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Comparing Nonsynergy Gamma Models and Interaction Models To Predict Growth of Emetic *Bacillus cereus* for Combinations of pH and Water Activity Values▽

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This research aims to test the absence (gamma hypothesis) or occurrence of synergy between two growth-limiting factors, i.e., pH and water activity (a_w), using a systematic approach for model selection. In this approach, preset criteria were used to evaluate the performance of models. Such a systematic approach is required to be confident in the correctness of the individual components of the combined (synergy) models. With *Bacillus cereus* F4810/72 as the test organism, estimated growth boundaries for the a_w-lowering solutes NaCl, KCl, and glucose were 1.13 M, 1.13 M, and 1.68 M, respectively. The accompanying a_w values were 0.954, 0.956, and 0.961, respectively, indicating that equal a_w values result in similar effects on growth. Out of the 12 models evaluated using the preset criteria, the model of J. H. T. Luong (Biotechnol. Bioeng. 27:280–285, 1985) was the best model to describe the effect of a_w on growth. This a_w model and the previously selected pH model were combined into a gamma model and into two synergy models. None of the three models was able to describe the combined pH and a_w conditions sufficiently well to satisfy the preset criteria. The best matches between predicted and experimental data were obtained with the gamma model, followed by the synergy model of Y. Le Marc et al. (Int. J. Food Microbiol. 73:219–237, 2002). No combination of models that was able to predict the impact of both individual and combined hurdles correctly could be found. Consequently, in this case we could not prove the existence of synergy nor falsify the gamma hypothesis.

The microorganism *Bacillus cereus* is associated with food spoilage as well as food poisoning (1, 34). The spores formed by *B. cereus* generally will resist treatments used to prolong the shelf life of food. Viable spores present in a food product may germinate, and the vegetative cells can subsequently grow if conditions are favorable, leading to spoilage of the food product (9, 14, 18). Several growth-limiting factors, collectively referred to as hurdles, can be used to ensure food stability and safety. Examples of such hurdles are low pH, low water activity (a_w), and low temperature (12). Combining hurdles to achieve food stability and safety, known as hurdle technology, can be used to achieve an overall level of protection in food while minimizing detrimental impacts on food quality (19).

Improved quantification of the combined impact of hurdles on growth of microorganisms is an ongoing endeavor, but there are different views of how antimicrobial factors combine. One view is that there are interactive effects between hurdles. When combinations of hurdles are used, they might give significantly greater protection than expected on the basis of the application of the individual hurdles, so called synergy (19). The other view follows the gamma hypothesis (39) in which there is no synergy, but inhibitory environmental factors combine in a multiplicative manner to produce the observed overall microbial inhibition. Evidently, it is important in the selection of hurdles to know whether either the gamma hypothesis is valid or synergy occurs between factors. Assuming synergy where this does not occur can lead to wrong estimations of growth boundaries, which in turn can lead to unsafe food products.

Our previous study of testing the combined effect of pH and undissociated acid concentration did not confirm that there were synergistic effects between these two hurdles, which by definition are closely related (6). This finding was in line with several other studies (15, 16, 24, 36, 38). However, there have also been studies showing that interaction occurs when various hurdles are combined, and for these interactions, gamma models, including a synergy factor, were developed (4, 20, 29).

It is evident that quite different conclusions have been drawn in the studies in the field of quantification of the microbial growth impact of combined hurdles. The underlying variation in test organisms and preservative factors as well as the different experimental approaches employed may well have contributed to the different conclusions. In our previous study we advocated a systematic approach for model selection. This approach was based on using a set of predetermined criteria to more objectively judge the performance of individual models. In the current study, we used this systematic approach for another combination of hurdles, i.e., pH- and a_w-lowering solutes. The validity of the gamma hypothesis for the hurdles pH- and a_w-lowering solutes was judged by comparing the predictive performance of the newly constructed gamma model with that of two gamma models, including a synergy factor reported in the literature.

MATERIALS AND METHODS

Bacterial strain, preculturing conditions, and growth rate determination. *B. cereus* F4810/72, an emetic toxin producer, was originally isolated from human vomit (35). A preculture of the strain was prepared by adding a loopful from a frozen (−80°C) culture of microorganisms to a 500-ml Erlenmeyer flask containing 100 ml brain heart infusion (BHI) broth (Becton Dickinson and Com-

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TABLE 1. Singular models describing the effect of a_w-lowering solutes on the maximum specific growth rate, fitting performance, and optimal parameter estimates

No.	Model formula[a]	Ref.	# par.[b]	Solute	MSE	MSE_{sum}	μ_{opt} (h^{-1}) (SE)	$[s]_{max}$ (M) (SE)	$[s]_{opt}$ (M) (SE)	*a* (SE)	*b* (SE)	*c* (SE)
1	$\mu_{max} = \mu_{opt}\left(\frac{[solute]_{max} - [solute]}{[solute]_{max}}\right)$	28	2	NaCl	0.0124	0.0427	2.63 (0.012)	1.20 (0.006)				
				KCl	0.0101		2.58 (0.010)	1.15 (0.005)				
				Glucose	0.0202		2.64 (0.014)	1.81 (0.009)				
2	$\mu_{max} = \mu_{opt}\left(\frac{([s]_{max} - [s])^a ([s]_{min} - [s])}{([s]_{max} - [s]_{opt})^{a-1}\{([s]_{max} - [s]_{opt})([s]_{opt} - [s]) + ([s]_{min} - [s]_{opt})[(a-1)[s]_{opt} + [s]_{max} - a[s]]\}}\right)$ $[s]_{min} = -0.322$ $a = 2$	32	3	NaCl	0.0120	0.0344	2.37 (0.013)	1.41 (0.012)	0.08 (0.006)			
				KCl	0.0088		2.39 (0.013)	1.39 (0.010)	0.04 (0.005)			
				Glucose	0.0136		2.51 (0.011)	2.12 (0.015)	0.16 (0.005)			
3	$\mu_{max} = a + b\sqrt{[solute]} + c[solute]$	10	3	NaCl	0.0468	0.1306				0.80 (0.021)	1.47 (0.088)	−2.97 (0.093)
				KCl	0.0320					0.80 (0.017)	1.36 (0.074)	−2.95 (0.078)
				Glucose	0.0518					0.84 (0.014)	1.30 (0.060)	−2.07 (0.057)
4	$\mu_{max} = \mu_{opt}\left(1 - \sqrt{\frac{[solute]}{[solute]_{max}}}\right)$	19	2	NaCl	0.0791	0.2737	3.18 (0.040)	1.46 (0.023)				
				KCl	0.0690		3.16 (0.035)	1.39 (0.021)				
				Glucose	0.1256		2.93 (0.041)	2.30 (0.045)				
5	$\mu_{max} = \mu_{opt}\left(\frac{a([solute]_{max} - [solute])}{[solute]_{max}(a - [solute])}\right)$	13	3	NaCl	0.0125	0.0433	2.63 (0.014)	1.19 (0.007)		-2.11×10^{20} ($8.89*10^{27}$)		
				KCl	0.0102		2.57 (0.011)	1.16 (0.005)		-2.98×10^{20} (1.49×10^{28})		
				Glucose	0.0206		2.61 (0.015)	1.83 (0.010)		-8.07×10^{18} (5.12×10^{26})		
6	$\mu_{max} = \mu_{opt}\left(1 - \frac{[solute]}{[solute]_{max}}\right)^a$	21	3	NaCl	0.0062	0.0265	2.50 (0.011)	1.05 (0.004)		0.67 (0.010)		
				KCl	0.0100		2.56 (0.013)	1.13 (0.011)		0.94 (0.022)		
				Glucose	0.0103		2.50 (0.012)	1.54 (0.011)		0.61 (0.014)		
7	$\mu_{max} = \mu_{opt}\left(1 + \frac{a[solute]}{b + [solute]}\right)\left(1 - \frac{[solute]}{[solute]_{max}}\right)$	27	4	NaCl	0.0064	0.0250	2.47 (0.012)	1.12 (0.004)		-3.27*10^-6	-8.11*10^-6	
				KCl	0.0122		2.54 (0.016)	1.13 (0.009)		$9.75*10^6$ ($2.05*10^{14}$)	$2.16*10^9$ ($4.53*10^{16}$)	
				Glucose	0.0064		2.46 (0.011)	1.67 (0.007)		-471927 ($5.34*10^{10}$)	$-1.41*10^6$ ($1.59*10^{11}$)	
8	$\mu_{max} = \mu_{opt}\exp\left[-\left(\frac{[solute]}{10^{-a}}\right)^b\right]$	15	3	NaCl	0.0132	0.0366	2.32 (0.015)			0.10 (0.003)	2.51 (0.053)	
				KCl	0.0110		2.37 (0.002)			0.14 (0.003)	2.11 (0.037)	
				Glucose	0.0124		2.37 (0.012)			-0.07 (0.002)	2.67 (0.044)	
9	$\mu_{max} = \mu_{opt}\left[1 - \left(\frac{[solute]}{[solute]_{max}}\right)^a\right]$	22	3	NaCl	0.0063	0.0216	2.43 (0.013)	1.13 (0.004)		1.38 (0.022)		
				KCl	0.0091		2.50 (0.016)	1.13 (0.006)		1.13 (0.021)		
				Glucose	0.0062		2.45 (0.10)	1.68 (0.006)		1.47 (0.019)		
10	$\mu_{max} = \mu_{opt}\exp(-a[solute])$	2	2	NaCl	0.0889	0.3096	2.90 (0.045)			1.62 (0.040)		
				KCl	0.0656		2.84 (0.036)			1.63 (0.034)		
				Glucose	0.1551		2.74 (0.040)			0.99 (0.024)		
11	$\mu_{max} = \mu_{opt}\left(\frac{a}{a + [solute]}\right)$	27	2	NaCl	0.2108	0.5781	2.94 (0.087)			0.33 (0.022)		
				KCl	0.1532		2.90 (0.069)			0.35 (0.020)		
				Glucose	0.2141		2.68 (0.061)			0.60 (0.034)		
12	$\mu_{max} = \mu_{opt}\left(\frac{a}{a + [solute]} - b[solute]\right)$	27	3	NaCl	0.0394	0.0761	2.65 (0.032)			1.45 (0.141)	0.37 (0.020)	
				KCl	0.0127		2.57 (0.016)			5.24 (1.97)	0.69 (0.050)	
				Glucose	0.0240		2.61 (0.018)			5235.9 ($5.65*10^8$)	0.54 (20.58)	

[a] $[s]_{min}$ is the minimum solute concentration at parameter $a_{w\ max}$ of 1, as set by Rosso and Robinson (32). $a_{w\ max}$ is converted to $[s]_{min}$ using $a_{w\ min}$ of 0.955 and a_w (BHI) of 0.990. *a* is a shape parameter set for a_w in the original model of Rosso and Robinson (32).

[b] Number of parameters used.

pany, Le Pont de Claix, France). The flask was incubated for 16 h at 30°C while shaking at 200 rpm (Julabo SW20; Julabo Labortechnik GmbH, Germany), this way affording an overnight culture of approximately 10^9 cells ml^{-1}, which was used for further experiments.

Effect of pH, a_w-lowering solutes, and combinations of both on the maximum specific growth rate (μ_{max}). The experiments were divided into the following three groups: testing the pH effect (tested in our previous research [6]) (group 1), testing the effect of different concentrations of selected water activity-lowering solutes (group 2), and testing the combined effect of pH and different concentrations of selected a_w-lowering solutes (group 3). The solutes tested for the a_w effect were sodium chloride (NaCl; VWR International, Leuven, Belgium), potassium chloride (KCl; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and glucose (Merck KGaA, Darmstadt, Germany).

The data for the pH experiments (group 1) were copied from our previous research. For the experiments of groups 2 and 3, the selected a_w-lowering solute was added in the desired amount to the BHI broth, whereupon the bottles were autoclaved. For the experiments of group 3, the pH was subsequently adjusted to the desired value (being pH 7, 6.5, 6, 5.5, or 5) after autoclaving by adding 0.5 M sulfuric acid (H_2SO_4; Riedel-de Haën, Seelze, Germany) until the desired pH was reached. The broth was filter sterilized thereafter, according to the manu-

facturer's instructions (Steritop/Steriflip; Millipore Corporation, MA). For every individual test condition, dilution tubes of the overnight culture were prepared with the adjusted corresponding broths, and the overnight bacterial suspension was diluted, aiming at an initial cell concentration of approximately 10^4 CFU ml^{-1}. The level of 10^4 cells was chosen to be far enough away from the stationary phase, preventing effects of high levels, but on the other hand, the level had to be high enough to prevent too high variability as a result of individual cells. The content of the tube of pH 7 without additional solute was spiral plated on BHI agar plates for determination of the exact start level of microorganisms.

Two honeycomb well plates (Oy Growth Curves AB Ltd., Helsinki, Finland) were filled per experiment, as previously described (7), and the experiment was repeated at least once. The honeycomb plates were incubated in Bioscreen C at 30°C for 3 days while continuously shaking at the medium setting. The optical density at 600 nm (OD_{600}) was measured every 10 min. The OD_{600} data obtained from the Bioscreen were imported in Microsoft Excel for data capturing. Wells with an initial OD_{600} above 0.2 (<1% of all wells measured) were removed from the data set, as they were likely to have an incidentally too high inoculum level. For all relevant data series, the time to detection (TTD), defined as the time (h) to reach an OD_{600} of 0.2, was determined. For wells not reaching an OD_{600} of 0.2 within the time frame of the experiment, viability of bacteria was determined, and if no viable bacteria were detected, μ_{max} was set to 0 h^{-1}. In case occasionally viable bacteria were detected (<1% of all wells measured), the data point was removed from the data set, since no μ_{max} was determined, but μ_{max} also could not be considered 0 h^{-1}. The a_w experiments of group 2 were repeated once, and the pH experiments of group 1 and combined pH and a_w experiments of group 3 were repeated twice. The amount of repetitions was determined by the ability to visually estimate the growth boundary from the obtained data. If this was not possible using one repetition, as for the experiments in groups 1 and 3, another experiment was performed.

The maximum specific growth rate for the different test conditions, μ_{max} (h^{-1}), was determined using the relative rate to detection (RRD) method by measuring the time to detection (TTD) (6, 7) using Bioscreen C (Oy Growth Curves AB Ltd., Helsinki, Finland). The TTD, defined as the time (h) until the OD_{600} of a culture reaches 0.2, was determined for every test condition (TTD_i) and was related to the TTD under the optimal condition (TTD_{opt}), with TTD_{opt} being pH 7 with no additional a_w-lowering solutes. The specific growth rate for every test condition ($\mu_{max,\,i}$) was calculated according to equation 1:

$$\mu_{max,\,i} = \mu_{opt} \cdot RRD_i = \mu_{opt} \cdot \frac{TTD_{opt}}{TTD_i} \quad (1)$$

The μ_{opt} value was estimated independently by plating viable cells, enumeration, and subsequent fitting of the Gompertz model to the counts (7). In assessing TTD_{opt} and TTD_i for use in equation 2, care was taken to always start with equal inoculum levels. The obtained μ_{max} values were studied in more detail, and in case outliers in replicate experiments were observed (e.g., one replicate showed no growth, while the others showed considerable growth), these were evaluated based on criteria as previously described (6), and if not applying with the criteria, the points were removed from the data set.

To assess whether an incubation time of 3 days would be long enough to detect all possible growth in the wells, inoculated incubation experiments were conducted under near-growth boundary conditions. BHI broths adapted to pH 6 with additional NaCl in the range of 0.8 to 1.3 M or additional glucose in the range of 1.5 to 2.2 M were selected for this experiment. The cultures, with an initial cell level of 10^4 cells ml^{-1}, were incubated at 30°C while shaking at 200 rpm for 40 days. Growth of cells was determined by visually inspecting the turbidity of the broth.

The water activity of the BHI broth with additional a_w-lowering solute was measured using a water activity measuring device (LabMaster-aw; Novasina, Lachen, Switzerland). The samples were prewarmed to 30°C prior to measuring and also measured at 30°C, the same temperature used for the growth experiments. The a_w value was determined in triplicate.

Model selection and performance. Three criteria were used to select the best-fitting models: (i) the mean square error (MSE) value for the model fit should be below 0.01, ensuring a high level of fit; (ii) the standard deviations for individual model parameters should be smaller than the parameter estimates themselves, since standard deviations greater than the respective parameter estimate indicate large variation; and (iii) the model parameters should preferably have biological significance or be interpretable.

Secondary models for growth rate, which actually included or could be amended to include a pH term, were previously selected from the literature (6). Equation 2 proved to be the best-fitting model to describe the pH effect on μ_{max} and to predict gamma factors, as follows:

$$\mu_{max} = \mu_{opt} \frac{(pH - pH_{max})(pH - pH_{min})}{(pH - pH_{min})(pH - pH_{max}) - (pH - pH_{opt})^2} \quad (2)$$

where μ_{opt} is the maximum specific growth rate under optimal conditions, pH_{max} is the maximum pH just not allowing growth, pH_{min} is the minimum pH just not allowing growth, and pH_{opt} is the optimum pH for growth.

Secondary models for growth rate, which actually included or could be amended to include a term for a_w-lowering solute concentration, were selected from the literature and summarized in Table 1. The names of the parameters of all models were standardized to improve transparency and comparability throughout this research, and the models were made relative if possible and converted to gamma models. All outcomes of the models were expressed as μ_{max} values. Originally a_w-based models were transferred to solute concentration models, which were expected to give equal results since a_w and the solute concentration were linearly related in the range tested (8). Whether this transfer was valid was assessed by testing model 1 of Table 1 both as an a_w model and as a concentration model. This was done by transferring the concentration data to a_w data using the linear transformation proposed by Buchanan and Bagi (8).

The water activity models were fitted to the μ_{max} data of group 2. Model performance (MSE values) and parameter estimates for the two types of models are included in Table 1. The model selected on the basis of the three criteria stated above was tested against the best-performing model with one parameter less, using an F test to evaluate whether the reduction of one parameter was still statistically acceptable (11). The f value was tested against the 95% confidence F table value (F^1_∞ [F at 1 and infinite degrees of freedom] = 3.84). If the f value was smaller than the F table value, the F test was accepted, and the model with the smallest number of parameters was accepted.

Evaluating the gamma hypothesis. The selected models for pH and solute concentration were combined in a gamma model according to equation 3 (39):

$$\mu_{max} = \mu_{opt} \cdot \gamma(pH) \cdot \gamma([solute]), \text{ with } \gamma = \frac{\mu_{max}\,(pH, [solute])}{\mu_{opt}\,(7,0)} \quad (3)$$

where μ_{max} is the maximum specific growth rate at the tested condition and μ_{opt} is the maximum specific growth rate as determined by plate count (2.42 h^{-1}) in medium of pH 7 when no a_w-lowering solute is present. Parameter estimates derived by fitting single models were incorporated into the gamma model, and predictions about the combined effect were made. These predictions were compared to the experimental data of group 3, which included both acid effects and a_w-lowering solute effects. The differences between predictions and experimental data were expressed as MSE values.

Two gamma models, including a synergy factor from the literature, were compared with the newly composed gamma models combining pH and a_w-lowering solute concentration. The first synergy model was that described by Le Marc et al. (20):

$$\mu_{max} = \mu_{opt} \cdot \gamma(pH) \cdot \gamma([solute]) \cdot \xi(pH, [solute]) \quad (4)$$

in which ξ is the synergy factor, calculated according to the model of Le Marc et al. (20). The second synergy model was that of Augustin and Carlier (3, 4). This model does not include a synergy factor, but the different inhibitory factors were corrected independently for synergy by estimating new minimal growth values, which were then used in the nonsynergistic gamma model according to equations 5A, B, and C.

$$\mu_{max} = \mu_{opt} \cdot \gamma_{new}(pH) \cdot \gamma_{new}([solute]) \quad (5A)$$

with

$$pH_{min,new} = pH_{opt,fit} - (pH_{opt,fit} - pH_{min,fit}) \cdot \left(1 - \frac{[solute]}{[solute]_{max,fit}}\right)^{1/3} \quad (5B)$$

and

$$[solute]_{max,new} = [solute]_{max} \cdot \left(1 - \left(\frac{pH_{opt,fit} - pH}{pH_{opt,fit} - pH_{min,fit}}\right)^3\right) \quad (5C)$$

Apart from the MSE values determined, the bias and accuracy factors of models were also determined, which also can be used for performance evaluation of predictive models (25, 30).

The effect of other model combinations incorporated in a gamma model was tested by combining the different a_w-lowering solute models of Table 1 with the pH model of equation 2. The difference between the model prediction and the data points obtained for a combination of both hurdles was expressed as an MSE value.

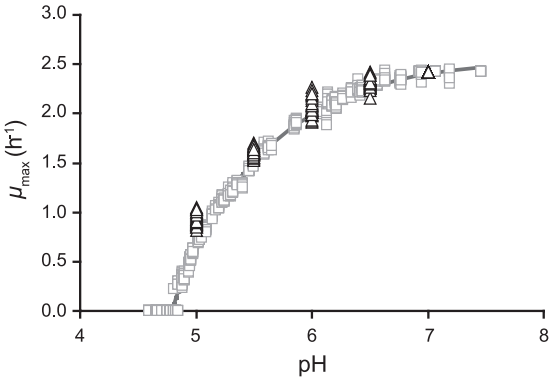

FIG. 1. Maximum specific growth rate of emetic *B. cereus* as a function of pH, in which the gray squares represent the experimental data and the gray line depicts the fit of the most optimal pH model (equation 2) to the original data sets; the black triangles represent the data points of the experiments of group 3, in which no a_w-lowering solutes were added to the BHI broth.

RESULTS

Effect of a_w-lowering solute concentrations on μ_{max} and growth boundary. The effects of pH on μ_{max} and the growth boundary, as obtained in previous research (6) and complemented with additional data from the experiments of group 3 without a_w-lowering solutes, are displayed in Fig. 1. The effects of the concentration of a_w-lowering solutes NaCl, KCl, and glucose on μ_{max} and growth boundary are shown in Fig. 2. The increase of solute concentration resulted in a decrease of the growth rate. The visually determined boundaries were 1.2 M, 1.2 M, and 1.7 M solute for NaCl, KCl, and glucose, respectively.

Selection of the best-fitting model to describe growth in the presence of a_w-lowering solutes. Twelve a_w-lowering solute models (Table 1) were fitted to the growth rate curves of *B. cereus* F4810/72 cultured in BHI broth with various concentrations of NaCl, KCl, or glucose. Table 1 reports the fitting performance of all models by means of MSE values and their standard deviations. Fitting model 1 to the NaCl data resulted in an MSE value of 0.0124. When this model was fitted as an a_w-based model to the a_w data of NaCl, the MSE value was almost identical at 0.0125. Identical results were also obtained when fitting model 1 as a solute concentration model and as an a_w model to the KCl and glucose data. It is therefore considered valid to transfer the a_w models to concentration models, since a linear relationship between a_w values and solute concentrations appears to be present (8).

Based on the criterion that the MSE value between the data points and the fit should be below 0.01 for every solute tested, only model 9 (with three parameters) remained for further analysis. Using a slightly different approach, in which the MSE value selection criterion was not taken as the first step, the best-fitting model was assessed based firstly on the criterion that the standard deviation should not exceed the parameter estimate. Nine models were identified as meeting the criterion, i.e., models 1, 2, 3, 4, 6, 8, 9, 10, and 11. Model 9, with the lowest MSE value, was compared to models 1, 4, 10, and 11, with less parameters than model 9, using an *F* test to see if the difference in the MSE values between each of the four models

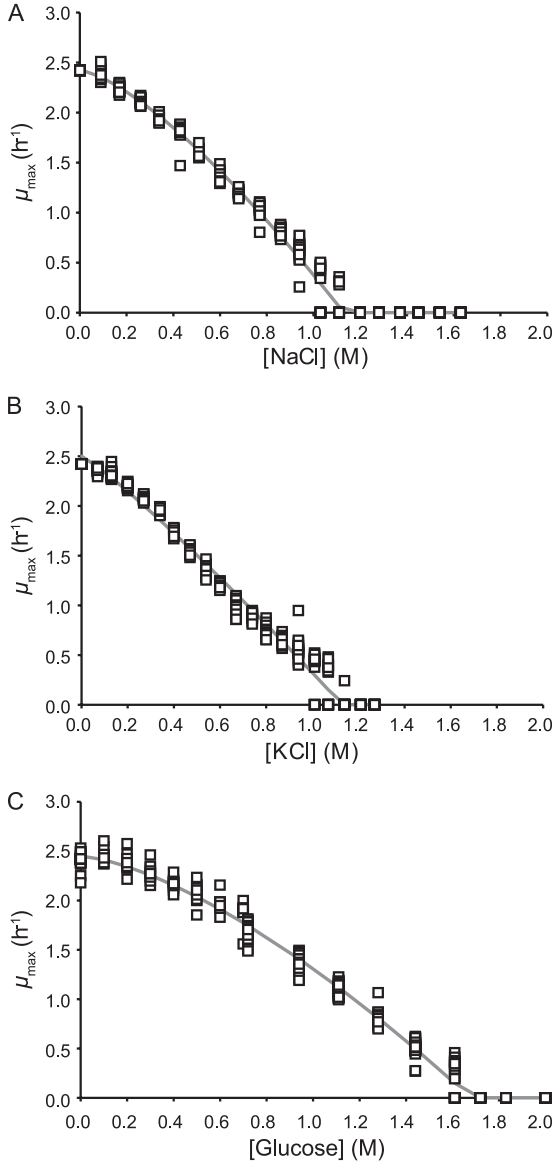

FIG. 2. Maximum specific growth rate of emetic *B. cereus* as a function of a_w-lowering solute concentration for NaCl (A), KCl (B), and glucose (C). The black squares represent experimental data, and the solid gray lines show the fits of the most optimal solute concentration model (model 9; see the text) to the data sets.

individually and model 9 was significant. The *F* test values ranged between 43 and 12,562 (data not shown), all considerably exceeding the *F* table value, which indicated significant improvement of model 9, with three parameters, over any of the two-parameter models. Therefore, also following this approach, model 9, with three parameters, was considered the best-fitting model.

The growth boundaries were estimated using model 9. They were found to be 1.13 M, 1.13 M, and 1.68 M for NaCl, KCl, and glucose, respectively. The a_w values determined experimentally ($n = 3$) for the three solutes, NaCl, KCl, and glucose, were 0.954 (±0.001), 0.956 (±0.001), and 0.961 (±0.000), re-

TABLE 2. MSE values for predictions obtained with the nonsynergistic gamma model, the model of Le Marc et al., and the model of Augustin and Carlier for NaCl, KCl, and glucose[b]

Model	Solute	Model formula	MSE value[a]				
			pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0
A	NaCl	$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-4.79)}{(pH-4.79)(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{1.13}\right)^{1.38}\right]$	0.0625 (n = 105)	0.0716 (n = 119)	0.0797 (n = 118)	0.1096 (n = 119)	0.0760 (n = 128)
B		$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-4.79)}{(pH-4.79)(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{1.13}\right)^{1.38}\right] \cdot \xi(pH,[solute])$	0.0531	0.0700	0.0793	0.1096	0.0760
C		$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-pH_{min,new})}{(pH-pH_{min,new})(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{[solute]_{max,new}}\right)^{1.38}\right]$	0.1531	0.2032	0.1303	0.0835	0.0642
A	KCl	$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-4.79)}{(pH-4.79)(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{1.13}\right)^{1.13}\right]$	0.0477 (n = 106)	0.0496 (n = 119)	0.0369 (n = 120)	0.0294 (n = 118)	0.0350 (n = 130)
B		$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-4.79)}{(pH-4.79)(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{1.13}\right)^{1.13}\right] \cdot \xi(pH,[solute])$	0.0372	0.0457	0.0369	0.0297	0.0350
C		$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-pH_{min,new})}{(pH-pH_{min,new})(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{[solute]_{max,new}}\right)^{1.13}\right]$	0.1397	0.2282	0.2260	0.0968	0.0460
A	Glucose	$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-4.79)}{(pH-4.79)(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{solute}{1.68}\right)^{1.47}\right]$	0.1874 (n = 103)	0.1796 (n = 116)	0.2230 (n = 150)	0.1937 (n = 140)	0.0920 (n = 132)
B		$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-4.79)}{(pH-4.79)(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{1.68}\right)^{1.47}\right] \cdot \xi(pH,[solute])$	0.2207	0.1907	0.2283	0.1937	0.0920
C		$\mu_{max} = 2.42 \cdot \frac{(pH-19.16)(pH-pH_{min,new})}{(pH-pH_{min,new})(pH-19.16)-(pH-8.00)^2} \cdot \left[1-\left(\frac{[solute]}{[solute]_{max,new}}\right)^{1.47}\right]$	0.5264	0.7750	0.7223	0.2365	0.1266

[a] MSE_{sum} for model A, 1.3977; MSE_{sum} for model B, 1.7652; MSE_{sum} for model C, 3.7578.
[b] Model A, nonsynergistic gamma model; model B, model of Le Marc et al. (20); model C, model of Augustin and Carlier (3, 4).

spectively. The a_w value of 1.13 M glucose in BHI broth had been separately determined to be 0.972 (±0.001) (n = 3).

Evaluating the gamma hypothesis. The pH model and model 9 were combined into a gamma model, as per equation 3, resulting in the models displayed in Table 2, describing the combined effect of pH and NaCl, KCl, or glucose. In addition, the pH model and model 9 were combined into the synergy models of Le Marc et al. (20) (equation 4; Table 2) and Augustin and Carlier (3, 4) (equation 5; Table 2). Using these three equations, the growth rates and the growth boundaries of *B. cereus* in BHI broth were predicted for combinations of the two hurdles pH and a_w.

Figure 3I, II, and III show the predictions of the three models for various combinations of concentrations of NaCl, KCl, or glucose and pH values next to the experimental data. Overall, the MSE values between prediction and experiments were found to be the lowest for the gamma model not assuming a synergy factor, which had a sum of the MSE values between data and prediction for all three solutes used (NaCl, KCl, and glucose), for all concentrations, and for all 5 pH values tested (pH 5.0, 5.5, 6.0, 6.5, and 7.0) (MSE_{sum}) of 1.3977. The synergy model of Le Marc et al. (20) was considered the next best model, having an MSE_{sum} of 1.7652, followed by the model of Augustin and Carlier (3, 4), with an MSE_{sum} of 3.7578. All accuracy factors (data not shown) were higher than the accepted factor of 1.2 (10% per number of environmental parameters in the model), indicating that for all models, the estimate is not very accurate. In general, the accuracy factors for the gamma model and the model of Le Marc et al. (20) were the lowest ones, ranging from 1.226 to 2.337. The accuracy factors for the model of Augustin and Carlier (3, 4) were higher and ranged from 1.269 to 3.852, with most factors in the high range. The bias factors for the models of Augustin and Carlier were all indicating that the estimates were unacceptable and that the predictions were fail dangerous. The gamma model and the model of Le Marc et al. (20) had bias factors ranging mainly from good to acceptable, and no preference for one or the other model could be made based on the bias factors.

For most combinations of a_w-lowering solutes with pH, and especially in the case of low pH conditions, the data points were higher than the values predicted by the gamma model. This resulted in increased MSE values for model fit and a poorer performance of the gamma model compared to our previous study, in which pH and undissociated acid concentrations were combined (6). For NaCl and KCl, the growth boundary at pH 5 was also considerably overestimated by the gamma model, which also contributed to an increase of the MSE values. The model of Le Marc et al. (20) had lower MSE values for these conditions, which is likely due to its assumed synergy, which shifts the growth boundary to lower concentrations of a_w-lowering solute. The reduction in MSE values was actually from 0.0625 to 0.0531 for NaCl and from 0.0477 to 0.0372 for KCl. For the other conditions evaluated, i.e., at higher pH, and for glucose, this reduction in MSE values was not observed. For glucose, the growth boundary is underestimated by the model of Le Marc et al. (20), therefore not reducing the difference between prediction and data. The model of Augustin and Carlier (3, 4) also assumed synergy, but this model underestimated the growth boundary considerably at pH values of 5 and 5.5, causing the MSE values to increase to 0.1531 for pH 5 (NaCl) and to 0.1397 (KCl).

Retesting of the growth boundary to check whether 3 days of incubation was enough to determine the growth boundary revealed that if cultures did not show growth within 3 days, growth was also not commencing in the following 37 days. This

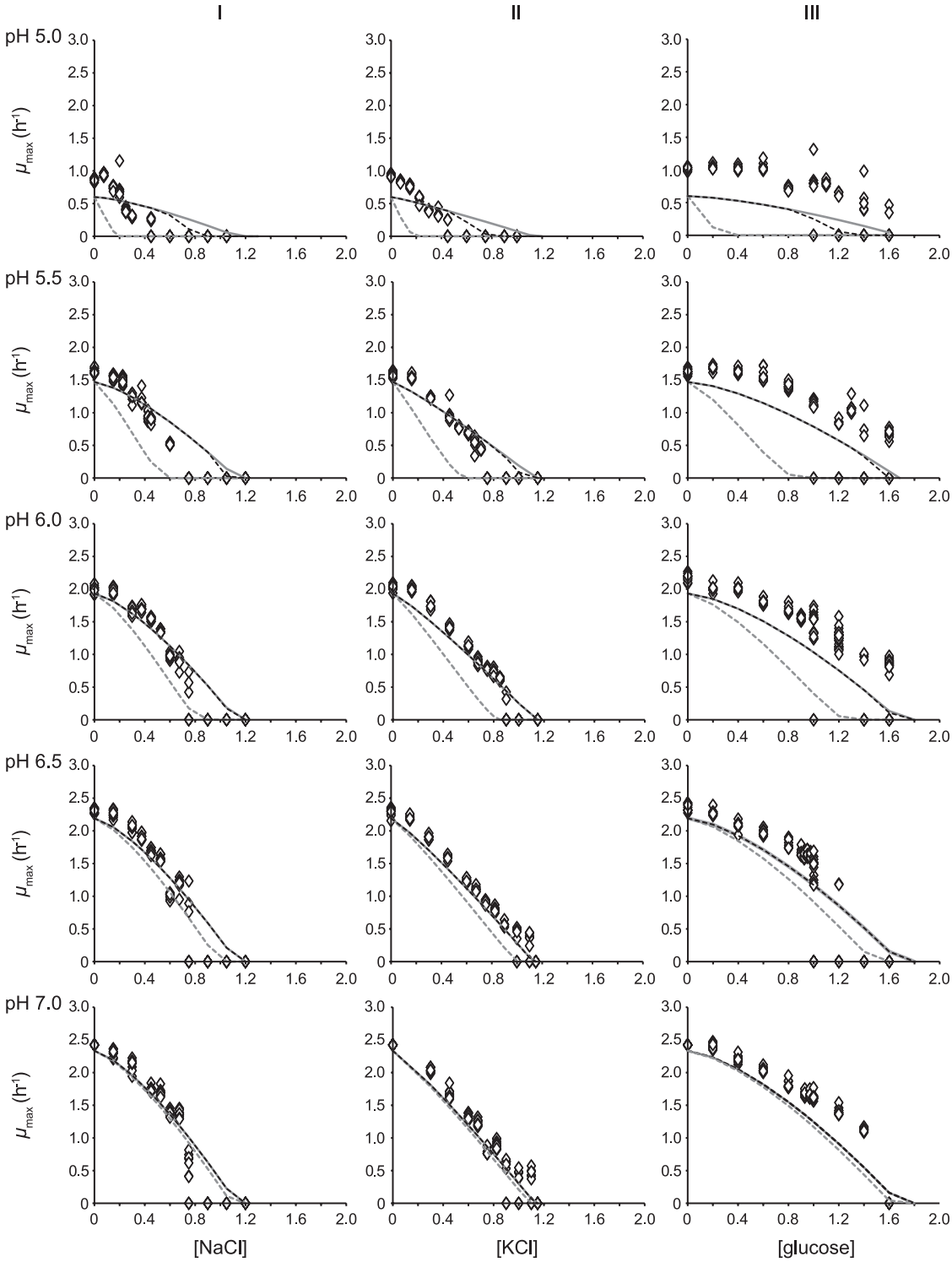

FIG. 3. Experimental data (black diamonds) and predictions using equation 2 and model 9 for the combined effect of pH- and a_w-lowering solute concentrations (in M) on μ_{max} without (solid gray line) and with (dashed black line) an interaction factor, according to the model of Le Marc et al. (20) and predictions using the model of Augustin and Carlier (3, 4) (dashed gray line) for NaCl (I), KCl (II), and glucose (III) at pH 5.0, 5.5, 6.0, 6.5, and 7.0.

indicates that the boundary found after 3 days of incubation corresponds well to the real growth boundary of the tested *B. cereus* strain.

The result of retesting model combinations is displayed in Table 3, comparing MSE values between experimental data and predictions. The initial combination of model 9 and the pH model is highlighted by shading. For all combinations that perform better than the combination of model 9 and equation 2, as indicated by a lower MSE value, the MSE value is in boldface. Evidently, no model was much better overall than

TABLE 3. MSE values for NaCl, KCl, and glucose between experiments and predictions obtained with various combinations of the a_w-lowering solute models and the pH model (equation 2) combined into the nonsynergistic gamma model[a]

Model	MSE value															No. of times model performed better than model 9	MSE_{sum}
	NaCl					KCl					Glucose						
	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0		
1	**0.0553**	0.0792	0.0970	0.1239	0.1203	**0.0431**	0.0575	**0.0294**	**0.0176**	0.0401	**0.0729**	0.2610	0.3660	0.7610	1.0773	5	3.2016
2	0.0621	**0.0634**	**0.0671**	**0.0930**	**0.0712**	**0.0464**	**0.0423**	**0.0300**	**0.0233**	**0.0318**	0.1939	0.1868	0.2541	0.2150	0.1493	9	1.5297
3	**0.0413**	**0.0549**	0.2566	0.3751	0.4427	**0.0354**	0.1230	0.3578	0.4615	0.5021	0.3040	0.4688	0.8689	0.7962	0.8439	3	5.9323
4	0.0640	0.1309	0.2386	0.3383	0.2766	0.0527	0.0875	0.1743	0.1939	0.1578	0.2791	0.3844	0.4866	0.6898	1.1474	0	4.7020
5	**0.0564**	**0.0632**	0.0937	0.1358	0.1019	**0.0464**	**0.0485**	0.0485	0.0412	0.0436	0.2166	0.2331	0.2884	0.2767	0.1886	4	1.8827
6	0.0618	**0.0715**	0.0852	0.1194	0.0814	**0.0468**	**0.0487**	0.0446	0.0389	0.0461	0.1962	0.2073	0.2639	0.2102	0.1057	3	1.6276
7	**0.0531**	**0.0541**	0.0897	0.1336	0.1021	0.0450	**0.0436**	0.0496	0.0490	0.0499	0.1894	0.1845	0.2264	0.1979	0.0935	3	1.5613
8	0.0660	0.0778	**0.0740**	**0.0989**	**0.0708**	**0.0471**	**0.0416**	**0.0300**	**0.0241**	**0.0318**	**0.1792**	**0.1615**	**0.2165**	**0.1707**	0.1016	12	1.3914
9	0.0625	0.0716	0.0797	0.1096	0.0760	0.0477	0.0496	0.0369	0.0294	0.0350	0.1874	0.1796	0.2230	0.1937	0.0920		1.4737
10	0.0540	0.0984	0.1909	0.2719	0.2255	0.0448	0.0711	0.1281	0.1306	0.1217	0.2520	0.3138	0.3914	0.4350	0.3470	0	3.0763
11	0.0637	0.1602	0.2880	0.4010	0.3331	0.0535	0.1101	0.1871	0.1935	0.1727	0.2658	0.3493	0.4127	0.5100	0.4009	0	3.9016
12	**0.0583**	0.0808	0.1260	0.1779	0.1398	**0.0468**	0.0507	0.0507	0.0407	0.0435	0.2149	0.2285	0.2787	0.2742	0.1808	2	1.9922

[a] MSE values that are lower for the solute/pH using the model indicated are highlighted in boldface, in comparison to the combination with model 9 (highlighted with shading).

model 9, although other models were better in some specific combinations. For example, a combination with model 1 performed better 5 out of 15 times. Model 8 of Lambert and Bidlas (15) gives better predictions in 12 out of 15 cases, though the improvements were relatively small.

DISCUSSION

Model criteria and selection. This research aimed to test the gamma hypothesis for two independent hurdles, i.e., pH and various a_w-lowering solutes. As part of the systematic approach deployed for testing of the gamma hypothesis for pH and various concentrations of undissociated acids (6), the most optimal model to predict the effect of the solute on the growth rate was selected. Relatively few models are available in the literature that incorporate the parameter a_w or the concentration of a_w-lowering solutes. Where this was the case, model 1 was predominantly used (17, 23, 26, 28, 31, 38). However, many undissociated acid concentration models could be interpreted as a_w-lowering solute models and were of equal performance as their original a_w models. The model selected as best fitting the data on the basis of having the lowest MSE value and the least number of parameters, model 9, was not among the original a_w-lowering solute models. Model 9 was adapted from a model describing the effect of undissociated acid on bacterial growth (22). The previously established selection procedures for model selection were also applied for selection of the best-performing a_w-lowering solute model and allowed a considerable reduction in models to be used while not eliminating all.

Solute-specific effect versus a_w effect for single hurdles. The differences found in the accuracy of the prediction of the boundary between the two salts and glucose may have two reasons. First, it may be because interactive effects occur between the salts and the acid, which is not occurring between the glucose and the acids. Second, it may be because of certain a_w-specific effects of the solutes. NaCl and KCl have almost equal a_w values (0.954 and 0.956, respectively) at equal concentrations (1.13 M) of the solute. These values correspond to a_w values for various concentrations of NaCl and KCl in water previously reported by Samapundo et al. (33) and confirm the conclusion of Bidlas and Lambert (5) that NaCl and KCl have equal antimicrobial effects when calculated on a molar basis. This would encourage the view that these solutes have an a_w effect and not a solute-specific effect. In our study, we found that glucose had a much higher a_w value (0.972) at a solute concentration of 1.13 M, whereas the a_w value at the growth boundary concentration for glucose (1.68 M) was found to be 0.961. The latter is in the same range as the growth boundary a_w values of NaCl and KCl. Although NaCl and KCl have identical growth boundaries, for the NaCl and KCl graphs at pH 6.5 and pH 7 (Fig. 3I and II), it can be observed that the shape of the curve at near-growth boundary conditions is different. Where KCl shows tailing toward the growth boundary, NaCl shows an acute and rapid decrease toward the growth boundary, a so-called cliff edge. A reason for this difference could not be found and is of interest for future studies.

Evaluation of the gamma hypothesis. Model 9 and the pH model of equation 2 were combined into a gamma model, which was used to assess the combined effect of a_w-lowering solutes and pH on μ_{max} and the growth boundary of *B. cereus* F4810/72. This newly constructed gamma model was compared to the synergy models of Le Marc et al. (20) and Augustin and Carlier (4). The maximum specific growth rate was underestimated for the three solutes tested when predictions on the effect of combined low pH values (pH 5 and pH 5.5) and low concentrations of a_w-lowering solute were made. The underlying cause for this low prediction compared to the real data is as yet unknown. No systematic differences in pH of the broth were measured when remeasuring the pH with other pH meters and before and after filter sterilizing. The same batch of BHI broth was used for all experiments, but the time frame between the first experiments, determining the μ_{max} for pH effects, and the last experiments, determining the effect of different concentrations of a_w-lowering solute on μ_{max}, was 2 years. Whether it is possible that the μ_{max} had changed over this period of time is speculative but an option to consider. However, when plotting the μ_{max} data of the combination

experiments, performed when the solute concentrations were 0 M, in the same graph as the original pH experiments (Fig. 1), it can be seen that small deviations in pH under near-growth boundary conditions can cause very significant changes in μ_{max} values. For instance, a two-fold increase in μ_{max} value can be caused by a 0.14 pH point increase. This pH increase is two times bigger than the measuring error of a pH meter and the measured differences between the pH meters in the laboratory. This observation of structural differences in μ_{max} values also confirms the differences between data points and the models shown in Fig. 3 when no a_w-lowering solute is present.

Although not considered optimal data sets with respect to absolute μ_{max} values, the data sets obtained in our study could still be used to draw conclusions about synergy, since the three models evaluated in this assessment differed in their growth boundary estimates and not in their μ_{max} values when the a_w-lowering solute was absent. For all models, higher MSE values were noted, but this was consistent for all three models tested and thus would not influence any further conclusions about the validity of the gamma hypothesis.

Different trends for the various predictions of the salt and glucose curves combined with different pH values could be seen. The glucose curves clearly had a different shape compared to those of the salts. For the two salts evaluated, the gamma model extensively overestimated the growth boundary. For glucose, the growth boundary was underestimated by the gamma model. This could be explained by the fact that glucose may have a growth-stimulating effect as well as a growth-limiting effect and that the two opposing effects balanced out each other's impact. As a consequence, a shift of the growth boundary was not apparent, and the nonsynergistic gamma model was able to predict the growth rate curve well. Since the curves of the salts and glucose were so different, drawing conclusions on the MSE_{sum} values appeared not to be specific enough to judge the quality of predictions. However, the use of MSE values seems unavoidable when an objective, quantitative judgment about the model performance is to be made.

When assessing the MSE_{sum}, the sum of the MSE values between data and prediction for all three solutes used (NaCl, KCl, and glucose), for all concentrations, and for all 5 pH values tested (pH 5.0, 5.5, 6.0, 6.5, and 7.0), the newly composed gamma model had the lowest value and was judged to be performing the best. When focusing on specific, single MSE values and the visual analysis shown in Fig. 3, this conclusion was not unambiguously proven. As could be seen, the gamma model overestimated the boundaries for NaCl and KCl, especially for low pH values, i.e., pH 5 and 5.5, and the introduction of a synergy factor as shown in the model of Le Marc et al. (20) improved the estimate of the growth boundary. Assessing the MSE_{sum} values for the two salts only and neglecting the glucose data ($MSE_{sum,\ NaCl+KCl}$) actually revealed that the synergy model performed better ($MSE_{sum,\ NaCl+KCl} = 0.5980$ for the gamma model and $MSE_{sum,\ NaCl+KCl} = 0.5725$ for the synergy model of Le Marc et al.). This would indicate that there was indeed a shift of the growth boundary which might have been caused by synergy. Notably, the synergy model of Augustin and Carlier (3, 4) underestimated the growth boundary by assuming synergy ($MSE_{sum,\ NaCl+KCl} = 1.371$).

Since the growth boundary was estimated differently by all three methods, it was decided to retest the growth boundaries. Retesting has elsewhere been found to give useful insights. According to Vermeulen et al. (37), *Listeria monocytogenes* showed a significant increase in detection time (determined using optical density) of up to 30 days when both pH and a_w were lowered simultaneously. This finding stresses the importance of retesting the boundary for the most stringent conditions, i.e., low pH and high concentrations of a_w-lowering solute. The experiment validating the growth boundary for the strain of emetic *B. cereus* used in the current study revealed that if cultures did not show growth within 3 days, growth was also not commencing in the following 37 days. So the experimental setup used was sufficient to correctly determine the growth boundary.

All of the a_w-lowering solute models were retested as well (Table 3), since the incorrect prediction of the boundary might be caused by failure of the model selection criteria, and another combination of models possibly would improve the prediction of the combined pH- and a_w-lowering solute effect. Evidently, no model was much better overall than model 9, although other models were better in some specific combinations. The improved performance of a combination was observed mostly under near-growth boundary conditions. The improvement can be caused by a better estimation of the growth boundary by the gamma model. The improvements of model 8 may have been due to a better shape of the curve, since the curve was first parabolic and showed tailing toward the growth boundary.

In previous work of modeling the combined effects of hurdles on the growth rate of microorganisms, good results often have been achieved using models without a synergy factor (15, 31, 38). There also have been studies showing that interaction occurs when various hurdles are combined, and for these interactions, gamma models including a synergy factor were developed (4, 20, 29). The underlying variation in test organisms and preservative factors as well as the different experimental approaches employed may well have contributed to the different conclusions. In our study, predictions made using synergy models approached the growth boundary best for low pH conditions combined with different concentrations of a_w-lowering solutes. For less stringent combinations of growth-limiting factors, it was clearly found that the introduction of a synergy factor did not improve the predictions. A discussion on whether nonoptimal models predicting the effect of single hurdles should be incorporated in models predicting combined effects is warranted. Where the best performance in predictions of a combined effect is driving the creation of synergy models, in effect, poorer performance of the individual hurdles is generally accepted. This may result in a bias toward the synergy factor. Our study suggests that it may not be always possible to achieve the same level of good performance with the combined models and with the models of the single hurdles. However, as a best practice, one should follow a systematic and quantitative approach to objectively identify the most optimal model for single effects that is to be used in assessing a combined effect. Such an approach was followed here, and the fact that one model was eliminated that had very good performance in a gamma model proved that in order not to be biased for synergy, it is necessary to additionally use preset criteria for single-factor effects first.

In general, in other researches, single-hurdle models are not

systematically selected from a list of available models, as was done in this research. They are chosen because they have a (reasonably) good fit, and no selection is made based on preset criteria such as the lowest MSE value for the fit to the data across available models. It is recommended that for future studies, a systematic approach is indeed used for model selection, which may allow for more conclusive results about the occurrence of synergy. In conclusion, no combination of models could be found that was able to predict the impact of both individual and combined hurdles correctly in this study. Consequently, in this case we could not prove the existence of synergy nor falsify the gamma hypothesis.

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