Thermal resistance of *Salmonella enterica*, *Escherichia coli* and *Staphylococcus aureus* isolated from vegetable feed ingredients

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Running title: Foodborne pathogen inactivation in feed
Abstract

BACKGROUND: Cattle feed is at the beginning of the food chain in the “farm-to-fork” model and might serve as a source of contamination with pathogenic bacteria. Heat treatments are one of the most effective methods utilized to ensure the microbial safety of feeds. In this work, the thermal resistance of Salmonella enterica, Escherichia coli and Staphylococcus aureus isolated from vegetable feed ingredients was investigated in phosphate buffer saline (PBS) and in cattle feed.

RESULTS: Mean D values calculated in PBS ranged from 34.08 to 5.70 min at 55°C decreasing to 0.37 and 0.22 min at 65°C for E. coli and S. enterica, respectively. No relationship was found between thermoresistance and source of isolation. D values in feed were calculated from the adjustment of two nonlinear models to the inactivation data. Thermal resistance of E. coli and S. enterica in cattle feed showed similar results to liquid medium however, a 5-fold increment of S. aureus thermoresistance in feed was observed. Our results also revealed an increase of microbial thermoresistance with the mean feed particle diameter.

CONCLUSION: These results provide relevant information for the improvement in the safety of cattle feed regarding its process conditions (i.e. time, temperature and particle size).

Keywords: food safety; thermal processing; foodborne microorganism; cattle feed; mathematical modeling.
Abbreviations: AIC, Akaike’s information criterion; LIA, Lysine Iron Agar; PBS, phosphate buffer saline; TSB, tryptic soy broth; TSI, Triple Sugar Iron Agar; XLD, xylose lysine deoxycholate.

INTRODUCTION

Despite the development of new food processing technologies, microbial contamination of feed continues to be a global concern since it is at the beginning of the food chain in the “farm-to-fork” model. Contamination with pathogenic bacteria in the animal production industry has been linked to the consumption of contaminated feed, being considered a vehicle for the transmission of pathogens, some of great health significance for humans such as Salmonella enterica or Escherichia coli, including E. coli O157:H7. One of the sources of such contamination is feed ingredients, which are susceptible to contamination by pathogens at several stages from the growth and harvesting to transport and storage. Among the wide number of preservation methods available to reduce the microbial contamination of feeds, heat treatments are one of the most effective methods utilized. Although the effectiveness of heat treatments is usually high, the resulting pelleted feeds are sensitive to post-processing recontamination. In this regard, Bucher et al. suggested that the most thermoresistant Salmonella strains could survive the heating process during feed pelleting. Besides, several studies have reported increased microbial thermal resistance due to adverse environmental conditions such as low \( a_w \), acidity and even food structure. Therefore, a critical point to ensure the microbial safety of feeds is defining a heat treatment designed to achieve a specific lethality of target microorganisms. Nevertheless, few published papers deal with microbial heat resistance in animal feed,
since its low $a_w$ limits the proliferation of remaining bacteria after heat treatment.  

Finally, different experimental conditions referred in the literature to quantify decimal reduction times make it difficult to compare the effectiveness of heat treatments, particularly because frequent deviations from the classical semi-logarithmic linear behavior (presence of shoulders and tail-effects) are widely reported in the literature.  

The aim of this study was to characterize the thermal inactivation of *Salmonella*, *E. coli* and *S. aureus* isolated from cereals and vegetable thermally-treated feed ingredients in feed. For this purpose, thermal inactivation kinetics of 21 isolates of *Salmonella*, *E. coli* and *S. aureus* were carried out in liquid medium (PBS). The effect of feed matrix was also studied with selected isolates. The linear model and two nonlinear models (biphasic linear and biphasic logistic) were fitted to survival curves, comparing their goodness-of-fit and predicted parameters.

**MATERIALS AND METHODS**

**Bacterial isolates and culture conditions**

Bacteria were isolated in our laboratory from vegetable feed ingredients (Table 1). Detection and isolation were performed using the ISO methods for *Salmonella* spp. (ISO 6579: 2002), *E. coli* (ISO 4831:2006 and ISO 4832:2006) and coagulase-positive *Staphylococcus* (ISO 6888-1:1999) detection in food and animal feed. Biochemical confirmative tests were performed following preliminary identification based on colony morphology on selective media. Isolates were preserved as frozen stocks at −80°C in Tryptic Soy Broth (Cultimed Panreac Química S.A., Barcelona, Spain), containing 300 $\mu$L ml$^{-1}$ of glycerol, and propagated twice in appropriate media before use. All cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of TSB on a rotary shaker, at
37°C for 24 h.

**Thermal inactivation in liquid medium**

Cells were harvested by centrifugation (13000 g, 10 min, 4°C), washed twice in 8 g L\(^{-1}\) sterile buffered saline solution (PBS) and suspended in 5 mL of PBS. A sample of working bacterial suspension (50 µl) was dispensed in glass capillary tubes (Micro haematocrit capillary 1.15×75mm, BRAND GmBH, Germany) in duplicate. Tubes were heat sealed and immediately incubated in a thermostatically controlled water bath at 55, 57.5, 60, 62.5 and 65°C. At each sampling time, samples were removed, immediately cooled and sanitized with 100 mL L\(^{-1}\) sodium hypochlorite. After rinsing, the content of each capillary tube was diluted with PBS, obtaining the count suspension (\(S_c\)). Then, 0.1 mL of appropriate dilutions of \(S_c\) was plated, in duplicate, using the following media: Levine (\(E.\ coli\)), Xylose lysine deoxycholate (\(Salmonella\)) and Baird-Parker (\(S.\ aureus\)), purchased from Cultimed Panreac Química S.A. (Barcelona, Spain). Plates were aerobically incubated at 37°C for 48 h, and colonies were counted and recorded as numbers of cfu mL\(^{-1}\).

**Preparation of contaminated feed**

The composition of the antibiotic and acid-free pelleted cattle feed utilized is shown in Table 2. The feed was previously milled using a laboratory batch mill (IKA-Werke GmbH & Co. KG, Staufen, Germany) and sterilized by autoclaving. Cultures were centrifuged (13000 g, 10 min, 4 °C), cells resuspended in PBS and added (20 mL kg\(^{-1}\)) to the feed at a concentration of approximately \(1 \times 10^5\) cfu g\(^{-1}\) in case of \(Salmonella\) and \(1 \times 10^7\) cfu g\(^{-1}\) for \(E.\ coli\) and \(S.\ aureus\) isolates. Cultures were sprayed and then agitated end-over-end in a 1.5 L plastic beaker for 4 minutes, as previously optimized in
Thermal inactivation in cattle feed

For thermal inactivation experiments, one gram of acidified feed was used to fill devices specifically designed to perform the kinetics. These devices (3 mm thick and 45 mm of internal diameter) consisting of a flat rubber O-ring completely sealing two aluminum layers, were submerged in a thermostatically controlled water bath at the same temperatures assayed in PBS. After heat challenges, the procedure was identical to that described in the previous section, determining the number of surviving bacteria (cfu g\(^{-1}\)) in the contaminated feed after incubation at 37ºC for 48 h. Experiments were performed in triplicate.

To analyze the effect that cattle feed structure had on bacterial survival, inactivation kinetics were carried out at 60ºC, using feed with different particle diameters (mm): 1<\(\phi\)<2, 0.5<\(\phi\)<1 and \(\phi\)<0.5.

Mathematical modeling

Survival kinetics in PBS

Survival data were transformed onto their base-10 logarithms (log (cfu mL\(^{-1}\))) and a linear equation was fitted to the time course of surviving bacteria:

\[
\log N(t) = \log N_0 - \frac{t}{D}
\]

[1]

where, \(N_0\) and \(N(t)\) are the initial and final number of cells (cfu mL\(^{-1}\)) after a treatment time of \(t\) (min), respectively. \(D\) is the decimal reduction time (min).

The decimal reduction temperature (\(z_D\)) or the temperature increase required to reduce
the $D$ value in one logarithm unit, was obtained using the following linear relationship:

$$\log D = \alpha - \frac{T}{z_D}$$ \[2\]

where, $\alpha$ is the intercept and $T$ is the temperature ($^\circ$C).

Survival kinetics in cattle feed

Two types of equations were used to fit the survival kinetics in cattle feed:

i) a biphasic linear model proposed by Cerf & Metro \(^{16}\) considering a heat-sensitive and a heat-resistant population and formulated based on the equation of Den Besten et al. \(^{17}\)

$$\log N_T(t) = \log N_{T0} + \log \left(1 - f\right) e^{-\frac{2.3t}{D_1}} + fe^{-\frac{2.3t}{D_2}}$$ \[3\]

where, $N_{T0}$ is the initial number of cells (cfu g\(^{-1}\)), $N_T$ is the number of survivors (cfu g\(^{-1}\)) after a treatment time of $t$ (min) and $f$ is the fraction of bacteria in the subpopulation – 2–. $D_1$ and $D_2$ are the decimal reduction times (min) of the two subpopulations, respectively. When the value of $\log N_{T0}$ is reduced in one logarithmic unit, then $t$ is equal to $D$ and so, the $D$ value can be estimated by means of numerical optimization, after substituting in equation [3] the values of $f$, $D_1$ and $D_2$ previously calculated by nonlinear regression.
ii) a biphasic logistic model, describing survival profiles of two distinct subpopulations with different specific mortality rates. The equation described for biphasic survival curves by Kamau et al.\textsuperscript{18} was utilized in the form of Xiong et al.,\textsuperscript{19} parameterized to have explicit $D_1$ and $D_2$:

\[
\log N_T(t) = \log N_{T0} + \log \left[ \frac{2f}{1 + e^{\left(-2.31/D_1\right)}} + \frac{2(1-f)}{1 + e^{\left(-2.31/D_2\right)}} \right]
\]

where, $N_{T0}$ is the initial number of cells (cfu g\textsuperscript{-1}), $N_T$ is the number of survivors (cfu g\textsuperscript{-1}) after a treatment time of $t$ (min) and $f$, $D_1$ and $D_2$ have the same meaning as described above. The $D_T$ value can be estimated by means of numerical optimization, as previously described, after substituting in equation [4] the values of $f$, $D_1$ and $D_2$ calculated by nonlinear regression.

**Numerical and statistical analysis**

Fitting procedures and parametric estimations were carried out by minimizing the sum of quadratic differences between observed and model predicted values using the nonlinear least-squares (quasi-Newton) method provided by the Solver macro of the Microsoft Excel 2007 spreadsheet (Microsoft, Redmond, WA). Confidence intervals from the parametric estimates (Student’s $t$-test) and consistence of mathematical models (Fisher’s $F$ test) were evaluated using DataFit 9 (Oakdale Engineering, Oakdale, PA). Also the Akaike’s information criterion (AIC) was also used for equation comparison.\textsuperscript{20,21}

A one-way analysis of variance (ANOVA) with the Tukey post hoc test ($P = 0.05$) was used to determine whether there were significant differences between $D$ and $zD$ mean
values. Statistical analysis was performed using the general linear model (GLM) procedure of the software package IBM® SPSS® Statistics 20 for Windows (Release 20.0.0, IBM SPSS Inc., Armonk, NY, 2011).

RESULTS

Thermal inactivation in liquid medium

Survival curves of *Salmonella*, *E. coli* and *S. aureus* in PBS at different temperatures are shown in Figure 1. Due to the linear behavior of the logarithmic representation of the counts, equation [1] acceptably fitted the data ($R^2>0.9$). As expected, $D$ values decreased with increasing temperature (Table 3). Microbial viability fell at temperatures above 57.5°C, however after 2 min of heat treatment, drops of viability varied from 1 log-unit at 55°C and 57.5°C to reductions of 4-5 log-units (*Salmonella*) and 2-3 log-units (*E. coli* and *S. aureus*) at the highest temperatures assayed.

Thermal inactivation in cattle feed

Isolates showing the highest $D$ values among assayed temperatures (*slSAL-1*, *ecSJ4-2* and *stSAL-7*) were selected to carry out survival kinetics in cattle feed. Equation [1] was used for modeling the semi-logarithmic plots of the counts (Figure 2). Results showed thermal resistance decreased in the order *S. aureus* > *E. coli* > *Salmonella*, although the time required for reducing the viability equivalently was rather different. After 5 min of heating at 55°C and 57.5°C, *Salmonella* and *E. coli* counts were reduced in 1 log-unit, while 2 and 1 h were necessary to ensure consistent reductions of *S. aureus* counts at those temperatures. At 65°C, heating for 2 or 5 min resulted in around 2 log-units reductions in *Salmonella* and *E. coli* numbers, respectively. By contrast, at this temperature, 30 min of heat treatment were required to achieve reductions of 4 log-units.
in *S. aureus* counts. Decimal reduction times *(D)* from [1] were calculated using the linear portion of the inactivation curves (Figure 2). Generally, *D* values were higher than those observed in PBS, with differences particularly relevant for *stSAL-7*. Although calculating *D* values from the linear portion of the semi-logarithmic plots of survival curves is a common practice in thermobacteriology, non-linear models must be applied to correctly describe tailing curves. In the present study two equations commonly used to describe biphasic profiles, the Cerf model [3] and the Kamau model [4], were compared using the logarithmic counts as survival response. Figure 3 shows the experimental results and descriptions according to both equations. Parameter estimates and statistical analysis are also listed in Table 4. The results showed that both equations were statistically robust *(p < 0.01 from Fisher’s *F* test) and parameter estimations were almost always significant (Student’s *t* test, *α* = 0.05). Besides, all the adjusted coefficients of multiple determination between predicted and observed values were higher than 0.97. Comparison of the *r*² and Akaike’s information criterion (data not shown) indicated that both models adequately described the inactivation data in cattle feed, though differences were found for each species. For most of the experimental conditions, the Kamau model was most likely to be correct for fitting experimental data (probability higher than 65%) of *Salmonella* and *S. aureus* isolates. While for *E. coli* isolate, the Cerf model described better the inactivation data, with a probability higher than 65% at all temperatures tested.

As can be seen in Table 4, *D* values calculated from equations [3] and [4] show very close values due to the suitability of both models to describe the experimental data. In addition, thermostability of *stSAL-7* was clearly higher than that observed for *E. coli* and *Salmonella* isolates. Specifically, *D*₅₅.₀ values increased from 12 min in PBS to more than 2 h for the thermostable subpopulation, *i.e.* nearly a 9-fold increment of
microbial viability in cattle feed.

Effect of feed structure in thermal inactivation

To assess the effect of cattle feed structure on bacterial survival, inactivation kinetics were carried out at 60°C using feed with different particle size (mm): 1<φ<2, 0.5<φ<1 and φ<0.5. Our results showed that particle size influenced the specific mortality rate with an increase of microbial thermostability (D values) with the mean feed particle diameter (Figure 4). E. coli D_{60.0} values increased from 4 min in fine feed particles (<0.5 mm) to 10 min in coarser feed (1<φ<2 mm), i.e. a 2.5-fold increment of microbial viability. A lesser effect was observed for Salmonella and S. aureus, showing in both cases a 1.6-fold greater D_{60.0} values in feed with larger particle size.

DISCUSSION

Bacteria isolation sources shown in Table 1 include cereals and thermally treated ingredients (soybean meals, wheat bran and corn distillers dried grains with solubles). This selection followed a double goal, to include microbial indicators of good manufacturing practices and to investigate whether heat treatment influenced the thermostability of isolates from processed ingredients.

The average D values of Salmonella isolates in PBS (Table 3) were similar to previously reported for multiantimicrobial-resistant strains in TSB and slightly higher than those of Salmonella Enteritidis and Typhimurium in PBS. Lower D_{55.0} values were obtained than those reported by Stopforth et al. in peptone water, while higher thermostabilities at 60 and 65°C were observed in the present study. Otherwise, the resistance of E. coli isolates used in this work (57.5°C) was similar to that described by Buchanan & Edelson for three strains of E. coli O157:H7 in TSB at 58°C. Gabriel &
Nakano \textsuperscript{26} reported significantly lower $D_{55.0}$ values for \textit{E. coli} O157:H7 and \textit{E. coli} K-12 in PBS. Except for \textit{ecSJ4-2}, $D_{55.0}$ values were among those reported (2.6 and 21.5 min) for 17 different strains of \textit{E. coli} O157:H7 in BHI broth. \textsuperscript{27} Nevertheless, at 60\(^{\circ}\)C, higher thermoresistance was observed in the isolates assayed in the present work, since the highest $D_{60.0}$ value reported by these authors was 2.1 min. Thermal resistance data of \textit{S. aureus} available in the bibliography are not as abundant as $D$ values of \textit{Salmonella} and \textit{E. coli}. In general, $D$ values obtained in this study were lower than those previously reported for \textit{S. aureus} in TSB \textsuperscript{28}. These authors reported $D_{55}$ and $D_{60}$ values ranged from 13.7 to 21 min and 4.8 to 6.5 min after direct selective plating onto Baird-Parker agar.

The average $z_D$ values obtained in this study ranged from 7\(^{\circ}\)C to 14\(^{\circ}\)C (Table 3), being greater than those reported by Juneja & Eblen \textsuperscript{29} for \textit{Salmonella} in chicken broth at temperatures ranging from 58 to 62\(^{\circ}\)C and by Bacon et al. \textsuperscript{22} in TSB. On the other hand, Buchanan & Edelson \textsuperscript{25} reported a decimal reduction temperature of 4.3\(^{\circ}\)C for \textit{E. coli} O157:H7 in TSB (56–62\(^{\circ}\)C).

Differences in $D$ and $z_D$ values reported in this study compared to those described in the literature can be due to variations in experimental conditions, both in terms of strain and medium in which the thermoresistance is studied (\textit{i.e.}, pH, $a_w$), conditions of microbial growth, etc. \textsuperscript{30} Besides, from our results no relationship was found between thermoresistance and source of isolation. So, we cannot conclude that isolates from thermally processed ingredients are a selection of the most heat-resistant microorganisms, as suggested by some authors. \textsuperscript{5,13}

Thermal inactivation curves of isolates showing the highest $D$ values in PBS (slSAL-1, \textit{ecSJ4-2} and \textit{stSAL-7}) had a tailing effect in cattle feed at all temperatures (Figure 2). Profiles with tailing effects and lag phases have been widely reported for thermal inactivation kinetics. \textsuperscript{31,32} Different causes can explain non-linear kinetic data, including
the need of certain damage before inactivation follows a first order kinetics \(^3^3\) or the presence of subpopulations with different death mechanisms or different sensitivities to heat. \(^1^6\) Also this tailing effect was reported to be a consequence of using dry heat in thermal inactivation studies of *Escherichia coli* O157:H7 in cattle feeds. \(^1^2\)

In these cases, \(D\) values were obtained from the adjustment of two nonlinear models (biphasic linear and biphasic logistic) to the inactivation data (Figure 3). Both biphasic equations accurately described the tailing-survival curves (Table 4), suggesting the existence of two subpopulations with different thermoresistance. In fact, *S. aureus* isolate showed a markedly tailing behavior at 57.5, 60 and 62.5\(^\circ\)C (Figure 3), indicating the presence of a highly heat-resistant subpopulation. Although this group of cells is a minor fraction of the population (Table 4), might be responsible of the enhanced thermoresistance observed in cattle feed. \(D\) values calculated using this approach were comparable to results reported by Hutchison et al., \(^1^2\) who found reductions of 2 log units of a mixture of *E. coli* O157 after thermal treatment at 70\(^\circ\)C for 2 min in cattle feed.

As mentioned in the introduction, other factors like \(a_w\), acidity and structure of foods influence the heat resistance of foodborne microorganisms. In regards to feed, Liu et al. \(^7\) reported greater thermal resistance (52-85\(^\circ\)C) of *Salmonella* Senftenberg 775W in dry feeds with lower moisture content. However, despite being particle size a relevant variable in feed pelletization technology and on the thermal resistance of foodborne microorganisms, \(^9\) to our knowledge, its effect on microbial heat inactivation parameters has not been investigated.

Laroche et al. \(^6\) observed a significant effect of food powders size on the heat resistance of *Saccharomyces cerevisiae*. These authors attributed the higher thermal resistance to an increase in the time required for the diffusion of heat into the food particles, reducing
the temperature at which cells are exposed and increasing the time or temperature
needed to achieve an equivalent level of decontamination. Likewise, the protective
effect of feed observed on the thermal resistance of *Salmonella, E. coli* and *S. aureus*
can be due the lower heat conductivity into the feed particle when the mean diameter is
increased. In general, larger particle size has yielded higher microbial heat resistance in
solid food matrices as diverse as wheat flour and meats such as beef and turkey.  

**CONCLUSIONS**

This study focused on the characterization of *Salmonella enterica, Escherichia coli* and
*Staphylococcus aureus* thermal resistance in liquid medium (PBS) and in cattle feed.
The bacteria utilized in the present work were isolated in our laboratory from cereals
and thermally treated ingredients. Mean *D* values calculated in PBS ranged from 34.08
to 5.70 min at 55ºC decreasing to 0.37 and 0.22 min at 65ºC for *E. coli* and *S. enterica*,
respectively. Furthermore, from our results we found no association between the
thermoresistance and the source of isolation, suggesting that isolates from thermally
processed ingredients are not a selection of the most heat-resistant microorganisms.

Thermal inactivation curves of isolates showing the highest *D* values in PBS had a
tailing effect in cattle feed at all temperatures and so, *D* values were calculated from the
adjustment of two nonlinear models to the inactivation data. According to this approach,
thermal resistance of *E. coli* and *S. enterica* in cattle feed showed similar results to PBS,
however, a 5-fold increment was observed for *S. aureus D* values. Our results also
revealed an increase of microbial thermoresistance with the mean feed particle diameter.
Overall, these results provide relevant information for the improvement in the safety of
cattle feed regarding its process conditions (*i.e.* time, temperature and particle size).
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