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Effect of pomegranate powder on the heat inactivation of *Escherichia coli* O104:H4 in ground chicken☆

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ABSTRACT

Health concerns have led to a search for natural plant-based antimicrobials. Ellagic acid has been shown to have antimicrobial activity against foodborne pathogens. The objective of this study was to assess the effect of a high-ellagic acid commercial pomegranate on the heat resistance of *Escherichia coli* O104:H4 in ground chicken. A full 2^4 factorial design was used, consisting of temperature treatment with four levels (55.0, 57.5, 60.0, and 62.5 °C) and pomegranate with four levels (0.0, 1.0, 2.0, and 3.0 wt/wt. % containing 70% ellagic acid). Experiments were conducted twice, providing a total of 32 survival curves. A three-parameter Weibull primary model was used to describe survival kinetics. Secondary models were then developed to estimate the shape parameter β (i.e., curvature representing susceptibility of cells to stress), scale parameter γ (i.e., time to reach the first decimal reduction) and the 5.0-log lethality time $t_{5.0}$ (i.e., time to reach a 5.0-log reduction), all as polynomial functions of temperature and pomegranate powder concentration. The positive effect of pomegranate concentration on both β and γ demonstrated that the phenolic-rich pomegranate powder causes *E. coli* O104:H4 cells to become more susceptible to heat, increasing the steepness and concavity of the isothermal survival curves. It was estimated that the 5.0-log reduction time would reach a minimum at a pomegranate powder concentration of 1%, producing a 50% decrease in lethality time, in comparison to that without added pomegranate powder. Nonetheless, a mixed-effect omnibus regression further confirmed that the greatest difference in the thermal resistance of *E. coli* O104:H4 happened between tests with and without pomegranate powder. In fact, adding more than 1.0% pomegranate powder, at a constant temperature, resulted only in a marginal decrease in thermal resistance. Meat processors can use the model to design lethality treatments in order to achieve specific reductions of *E. coli* O104:H4 in ground chicken.

1. Introduction

The application of adequate heat treatment continues to be known as an effective intervention strategy to guard against the potential hazards associated with the contaminating pathogens in cooked foods. It is, therefore, a critical control point to ensure elimination of pathogens in ready-to-eat (RTE) formulated foods in the food processing industry. Although microbiological safety of these products depends on ensuring that the contaminating pathogens are inactivated during heating, a number of extrinsic and intrinsic factors have been documented to render the pathogens more sensitive or resistant to the lethal effect of heat. These factors, in addition to inherent genetic factors, include growth phase, growth temperature and growth medium of the cultures; heat shock, acid shock or other stresses encountered by the pathogen; the fat level, pH and water activity of foods formulated with preservatives; and the methodology used for recovery of survivors (Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001; Juneja, 2002). Although excessive heating adversely affects the color, flavor,

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texture, and nutrient value of the foods, inadequate heating increases the likelihood of survival and persistence of the contaminating pathogens in processed RTE foods. Thus, it is critical to precisely define the heat resistance of pathogens in formulated foods and explore strategies to quantify a reduced time and temperature that could be employed to inactivate pathogens in formulated foods to ensure microbiological safety as well as to retain the sensory attributes.

There has been a growing demand for processed foods with fewer synthetic additives (David, Steenson, & Davidson, 2013). The food process industry, restaurants, and consumers have been increasingly aiming for "green" labels. Preservatives that comply with this description can be supplemented in thermally processed foods designed to determine if the addition of these natural additives would render pathogens sensitive to the lethal effect of heat. If the response is positive, reduced heat may be employed to inactivate pathogens in cooked foods and thereby, retaining desirable attributes that may otherwise be negatively impacted by excessive cooking temperatures.

In previous publications, we have described the development and application of predictive models for the enhancement of thermal inactivation of foodborne pathogens by natural, food-compatible compounds in ground chicken, turkey, ham, and beef. These include the inactivation of: *Salmonella* serotypes in ground chicken by cinnamaldehyde and carvacrol (Juneja et al., 2012; Juneja, Gonzales-Barron, Butler, Yadav, & Friedman, 2013); *Listeria monocytogenes* in ground turkey by the combined effects of temperature, sodium chloride, and green tea (Juneja et al., 2014); *Escherichia coli* O157:H7 in sous-vide cooked ground beef by tea leaf and apple skin powders (Juneja, Bari, Inatsu, Kawamoto, & Friedman, 2009); *L. monocytogenes* in ground beef by the combined effects of sodium chloride and apple polyphenols (Juneja, Altuntas, et al., 2013); and *Salmonella* on sliced cooked ham as a function of apple skin polyphenols, acetic acid, oregano oil, and carvacrol (Zhang, Mukhopadhyay, Hwang, Xu, & Juneja, 2015). These findings indicate that food processors can use the predictive models to design appropriate heat treatments to maximize the inactivation of foodborne pathogens in ground poultry and meat products without adversely affecting the quality of the heated products.

Recent reports indicate that *E. coli* O104:H4 strongly adheres to the surfaces of spinach (Nagy, Xu, Bauchan, Shelton, & Nou, 2016) and is present in feedlot cattle feces (Shridhar et al., 2016), suggesting that the pathogen has the potential to contaminate produce and meat derived from food-producing animals. Related studies (Navarro-Garcia, 2014; Tietze et al., 2015) suggest that the virulent pathogen might originate from yet unknown reservoirs.

The present study on the kinetics of inactivation of multiple *E. coli* O104:H4 strains by a commercial pomegranate (*Punica granatum*) extract was conducted in the context of reported studies of the antimicrobial activities of pomegranate products against different *E. coli* serotypes. For example, Duman, Ozgen, Dayisoylu, Erbil, and Durgac (2009) found that the minimum inhibitory concentration (MIC) values of extracts from six pomegranate varieties grown in the Mediterranean region of Turkey against seven bacteria including *E. coli* ranged between 30 and > 90 μg/mL, suggesting that different *Punica granatum* varieties and bioactive components act as broad-spectrum antibiotics. Asadishad, Hidalgo, and Tufenkji (2012) discovered that a pomegranate rind extract and fractions with a molecular weight between 1000 and 3000 kDa strongly inhibited flagellin gene expression on motility of uropathogenic *E. coli* strain CFT073, suggesting that they might be therapeutically beneficial in the treatment and prevention of uncomplicated urinary tract infections. It has also been found by Zhong et al. (2014) that polyphenol extracts from pomegranates protected against inflammation and increased the survival rate of chickens challenged with avian pathogenic *E. coli* that cause inflammation in organs called colibacillosis, suggesting the potential of the extracts as an alternative medicine for the prevention or treatment of avian colibacillosis. Pagliarulo et al. (2016) reported that both pomegranate aril and peel extracts containing multiple bioactive molecules (anthocyanins, catechins, tannins, gallic and ellagic acids) inhibited the bacterial growth of two clinical isolates of *E. coli* and *Staphylococcus aureus* that are involved in foodborne illness. Finally, pomegranate juice and polyphenolic extracts also exhibited antiviral properties against medical and foodborne viruses (Su, Sangster, & D'Souza, 2011).

These observations and related studies show that pomegranate extracts and bioactive compounds exhibited antibacterial activity against both susceptible and resistant Gram negative and Gram positive bacteria that cause food poisoning (Al-Zoreky, 2009; Braga et al., 2005; Dey et al., 2012; Haghayeghi, Shetty, & Labbe, 2013; Lucas & Were, 2009; Mahboubi, Asgarpanah, Sadaghiyani, & Faizi, 2015), as well as against drug-resistant *Mycobacterium tuberculosis* and β-lactamase producing *Klebsiella pneumoniae* (Dey, Ray, & Hazra, 2015). The objective of this study was to determine the effect of pomegranate powder on the thermal inactivation of *Escherichia coli* serotype O104:H4 in ground chicken. This virulent Verotoxin (Vtx)-producing *E. coli* strain has been associated with an outbreak of hemolytic-uremic syndrome and bloody diarrhea in humans in Germany and 14 other European countries, in the United States and in Canada (Piérard, De Greve, Haesebrouck, & Mainil, 2012).

2. Materials and methods

2.1. Additives, bacterial strains and preparation of inocula

Pomegranate Powder (70% Ellagic Acid) was obtained from Super Organic Foods (Clifton, NJ). The cocktail of three *Escherichia coli* O104:H4 strains, obtained from CDC, used in the study comprised 2009 EL-2050, 2009 EL-2071 (both clinical isolates, Republic of Georgia, 2009) and 2011C-3493 (clinical isolate, Germany outbreak, 2011). The strains were maintained as frozen stock cultures at −80 °C in brain heart infusion broth (BHI; Becton Dickinson & Co., Sparks, MD) supplemented with 15% (v/v) glycerol (Sigma-Aldrich Co., St Louis, MO). To propagate the cultures, vials were partially thawed at room temperature. An aliquot (1.0 ml) was added to 10 ml of sterile brain heart infusion broth (BHI; Difco) in 50 ml tubes and incubated for 24 h at 37 °C. These cultures containing freeze-damaged cells were not used for thermal inactivation studies. The inocula for heating studies were prepared by conducting a second transfer of 0.1 ml of each culture into BHI (10 ml) and incubating for 24 h at 37 °C. These cultures were maintained in BHI at 4 °C for two weeks when a new series of cultures were activated individually from the frozen stock.

To prepare the cell suspensions for inoculation in meat, each culture (0.1 ml) was transferred into 50 ml BHI in 250 ml flasks, and incubated for 37 °C for 18 h to provide late stationary phase cells. The cultures were harvested by centrifugation (4 °C) at 2800 × g for 15 min and washed twice in 0.1% (w/v) peptone water (PW). The cell pellets (about 8–9 $\log_{10}$ CFU/ml) re-suspended in PW (2 ml) were enumerated by surface plating appropriate dilutions in PW, in duplicate, onto tryptic soy agar (TSA, Difco) plates, which were subsequently incubated for 24 h at 37 °C before counting colonies. Equal volumes (2 ml) of each culture were combined in a sterile conical vial, mixed thoroughly to obtain a three-strain mixture of *E. coli* O104:H4 (ca. 8 $\log_{10}$ CFU/ml) prior to inoculation of chicken meat.

2.2. Preparation and inoculation of meat

Raw ground chicken, used as the heating menstruum, was obtained from a retail supermarket and stored frozen until used. The meat (100 g) was mixed thoroughly with pomegranate (0.0–3.0%; w/w) using a Kitchen Aid Mixer, Model K5SSDWH, (Kitchen Aid Co., St. Joseph, MI, USA) and then, 3 g portions were weighed into filter stomacher bags and vacuum sealed (Multivac Model A300/16, Sepp Haggenmuller GmbH & Co., Wolfertschwenden, Germany). Thereafter, these bags were vacuum-sealed in barrier pouches (25 bags/pouch; Bell Fibre Products, Columbus, GA, USA), frozen and irradiated (25 kGy) to eliminate background microflora. Irradiation was performed using a self-contained 137Cs Irradiator (Lockheed Georgia Co., Marietta, GA, USA) at the Eastern Regional Research Center, ARS, USDA, Wyndmoor, PA. Random samples were tested to verify elimination of indigenous microflora, i.e., for sterility, by surface plating.

The cocktail of three-strain mixture of *E. coli* O104:H4 (0.1 ml) was added to completely thawed ground chicken (3 g) in bags to obtain a final concentration of cells of approximately 6.5 $\log_{10}$ CFU/g. The inoculated meat was pummeled with a Mini Mix stomacher for 2 min to ensure homogeneous distribution of the organisms. Negative controls included meat sample bags inoculated with only 0.1 ml of 0.1% sterile PW. Thereafter, the sample bags were compressed into a thin layer by pressing them against a flat surface to prevent air pockets, excluding most of the air and achieving approximately 1–2 mm thickness. These compressed bags were then heat sealed.

2.3. Experimental design

A complete factorial design (4 × 4) was employed to assess the effects of arbitrarily chosen four pomegranate concentration (0.0, 1.0, 2.0, 3.0%) at different heating temperatures (55.0, 57.5, 60, 62.5 °C). All 16 variable combinations were selected at random and repeated twice. Thus, the experimental data resulted in a total of 32 survivor curves. Subsequently, the data collected were used to develop a predictive model that describes the combined effects of various concentrations of pomegranate and heating temperature on the heat resistance of *E. coli* O104:H4 cells in chicken.

2.4. Procedure for thermal inactivation and enumeration of survivors

Thermal inactivation studies were conducted in a temperature-controlled water bath (Neslab RTE 17 Digital One, Thermo Electron Corp, Newington, NH, USA) stabilized at 55, 57.5, 60, or 62.5 °C. The time of heat treatments began immediately after samples were submerged in the water bath since the come-up times (<30 s) were negligible, as observed in preliminary studies. In other words, come up times were included as part of the total heating time when these were used to calculate the D-values. The frequency of sampling times was based on the heating temperature: 10–20 min at 55 °C, 5–10 min at 57.5 °C, 2–5 min at 60 °C and 0.5–1 min at 62.5 °C. Bags for each replicate were removed at each sampling time and instantaneously plunged in an ice-water bath to stop the effect of heat on microbial inactivation, if any. Total heating times were 80–170 min at 55 °C, 25–60 min at 57.5 °C, 10–25 min at 60 °C and 3–8 min at 62.5 °C.

To determine the number of surviving CFU per gram, 3 ml of sterile 0.1% peptone was added to each meat sample to obtain a 1:1 (wt./vol) slurry and pummeled for 2 min with a Bag Mixer 100 MiniMix (Interscience, St. Nom, France). Decimal serial dilutions prepared in 0.1% peptone water were surface plated onto duplicate dishes containing TSA-YP (tryptic soy agar with added 0.6% yeast extract and 1% pyruvate) Control samples not inoculated with *E. coli* O104:H4 were plated as controls. When low microbial numbers were expected at longer heating times and/or at higher temperatures, 0.1 ml of the undiluted cell suspensions were also manually plated onto agar plates. The plates were incubated for 48 h at 30 °C before surviving *E. coli* O104:H4 cells were counted and recorded as CFU/g.

2.5. Predictive modeling

By conducting a preliminary analysis of the shape of the 32 experimental curves, it was concluded that the modified Weibull equation was the most parsimonious model to appropriately describe the population decay curves with upward concavity encountered in this study. The three-parameter Weibull primary model is defined as,

$$\log N = \log N_0 - \frac{1}{2.303}\left(\frac{t}{\chi}\right)^{\beta} \quad (1)$$

The scale and shape parameters of the underlying Weibull distribution are χ and β, respectively. If the shape parameter $\beta > 1$, convex curves are obtained, and for $\beta < 1$, concave curves are represented. Although the Weibull model is basically of an empirical nature, Van Boekel (2002) suggested that $\beta < 1$ presumes that the surviving microorganisms at any point in the inactivation curve have the capacity to adapt to the applied stress, whereas $\beta > 1$ indicates that the remaining cells become increasingly susceptible to heat. The base 10 logarithm of the microbial concentration (CFU/g) at the time t (min) is represented by log N. Log N_0 is a third model parameter which represents the initial microbial concentration at time $t = 0$.

2.6. Fitting of primary models

The Weibull primary model was separately fitted to each of the 32 survival curves, and the parameters Log N_0, χ and β were extracted. In addition, for each of the survival curves, the lethality time needed to obtain a 5-log relative reduction ($t_{5.0}$) was estimated as,

$$t_{5.0} = \chi\,(5 \ln 10)^{1/\beta} \quad (2)$$

2.7. Fitting of secondary models

Initially, the 32 estimates of the shape and scale parameters from the modified three-parameter Weibull model were logarithmically transformed (Ln χ and Ln β). The two transformed variables underwent stepwise regressions to identify the statistically significant environmental conditions that could predict them. In each stepwise regression, the predictors were entered as linear terms (Temperature, Pomegranate), quadratic terms (Temperature^2, Pomegranate^2) and in interaction (Temperature × Pomegranate). To determine the terms to be included in the model, the regressions were performed with an entry significance of 0.10 and a required significance of 0.025 to stay in the model. At each step, the studentized residuals were examined for identifying spurious data points. Once the terms to be included in the secondary models were determined, the parameter coefficient values for the selected terms were estimated by fitting an omnibus model (see next Subsection). Following the same methodology, another stepwise regression was conducted to find the best polynomial model that could predict the log-transformed lethality time $t_{5.0}$ (Ln $t_{5.0}$) in terms of temperature

and pomegranate powder concentration.

2.8. Fitting of omnibus model

An omnibus or a global model is a model type that fits the primary and secondary model at the same time using all the data from the experimental curves (Juneja, Gonzales-Barron et al., 2013; Pradhan et al., 2012), and as such, they can predict survival curves for any specified value of the environmental conditions. The independent variables (i.e., temperature and pomegranate powder concentration) predicting the Weibull's model parameters were selected by the previous stepwise-regressions, and were added to the omnibus model one by one while assessing the improvement in the goodness-of-fit measures (log-likelihood, Akaike Information Criterion [AIC] and BIC) and the behavior of the residuals.

The omnibus mixed-effects model based on the Weibull model assumed that both parameters χ and β could be expressed as a function of the environmental variables: temperature (*Temp*), pomegranate concentration (*Pmg*) and their interaction (*Temp* × *Pmg*). The random-effects terms u and v were added to the mean of the intercepts a_1 and b_1 of the polynomial expressions predicting Ln χ and Ln β, respectively. This was done because some fraction of the variability in the scale and shape parameters could not be explained by their fixed-effects predictors. Although the initial microbial concentration Log N_0 was slightly variable from condition to condition, a third random-effects term w located on Log N_0 turned out to be non-significant. Thus, only two random effects u, v remained in the omnibus model, and were assumed to take in random shifts subject to a given set j of experimental conditions. They follow normal distributions with means zero and covariance matrix [s^2_u, s^2_{uv}; s^2_{uv}, s^2_v]. The residual error ε_{ijk} followed a normal distribution with mean zero and variance s^2. The log CFU concentration taken at the time k in the food sample i exposed at the environmental condition j ($j = 1, \ldots 16$) was estimated as,

$$\log N_{ijk} = \log N_0 - \frac{1}{2.303}\left(\frac{t}{\chi_j}\right)^{\beta_j} + \varepsilon_{ijk}$$

$$\begin{aligned} Ln\ \chi_j &= a_1 + a_2 Temp + a_3 Pmg + a_4 Temp \times Pmg + u_j \\ Ln\ \beta_j &= b_1 + b_2 Temp + b_3 Pmg + b_4 Temp \times Pmg + v_j \end{aligned} \quad (3)$$

Finally, for the evaluation of the performance of the model, two statistical internal-validation indices proposed by Ross (1996), the bias factor (Bf) and the accuracy factor (Af), were computed from the observed and predicted values. All independent non-linear regressions, stepwise regressions, and non-linear mixed-effects models were adjusted in R software (version 2.14.2; R Development Core Team) using the 'MASS' and 'nlme' packages.

3. Results and discussion

In the present study, *E. coli* O104:H4 followed, in most cases, non-log linear inactivation displaying mainly concave curves, which suggested that sensitive members of the bacterial population perish rapidly (Mattick, Legan, Humphrey, & Peleg, 2001), leaving behind progressively more resistant microorganisms that may adapt to the combined stress of heat and pomegranate's antioxidant activity. Preliminary analysis showed that a three-parameter Weibull decay function (Eq. (1)) was capable of describing the raw data from each of the experimental curves with coefficients of correlation (between observed and predicted concentrations) ranging between 0.970 and 0.998.

The relationships between the primary model parameters (Ln χ and Ln β) and the environmental conditions (temperature and pomegranate concentration) were explored by scatter plots and, subsequently, by separate stepwise regression analyses (i.e., secondary models). For the Weibull's shape parameter (ln β, a parameter related to concavity), it was clear that, when no pomegranate was added, the higher the temperature, the lower the ln β and the greater the concavity of the survival curve (Fig. 1, top). However, when pomegranate powder was added, some interaction between temperature and pomegranate concentration was evident. Certainly, for the lowest temperature of 55 °C whereby ln β decreased steadily with increasing pomegranate concentration, the addition of higher concentrations of pomegranate powder in ground chicken resulted either in an increase or decrease of ln β

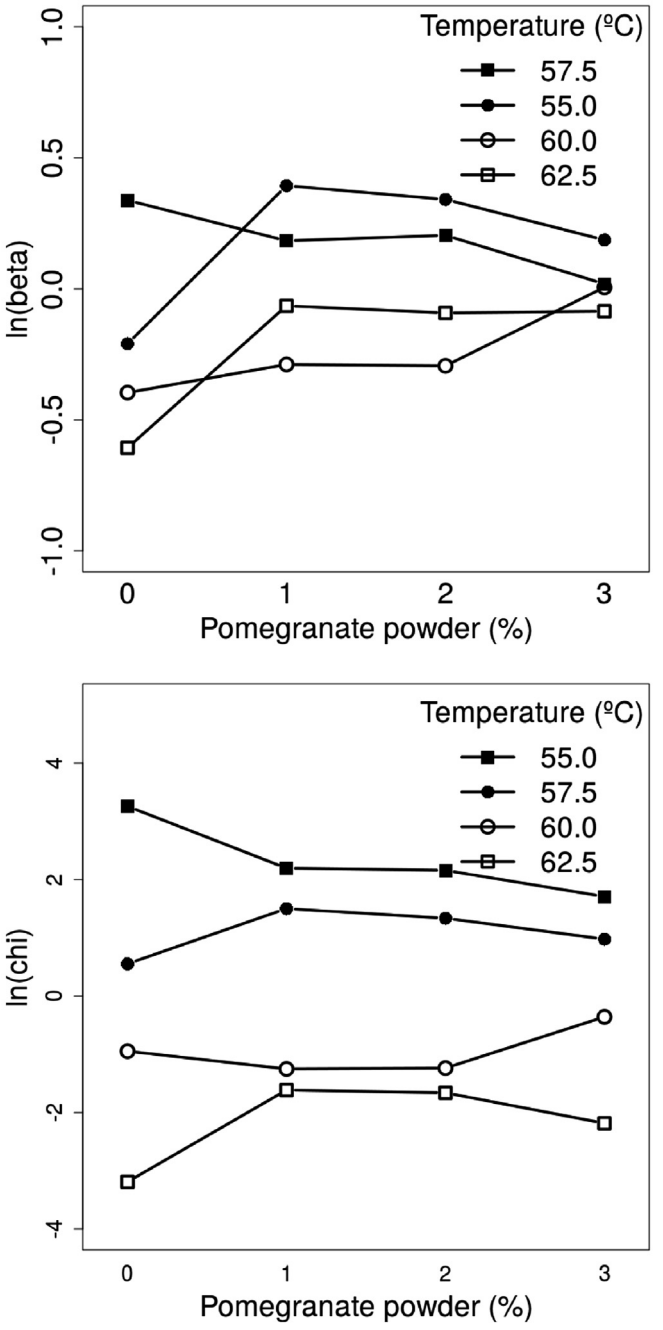

Fig. 1. Effects of temperature (°C) and pomegranate powder concentration (% w/w) on the log-transformed Weibull model's parameters β (top) and χ (bottom) describing the inactivation of *E. coli* O104:H4 in ground chicken.

value. For this reason, in the stepwise regression model predicting ln β, the interaction term temperature × pomegranate was significant ($p < 0.001$), as well as the main effects of temperature and pomegranate concentration ($r^2 = 0.923$). The same three variables were also selected by the stepwise procedure when fitting the secondary model for the Weibull's scale parameter ln χ ($r^2 = 0.632$). Fig. 1 (bottom) shows that, as occurred with ln β, at the lowest evaluated temperature of 55 °C, the ln χ (or first decimal reduction time) values neatly decreased as the pomegranate concentration increased from 0% to 3%. However, for the other (higher) fixed temperatures, a more variable behavior was identified. Nevertheless, in general terms, the change in the first decimal reduction time (ln χ) did not appear meaningful for pomegranate concentrations from 1.0 to 3.0% for all isotherms (Fig. 1, bottom). Tukey's mean comparisons from a two-way analysis of variance further confirmed that there were no significant differences in ln β among the pomegranate concentrations of 1.0, 2.0 and 3.0%. The only significant difference in first decimal reduction times was found between the treatments with 0% pomegranate (no addition) and the treatments with pomegranate powder added at different levels.

As a consequence, the addition of pomegranate powder to ground chicken also decreases the time required to reach a 5.0-log reduction (ln $t_{5.0}$). Notice in Fig. 2 (left) that the main decrease in ln $t_{5.0}$ takes places between 0% and 1.0% pomegranate powder concentration. Generally, further increments in pomegranate concentration only decrease marginally the 5.0-log lethality times (except for the difference between 2.0% and 3% pomegranate concentration at 62.5 °C). This behavior becomes more evident in Fig. 2 (right) which clearly shows that, across the studied temperatures, pomegranate powder concentrations beyond 1.0% led to similar reductions in ln $t_{5.0}$ values. To support this outcome, Tukey's comparison of means failed to reveal any significant differences in ln $t_{5.0}$ among the treatments with 1.0, 2.0 and 3.0% pomegranate powder. Thus, the addition of 1.0% (or higher) pomegranate powder in ground chicken can attain a mean decrease of ~0.60 $\log_e$ (i.e., a factor of ~0.55) in the time to achieve a 5.0-log reduction.

The parameters to predict the 5.0-log lethality time of *E. coli* O104:H4 in ground chicken are compiled in Table 1. As Fig. 2 (left) visually suggested, the pomegranate concentration had a quadratic effect ($p = 0.002$; Table 1) on ln $t_{5.0}$. The negative linear effects of temperature (−0.452; $p < 0.0001$) and pomegranate concentration (−0.508; $p < 0.0001$) were anticipated as higher values of any of those environmental variables should lead to shorter times to achieve a 5.0-log reduction. However, unlike the secondary models for ln χ and ln β, the secondary model for ln $t_{5.0}$ did not include a significant interaction term temperature × pomegranate. Still, the fitting capacity of the secondary model for ln $t_{5.0}$ (Table 1) was high ($r^2 = 0.986$), as can be visually inferred from Fig. 3.

Table 1
Parameter estimates of the polynomial secondary model predicting the natural logarithm of the time to reach a 5-log reduction of *E. coli* O104:H4 in ground chicken as a function of temperature (°C) and pomegranate powder concentration (% w/w).

Parameters	Mean	Standard error	Pr > |t|	AIC/BIC
Predictors of ln ($t_{5.0}$)				
a_1 (Intercept)	29.64	0.584	<0.0001	−22/−14
a_2 (Temperature)	−0.452	0.010	<0.0001	
a_3 (Pomegranate)	−0.508	0.087	<0.0001	
a_4 (Pomegranate^2)	0.096	0.028	0.002	
Variance				
s^2 (residual)	0.024		Adj. R^2	0.986

Table 2 lists the parameter estimates for the omnibus mixed-effect model based on the three-parameter Weibull decay function. Analyzing the environmental predictors and their p-values, it can be stated that the mild temperature (range evaluated in this study) had a larger impact on the inactivation kinetics of *E. coli* O104:H4 than pomegranate powder concentration. Nonetheless, the fact that the term temperature × pomegranate was significant for both Weibull's model parameters ln χ ($p = 0.001$) and ln β ($p = 0.003$; Table 2), and had in both cases positive slopes, seems to suggest that temperature itself has an effect on the antimicrobial properties of pomegranate. With regards to the model's random effects, the two variances s^2_u and s^2_v were significant ($p < 0.05$), and the correlation coefficient between the intercept of the linear predictor of ln χ and the intercept of the linear predictor of ln β was 0.887. In a Weibull model, a high correlation between its parameters is not unexpected as shifts in ln χ are normally compensated by shifts in ln β (Juneja, Gonzales-Barron et al., 2013). Random effects were not placed on log N_0 because there was no significant variation in log N_0 (related to the inoculum size used) among experimental conditions.

The omnibus mixed-effects model consisted of thirteen parameters – nine fixed-effect terms and four variances, and was capable of describing well all the inactivation experimental curves

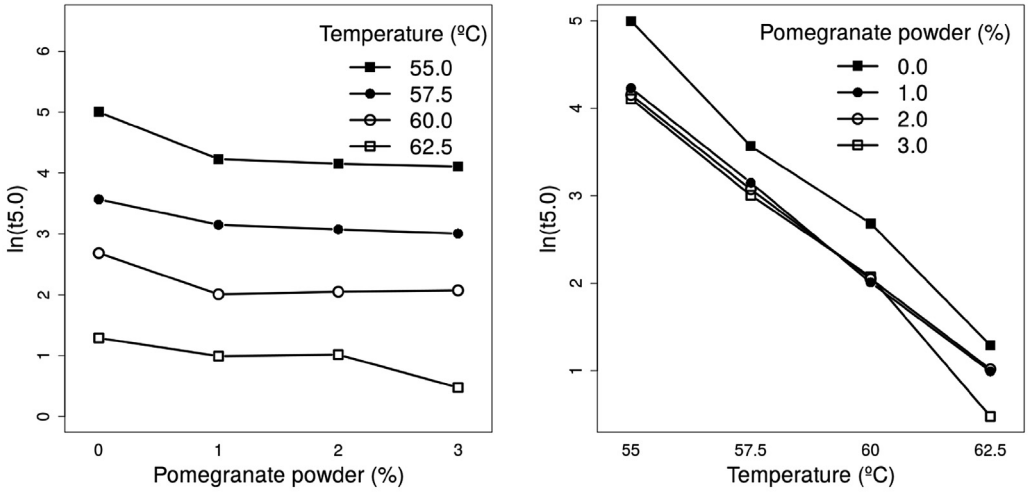

Fig. 2. Effects of temperature (°C) and pomegranate powder concentration (% w/w) on the log-transformed time (min) to achieve a 5.0 log-reduction in *E. coli* O104:H4 in ground chicken.

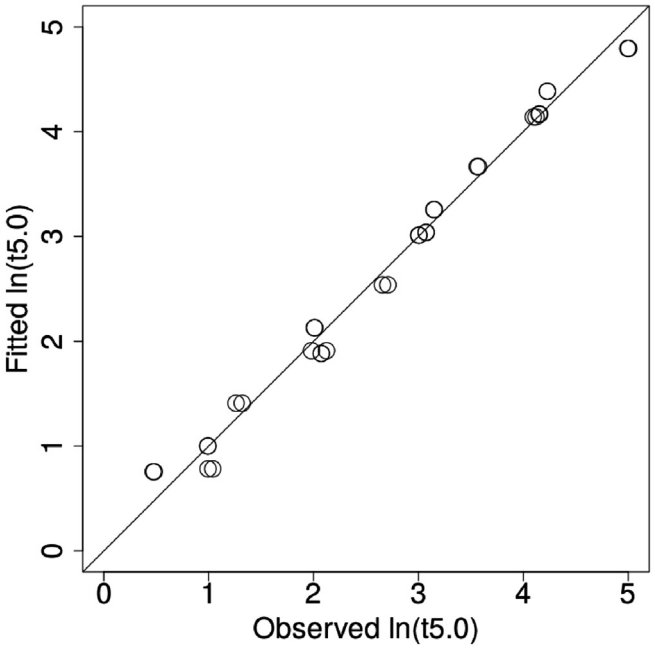

Fig. 3. Linear correlation of time to achieve a 5.0-log reduction (ln $t_{5.0}$) of *E. coli* O104:H4 in ground chicken, in comparison with values fitted by the secondary model from Table 1.

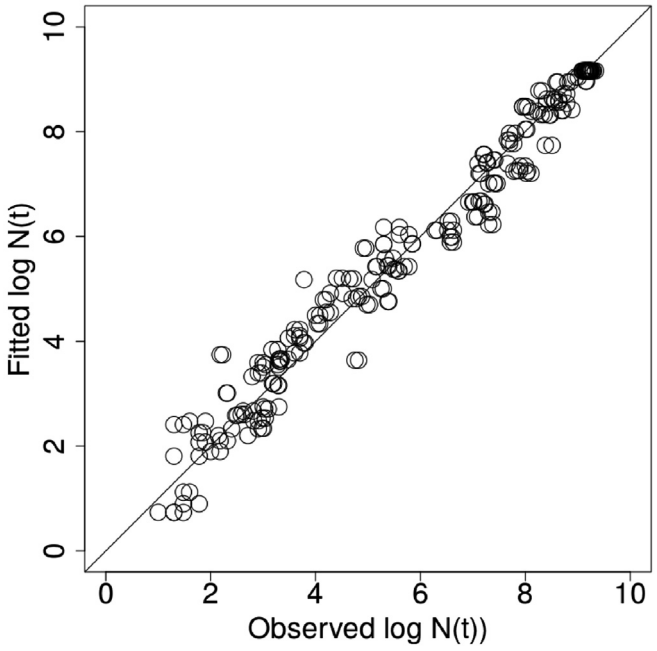

Fig. 4. Observations of the concentrations in time (log N(t)) of *E. coli* O104:H4 in ground chicken from the 32 survival curves, in comparison with values fitted by the mixed-effects omnibus regression model from Table 2.

Table 2
Parameter estimates of the mixed-effects omnibus model predicting the non-log-linear decline of *E. coli* O104:H4 in ground chicken as a function of temperature (°C) and pomegranate powder concentration (% w/w).

Parameters	Mean	Standard error	Pr > |t|	AIC/BIC
Predictors of Ln χ				
a_1 (Intercept)	45.64	3.931	<0.0001	467/512
a_2 (Temperature)	−0.773	0.067	<0.0001	
a_3 (Pomegranate)	−6.994	2.077	0.0009	
a_4 (Temp × Pomeg)	0.118	0.035	0.0010	
Predictors of Ln β				
b_1 (Intercept)	7.414	1.598	<0.0001	
b_2 (Temperature)	−0.127	0.027	<0.0001	
b_3 (Pomegranate)	−2.519	0.849	0.0033	
b_4 (Temp × Pomeg)	0.044	0.014	0.0028	
Log N_0	9.203	0.066	<0.0001	
Variances				
s^2_u (a_1)	0.148			
s^2_v (b_1)	0.026	Correlation		
s^2_{uv} (Cov(a_1, b_1))	0.055	ρ(a_1, b_1)	0.887	
s^2 (residual)	0.211			

that arose from the combination of environmental factors. Notice in Fig. 4 that overall the omnibus model had a good fitting capacity. For each of the 32 experimental curves, the omnibus model provided a good coverage of the observed data points as all observations were well within the 95% confidence intervals (Fig. 5). Such agreement was supported by the bias factor (Bf) of 1.002. The accuracy factor (Af = 1.230) indicated that on average predictions are 1.23 factors of the difference with respect to observations. For this omnibus model, the studentized residuals fell between −3 and 3, and according to the Shapiro-Wilk test, their distribution did not deviate from a normal distribution. Furthermore, the studentized residuals versus the fitted values (i.e., microbial concentrations in time) did not display any singular pattern; they were randomly spread with a coefficient of correlation of −0.001 (Fig. 6).

4. Conclusions and research needs

An omnibus mixed-effects model based on the three-parameter Weibull decay function was selected for evaluation based on our previously reported successful use of this model. The model was used to describe the combined effects of heating temperature and pomegranate powder concentration on the inactivation kinetics of *E. coli* O104:H4 in ground chicken. The positive effect of pomegranate concentration on both the shape (β) and the scale (γ) factors demonstrated that the bioactive compounds in the pomegranate powder cause *E. coli* O104:H4 cells to become more susceptible to heat, increasing the steepness and concavity of the isothermal survival curves, so that a target inactivation level can be achieved in shorter time.

It was estimated that adding 1.0% pomegranate powder to ground chicken decreases the 5.0-log reduction time by half, in comparison to when no pomegranate powder is added in the formulation. Nonetheless, adding pomegranate powder to ground chicken in concentrations higher than 1.0% (w/w) results only in a marginal decrease in thermal resistance at a constant heating temperature, as measured by the 5.0-log lethality time. A possible explanation of why at low concentration, the effective compounds are more competitive than at high concentrations could be that the binding sites of the effective compounds could be saturated by the compounds themselves.

We do not know whether individual or combinations of bioactive compounds reported to be present in pomegranates are responsible for the observed reduced thermal death time for *E. coli* O104:H4 inactivation in the ground chicken. A review by Akhtar, Ismail, Fraternale, and Sestili (2014) reports the presence of the following 20 characterized compounds in pomegranate peel: ellagic acid, caffeic acid, casuarinin, catechin, corilagin, cyanidin, *p*-coumaric acid, delphinidin, ellagic acid, gallagic acid, gallic acid, gallocatechin, granatin, luteolin, kaemferol, pelagonidin, pendunculagin, punicalgin, punicalin, and telimangrandin. A possible explanation for the observed low efficacy of high levels (>1%) of the

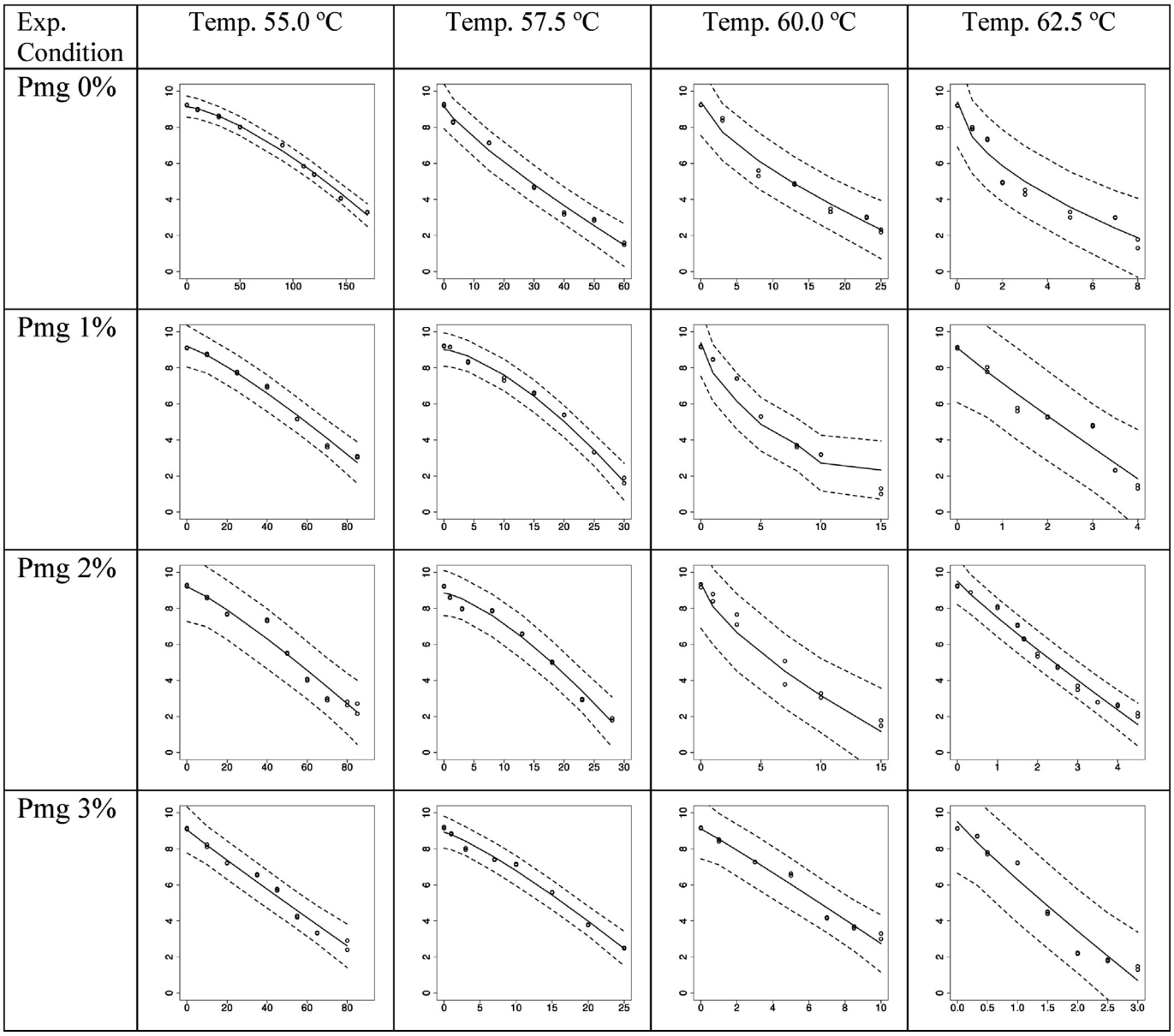

Fig. 5. Survival curves of *E. coli* O104:H4 in ground chicken for the different combinations of values of temperature (°C) and pomegranate powder (%) as modelled by a mixed-effects omnibus regression based on the modified three-parameter Weibull inactivation model. Mean predicted values and 95% confidence intervals are shown for the omnibus regression. x-axis: time in min., y-axis: microbial concentration in $\log_{10}$ CFU/g.

pomegranate powder observed in the present study is that some of the mentioned molecules are not antimicrobial but compete with antimicrobial compounds such ellagic acid for binding sites on the *E. coli* cell membranes. These aspects and the antimicrobial properties of individual and multiple combinations of the twenty characterized pomegranate compounds merit further study.

The following additional relevant considerations show that natural compounds have the potential to inhibit both antibiotic-susceptible and antibiotic-resistant foodborne and medical pathogenic microorganisms as described in detail elsewhere (Friedman, 2015). This is because the destruction of the bacteria occurs via the disruption of cell membranes, which do not differ structurally between susceptible and resistant organisms.

Because of the current concern that processed meats seem to contribute to the reported incidence of human cancers (Bernstein et al., 2015; Bouvard et al., 2015), it is also relevant to note that food-compatible natural antimicrobials can simultaneously inhibit the growth of both foodborne pathogens such as *E. coli* as well as the heat-induced formation of carcinogenic heterocyclic amines in meat as described in detail elsewhere (Friedman, Zhu, Feinstein, & Ravishankar, 2009; Rounds, Havens, Feinstein, Friedman, & Ravishankar, 2012, 2013). The results of the present study and the reported inhibition of formation of carcinogenic aromatic amines by a pomegranate seed extract (Keşkekoğlu & Üren, 2014) imply that pomegranate formulations might also concurrently inhibit both pathogens and heterocyclic amines in processed meat and poultry products. This aspect also merits study.

Finally, the results of the present study suggest that meat processors can use the described model to design lethality treatments in order to achieve specific reductions of *E. coli* O104:H4 in ground chicken.

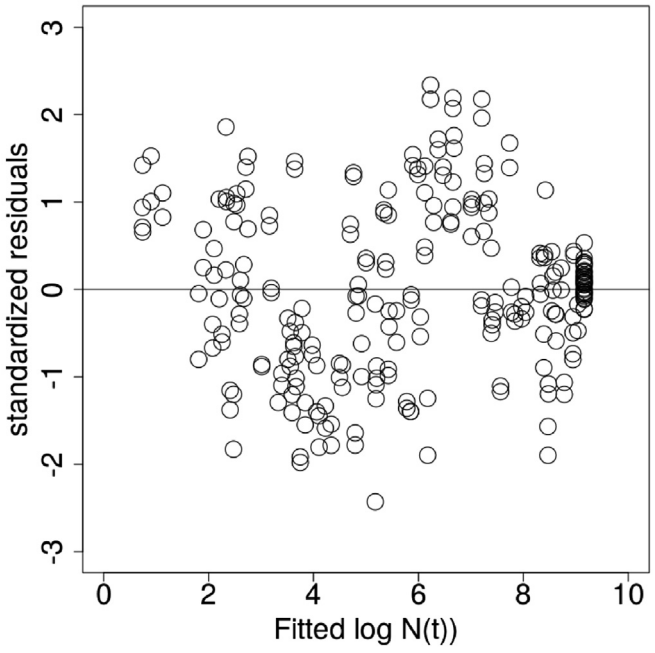

Fig. 6. Scatter plot of standardized residuals versus microbial concentration values (log_{10} CFU/g) fitted by the mixed-effects omnibus regression model for the 32 survival curves of *E. coli* O104:H4 in ground chicken.

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