Prediction of *Listeria monocytogenes* Growth Kinetics in Sausages Formulated with Antimicrobials as a Function of Temperature and Concentrations

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Abstract This study was conducted to develop a model to describe the effect of antimicrobials [potassium sorbate (PS), potassium lactate (PL), and combined PL and sodium diacetate (SDA, PLSDA)] on the growth parameters of *Listeria monocytogenes* such as specific growth rate (SGR) and lag phase periods (LT) in air-dried raw sausages as a function of storage temperature (4, 10, 16, and 25°C). Results showed that the SGR of *L. monocytogenes* was dependent on the storage temperature and level of antimicrobials used. The most effective treatment was the 4% PLSDA, followed by the 2% PLSDA and 4% PL and 0.2% PS exhibited the least antimicrobial effect. Increased growth rates were observed with increasing storage temperatures from 4 to 25°C. The growth data were fitted with a Gompertz equation to determine the SGR and LT of the *L. monocytogenes*. Six polynomial models were developed for the SGR and LT to evaluate the effect of PS (0.1, 0.2%) and PL (2, 4%) alone and PLSDA (2, 4%) on the growth kinetics of *L. monocytogenes* from 4 to 25°C.

Key words: *Listeria monocytogenes*, predictive model, antimicrobial, storage temperature, sausage

Introduction

Predictive microbiology aims to predict the microbial behavior in foods over time as a function of different influencing factors (1). Primary models describe changes in microbial numbers over time under certain conditions, whereas secondary models describe the effect of environmental factors on the growth kinetic parameters (e.g., the lag time (LT) and specific growth rate (SGR)) of the primary model (2). Microbial models are valuable tools in planning hazard analysis critical control point (HACCP) programs and making decisions and regulating plans and policies for the food industry, as they provide the first estimates of expected changes in microbial populations when exposed to a specific set of conditions (2). Because microbial testing in foods is expensive and time-consuming, mathematical models might be useful alternatives that facilitate constructing a matrix of microbial growth responses to a broad range of storage conditions.

The presence of the psychrotropic bacterium *Listeria monocytogenes* in meat products is a food safety concern when very high doses of the organism are consumed due to its high mortality rate, especially for susceptible populations such as young, old, pregnant, and immunocompromised

patients. Ready-to-eat (RTE) meat and poultry foods that are commonly consumed without further cooking are of particular concern because *L. monocytogenes* contaminates products mainly after thermal processing (3). Rocourt *et al.* (4) estimated the risks of serious illness and death associated with the consumption of RTE foods possibly contaminated with *L. monocytogenes*.

In response to public concern, the food industry is showing increased interest in the antilisterial activity of various salts of lactic, acetic, or other organic acids for food safety and shelf life extension. Commonly applied organic acids that are generally recognized as safe (GRAS; i.e., have low toxicity to humans) for meat and poultry products include sorbate, lactate, and a mixture of lactate and diacetate (5-9). Numerous studies have reported the effects of these preservatives, used individually or in combination to inhibit *L. monocytogenes* growth in various meat products (3,10-13). Moreover, the use of GRAS antimicrobial agents for control of *L. monocytogenes* in RTE meat or poultry products is encouraged in the interim final rule issued by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) in response to recent listeriosis outbreaks associated with RTE meat and poultry products (14-17). As a result, the RTE product industry now uses antimicrobials as product ingredients for *L. monocytogenes* control.

In such a susceptible and highly regulated environment, a mathematical model would be of considerable practical importance to predict the combined effects of antimicrobials

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on the growth of *L. monocytogenes* and assist in the formulation of safe food products under various conditions without altering their sensory characteristics. The purpose of this study was to evaluate and model the effect of storage temperatures and antimicrobial agents on the growth kinetics of *L. monocytogenes* in sausages separately using a response surface model.

Materials and Methods

Bacterial culture Three strains of *L. monocytogenes* were used throughout this study; 1 strain isolated from a human outbreak (Scott A) and 2 strains isolated from poultry (ATCC 19111 and ATCC 19116). All strains were stored at −70°C in tryptic soy broth (TSB, Difco, Sparks, MD, USA) with a 0.6% yeast extract (YE, Difco) containing 20% glycerol. The strains were activated by transferring 10 μL of the stock culture into 10 mL of TSBYE, and then incubated at 35°C for 24 hr, allowing the cultures to reach the late stationary phase. This was followed by a consecutive transfer in the medium and incubation under the conditions just indicated. Following the incubation, the cells were harvested by centrifugation (3 min at 13,000×g) at 4°C and washed twice in 0.1%(w/v) buffered peptone water (Difco). After a second washing, the cell fractions of *L. monocytogenes* were resuspended in 0.1% buffered peptone water to reach approximately 10^6 CFU/mL. A mixture containing equal numbers of cells from each strain was used as a cocktail.

Inoculation of *L. monocytogenes* in sausages prepared with different antimicrobials Air-dried raw sausages requiring cooking before consumption were prepared at the pilot plant in the Department of Animal Food Science in Kangwon National University according to a standard method of the Dongwon Company (Seongnam, Korea). The sausage batter was composed of 1,000 g of ground pork (72% lean pork, purchased locally), 17.70 g of table salt, 4.41 g of sage (McCormick, Hung Valley, MD, USA), 2.20 g of red pepper flake (Tone's, Ankeny, IA, USA), and 6.61 g of black pepper (Tone's). All of the ingredients were mixed for 10 min with an electric hand/stand mixer (GE Electronic Inc., Bentonville, AK, USA).

For the preparation of antimicrobials, stock solutions of 2% potassium sorbate (PS, Junsei Chemical Co., Tokyo, Japan), 20% potassium lactate (PL, Purac; America, Inc., Lincolnshire, IL, USA) and 20% combined PL and sodium diacetate (SDA) (PLSDA, Purac; 56% PL and 4% SDA) in distilled water were prepared fresh before each experiment. The stock solutions were filter sterilized using a millipore membrane filter of 0.45-μM porosity (Sigma-Aldrich, St. Louis, MO, USA) and added to the sausage mixture to give final concentrations of 0 (control), 0.1, and 0.2% PS, 2 and 4% PL and 2 and 4% PLSDA, respectively. The mixed ingredients containing different concentrations of antimicrobials were then placed in natural casings (Natural Best, Chicago, IL, USA) using a hand-cranked stuffer. Then, the 7 batches of sausages were chopped aseptically into 10.0±0.2 g slices and inoculated with 100 mL of *Listeria* cocktails on several locations of the sausage surface to make a desired inoculum level of 10^3 CFU/g. Finally, the inoculated samples were air-dried for 30 min. Preliminary experiments confirmed

that the *L. monocytogenes* cells were attached on the surface during this time. The inoculated sausages were placed in a whirl-pack (Nasco, WI, USA) and stored for 27 day at 4°C, 10 day at 10°C, 2, 3, 5, 7 day at 16°C, and 1 day at 25°C. Microbiological analysis (2 replicates of 2 samples/sampling time and treatment at each storage temperature) were conducted at predetermined time intervals.

Microbiological analysis Product samples of 10.0±0.2 g were aseptically mixed with 90.0 mL of sterile 0.1% peptone water (Difco) and homogenized for 2 min in a stomacher (Interscience, Weymouth, MA, USA) at 200 rpm at room storage temperature. Further 10-fold serial dilutions of homogenates were prepared and 100 μL of appropriate dilutions were spread-plated onto a modified Oxford medium base (Difco) in duplicate, which were then incubated at 35°C for 24 hr. After incubation, colonies of *L. monocytogenes* were enumerated and expressed as log CFU/g.

Model development and validation At each storage temperature, the mean values of the microbial counts obtained from the 2 replicated experiments over time were used to generate growth curves using Sigma Plot (version 8, Systat Software, Inc., Richmond, CA, USA). The growth parameters were estimated for fitting the data to the modified Gompertz equation (18) using GraphPad prism software (version 4, GraphPad Software, Inc., San Diego, CA, USA) to generate the SGR (log units/day) and LT (log units/day) from each growth curve. Also, generation time in sausages experiments was generated to compare with pathogen modeling program (PMP) for analogous growth conditions. The SGR or LT values were used to develop the response surface model as a secondary model using the equation described below (19):

$$Ln\ X = A + b_1\ (T) + b_2\ (P) + b_3\ (T*T) + b_4\ (P*P) + b_5\ (T*P)$$

where X is the SGR or LT, A is the intercept, b_1-b_5 are the regression coefficients, T is the storage temperature, and P is the concentration of various antimicrobials.

The goodness-of-fit of the models was evaluated by coefficients of determination (R^2). The performance of the predictive equations was assessed by median relative error (MRE) and mean absolute relative error (MARE). The MRE of the model predictions was used as a measure of bias by estimating the mean difference between the observed and predicted values. MARE was used to measure the model prediction accuracy, which assesses how close the predicted values are to the observed values. MARE was calculated using the following equation by Delignette-Muller *et al.* (20):

$$MARE = (1/n)\Sigma_i |((X_p - X_o)/X_o)_i|$$

where X_p and X_o are the predicted and observed values, respectively, and n is the number of experiments.

Results and Discussion

Growth kinetics of *L. monocytogenes* in sausages The initial populations of *L. monocytogenes* cocktails used in the experiment were 2-4 log CFU/g in 7 batches of sausages at all storage temperatures tested. At 4 and 10°C, growth of *L. monocytogenes* was observed in the control
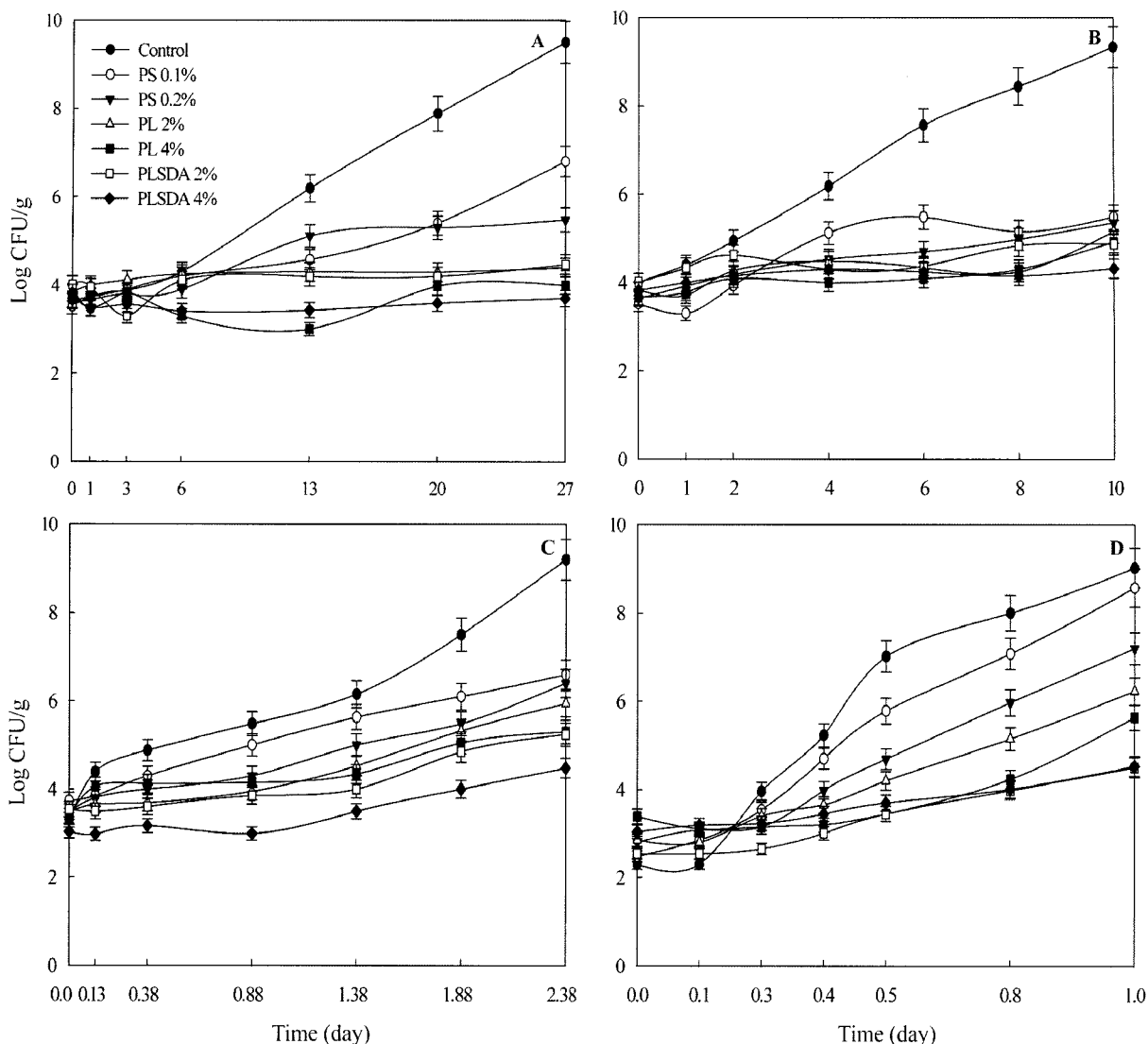
Fig. 1. Growth of *L. monocytogenes* in sausages containing different concentrations of potassium sorbate (PS), potassium lactate (PL), and combined PL and sodium diacetate (PLSDA) at 4 (A), 10 (B), 16 (C), and 25°C (D).

containing no preservatives (Fig. 1A and 1B). In the sausages containing additives stored at 4 and 10°C, no obvious growth of *L. monocytogenes* was observed except in samples treated with 0.1 and 0.2% of PS. The effect of organic acid salts individually or in combination with the formulated products on inhibiting *L. monocytogenes* growth was observed during 27 and 10 day at 4 and 10°C, respectively (Fig. 1A and 1B).

At 16°C, the growth patterns of the pathogen with different concentrations of preservatives were more pronounced than those at lower storage temperatures with increased growth rates and decreased storage time (Fig. 1C). Overall, the most effective treatment was the 4% PLSDA, followed by the 2% PLSDA and 4% PL at 16°C (Fig. 1C). Similar growth patterns were observed at 25°C (Fig. 1D). The growth of *L. monocytogenes* was inhibited by 1-4.5 log CFU/g for preservative-treated groups compared to the control at 16 or 25°C. These results are consistent with others who have conducted experiments with antimicrobials against *L. monocytogenes* in different types of meat products (8,10-12). For example, Stekelenburg and Kant-Muermans (21) reported an addition of 2.5-3.3% sodium

lactate to a cooked ham product inhibited the growth of *L. monocytogenes* for up to 40 day at 4°C, while Glass *et al.* (10) used a mixture of 0.1% SDA and 1.0% sodium lactate to prevent the growth of *L. monocytogenes* in smoked frankfurters for 60 day at 4.5°C. In frankfurters and smoked-cooked ham treated with 0.15% SDA and 1.5% PL, comparable antimicrobial effects were observed against *L. monocytogenes* for 72 day at 4°C (11). The growth of *L. monocytogenes* was prevented for 45 day at 4°C in the presence of 0.2% SDA and 2.5% sodium lactate in beef bologna (8). Stekelenburg (12) showed that the growth of *L. monocytogenes* was inhibited at 4°C in frankfurter sausage treated with 0.1% SDA, and with mixture of 2-3% lactate and diacetate. Although both SDA and sodium lactate alone have been shown to suppress the growth of *L. monocytogenes* (22,23), lactate and diacetate blends extend the shelf life of meat more effectively than either of the 2 alone due to the synergistic effect of lactate and diacetate in combination (7,8).

SGR, LT, and generation time (GT) of *L. monocytogenes* in sausages The effect of antimicrobials on the SGR and

Table 1. Specific growth rate (SGR) and lag time (LT) obtained using a modified Gompertz equation

Treatment[1] (%)	4°C		10°C		16°C		25°C	
	SGR[2]	LT	SGR	LT	SGR	LT	SGR	LT
Control	0.34	6.84	0.66	0.46	2.29	0.18	9.87	0.05
PS 0.1	0.21	8.82	0.35	1.14	2.12	0.22	9.12	0.12
PS 0.2	0.19	23.63	0.25	1.71	1.49	0.3	8.22	0.14
PL 2	0.13	36.81	0.32	3.75	1.47	0.69	8.18	0.15
PL 4	NG[3]	NG	0.23	4.18	1.25	0.76	6.99	0.18
PLSDA 2	0.12	53.46	0.17	4.87	1.18	0.87	4.18	1.11
PLSDA 4	NG	NG	0.12	14.8	1.09	1.07	3.96	1.67

[1]PS, Potassium sorbate; PL, potassium lactate; PLSDA, combined PL and sodium diacetate.
[2]Maximum SGR in log units/day.
[3]No growth.

LT of *L. monocytogenes* is summarized in Table 1. In general, lower SGR was observed in sausages with higher concentrations of antimicrobial agents, with the lowest SGR from PLSDA, followed by PL and PS. Storage temperatures also affected *L. monocytogenes* growth. Higher storage temperatures resulted in higher SGR values for all treatments (from 3.96 to 9.87 log CFU/day at 25°C) compared to lower storage temperatures (from no growth to 0.34 log CFU/day at 4°C). In contrast, the effect of antimicrobials on the growth kinetics of *L. monocytogenes* was more pronounced at lower storage temperatures with decreased SGR. In addition, extended lag phases of *L. monocytogenes* were observed at lower storage temperatures for all treatments because the cells need more time to adjust to the lower storage temperature, except for 4% PL and 4% PLSDA (no growth observed at 4°C) (Table 1). As storage storage temperatures increased, the effect of antimicrobials was reduced; increased SGR and decreased LT were observed for all treatments. Overall, PLSDA at 4% prevented *L. monocytogenes* growth much longer than any other treatments at all storage temperatures.

Another parameter that influenced the growth of *L. monocytogenes* in sausages was GT, derived from the SGR as GT=log$_{10}$ (2)/SGR. Because GT is often fixed to a single value when environmental conditions remain constant (23), the mean GT values were calculated for each set of environmental conditions (Table 2) and compared to predictions made by the USDA-PMP for analogous growth conditions. Although the PMP is not food-oriented, PMP software is used in the field of predictive microbiology. Compared to the GT predicted in sausages from our experiments, the PMP underestimated the GT at all storage temperatures except 25°C (Table 2). There was a tendency that as storage temperature increased, the predictions from the PMP and control decreased (Table 2).

The discrepancy observed between the experimental and PMP-simulated GT can be explained by the following: firstly, the data on which the PMP (aerobic) was based was generated from growth media rather than food, which can contribute a substantial error to predictions due to interactions between food components. Moreover, the PMP was not able to take product structure and composition or antimicrobials into consideration (24,25).

Polynomial modeling of *L. monocytogenes* growth Six polynomial models were developed for the SGR and LT to

Table 2. Comparison of the generation time obtained for *L. monocytogenes* under experimental conditions with that obtained from the pathogen modeling program (PMP)

Treatment[1] (%)	Generation time (day)			
	4°C	10°C	16°C	25°C
PMP	0.65	0.21	0.09	0.03
Control	0.88	0.45	0.13	0.03
PS 0.1	1.43	0.86	0.14	0.03
PS 0.2	1.58	1.2	0.2	0.04
PL 2	2.31	0.94	0.2	0.04
PL 4	NG[2]	1.3	0.24	0.04
PLSDA 2	2.5	1.76	0.25	0.07
PLSDA 4	NG	2.5	0.28	0.08

[1]PMP, Data from the USDA-PMP (pH 6, 1.4% NaCl)-aerobic; control, data from the present study (pH 6, 1.36 % NaCl); PS, potassium sorbate; PL, potassium lactate; PLSDA, combined PL and sodium diacetate.
[2]No growth.

take into account the effect of PS, PL, and a combination of PLSDA on the growth kinetics of *L. monocytogenes*. Each growth curve was fitted to a modified Gompertz function with R^2>0.951 (data not shown). In order to produce a more homogeneous error structure over the region of experimental design, the natural logarithm (ln) of the SGR and LT were also considered (17). The polynomial models developed for the SGR and LT with the coefficient of determination (R^2) are shown in Table 3.

Validation is a step to evaluate the ability of a new model to interpolate and it is a critical step in their development. The first stage is internal validation that evaluates the model against the same data used for building the model (26-29) to ensure that the model accurately describes the data from which it was generated and represents any biological trend in those data. A graphical comparison was performed using a scatter plot to illustrate the fit of the proposed response surface model by plotting observed SGR and LT against predictive values. As shown in Fig. 2, both values are similar, indicating that the models provided a good description for the data used to generate them. In general, the scatter about the line is quite small, indicating a strong linear relationship. In our experiments, each model was developed separately for different antimicrobials. The upward slope of the line corresponds to a positive correlation

Table 3. Polynomial models for the specific growth rate (SGR) and lag time (LT) developed for *L. monocytogenes* with different preservatives

Preservatives	Predictive model
Potassium sorbate (PS)	Ln SGR=-1.613+0.108*(T)-5.102*(PS)+0.002*(T*T)+3.5*(PS*PS)+0.117*(T*PS) R^2=0.98
	Ln LT=3.792-0.546*(T)+7.119*(PS)+0.11*(T*T)-4.5 *(PS*PS)-0.074*(T*PS) R^2=0.99
Potassium lactate (PL)	Ln SGR=-1.848+0.157*(T)-0.555*(PL)+0.034*(PL*PL)+0.014*(T*PL) R^2=0.99
	Ln LT=3.639-0.494*(T)+1.2701*(PL)+0.009*(T*T)-0.170*(PL*PL)-0.011*(T*PL) R^2=0.99
Combined PL and sodium diacetate (PLSDA)	Ln SGR=-1.822+0.162*(T)-0.844*(PLSDA)+0.079*(PLSDA*PLSDA)+0.014*(T*PLSDA) R^2=0.96
	Ln LT=4.221-0.629*(T)+1.498*(PLSDA)+0.014*(T*T)-0.211(PLSDA* PLSDA)+0.004*(T*PLSDA) R^2=0.97
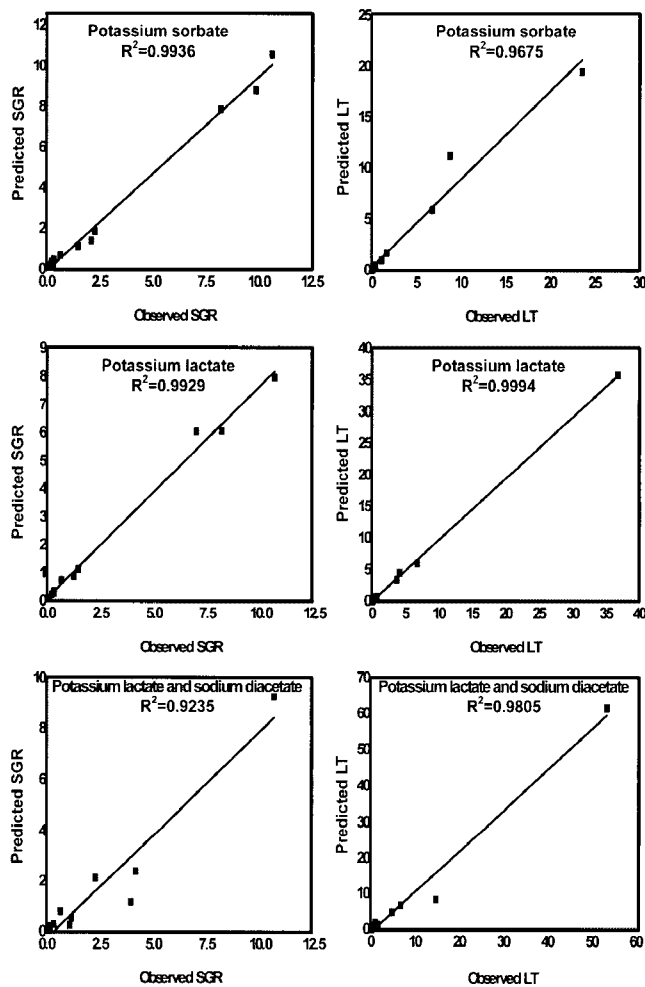
Fig. 2. Observed vs. predicted growth rates (SGR) and lag times (LT) of *L. monocytogenes* in sausages containing different preservatives.

(good agreement) between the observed and predicted SGR and LT for all 6 models, and can be confirmed from the coefficient of correlation ($R^2 \geq 0.92$) (Fig. 2).

Eifert *et al.* (30) and Fernandez *et al.* (31) also used scatter plots of observed vs. predicted data to evaluate their models in predicting the data used to generate them. A

Table 4. Comparison of the accuracy and bias of the specific growth rate (SGR) and lag time (LT) of *L. monocytogenes* on sausages treated with different antimicrobials[1]

Model developed for	SGR		LT	
	MRE	MARE	MRE	MARE
PS	-1.13	20.55	-6.34	18.73
PL	-9.15	17.67	2.86	12.32
PLSDA	-24.38	33.07	1.42	28.88

[1]MRE, Median relative error; MARE, mean absolute relative error; PS, potassium sorbate; PL, potassium lactate; PLSDA, combined PL and sodium diacetate.

model is generally regarded as safe when it predicts faster growth than that observed (32). In the 6 models described here, most points fell close to or slightly above the line, indicating that the predicted values were slightly higher compared to the observed data. This might be due to the different growth patterns of different strains of the same bacteria species with SGT or LT (33). Taking into account the very large strain variation in the physiological properties of *L. monocytogenes* (34), evaluation of a greater number of strains might be required to confirm these findings.

The 6 developed models were validated by assessing the accuracy (MARE) and bias (MRE) of their predictions against the data obtained from the literature (Table 4). MARE indicated that the average deviation for the SGR was higher than that for the LT for the 3 tested preservatives. In the models described here for different preservatives, the MARE ranged from 17.7 to 33.1% for the SGR and 12.3 to 28.9% for the LT, while the MRE ranged from 24.4 to 1.13% for the SGR and -6.3 to 2.86% for the LT. The overall performance of the predicted polynomial for the SGR was better than that of the LT (Table 4).

Delignette-Muller *et al.* (20) pooled 468 prediction cases from 7 predictive models and calculated a MARE of 40.3% for LT and 36.2% for GT. Moreover, Oscar (35) reported that MARE ranged from 28.1 to 74.8% for LT and 18.5 to 72.0% for GT when MARE was calculated for 16 predictive models representing 823 prediction cases in 9 modeling papers for data used in model development. Compared to most published models, the MARE of our

models was low, suggesting better prediction accuracy in most cases. In addition, the average deviation of our models was in the acceptable range. Therefore, the models developed in this study can be considered to provide a reliable prediction of *L. monocytogenes* in sausages.

Acknowledgments

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