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Performance of response surface model for prediction of *Leuconostoc mesenteroides* growth parameters under different experimental conditions

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Abstract

The combined effects of different temperatures (10.5–24.5 °C), pH level (5.5–7.5), sodium chloride levels (0.25–6.25%) and sodium nitrite levels (0–200 ppm) on the predicted growth rate and lag-time of *Leuconostoc mesenteroides* under aerobic and anaerobic conditions was studied. The response surface (RS) model developed provided reliable estimates of the three kinetic parameters studied, with a bias factor between 0.86 and 1.18 and an accuracy factor between 1.13 and 1.31, in aerobic and anaerobic conditions, respectively. For both conditions, SEP values ranged between 15.62% and 27.63%. The developed models are a valuable tool, enabling its application for shelf-life estimation of a food product.

Keywords: Predictive microbiology; *Leuconostoc mesenteroides*; Response surface model

1. Introduction

The deterioration of food products owing to spoilage microorganisms is a highly important social and economic problem, which affects both the food industry and consumers. Specifically, in the case of cooked meat products that are vacuum-packed, alterations in the product are chiefly caused by lactic acid bacteria, such as *Leuconostoc mesenteroides*. (Huis in't Veld, 1996; Zhang & Holley, 1999). These bacteria contribute to the alteration process of food products via the fermentation of sugars, thus forming lactic acid, and producing slime and CO_2, which cause pH levels to drop and result in the appearance of strange smells and flavors. This affects the sensorial qualities of the food product, and its acceptability to the consumer (Huis in't Veld, 1996), resulting in significant economic losses for the food industry. It is therefore important to know the growth capacity of this microorganism to multiply in the food product under the conditions experienced during processing, preservation, storage and distribution.

Predictive microbiology is an important tool in the food industry to predict the behavior of microorganisms. The main objective is to use mathematical models to describe the evolution of food-based microorganisms under the influence of intrinsic environmental factors (pH, a_w) and extrinsic factors (temperature, gaseous atmosphere).

The development of predictive models requires a large amount of growth data. The time-consuming nature of traditional plate-count techniques has prompted a need for swifter and more convenient data-collection methods, which would represent a considerable saving

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in effort and resources (Cole, 1991). One proposed alternative is based on absorbance measurements (Begot, Desnier, Daudin, Labadie, & Lebert, 1996; Dalgaard, Ross, Kamperman, Neumeyer, & McMeekin, 1994): predictive models derived from automated optical density data are reliable, generally validate well against models based on traditional methods, and provide a favorable estimation of microbial response (Dalgaard & Koustsoumanis, 2001; Dalgaard, Mejlholm, & Huss, 1997; Nerbrink, Borch, Blom, & Nesbakken, 1999; Neumeyer, Ross, Thomson, & McMeekin, 1997b).

Growth predictive models are currently accepted as informative tools that assist in rapid and cost-effective assessment of microbial growth for product development, risk assessment and education purposes (Ross, 1999). Although, over the past few years, much effort has been directed towards developing models describing the combined effects of environmental factors on microbial growth of pathogens in foods (Devlieghere et al., 2001; García-Gimeno, Hervás-Martínez, Barco-Alcalá, Zurera-Cosano, & Sanz-Tapia, 2003; Ross, Dalgaard, & Tienungoon, 2000; Zurera-Cosano, Castillejo-Rodríguez, García-Gimeno, & Rincón-León, 2004), predictive microbiology has been used to forecast the growth of spoilage microorganisms in order to study the shelf life of a food product. Specific spoilage organisms are selected for certain food products and used as test organisms such as *Brochothrix thermosphacta* (Baranyi, Robinson, Kaloti, & Mackey, 1995), *Pseudomonas* (Neumeyer, Ross, & McMeekin, 1997a), *Lactobacillus sake* (Devlieghere, Debevere, & Van Impe, 1998), *Lactobacillus curvatus* (Wijtzes, Rombouts, Kant-Muermans, van't Riet, & Zwietering, 2001), or *Lactobacillus plantarum* (García-Gimeno, Hervás-Martínez, & de Silóniz, 2002).

The relationships between the combination of factors and the growth curve parameters are most frequently described using response surface methodology (Devlieghere et al., 1998). Given the lack of a mathematical model for *L. mesenteroides* in current scientific literature, the aim of the present study was to elaborate models for predicting the combined effects of temperature, pH, salt and nitrite concentrations in aerobic and anaerobic conditions on the growth rate, lag-time and maximum population density of *L. mesenteroides* growth and to evaluate the relative importance of these environmental factors in controlling the growth of this microorganism.

2. Material and methods

2.1. Inoculum

For the preparation of the inoculum of *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (Spanish Collection of Strain Types, Valencia), the strain was inoculated in flasks with 10 ml of MRS broth (pH 6.2; no added NaCl), incubated at 30 °C for 24 h, and subcultured on three successive days. The third subculture was grown for 18 h until the stationary stage of growth. Subsequently, the necessary dilutions were made in MRS broth to obtain an inoculum size of 10^6 cfu/ml, above the detection level.

2.2. Experimental design

A central composite design (CCD) was employed, incorporating the following variables and levels: temperature (10.5, 14, 17.5, 21 and 24.5 °C), pH (5.5, 6, 6.5, 7 and 7.5), concentrations of sodium chloride (0.25%, 1.75%, 3.25%, 4.75% and 6.25 %) and concentrations of sodium nitrite (0, 50, 100, 150, and 200 ppm) under aerobic and anaerobic conditions shown in Tables 1 and 2. Each of the different factor combinations thus obtained was replicated seven times (five of these were used for model development and two for internal validation or testing), and six center point replications were performed to estimate experimental variance. Additional conditions were selected randomly within the ranges indicated and used for model validation (Tables 4 and 5).

2.3. Media preparation

Sodium chloride concentrations ranging from 0.25% to 6.25% were obtained by adding the appropriate amount of NaCl to a series of flasks containing 100 ml TSB. Next, pH was adjusted using HCl (5 N) and NaOH (5 N) solutions, to values of between 5.5 and 7.5. Aliquots of 9.9 ml were then autoclaved (121 °C for 15 min), and adjusted pH was checked. Sodium nitrite solutions were prepared in 10 ml volumes and sterilized by filtration due to nitrite loss of heat stability. Aliquots of 0.1 ml of these solutions were then pipetted into the 9.9 ml TSB obtained earlier, to give final concentrations from 0 to 200 ppm.

2.4. Data collection and curve fitting

To obtain *L. mesenteroides* growth data, the Bioscreen C analyser (Labsystem, Helsinki, Finland) was used, with which optical density measurements were taken. 200 μl of sterile MRS broth from the different test conditions were transferred into each well of the Bioscreen C plates, along with 50 μl of *L. mesenteroides* inoculum with a concentration close to 10^6 ufc/ml. Optical density measurements were taken each hour until the microorganism had reached the stationary stage of growth. To simulate the anaerobic environment, the wells were covered with 200 μl of liquid paraffin. For each atmospheric condition, 150 growth curves were obtained for further development of the model, and

Table 1
Average of observed (OBS) and estimated growth rate (Gr, h^{-1}), lag time (lag, h) and maximum population density (yEnd, OD) by response surface model (RS) of *Leuconostoc mesenteroides* in aerobics conditions

				Gr (h^{-1})		lag (h)		yEnd (OD)	
T (°C)	pH	NaCl (%)	$NaNO_2$ (ppm)	OBS	RS	OBS	RS	OBS	RS
10.5	6.5	3.25	100	0.141	0.150	13.170	11.478	0.391	0.359
14	6	1.75	50	0.178	0.190	6.446	5.329	0.583	0.633
14	6	1.75	150	0.160	0.174	5.742	7.229	0.334	0.369
14	6	4.75	50	0.147	0.140	9.113	12.411	0.262	0.306
14	6	4.75	150	0.138	0.124	23.169	20.157	0.171	0.178
14	7	1.75	50	0.200	0.226	4.805	6.888	0.856	0.792
14	7	1.75	150	0.183	0.210	7.980	7.063	0.494	0.462
14	7	4.75	50	0.153	0.152	9.083	8.427	0.546	0.521
14	7	4.75	150	0.146	0.136	8.304	10.344	0.270	0.303
17.5	5.5	3.25	100	0.114	0.159	10.372	8.810	0.425	0.353
17.5	7.5	3.25	100	0.180	0.207	6.614	5.884	0.627	0.751
17.5	6.5	3.25	0	0.194	0.232	5.112	5.240	0.971	0.864
17.5	6.5	3.25	200	0.165	0.200	8.212	8.726	0.314	0.294
17.5[a]	6.5	3.25	100	0.177	0.194	6.579	6.762	0.453	0.504
17.5[a]	6.5	3.25	100	0.178	0.194	6.682	6.762	0.485	0.504
17.5[a]	6.5	3.25	100	0.176	0.194	6.709	6.762	0.514	0.504
17.5[a]	6.5	3.25	100	0.176	0.194	6.408	6.762	0.541	0.504
17.5[a]	6.5	3.25	100	0.176	0.194	6.233	6.762	0.565	0.504
17.5[a]	6.5	3.25	100	0.177	0.194	6.553	6.762	0.500	0.504
17.5	6.5	6.25	100	0.152	0.236	15.176	12.488	0.262	0.230
17.5	6.5	0.25	100	0.369	0.359	3.371	3.661	0.716	0.725
21	6	1.75	50	0.347	0.360	3.522	3.139	0.861	0.888
21	6	1.75	150	0.317	0.344	4.183	4.259	0.505	0.517
21	6	4.75	50	0.324	0.311	6.805	7.311	0.423	0.429
21	6	4.75	150	0.294	0.295	10.385	11.874	0.246	0.250
21	7	1.75	50	0.380	0.396	4.615	4.058	1.026	1.112
21	7	1.75	150	0.362	0.380	4.516	4.160	0.730	0.648
21	7	4.75	50	0.328	0.323	5.290	4.964	0.707	0.731
21	7	4.75	150	0.308	0.307	4.983	6.093	0.440	0.426
24.5	6.5	3.25	100	0.422	0.492	3.333	3.983	0.710	0.707
				RMSE	**0.022**		**0.169**		**0.054**
				SEP	**9.54**		**8.89**		**10.27**
				B_f	**1.01**		**1.03**		**1.00**
				A_f	**1.08**		**1.14**		**1.09**

[a] Center point conditions; RMSE: root mean square error; SEP: % standard error of prediction; B_f: Bias factor; A_f: accuracy factor; OD: optical density units.

another 60 curves for the test itself, giving a total of 420 growth curves.

To find out with some accuracy the number of cells injected into the samples, a calibration line was drawn by taking previous calibrations made with the same instrument, with readings at 600 nm and under an optimal temperature condition of 30 °C. For this, double dilutions were made in MRS broth (Man Rogosa Sharpe, Scharlau) at different initial microorganism concentrations. At the same time, they were plated on MRS Agar (Oxoid, CM361) and incubated at 30 °C for 48 h.

$$\text{Log } N = 2.9793(\text{OD}) + 7.2884 \quad R^2 = 0.958 \tag{1}$$

where N = cfu/g; OD = optical density.

The DMFit curve fitting Program designed by Baranyi (IFR, Norwich) was used for the optical density (Ln (OD)) data fit, applying the Baranyi function (Baranyi & Roberts, 1994) and the estimation of growth rate (*Gr*), lag-time (*lag*) and maximum population density (*yEnd*).

2.5. Response surface model development

The combined effect of different levels of the variables studied (temperature, pH, salt and nitrite concentration) was correlated with the kinetic growth parameters (Gr, Lag and yEnd) in aerobic and anaerobic conditions using a second degree polynomial equation such as:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{j<l} \sum_{l=2}^{k} \beta_{jl} x_j x_l + \varepsilon \tag{2}$$

where y is the response variable, β_0 (intercept y-axis) and β_j, β_{jj} and β_{jl} are the different coefficients of the full model, X_j and X_l are the independent variables related to

Table 2
Average of observed (OBS) and estimated growth rate (Gr, h^{-1}), lag time (lag, h) and maximum population density (yEnd, OD) by response surface model (RS) of *Leuconostoc mesenteroides* in anaerobic conditions

				Gr (h^{-1})		lag (h)		yEnd (OD)	
T (°C)	pH	NaCl (%)	$NaNO_2$ (ppm)	OBS	RS	OBS	RS	OBS	RS
10.5	6.5	3.25	100	0.106	0.112	16.919	15.022	0.382	0.326
14	6	1.75	50	0.161	0.167	7.535	6.386	0.706	0.750
14	6	1.75	150	0.149	0.149	7.855	8.883	0.286	0.303
14	6	4.75	50	0.139	0.107	12.446	15.410	0.297	0.332
14	6	4.75	150	0.120	0.089	21.743	21.435	0.094	0.112
14	7	1.75	50	0.180	0.187	6.571	7.401	0.978	0.795
14	7	1.75	150	0.168	0.169	7.854	8.098	0.487	0.471
14	7	4.75	50	0.142	0.127	12.817	11.461	0.617	0.618
14	7	4.75	150	0.130	0.109	11.168	12.540	0.305	0.305
17.5	5.5	3.25	100	0.103	0.128	12.914	11.885	0.544	0.337
17.5	7.5	3.25	100	0.169	0.170	6.122	6.487	0.824	0.972
17.5	6.5	3.25	0	0.191	0.200	5.335	5.455	1.028	0.958
17.5	6.5	3.25	200	0.157	0.164	9.419	8.303	0.233	0.191
17.5[a]	6.5	3.25	100	0.172	0.161	6.475	6.730	0.548	0.572
17.5[a]	6.5	3.25	100	0.172	0.161	6.602	6.730	0.529	0.572
17.5[a]	6.5	3.25	100	0.170	0.161	6.356	6.730	0.539	0.572
17.5[a]	6.5	3.25	100	0.176	0.161	6.498	6.730	0.537	0.572
17.5[a]	6.5	3.25	100	0.178	0.161	6.679	6.730	0.536	0.572
17.5[a]	6.5	3.25	100	0.167	0.161	6.063	6.730	0.542	0.572
17.5	6.5	6.25	100	0.141	0.201	14.864	14.429	0.269	0.195
17.5	6.5	0.25	100	0.363	0.321	3.589	3.861	0.632	0.679
21	6	1.75	50	0.336	0.319	3.793	3.712	0.783	0.890
21	6	1.75	150	0.312	0.301	4.259	5.163	0.366	0.360
21	6	4.75	50	0.323	0.258	9.088	8.958	0.371	0.395
21	6	4.75	150	0.269	0.240	12.648	12.460	0.129	0.133
21	7	1.75	50	0.363	0.339	3.630	3.463	1.049	0.994
21	7	1.75	150	0.337	0.321	4.272	3.789	0.634	0.559
21	7	4.75	50	0.313	0.279	5.880	5.362	0.696	0.733
21	7	4.75	150	0.296	0.261	5.301	5.867	0.367	0.362
24.5	6.5	3.25	100	0.409	0.416	3.658	4.086	0.480	0.459
				RMSE	**0.022**		**0.120**		**0.087**
				SEP	**10.48**		**6.02**		**16.35**
				B_f	**1.00**		**1.02**		**0.98**
				A_f	**1.09**		**1.10**		**1.14**

[a] Center point conditions; RMSE: root mean square error; SEP: % standard error of prediction; B_f: Bias factor; A_f: accuracy factor; OD: optical density units.

factors and ε the error of model. The values of the coefficients were estimated by the least-squares method. For the estimation of the parameters of the fitting function, SPSS version 11.0 (SPSS) software was used, considering the Levenberg–Marquardt algorithm as suitable for the optimization of the error function. Since variation usually decreases with increasing growth rate and decreasing lag-time and maximum population density, log transformations of these parameters were checked to achieve homogeneous variances.

2.6. Evaluation criteria

To evaluate the fitting and prediction accuracy of RS model, the following evaluation criteria were employed: root-mean-squares error (RMSE); standard error of prediction (SEP) (Hervás, Zurera, García, & Martínez, 2001); Bias factor (B_f) and Accuracy factor (A_f) (Ross, 1996).

$$\mathrm{RMSE} = \sqrt{\frac{\sum(\mathrm{obs} - \mathrm{pred})^2}{n}} \tag{3}$$

$$\%\mathrm{SEP} = \frac{100}{\text{mean obs}} \sqrt{\frac{\sum(\mathrm{obs} - \mathrm{pred})^2}{n}} \tag{4}$$

$$B_f = 10^{\left(\frac{\sum \log\left(\frac{\mathrm{pred}}{\mathrm{obs}}\right)}{n}\right)} \tag{5}$$

$$A_f = 10^{\left(\frac{\sum \left|\log\left(\frac{\mathrm{pred}}{\mathrm{obs}}\right)\right|}{n}\right)} \tag{6}$$

where obs: observed value; pred: predicted value; mean obs: mean of observed values.

2.7. Model validation

The model was tested against a growth data set obtained under the same experimental conditions (30% of the total data set), but not included in the development of the model (internal validation or testing) (Table 3) and against a new data set obtained under different experimental conditions, but included in the experimental design range (external validation) (Tables 4 and 5) in order to evaluate the predictive capacity of the proposed model by calculating the same error criteria described above.

3. Results and discussion

The DMFit program was used to adjust the *L. mesenteroides* growth data to the Baranyi and Roberts (1994) mathematical model, thus obtaining the kinetic parameters growth rate, (*Gr*), lag-time (*lag*) and maximum population density (*yEnd*) in aerobic (Table 1) and anaerobic (Table 2) conditions. Comparison of observed growth data, in aerobic and anaerobic conditions, from the six experimental replications revealed no significant differences ($p > 0.05$) of experimental variance.

Under the experimental conditions, significant differences were observed for the growth rate and the adaptation stage of *L. mesenteroides* between aerobic and anaerobic conditions ($p < 0.05$), whereas no differences were observed for maximum population density. The facultative anaerobic nature of *L. mesenteroides* enables it to develop in the absence of oxygen, although, in general, aerobic conditions are more favorable to the growth of this microorganism, producing higher growth rates and a shorter adaption stage (Tables 1 and 2). Scientific literature contains several references that highlight the behavior of LAB in aerobic and anaerobic conditions. For example, in the comparative studies carried out on meat products packed in aerobic conditions, a modified atmosphere or vacuum-packed, LAB has a lower growth rate in the latter two gaseous atmospheres, thereby increasing the average shelf-life of the product (Cegielska-Radziejewska & Pikul, 2000, 2001; Pikul, Holownia, Cegielska-Radziejewska, & Kijowski, 1997).

3.1. Effect of environmental variables on the growth of L. mesenteroides

To determine which of the variables has the greatest influence on the development of *L. mesenteroides*, a statistical study was carried out using correlation matrices. In both aerobic and anaerobic conditions, temperature was the most decisive factor for Growth rate (*Gr*), with a statistically significant correlation ($p < 0.01$), followed by the concentration of NaCl and pH. When temperature and pH are increased, there is a corresponding increase in the maximum specific growth rate, whereas when the concentration of NaCl and $NaNO_2$ is increased in the culture medium, this growth parameter decreases (Tables 1 and 2). Other authors have observed a greater influence of temperature as opposed to other environment factors (CO_2, pH, etc.) on this same parameter for several lactic acid bacteria (Devlieghere et al., 1998; Giannuzzi, Pinotti, & Zaritzky, 1998). However, some researchers (Pin & Baranyi, 1998; Schepers, Thibault, & Lacroix, 2002) assert that pH is the most decisive factor in the growth of this group of microorganisms. For García-Gimeno et al. (2002), the concentration of NaCl, pH levels and temperature had the most significant effect on the *Gr* of *Lactobacillus plantarum*.

As for lag-time (*lag*), temperature and the concentration of NaCl had the greatest influence on the growth of *L. mesenteroides*; both of these factors had a significant effect ($p < 0.01$) both in aerobic and anaerobic conditions. An increase in temperature or pH produces a decrease in the adaptation stage. However, an increase in the level of salt or nitrites has the opposite effect (Table 1). Other scientific studies agree that the most significant environmental factors for this kinetic parameter are temperature (Devlieghere et al., 1998; Giannuzzi et al., 1998) and, to a lesser degree, the concentration of NaCl (García-Gimeno et al., 2002).

For the kinetic parameter maximum population density (*yEnd*), in both aerobic and anaerobic conditions, a significant correlation was observed between the concentration of NaCl and nitrites ($p < 0.01$) and the pH level ($p < 0.05$). Nitrite concentration was the most important factor with a negative effect on *yEnd,* followed by NaCl

Table 3
Internal validation estimation errors for growth parameter by response surface model of *Leuconostoc mesenteroides* in aerobic and anaerobic conditions

		RMSE	SEP(%)	B_f	A_f
Aerobiosis	Ln lag (h)	0.198	**8.28**	1.01	1.09
	Gr (h^{-1})	0.028	**9.95**	0.98	1.16
	Ln yEnd (OD)	0.057	**10.59**	0.98	1.09
Anaerobiosis	Ln lag (h)	0.119	**6.58**	1.01	1.09
	Gr (h^{-1})	0.025	**11.36**	0.98	1.11
	Ln yEnd (OD)	0.086	**16.31**	0.95	1.13

RMSE: root mean square error; SEP % standard error of prediction of model; B_f: Bias factor; A_f: accuracy factor; OD: optical density units.

Table 4
Values of observed (OBS) and estimated growth rate (Gr, h^{-1}), lag time (lag, h) and maximum population density (yEnd, OD) by response surface model (RS) of *Leuconostoc mesenteroides* for mathematical validation in aerobic conditions

				Gr (h^{-1})		lag (h)		yEnd (OD)	
T (°C)	pH	NaCl (%)	$NaNO_2$ (ppm)	OBS	RS	OBS	RS	OBS	RS
10.5	6.5	0.25	50	0.190	0.323	10.65	5.99	0.628	0.677
10.5	6.5	1.75	0	0.182	0.239	10.35	7.16	0.950	0.779
10.5	6.5	1.75	50	0.172	0.215	10.25	7.78	0.547	0.594
10.5	6.5	1.75	100	0.161	0.201	9.90	8.45	0.411	0.454
10.5	6.5	3.25	0	0.162	0.183	11.05	8.89	0.895	0.616
10.5	6.5	3.25	50	0.151	0.158	10.12	10.10	0.518	0.470
10.5	6.5	3.25	100	0.141	0.145	9.76	11.48	0.368	0.359
14	7	1.75	0	0.230	0.240	3.24	6.80	1.104	1.038
14	7	4.75	0	0.161	0.167	10.19	7.61	0.910	0.682
17.5	6.5	0.25	50	0.382	0.357	2.50	3.53	0.976	0.950
17.5	6.5	1.75	50	0.350	0.249	3.61	4.58	0.882	0.834
17.5	6.5	1.75	100	0.341	0.235	4.11	4.98	0.632	0.637
17.5	6.5	3.25	50	0.177	0.192	6.16	5.95	0.864	0.660
17.5	6	1.75	50	0.290	0.228	4.42	4.09	0.838	0.749
17.5	6	3.25	50	0.172	0.177	5.56	6.24	0.820	0.549
17.5	7	3.25	50	0.268	0.201	3.64	5.85	0.955	0.801
21	6	3.25	50	0.339	0.288	4.92	4.79	0.637	0.650
21	7	3.25	50	0.352	0.312	4.27	4.49	1.011	0.949
21	6	0.25	0	0.383	0.465	1.90	1.85	1.135	1.430
21	6	1.75	0	0.372	0.363	2.01	2.70	1.052	1.163
24.5	6.5	3.25	50	0.432	0.475	2.69	3.51	0.903	0.926
24.5	6.5	3.25	150	0.394	0.459	4.30	4.52	0.520	0.540
24.5	6	1.75	150	0.437	0.495	3.43	3.27	0.519	0.613
24.5	6	4.75	50	0.386	0.462	5.58	5.61	0.539	0.508
24.5	6	4.75	150	0.308	0.446	4.49	9.11	0.281	0.296
24.5	7	4.75	50	0.350	0.474	2.24	3.81	0.812	0.865
24.5	7	4.75	150	0.322	0.458	3.87	4.68	0.536	0.504
				RMSE	**0.070**		**0.322**		**0.130**
				SEP	**22.88**		**27.63**		**15.62**
				B_f	**1.07**		**1.10**		**0.96**
				A_f	**1.20**		**1.28**		**1.13**

RMSE: root mean square error; SEP: % standard error of prediction; B_f: Bias factor; A_f: accuracy factor; OD: optical density units.

concentration and positively by pH and temperature. Just as with the maximum specific growth rate, when temperature and pH are increased, there is a corresponding increase in *yEnd*, whereas when the concentrations of NaCl and $NaNO_2$ are increased in the culture medium, this growth parameter decreases (Table 2). Zhang and Holley (1999) describe the significant effect of pH on the parameter *yEnd*, although they did not observe a significant influence of the concentration of nitrites on microbial levels.

3.2. Response surface model

The response surface models were elaborated following various different mathematical transformations such as the use of logarithms, and the equations that produced the best fit and prediction accuracy were selected. The terms whose coefficients were non-significant were deleted backward, stepwise, and finally, only the terms that had significantly affected the model remained in the equation. The results show a second-order response surface model since the third-order model displayed regression coefficients, whose differences were not significant ($p > 0.05$).

For aerobic conditions, the following equations were selected:

$$\begin{aligned} Gr_{\text{aero}} = {} & -0.0666 \times T + 0.1929 \times \text{pH} - 0.0439 \\ & \times \text{NaCl} - 0.0006 \times \text{NaNO}_2 + 0.0026 \\ & \times T^2 - 0.0110 \times \text{pH}^2 + 0.0115 \\ & \times \text{NaCl}^2 + 0.0000022 \\ & \times \text{NaNO}_2^2 - 0.0079 \times (\text{pH} \times \text{NaCl}) \\ R^2 = {} & 0.942 \end{aligned} \tag{7}$$

$$\begin{aligned} Ln\ lag_{\text{aero}} = {} & -0.0756 \times T + 1.5394 \times \text{NaCl} + 0.0188 \\ & \times \text{NaNO}_2 + 0.0594 \times \text{pH}^2 - 0.2146 \\ & \times (\text{pH} \times \text{NaCl}) - 0.0028 \\ & \times (\text{pH} \times \text{NaNO}_2) + 0.0006 \\ & \times (\text{NaCl} \times \text{NaNO}_2) \\ R^2 = {} & 0.859 \end{aligned} \tag{8}$$

Table 5
Values of observed (OBS) and estimated growth rate (Gr, h^{-1}), lag time (lag, h) and maximum population density (yEnd, OD) by response surface model (RS) of *Leuconostoc mesenteroides* for mathematical validation in anaerobic conditions

				Gr (h^{-1})		lag (h)		yEnd (OD)	
T (°C)	pH	NaCl (%)	$NaNO_2$ (ppm)	OBS	RS	OBS	RS	OBS	RS
10.5	6.5	0.25	50	0.172	0.286	10.58	7.76	0.688	0.491
10.5	6.5	1.75	0	0.161	0.206	11.39	8.99	1.009	0.608
10.5	6.5	1.75	50	0.153	0.181	12.91	9.98	0.601	0.528
10.5	6.5	1.75	100	0.141	0.167	11.29	11.09	0.378	0.397
10.5	6.5	3.25	0	0.128	0.151	13.44	12.18	0.978	0.545
10.5	6.5	3.25	50	0.112	0.126	11.01	13.52	0.562	0.453
10.5	6.5	3.25	100	0.106	0.112	15.06	15.02	0.385	0.326
14	7	1.75	0	0.214	0.212	3.53	7.08	1.154	0.832
14	7	4.75	0	0.149	0.152	14.36	10.96	0.979	0.707
17.5	6.5	0.25	50	0.374	0.335	2.42	3.48	0.994	0.863
17.5	6.5	1.75	50	0.305	0.230	4.06	4.47	0.913	0.928
17.5	6.5	1.75	100	0.297	0.216	4.41	4.97	0.532	0.698
17.5	6.5	3.25	50	0.175	0.175	5.93	6.06	0.890	0.796
17.5	6	1.75	50	0.274	0.217	3.24	4.69	0.853	0.901
17.5	6	3.25	50	0.157	0.162	3.66	7.10	0.769	0.672
17.5	7	3.25	50	0.258	0.183	2.26	5.91	1.018	0.943
21	6	3.25	50	0.332	0.264	5.24	5.62	0.543	0.663
21	7	3.25	50	0.350	0.284	4.52	4.20	1.004	0.932
21	6	0.25	0	0.380	0.448	2.67	2.19	1.250	1.151
21	6	1.75	0	0.369	0.343	3.11	3.15	1.128	1.126
24.5	6.5	3.25	50	0.416	0.430	2.66	3.68	0.763	0.638
24.5	6.5	3.25	150	0.389	0.412	3.56	4.54	0.383	0.285
24.5	6	1.75	150	0.402	0.454	4.08	4.41	0.346	0.292
24.5	6	4.75	50	0.383	0.412	5.81	7.65	0.500	0.320
24.5	6	4.75	150	0.334	0.394	5.96	10.65	0.122	0.108
24.5	7	4.75	50	0.350	0.432	1.93	4.11	0.747	0.595
24.5	7	4.75	150	0.332	0.414	4.46	4.50	0.406	0.294
				RMSE	**0.052**		**0.371**		**0.169**
				SEP	**17.65**		**27.28**		**20.56**
				B_f	**1.03**		**1.18**		**0.86**
				A_f	**1.17**		**1.31**		**1.22**

RMSE: root mean square error; SEP: % standard error of prediction; B_f: Bias factor; A_f: sccuracy factor; OD: optical density units.

$$\begin{aligned} Ln\ yEnd_{\text{aero}} &= 0.0484 \times T - 0.2353 \times \text{pH} - 0.7059 \\ &\times \text{NaCl} - 0.0054 \times \text{NaNO}_2 + 0.0216 \\ &\times \text{pH}^2 - 0.0232 \times \text{NaCl}^2 + 0.1024 \\ &\times (\text{pH} \times \text{NaCl}) \\ R^2 &= 0.935 \end{aligned} \quad (9)$$

And for anaerobic conditions:

$$\begin{aligned} Gr_{\text{anaero}} &= -0.0518 \times T + 0.1780 \times \text{pH} - 0.0922 \\ &\times \text{NaCl} - 0.0006 \times \text{NaNO}_2 + 0.0021 \\ &\times T^2 - 0.0121 \times \text{pH}^2 + 0.0111 \\ &\times \text{NaCl}^2 + 0.0000021 \times \text{NaNO}_2 \\ R^2 &= 0.936 \end{aligned} \quad (10)$$

$$\begin{aligned} Ln\ lag_{\text{anaero}} &= 8.6726 - 2.4963 \times \text{pH} + 1.1063 \\ &\times \text{NaCl} + 0.0177 \times \text{NaNO}_2 + 0.0031 \\ &\times T^2 + 0.2659 \times \text{pH}^2 + 0.0115 \\ &\times \text{NaCl}^2 - 0.0310 \times (T \times \text{pH}) - 0.1479 \\ &\times (\text{pH} \times \text{NaCl}) - 0.0024 \\ &\times (\text{pH} \times \text{NaNO}_2) \\ R^2 &= 0.940 \end{aligned} \quad (11)$$

$$\begin{aligned} Ln\ yEnd_{\text{anaero}} &= 0.3045 \times T - 0.4573 \times \text{pH} - 1.0349 \\ &\times \text{NaCl} - 0.0250 \times \text{NaNO}_2 - 0.0080 \\ &\times T^2 - 0.0502 \times \text{NaCl}^2 - 0.000029 \\ &\times \text{NaNO}_2^2 + 0.1867 \times (\text{pH} \times \text{NaCl}) \\ &+ 0.0038 \times (\text{pH} \times \text{NaNO}_2) - 0.0006 \\ &\times (\text{NaCl} \times \text{NaNO}_2) \\ R^2 &= 0.902 \end{aligned} \quad (12)$$

These equations were used to estimate the predicted values of each kinetic parameter in aerobic and anaerobic conditions, shown in Tables 1 and 2, respectively. They also show the statistical factors that indicate the average deviations between observed and predicted values for each of the models, in aerobic and anaerobic conditions. In both cases, the polynomial equations produced a high value for the multiple regression coefficient (R^2) and a

low value for the RMSE statistic, which indicates a good fit of the experimental data, and better values than those observed in other scientific studies (García-Gimeno et al., 2003; Juneja, Eblen, & Marks, 2001; Lou & Nakai, 2001; Zurera-Cosano et al., 2004).

The standard error of prediction, SEP, produced low values for the three parameters, less than 11% in aerobic conditions and 16% in anaerobic conditions, thus confirming the concordance between the observed and predicted values (Tables 1 and 2). Although there are few scientific studies that reflect the SEP values, García-Gimeno et al. (2002) obtained approximate values of between 36% and 39% for *Gr* and *lag-time*, respectively for the spoilage microorganism *Lactobacillus plantarum*. For *E. coli* O157:H7, these values ranged from between 22% and 31% (García-Gimeno et al., 2003). Thus, comparing the results obtained in these studies and our own, we have observed that our model produced a better fit between the experimental and estimated data.

To determine the goodness-of-fit of the response surface models elaborated, the calculated bias factor (B_f) and accuracy factor (A_f) are shown (Tables 1 and 2). We can see how these factors are close to unity, which would mean a perfect concordance, since there is a good fit between the observed and predicted values obtained from the developed models. The perfect value for these factors should be unity, $B_f = 1 = A_f$; however, Ross et al. (2000) consider a A_f value to be acceptable with an increase of up to 0.15 (15%) for each variable included in the model. Therefore, in our study, with four variables, (temperature, pH, concentration of salt and nitrites) we should expect A_f values of up to 1.6.

For the three growth parameters, the B_f is very close to 1 (Tables 1 and 2). For Growth rate (*Gr*) it is important for B_f to be greater than 1, because, in the case of spoilage microorganisms, it indicates that the model produces adequate shelf-life predictions, since it will estimate beforehand any sensorial alteration in the product. However, the shelf-life estimation should not be substantially shorter than the observed period, since this could result in important losses for the manufacturers if they are forced to withdraw a product from the shelves when it it is still suitable for consumption.

Other authors, such as García-Gimeno et al. (2003), have developed predictive models for *E. coli* and obtained similar values to those produced in our study for both mathematical factors, where B_f ranged from between 0.93 and 1.10 and A_f from between 1.17 and 1.45.

3.3. Internal validation (testing)

The values obtained for the mathematical factors used for the three kinetic parameters were very similar to the results produced with data from the model (Table 3). In all cases, B_f and A_f values were close to unity, which indicates a good fit between the observations and the predictions. In anaerobic conditions, the same trend was observed. All of this indicates that the model has a good generalization ability in its estimation of *L. mesenteroides* growth response.

Scientific literature contains few references about the internal validation of predictive models; however, the results found are very similar to those produced in our study. For example, the studies conducted by Hervás et al. (2001) obtained SEP values of around 9% for *Gr* in a model of Artificial Neural Networks for *Salmonella* spp. García-Gimeno et al. (2002), observed values of between 11% and 17% for *Gr* and *lag* for this same type of model, which was elaborated in this case for *L. plantarum*.

3.4. External validation

For the purposes of external validation, the observed values for each of the growth parameters described for *L. mesenteroides*, were compared with the estimations provided by the model, in aerobic and anaerobic conditions (Tables 4 and 5, respectively), quantified using the calculations of the RMSE, SEP, A_f and B_f factors described above. In both aerobic and anaerobic conditions, there was not a great deal of variance between the observed values and those predicted by the RS model for each of the kinetic parameters.

When validating the predictive models (Dalgaard, 2000), an acceptable value for B_f was considered to be between 0.80 and 1.30 for spoilage microorganisms in fish products. In our study, under both aerobic and anaerobic conditions, the models elaborated are suitable to describe the growth of *L. mesenteroides* in culture broth.

In our study, for the parameter *maximum growth rate*, we obtained a B_f greater than 1, which means that accurate predictions can be made regarding sensorial qualities and shelf-life, since any sensorial alteration in the product can be estimated beforehand. At the same time, values of close to 1 were observed (Tables 4 and 5), indicating only minimal differences between the predicted and observed data (B_f) and the general proximity of the predictions and the observations (A_f). The results obtained during our study were in general better than those described by other authors (Lebert, Robles-Olvera, & Lebert, 2000; Neumeyer et al., 1997b). García-Gimeno et al. (2003), obtained adequate values of $B_f = 1.09$ and $A_f = 1.27$ in the mathematical validation of their RS models developed for *E. coli* O157:H7, whereas Zurera-Cosano et al. (2004) described values of $B_f = 1.06–1.33$ and $A_f = 1.17–1.37$ for their RS models of *S. aureus*. In another study conducted by Valik and Pieckova (2001) on spoilage moulds, values very close to unity were observed, $B_f = 1.01$ and $A_f = 1.07$, demonstrating the fit and accuracy of the RS model elaborated. Arinder and Borch (1999) observed similar

values for these factors, $B_f = 1.02$ and $A_f = 1.36$, for the growth rate of *Pseudomonas sp.*

The prediction of the *lag-time* poses greater difficulties for the creation of a model than the other parameters, since it depends on many factors, such as the physiological state and size of the inoculum, and the previous growth conditions (Robinson, Ocio, Kaloti, & Mackey, 1998; Ross et al., 2000), which could explain possible differences between the observed and predicted values. Many scientific studies contain references to the mathematical validation factors, B_f and A_f, for the lag-time of various microorganisms. García-Gimeno et al. (2003) conducted a study with *E. coli* O157:H7, in which the estimations that provided the best fit were obtained using an RS model ($B_f = 0.98$ and $A_f = 1.17$). For this same parameter, Zurera-Cosano et al. (2004) observed values of $B_f = 0.87$–1.54 and $A_f = 1.52$–2.22 in a response surface model of *S. aureus*, in which, as in our study, they also considered both gaseous atmospheres. As shown in Tables 4 and 5, the factors obtained in our study fall into the range described by these authors, and are even better since they are closer to unity.

In predictive microbiology, models are not often elaborated for the kinetic parameter *maximum population density*; it is included in only a few models, such as those developed by McCann, Eifert, Gennings, Schilling, and Carter (2003) and Nauta, Litman, Barker, and Carlin (2003). In our study, particularly good values were obtained for B_f, which produced values close to unity, although the growth response of *L. mesenteroides* was slightly underestimated.

According to the results obtained for the statistics RMSE, SEP and the factors B_f and A_f during the development and mathematical validation of the model, we can conclude that the RS models elaborated in aerobic and anaerobic conditions are suitable for the estimation of the three growth parameters of *L. mesenteroides,* and can therefore be applied to shelf-life estimations for food products.

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