Establishing Equivalence for Microbial-Growth-Inhibitory Effects (“Iso-Hurdle Rules”) by Analyzing Disparate *Listeria monocytogenes* Data with a Gamma-Type Predictive Model

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Preservative factors act as hurdles against microorganisms by inhibiting their growth; these are essential control measures for particular food-borne pathogens. Different combinations of hurdles can be quantified and compared to each other in terms of their inhibitory effect (“iso-hurdle”). We present here a methodology for establishing microbial iso-hurdle rules in three steps: (i) developing a predictive model based on existing but disparate data sets, (ii) building an experimental design focused on the iso-hurdles using the model output, and (iii) validating the model and the iso-hurdle rules with new data. The methodology is illustrated with *Listeria monocytogenes*. Existing data from industry, a public database, and the literature were collected and analyzed, after which a total of 650 growth rates were retained. A gamma-type model was developed for the factors temperature, pH, a_w , and acetic, lactic, and sorbic acids. Three iso-hurdle rules were assessed (40 logcount curves generated): salt replacement by addition of organic acids, sorbic acid replacement by addition of acetic and lactic acid, and sorbic acid replacement by addition of lactic/acetic acid and salt. For the three rules, the growth rates were equivalent in the whole experimental domain (γ from 0.1 to 0.5). The lag times were also equivalent in the case of mild inhibitory conditions ($\gamma \geq 0.2$), while they were longer in the presence of salt than acids under stress conditions ($\gamma < 0.2$). This methodology allows an assessment of the equivalence of inhibitory effects without intensive data generation; it could be applied to develop milder formulations which guarantee microbial safety and stability.

Microbiological safety and stability of food products rely on a combination of carefully controlled environmental conditions and preservation methods such as storage and distribution conditions, product formulations, and manufacturing processes. The recent listeriosis cases in Europe and the United States (14, 15, 19, 39) are an important reminder that this organism remains a serious threat for public health and that appropriate designs and control measures must be in place. This is further endorsed by the recently published Codex Alimentarius *Guidelines on the Application of General Principles of Food Hygiene to the Control of Listeria monocytogenes in Foods* (8), which was developed for the purpose of providing guidance on controls and associated tools that can be adopted to minimize the risk of ready-to-eat foods containing harmful levels of this pathogen.

In addition, the growing consumer demand for more natural foods that are minimally processed and contain less preservatives (37) pushes food developers to design products under conditions that are closer to microbial growth/no-growth boundaries. Such conditions, if not properly determined and controlled, may result in the growth of spoilage microorganisms and pathogenic microorganisms at levels that may compromise the quality of the food product before the end of shelf-life and may cause adverse health effects to consumers following consumption.

Mildly preserved food products with minimal intervention during manufacture (e.g., those receiving a mild heat process) rely on a combination of different preservative factors (called hurdles) to achieve microbiological safety and stability by inhibiting growth of microorganisms. This approach is the basis of the “hurdle technology” concept, as originally introduced by Leistner and coworkers (20, 21). The quest for producing “healthy” foods with

less artificial (and more natural) preservatives requires a greater understanding of the individual and combined effect of the preservatives on the growth rate of microorganisms in foods.

The European Union regulation (13) states that predictive mathematical modeling can be used by food business operators as one of the studies to investigate compliance with microbiological criteria throughout shelf-life. This applies particularly to ready-to-eat foods that are able to support the growth of *L. monocytogenes* and that may pose a risk for public health. Several predictive growth models have been already developed (1, 24, 35). Among them, modular models such as the gamma-type models developed by Zwietering et al. (45) allow the quantification of individual and combined preservative factors or hurdle effects on the bacterial growth rate. Subsequently, different combinations of hurdles (formulations) can be compared to each other to derive inhibitory effect equivalences, namely, “iso-hurdle rules.” Likewise, the lag times of bacteria generated by equivalent inhibitory hurdles should be similar if the amount of work done by the cells during the lag time prior to growth is independent of the environmental conditions (32). Other modeling approaches dealing with the hurdle concept have been reported previously in the literature. For instance, several authors have developed growth/no-growth mod-

Received 26 August 2011 Accepted 28 November 2011

Published ahead of print 5 December 2011

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doi:10.1128/AEM.06691-11

FIG 1 Diagram illustrating the objectives of this study: new preservative combination assessment and iso-hurdle rules deduced from a model built with limited sets of data.

els with *Listeria* (10, 16, 22, 40, 41, 43, 44), while other authors have introduced the principle of equivalence in preservative systems for *Listeria monocytogenes* in the growth area (28).

The advantage of using the gamma structure to establish iso-hurdle rules is first of all that its structure, with multiplicative terms, enables the visualization of iso-hurdles instantaneously, since each gamma term is characterized directly by its associated factor-inhibitory effect (Fig. 1). Second, when reusing an existing but disparate data set, the gamma model structure enables the determination of “equivalent” preservative factors which were not initially studied together. For instance, it is theoretically possible to assess the NaCl versus acetic acid effects, based on a data set initially generated to investigate the effects of temperature, pH, and water activity (a_w) on the one hand, and, on a data set initially generated to investigate the effects of temperature, pH, and acetic acid on the other hand (Fig. 1). That is definitively an asset in comparison with a number of studies reported in the literature, designed to derive iso-hurdle rules from a unique substantial piece of work. Indeed, it is common for food companies to generate and accumulate disparate data, disparate in the sense that different data sets would have been generated for different and specific purposes. Such data may describe the growth of microbiological contaminants associated with their food production, whether the purpose is for safety or quality checks, and then might be combined and reused for future developments.

In this context (and unlike most of the studies reported in the literature), we present here a methodology aiming to assess equivalence of preservative system effects in the growth area, without generating many data. The methodology consists of three steps: (i)

building a gamma model on existing data, (ii) building an experimental design focused on the iso-hurdle using the model output, and (iii) validating the model and then consequently the iso-hurdle rules by generating a limited number of experimental data.

The methodology is illustrated with *L. monocytogenes*, on the basis that it remains a serious threat for the safety of ready-to-eat foods and numerous data have been published and are available. The environmental factors of interest are temperature, pH, a_w , and acetic, lactic, and sorbic acids. Once the methodology is successfully tested, a practical application of the iso-hurdle rules could consist of substituting artificial preservatives with natural ones or reducing the salt concentration, with the assurance that the same protective effect will be achieved. With this application in mind, the methodology has been developed for three specific iso-hurdle rules: (i) partial replacement of NaCl by a combination of organic acids, (ii) replacement of sorbic acid with a combination of acetic and lactic acids, and (iii) replacement of sorbic acid with a combination of either acetic or lactic acid and salt.

MATERIALS AND METHODS

Data. (i) Existing experimental data to build the predictive model. A total of 1,778 growth rates describing the influence of temperature, pH, water activity (a_w), and lactic, acetic, and sorbic acids on the growth of *L. monocytogenes* were collected from the literature, ComBase (<http://browser.combase.cc/BrowserHome/SearchOptions/SourceSearch.aspx>), and industrial trials. The choice of environmental factors was driven by the food type application. In our case, the foreseen application was an ambient stable dressing type product with a preservative system based upon mild acidity (pH and organic acids) and salt. The database was then treated according to four different quality criteria presented in Table 1, so that the remaining data set was appropriate for modeling purposes. The criteria were defined by experts in microbiology considering the following principles: since microbiological experimental error is commonly accepted to be $0.5 \log_{10}$ CFU/ml, it was considered that bacterial growth should be assessed with values strictly twice bigger than the error ($>1 \log_{10}$); the limit was rounded up to $2 \log_{10}$ (criterion 2). Since specific growth rate values are often reported to be less than 2 h^{-1} , higher values were considered rare and values above 3 h^{-1} were discarded (criterion 4).

The first step of data selection resulted in 650 growth data points corresponding to 25 data sets from multiple sources (Table 2). The growth data include growth logcount curves (90%) or maximum specific growth rates (10%). Growth was observed in laboratory culture media, milk and dairy products, egg products, and dessert foods for a number of different strains of *L. monocytogenes*. The data on other foods such as smoked salmon or processed meat were not eliminated on purpose but did not pass the data elimination process. The environmental factors were temperature (1 to 40°C), pH (4.5 to 8.2), water activity (0.911 to 0.997), and sorbic (0.025 to 0.3% of potassium sorbate), acetic (0.05 to 1% of sodium acetate), and lactic (0.05 to 2% of sodium lactate) acids. With the data sets used to estimate the temperature, pH, and a_w factors, a single maximum specific growth rate at optimum growth conditions (μ_{opt}) value was used for the broth medium, e.g., for all of the temperature data sets in culture medium, the same μ_{opt} value was used, regardless of the strains or the broth composition. However, for the data sets used to identify the acid-related parameters, a different μ_{opt} value was used for each medium and source, considering that the experimental protocol carried out to perform experiments with organic acids may vary with authors and studies (even if applied to the same medium).

(ii) Experimental design based on iso-hurdle rules to validate the predictive model and confirm the equivalence in growth inhibitory effects. To validate the model, a total of 20 experimental conditions (each in duplicate) were tested in broth at 23°C (temperature typically used in industrial challenge test studies for acidic ambient stable products sold in nontropical regions). The experimental design was also built to verify

TABLE 1 Four criteria with their conditions for data quality checks^a

Criterion	Conditions of elimination ^b
Criterion 1: extra factors	
Atmosphere	Vacuum packed, anaerobic conditions, modified atmosphere*
Process or formulation	Irradiated, benzoic acid*
Other flora in the medium	Microorganisms other than <i>Listeria monocytogenes</i> , medium not sterilized*
Criterion 2: lack of interpretability of the logcount curves	
Difference between the final concn and the initial concn	<100 CFU/ml
No. of points in the exponential phase	Not informative enough (e.g., one point in the lag time and one point in the stationary phase)
Criterion 3: data not suitable for estimation	
No. of data in the data set	Less than the no. of parameters to estimate
Nonvarying factors in the data set	Not close to the optimum
Criterion 4: high variation within a given experimental condition	
Same experimental condition of factors (<i>T</i> , pH, <i>a_w</i> , and acids): [$\sigma(\mu_{\max})/E(\mu_{\max})] \times 100^c$	>20%
Very high values of maximum specific growth rates	>3 h ⁻¹

^a The criteria were applied successively, i.e., if the data passed the conditions associated with the criterion 1, then the conditions associated with criterion 2 were reviewed, and so on.

^b *, nonexhaustive list of conditions.

^c Where $\sigma(\mu_{\max})$ is the standard deviation of the growth rate, $E(\mu_{\max})$ is the mean growth rate, and $\mu_{\max}$ is the maximum specific growth rate.

the inhibition equivalence (iso-hurdle rules) of various preservative formulations. Three iso-hurdle rules were selected based on current consumer preferences and industry demands for products formulated with lower concentrations of chemical preservative (such as sorbic acid) and

lower salt concentrations. They were (i) replacement of NaCl by a combination of organic acids (eight experimental conditions), (ii) replacement of sorbic acid with a combination of acetic and lactic acids (eight experimental conditions), and (iii) replacement of sorbic acid with a com-

TABLE 2 Sources, factors, range, and number of levels for the disparate datasets used in this study^a

Data set	No. of individual growth rates	Temp (°C) (levels)	pH (levels)	<i>a_w</i> (levels)	Acid salt		Source or reference ^b	Medium or food product	Estimated parameter in the "sequential" method	μ_{opt} no.
					Nature	Concn (%) (levels)				
1	8	5–30 (5)	7	0.997		0	Logcount curves	6	Culture medium	$T_{\min}$
2	52	4–11.6 (8)	7.1	0.997		0	Logcount curves	ENVA*	Culture medium	$T_{\min}$
3	6	4–40 (3)	7.1	0.997		0	Growth rates	11	Skimmed milk	$T_{\min}$
4	14	4–35 (5)	7.1	0.997		0	Growth rates	33	Milk and dairy products	$T_{\min}$
5	14	4–35 (5)	7.1	0.997		0	Growth rates	33	Cream	$T_{\min}$
6	13	4–35 (5)	7.1	0.997		0	Growth rates	33	Whole milk	$T_{\min}$
7	14	4–35 (5)	7.1	0.997		0	Growth rates	33	Skimmed milk	$T_{\min}$
8	22	2–15 (5)	5–7 (5)	0.997		0	Logcount curves	FSA CCFRA B165*	Culture medium	pH _{min}
9	22	2–15 (5)	4.5–7 (6)	0.997		0	Logcount curves	FSA CCFRA B166*	Culture medium	pH _{min}
10	9	5–10 (3)	5.4–7 (3)	0.997		0	Logcount curves	FSA CCFRA L11*	Milk	pH _{min}
11	9	4–20 (4)	5.5–7 (6)	0.997		0	Logcount curves	FSA IFR B117*	Culture medium	pH _{min}
12	44	4–20 (4)	4.5–7 (11)	0.997		0	Logcount curves	FSA IFR B288*	Culture medium	pH _{min}
13	7	4–35 (4)	5–5.6 (2)	0.997		0	Logcount curves	12	Culture medium	pH _{min}
14	5	21	7	0.911–0.929 (6)		0	Logcount curves	26	Culture medium	<i>a_w</i> min
15	6	5–19 (2)	7.2	0.964–0.997 (4)		0	Logcount curves	7	Culture medium	<i>a_w</i> min
16	79	5–35 (8)	4.5–7.4 (10)	0.950–0.997 (6)		0	Logcount curves	FSA IFR B113*	Culture medium	<i>a_w</i> min
17	70	1–10 (4)	5–7.1 (19)	0.924–0.997 (12)		0	Logcount curves	FSA IFR B413*	Culture medium	<i>a_w</i> min
18	29	4–20 (4)	4.5–7 (12)	0.95–0.997 (5)		0	Logcount curves	FSA IFR B428*	Culture medium	<i>a_w</i> min
19	9	5–15 (3)	5.8–6.6 (3)	0.992–0.995 (3)		0	Logcount curves	FSA IFR D0*	Egg or egg product	<i>a_w</i> min
20	9	5–15 (3)	5.8–6.6 (3)	0.992–0.995 (3)		0	Logcount curves	FSA IFR D11*	Dessert food	<i>a_w</i> min
21	3	28	7	0.93–0.99 (3)		0	Growth rates	42	Culture medium	<i>a_w</i> min
11	32	4–20 (4)	5–7 (6)	0.997	Sodium acetate	0.05–1 (9)	Logcount curves	FSA IFR B117*	Culture medium	MIC _{acetic}
22	13	4–20 (4)	5–7 (6)	0.997	Sodium acetate	0.1–0.85 (8)	Logcount curves	FSA IFR B412*	Culture medium	MIC _{acetic}
13	32	13–35 (3)	5–5.6 (2)	0.997	Potassium sorbate	0.05–0.3 (6)	Logcount curves	12	Culture medium	MIC _{sorbic}
23	13	8–20 (3)	5.1–6 (4)	0.997	Potassium sorbate	0.025–0.2 (5)	Logcount curves	FSA IFR B234*	Culture medium	MIC _{sorbic}
12	64	4–20 (4)	4.5–8.2 (13)	0.997	Sodium lactate	0.05–1.5 (12)	Logcount curves	FSA IFR B288*	Culture medium	MIC _{lactic}
23	5	16	5–6.1 (3)	0.997	Sodium lactate	0.05–0.25 (2)	Logcount curves	FSA IFR B234*	Culture medium	MIC _{lactic}
24	9	1–20 (5)	6.1–7.2 (4)	0.997	Sodium lactate	0.2–1 (3)	Logcount curves	FSA IFR B289*	Culture medium	MIC _{lactic}
25	38	2–20 (5)	5–7 (11)	0.997	Sodium lactate	0.25–2 (11)	Logcount curves	FSA IFR B292*	Culture medium	MIC _{lactic}

^a For each subset of data, the corresponding estimated parameter and μ_{opt} reference number are also indicated.

^b *, ComBase (www.combase.cc) source or key.

TABLE 3 Experimental design for model validation based on three iso-hurdle rules and results obtained in broth (growth rates and lag times)^a

Iso-hurdle rule	Condition	pH	NaCl (%) / a _w	Sorbic acid (%)	Lactic acid (%)	Acetic acid (%)	Π γ(.) from predictive model	μ _{max} observed (h ⁻¹)		Lag observed (h)	
								A	B	A	B
Salt vs. organic acids	1	6.5	0.997	0	2.5	0.1	0.41	0.462	0.460	4.8	5.4
	2	6.5	3.5/0.98	0	0	0	0.42	0.505	0.449	6.7	5.6
	3	5.4	0.997	0.04	0.1	0.1	0.08	0.126	0.147	16.2	18.3
	4	5.4	9.4/0.94	0	0	0	0.09	0.092	0.138	44.1	67.3
	5	5.7	0.997	0.1	0.9	0.1	0.09	0.125	0.126	18.1	8
	6	5.7	9.4/0.94	0	0	0	0.11	0.109	0.146	31.6	34
	7	5.8	0.997	0	2	0.1	0.21	0.196	0.235	12.3	10.6
	8	5.8	7.3/0.955	0	0	0	0.20	0.225	0.235	18.7	17.1
Sorbic acid vs. acetic and lactic acids	9	6.1	0.997	0	1.8	0.3	0.23*	0.263	0.211	7.4	12.3
	10	6.1	0.997	0.1	0	0	0.26*	0.309	0.332	6.2	5.2
	11	5.5	0.997	0	1.1	0.1	0.14	0.141	0.148	33.1	32.6
	12	5.5	0.997	0.09	0	0	0.14	0.136	0.111	10	14.2
	13	5.5	4.8/0.972	0	1.1	0.1	0.10	0.087	0.115	46.5	38.9
	14	5.5	4.8/0.972	0.09	0	0	0.10	0.135	0.199	17.5	19.6
	15	5.5	4.8/0.972	0	1.4	0.15	0.07	0.068	0.072	77.7	64.7
	16	5.5	4.8/0.972	0.15	0	0	0.07	0.062	0.078	57.5	48
Sorbic acid vs. lactic/acetic acid and salt	17	5.8	0.997	0.05	0	0	0.24†	0.308	0.315	6	5.7
	18	5.8	3.5/0.98	0	0	0.12	0.21†	0.398	0.311	14.3	6.1
	19	5.2	0.997	0.05	0	0	0.12	0.128	0.150	13.4	14.1
	20	5.2	6.6/0.96	0	0.4	0	0.13	0.107	0.120	58.9	46.4

^a "A" and "B" represent two independent replicates. Values in boldface indicate the level of factors changed to achieve a given iso-hurdle.

^b *, even if there was a slight difference in Π γ(.) (0.23 and 0.26), these two experimental conditions were considered equivalent in terms of inhibitory formulation. †, likewise, experimental conditions with Π γ(.) set at 0.21 and 0.24 were considered equivalent.

bination of either acetic or lactic acid and salt (four experimental conditions). To provide further flexibility in product formulation, each rule was tested at various pH conditions (four, two, and two pH levels for rules i, ii, and iii, respectively). The levels of environmental factors were set to cover a range of inhibition varying from harsh (low gamma values of the model, γ ≈ 0.1) to mild (γ ≈ 0.5) conditions, with gamma values equally distributed between these two limits. Moreover, the levels tested corresponded to levels either observed or foreseen in dressings type products. The experimental design is presented in Table 3.

(iii) Organism, preparation of the inoculum, and inoculation procedure. Eleven strains of *L. monocytogenes* were obtained from Unilever. The strains were a mix of product isolates, factory isolates, and outbreak strains. They were stored using cryotubes at -85°C until use. Each strain was spread onto a tryptone soy agar (TSA; Oxoid) plate. Plates were incubated at 30°C for 3 days, inspected for purity with Gram coloration and ALOA confirmation (AES), and stored at 5°C for a maximum of a week before use. Three colonies were taken from each plate (to ensure a sufficient inoculum) and suspended in 10 ml of tryptone soy broth (TSB; Oxoid). Cultures were incubated at 30°C for 24 h and enumerated using a Thoma slide under the microscope. Each strain was mixed equally to give approximately 2 × 10⁵ CFU/ml in the final cocktail. Each cocktail was prepared just before inoculation into 100 g of culture medium of defined composition. Duplicate compositions were each inoculated with 500 μl of the *L. monocytogenes* cocktail (to give an initial inoculum of approximately 1,000 CFU/ml in each 100-g sample). The inoculated samples were mixed well and enumerated immediately (as described below) to give a time = 0 reading.

(iv) Preparation of culture medium. The culture medium was TSB (Oxoid) supplemented with sodium chloride (Sigma), glacial acetic acid solution (Sigma), potassium sorbate (Sigma), and L-(+)-lactic acid solution (Sigma) according to the experimental design. Solutions were filter sterilized by using a presterilized filter apparatus containing a membrane with a pore diameter of 0.2 μm (Nalgene disposable filter unit, MF75

series). The salt concentration was converted into water activity by using the equations below, developed by Resnik and Chirife (31):

$$a_w = 1 - 0.0052471 \times WPS - 0.00012206 \times WPS^2 \quad (1)$$

$$WPS = \frac{100 \times \% \text{ NaCl}}{\% \text{ moisture} + \% \text{ NaCl}} \quad (2)$$

where WPS is the water phase salt estimated from the sodium chloride percent (wt/vol) concentration.

(v) Sampling and enumeration. The growth of *L. monocytogenes* was followed by plate count. At each specified time point, 10-fold serial dilutions were made from samples, using maximum recovery diluent (Oxoid). An aliquot (100 μl) of the neat sample and each dilution was spread onto TSA plates. All plates were incubated for at least 5 days at 30°C before colonies were counted (a preliminary count was performed at 2 days).

(vi) Logcount curves for determination of the parameter μ_{opt} in TSB. The parameter μ_{opt} was deduced from two experiments carried out under optimal conditions: the temperature was 37°C, the pH was 7.1, and no salt or acid was added.

Predictive models. (i) Primary model. All of the data extracted from ComBase or from the industrial trials were logcount curves, while data from literature were either reported as logcount curves or directly as maximum specific growth rates, μ_{max} (h⁻¹). For all logcount curves, μ_{max} values were obtained by fitting the growth curves with the logistic model with delay (4, 18). This model was also used to fit the new experimental curves generated to validate the model. For data consisting of maximum growth rates from the literature, since the primary model used to calculate μ_{max} was reported in the corresponding paper, corrective factors proposed by Augustin and Carlier (1) were applied, when the growth rates were not calculated with the logistic model with delay. Values of 1.00, 0.84, 0.86, and 0.97 were used for the exponential, Gompertz, logistic, and Baranyi models, respectively.

(ii) **Secondary model.** The secondary model is based on the gamma concept (45), which consists of independently quantifying the individual effect of each factor involved in the observed bacterial behavior. The gamma concept has been successfully applied on *L. monocytogenes* in the last 20 years (2, 10, 22, 24).

The model can be written as follows:

$$\sqrt{\mu_{\max}} = \sqrt{\mu_{\text{opt}} \times \gamma(T) \times \gamma(\text{pH}) \times \gamma(a_w) \times \gamma(\text{acids})} \quad (3)$$

where $\mu_{\max}$ is the maximum specific growth rate in a specific environmental condition. μ_{opt} is the maximum specific growth rate obtained at optimum environmental conditions for growth. This parameter only describes the effect of the medium, i.e., food or broth specificity plus possibly an unstudied matrix-dependent factor such as fat content. The terms $\gamma(T)$, $\gamma(\text{pH})$, $\gamma(a_w)$, and $\gamma(\text{acids})$ represent the effects of temperature, pH, water activity, and organic acids on bacterial growth, respectively. The $\gamma(\cdot)$ values vary between 0 and 1; for a given factor, a value of 0 indicates that microbial growth is prevented, and a value of 1 indicates that the potential growth is optimal. To homogenize the error variance, a square root transformation was applied to $\mu_{\max}$ before estimating the parameters.

The cardinal model (36) was used to quantify the influence of temperature, pH, and a_w on the bacterial growth rate. The effects were calculated as follows:

$$\gamma(X) = \begin{cases} 0 & \text{if } X_{\min} \leq X \\ \frac{(X - X_{\max}) \times (X - X_{\min})^n}{(X_{\text{opt}} - X_{\min})^{n-1} \times \{[X_{\text{opt}} - X_{\min}] \times [X - X_{\text{opt}}] - [X_{\text{opt}} - X_{\max}] \times [(n-1) \times X_{\text{opt}} + X_{\min} - n \times X]\}} & \text{if } X_{\min} < X < X_{\max} \\ 0 & \text{if } X \geq X_{\max} \end{cases} \quad (4)$$

where $X_{\min}$, X_{opt} , and $X_{\max}$ are the minimal, optimal, and maximal values, respectively, of the factor for bacterial growth. The n value was set to 2 for temperature, and 1 for pH and water activity (9).

Since no data were available in the range above the optimum for a_w and only limited data were available in the range above the optimum for temperature and pH, the parameters T_{opt} , $T_{\max}$, pH_{opt} , $\text{pH}_{\max}$, $a_{w\text{opt}}$, and $a_{w\max}$ were not estimated but fixed in the fitting procedure. $T_{\max}$ and T_{opt} were fixed at 45 and 37°C, respectively, $\text{pH}_{\max}$ and pH_{opt} were fixed at 9.4 and 7.1, respectively, and $a_{w\max}$ and $a_{w\text{opt}}$ were fixed at 1 and 0.997, respectively, (17).

For the acid model, the multiplicative effect of the weak acid model was used (9). It was calculated as follows:

$$\gamma(\text{acids}) = \gamma(\text{HA}_{\text{sorbic}}) \times \gamma(\text{HA}_{\text{acetic}}) \times \gamma(\text{HA}_{\text{lactic}}) \quad (5)$$

where $\gamma(\text{HA}_{\text{sorbic}})$, $\gamma(\text{HA}_{\text{acetic}})$, and $\gamma(\text{HA}_{\text{lactic}})$ are the effects of undissociated sorbic, acetic, and lactic acids, respectively. Each acid term was determined as follows:

$$\gamma([\text{HA}]) = 1 - \left(\frac{[\text{HA}]}{\text{MIC}} \right)^\alpha \quad (6)$$

where, [HA] is the undissociated concentration of the considered acid (i.e., sorbic, acetic, or lactic acid) and MIC is its minimal inhibitory concentration. The α parameter was fixed at 0.3, 0.5, and 1 for sorbic, acetic, and lactic acid, respectively, as in Zuliani et al. (44).

Finally, in the fitting procedure, the estimated parameters were $T_{\min}$, $\text{pH}_{\min}$, $a_{w\min}$, $\text{MIC}_{\text{sorbic}}$, $\text{MIC}_{\text{acetic}}$, and $\text{MIC}_{\text{lactic}}$.

Statistical methods. (i) Estimation procedure. The predictive models were built using the disparate existing data sets. Two fitting procedures were used, a “sequential” and a “simultaneous” method. The sequential method consists of estimating the effect of each gamma term successively, using its associated subset of data (Table 2). For example, to get $T_{\min}$, data sets with only T varying were used. To get $\text{pH}_{\min}$, data sets with T and pH

varying were used, with parameters for the model of temperature already estimated. Since more data are available in literature or in ComBase for the effects of temperature than for the other factors, the temperature-related parameters were estimated first; the second highest in terms of data (pH factor) were then identified, and so on. In Table 2, the factors are presented in the same order as they have been estimated. The “simultaneous” method means that all model parameters were estimated simultaneously using the whole data set.

(ii) **Indices for performance evaluation of predictive models.** To validate the predictive models built on existing data, a comparison of their predictions against the new data (growth rates extracted from the 40 log-count curves) was made using the bias factor, the accuracy factor (34), and the discrepancy factor (5).

The bias factor (B_f) can be written as follows:

$$B_f = 10^{\sum_i^n \left(\frac{\log \left(\frac{\mu_{i,\text{observed}}}{\mu_{i,\text{predicted}}} \right)}{n} \right)} \quad (7)$$

where μ_{observed} and $\mu_{\text{predicted}}$ are the observed and predicted maximum growth rates, respectively, n is the number of observations. A bias factor of 1 corresponds to a perfect agreement between predictions and observations. If the value is <1 , it means that the model is “fail-dangerous,” in the other case of a value >1 , the model is “fail-safe.”

The second criteria was the accuracy factor (A_f):

$$A_f = 10^{\sum_i^n \left(\left| \frac{\log \left(\frac{\mu_{i,\text{observed}}}{\mu_{i,\text{predicted}}} \right)}{n} \right| \right)} \quad (8)$$

The accuracy factor indicates the spread of results about the prediction. A value of 1 indicates perfect agreement. The discrepancy factor (D_f) was determined as follows:

$$D_f = \left[\exp \left(\sqrt{\frac{\sum_i^n \left[\ln \left(\frac{\mu_{i,\text{observed}}}{\mu_{i,\text{predicted}}} \right) \right]^2}{n}} \right) - 1 \right] \times 100 \quad (9)$$

In addition, the acceptable prediction zone method (27) was utilized to evaluate visually the model performance. The relative error (RE) was calculated as follows:

$$\text{RE} = \frac{(\mu_{\text{observed}} - \mu_{\text{predicted}})}{\mu_{\text{predicted}}} \quad (10)$$

(iii) **Validation of iso-hurdles.** To assess whether the microbial growth inhibitory effects due to salt and sorbic or organic acids were equivalent, logcount curves were compared visually and growth rates were analyzed by analysis of variance (ANOVA). For each iso-hurdle tested under various pH (and a_w) conditions, a two-factor ANOVA was performed. The first factor was the iso-hurdle rule: salt versus acids in rule i, sorbic acid versus acetic and lactic acids in rule ii, and sorbic acid versus acetic or lactic and salt in rule iii; the second factor was the pH (and a_w).

Software. Model estimation procedure was performed using R software (30). The “nls” function was used for conducting nonlinear regression, and the numerical process for parameter estimation was based on the Gauss-Newton algorithm. An additional package, “nlstools,” was used to calculate the confidence intervals of the parameters and the correlation matrix. Indices of performance were calculated in Excel (Microsoft), and validation of the iso-hurdle by ANOVA was carried out using the Excel add-in XLStat (version 2011.1.04; Addinssoft).

RESULTS

Building the predictive models using the existing data set. Building the predictive models using the existing data set. To assess the equivalence in preservative system, a three-step approach was carried out. First of all, a gamma model was built, using 650 growth rates. The data came from ComBase, literature, or industrial trials and were disparate since they came from different partial studies. All parameters ($T_{\min}$, $\text{pH}_{\min}$, $a_{w\min}$, $\text{MIC}_{\text{sorbic}}$, $\text{MIC}_{\text{acetic}}$, and

TABLE 4 Estimated parameter values and their confidence intervals obtained with the sequential and simultaneous methods

Parameter	Sequential method			Simultaneous method		
	Estimated parameters	Confidence interval		Estimated parameters	Confidence interval	
		2.50%	97.50%		2.50%	97.50%
$T_{\min}$ (°C)	−0.939	−1.41	−0.472	−0.904	−1.19	−0.613
$\text{pH}_{\min}$	4.14	4.02	4.26	4.19	4.12	4.26
$a_{w \min}$	0.921	0.918	0.924	0.921	0.919	0.924
$\text{CMI}_{\text{sorbic}}$ (mM)	5.84	5.30	6.39	6.35	5.50	7.20
$\text{CMI}_{\text{lactic}}$ (mM)	16.3	2.46	30.2	9.87	6.82	12.93
$\text{CMI}_{\text{acetic}}$ (mM)	11.3	10.1	12.4	10.91	9.42	12.4

$\text{MIC}_{\text{lactic}}$) were estimated with the two simultaneous and sequential methods. The comparison of the two methods on the parameter values with their 95% confidence interval limit is presented in Table 4. A large confidence interval for the $\text{MIC}_{\text{lactic}}$ parameter was observed when the sequential method was performed. To check whether this large confidence interval was due to the fitting method or to the lack of data, the fitting methods were also run on a simulated data set (based on a complete factorial design). For both fitting methods, the estimation of parameters was accurate (data not shown), suggesting that the difference observed with the data set analyzed in the present study is due to the scarcity of the existing data set and not to the fitting methods. The parameter correlation matrix is presented in Table 5 for the simultaneous method (all parameters are estimated together at once). Overall, no strong correlation was noticed. It should be mentioned that when the models are fitted sequentially, no correlation between parameters is assumed.

Building an experimental design focused on the iso-hurdle using the model output. The second step of the methodology involved building an experimental design focused on the iso-hurdle rules using the predictive model output (Table 3). The rules were (i) replacement of NaCl by a combination of organic acids, (ii) replacement of sorbic acid with a combination of acetic and lactic acids, and (iii) replacement of sorbic acid with a combination of either acetic or lactic acid and salt.

For the rule “replacement of NaCl by a combination of organic acids,” eight experimental conditions were tested, with condition 1 being associated with condition 2, condition 3 being associated with condition 4, condition 5 being associated with condition 6, and condition 7 with being associated 8. In detail, combination 1 was designed to achieve an overall inhibitory effect of 0.41, a value calculated using the predictive model built on existing data [$\Pi \gamma(.) = 0.41$] by the addition of lactic and acetic acids. Condition 2 was designed to achieve the

same inhibitory effect [$\Pi \gamma(.) = 0.42$] by the addition of 3.5% NaCl. The experimental design for the two other iso-hurdle rules was based on the same principle of comparison in pairs.

Validating the model and the iso-hurdle rules by generating experimental data. The last step of the methodology aimed at validating the iso-hurdle rules by generating new data. The iso-hurdle rules corresponded to 20 experimental conditions (40 log-count curves with the repetitions). The corresponding growth rates and lag times are presented in Table 3. The comparison between the observed and predicted growth rates (two fitting methods) is presented in Fig. 2. In Fig. 3, the relative error (RE) is plotted as a function of the environmental factors. These observations were completed by the indices of performance evaluation presented in Table 6.

Overall, the results obtained by the two fitting methods were very close (Fig. 2 and 3), and the bias factor values were similar: 0.98 for the sequential method and 1.00 for the simultaneous method. The analysis of the relative error plots (Fig. 3) reveals that all points but four fall within the acceptable prediction zone as defined by Oscar (27) ($\text{RE} > 0.15$). The four points outside correspond to the experimental conditions 3 (two repetitions) and 14 and 18 (one repetition each), as referred to in Table 3. The log-count curves associated with these experimental conditions were particularly scrutinized during our assessment of the equivalence of iso-hurdles rules (see below).

Equivalence in microbial-growth-inhibitory effect (“iso-hurdle rules”). For the first iso-hurdle rule, which focused on the

TABLE 5 Correlation matrix of estimated parameter factors for the simultaneous method

Parameter	Parameter				
	$T_{\min}$	$\text{pH}_{\min}$	$a_{w \min}$	$\text{MIC}_{\text{sorbic}}$	$\text{MIC}_{\text{lactic}}$
$T_{\min}$					
$\text{pH}_{\min}$	0.07				
$a_{w \min}$	0.02	0.04			
$\text{MIC}_{\text{sorbic}}$	−0.01	0.03	0.00		
$\text{MIC}_{\text{lactic}}$	−0.05	0.21	0.01	0.01	
$\text{MIC}_{\text{acetic}}$	−0.03	0.03	0.00	0.00	0.01

FIG 2 Model validation: comparison of observed and predicted growth rates (square root transformation). Sequential (○) and simultaneous (×) method data are indicated.

FIG 3 Relative error (RE) plots with an acceptable prediction zone from an RE of -0.3 (fail-safe) to 0.15 (fail-dangerous) for comparison of observed and predicted growth rate values obtained with sequential ($\circ$) and simultaneous ($\times$) method in function of independent environmental factors.

TABLE 6 Values of the bias, accuracy, and discrepancy factors for the sequential and simultaneous methods

Mathematical criterion	Sequential method	Simultaneous method
Bias factor (B_p)	0.98	1.00
Accuracy factor (A_p)	1.23	1.18
Discrepancy factor (%)	29.03	24.54

salt replacement, logcount curves obtained with $[\gamma(a_w) = k_1]$ or without salt $[\gamma(\text{acetic}) \times \gamma(\text{lactic}) \times \gamma(\text{sorbic}) = k_1 \text{ or } \gamma(\text{acetic}) \times \gamma(\text{lactic}) = k_1]$ at various pH levels were generated. As shown in Fig. 4, the trend of the exponential phase was very close for the same iso-hurdle [conditions which have the same $\Pi \gamma(\cdot)$]. This was confirmed by ANOVA in Table 7: there was no difference ($P > 0.05$) between growth rates from *L. monocytogenes* logcount curves obtained in the presence of salt or acids.

FIG 4 Results for the first iso-hurdle: logcount curves of *L. monocytogenes* in the presence of organic acids (+) or salt (O). (A) $\Pi \gamma(.) = 0.41/42$ (black symbols, experimental conditions 1 and 2) and $\Pi \gamma(.) = 0.08/0.09$ (gray symbols, experimental conditions 3 and 4). (B) $\Pi \gamma(.) = 0.21/0.20$ (black symbols, experimental conditions 7 and 8) and $\Pi \gamma(.) = 0.09/0.11$ (gray symbols, experimental conditions 5 and 6).

Moreover, as logcount curves were generated, it was also possible to assess whether the equivalence of preservative system could be applied to the lag time. Indeed, under the hypothesis of a constant amount of work (4, 32, 38), if the growth rates are equivalent, the lag times should be equivalent as well ($\mu_{max} \times lag = K$). In the case of salt replacement, under mild condition ($\gamma \geq 0.2$), the lag times observed in the presence of salt and in the presence of organic acids were similar (curves plotted in black symbols, Fig. 4). However, under more stressful conditions ($\gamma < 0.20$) the lag time observed in the presence of NaCl were significantly longer (curves plotted in gray symbols, Fig. 4). Finally, it should be noted that the pattern of the logcount curve associated with condition 3, indicated by gray “+” symbols in Fig. 4A, followed the same general trend as all curves analyzed within the first iso-hurdle rule.

For the second rule, sorbic acid replaced by organic acids, logcount curves obtained at various pH and a_w values with

$[\gamma(\text{sorbic}) = k_2]$ or without sorbic acid $[\gamma(\text{acetic}) \times \gamma(\text{lactic}) = k_2]$ were compared (Fig. 5), and growth rates were analyzed by ANOVA (Table 7). For equivalent inhibitory condition (iso-hurdle), growth rates were similar ($P > 0.05$). In the absence of NaCl (conditions plotted in Fig. 5A), the logcount curves were rather similar. In contrast, the lag time was relatively longer when salt was added to the broth, under stress conditions ($\gamma < 0.20$, Fig. 5B). This confirmed a specific effect of salt in lag time, questioning the hypothesis of a constant amount of work, i.e., $\mu_{max} \times lag = K$. The logcount curve associated with condition 14, indicated by black “+” symbols in Fig. 5B, did not present any particular pattern even if one of its repetition did not fall within the acceptable prediction zone.

A third rule, sorbic acid $[\gamma(\text{sorbic}) = k_3]$ replaced by a combination of acid (lactic or acetic) and salt $[\gamma(\text{acetic}) \times \gamma(a_w) = k_3 \text{ or } \gamma(\text{lactic}) \times \gamma(a_w) = k_3]$, was also tested. Logcount curves are de-

TABLE 7 Outputs of the ANOVA performed to assess the three iso-hurdle rules

Iso-hurdle rule	Source ^a	DF	Sum of square	F	Pr > F
Salt vs. organic acids	Model	4	0.291	109.5	<0.0001
	Error	11	0.007		
	Total error	15	0.299		
	R ²	0.98			
	Hurdle	1	0.000	0.002	0.965
	Other factors	3	0.291	146.000	<0.0001
Sorbic acid vs. acetic and lactic acids	Model	4	0.146	23.392	<0.0001
	Error	11	0.017		
	Total error	15	0.163		
	R ²	0.89			
	Hurdle	1	0.005	3.185	0.102
	Other factors	3	0.141	30.128	<0.0001
Sorbic acid vs. lactic/acetic acid and salt	Model	2	0.101	76.04	0.000
	Error	5	0.003		
	Total error	7	0.104		
	R ²	0.97			
	Hurdle	1	0.003	3.929	0.104
	Other factors	1	0.098	148.148	<0.0001

^a Hurdle, this factor corresponds to the iso-hurdle rule tested, for example, salt versus organic acids; other factors, the other factor correspond to the environmental factor not directly involved in the iso-hurdle, for example, pH.

FIG 5 Results for the second iso-hurdle rule: logcount curves of *L. monocytogenes* in the presence of lactic and acetic acids (○) or sorbic acid (+). (A) $\Pi \gamma(.) = 0.23/0.26$ (black symbols, experimental conditions 9 and 10) and $\Pi \gamma(.) = 0.14$ (gray symbols, experimental conditions 11 and 12). (B) $\Pi \gamma(.) = 0.10$ (black symbols, experimental conditions 13 and 14) and $\Pi \gamma(.) = 0.07$ (gray symbols, experimental conditions 15 and 16).

picted in Fig. 6, and the output of the ANOVA is displayed in Table 7. No significant difference ($P > 0.05$) between growth rates obtained in the presence of sorbic or acids/salt was noticed. Again, the lag times were equivalent under mild inhibitory conditions ($\gamma \geq 0.20$), while under more stressful conditions the lag times were longer in the presence of salt than in the presence of sorbic acid. This iso-hurdle rule analysis included the logcount curve associated with condition 18, indicated by the black “○” symbols in Fig. 6, for which one of the repetitions did not fall within the acceptable prediction zone.

In conclusion, the results presented here showed that the three iso-hurdle rules derived from a gamma-type model built with disparate data were confirmed experimentally with regard to the growth rates. This indicates that the gamma model built in a first step using existing data could be used for predictions even if four data points were not in the acceptable prediction zone (validation step). Moreover, as logcount curves were generated, it was also possible to assess whether the hypothesis $\mu_{\max} \times \text{lag} = K$ might be applied. The inverse of lag time is plotted against the growth rates, with a distinction between experimental conditions in the

presence of salt addition or not (Fig. 7). Under the hypothesis $\mu_{\max} \cdot \text{lag} = K$ (32), data will follow a linear pattern. This was the case with our set of experimental conditions, even if relatively longer lag times were observed in the presence of salt. To further investigate this point, we searched ComBase for experimental conditions where growth rates are very similar, and the main controlling factors were either salt or organic acids. A selection of data (Table 8) provides additional evidence (three of four sets of conditions examined) that the lag times observed for conditions for which NaCl is the main controlling factor are longer than lag times reported for conditions where organic acids are the main controlling factor.

DISCUSSION

We presented here a methodology for establishing iso-hurdle rules based on the modeling of disparate existing data sets. The methodology is illustrated with data collected on *L. monocytogenes* under various experimental conditions. After data selection, 650 growth rates were retained for further analysis. Existing data from

FIG 6 Results for the third iso-hurdle rule: logcount curves of *L. monocytogenes* in the presence of sorbic acid (+) or lactic/acetic acid and salt (○). $\Pi \gamma(.) = 0.24/0.21$ (black symbols, experimental conditions 17 and 18) and $\Pi \gamma(.) = 0.12/0.13$ (gray symbols, experimental conditions 19 and 20).

FIG 7 Comparison of observed growth rate and lag time (expressed as $1/\text{lag}$). Experimental conditions without any addition of salt (□, black symbol). Experimental conditions with an addition of salt (△, gray symbol). Linear regression for all conditions (—), $y = 0.614x$.

TABLE 8 Selected data for *L. monocytogenes* from ComBase for conditions that have the same or very similar growth rates, comparing lag times using NaCl or organic acids as the main controlling factors^a

ComBase ID	Temp (°C)	pH	NaCl (%)	Lactic acid (%)	$\mu_{\max}$ (h ⁻¹)	Lag (h)	K = $\mu_{\max} \times \text{lag}$
B292_77	20	5.5	5	0.5	0.20	65	13.0
B288_129	20	5.5	0.5	1.5	0.18	41.8	7.7
B113_45	25	4.9	7	0	0.22	58.8	12.8
B292_193	20	5.1	2.5	0.25	0.21	23.6	5.0
B113_64	20	6	4	0	0.32	34.3	11.0
B292_52	20	6	2.5	1.5	0.33	119.0	6.2
B113_65	20	6	6	0	0.28	45.8	13.0
B288_66	20	5.5	0.5	1	0.24	20.8	5.0

^a Values in boldface indicate the level of factors changed to achieve a given iso-hurdle.

the literature, databases, and/or industrial partners have already been used in the predictive microbiology area, but only for comparing several secondary models. For instance, Augustin et al. (1) collected 588 growth rates of *L. monocytogenes* for estimating parameters of five gamma-type secondary models, and Mejlholm et al. (24) used 1,014 growth rates to evaluate the performance of six secondary models. Conversely, our focus was not on the choice of the secondary model but on the methodology to fit a given model (i.e., to estimate the parameters of the model) using disparate data sets. The model chosen was derived from Coroller et al. (9) and was adapted to include the six following environmental factors: temperature, pH, a_w , and acetic, lactic, and sorbic acids. In terms of data selection, a systematic procedure was applied. The four criteria that we suggested are universal (i.e., they can be applied to other microorganisms) and simple to implement, allowing transparency and repeatability of the approach in terms of data management. However, we should emphasize that data selection is time-consuming and requires microbiology skills.

To fit the secondary model to the data, two methodologies were used. The first one, named “sequential method,” assessed the effect of each preservative factor using only the subset of data in which this factor was studied; the second one, “simultaneous method,” assessed the effect of the preservative factors altogether using the whole set of data. There was no significant difference between the model parameter values obtained from the two methods. However, the examination of the confidence interval boundaries revealed that the “simultaneous method” provided more accurate results in this case study. It should be mentioned that this conclusion cannot be applied generally since it depends on the data set (number of levels for each factor, number of repetitions, range of the levels, etc.). Both methods give the same results if the data come from a full factorial design. In the future, for applications of the methodology with other data sets (e.g., for other microorganisms), it is recommended to solve the model equations using both sequential and simultaneous methods and to check the parameter estimation relevance using statistical tools such as confidence interval boundaries and a parameter correlation matrix before drawing any final conclusions on the possibility of reusing an existing data set.

The secondary model was validated by generating new data. This validation procedure is highly recommended in predictive microbiology. It is performed by comparing predicted and observed growth rates visually and through indices of performance (3, 24, 29). In the present study, the new data generation was limited; only 20 experimental conditions were tested to assess the

inhibitory effect of five factors. This two-step procedure, i.e., combining the use of existing data for building the model and the generation of a limited set of new experimental data for validating it, enables the food industry, and other groups generating predictive models, to save time and money when assessing the effect of preservative systems on pathogenic and/or spoilage microorganisms.

Once the gamma model was built, the inhibitory effect of one environmental factor on the growth rate of *L. monocytogenes* could be quantified independently of the level of other environmental factors. This direct application of the gamma-type secondary model has not often been emphasized in the literature, although this model structure is definitely an asset when comparing preservative system efficiency. It enables the building of experimental designs in such a way that growth curves can be directly compared, for instance, the growth in the presence of salt or acids should be similar (e.g., experimental conditions of the first two rows of Table 3 designed to provide similar growth rates). The methodology developed here enables the testing of iso-hurdles involving factors which were not initially studied together. For example, in the existing data set used to build the model, the sorbic acid factor was not studied in combination with the acetic acid factor. Nevertheless, the secondary model based on the gamma concept enabled assessment of the effect of these two acids, and subsequently to suggest a rule such as “replacement of sorbic acid by acetic acid.”

This flexibility is definitively a real advantage when reanalyzing disparate data generated for different and specific purposes. Moreover, the fact that the validation of the model, and then the iso-hurdle rules, is done through generating logcount curves provides another advantage. A microbiologist without modeling skills might be much more convinced in the applicability of iso-hurdle rule by having access to the logcount curves rather than checking the model performance criteria. This helps food microbiologists and risk assessors to have confidence in model predictions and consequently could facilitate a change of practice when assessing safety and stability of food formulations.

In our study, the model was developed with growth rates as outputs (model responses) and, consequently, the experimental plan was designed to validate three iso-hurdle rules targeting the growth rates. That was successfully done with the three iso-hurdle rules. Examination of the lag times shows an effect of NaCl on the work to be done in stressful conditions ($\gamma < 0.20$). Our data, completed by a few sets of ComBase data, indicated that the hypothesis of a constant amount of work (32) might be questioned

when NaCl is used as a preservative factor, in stress conditions. That is in line with recent results reported in the literature showing a specific effect of NaCl on *L. monocytogenes* lag time (23, 25).

For practical application of the methodology developed in the present study and illustrated with *L. monocytogenes*, the following conclusions could be drawn. For sorbic replacement by organic acids, our results indicated that predictive growth models can be successfully used to determine the concentration of organic acids to be utilized in order to achieve an equivalent level of growth inhibition. Moreover, analysis of the lag times showed that the “sorbic replacement by acetic/lactic acids” rule could be applied not only to growth rates but also to shelf-life estimation. On the other hand, under stressful conditions ($\gamma < 0.20$), replacing salt by a combination of lactic and acetic acids may lead to design a food product with a shorter lag time and then consequently to a shorter shelf-life than expected. To tackle this drawback, an alternative to the simple equation $\mu_{\max} \times \text{lag} = K$ might be investigated. More generally, for future use of the methodology presented here, one may recommend to carry out the experimental validation work by generating logcount curves since they illustrate directly how the iso-hurdle rules might be utilized to achieve the same level of inhibition, applied to growth rate, and/or to shelf-life (calculated with or without including the lag time, the latter being a conservative option). That is particularly valuable if the methodology is transferred to spoilage bacteria where some limited growth is possible.

In conclusion, a methodology for assessing the effect of preservative factors on microbial growth has been developed. It includes three steps: (i) building a predictive model on existing data, (ii) building an experimental design focus on the iso-hurdle using the model output, and (iii) validating the model and the iso-hurdle rules with new data. The gamma model structure is appropriate for analyzing an existing data set, even if the data are disparate. It also enables direct visualization of the preservative equivalence (same γ values) and facilitates building easily an experimental design focused on the iso-hurdle rule of interest. A limited number of curves are generated to validate the model and then the iso-hurdle rules; this last step, important to research and development microbiologists, risk assessors, food product designers, illustrates that the same inhibitory effects can be obtained for their product formulation. The methodology was successfully applied to *L. monocytogenes* growth rates with the factors temperature, pH, a_w , and sorbic, acetic, and lactic acids. However, it has to be utilized with care to predict shelf-life since the lag time was not systematically well predicted. Nevertheless, the concept of iso-hurdle rules for the replacement of salt and sorbic acid, in the growth area, yielded promising results for *L. monocytogenes* and should be explored further, for instance, for spoilage microorganisms. This will help the food industry to develop milder formulations which guarantee microbial safety and stability, without generating too many data.

ACKNOWLEDGMENTS

We gratefully acknowledge Liliya Ivanova for her technical assistance and Alejandro Amézquita for his valuable comments and insights.

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