

Modelling the growth rate of *Listeria monocytogenes* in cooked ham stored at different temperatures

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Abstract

Introduction: The purpose of the study was to determine and model the growth rates of *L. monocytogenes* in cooked cured ham stored at various temperatures. **Material and Methods:** Samples of cured ham were artificially contaminated with a mixture of three *L. monocytogenes* strains and stored at 3, 6, 9, 12, or 15°C for 16 days. The number of listeriae was determined after 0, 1, 2, 3, 5, 7, 9, 12, 14, and 16 days. A series of decimal dilutions were prepared from each sample and plated onto ALOA agar, after which the plates were incubated at 37°C for 48 h under aerobic conditions. The bacterial counts were logarithmised and analysed statistically. Five repetitions of the experiment were performed. **Results:** Both storage temperature and time were found to significantly influence the growth rate of listeriae ($P < 0.01$). The test bacteria growth curves were fitted to three primary models: the Gompertz, Baranyi, and logistic. The mean square error (MSE) and Akaike's information criterion (AIC) were calculated to evaluate the goodness of fit. It transpired that the logistic model fit the experimental data best. The natural logarithms of *L. monocytogenes*' mean growth rates from this model were fitted to two secondary models: the square root and polynomial. **Conclusion:** Modelling in both secondary types can predict the growth rates of *L. monocytogenes* in cooked cured ham stored at each studied temperature, but mathematical validation showed the polynomial model to be more accurate.

Keywords: ham, storage, *Listeria monocytogenes*, predictive modelling.

Introduction

Listeria monocytogenes causes listeriosis, a disease highly fatal (30%–40%) to fetuses, newborns, infants, pregnant women, and elderly and immunocompromised individuals such as those with cancer, renal diseases, heart diseases, or AIDS. People receiving organ transplants and treated with immunosuppressive drugs are also susceptible to listeriosis (3, 27).

Over 10,000 cases of human infection were published in medical literature during 1960–1982. An estimated 23,150 cases (5463 fatal) of listeriosis occurred worldwide in 2010 (3). The incidence of listeriosis in the United States is approximately 0.7 per 100,000 people per year (3). In the European Union (EU), 1763 confirmed human cases of listeriosis were reported in 2013 by 27 member states (MS). The EU notification rate was 0.44 cases per 100,000 population, which indicated an 8.6% increase compared to 2012.

There was a statistically significant increasing trend of listeriosis in the EU and European Economic Area (EEA) during 2009–2013. A total of 191 deaths due to listeriosis were reported in 2013. The highest number of fatal cases (64) was reported in France. The mortality rate in the EU was established at 15.6% among cases with known outcome. In 2013, a total of 13 outbreaks caused by *L. monocytogenes* were reported by seven MS and one non-MS. It was observed that the number of listeriosis cases was slightly higher in 2013 than in previous years (13).

An increasing trend in the number of cases of listeriosis in humans has been also observed in Poland (8). The calculated median of listeriosis cases in Poland between 2007 and 2011 was 43 with an average incidence rate of 0.11 per 100,000 population. In total, 58 cases of listeriosis with an incidence rate of 0.15 were reported in Poland in 2013 (30).

L. monocytogenes has been isolated from many kinds of food, including ready-to-eat (RTE) meat products (1, 9, 10, 12). According to a European Food Safety Authority (EFSA) report, the non-compliance in 2013 for different categories of RTE food was generally at a comparable level to previous years (13). Nevertheless, a higher level of non-compliance was observed in case of fishery products at processing plant level (mainly smoked fish). Consistent with the results of the baseline study on the prevalence of *L. monocytogenes* in certain RTE foods in the EU between 2010 and 2011, the observed proportion of positive samples in retail products was the highest in fishery products, followed by soft and semi-soft cheeses, RTE meat products, and hard cheeses (12).

L. monocytogenes belongs to the psychrotrophic microorganisms able to multiply in food stored under refrigeration. Even a small number of listeriae present in some RTE products can grow to a level which threatens consumer health (3, 27, 28). The growth of *L. monocytogenes* can be particularly dynamic in food stored under temperature abuse conditions, which often occurs both in food trade and domestic refrigerators (19, 20).

Therefore, accurate information on the behaviour of *L. monocytogenes* in meat products constitutes crucial knowledge for the Microbiological Risk Assessment (MRA) process, as well as the Hazard Analysis and Critical Control Point (HACCP) system. The experimental data regarding survival, inactivation, and growth of this foodborne pathogen during production and storage of meat products at different temperatures can be described mathematically by predictive models (2, 15, 17, 32). Predictive microbiology is based on the assumption that the reaction of microorganisms to environmental factors is reproducible. By experimental determination of parameters having the most important effects on microorganism behaviour, it is possible to predict the response of microbial pathogens to processes applied in food hygiene and technology (4, 11).

Predictive microbiology has become an important tool supporting food safety management systems in recent years. Moreover, predictive microbial models are commonly being used by food inspectors and food technologists in their everyday work (2, 17, 33).

The aims of the present study were as follows: to determine the growth rates of *L. monocytogenes* in cooked cured ham stored at various temperatures; to describe the microbiological data by using primary and secondary mathematical models; and to generate equations enabling prediction of the behaviour of *L. monocytogenes* in cooked ham stored under different temperature conditions.

Material and Methods

Inoculum preparation. The three following reference strains of *L. monocytogenes* were used in the study: ATCC No. 7644 (isolated from human), ATCC

No. 15313 (isolated from rabbit), and ATCC No. 19112 (isolated from human spinal fluid). In order to prepare the inoculum, the organisms were cultured in 10 mL nutrient broth at 37°C for 24 h. The *L. monocytogenes* cultures were mixed together in the stationary phase of growth and diluted to obtain a suspension containing approx. 10⁴ cfu/cm³. The inoculum density was determined by surface plating of bacterial suspension onto ALOA - Agar *Listeria* Ottaviani & Agosti medium (Merck, Poland).

Material. Cooked ham was purchased from a local producer (meat processing plant, Kozenice, Poland). The production stages of the ham included wet-brine curing, light smoking, and cooking to internal temperature of 70°C. The composition of the ham declared by the producer was as follows: pig hind leg (75%), water, natural spices and their extracts, sodium chloride, E-250, E-316, E-407, E-452, and E-621. Polish standard PN-A-82007 lays down the chemical and microbiological requirements for this ham (26).

Inoculation and storage of ham. Samples of ham (10 g) were placed in polyethylene pouches and inoculated with *L. monocytogenes* by adding 0.1 cm³ bacterial suspension to each pouch. The samples were transferred to incubators directly after inoculation and stored at 3, 6, 9, 12 and 15°C for 384 h.

Bacteriological examination. Enumeration of *L. monocytogenes* in ham samples was performed after 0, 24, 48, 72, 120, 168, 216, 288, 336, and 384 h of storage. The samples were homogenised with dilution fluid for 2 min, ten-fold dilution series were prepared, and from each dilution 0.5 cm³ was taken and plated onto ALOA medium. The plates with culture medium were incubated under aerobic conditions at 37°C for 48 h. The colonies grown on plates were counted, bacterial counts were multiplied by the appropriate dilutions, and numbers of colony-forming units were calculated.

pH measurement. The pH of uncontaminated samples of ham stored at 3, 6, 9, 12, and 15°C was determined using a Schott Instruments digital pH meter equipped with a temperature sensor. During calibration of the equipment and measurements the effect of temperature on pH was taken into account.

Statistical calculations. Bacterial counts were transformed into decimal and natural logarithms. The calculations and modelling were conducted on transformed data using IBM SPSS Statistics 23, Statistica 10 (StatSoft, Polska) and Microsoft Office Excel 2007. The experiment was carried out in five replicates.

Primary modelling - curve fitting. The growth curves of *L. monocytogenes* were fitted to modified Gompertz, logistic, and Baranyi models. The bacterial growth in foods is most frequently described by the modified Gompertz (Eq. 1) and logistic models (Eq. 2) (15, 21, 24):

$$x(t) = C + A \exp\left(-\exp(-B(t - M))\right) \quad (1)$$

$$x(t) = C + \frac{A}{1 + \exp(-B(t - M))} \quad (2)$$

where $x(t)$ is \log_{10} (CFU/g) of cell concentration at time t ; C is the value of the lower asymptote in units of \log_{10} (cfu/g); A is equal to $\log_{10}(x_{max}/x_0)$; x_0 is the initial population density; x_{max} is the maximum population density; B is the maximum relative growth rate at M (1/h); and M is the time at which the absolute growth rate is maximum (hours). Baranyi and Roberts (2) introduced a mechanistic model that describes sigmoidal bacterial growth curves. It is referred to as the Baranyi model and described by the following equations:

$$y(t) = y_0 + \mu_{max}F(t) - \ln\left(1 + \frac{e^{\mu_{max}F(t)} - 1}{e^{(y_{max}-y_0)}}\right) \quad (3)$$

$$F(t) = t + \frac{1}{v} \ln(e^{-vt} + e^{-h_0} - e^{(-vt-h_0)}) \quad (4)$$

where $y(t)$ is the cell concentration at time t (ln CFU/g), y_0 is the initial cell concentration (ln CFU/g), y_{max} is the maximum cell concentration (ln CFU/g), μ_{max} is the maximum specific growth rate (1/h), v is the rate of increase in the limiting substrate, and h_0 is a product of $\mu_{max}\lambda$.

The modified Gompertz and logistic equations' parameters (A , B , C , M) were subsequently used to calculate: lag phase duration (h) = $M - (1/B)$, generation time (h) = $(\log 2e)/BC$ (log denotes the decimal basis of the logarithm), exponential growth rate $[(\log \text{cfu/g})/h] = BC/e$, and maximum population density $(\log \text{cfu/g}) = A + C$ (15, 21, 24).

The goodness of fit was evaluated by calculating the mean square error (MSE) and Akaike's information criterion (AIC) for each of the studied primary models according to the following equations:

- the mean square error (MSE):

$$MSE = \frac{RSE}{df} \quad (5)$$

where RSE is the residual sum of errors and df is degrees of freedom.

- Akaike's Information Criterion with bias adjustment for small sample size (6).

$$AIC_c = -2 * \ln(\text{likelihood}) + 2 * K + (2 * K * (K + 1)) / (n - K - 1) \quad (6)$$

where n is number of observations and K is the number of model parameters.

Secondary modelling. The natural logarithms of mean growth rates of *L. monocytogenes* were calculated from the primary logistic model and fitted to the secondary models, namely the square root (7) and polynomial (8) according to the following functions:

$$\sqrt{\mu_{max}} = a(T - T_{min}) \quad (7)$$

$$\mu_{max} = a_0 + a_1T + a_2T^2 \quad (8)$$

where μ_{max} is growth rate (1/h), a_0 , a_1 , and a_2 are adjustment factors, T is temperature ($^{\circ}\text{C}$), and T_{min} is the determined minimum temperature for the growth of microorganisms ($^{\circ}\text{C}$).

The internal mathematical validation of the secondary models was conducted by calculating accuracy (A_f) and bias (B_f) factors as suggested by Ross (29):

$$A_f = 10 \left(\frac{\sum |\log \mu_{predicted} / \mu_{observed}|}{n} \right) \quad (9)$$

$$B_f = 10 \left(\frac{\sum \log \left(\frac{\mu_{observed}}{\mu_{predicted}} \right)}{n} \right) \quad (10)$$

where n is the number of observations, $\mu_{predicted}$ is the predicted specific growth rate (1/h), and $\mu_{observed}$ is the observed specific growth rate (1/h).

The bias factor B_f indicates consistent over- and under-prediction, whereas the coefficient of accuracy A_f shows the mean difference between the logarithms of the observed and predicted values (10).

Results

The raw experimental data on changes in the number of listeriae in the samples of ham stored at 3, 6, 9, 12, and 15 $^{\circ}\text{C}$ and fitted to the primary logistic model are presented in Fig. 1.

The number of listeriae remained almost the same for the entire period of the experiment in the samples kept at 3 $^{\circ}\text{C}$. In the samples stored at higher temperatures, growth of *L. monocytogenes* was observed (Fig. 1).

Analysis of variance showed that the effect of storage time on the number of listeriae in samples stored at 3 $^{\circ}\text{C}$ was not statistically significant ($P < 0.01$), whereas in samples stored at 6, 9, 12, and 15 $^{\circ}\text{C}$ storage time exerted statistically significant influence ($P < 0.01$) on the number of tested microorganisms (Fig. 1). The growth rate of *L. monocytogenes* increased simultaneously with incubation temperature, being the most dynamic at the highest applied temperature of 15 $^{\circ}\text{C}$.

The results of calculations given in Table 1 allow detailed comparison of the performance of the applied primary models. The mean square error (MSE) as well as Akaike's information criterion (AIC) indicate that the logistic model showed the best fit to the experimental *L. monocytogenes* growth rate results in the ham samples stored at 6, 9, 12, and 15 $^{\circ}\text{C}$. Both the logistic and modified Gompertz models gave the same fit to data related to the samples stored at 3 $^{\circ}\text{C}$ in terms of MSE and AIC values. It should be emphasised that the differences in the goodness of fit between the logistic and modified Gompertz models were very low. In all groups of samples the Baranyi model provided the worst fit (Table 1).

The growth kinetics presented in Table 2 were calculated from logistic equations. The values demonstrated that together with increasing storage temperature, decreases in lag time and generation time as well as an increase in the exponential growth rate of the tested microorganisms regularly occurred.

Because comparison of three primary models showed that the logistic model yielded the best goodness of fit, the mean growth rates of *L. monocytogenes* calculated from logistic equations were fitted to two secondary models. The results related to the square root and polynomial models are presented in Figs 2 and 3, respectively.

The results shown in Figs 2 and 3 demonstrate that both secondary models, namely the square root and polynomial, can be efficiently used to predict the growth rate of listeriae in ham during storage at temperatures in the range 3–15 $^{\circ}\text{C}$. The accuracy (A_f) and bias (B_f) factors

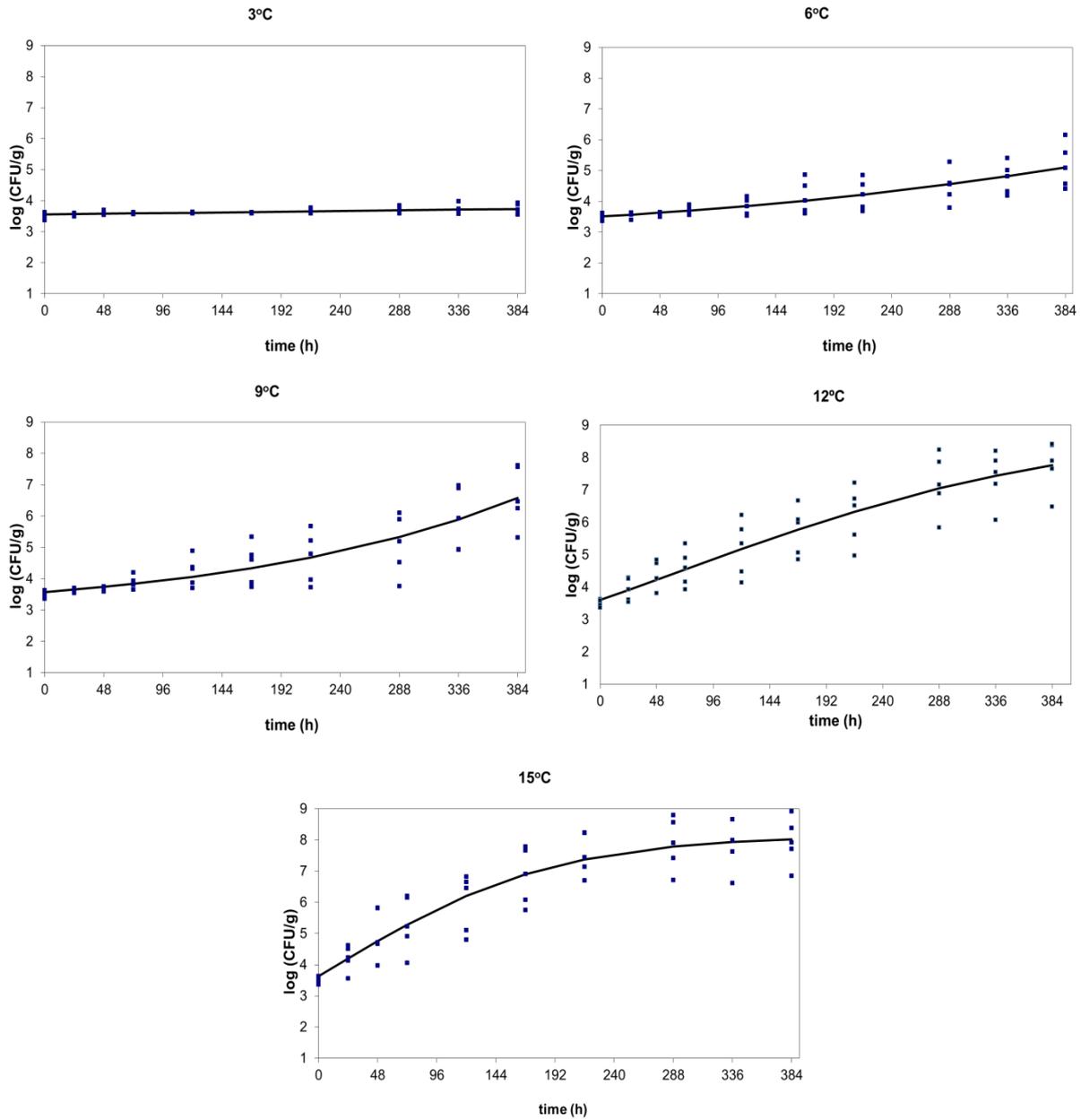


Fig. 1. Growth of *Listeria monocytogenes* in cooked ham stored at 3, 6, 9, 12, and 15°C. Raw data from five replicates of the experiment are represented by black squares. Predicted values calculated from the logistic model are represented by the solid line

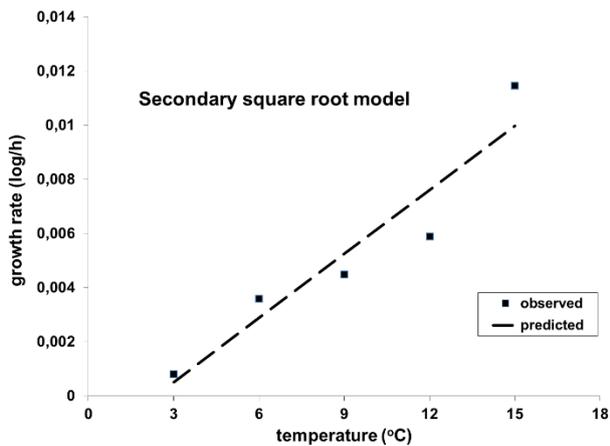


Fig. 2. The relationship between storage temperature and growth rate of *Listeria monocytogenes* in cooked ham. Observed mean values from five replicates fitted into secondary square root model

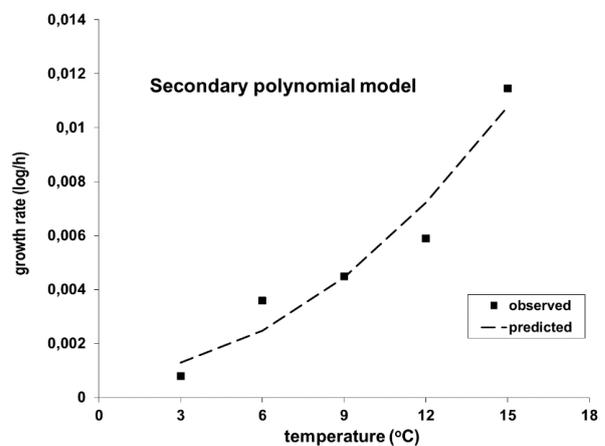


Fig. 3. The relationship between storage temperature and growth rate of *Listeria monocytogenes* in cooked ham. Observed mean values from five replicates fitted into secondary polynomial model

Table 1. Statistics obtained from three compared primary models. The goodness of fit was estimated on the basis of the mean square error (MSE) and Akaike's information criterion (AIC)

Storage temperature	Model	MSE	AIC
3°C	Modified Gompertz	0.0077*	-304.64*
	Logistic model	0.0077*	-304.64*
	Baranyi model	0.0178	-260.74
6°C	Modified Gompertz	0.141	-159.26
	Logistic model	0.1404*	-159.48*
	Baranyi model	0.3237	-115.71
9°C	Modified Gompertz	0.3697	-111.07
	Logistic model	0.3542*	-113.21*
	Baranyi model	0.9679	-62.95
12°C	Modified Gompertz	0.4128	-105.55
	Logistic model	0.4065*	-106.32*
	Baranyi model	0.9761	-62.52
15°C	Modified Gompertz	0.4992	-96.05
	Logistic model	0.4862*	-97.37*
	Baranyi model	1.6648	-35.83

* The model best fitting (the lowest value of MSE and AIC)

Table 2. Growth kinetics values calculated from the logistic equations

Storage temperature	Growth kinetics	Logistic model
3°C	Lag time (h)	384
	Generation time (h)	288.67
	Exponential growth rate (log/mL/h)	0.0008
	Max. pop. density (log/mL)	3.725
6°C	Lag time (h)	150
	Generation time (h)	71.01
	Exponential growth rate (log/mL/h)	0.0036
	Max. pop. density (log/mL)	5.16
9°C	Lag time (h)	100
	Generation time (h)	37.3
	Exponential growth rate (log/mL/h)	0.0047
	Max. pop. density (log/mL)	6.64
12°C	Lag time (h)	50
	Generation time (h)	27.4
	Exponential growth rate (log/mL/h)	0.006
	Max. pop. density (log/mL)	7.767
15°C	Lag time (h)	20
	Generation time (h)	26.27
	Exponential growth rate (log/mL/h)	0.0115
	Max. pop. density (log/mL)	7.951

Table 3. Comparison of accuracy (A_f) and bias (B_f) factors calculated for validation of the square root and polynomial secondary models describing the growth rate of *L. monocytogenes* in the samples of ham

Secondary models	Equations	A_f	B_f
Square root	$\sqrt{\text{growth rate (log)}} = 0.000787 * T - 2.345932$	1.392	1.116
Polynomial	$\text{growth rate (log)} = 0.000893 - 0.00\text{temp} + 0.0000439\text{temp}^2$	1.332*	1.086*

* The model better fitted (A_f and B_f values closer to 1.0)

(29) presented in Table 3 used for mathematical validation purposes suggest that in comparison to the square root model, the polynomial secondary model gave a better fit to the observed microbial data characterising the growth of *L. monocytogenes* in cooked ham (Table 3).

The obtained results demonstrated that contamination of cooked ham with *L. monocytogenes* after heat treatment poses a significant risk to consumer health, particularly high in cases of storage under temperature abuse conditions.

Discussion

Among the food safety microbiological criteria which are given in EU Regulation 2073/2005 (14), particularly important for the manufacturers of meat products and food inspectors are those concerning *L. monocytogenes* in ready-to-eat (RTE) foodstuffs. Certain food categories are no longer under a zero tolerance regime but have a specified maximum allowed concentration of 100 CFU/g or ml for products placed on the market during their shelf-life. Therefore, many studies on modelling the effects of various factors on the growth of *L. monocytogenes* have been published in recent years (5, 18, 22, 23, 35). This research allows the behaviour of *L. monocytogenes* in RTE meat products to be predicted.

In our previously conducted experiments, yogurt and other fermented dairy products were contaminated with *L. monocytogenes* and stored at temperatures of 3, 6, 9, 12, and 15°C. A linear decrease in the number of these bacteria was observed during storage (34, 35). This phenomenon was mainly explained by the low pH of the samples (range 4.1–4.4), because the pH value of about 4.6 is generally considered as the minimum enabling the growth of *L. monocytogenes* (7, 28). The initial average pH value of ham samples used in present study was 6.43 and increased to the range of 6.75 – 7.99 at the end of storage.

A direct comparison of our results with other published studies is difficult because of differences in the experimental conditions. Comparison of the primary models applied in our study (Table 1) indicated that the logistic model best fitted the experimental results describing the growth of *L. monocytogenes* in the cooked ham samples. In a similar study, Luo *et al.* (22) modelled the growth kinetics of *L. monocytogenes* in RTE ham and sausage at temperatures from 4 to 35°C. The observed data fitted four primary models well (the Baranyi, modified Gompertz, logistic, and Huang). The square root and the natural logarithm models were used to describe the relationship between temperature and lag time as well as the specific growth rate derived from the primary models. Mataragas *et al.* (23) determined the growth limits of *L. monocytogenes* in a vacuum-packaged sliced cooked cured meat product. The authors found that the Baranyi model appeared to fit the overall experimental data better than the modified logistic or modified Gompertz models.

The growth kinetics values calculated in the present study (Table 2) for *L. monocytogenes* in ham were compared to predicted values generated by the Pathogen Modeling Program (PMP) and ComBase Predictor (6). Lag times and generation times obtained from the PMP for *L. monocytogenes* incubated at 6, 9, 12 and 15°C in optimum laboratory culture medium were much shorter than those observed in our studies (Table 2). Generation times obtained from ComBase were very similar to those obtained from the PMP. Predicted values for exponential growth rates given by ComBase were conspicuously

higher than observed data at each of the applied storage temperatures in our experiment. All conducted comparisons demonstrated that the growth of *L. monocytogenes* in real meat product is much slower than in a laboratory culture medium assuming similar temperature, pH, water activity, and concentration of NaCl and NaNO₂.

It should be recognised that the results presented in this paper are specific to a particular product and also to the *L. monocytogenes* strains used in this study. Different growth rates might be obtained by testing in other environments and with *L. monocytogenes* from other sources (31). However, the cured, cooked ham used in this study met the requirements laid down in the Polish standard (26) and its manufacturing and chemical composition was typical for this kind of product. To obtain a more representative microorganism response, a mixture of three different strains of *L. monocytogenes* was used for sample inoculation. Therefore, we believe that the results obtained in the present study can be useful not only for the meat processing plant in Kozenice which produced the ham used in our experiments, but also for other Polish producers of cured, cooked ham.

The results demonstrated that the storage of food of animal origin at low temperatures is one of the most important factors limiting the number of food-borne illness outbreaks caused by bacterial pathogens, including *L. monocytogenes*, and even a minor breach of the provisions on required storage temperature and time can significantly increase the risk to consumer health.

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