Influence of water activity on the heat resistance of *Salmonella enterica* in selected low-moisture foods

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ABSTRACT

Low-moisture foods (LMF with water activity, a_w < 0.85) including pet foods and black pepper powder have consistently been associated with foodborne disease caused by *Salmonella enterica*. Increased heat resistance and prolonged survival at low-moisture conditions, however, remain major challenges to achieve effective inactivation of *Salmonella* in low-moisture foods. At low water activity (a_w) conditions, heat resistance of *Salmonella* is greatly enhanced when compared to high- a_w conditions. This study aimed to quantify the effect of a_w on the heat resistance of *Salmonella enterica* in pet food pellets and black pepper powder. Pet food pellets were inoculated with two strains of heat resistant *S. enterica* and black pepper powder was inoculated with a 5-strain cocktail of *Salmonella*. Both inoculated food samples were equilibrated at 0.33, 0.54, and 0.75 a_w in controlled humidity chambers. Inoculated pet food pellets and black pepper powder in closed aluminum cells were heat treated at specific temperatures for selected times. The results showed that the Weibull model fitted well the inactivation data. At a specific temperature, the rate of inactivation increased with the increase in the a_w from 0.33 to 0.75, and the 3-log reduction times decreased for *Salmonella* in both food samples with the increase in a_w. Water adsorption isotherms of pet food pellets and black pepper powder at initial and treatment temperatures were developed to understand the change in a_w during heat treatments. The change in a_w during heat treatment was dependent on the type of food matrix, which possibly influenced the thermal inactivation of *Salmonella* in pet food pellets and black pepper powder. The quantitative analysis of heat reduction of *Salmonella* with respect to a_w aids in selection of the appropriate initial a_w to develop effective heat treatment protocols for adequate reduction of *Salmonella* in pet foods and black pepper powder.

Keywords: pet foods, black pepper powder, Weibull model, water sorption isotherm, heat treatment, water activity.

1. Introduction

Low-moisture foods (LMFs) and intermediate moisture foods with water activity (a_w) below 0.85 cover a wide range of food products with a long shelf life at room temperature (Beuchat et al., 2013; Santillana Farakos et al., 2013). Foodborne bacterial pathogens can survive in low-moisture environment for long periods of time and cause outbreaks (Ray et al. 1971; Tamminga et al., 1976; Beuchat et al. 2011). Recent recalls show that issues related to LMF safety constitute a persistent problem (Santillana Farakos et al., 2013; Smith et al., 2016; WHO, 2014), posing risk on consumer health (Beuchat et al., 2013). Human salmonellosis is caused by *S. enterica* subsp. *enterica*, which consists of more than 2600 serotypes. Non-typhoidal serovars of *Salmonella* are a main cause of foodborne illnesses and death globally (De Cesare, 2018; Graziani et al., 2017; Santos, 2015). Among the foodborne outbreaks that occurred between 1996 to 2005, 6.6% were caused by *S.* Typhimurium (Greig and Ravel, 2009). *Salmonella* outbreaks in the past decades have consistently been linked to LMFs, including *S.* Agona in almonds, *S.* Typhimurium in chocolate, *S.* Typhimurium in dried coconut, *S.* Senftenberg in pistachios, and *S.* Infantis in dry dog foods (Beuchat et al. 2011; CDC, 2018).

Heat treatment is among the most widely used processes in food preservation as it is relatively easy to perform and is an effective and reliable treatment method (Villa-Rojas et al., 2013). High a_w food products are decontaminated effectively by heat treatment (Lang et al., 2017). The heat resistance of foodborne pathogens is higher at low a_w compared to higher a_w (Beuchat et al., 2011; Lang et al., 2017; Smith et al., 2016). Mechanisms of adaptation of *Salmonella* to the dry state, particularly the accumulation of compatible solutes (Finn et al., 2013), also mediate heat resistance. However, only a few studies report the influence of initial a_w on heat resistance of *Salmonella* in LMFs.

Modeling of inactivation kinetics helps to determine the critical parameters required for the thermal process to achieve desired reduction levels in LMFs (US FDA, 2018). Primary models, particularly first order kinetics or the Weibull model, have been used to fit the *Salmonella* inactivation kinetics at selected temperatures and a_w values in different types of foods (Forghani et al. 2019; Santillana Farakos et al. 2013; Zhang et al. 2020). The log linear model assumes that each cell is equally susceptible to heat regardless of treatment time (Peleg, 2006; van Boekel, 2002). The Weibull model can be used for fitting inactivation data for both linear and non-linear cases (Peleg, 2006). Upward convexity of survival curve fitted with the Weibull model indicates an increase in inactivation rate over extended treatment times, showing a shoulder effect; upward concavity of the survival curve indicates a decrease in inactivation rate with time and shows the presence of tailing effect (Bevilacqua et al., 2015; Peleg, 2006).

In a closed system with a fixed water content, the a_w of food products changes with a change in temperature and this change depends on the food constituents, physicochemical state, and physical structure of food products (Syamaladevi et al., 2016a&b). Hence, it is necessary to determine the a_w of food products at the treatment temperatures and analyze the difference in the effectiveness of the heat treatments based on the changes in a_w of food products during the treatment (Tadapaneni et al., 2017; Tadapaneni et al., 2018). The increase or decrease in a_w at elevated temperatures depends on the type and nature of food ingredients and this specific information on the a_w change in different food products at elevated temperatures is limited. Water sorption isotherms of LMFs generated at heat treatment temperatures provide the information about a_w change during heat treatments. Along with the a_w of the food products, the heat resistance of *Salmonella* is also affected by the type of food products and the nature of food constituents (Podolak et al., 2010; Syamaladevi et al., 2016a).

Understanding the influence of a_w on the heat resistance of target foodborne pathogens in LMFs is important for processors to select appropriate treatment conditions, depending on the initial a_w of their products. Also, data on heat inactivation kinetics for target foodborne pathogens in LMFs supports development of new heat treatments. The main objective of this study was to investigate the importance of initial a_w on the inactivation kinetics of *Salmonella* cocktail in pet food pellets and black pepper powder, equilibrated to different a_w and treated at specific temperatures in a closed system. This study also related the heat inactivation of *Salmonella* cocktails with change in a_w, determined from water sorption isotherms of pet food pellets and black pepper powder. Pet food pellets and black pepper powder were selected as the food products as these products are associated with recent recalls and only very limited studies have been conducted on determining the heat inactivation kinetics of foodborne pathogens in these products at different a_w. Studies were performed with a *Salmonella* cocktail to reflect the strain-to-strain variability in dry heat resistance in the species *Salmonella*.

2. Materials and methods

2.1. Sample preparation

Small breed dog food pellets and organic whole black peppercorn were bought at a supermarket in Edmonton, Alberta, Canada. The a_w values of samples were measured using a water activity meter (Model 3TE, Decagon Devices, Pullman, WA). The average initial a_w of pet food pellet was 0.641 ± 0.001 at 25.1 °C. The whole black peppercorns were autoclaved at 121°C for 30 min to eliminate background microbiota. The average a_w of autoclaved black peppercorn was 0.504 ± 0.001 at 25.0°C. Prior to use, approximately, 25 g of the autoclaved black peppercorn was ground in a coffee grinder for 45 seconds. Initially, to determine the total viable cell counts of the pet food pellets and black pepper powder, 0.3 g of sample was homogenized in 100 mL of sterile 0.1%

peptone water in a stomacher bag with a filter using a stomacher (Seward™ Stomacher™ Model 400C, Fisher Scientific, Ottawa, ON). The obtained homogenized suspension was surface plated on tryptic soy agar with 0.6% yeast extract (TSAYE) plates after serial tenfold dilutions and incubated for 24 h at 37°C.

2.2. Bacterial strain and inoculum preparation

The two strain *Salmonella* cocktail composed of *S. enterica* serovar Typhimurium ATCC 13311 and *S. enterica* serovar Senftenberg ATCC 43845 was used for all heat treatments of pet food pellets. The two-strain cocktail was used for all heat treatments of pet food pellets. These strains were selected in this study as they exhibit average and exceptionally high resistance to wet heat (Ng et al., 1969; Mercer et al., 2017). A five strain *Salmonella* cocktail was used for heat treatments of black pepper powder. The strain cocktails of *Salmonella* were used to support the validation of novel food preservation processes ensuring that the diversity of resistance in each target species or genus is represented (Fugett et al., 2006; Garcia-Hernandez et al., 2015; Hsu et al., 2014). In addition to *S.* Typhimurium ATCC13311 and *S.* Senftenberg ATCC43845, the five-strain cocktail included *S. enterica* FUA1934, FUA 1946, and FUA 1955. These isolates of unknown serotype are isolates from treated municipal wastewater. These strains exhibit resistance to drying and dry heat treatment (Seeras, 2017). Lawn-based liquid inoculum (LLI) of *Salmonella* was used as LLI was reported to be more stable and repeatable (Hildebrandt et al., 2016). *Salmonella* was recovered from the frozen stock culture by streaking on TSAYE plates and incubating at 37°C for 24 h.

Single colonies from TSAYE plates were transferred to tryptic soy broth with 0.6% yeast extract (TSBYE) and incubated with shaking at 37°C for 24 h, sub-cultured with 0.1% inoculum and incubated with shaking at 37°C. After 18 h of incubation, 100 µL of broth culture of each strain was spread plated on TSAYE agar plates and incubated at 37°C for 24 h. The bacterial lawn was

scraped out from each TSAYE agar plates in two mL of sterile 0.1% peptone water and collected in centrifugation tubes. Cells were harvested by centrifugation, washed in one mL fresh sterile 0.1% peptone water, and resuspended in one mL of 0.1% peptone water in each centrifugation tube. Equal volumes of inoculum of each strain of *Salmonella* were mixed to prepare the strain cocktails as inoculum for food samples.

2.3 Inoculation and drying of food products

Inoculation and drying of samples were conducted according to Bowman et al. (2015) with modifications. In brief, 50 µL of the two-strain cocktail was inoculated on the flat side of a pellet. Inoculated pet food pellets were air-dried for 24 h in a biosafety cabinet, followed by drying in a desiccator over silica gel (ACROS Organics™, Fisher Scientific, Ottawa, ON). For the inoculation of whole black peppercorn, one mL of the five-strain cocktail was added to 12 g of peppercorns in a centrifugation tube, mixed on a vortex, and stored for 20 min to absorb the inoculum. The procedure was repeated until a total volume of 2.5 mL of the strain cocktail was added to the 12 g of peppercorns. Inoculated peppercorns were air-dried for 24 h in a biosafety cabinet and ground in a coffee grinder for a total of 45 min. Grinding was performed in intervals to avoid heating of the sample, followed by drying over silica gel.

2.4 Equilibration of samples at selected relative humidity conditions

The inoculated and non-inoculated samples were equilibrated in humidity chambers separately for one week at room temperature (20-25°C). Three different chambers were filled with 300 ml of supersaturated solutions of NaCl (Fisher Scientific, Ottawa, ON), $MgCl_2$ and $Mg(NO_3)_2$ (ACROS Organic, Fisher Scientific, Ottawa, ON) to maintain the relative humidity (RH) at 33%, 54% and 75%, respectively. After equilibration, the a_w values of samples were confirmed as 0.33, 0.54 and 0.75, corresponding to the respective RH values during equilibration.

2.5 Heat treatments of samples inside thermal death time test cells

Aluminum thermal death time (TDT) test cells (Chung et al., 2008) were used for modeling the inactivation of *S. enterica* in pet food pellets and black pepper powder. Inoculated product samples i.e., 0.3 g of pet food pellet and 0.33 g of black pepper powder, were hermetically sealed in a TDT test cell to avoid loss of moisture during heat treatments. The inoculated and equilibrated pet food pellets and black pepper powder samples in TDT cells were treated at different temperatures using a water bath (Isotemp GPD10, Fisher Scientific, Ottawa, ON).

The come-up time (CUT) values i.e., the time required to achieve the target temperatures (0.5^{o}C less than 75, 85 or 95^{o}C) at the center of the pet food pellet and black pepper powder, were obtained using a Type-T thermocouple probe attached to the digital thermometer (Fisher Scientific, Ottawa, ON). The thermocouple sensor probe was placed directly in the center of the black pepper powder. To determine the CUT for a pet food pellet, a small hole was made at the geometrical center of a pet food pellet and the tip of a thermocouple was placed at the center of the pellet (Villa-Rojas et al., 2013). The food sample in the TDT cell was heated in a water bath and the temperature at the center of the samples was monitored to determine the CUT. Triplicate samples were selected for CUT measurements.

Food samples with defined a_w were treated at temperatures ranging from 60 to 85°C; treatment times ranged from 0.5 to 50 min. Thermal treatments of pet food pellets and black pepper powder were carried out at different conditions and this information is included in the supplementary file. Treatment times mentioned in the supplementary file are after the come-up time of 3 min for pet food pellets and 2 min for black pepper powder. After treatments, the TDT cells were placed in ice-water for 45 seconds prior to opening and removal of the food samples.

All the experiments were performed in three independent replicates representing *Salmonella* cocktails that were prepared, dried, equilibrated, and heat treated independently.

2.6 Recovery and enumeration of Salmonella

Samples were mixed with sterile 0.1% peptone water in a stomacher bag with filter (Fisherbrand™ Lab Blender Bag, Fisher Scientific, Ottawa, ON). Samples were soaked for 30-45 min, massaged manually and then stomached in a stomacher for 3 min. Surviving *Salmonella* cells were enumerated by surface plating of serial tenfold dilutions in 0.1% peptone water on TSAYE, followed by incubation at 37°C for 24 h. The reduction of cell counts was calculated as $\log_{10}(N/N_0)$ where N represents the cell count at specific treatment times and N_0 is the cell count at a treatment time of 0min, i.e. heat treatment for the CUT, followed by immediate cooling.

2.7 Heat inactivation kinetics of Salmonella *in pet food pellets and black pepper powder*

The Weibull model (Peleg, 2006) was used to describe the heat inactivation kinetics of *Salmonella* on pet food pellets and black pepper powder:

$$\ln\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\alpha}\right)^{\beta} \qquad (1)$$

Here, N and N_0 are the populations (CFU/g) of *Salmonella* at time t and 0, respectively, t is the time of isothermal treatment, α is the first "ln" reduction time, which is a scale factor determining the overall steepness of the slope and β is the shape parameter determining the shape of the curve. Concavity of the inactivation curve or decreasing inactivation rate with time is reflected by a $\beta < 1$ while $\beta > 1$ reflects convexity of the inactivation curve or increasing inactivation rate (Peleg, 2006). The Microsoft Office Excel solver software was used to fit the model parameters α and β to experimental inactivation data at the selected temperatures and a_w. The 3 and 5-log reduction times were predicted by Weibull model using the equation:

236 $$t_d = \alpha\left(-\ln\left(10^{-d}\right)^{\frac{1}{\beta}}\right) \quad (2)$$

237 where d is the number of decimal reductions (Van Boekel, 2002).

238 A fixed value approach was used to re-estimate α values with improved stability by keeping the
239 average of β values as a fixed β value, obtained from all inactivation curves for pet food or black
240 pepper powder (Mafart, et al., 2002; Zhang et al. 2020).

241 The secondary models were used to evaluate the influence of treatment temperature or
242 water activity on α values and the z_T and z_{aw} values were determined using following equations
243 similar to the relationship between temperature and conventional D values (Coroller et al., 2001;
244 Mafart et al., 2002).

245 $$\log\frac{\alpha}{\alpha^*} = \frac{(T^*-T)}{z_T} \quad (3)$$

246 $$\log\frac{\alpha}{\alpha^*} = \frac{(1-a_w)}{z_{a_w}} \quad (4)$$

247

248 where α^* is the time of first "ln" reduction at the reference temperature T^* (85 ^oC). Reference a_w
249 was selected as 1, and z_T and z_{aw} are the temperature (°C) and water activity increments needed to
250 reduce α value of the target microorganisms by 90%, respectively.

251 *2.8 Water adsorption isotherms at different temperatures*

252 A vapor sorption analyzer (VSA) (METER group, Pullman, WA, USA) was used to generate
253 sorption isotherms of pet food pellets and black pepper powder at 20 and 60°C, following the
254 dynamic vapor sorption (DVS) method (Syamaladevi et al., 2016a). Water sorption isotherm of
255 black pepper powder at 60°C could not be completed due to contamination of mirror of VSA by
256 fine powder and the presence of volatile components. Since VSA could not be used to generate a
257 adsorption isotherm curve above 60°C, a sealed thermal cell containing a commercial relative

humidity sensor was used to measure a_w of food sample at 81°C to generate the water adsorption isotherm for pet food pellets and black pepper powder (Syamaladevi et al. 2016a). Dry samples (a_w<0.1) were equilibrated at various relative humidity conditions (11.3%, 23%, 33%, 43%, 54%, 75%) using supersaturated salt solutions of LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, $MgNO_3$, NaBr and NaCl for 2 weeks. The equilibrated samples were sealed in the TDT cell and kept in a hot water bath set at 80°C. Once the a_w of each sample at elevated temperature was constant when equilibrium was achieved, the a_w value was recorded. The TDT cells were then removed and cooled to 25°C in ice-bath. The water content of each sample was determined by oven drying method by heating at 105°C for 8 to 12h in an oven until constant weight was obtained. The a_w and water contents were used to develop isotherms of pet food pellets and black pepper powder at the selected temperatures.

2.9 Modeling of water adsorption isotherms at selected temperatures

The GAB model was used to describe the water adsorption isotherms of pet food pellets and black pepper powder. The GAB model is expressed as (Maroulis et al., 1988):

$$\frac{X}{X_m} = \frac{CKa_w}{(1- Ka_w)(1- Ka_w+CKa_w)} \qquad (5)$$

where X is the dry basis water content of the material, X_m is the monolayer water content (dry basis). The parameter C is a measure of the strength of binding water to the primary binding sites of the food, and the parameter K is a correction factor (Quirijns et al., 2005). X_m is the monolayer water content which is regarded as a constant.

2.10 Statistical analysis

ANOVA for the response variable was conducted with the SAS software (SAS Institute, 2011). Each of the different combinations of the two factors (a_w and temperature) for multiple time points were considered as separate treatments in the analysis. There were three replicates for each

281 treatment. The level of significance was set at a *P* value of <0.05. Post hoc multiple pairwise
282 comparisons of treatment group means were performed with the Tukey-Kramer adjustment
283 (Tukey's honestly significant difference test) to control for the type I error rate.

284 **3 Results**

285 *3.1. Heat inactivation of* Salmonella *in pet food pellets and black pepper powder*

286 The average cell counts of the inoculum of *Salmonella* cocktails were between 9.3 to 10.8 ± 0.19
287 log (CFU/g). Cell counts of uninoculated pet food pellets were below the detection limit of 100
288 cfu/g. After inoculation and air drying, the average *Salmonella* count was 9.7 log CFU/g,
289 indicating that viable cell counts of *Salmonella* were not reduced by drying. After equilibration at
290 0.33, 0.54 and 0.75 a_w, the average *Salmonella* counts in pet food pellets were 9.3, 9.4, and 8.8 log
291 CFU/g, respectively. The cell count of autoclaved black peppercorns was less than 100 cfu/g. The
292 average *Salmonella* population in air-dried dried black pepper powder was 9.4 log CFU/g. After
293 equilibration at 0.33, 0.54 and 0.75 a_w, the average *Salmonella* counts in black pepper powder were
294 8.8, 8.9, and 8.7 log CFU/g, respectively.

295 For pet food pellets, the CUTs were 3.0, 3.1 and 3.1 min for 75, 85 and 95°C, respectively.
296 For convenience, 3 min was considered as the CUT regardless of treatment temperature. The
297 cooling time required after heat treatment was 45 seconds for the pet food pellets at all
298 temperatures and water activities. For black pepper powder, the average CUTs were 2.2, 2.4 and
299 2.4 min for 75, 85, and 95°C and 2 min was considered as the CUT regardless of a_w and
300 temperature. Considerable decrease in *Salmonella* population was observed, specifically at high
301 a_w and treatment temperatures in pet food pellets and black pepper powder after CUT of 3 min and
302 2 min, respectively (Table 1).

The *Salmonella* population after CUTs were considered as the initial population or the population corresponding to treatment time of 0 min. Surviving *Salmonella* populations in heat treated and control samples were determined from isothermal inactivation kinetics of the *Salmonella* on inoculated and equilibrated pet food pellets and black pepper powder at different experimental conditions (specific a_w, temperature, and time). Treatment times for the different combinations of water activity and temperature were selected after preliminary treatments. Treatments at combinations of a_w and temperatures were not evaluated when CUT inactivated more than 2 log of *Salmonella*, or when treatment time for maximum inactivation of *Salmonella* was more than 1 h. The only common temperature used to treat the pet food pellets at all three a_w levels was 75ºC.

3.2 Modeling of the heat inactivation kinetics and 3- and 5-log reduction times

In both samples, *Salmonella* inactivation kinetics at 0.33, 0.54 and 0.75 a_w exhibited non-linear trend with tailing effect and an upward concavity (Figs. 1 & 2). The Weibull model was used to fit the inactivation trend for all data (Equation 1) due to the non-linearity of the inactivation data. At higher a_w and temperatures, curves were characterized by tailing or a decrease in the slope over time. The values of Weibull parameter β were less than one for all treatment conditions in both samples, except for black pepper powder with 0.75 a_w, treated at 70°C, where the inactivation curve was downward concave. The $\beta<1$ indicates tailing, i.e. slower inactivation of *Salmonella* with increasing treatment time (Bevilacqua et al., 2015; Van Boekel, 2002). In the case of 0.75 a_w black pepper powder treated at 70°C, the presence of a shoulder in the curve was observed with a value of β greater than one, corresponding to an increase in inactivation rate of *S. enterica* with an increase in treatment time.

Weibull model was used to determine the times required for 3-log reduction in *Salmonella* population. Predicted values show that comparatively less time is required to achieve the 3-log reduction of *S. enterica* in the sample with high a_w and temperature (Table 2 & 4). For pet food pellets, the highest value of the time required to achieve the 3-log reduction in *Salmonella* was 37 min in 0.33 a_w at 75°C. The temperature had a significant effect on the time required for 3-log reduction in *Salmonella*, regardless of a_w. The 3-log reduction times for *Salmonella* in pet food pellets increased from 10 min to 24 and 37 min when a_w was reduced from 0.75 to 0.54 and 0.33, respectively (Table 2). In the case of black pepper powder, the highest 3-log reduction time value was 138 min for 0.75 a_w sample treated at 60°C (Table 4). The relationship between log (3-log reduction times) and treatment temperatures for pet food pellets and black pepper powder with the selected a_w followed a log-linear trend (Fig. 3).

The validation of pasteurization process for different types of food products may require the reduction of foodborne pathogens like *S. enterica* by 5-log to ensure the safety of food products (US FDA, 2018). Although, the time-a_w-temperature combinations inactivated the *Salmonella* cocktails by less than 3 log (Fig. 2), 5-log reduction times can be extrapolated from the Weibull model and are additionally indicated in Tables 2 and 4. Because the 5-log reduction times were calculated with the same model as the 3-log reduction times, both follow the same pattern (Tables 2 and 4).

Tables 3 and 5 present the re-estimated α values when the β values were fixed to average values as 0.681 and 0.638 for the inactivation kinetics of *Salmonella* in pet food pellet and black pepper powder, respectively, which eliminated the structural correlation between α and β values (Mafart et al., 2002). Using secondary models, the z_T values for *Salmonella* were determined for pet food and black pepper powder with different water activities (Table 3 & 5). The R^2-values

were greater than 0.972, indicates goodness of fit of the secondary models and confirmed their suitability for estimation of z_T-values.

3.3 Adsorption isotherms of pet food pellets and black pepper powder

Water adsorption isotherms of pet food pellets and black pepper powder at selected temperatures (20, 23.5, 60 and 81ºC) were generated (Fig. 4) to understand the change in a_w during heat treatments. The GAB model was used to fit the isotherm data of pet food pellets and black pepper powder. Considerable changes in initial a_w values of pet food pellets from 0.33 to 0.50 and 0.54 to 0.62 were observed when the temperature was increased from 20 to 81ºC (red lines in panel A in Fig. 4) at constant water content, however, temperature did not change the a_w of pet food pellets with 0.75 a_w (red lines in panel A in Fig 4). For black pepper powder, an increase in a_w from 0.33, 0.54 and 0.75 at 20ºC to approximately 0.55, 0.70, and 0.83 a_w, respectively, at 81ºC at constant water content (red lines in panel B in Fig. 4) were observed. The increase in a_w at a specific water content was higher in black pepper powder compared to pet food pellets. The change in a_w with temperature increase was lower at higher a_w, especially above 0.7 a_w in pet food pellets (panel A in Fig. 4). The isotherm curve for 81°C in pet food pellets tapered towards 20°C adsorption isotherm with a rise in the water content of sample over 0.1 kg water/kg dry pet food pellets, and this phenomenon was not observed in the case of black pepper powder. In order to determine the effect of a_w change with temperature increase on heat resistance of *Salmonella* in pet food pellets, future experiments should be conducted at controlled a_w conditions with no change in a_w when the temperature is increased.

4. Discussion

The type of strain, the growth conditions of pathogens, food composition, a_w, and temperature affect the survival of pathogens in food products during heat treatments (Santillana Farakos and

371 Frank, 2014). This study evaluated the effect of initial a_w and the change of the a_w during heat
372 treatments on the heat resistance of cocktails of *Salmonella* in two food products with different
373 composition and structure. The use of strain cocktails is necessary to ensure that novel processes
374 eliminate resistant and sensitive strains in the target organisms (Fugett et al., 2006; Garcia-
375 Hernandez et al., 2015; Hsu et al., 2014). For pet food pellets and black pepper powder, low a_w
376 samples required more time than high a_w samples to achieve the same log reduction of *Salmonella*
377 at any treatment temperature. The increase in heat resistance of *Salmonella* with decrease in a_w
378 matches prior observations in LMFs including chicken seasoning, pet food formulation, chicken
379 litter, whey protein, peanut butter, all-purpose wheat flour, and almond (Chen et al., 2013; Rachon
380 et al., 2016; Santillana Farakos et al., 2013; Song and Kang, 2016; Syamaladevi et al., 2016a;
381 Villa-Rojas et al., 2013).

382 Development and validation of food treatments that can ensure 4 to 5 log reduction of
383 foodborne pathogens is generally recommended. The assessment of the bactericidal effect of heat
384 treatments at controlled water activity, however, requires drying and equilibration to the desired
385 a_W value. The reduction of cell counts after drying for 24 h, equilibration for 7 days in combination,
386 and the come-up-time of heat treatment in combination with the detection limit of standard plate
387 counts, prevented experimental validation of a 5-log reduction of cell counts. We therefore report
388 3-log reduction values, which allow a comparative assessment for the effect of temperature and
389 a_W although, they were extrapolated with the Weibull model in many cases in our study (Fig 1 and
390 2). The 5-log reduction values were also extrapolated with the Weibull model, adding uncertainty
391 to the prediction, which should be carefully considered.

392 Inactivation on pet food pellets was studied with a two-strain cocktail that comprises a stain
393 of *S.* Senftenberg that is known for its exceptional (wet) heat resistance (Mercer et al., 2017).

Inactivation on black pepper powder was conducted with a cocktail of 5 strains of *Salmonella*, which additionally includes wastewater isolates of *S. enterica* to match the number of strains included in cocktails of *Listeria monocytogenes* and enterohaemorrhagic *E. coli* that were used for challenge studies in foods (Fugett et al. 2006). Although the addition of three wastewater isolates to the cocktail used for treatment of black pepper power confounds the direct comparison, this study nevertheless demonstrates that the *Salmonella* survival during heat treatments depends on the food components. At a water activity of 0.75, the 5-strain cocktail was more heat-sensitive in black pepper powder than the two-strain cocktail in pet food pellets; this increased sensitivity cannot be explained by the addition of three strains to the cocktail. Moreover, at 75^{o}C and 0.33 a_w, the 3-log reduction times for *Salmonella* in pet food pellets were 37 and 65 min while in black pepper powder, they were 77 and 198 min, respectively. However, for pet food pellets and black pepper powder with 0.75 a_w, an opposite trend was observed. For instance, the predicted 3- and 5-log reduction times for *Salmonella* were 20 and 54 min for pet food pellets with 0.75 a_w at 70^{o}C in comparison to 5.3 and 8 min for black pepper powder with same a_w at 70^{o}C. Again, direct comparison is confounded using different strains but clearly supports the role of the food matrix on thermal resistance of dry *Salmonella*.

The z_T value for *Salmonella* in 0.75 a_w pet food (Table 3) differed substantially from the corresponding values obtained at lower water activities. A high z_T value, reflecting a small effect of increasing treatment temperatures, was also observed in whole milk powder with a_w between 0.11 to 0.58, treated at temperatures ranging from 85°C to 100°C, where the z_T value for *S.* Typhimurium was 62.2°C and for *S.* Senftenberg, it was 44.3°C (Lang et al., 2017). However, for black pepper powder, the z_T values were similar for all the tested water activities (Table 5). These values are comparable to the z_T value for *S.* Enteritidis PT 30 in almond kernel flour (Villa-Rojas

et al., 2013). Similarly, z_T -value of 12.7 was reported for *E. coli* in red pepper powder with 0.55 a_w (Zhang et al., 2020). The z_T -value for *Salmonella* in cocoa powder at 0.30 and 0.445 a_w were estimated as 16.9°C and 17.5°C respectively (Tsai et al., 2019). The z_{aw} of *Salmonella* was 0.594, which is the water activity change to achieve 90% change in α value at 75°C. The z_{aw} was determined only for pet food pellets for 75°C treatment, as that was the only common temperature used for pet food samples with the selected a_w values. The z_{aw} of *S. enterica* in pet food pellets at 75°C was similar to the z_{aw} value for *E. coli* in red pepper powder, which was estimated as 0.42 at a treatment temperature of 65°C (Zhang et al., 2020). Similarly, z_{aw} for *S.* Enteritidis PT30 in almond kernel was 0.218 at 71°C (Villa-Rojas et al., 2013).

The adsorption isotherms at selected temperatures for pet food pellets and black pepper powder developed in this project provide a rationale for the impact of the food matrix on the thermal resistance of *Salmonella* and other pathogens. Low a_w foods are not at equilibrium during heat treatments. During heat treatments in a closed environment, the water content of the food remains constant but the a_w of the food, which is a thermodynamic property, changes in response to the change in temperature (Syamaladevi et al. 2016a&b). The change in a_w depends on the sorption isotherm of the specific food product. The a_w of pet food pellets and black pepper powder is affected differentially by heating, therefore, the a_w after heating differs even though the equilibrium a_w at ambient temperature was identical (Syamaladevi et al., 2016a; Taylor et al., 2018; Xu et al., 2019). The degree of change in a_w of food sample is influenced by various factors, including water content, temperature and the interaction of the food constituents with water molecules (Labuza and Altunakar, 2007; Liu et al., 2018). Current knowledge makes it impossible to predict the change in a_w of different foods in response to heating. To progress from empirical studies to a mechanistic understanding of the role of a_w on the resistance of pathogens in food, it

440 is necessary to combine model studies that control the a_w during heat treatments (Syamaladevi et
441 al. 2016a&b) with challenge studies in foods, that relate the effect of the a_w to the sorption
442 isotherm.

443 The current understanding of the change in a_w during heat treatments, determined from
444 sorption isotherms of different food products and its relationship to thermal inactivation of
445 pathogens in LMFs is extremely limited (Syamaladevi et al. 2016a; Liu et al. 2018; Xu et al. 2019;
446 Tadapaneni et al. 2017). For pet food pellets in our study, the influence of temperature on heat
447 inactivation kinetics was more noticeable at initial a_w values of 0.54 and 0.33 than at a_w 0.75. This
448 peculiar observation also relates to the change in a_w during heat treatments. Heating of pet food
449 pellets equilibrated to an a_w of 0.33 or 0.54 changed the water activity, however, the a_w remained
450 essentially unchanged after heating when the initial a_w was >0.70 (Fig. 4A). The relatively small
451 incremental lethality of treatments at 75°C when compared to treatments at 65 or 70 °C (Table 2
452 and Fig. 1) for 0.75 a_w pet food pellets indicates that the a_w is a more important parameter than the
453 temperature. Heating increased the initial a_w of black pepper powder for all a_w values that were
454 investigated (red lines in panel B in Fig. 4), accordingly, an increase of the treatment temperature
455 strongly influenced the heat inactivation kinetics (Fig. 2 and Table 3). Syamaladevi et al. (2016a)
456 reported that the influence of change in a_w on the thermal resistance of pathogens in LMFs such
457 as all-purpose wheat flour and peanut butter by developing the water sorption isotherms of these
458 products at treatment temperatures. The change in a_w was different for all-purpose flour and peanut
459 butter at elevated treatment temperatures (Syamaladevi et al. 2016a). Tadapaneni et al. (2017)
460 developed a novel thermal water activity cell to control the change in a_w during thermal treatments.
461 The D-value of *Salmonella* in 0.46 a_w organic wheat flour at controlled a_w condition at 80^{o}C using
462 thermal water activity cell was 7.3 min, which was significantly greater than the D-value at

uncontrolled a_w condition at 80°C (Tadapaneni et al. 2017). Here, during heating, the a_w increased to 0.73 at 80°C from 0.46 at ambient condition, which was obtained from the water sorption isotherm of wheat flour at 80°C. These studies show the importance of determining the a_w at treatment temperatures using sorption isotherms and relating them with thermal resistance of pathogens in LMFs.

In conclusion, this study showed that a decrease in a_w required an increase in the inactivation time and treatment temperature to achieve the required reduction levels. Modification of the a_w by food formulation could be a strategy to achieve the required level of pathogen inactivation in a relatively short time at low treatment temperatures. Heat inactivation kinetics of *S. enterica* cocktails in pet food pellets and black pepper powder were non-linear and the Weibull model was used to fit the inactivation data for both products. The a_w of food products has a decisive role in the thermal destruction of foodborne pathogens. Because the a_w of different foods is differentially affected by heating, the comparison of the heat resistance of *Salmonella* necessitates the knowledge of the sorption isotherms of specific foods at low and high temperatures. Adsorption isotherms developed at elevated treatment temperature (81 °C) for both products indicate that the change in a_w could be different during thermal treatments, depending on the type of product and this change in a_w could be related to the heat resistance of *Salmonella*. Overall, the results from this study could be used to select the appropriate initial a_w, treatment temperature, and time or develop or validate heat treatment protocols to achieve adequate reduction of *Salmonella* in pet food pellets and black pepper powder depending on the product a_w. Also, this information may be used by food companies handling these products or similar products to reduce the number of experimental trials for process development and validation to reduce the occurrence of *Salmonella*.

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Figure Captions

Fig. 1. Thermal inactivation kinetics of *S. enterica* cocktail inoculated on pet food pellets and equilibrated to an a_w of 0.33 **(Panel A)**, 0.54 **(Panel B)**, and 0.75 **(Panel C)**. Samples were treated at 65ºC (○); 70ºC (■); 75ºC (□); 80ºC (▲); or 85ºC (Δ). Data are shown as means ± standard deviation of triplicate independent experiments. Lines represent values predicted by the Weibull model.

Fig. 2. Thermal inactivation kinetics of *S. enterica* cocktail inoculated on ground black pepper and equilibrated to an a_w of 0.33 (**Panel A),** 0.54 (**Panel B**), and 0.75 (**Panel C**). Samples were treated at 60ºC (●); 65ºC (○); 70ºC (■);75ºC (□); 80ºC (▲); or 85ºC (Δ). Data are shown as means ± standard deviation of triplicate independent experiments. Lines represent values predicted by the Weibull model.

Fig. 3. Effect of temperature on 3-log reduction times of *S. enterica* cocktails on pet food pellets (**Panel A)** and black pepper powder (**Panel B)** with a water activity of 0.33 (●), 0.54 (○) and 0.75 (▼). Solid lines represent the log linear trend lines obtained by linear regression.

Fig. 4. Adsorption isotherms of pet food pellets (**Panel A)** and black pepper powder (**Panel B)** at 20°C (●), 23.5°C (○), 60°C (■), and 81°C (□). Data are shown as means ± standard deviation of triplicate independent experiments. Values predicted by the GAB model are shown as lines. Red lines show the change in a_w of sample when temperature increased from 20 to 81ºC at a specific water content.

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695 # Supplementary file

696 Heat treatment conditions for pet food pellets and black pepper powder.

Sample	a_w	Temperature (°C)	Treatment time (min)
Pet food pellet	0.33	75	3, 7, 11, 15, 23
		80	3, 7, 11, 15, 19
		85	1, 2, 3, 5, 7
	0.54	75	3, 11, 15, 19, 23
		80	1, 3, 5, 7, 9
		85	0.5, 1, 1.5, 2, 3
	0.75	65	3, 5, 7, 11, 15
		70	1, 3, 5, 7, 9
		75	1, 2, 3, 4, 5
Black pepper powder	0.33	75	16, 24, 32, 40, 48
		80	2, 4, 6, 8, 10, 12, 16
		85	1, 2, 4, 6, 8
	0.54	70	8, 16, 24, 32, 40
		75	4, 8, 12, 16, 20
		80	1, 2, 3, 4, 6, 8
	0.75	60	4, 8, 16, 20, 24, 32
		65	1, 2, 4, 6, 8, 12
		70	0.5, 1, 2, 3, 4

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Table 1: The reduction in *Salmonella* population in pet food pellets after 3 min come up time (CUT) and in black pepper powder after 2 min CUT

Pet food pellets			**Black pepper powder**		
a_w	**Temperature (°C)**	**log(N/No)**	**a_w**	**Temperature (°C)**	**log(N/No)**
0.33	75	-0.41±0.29	0.33	75	-0.29±0.22
0.33	80	-0.48±0.18	0.33	80	-0.31±0.23
0.33	85	-0.69±0.24	0.33	85	-1.49±0.01
0.54	75	-0.46±0.27	0.54	70	-0.19±0.01
0.54	80	-0.82±0.24	0.54	75	-0.88±0.05
0.54	85	-1.32±0.05	0.54	80	-1.24±0.60
0.75	65	-0.38±0.12	0.75	60	-0.24±0.25
0.75	70	-1.46±0.12	0.75	65	-0.56±0.10
0.75	75	-1.88±0.28	0.75	70	-1.35±0.05

Table 2. Calculated values of α, β parameters, and 3- and 5-log reduction times from the Weibull model for the thermal inactivation of the cocktail strain of *S. enterica* in pet food pellets.

Sample a_w	**Temp (°C)**	**α-value**	**95% confidence interval of α-value**	**β-value**	**95% confidence interval of β -value**	**R^2-value**	**Predicted 3-log reduction time (min)**	**Predicted 5-log reduction time (min)**
0.33 ±	75	4.43	0.20 to 8.67	0.91	0.45 to 1.38	0.926	37	65
0.01	80	2.12	1.27 to 2.96	0.91	0.76 to 1.07	0.979	18	31
	85	0.38	0.18 to 0.59	0.70	0.53 to 0.88	0.960	6	12
0.54 ±	75	0.47	-0.01 to 0.94	0.49	0.37 to 0.61	0.976	24	69
0.01	80	0.51	0.08 to 0.94	0.70	0.48 to 0.92	0.979	8	17
	85	0.21	0.11 to 0.31	0.69	0.54 to 0.83	0.918	3.5	7.2
0.75 ±	65	0.52	-0.10 to 1.14	0.51	0.31 to 0.71	0.979	23	63
0.01	70	0.41	0.09 to 0.72	0.50	0.32 to 0.62	0.971	20	54
	75	0.64	0.11 to 1.18	0.71	0.37 to 1.04	0.918	10	20

Table 3. Re-estimated α values, predicted α^* values and z_T values from Weibull model and secondary model for the thermal inactivation of the cocktail strain of *S. enterica* in pet food pellets.

Sample a_w	**Temp. (°C)**	**Re-estimated α-value**	**R^2-value**	**α^*-value**	**z_T value**
0.33 ± 0.01	75	2.99	0.895		
	80	1.10	0.964	0.354	10.6
	85	0.344	0.952		
0.54 ± 0.01	75	1.14	0.954		
	80	0.426	0.967	0.193	13.3
	85	0.201	0.912		
0.75 ± 0.01	65	0.989	0.944		
	70	0.812	0.950	0.359	44.4
	75	0.590	0.895		

To determine the z_T in pet food pellet, reference temperature (T*) = 85°C and 65° ≤ treatment temperatures (T) ≤ 85°C

Table 4. Calculated values of α, β parameters, and 3- and 5-log reduction times from the Weibull model for the thermal inactivation of the cocktail strain of *S. enterica* in black pepper powder.

Sample a_w	**Temp (°C)**	**α-value**	**95% confidence interval of α-values**	**β-value**	**95% confidence interval of β -values**	**R^2-value**	**Predicted 3-log reduction time (min)**	**Predicted 5-log reduction time (min)**
0.33 ± 0.01	75	2.15	-0.83 to 5.14	0.54	0.24 to 0.85	0.981	77	198
	80	1.60	0.99 to 2.2	0.76	0.61 to 0.92	0.946	20	40
	85	0.41	0.32 to 0.51	0.61	0.57 to 0.64	0.994	10	23
0.54 ± 0.01	70	1.34	0.32 to 2.36	0.46	0.33 to 0.59	0.978	89	272
	75	0.12	0.05 to 0.18	0.36	0.30 to 0.42	0.980	26	106
	80	0.19	0.08 to 0.31	0.45	0.37 to 0.53	0.939	14	43
0.75 ± 0.01	60	0.85	0.44 to 1.26	0.38	0.33 to 0.42	0.973	138	530
	65	2.19	1.46 to 2.92	0.94	0.91 to 0.97	0.891	17	29
	70	1.12	1.10 to 1.14	1.24	1.06 to 1.41	0.976	5.3	8

Table 5. Re-estimated α values, predicted α^* values and z_T values from Weibull model and secondary model for the thermal inactivation of the cocktail strain of *S. enterica* in black pepper powder.

Sample a_w	**Temp. (°C)**	**Re-estimated α-value**	**R^2-value**	**α^*-value**	**z_T value**
0.33 ± 0.01	75	2.87	0.955		
	80	1.11	0.935	0.458	12.6
	85	0.464	0.993		
0.54 ± 0.01	70	3.03	0.954		
	75	0.936	0.923	0.182	12.7
	80	0.492	0.905		
0.75 ± 0.01	60	3.06	0.918		
	65	1.22	0.855	0.028	12.2
	70	0.461	0.904		

To determine the z_T in black pepper powder, reference temperature (T*) = 85°C and 60° ≤ treatment temperatures (T) ≤ 85°C

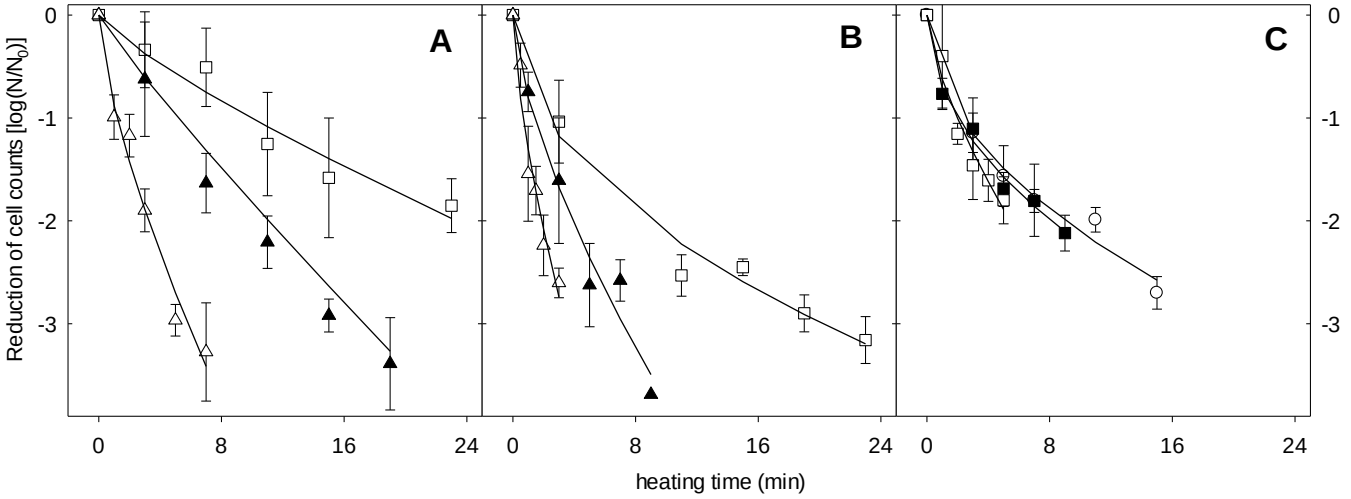

Figure 1. Thermal inactivation kinetic of a *S. enterica* cocktail inoculated pet food pellets and equilibrated to an a_w of 0.33 **(Panel A)**, 0.54 **(Panel B)**, and 0.75 **(Panel C)**. Samples were treated at 65ºC (○); 70ºC (■); 75ºC (□); 80ºC (▲); or 85ºC (Δ). Data are shown as means ± standard deviation of triplicate independent experiments. Lines represent values predicted by the Weibull model.

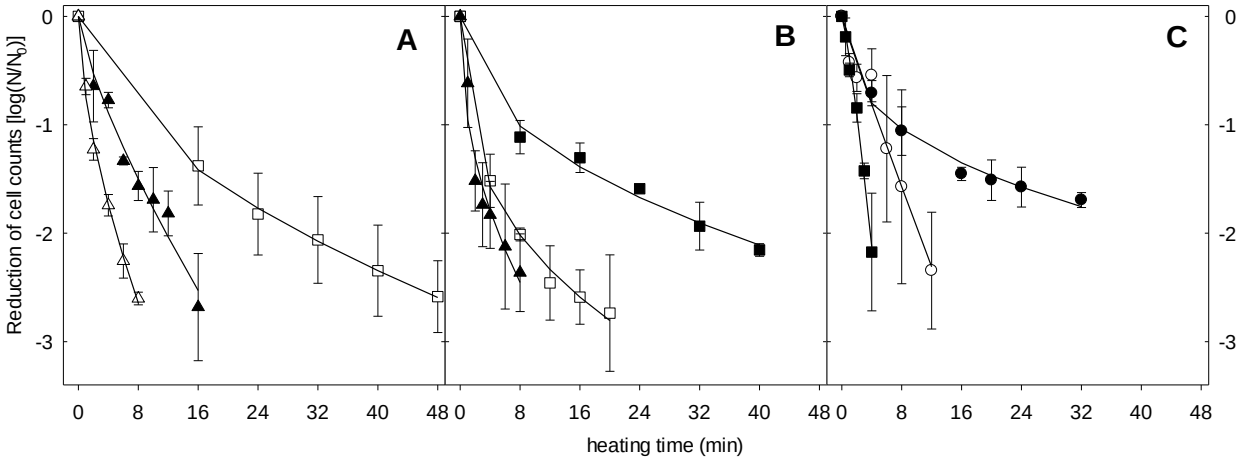

Figure 2. Thermal inactivation kinetic of a *S. enterica* cocktail inoculated ground black pepper and equilibrated to an a_w of 0.33 (**Panel A),** 0.54 (**Panel B**), and 0.75 (**Panel C**). Samples were treated at 60ºC (●); 65ºC (○); 70ºC (■);75ºC (□); 80ºC (▲); or 85ºC (Δ). Data are shown as means ± standard deviation of triplicate independent experiments. Lines represent values predicted by the Weibull model.

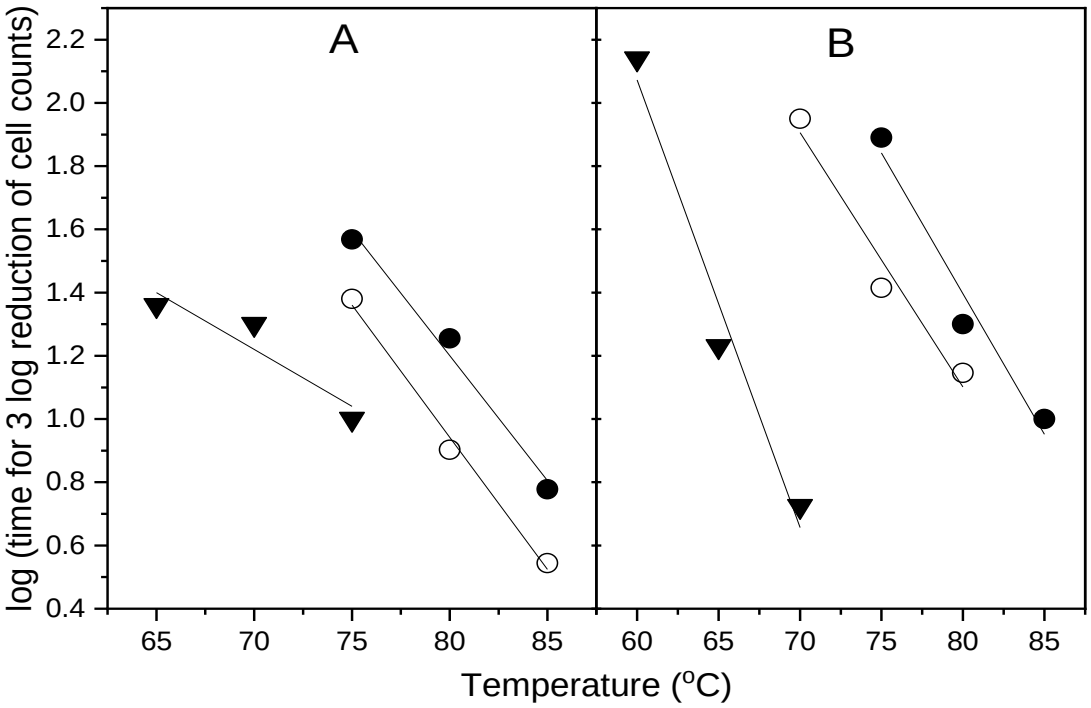

Figure 3. Effect of temperature on 3-log reduction times of *S. enterica* cocktails on pet food pellets (**Panel A)** and black pepper powder (**Panel B)** a water activity of 0.33 (●), 0.54 (○) and 0.75 (▼). Solid lines represent the log linear trend line obtained by linear regression.

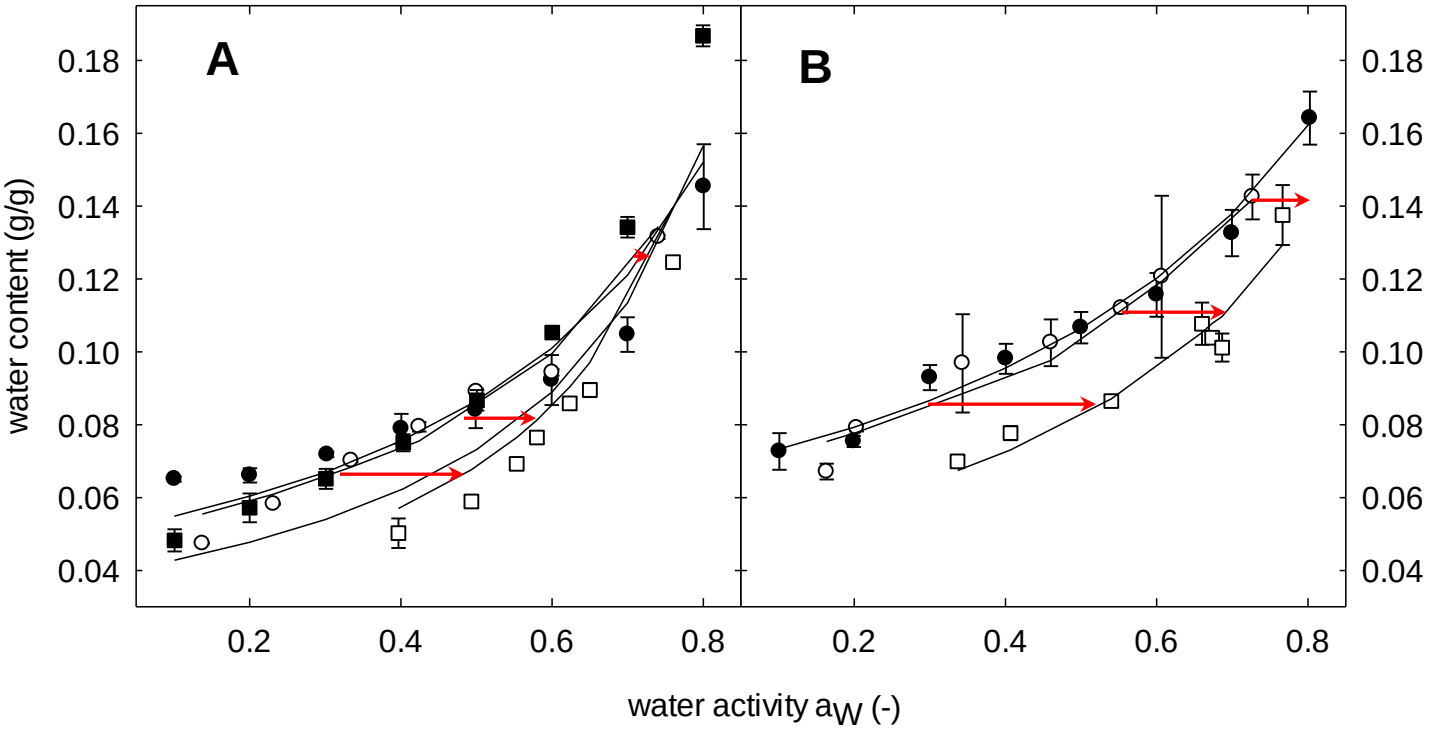

Figure 4. Adsorption isotherms of pet food pellets (**Panel A)** and black pepper powder (**Panel B)** at 20°C (●), 23.5°C (○), 60°C (■), and 81°C (□). Data are shown as means ± standard deviation of triplicate independent experiments. Values predicted by the GAB model are shown as lines. Red lines show the change in a_w of sample when temperature increased from 20 to 81ºC at a specific water content.