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Research Paper

Validation of a High-Throughput Sausage Casing Model for the Assessment of Bacterial Inactivation Affected by Salt Concentration, pH, and Temperature

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ABSTRACT

Previous studies have shown the efficacy of high concentrations of salt as the main preservative against vegetative bacteria present on natural sausage casings. These studies were limited in the number of variables and the interactions between these variables that were assessed. To remedy this situation, a MicroCasing high-throughput model was developed and validated to study the inactivation kinetics of various combinations of parameters (salt concentration, pH, and temperature) on eight bacterial isolates of *Salmonella enterica, Staphylococcus aureus, Escherichia coli,* and *Listeria monocytogenes* over a prolonged period. A Weibullian power model was the best fit to show the trends in sensitivity of each bacterial isolate to salt, pH, and temperature over time. The inactivation kinetics generated with this novel approach could serve as a predictive model for the required salting period for casings. The actual bacterial contamination of the product can vary with the respective production step during processing from animal intestine into sausage casings (initial level, $\sim 10^5$ CFU/g; level after salting, $< 10^2$ CFU/g). Subsequent selection and grading of these casings will require complete removal of all salt, and upon completion of this production step, the casings will be resalted. By determining the actual contamination level before the salting process, the minimum storage period in salt can be calculated and potentially optimized by adjusting the pH and temperature. As a result, a standard holding period of at least 30 days may no longer be necessary to produce salted natural casings in accordance with validated quality and food safety criteria.

HIGHLIGHTS

- A new model system was developed for analysis of bacterial inactivation kinetics in foods.
- The novel model allows determination of product-specific bacterial inactivation over time.
- Effects of time, temperature, pH, and salt on casing preservation can be clarified with the model.
- Prediction of inactivation parameters allows casing production to meet HACCP criteria.

Key words: High-throughput model; Inactivation; Mathematical model; Sausage casings; *Staphylococcus aureus*; Weibullian model

The intestinal tracts of pigs, cattle, and small ruminants are used for the production of edible sausage containers. Although these intestines are harvested from animals considered fit for human consumption, by their very nature these intestines are covered with bacteria originating from the animal's intestinal microbiome *(2)* at levels up to 10^6 CFU/g *(3)*. To become an edible sausage casing, these intestines need to be thoroughly cleaned, scraped, washed, and preserved *(12, 20, 25, 29)*. The preservation of casings is generally done by applying sodium chloride (NaCl) as the main antimicrobial agent, either as dry salt or as a fully saturated brine. In several studies *(3, 9, 11, 30),* the efficacy of salt as a preservative has been shown, and a better understanding has been obtained of how various bacteria inoculated onto casings are inactivated when exposed over time to different salt concentrations and temperatures.

However, these studies have been performed using classical manual inoculation and plating techniques, which limit the overall number of variables that can be included into one study design. Therefore, potential combinatory effects of temperature, salt concentration, and/or pH for casing preservation have not been studied in detail. To fill this data gap, a fully automated MicroCasing high-throughput model (MC-HTM) was developed to study the inactivation of various bacteria using combinations of parameters over a prolonged period of time.

The international industry standard *(7)* for the preservation of casings is currently based on the application of either dry salt or fully saturated brine (NaCl) at ambient

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TABLE 1. *Bacterial strains, ID, and origin*

Strain	ID	Origin
Escherichia coli ETEC O149:K91	NIZO2254	Pig intestine
E. coli ETEC E24377A	NIZO4415	Unknown
Listeria monocytogenes	NIZO2394	Meat processing plant
L. monocytogenes	NIZO2625	Smoked meat
Salmonella enterica	NIZO1287	Pig
S. enterica	NIZO1290	Pig
Staphylococcus aureus	NIZO3945	Cow
S. aureus	NIZO4018	Raw milk

TABLE 2. *Equations for statistical modeling*[a]

Equation	Description
1	$\frac{dN}{dt} = -k \cdot N$
2	$\ln \frac{N}{N_0} = -k \cdot t$
3	$\log N = \log N_0 - \frac{t}{D}$
4	$\log N = \log N_0 - \frac{1}{\ln 10} \cdot \left(\frac{t}{\alpha}\right)^{\beta}$
5	$\log N = \log N_0 - a \cdot t^b$
6	$a = a_0 + C_{\text{salt}} \cdot \text{salt} + C_{\text{temp}} \cdot \text{temp} + C_{\text{pH}} \cdot \text{pH}$

[a] For fitting parameters a_0 and b per bacterial species, relating to equations 5 and 6, see the supplemental material.

temperatures and a neutral pH for 30 days. Using the MC-HTM to clarify the effects of these parameters on bacterial reduction would provide a more precise bacterial inactivation profile over time and would allow for the interval required for 1-log inactivation of bacteria to be calculated. Therefore, the main objective of this study was to validate the micromodel concept and its application to casings by producing data comparable to those of previous studies. The relative contributions of pH, salt concentration, temperature, and time to bacterial inactivation should be determined. This data set can then be used to create a practical mathematical model and a better understanding of bacterial inactivation by influencing known parameters in salted casings during industrial production.

MATERIALS AND METHODS

Bacterial strains. Eight strains of four bacterial species were used in this study, including representatives of gram-negative and gram-positive species. These bacteria, which are all relevant to food safety, represent the normal bacteria that can be found during the cleaning process of animal intestines for manufacture of sausage casings. All strains used in this study (Table 1) are well documented and were made available by NIZO (Ede, The Netherlands) from its own collection.

Sample preparation. Commercially available dry salted Australian sheep casings (AA quality; Van Hessen B.V., IJssel, The Netherlands) were taken from stock and rinsed with potable water to remove any salt crystals. These casings were subsequently divided into pieces approximately 2 m in length, tied in bundles, rinsed, and stored in a fully saturated salt (NaCl) solution (water activity [a_w] < 0.79) using suitable plastic containers (maximum of 1 kg). These containers were then sterilized by exposure to gamma radiation (Cobalt-60, 10 kGy; Synergy Health, Ede, The Netherlands) using a validated method for casings *(31)*.

The sterilized casings were frozen in liquid nitrogen and then ground while frozen into small particles with a cryogenic tissue grinder (no. CTG111, BioSpec, Bartlesville, OK). The particulates were subsequently freeze-dried into a fine powder to allow the powder to be mixed in 10 mM phosphate buffer as a 5% final solution. This mixture represents the actual MC-HTM, retaining the relevant properties of the original casing *(25)*.

Inoculation procedure pilot study and full analysis using the MC-HTM. For the pilot study, which was developed to test the MC-HTM as proof of principle, and the main study, the following parameters and procedures were used. All bacterial strains listed in Table 1 were pregrown in brain heart infusion (BHI) broth (Sigma-Aldrich, St. Louis, MO). All cultures were normalized to 1.0 as their optical density at 600 nm and were inoculated as a 1% inoculum. The prepared ground casing powder was used as a 5% mass percentage in a 10 mM phosphate solution.

For the pilot study the pH was set at 7.0, whereas for the main study the pH was set at 4.5, 7.0, 8.0, or 10.0. The pH for both studies was adjusted with HCl or NH_4OH.

Food-grade salt (NaCl, AKZO Nobel Salt B.V., Farmsum, The Netherlands) was added at various concentrations, with 350 g/L used as a 100% saturated salt solution. For the pilot study only the 100% salt solution was used, whereas for the main study 0, 25, 50, 75, and 100% salt solutions were used.

For the pilot study, all bacteria were inoculated with approximately 10^7 CFU/mL and tested at three temperatures (4, 12, and 30°C). Inactivation over time was determined by ascertaining bacterial populations on day 0 and day 7. Experiments in the pilot study were performed as biological duplicates to determine reproducibility of the population analysis in the MC-HTM.

For the main study, all samples were kept at four temperatures (3, 12, 20, and 30°C) during the entire study. The high-throughput population analysis used for both the pilot and the main study has a lower quantification limit of 10^3 CFU/mL. Therefore, after completion of the pilot study, the decision was made for the main study to inoculate the model with at least 10^9 CFU/mL for each of the strains to study inactivation over time for at least four orders of magnitude. This inoculation level is much higher than previously recorded or applied bacterial levels in casing models and therefore can represent a worst-case scenario *(3, 6, 30)*. At various time points (days 0.5, 1, 2, 3, 6, 10, 13, 17, 20, 27, 34, 46, and 60), 10-fold serial dilutions from 10^0 to 10^{-7} were prepared in 96-well plates (10 μL of coculture sample plus 90 μL of coincubation medium). BHI agar plates were used for determination of all bacterial levels. For both the pilot and main study, pipetting was done with a semiautomatic 96-fold pipettor (CyBio SELMA, Analytic Jena AG, Jena, Germany). Negative control samples were included in the MC-HTM and stored at pH 7.0, 0% salt, and 20°C. No visual spoilage from molds, yeasts, or bacteria was observed after 60 days.

Statistical analysis and development of the mathematical model. Several models were available for describing the inactivation of microorganisms. For this project, two models were evaluated for their ability to describe the inactivation data obtained.

For first-order inactivation, a much applied model for microbial inactivation, also called the *D-z* model (with regards to the fitting parameters), was used. A first-order kinetics model assumes that the rate of change of a variable is described by the variable itself multiplied by a kinetic constant.

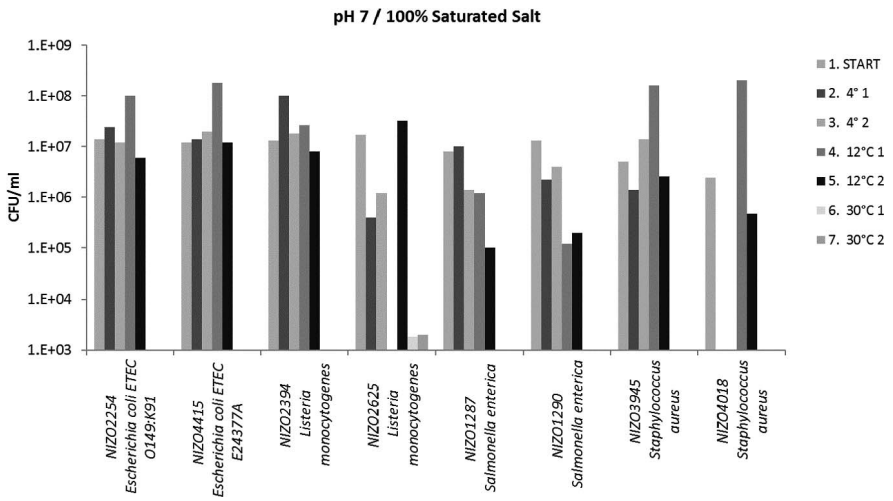

FIGURE 1. *Results of validation pilot study with MC-HTM for inactivation of all bacteria at* t = *0 (start) and* t = *7 days after storage at three temperatures, pH 7, and 100% saturated salt solution.*

For microbial inactivation (Table 2, equation 1), N is the number of live cells (CFU), t is the time (days), and k is a kinetic constant. This equation can be integrated from $t = 0$ to a variable time to form equation 2 (Table 2), in which N_0 is the number of live cells at $t = 0$. Because in practice the number of live cells is generally presented in log form, equation 2 was rewritten as equation 3 (Table 2), where D is the D-value, which is the amount of time required for a 1-log inactivation.

A Weibullian model was used as the power model. The first-order model assumes that all microorganisms in a population share the same inactivation time, and the log inactivation is therefore linear in time. In practice however, nonlinear log inactivation is more often observed because the inactivation time of a microbial population is not a single value but a frequency distribution. The Weibull distribution is generally applied to microbial inactivation, and the cumulative form represents the rate of inactivation (Table 2, equation 4), in which α is a scale parameter and β is a shape parameter. In practice, the bacterial population is more commonly expressed in log form. For this purpose, the Weibull model can be rewritten into a Weibullian power model (Table 2, equation 5). The fitting parameters a_0 and b have no direct physical meaning and are purely empirical. However, the a_0 value can be used as a relative number for inactivation rate, i.e., indicating whether inactivation rates are increased or decreased under a specific condition as compared with another condition. A link can be made between the value b and inactivation kinetics. When $b < 1$, the surviving cells are less likely to die, indicating the presence of persisters or cells that are adapting to the stress *(8)*. When $b > 1$, the surviving cells are dying at an increased rate, indicating some sort of cumulative damage over time under the applied stress *(4)*. The effects of environmental variables can be incorporated in the a parameter using independent multilinear contributions (Table 2, equation 6).

Data analysis. All data analyses were performed using Rstudio, running R v.3.2 (Rstudio, Boston, MA). The fitting procedures were performed based on least-square fitting using the Gauss-Newton algorithm. Equation 3 and the combination of equations 5 and 6 were used to fit the experimental data. N_0 was always treated as a fitting parameter and not as an input variable.

RESULTS AND DISCUSSION

Model validation, step 1: pilot study. To validate the newly developed MC-HTM, a limited pilot experiment was conducted to confirm the inactivation over time of the bacteria chosen for this study (Table 1). Figure 1 presents an overview of the data obtained from this pilot study. The bacterial populations for each of the duplicate experiments are shown as two individual bars for all eight strains at three temperatures at $t = 0$ (START, bar 1) and after 7 days (4°C, bars 2 and 3; 12°C, bars 4 and 5; 30°C, bars 6 and 7).

Within the 7-day period at pH 7, 100% saturated salt solution, and 30°C, all inoculated bacteria were quickly inactivated to below or slightly above the detection level. After 7 days at 4 and 12°C, most cultures seemed relatively stable with no apparent inhibitory effect of the 100% saturated salt solution (Fig. 1). However, after 7 days at 12 or 30°C with 0% salt solution, all strains increased in growth ($>10^9$ CFU/mL, data not shown). These results indicate that bacteria are able to cope with osmotic stress better under cooler conditions *(5)* than under hotter conditions and survival is prolonged *(18)* during the initial period of exposure to a high salt concentration. Overall, the duplicates per strain has less than a twofold variation from the mean for each duplicate. Because the differences were a 3- to 4-log order of magnitude and a large number of analyses in time were performed, the accuracy was deemed sufficiently high.

Model validation, steps 2 and 3: main study. The newly developed MC-HTM was applied to a full screen of all chosen parameters for inactivation over time of all bacterial strains (Table 1), resulting in 80 preservation conditions (combinations of four pH values, five salt concentrations, and four temperatures). The data for *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella enterica* are the mean inactivation rate of the two strains per species, which were studied as biological singles. An exception to this approach was *S. aureus* NIZO4018, which was analyzed as a biological triplicate for validation purposes.

The second step in the validation process was determination of whether the data generated using the MC-HTM was comparable to other known inactivation characteristics under similar conditions. A comparison was made between the current data set for *S. aureus* (strain NIZO4018, in triplicate) and a previously published *S.*

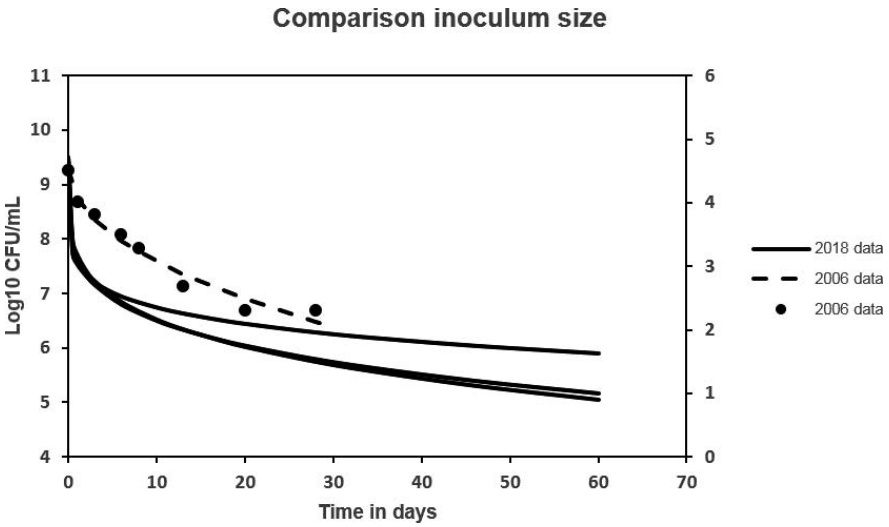

FIGURE 2. *Comparison of the inactivation kinetics for* S. aureus *(in triplicate) from the current 2018 data using different inoculum levels (solid lines, left* y *axis) compared with the data from 2006 (30) (dashed line, right* y *axis). In both graphs, data are from experiments with 100% saturated salt concentration at 20°C and pH 7.*

aureus data set (strain ATCC 29213, in duplicate) applying similar testing parameters for pH, salt concentration, and temperature *(30)*. The respective individual data sets were modeled to allow for a direct comparison.

Although the inoculum level of *S. aureus* in the present study was considerably higher than that in the 2006 data set (10^9 versus $10^{4.5}$ CFU/mL), no apparent effect of inoculum level was observed (Fig. 2); the inactivation over time strongly overlapped between the two sets of experiments over 30 days. The three separate biological *S. aureus* replicates in the present study were highly similar in their inactivation kinetics.

The third step in the validation process, again using the data from the main model, was a comparison between the present data and the 2006 data set using a separate Weibullian model (Fig. 3). The inactivation kinetics were comparable for the *L. monocytogenes* and *S. enterica,* especially during the initial 15 days, whereas after a more prolonged period faster inactivation was observed in the model based on the 2006 data set. This difference may be due to the lack of confident modeling of the later time points (after day 15). For these strains, no data were available above the 2-log detection limit in the 2006 data set. For the averaged *S. aureus* data, the initial rate of inactivation was much faster in the present model than in the model based on the 2006 data set. However, long-term rates seemed to be similar. Previous work with *L. monocytogenes (14)* suggested how the inoculum level and the combined temperature, pH, and a_w limits could have an effect on bacterial growth. Temperature was identified as the most important factor influencing the inactivation rate. In the present study, temperature also was the most important determinant of bacterial inactivation (Table 3).

Modeling of the inactivation kinetics. Data analysis was initiated at $t = 0.5$ to limit any effects of changes in cell viability caused by the change of the preculture medium to the MC-HTM directly at $t = 0$. Because the inactivation kinetics do not show continuous logarithmic inactivation over time (Fig. 2), a *D-z* model would not allow for accurate modeling of the inactivation kinetics. The Weibullian model (power model) did allow for an accurate fit to the results, a conclusion shared by other authors who have applied multiple parameters to determine bacterial inactivation kinetics *(22, 24)*.

The full data set per bacterial species was used to determine the sensitivity of each species to salt (saturation percentage), pH, and temperature. From the fitting procedure, the linear coefficients for salt, pH, and temperature were multiplied by the average of their corresponding variables in the data set, resulting in a relative contribution. These contributing values (Table 3) thus represent the relative effect of each variable on the inactivation rate.

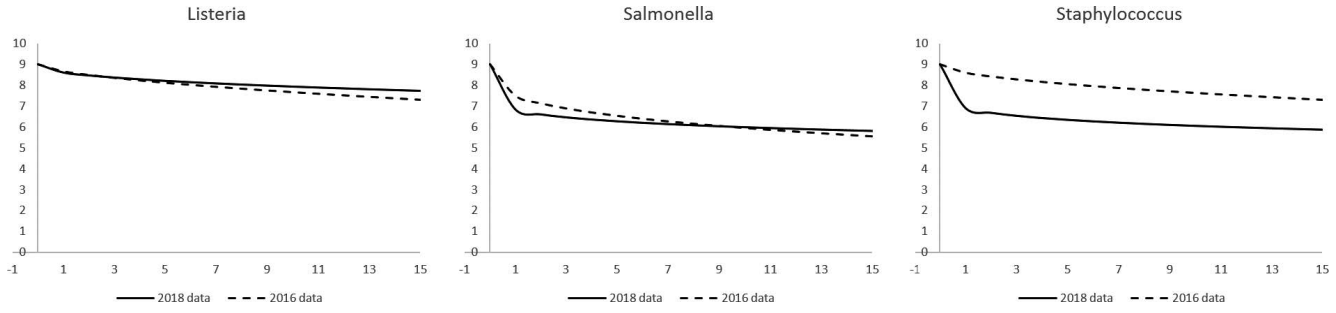

FIGURE 3. *Overview of the inactivation kinetics (Weibullian model) from the current 2018 data (solid line) compared with the data from 2006 (30) (dashed line). In all graphs, data are from experiments with 100% saturated salt concentration at 20°C and pH 7.* y *axis, log CFU/mL;* x *axis, time in days.*

TABLE 3. *Overview of the basic relative effect of the various parameters on the inactivation kinetics per bacterial strain*[a]

Species	Salt	Temp	pH
S. aureus	0.135	0.000	0.026
E. coli	0.198	0.000	0.174
L. monocytogenes	0.063	0.182	0.000
S. enterica	0.201	0.000	0.093

[a] High numbers indicate faster inactivation rates when the value of the parameter increases. The numbers are normalized to allow for a comparison among the parameters.

In general, the bacterial inactivation over time was clearly dependent on salt concentration, temperature, and pH (Table 3). For the *L. monocytogenes* strains, the current model shows limited modulation of the inactivation kinetics by salt concentration, which is in agreement with previous results with high salt concentrations *(24, 28, 30)*. The determined sensitivity of the inactivation kinetics for *S. aureus* indicated some dependence on salt concentration, in contrast to the known high osmotolerance usually displayed by gram-positive organisms *(18, 26, 30)*. However, this calculated dependence (Table 3) was possibly slightly augmented by the sharp decrease in bacterial populations over the first 2 days, whereas the overall profile of inactivation over a longer period is more in line with existing data *(30)*. *S. aureus* is very tolerant of high salt concentrations *(1, 10, 30)*; therefore, the actual salt effect is quite likely lower than that calculated.

The *E. coli* and *S. enterica* strains were relatively sensitive to pH. The higher the pH, the faster was the inactivation over time, indicating that these strains may be effectively inactivated by incubation at pH 10.0. In contrast, the *S. aureus* and *L. monocytogenes* strains had either low or no sensitivity to pH (Table 3). *L. monocytogenes* was the only species that was highly sensitive to temperature. This pathogen was inactivated more quickly at higher temperatures than at lower temperatures, as was described previously in a study on the nonthermal inactivation of *L. monocytogenes* in fermented sausages relative to temperature, pH, and a_w *(16)*. However, to fully understand the inactivation kinetics under these extreme a_w and pH conditions, additional research is needed.

The combined effects on bacterial inactivation of various salt concentrations, pH levels, and temperatures measured over time, as illustrated in the efficacy test of the MC-HTM, give clear examples of how synergistic interactions can have a greater effect than the sum of the constraints applied individually *(15, 17)*. Apart from 0% salt, the salt concentrations used in this study were 25 to 100%, which translates into an a_w of 0.96 (25% salt) to 0.79 (100% salt). Most bacteria require an a_w of >0.90 for minimal growth *(28),* with clear exceptions for *S. aureus* and *L. monocytogenes,* which are known to be halotolerant and able to grow at a relatively low a_w *(1, 10, 23, 30)*. Multiple studies have been conducted on the osmotic stress or desiccation response of specific bacteria, often combined with or relative to temperature and pH *(5, 13, 16, 18, 19, 27)*. Although these studies have provided detailed descriptions of the various coping mechanisms of bacteria, the explanatory power in the present study is limited because the a_w values are much lower than those previously described. Other authors have suggested that bacterial viability studies with reduced a_w values are greatly affected by the composition of the foods tested *(21)*. Therefore, only the original 2006 study exposing different bacteria to low a_w conditions in a model for the preservation of casings *(30)* could serve as a true reference point for any data generated in the present study.

Three steps were taken to validate the newly developed MC-HTM, based on the reproducibility of the results using the model itself and by comparing the generated data sets to applicable references. Overall, the newly developed MC-HTM can serve as a validated model for studying the inactivation over time of various bacteria relevant to the preservation of natural sausage casings. For practical application, the inactivation kinetics defined in this study could serve as a prediction model for the required salting period for casings. The actual bacterial contamination of the product can vary with the respective production step during processing from animal intestine into sausage casings (initial level, $\sim 10^5$ CFU/g; level after salting, $<10^2$ CFU/g). Subsequent selection and grading of these casings will require complete removal of all salt, and upon completion of this production step, the casings will need to be resalted. By determining the actual level of contamination before the salting process, the minimum storage period in salt can be calculated and potentially optimized by variation of the pH and temperature. As a result, a standard holding period of at least 30 days may no longer be necessary to produce salted natural casings to comply with validated quality and food safety criteria.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/0362-028X.JFP-19-197.s1

REFERENCES

1. Abee, T., and J. A. Wouters. 1999. Microbial stress response in minimal processing. *Int. J. Food Microbiol*. 50:65–91.
2. Bahrndorff, S., T. Alemu, T. Alemneh, and J. L. Nielsen, 2016. The microbiome of animals: implications for conservation biology. *Int. J. Genom*. 2016:5304028.
3. Bakker, W. A. M., J. H. Houben, P. A. Koolmees, U. Bindrich, and L. Sprehe. 1999. Effect of initial mild curing, with additives, of hog and sheep sausage casings on their microbial quality and mechanical properties after storage at difference temperatures. *Meat Sci*. 51:163–174.
4. Boekel, M. A. J. S. 2008. Kinetic modeling of reactions in foods. CRC Press, Boca Raton, FL.
5. Burgess, C. M., A. Gianotti, N. Gruzdev, J. Holah, S. Knøchel, A. Lehner, E. Margas, S. Schmitz Esser, S. Sela (Saldinger), and O. Tresse. 2016. The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. *Int. J. Food Microbiol*. 221:37–53.

6. Chawla, S. P., R. Chander, and A. Sharma. 2006. Safe and shelf-stable natural casing using hurdle technology. *Food Control* 17:127–131.
7. European Natural Sausage Casings Association. 2018. Community guide to good practice for hygiene and the application of the HACCP principles in the production of natural sausage casings. Available at: http://www.ensca.eu/index.php?/eng/DOWNLOADS. Accessed 9 March 2019.
8. Franco-Vega, A., N. Ramírez-Corona, A. López-Malo, and E. Palou. 2015. Estimation of *Listeria monocytogenes* survival during thermoultrasonic treatments in non-isothermal conditions: effect of ultrasound on temperature and survival profiles. *Food Microbiol*. 52:124–130.
9. Gabis, D. A., and J. H. Silliker. 1974. *Salmonella* in natural animal casings. *Appl. Microbiol*. 27:66–71.
10. Gutierrez, C., T. Abee, and I. R. Booth. 1995. Physiology of the osmotic stress response in microorganisms. *Int. J. Food Microbiol*. 28:233–244.
11. Houben, J. H. 2005. A survey of dry-salted natural casings for the presence of *Salmonella* spp., *Listeria monocytogenes* and sulphite-reducing *Clostridium* spores. *Food Microbiol*. 22:221–225.
12. Koolmees, P. A., M. H. G. Tersteeg, G. Keizer, J. van den Broek, and R. Bradley. 2004. Comparative histological studies of mechanically versus manually processed sheep intestines used to make natural sausage casings. *J. Food Prot*. 67:2747–2755.
13. Koutsoumanis, K. 2008. A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations. *Int. J. Food Microbiol*. 128:116–121.
14. Koutsoumanis, K. P., and J. N. Sofos. 2005. Effect of inoculum size on the combined temperature, pH and a_w limits for growth of *Listeria monocytogenes*. *Int. J. Food Microbiol*. 104:83–91.
15. Leistner, L., and L. G. M. Gorris. 1995. Food preservation by hurdle technology. *Trends Food Sci. Technol*. 6:41–46.
16. Mataragas, M., K. Rantsiou, V. Alessandria, and L. Cocolin. 2015. Estimating the non-thermal inactivation of *Listeria monocytogenes* in fermented sausages relative to temperature, pH and water activity. *Meat Sci*. 100:171–178.
17. McMeekin, T. A., K. Presser, D. Ratkowsky, T. Ross, M. Salter, and S. Tienungoon. 2000. Quantifying the hurdle concept by modelling the bacterial growth/no growth interface. *Int. J. Food Microbiol*. 55:93–98.
18. Mellefont, L. A., T. A. McMeekin, and T. Ross. 2003. The effect of abrupt osmotic shifts on the lag phase of foodborne bacteria. *Int. J. Food Microbiol*. 83:281–293.
19. Nolan, D. A., D. C. Chamblin, and J. A. Troller. 1992. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. *Int. J. Food Microbiol*. 16:323–335.
20. Ockerman, H. W., and C. L. Hansen. 2000. Sausage containers, p. 285–323. *In* Animal by-product processing and utilization. CRC Press, Boca Raton, FL.
21. Park, C. M., and L. R. Beuchat. 2000. Survival of *Escherichia coli* O157:H7 in potato starch as affected by water activity, pH and temperature. *Lett. Appl. Microbiol*. 31:364–367.
22. Raffellini, S., M. Schenk, S. Guerrero, and S. M. Alzamora. 2011. Kinetics of *Escherichia coli* inactivation employing hydrogen peroxide at varying temperatures, pH and concentrations. *Food Control* 22:920–932.
23. Roberts, M. F. 2005. Organic compatible solutes of halotolerant and halophilic microorganisms. *Saline Systems* 1. https://doi.org/10.1186/1746-1448-1-5.
24. Rodríguez-González, O., M. Walkling-Ribeiro, S. Jayaram, and M. W. Griffiths. 2011. Cross-protective effects of temperature, pH, and osmotic and starvation stresses in *Escherichia coli* O157:H7 subjected to pulsed electric fields in milk. *Int. Dairy J*. 21:953–962.
25. Savic, Z., and I. Savic. 2016. Sausage casings, 2nd ed. Victus, Vienna.
26. Sperber, W. H. 1983. Influence of water activity on foodborne bacteria—a review. *J. Food Prot*. 46:142–150.
27. Tsai, M., R. Ohniwa, Y. Kato, S. L. Takeshita, T. Ohta, S. Saito, H. Hayashi, and K. Morikawa. 2011. *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiol*. 11:13.
28. Uzelac, G., and B.Stille.1977. Survival of bacteria of faecal origin in dry foods, in relation to water activity. *Dtsch. Lebensm. Rundsch*. 73:325–329.
29. Wijnker, J. J. 2009. Aspects of quality assurance in processing natural sausage casings. Ph.D. dissertation. Utrecht University, Utrecht, The Netherlands. Available at: https://dspace.library.uu.nl/handle/1874/31822. Accessed 6 March 2019.
30. Wijnker, J. J., G. Koop, and L. J. A. Lipman. 2006. Antimicrobial properties of salt (NaCl) used for the preservation of natural casings. *Food Microbiol*. 23:657–662.
31. Wijnker, J. J., E. A. W. S. Weerts, E. J. Breukink. J. H. Houben, and L. J. A. Lipman. 2011. Reduction of *Clostridium sporogenes* spore outgrowth in natural sausage casings using nisin. *Food Microbiol*. 28:974–979.