Quantification of persistence of the food-borne pathogens Listeria monocytogenes and Salmonella enterica during manufacture of Italian fermented sausages

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Quantification of persistence of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* during manufacture of Italian fermented sausages

Mataragas M.[1,2], Bellio A.[3], Rovetto F.[1], Astegiano S.[3], Greci C.[4], Hertel C.[5], Decastelli L.[3], Cocolin L.[1*]

[1] Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Via Leonardo da Vinci 44, Grugliasco, 10095 Turin, Italy

[2] Agriculture University of Athens, Department of Food Science and Technology, Laboratory of Food Quality Control and Hygiene, Iera Odos 75, 11855, Athens, Greece

[3] Istituto Zooprofilattico Sperimentale del Pimonte, Liguria e Valle d'Aosta. - S.C. Controllo Alimenti e Igiene delle Produzioni

[4] Frutarom Italy S.R.L., Via Cicerone 2/4, 43100 Parma, Italy

[5] Frutarom Savory Solutions GmbH, Dinklager Str. 8F, 49451 Holdorf, Germany

[*] Corresponding author: L. Cocolin, Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Via Leonardo da Vinci 44, Grugliasco, 10095 Turin, Italy, phone: +39-011-6708553, fax: +39-011-6708549, e-mail: lucasimone.cocolin@unito.it

Running title: *L. monocytogenes* and *Salmonella* spp. survival in Italian salami

Abstract

The objective was to evaluate the survival capability and quantify the persistence of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* in the Cacciatore, Felino and Milano type salami preparation. The batter of each sausage was inoculated with a five-strain cocktail of *L. monocytogenes* or *S. enterica* (*ca*. 10^4-10^5 CFU/g) and their progression was monitored at specific time intervals during preparation of the sausages. Four different batches were prepared at different times for each sausage/pathogen combination. Different models were used to fit the experimental data and to calculate the kinetic parameters. The best model was chosen based on statistical comparisons. *S. enterica* proved to be more sensitive susceptible to fermentation and ripening processes than *L. monocytogenes*. Both pathogens, however, survived relatively well as the result of the conditions (pH, a_w and fermentation temperature) prevailing during fermentation and ripening of the sausages. Water activity proved to be a key factor in the survival of the microorganisms. The statistical analysis of quantitative data gathered from challenge tests is useful for the food business operators as it can provide practical information on the process parameters combinations that could lead to a better control of the pathogens.

Keywords: Fermentation, food safety, modeling, nonthermal inactivation, risk-based control

1. Introduction

In fermented sausages a sequence of hurdles leads to stable and safe products (Leistner, 2000). The only killing steps interfering during their manufacture are the fermentation and ripening processes during which lowering of pH and water activity (a_w) is achieved. Although the general accepted stability of these products, food-borne illnesses due to consumption of contaminated with food-borne pathogens fermented foods have occurred (Adams & Mitchell, 2002). Main reasons could be failure in fermentation and ripening, high initial contamination of the raw material such as meat, secondary contamination or combination of all.

Listeriosis accounted for 1,642 reported cases in 2012 within the EU, estimated from all the food products; 10.5% more than in 2011 and has been gradually increasing over the past five years (EFSA, 2014). The incidence of listeriosis is particularly high in elderly people, aged over 74 years. Other risk groups include pregnant women and persons with weakened immune systems. *Listeria monocytogenes*, the bacterium that causes listeriosis in humans and animals, was mostly found in ready-to-eat fish and meat products (EFSA, 2014). Over the years, salmonellosis has been decreasing, with 91,034 reported cases in 2012 within the EU, irrespective the causative food product. However, evidence shows that any *Salmonella* serovar can cause human illness, which requires continued surveillance and vigilance (EFSA, 2014). *L. monocytogenes* and *Salmonella* ssp. have been detected in final products of fermented sausages with concentrations exceeding in some cases the legal safety limit for ready-to-eat foods and therefore can potentially cause foodborne illness (De Cesare et al., 2007; Martin et al., 2011; Thevenot et al., 2005; USDA, 2003).

Modeling may provide the required information on the changes in pathogen counts in fermented sausages during the manufacturing process. The application, however, of

modeling in such foods is more difficult compared to other because pH and a_w are not constant and are not attained at once in fermented sausages. Therefore, challenge tests with pathogens inoculated into the batter of the sausages will provide more reliable data by determining their fate throughout the actual manufacturing process (Adams & Mitchell, 2002).

Therefore, the objective of this study was to evaluate the survival capability and quantify the persistence of the food-borne pathogens *L. monocytogenes* and *S. enterica* in three fermented sausages of Italian origin with different maturation times, i.e. Cacciatore (20 days), Felino and Milano (40 days each) type salami. An understanding of the fate of a pathogen during the production process is important for both, Food Business Operators (FBOs) and evaluators of the microbial risk in foods (risk assessment studies), as it can provide practical information on the process parameters combinations that could lead to a better control of the pathogens.

2. Materials and methods

2.1. Sausage preparation and inoculation of pathogens

Meat batter for the production of Cacciatore, Felino and Milano type salami were prepared at the premises of Fructarom Italy (Parma, Italy) using industrial equipment for sausage preparation, and transported, at refrigerated temperature, to the Istituto Zooprofilattico for the pathogen contamination, stuffing and maturation. The transportation in all cases lasted less than 3 hours. Starter cultures BITEC STARTER R4, B1 and R3 (Frutarom Savory Solutions GmbH, Korntal-Münchingen), were used to inoculate Cacciatore, Felino and Milano type salami. The cultures consist of strains of species *Lactobacillus sakei* and *Staphylococcus carnosus* (R4 and R3) as well as of *L. sakei*, *S. carnosus* and *Kocuria salsicia* (B1). The *L. sakei* strain of starter culture

B1 produces a bacteriocin, active against *Listeria monocytogenes*. The standard recipes shown in Table 1 were followed. A cocktail made up of five strains of *L. monocytogenes* (the strain #5 isolated from minced beef meat, 4b; the strain #19 isolated from fresh salami, 1/2b; the strain #36 isolated from pork meat, 1/2a; the reference strain EGDe, 1/2a; and the human clinical isolate V7, not serotyped) or *S. enterica* (the strain 22754 isolated from pig feces, *S.* Derby; the strain 56596 isolated from pig feces, *S.* Typhimurium; the strain 57002 isolated from minced pork meat, *S.* Typhimurium; the strain 54398 isolated from packaged meat, *S.* Typhimurium; and the reference strain ATCC 14028, *S.* Typhimurium) (Mataragas et al., 2014) was introduced in the batter of the sausages. All strains were resuscitated twice in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, UK) (1% inoculum) and then incubated at 37°C for 24 h up to a final concentration *ca*. 10^9 CFU/ml.

The inoculation procedure is described in Mataragas et al. (2014). Briefly, the cells were harvested (13,400 ×*g* for 15 min at 4°C) by centrifugation (Eppendorf 5417R refrigerated centrifuge, Eppendorf, Milan, Italy), washed twice, and re-suspended with sterile Ringer solution (Oxoid) to give a similar final concentration. Bacterial suspensions were gradually distributed into the batter during mixing to achieve a uniform distribution of the inoculum and a final concentration *ca*. 10^4 to 10^5 CFU/g. After preparation, the mixtures were stuffed into casings (diameters of 46 mm, 60 mm and 100 mm for Cacciatore, Felino and Milano type salami, respectively) and inoculated with the mold culture BITEC MOLD SK 30 containing *Penicillium nalgiovensis*. Then, sausages were ripened following the fermentation and drying program displayed in Table 2. During ripening the mold grew superficially until the whole surface of the sausages was covered. For each food-borne pathogen and fermented sausage, two different batches were prepared at different time period (2

independent trials × 2 food-borne pathogens × 3 fermented sausages = 12 batches in total). Two replicates were collected from each batch (12 batches × 2 replicates per sampling day = 24 samples per time point in total) on days 0, 2, 5, 10 and 20 (Cacciatore), and 0, 3, 7, 10, 20 and 40 (Felino and Milano) after formulation. The samples were transported to the laboratory under refrigeration conditions (4^{o}C), and subjected to microbiological and physicochemical analyses within 24 h.

2.2. Microbiological analysis

The same procedure described in Mataragas et al. (2014) was also followed here. A unit of 10 g from the sausage was aseptically weighted and placed into a sterile stomacher bag with 90 ml of sterile Ringer solution. The sample was homogenized in a stomacher (BagMixer, Interscience, France) for 2 min at normal speed at room temperature. Serial decimal dilutions in sterile Ringer solution were prepared from this 10^{-1} dilution, and samples of 1 or 0.1 ml from three appropriate solutions were poured in or spread on selective agar plates in duplicate. Lactic acid bacteria (LAB) were enumerated in de Man, Rogosa and Sharpe agar (MRS agar, LABM, Heywood, Lancashire, UK), overlayed with 5 ml of the same medium and incubated at 30^{o}C for 72 h; Gram-positive/coagulase-negative cocci (CNC) on mannitol salt agar (MSA agar, LABM), incubated at 30^{o}C for 72 h; *L. monocytogenes* on Agar Listeria acc. to Ottaviani and Agosti (ALOA, Oxoid), incubated at 37^{o}C for 24-48 h; and *S. enterica* on Xylose Lysine Deoxycholate agar (XLD agar, Oxoid), incubated at 37^{o}C for 24-48 h.

Enrichment was also performed in parallel with enumeration to confirm the presence of the pathogens if their concentration was below the detection limit (10^{2} CFU/g). Primary enrichment for the detection of *L. monocytogenes* was carried out by

suspending 10 g of sample in 90 ml of half-concentrated Fraser broth (Oxoid) followed by incubation at 30°C for 24 h. Then, 0.1 ml of the primary culture were transferred in 10 ml of Fraser broth, incubated at 37°C for 48 h, 0.1 ml of the secondary enrichment were streaked on ALOA and OXFORD (Oxoid) agars and incubated at 37°C for 24-48 h. The plates were examined visually for typical colony characteristics associated with each growth medium. For *S. enterica*, pre-enrichment was performed by suspending 10 g of sample in 90 ml of buffered peptone water (BPW, Oxoid) followed by incubation at 37°C for 16-20 h. Selective enrichment was done by transferring 0.1 ml of pre-enrichment culture in 10 ml of Rappaport-Vassiliadis Soya broth (RVS broth, Oxoid) followed by incubation at 42°C for 24 h. After incubation samples were streaked on XLD and modified Brilliant-green Phenol-red Lactose Sucrose (mBPLS agar, LABM) agars and incubated at 37°C for 24-48 h. The selectivity of all growth media was checked with rapid tests (e.g., Gram, catalase reaction or motility) for about 10% of the countable colonies (Harrigan and McCance, 1976).

2.3. Physicochemical analysis

The pH was measured by immersing the pH probe of a digital pH meter (micropH 2001, Crison, Barcelona, Spain) in a diluted and homogenized sample containing 10 g of sausage and 90 ml of distilled water. Water activity (a_w) was measured with a calibrated electric hygrometer (HygroLab, Rotronic, Bassersdorf, Switzerland) according to the manufacturer's instructions.

2.4. Description of the nonthermal inactivation of L. monocytogenes and S. enterica

Various models describing the inactivation trend of both pathogens were used (Bigelow & Esty, 1920; Cerf, 1977; Mafart et al., 2002; Geeraerd et al., 2000; Geeraerd et al., 2005). The log-linear model is

$$N_t = N_0 \times e^{-k_{max} \times t} \qquad (1)$$

where N_t is the cell counts ($\log_{10}$ CFU/g) at time t; N_0 is the initial population ($\log_{10}$ CFU/g); t, the time (days); and k_{max}, the specific inactivation rate (per day) of each pathogen.

The Weibull model is

$$\frac{N_t}{N_0} = 10^{-\left(\frac{t}{\delta}\right)^p} \qquad (2)$$

where δ is the time for the first log-unit decrease (days); and p is a shape parameter. When p is below, above or equal to 1 indicates a downward or upward concave curve, or a straight line equal to the log-linear model, respectively.

The biphasic model is

$$N_t = N_0 + log_{10}\{(f \times e^{-k_{max\ 1} \times t}) + [(1-f) \times e^{-k_{max\ 2} \times t}]\} \qquad (3)$$

where f is the fraction of initial population in the major population, 1 - f is the fraction of population in the subpopulation, and k_{max1} and k_{max2} are the inactivation rates of the major population and subpopulation, respectively (per day).

The log-linear with tail model is

$$N_t = [(N_0 - N_{res}) \times e^{-k_{max} \times t}] + N_{res} \qquad (4)$$

where N_{res} is the resistant population (tail) ($\log_{10}$ CFU/g).

All inactivation models were fitted to the experimental data by nonlinear regression using the Microsoft Excel 2007 (Microsoft, Redmond, WA, USA) add-in GInaFiT

v1.6 (Geeraerd et al., 2005) and the software GraphPad Prism 5.04 (GraphPad Software, Inc., San Diego, CA, USA).

Various statistical indices were employed to identify the best model describing adequately the inactivation data (Zwietering et al., 1994 Ross, 1996; te Giffel & Zwietering, 1999; den Besten et al., 2006; Motulsky, 2007): number of model parameters, Lack-of-Fit (*LoF*) based on the *F*-test, accuracy factor (A_f), normality of residuals, Root Mean Square Error (*RMSE*), replicates tests, number of outliers, dependency between model parameters and coefficient of multiple determination (R^2). Replicates test shows if the data are too far from the fitted curve compared to the scatter among replicates. Dependency is reported for each parameter and quantifies the degree to which that parameter is intertwined with others. If it is high (> 0.90-0.95) then the same curve over the range of X values, for which data have been collected, can be constructed with multiple sets of parameter values. This means that the data do not define all the parameters in the model.

A one-way analysis of variance (ANOVA) comparing the calculated parameters of interest among the different experimental trials within each pathogen was performed using the SPSS v15.0 program (SPSS, Inc., Chicago, Ill., USA) to determine whether there was a significant influence of these parameters (composition and type of fermented sausage) on *L. monocytogenes* and *S. enterica* inactivation.

3. Results and Discussion

The LAB counts increased rapidly during fermentation in all sausages (increase of 2.3-2.5 $\log_{10}$ CFU/g in Cacciatore, 2.0-2.1 $\log_{10}$ CFU/g in Felino and 1.3-1.5 $\log_{10}$ CFU/g in Milano), dominating the micro-flora of the products (*ca*. 8.0 to 9.0 $\log_{10}$ CFU/g) within the first 48-72 h of fermentation (Fig. 1). During the same period,

Staphylococcus/*Micrococcus* counts displayed in general no change (Felino) or a slight decrease (Cacciatore and Milano) with a final population approximately at 6.5-6.7 $\log_{10}$ CFU/g (Felino) and 5.6-5.7 $\log_{10}$ CFU/g (Cacciatore and Milano) (Fig. 1).

The pH decreased during fermentation and displayed a slight increase during ripening in all sausages (Fig. 2). Drop by *ca*. 0.80-0.95 units in the pH value coincided with the increase in LAB counts. Felino product displayed the slowest (*ca*. 0.11 units/day) and least (from 5.65 to 4.85 on the day 7 of fermentation) pH decrease during fermentation. In contrast, the Cacciatore product had the fastest (*ca*. 0.43 units/day) pH decrease whereas the Milano product had the most dramatic (from 5.85 to 4.90 on the day 3 of fermentation). The pH in the Cacciatore went from 5.85 to 5.00 on the day 2 of fermentation while the pH decrease rate in the Milano was *ca*. 0.32 units/day.

The a_w decreased slowly (0.001-0.002 units/day) in all products from its initial value of *ca*. 0.970-0.980 (Fig. 3). The extent of reduction was small (decrease by 0.05-0.06 units in Cacciatore, 0.04 units in Felino and 0.03 units in Milano), achieving a final mean value not lower than 0.930-0.940. The same microbiological and physicochemical pattern was observed for the batches of Cacciatore, Felino and Milano inoculated with *S. enterica*. A similar trend relative to physicochemical and microbiological properties of the products was observed in a previous study of the authors (Mataragas et al., 2014) with the Cacciatore and Felino sausages irrespective whether they were inoculated with *L. monocytogenes* or *S. enterica*.

The log-linear, Weibull, biphasic and log-linear with tail models were fitted to the experimental data of *L. monocytogenes* and *S. enterica* inactivation. Based on a statistical comparison of the models (Tables 3 and 4), the log-linear with tail and Weibull models were chosen to estimate the kinetic parameters (Table 5) of the nonthermal inactivation of *L. monocytogenes* and *S. enterica*, respectively. These

models fitted the data better than did the other models in most of the cases. In total, three experimental trials were examined for each pathogen, and the log-linear with tail and Weibull models were accepted in all cases (100%) for *L. monocytogenes* and *S. enterica*, respectively. For *L. monocytogenes*, the log-linear and biphasic models were excluded because in one experimental trial (Cacciatore) the fit of the first model, based on the *LoF*, was not accepted whereas the fit of the second model was ambiguous in two cases (Cacciatore and Milano). The other two models were in general accepted. Only Weibull showed non-normal distribution of the residuals in one occasion (Felino). Therefore, the log-linear with tail model was considered further. The same reasoning was followed for *S. enterica*. The biphasic model was rejected because of its ambiguous fit in most of the cases (Felino and Milano). The log-linear was also excluded since the distribution of residuals was not normal in one case (Felino), displaying two data points as potential outliers. The log-linear with tail model was not accepted (ambiguous fit) in one trial (Felino) and thus the Weibull model was finally selected.

The inactivation of both pathogens in the fermented sausages was nonlinear (Figs 3 and 4). *L. monocytogenes* survived better than *S. enterica* during production of the sausages as reflected by the total reduction ($\log_{10}$ CFU/g). Gram-negative bacteria like *Salmonella* spp. are more susceptible to fermentation and ripening of the raw sausages compared to Gram-positive bacteria (Adams and Nicolaides, 1997; Ockerman and Basu, 2007). Within the production period of Cacciatore (20 days), Felino (40 days) and Milano (40 days), *S. enterica* may decrease by 1 log or more, but *L. monocytogenes* never reached 1 log cycle reduction. This is in accordance with the results of another study in which *L. monocytogenes* and *S. enterica* exhibited a similar ability of survival in Cacciatore and Felino (Mataragas et al., 2014). In that work, total

inactivation of *L. monocytogenes* at the end of the production process of Cacciatore and Felino was much lower than 1 log (*ca*. 0.4-0.5 $\log_{10}$ CFU/g). On the other hand, total inactivation of *S. enterica* achieved in Cacciatore and Felino was 1.1 and 1.6 $\log_{10}$ CFU/g, respectively.

The inactivation of *L. monocytogenes* was quicker in the short-maturated fermented sausage (Cacciatore, rapid fermentation) compared to the long-maturated fermented sausages (Felino and Milano, slow fermentation) as reflected by the different inactivation rates (Table 5). Most probably the rapid decrease of pH in Cacciatore, as it can be seen from the reduction rate of pH, caused the quicker inactivation of the pathogen, probably due to the fact that they were longer exposed to low pH especially at the beginning of fermentation when the temperature was high. The rapid decrease of pH, however, should be probably combined with elevated temperatures (i.e., 21-22^{o}C) at least the first 48-72h of fermentation to achieve higher *L. monocytogenes* inactivation. This, and the slow rate of moisture loss, could be the reasons of observing no differences in the total inactivation of *L. monocytogenes* between the fermented sausages despite the differences in the inactivation rates (Table 5). During fermentation of the products, the temperature was mainly below or at 20^{o}C. At elevated fermentation temperatures (>20^{o}C), the crucial factor for *L. monocytogenes* inhibition is the pH decrease while at lower temperatures the significant factor is a_w (Gounadaki et al., 2005). Similar results were obtained in the study with Cacciatore and Felino (Mataragas et al., 2014). In the current study, *L. monocytogenes* inactivation exhibited a tail implying the presence of a more resistant subpopulation (N_{res}). The explanation for the existence of a resistant subpopulation is summarized in the vitalistic or mechanistic theory (Cerf, 1977).

S. enterica displayed differences in the total inactivation between fermented sausages, but not in the time needed for the first decimal reduction of the pathogen (Table 5). Overall reduction was higher in the long-maturated fermented sausages (Milano and Felino) relative to the short-maturated fermented sausage (Cacciatore). Probably the a_w was the important factor for this higher inactivation of *S. enterica* in Felino and Milano (long maturation period). The rate of a_w reduction, however, was very slow and subsequently in order to achieve greater total inactivation higher dehydration rates are required. As already mentioned earlier, at fermentation temperatures $\leq 20^oC$, like those mostly applied during production of Cacciatore, Felino and Milano, a_w become more important than pH decrease (Gounadaki et al., 2005). Total inactivation of *S. enterica* was also higher in Felino compared to Cacciatore in the study of Mataragas et al. (2014), but the total inactivation was below 2 logs. The authors found by multiple regression that a_w played a key role in the *S. enterica* inactivation. In another study, *Salmonella* spp. was inactivated by 3 logs in an Australian-style fermented sausage. The product characteristics were similar to Cacciatore, i.e., relatively narrow and slow a_w reduction, rapid reduction of pH during fermentation and short maturation time. The main difference, however, was the applied fermentation temperature, i.e., 27^oC for 2 days (Shay, 1993).

Another evidence that supports the role of a_w in the inactivation of both pathogens is relative to the critical control points (CCPs) of the production process of dry fermented sausages for controlling the foodborne pathogens *L. monocytogenes*, *S. enterica* and *Staphylococcus aureus* as published by Lucke (2000) (Table 6). The a_w of Cacciatore, Felino and Milano was above the recommended limits during comminution and mixing, and at the end of the process allowing better survival of *L. monocytogenes* and *S. enterica*. Hence, challenge tests and quantification of the

inactivation data provide critical information on the factors influencing the survival of foodborne pathogens during a process. By making available such evidences, support for corrective actions is provided leading to better control of these microorganisms.

4. Conclusions

L. monocytogenes and *S. enterica* responded differently to the environmental changes during production of Cacciatore, Felino and Milano sausages. *S. enterica* proved to be more susceptible to fermentation and ripening processes than *L. monocytogenes*. The extent of inactivation of both pathogens, however, can be considered rather small since it was rarely higher than 1.0-1.5 log. Only in Felino the inactivation of *S. enterica* was above 2 log. The observed small reduction of *L. monocytogenes* and *S. enterica* in the Italian sausages was mainly attributed to the slow dehydration of the sausages and the small reduction of a_w in combination with the fermentation temperatures (mainly <20^{o}C), especially during the first 48-72h of fermentation. The environmental conditions prevailing in the first 48h are critical with regard to the growth and subsequent survival rate of these pathogens. Although, decline of pH was relatively rapid, the pH contribution to *L. monocytogenes* and *S. enterica* inactivation was only marginal. Fermentation temperatures above 20^{o}C are needed at least the first 48-72h of fermentation, in order for the pH to become an important contributing factor to the inactivation of both pathogens. For such short maturation times (Cacciatore) and/or slow a_w reduction (Cacciatore, Felino and Milano), pH is a critical parameter for controlling the pathogens in the products. The results of this work indicated that both pathogens were able to survive relatively well at the conditions prevailing during manufacture of Cacciatore, Felino and Milano. Therefore, it should be noted the importance of using raw materials contaminated by *L. monocytogenes*

and *S. enterica*, during manufacture of Cacciatore, Felino and Milano, as low as possible taking also into consideration potential cross-contamination of the products during its distribution, handling and/or before consumption.

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References

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control, 8,* 227-239.

Adams, M., & Mitchell, R. (2002). Fermentation and pathogen control: a risk assessment approach. *International Journal of Food Microbiology, 79,* 75-83.

Bigelow, W. D., & Esty, J. R. (1920). The thermal death point in relation to typical thermophylic organisms. *Journal of Infectious Diseases,* 27, 602-617.

Cerf, O. (1977). Tailing of survival curves of bacterial spores. *Journal of Applied Bacteriology, 42,* 1-19.

De Cesare, A., Mioni, R., & Manfreda, G. (2007). Prevalence of *Listeria monocytogenes* in fresh and fermented Italian sausages and ribotyping of contaminating strains. *International Journal of Food Microbiology, 120,* 124-130.

den Besten, H. M. W., Mataragas, M., Moezelaar, R., Abee, T., & Zwietering, M. H. (2006). Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. *Applied and Environmental Microbiology, 72,* 5884-5894.

EFSA (2014). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. *The EFSA Journal, 12,* 3547.

Geeraerd, A. H., Herremans, C. H., & Van Impe, J. F. (2000). Structural model requirements to describe microbial inactivation during a mild heat treatment. *International Journal of Food Microbiology, 59,* 185-209.

Geeraerd, A. H., Valdramidis, V. P., & Van Impe, J. F. (2005). GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *International Journal of Food Microbiology, 102,* 95-105.

Gounadaki, A., Skandamis, P., Drosinos, E. H., & Nychas, G. J. E. (2005). Survival of *Listeria monocytogenes* during the fermentation and ripening process of sausages, p. 436-441. In Proceedings of the 1st Hellenic Symposium in Food Biotechnology and Technology. Association of Greek Chemists and Hellenic Association of Chemical Engineers, Athens, Greece.

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology, 55,* 181-186.

Lucke, F.-K. (2000). Quality and safety issues in fermented meat products. Lecture presented at the Joint Meeting of the Society of Applied Microbiology (UK) and the Estonian Society for Microbiology on "Microbiological Safety of Food", Tartu, Estonia.

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology, 72,* 107-113.

Martin, B., Garriga, M., & Aymerich, T. (2011). Prevalence of *Salmonella* spp. and *Listeria monocytogenes* at small-scale Spanish factories producing traditional fermented sausages. *Journal of Food Protection, 74,* 812-815.

Mataragas, M., Bellio, A., Rovetto, F., Astegiano, S., Decastelli, L., & Cocolin, L. (2014). Risk-based control of food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* in the Italian fermented sausages Cacciatore and Felino. *Meat Science* (submitted).

Motulsky, H.J. (2007). Prism 5 Statistics Guide. GraphPad Software Inc., San Diego, CA. URL: www.graphpad.com.

Ockerman, H. W., & Basu, L. (2007). Production and consumption of fermented meat products. In Handbook of Fermented Meat and Poultry, F. Toldra (ed.), pp. 9-16. Blackwell Publishing Ltd, Oxford, UK.

Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology, 81,* 501-508.

Shay, B. J. (1993). Factors affecting growth and survival of *Salmonella* in fermented salami manufactured under Australian conditions. Australian Veterinarians in Public

Health and Australian Veterinarians in Industry, Proceedings of the Gold Coast Scientific Program, pp. 23-32.

te Giffel, M. C., & Zwietering, M. H. (1999). Validation of predictive models describing the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology, 46,* 135-149.

Thevenot, D., Delignette-Muller, M. L., Christieans, S., & Vernozy-Rozand, C. (2005). Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. *International Journal of Food Microbiology, 102,* 85-94.

U.S. Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS), & Food and Drug Administration (FDA) (2003). Quantitative assessment of the relative risk to public health from food-borne *Listeria monocytogenes* among selected categories of ready-to-eat foods. USDA, Washington, D.C.

Zwietering, M. H., Cuppers, H. G., de Wit, J. C., and van't Riet, K. (1994). Evaluation of data transformations and validation of a model for the effect of temperature on bacterial growth. *Applied and Environmental Microbiology, 60,* 195-203.

Figure legends

Fig. 1. Changes in lactic acid bacteria (LAB) (closed circles) and *Staphylococcus*/*Micrococcus* (closed squares) counts during production of the a) Cacciatore, b) Felino and c) Milano sausages inoculated with *L. monocytogenes*. The data points are the mean values (n = 4) and the bars (if visible) represent the standard deviation (SD) of each mean value.

Fig. 2. Changes in pH (closed circles) and a_w (closed squares) during production of the a) Cacciatore, b) Felino and c) Milano sausages inoculated with *L. monocytogenes*. The data points are the mean values (n = 4) and the bars (if visible) represent the standard deviation (SD) of each mean value.

Fig. 3. Nonthermal inactivation of *L. monocytogenes* in a) Cacciatore, b) Felino and c) Milano as described by the log-linear with tail model (solid line). Solid circles are the observed data.

Fig. 4. Nonthermal inactivation of *S. enterica* in a) Cacciatore, b) Felino and c) Milano as described by the Weibull model (solid line). Solid circles are the observed data.

Fig. 1

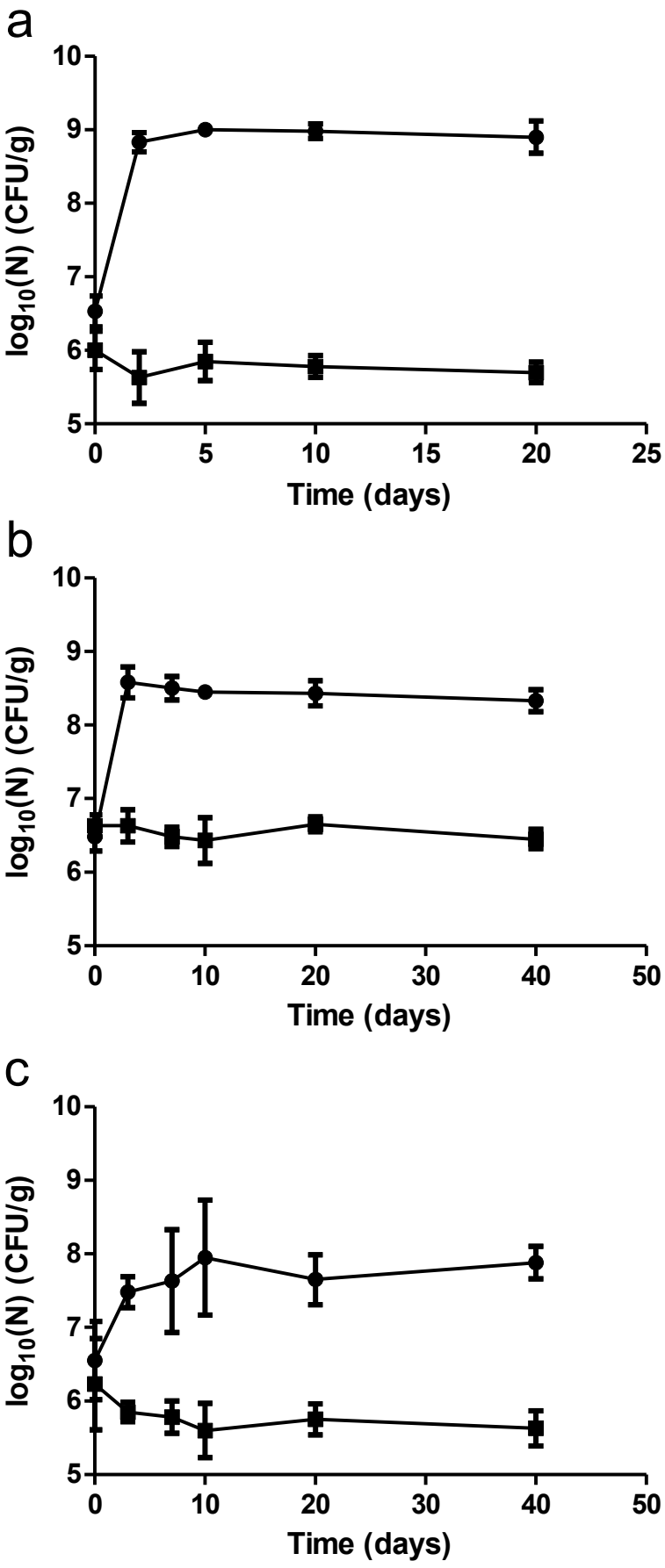
a
10
9
8
7
6
5
log10(N) (CFU/g)
0
5
10
15
20
25
Time (days)
b
10
9
8
7
6
5
log10(N) (CFU/g)
0
10
20
30
40
50
Time (days)
c
10
9
8
7
6
5
log10(N) (CFU/g)
0
10
20
30
40
50
Time (days)

Fig. 2

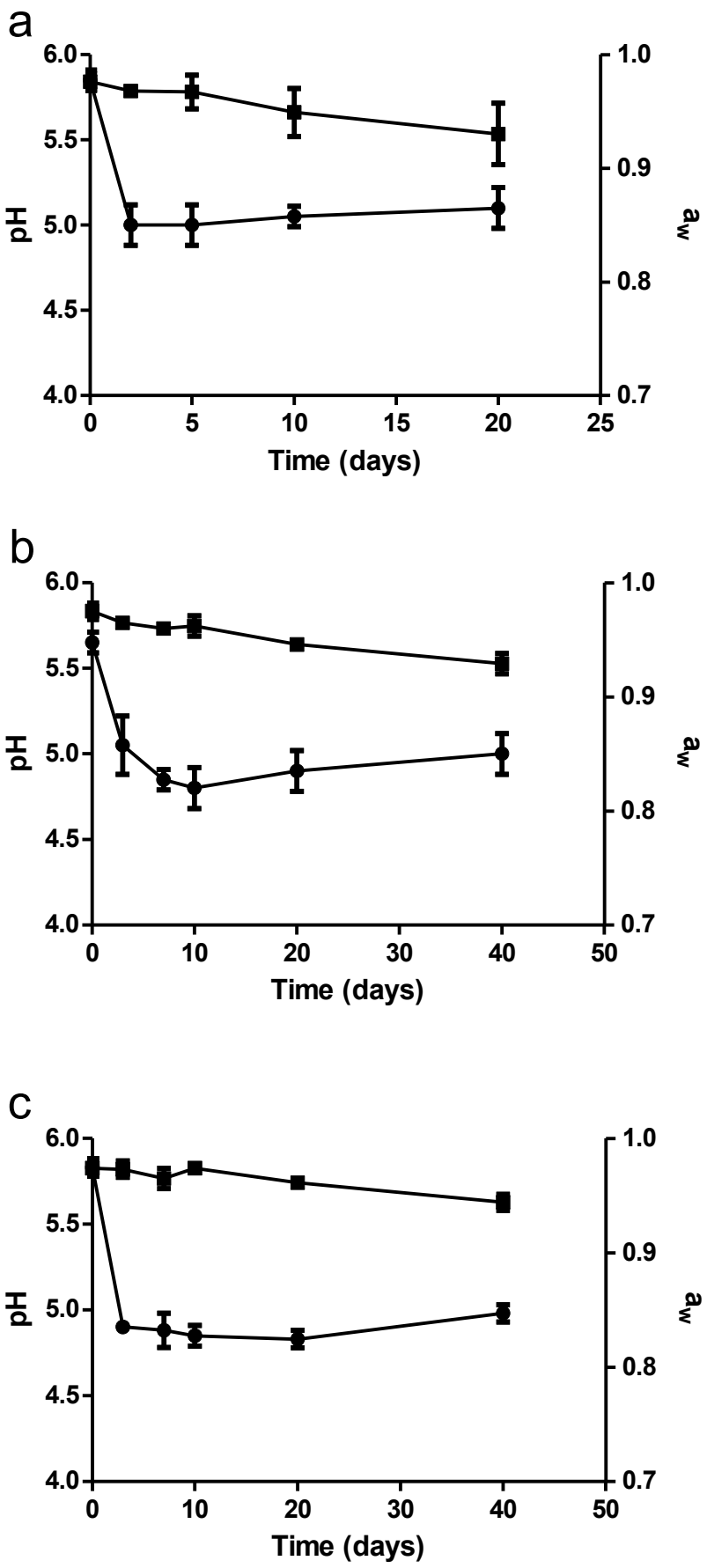
a
6.0
5.5
5.0
4.5
4.0
pH
1.0
0.9
0.8
0.7
a_w
0
5
10
15
20
25
Time (days)
b
6.0
5.5
5.0
4.5
4.0
pH
1.0
0.9
0.8
0.7
a_w
0
10
20
30
40
50
Time (days)
c
6.0
5.5
5.0
4.5
4.0
pH
1.0
0.9
0.8
0.7
a_w
0
10
20
30
40
50
Time (days)

Fig. 3

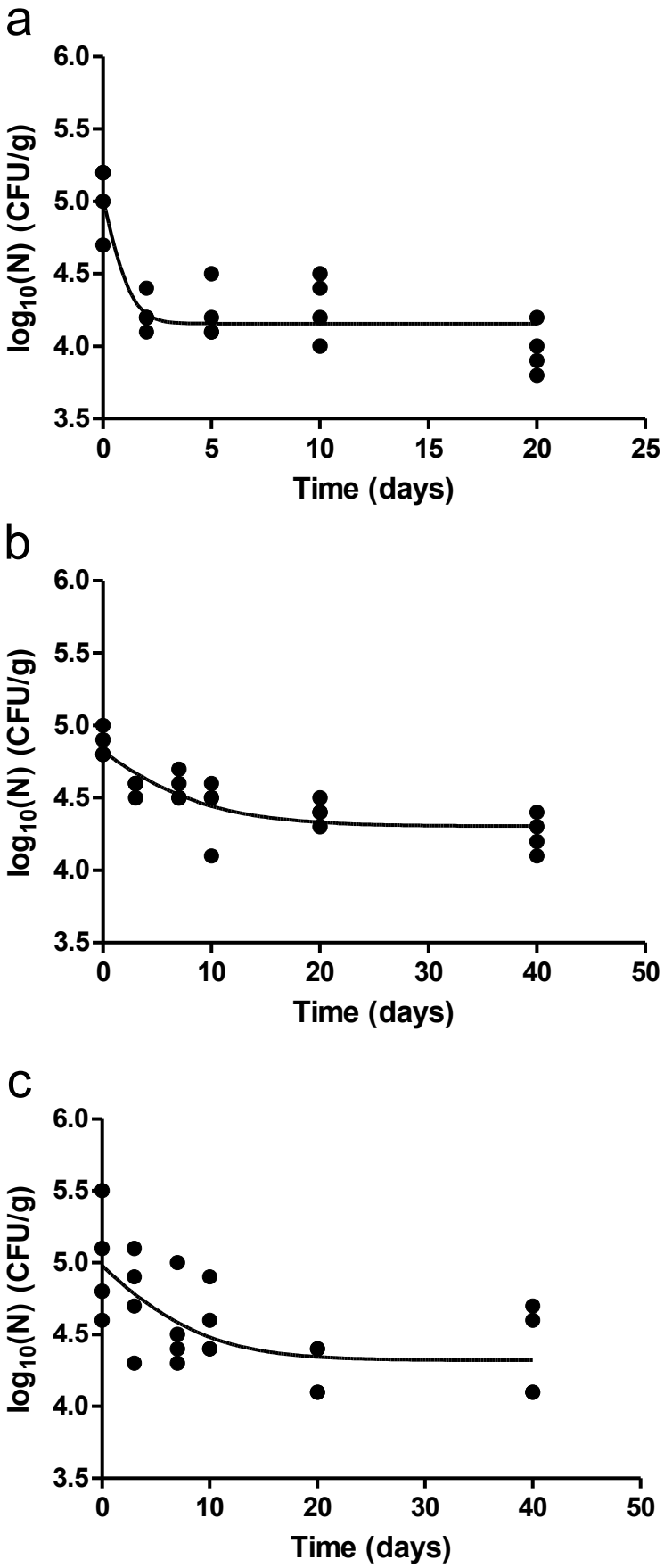
a
6.0
5.5
5.0
4.5
4.0
3.5
log10(N) (CFU/g)
0
5
10
15
20
25
Time (days)
b
6.0
5.5
5.0
4.5
4.0
3.5
log10(N) (CFU/g)
0
10
20
30
40
50
Time (days)
c
6.0
5.5
5.0
4.5
4.0
3.5
log10(N) (CFU/g)
0
10
20
30
40
50
Time (days)

Fig. 4

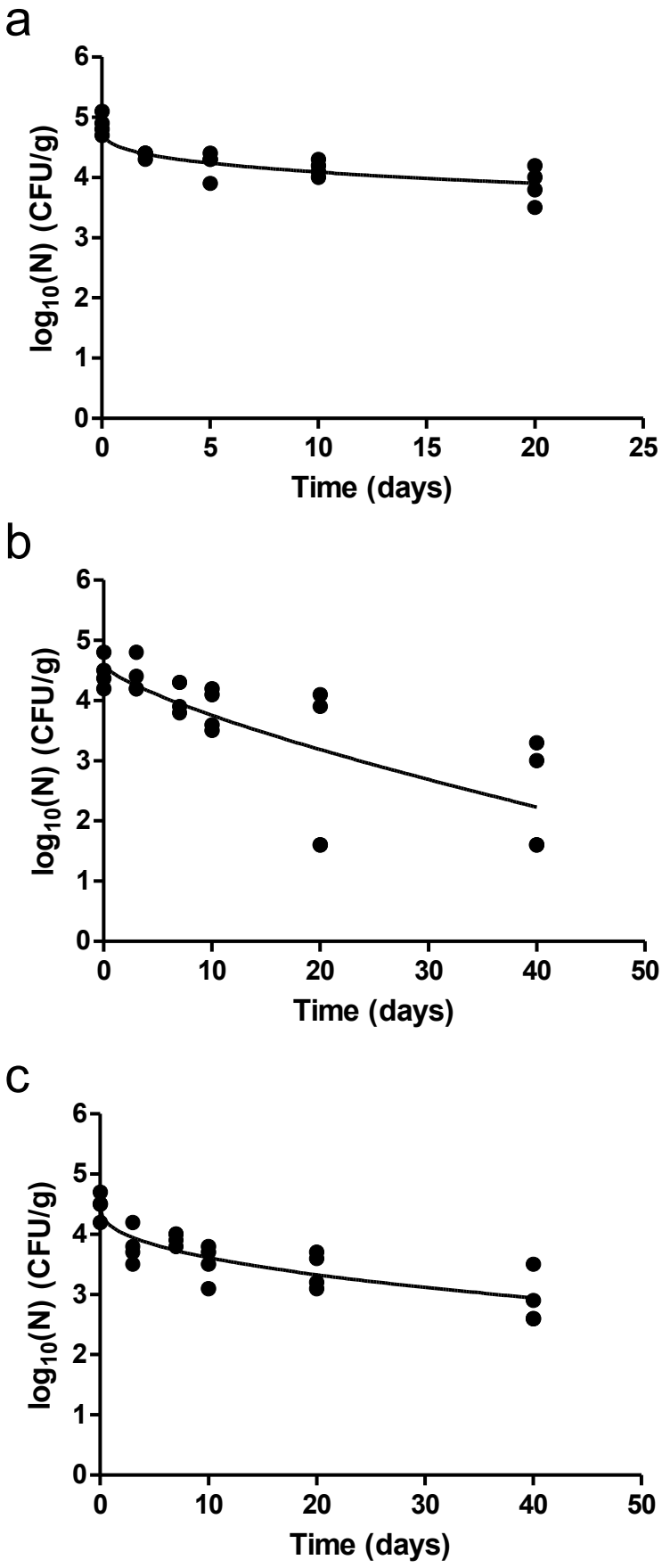
a
log10(N) (CFU/g)
Time (days)
b
log10(N) (CFU/g)
Time (days)
c
log10(N) (CFU/g)
Time (days)

Table 1

Recipes for the production of the fermented sausages Cacciatore, Felino and Milano.

Ingredients	Cacciatore (%)	Felino (%)	Milano (%)
Meat and fat			
Defatted boneless pork shoulder	50.0	67.0	34.0
Pork leg (bottom side)	32.5		
Pork throat fat	17.5		
Pork trimmings (80/20)			40.0
Pork skinned belly		30.0	26.0
Pork lard (Fatback)		3.0	
Additives			
Salt	2.200	2.400	2.500
Sodium nitrite	0.020		0.030
Potassium nitrate	0.014	0.015	0.015
Dextrose	0.400	0.400	0.500
Pepper 1/2	0.050	0.050	
Pepper powder	0.040	0.050	0.050
Ascorbate	0.070	0.050	0.050
Flavor (natural flavor and spices)	0.030	0.200	0.300
Garlic	0.030	0.020	0.030
Wine	0.500	0.020	0.030

Table 2

Fermentation and drying program followed for the production of the fermented sausages Cacciatore, Felino and Milano.

Phase	Time (h)	Temperature (^{o}C)	Relative Humidity (%)
Cacciatore			
Heating	-	19-22	Till 19^{o}C core temperature
Fermentation	10	20-22	58-68
Fermentation	10	19-21	55-77
Fermentation	24	18-20	55-77
Fermentation	24	17-19	65-80
Drying	Till to end (20 d)	15-17	72-82
Felino			
Heating	8	20-22	92-95
Fermentation	24	19-21	80-88
Fermentation	24	19-21	72-84
Fermentation	24	18-20	68-76
Fermentation	24	17-19	72-78
Fermentation	24	16-18	74-82
Fermentation	24	15-17	70-78
Drying	Till to end (40 d)	12-14	74-88
Milano			
Heating	8	23-25	92-95
Fermentation	24	21-23	80-88
Fermentation	24	19-21	76-84
Fermentation	24	17-19	65-75
Fermentation	24	16-18	68-76
Fermentation	24	15-17	74-82
Fermentation	24	15-17	70-78
Drying	Till to end (40 d)	12-14	70-84

Table 3

Statistical comparison of the models used to describe the nonthermal inactivation of *L. monocytogenes* in Cacciatore, Felino and Milano.

Experimental trial/Statistical indices	Models[a]			
	log-linear (2)	Weibull (3)	Biphasic (4)	log-linear with tail (3)
Cacciatore				
LoF[b]	no	yes	AF[e]	yes
RMSE	0.319	0.200		0.212
R^2	0.41	0.78		0.75
A_f	1.06	1.04		1.04
Normality of residuals	yes	yes		yes
Replicates test[c]	no	no		no
Dependency of model parameters[d]	no	no		no
No. of outliers	0	0		0
Felino				
LoF[b]	yes	yes	yes	yes
RMSE	0.157	0.121	0.125	0.133
R^2	0.55	0.74	0.74	0.69
A_f	1.02	1.02	1.02	1.02
Normality of residuals	no	no	no	yes
Replicates test[c]	no	no	no	no
Dependency of model parameters[d]	no	no	no	no
No. of outliers	0	0	0	0
Milano				
LoF[b]	yes	yes	AF	yes
RMSE	0.323	0.297		0.289
R^2	0.26	0.40		0.43
A_f	1.06	1.05		1.05
Normality of residuals	yes	yes		yes
Replicates test[c]	no	no		no
Dependency of model parameters[d]	no	no		no
No. of outliers	0	0		0

[a] The number in parentheses shows the number of parameters of the model

[b] *LoF* was either accepted (yes) or not accepted (no)

[c] It shows if the data are too far from the fitted curve (yes) or not (no) compared to the scatter among replicates

[d] It is reported for each parameter and quantifies the degree to which that parameter is intertwined (yes) or not (no) with others. If it is high (> 0.90-0.95) then the data do not define all the parameters in the model

[e] Ambiguous fit

Table 4

Statistical comparison of the models used to describe the nonthermal inactivation of *S. enterica* in Cacciatore, Felino and Milano.

Experimental trial/Statistical indices	Models[a]			
	log-linear (2)	Weibull (3)	Biphasic (4)	log-linear with tail (3)
Cacciatore				
LoF[b]	yes	yes	yes	yes
RMSE	0.247	0.185	0.188	0.213
R^2	0.60	0.79	0.79	0.72
A_f	1.04	1.03	1.03	1.04
Normality of residuals	yes	yes	yes	yes
Replicates test[c]	no	no	no	no
Dependency of model parameters[d]	no	no	no	no
No. of outliers	0	0	0	0
Felino				
LoF[b]	yes	yes	AF[e]	AF
RMSE	0.691	0.696		
R^2	0.57	0.58		
A_f	1.18	1.19		
Normality of residuals	no	yes		
Replicates test[c]	no	no		
Dependency of model parameters[d]	no	no		
No. of outliers	2	0		
Milano				
LoF[b]	yes	yes	AF	yes
RMSE	0.340	0.293		0.326
R^2	0.65	0.75		0.69
A_f	1.08	1.07		1.07
Normality of residuals	yes	yes		yes
Replicates test[c]	no	no		no
Dependency of model parameters[d]	no	no		no
No. of outliers	0	0		0

[a] The number in parentheses shows the number of parameters of the model

[b] *LoF* was either accepted (yes) or not accepted (no)

[c] It shows if the data are too far from the fitted curve (yes) or not (no) compared to the scatter among replicates

[d] It is reported for each parameter and quantifies the degree to which that parameter is intertwined (yes) or not (no) with others. If it is high (> 0.90-0.95) then the data do not define all the parameters in the model

[e] Ambiguous fit

Table 5

Estimation of the kinetic parameters of nonthermal inactivation of *L. monocytogenes* and *S. enterica* in Cacciatore, Felino and Milano according to log-linear with tail and Weibull models, respectively.

Experimental trial/Parameters	Models	
	log-linear with tail	Weibull
L. monocytogenes/Cacciatore		
N_0 ($\log_{10}$ CFU/g)	5.03	
N_{res} ($\log_{10}$ CFU/g)	4.16	
k_{max} (per day)	1.81^{A}	
Total inactivation ($\log_{10}$ CFU/g)	0.87^{C}	
L. monocytogenes/Felino		
N_0 ($\log_{10}$ CFU/g)	4.82	
N_{res} ($\log_{10}$ CFU/g)	4.31	
k_{max} (per day)	0.18^{B}	
Total inactivation ($\log_{10}$ CFU/g)	0.51^{C}	
L. monocytogenes/Milano		
N_0 ($\log_{10}$ CFU/g)	4.98	
N_{res} ($\log_{10}$ CFU/g)	4.32	
k_{max} (per day)	0.21^{B}	
Total inactivation ($\log_{10}$ CFU/g)	0.66^{C}	
S. enterica/Cacciatore		
N_0 ($\log_{10}$ CFU/g)		4.87
δ (days)		22.3^{D}
p		0.31
Total inactivation ($\log_{10}$ CFU/g)		0.97^{E}
S. enterica/Felino		
N_0 ($\log_{10}$ CFU/g)		4.59
δ (days)		12.8^{D}
p		0.75
Total inactivation ($\log_{10}$ CFU/g)		2.36^{F}
S. enterica/Milano		
N_0 ($\log_{10}$ CFU/g)		4.46
δ (days)		15.1^{D}
p		0.42
Total inactivation ($\log_{10}$ CFU/g)		1.51^{F}

For each parameter (k_{max}, δ and total inactivation) and within each foodborne pathogen, values with the same letter are not significantly different ($P > 0.05$)

Table 6

Preventive measures to control growth of *L. monocytogenes*, *S. enterica* and *St. aureus* during production of dry fermented sausages indicating the critical control points (CCPs) of the process.

Parameter	Target
pH during butchering	≤5.8
a_w during comminution and mixing	0.955-0.965
Sugars concentration during comminution and mixing	0.3-0.5%
Starters addition during comminution and mixing	yes
Fermentation	at 18-22^{o}C and target pH ≤5.3 after 3 days of fermentation
Ripening	at 10-15^{o}C and target a_w ≤0.90