	5.7 (5.78 ± 0.10)	-8.74 ± 0.88
21	750	3.5	3.6 (6.09 ± 0.05)	-9.33 ± 0.00

[a] Mean ± standard deviation of the pH of samples are reported between parentheses

[b] Mean of three replicates ± standard deviation

Table 3. Results of the multivariate regression analysis describing the effect of pressure intensity, pressure-holding time and lactic acid concentration on the inactivation of *Salmonella* spp. in raw pet food.

Terms[a]	**Regression coefficients**	**Standard Error**	***t*-value**	***p*-value**	**RMSE**[b]
Intercept	15.1380	0.6545	23.1293	<0.0001	0.677
P (MPa)	-0.0255	0.0010	-25.9814	<0.0001	
t (min)	-1.5467	0.1412	-10.9741	<0.0001	
LA (g/kg)	-0.3795	0.0410	-9.2495	<0.0001	
t^2 (min)	0.1219	0.0191	6.3613	<0.0001	

[a]: *P*, pressure; *t*, holding time; *LA*, lactic acid concentration

[b]: root mean square error (RMSE)

Table 4. Results of additional HPP experiments conducted for the evaluation of the model performance to describe de pressure-induced inactivation of *Salmonella* in raw pet food.

Pressure (MPa)	Time (min)	Lactic acid (g/kg)	Observed inactivation (log N/N_0)	Predicted inactivation (log N/N_0)	-95 % PI (log N/N_0)	+95 % PI (log N/N_0)
500	4	0	-1.64 ± 0.12	-1.86	-3.05	-0.67
500	6	0	-2.09 ± 0.06	-2.51	-3.73	-1.31
500	4	3.6	-2.25 ± 0.24	-3.22	-4.37	-2.07
500	6	3.6	-2.83 ± 0.19	-3.88	-5.06	-2.71

PI = Prediction interval

Table 5. Stochastic evaluation of zero tolerance compliance regarding *Salmonella* spp. (i.e. no detection in 25g) in high pressure processed raw pet food.

Pressure (MPa)	Time (min)	Lactic acid (g/kg)	Initial contamination (H_0, log cfu/g)	Observed inactivation (ΣR, log N/N_0)	H_0-ΣR+ΣI (log cfu/g)	P (x > FSO) %
500	4	0	-1.55 ± 0.51	-1.64 ± 0.12	-3.19 ± 0.52	0.0313
500	6	0	-1.55 ± 0.51	-2.09 ± 0.06	-3.64 ± 0.51	0.0006
500	4	3.6	-1.55 ± 0.51	-2.25 ± 0.24	-3.80 ± 0.56	0.0010
500	6	3.6	-1.55 ± 0.51	-2.83 ± 0.19	-4.38 ± 0.54	0.0000

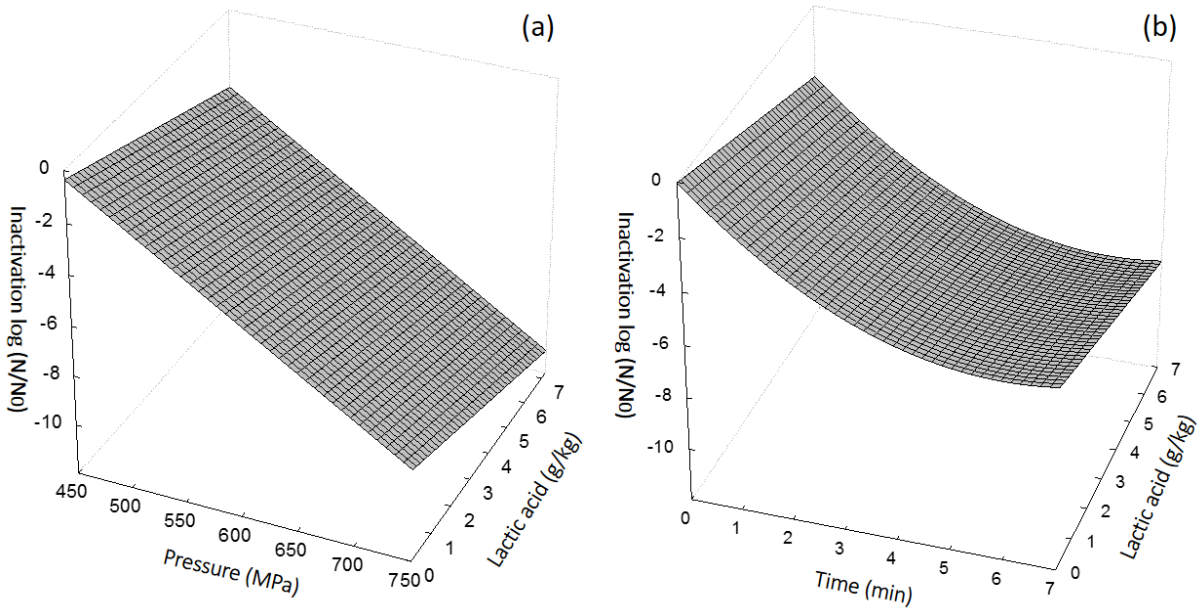
(a)
Inactivation log (N/No)
0
-2
-4
-6
-8
-10
450
500
550
600
650
700
750
Pressure (MPa)
0
1
2
3
4
5
6
7
Lactic acid (g/kg)
(b)
Inactivation log (N/No)
0
-2
-4
-6
-8
-10
0
1
2
3
4
5
6
7
Time (min)
0
1
2
3
4
5
6
7
Lactic acid (g/kg)

Figure 1

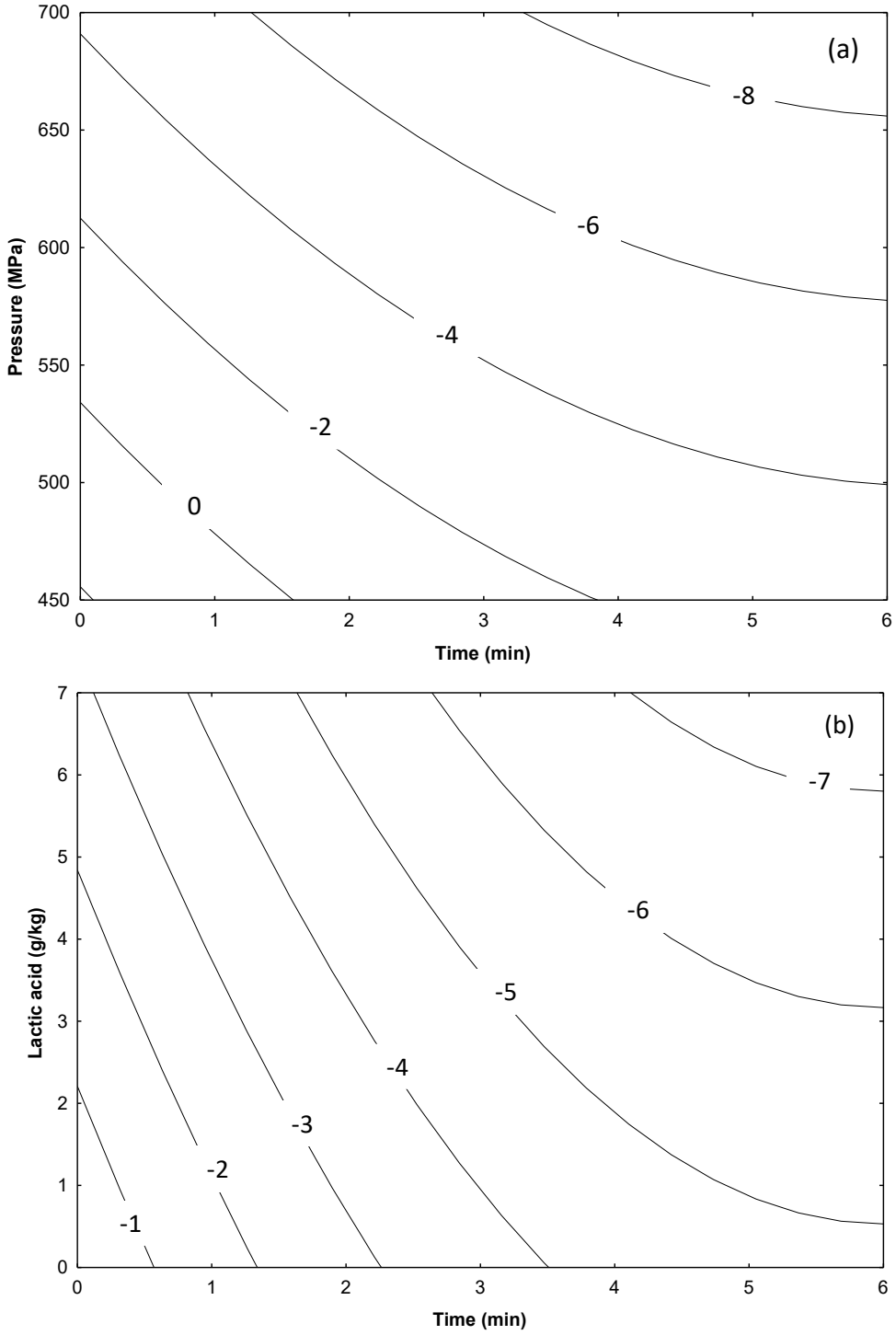
(a)
Pressure (MPa)
Time (min)
-8
-6
-4
-2
0
(b)
Lactic acid (g/kg)
Time (min)
-7
-6
-5
-4
-3
-2
-1

Figure 2

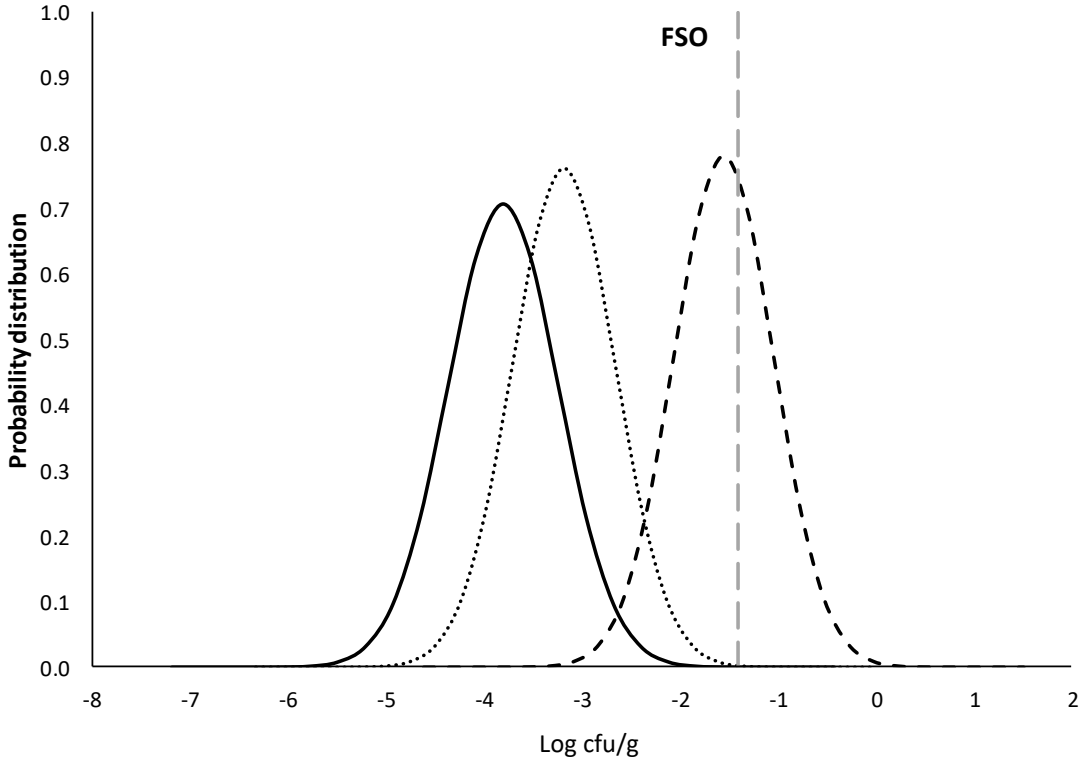
FSO
Probability distribution
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
Log cfu/g

Figure 3