Multiple response optimization of the effect of thyme essential oil against *Listeria monocytogenes* in ground meat at different times and temperatures

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Summary

Response surface methodology was used to optimize conditions (e.g., Thyme oil concentration [0.0-0.57%] and storage temperature [0.0-14.14°C]) for inhibiting the growth of *L. monocytogenes* (log cfu/g) in ground meat. Additionally, the effect of the variables; namely, temperature and concentration on $\mu_{\max}$ (maximum specific growth rate, ln cfu/g/h) values was also evaluated using a proposed combined model. The best fitting second order polynomial models were developed for each response using multiple linear regression analysis with backward elimination regression (BER) procedure. In this paper, multi-response surfaces using desirability function approaches were successfully applied to determine optimum operating conditions. Under these optimum treatment and storage parameters, *L. monocytogenes* populations at hours 6, 24, 48, 72 and 96 were 6.12, 5.96, 5.88, 5.81 and 5.41 log cfu/g and 0.001 ln cfu/g/h, respectively and $\mu_{\max}$ value 0.001 ln cfu/g/h. At the end, the proposed combined model is described numerically and experimentally.

Keywords: Baranyi model, central composite design, *Listeria monocytogenes*, ground meat, thyme essential oil

The presence of *L. monocytogenes* is unacceptable in ground beef; therefore, it should be inactivated/decontaminated in such meat products. In this respect, there are a great number of studies focused on reduction of the number of *L. monocytogenes* with essential oil applications. The utilization from antimicrobial activity of essential oils and extracts of many plant species have recently become popular for many pharmaceutical and food processing applications (11). Essential oil treatment is one of the effective methods for elimination of *L. monocytogenes* from food products (40). In the literature, there are a number of references highlighting the inhibitory effect of *Thymus vulgaris* on *L. monocytogenes* growth (14, 33, 36).

Response surface methodology (RSM) is a useful statistical tool, allowing to improve and optimize processing conditions by finding the experimental relationship between input and output variables that are considered in an experimental design (20). Using

an RSM design of experiment, it is possible to determine the relationship between the response and the independent parameters; to develop first- or second-order mathematical models of responses as a function of the parameters studied; to map a response surface over a particular region of interest and optimize the response, and to select operating conditions to achieve target specifications or customer requirements (6, 23, 32). Like RSM, predictive microbiological models are also widely used in the modeling of food processing to eliminate the complexity of reactions and heterogeneous structure of food products. Among the predictive models used, Baranyi is known to be capable of showing a good fit for growth curves of some pathogenic bacteria, such as *Bacillus* spp., *Brochothrix thermospacta*, *Clostridium* spp., *Escherichia coli* O157:H7, *Staphylococcus* spp., *Yersinia enterocolitica* and *Salmonella Typhimurium* as well as *Listeria monocytogenes* under different conditions (2, 4, 16, 38, 45).

Accurate assessments of *Listeria* growth inhibition, to a large extent, relies on technical expertise and experience that are necessary to determine the simultaneous effects of multiple factors, as opposed to conclusions based on a single factor. However, in the literature, a considerable number of studies based on RSM have been conducted using an approach with a single-response problem; conversely, limited attention has been paid to multi-response problems. Previous studies have indicated that the optimal factor settings for one response characteristic may not necessarily be compatible with those of the other response characteristics. In this respect, one of the most efficient ways to obtain desirable results is to use an appropriate statistical tool, allowing optimization of the levels of process variables. This necessitates a detailed experimental design to establish the most appropriate levels of essential oil and storage temperature to maximize growth inhibition of *L. monocytogenes*.

In order to achieve the best inactivation conditions, a multiple-response optimization study should be conducted, aiming to determine growth/inactivation kinetics of the bacterium in a model food system; for example, ground meat under optimum treatment and storage parameters. However, to the best of our knowledge, it seems that determination and optimization of simultaneous effects of essential oil concentration and storage temperature on the growth of *L. monocytogenes* by RSM based on the Baranyi model have heretofore not been reported. Therefore, this study was aimed to characterize and model the growth/survival of *L. monocytogenes* at different storage times in ground beef in order to establish optimum operating conditions. RSM and Baranyi model were used for modeling and optimization procedures.

Material and methods

Beef used in microbiological analysis was aseptically obtained from a commercial abattoir in Kayseri, Turkey. Beef including 10% to 12% fat was excised from loin muscle (cattle) of the carcasses kept in chilling room at $0 \pm 1^\circ\text{C}$ until reaching 24 h of postmortem. Next, the meat was immediately transferred to the laboratory under cold chain conditions, then ground aseptically through a previously autoclaved grinder (Tefal, China).

Dried thyme (500 g) (*Thymus vulgaris* L.) identified by the scientists of botany in Erciyes University in Kayseri, Turkey were obtained from a local retail spice market. The essential oil (EO) of the plant was extracted using Clavenger apparatus by the hydro distillation method described by Baydar et al. (5). For this aim, plant material (100 g) was cut into small pieces, placed in the distillation apparatus with 2 L of double distilled water and hydro distilled for 3 h. After dried over anhydrous sodium sulphate, the oils were stored at 4°C until analyses.

As a test bacterium, *Listeria monocytogenes* ATCC 7644 was used in this study. The bacterial culture was obtained

Tab. 1. Two-factor-five-level central composite rotatable design (ccrd) matrix indicating the levels of coded and actual for two variables

Experimental runs	Coded level of variables		Actual level of variables	
	Temperature (X_1)	Concentration (X_2)	Temperature ($^\circ\text{C}$)	Concentration (%)
Factorial points				
1	1	1	12.07	0.48
2	1	-1	12.07	0.08
3	-1	-1	2.07	0.08
4	-1	1	2.07	0.48
Axial points				
5	0	$+\alpha$ (+1.414)	7.07	0.57
6	0	$-\alpha$ (-1.414)	7.07	0.00
7	$+\alpha$ (+1.414)	0	14.14	0.28
8	$-\alpha$ (-1.414)	0	0.00	0.28
Center points				
9	0	0	7.07	0.28
10	0	0	7.07	0.28

from Kayseri Agriculture Control Protection Management, Turkey. At the first step, stock culture of *L. monocytogenes* ATCC 7644 was inoculated to Nutrient Broth (Merck, Germany) for obtaining fresh culture and grown at 37°C for 24 h. At the second step, the fresh culture of *L. monocytogenes* ATCC 7644 was again inoculated and activated ($\sim 10^8$ cfu/ml) in Nutrient Broth, after a second incubation at 37°C for 24 h and finally inoculated on the ground meat samples up to a final population of 10^6 cfu/g. Such a high inoculation level was selected as being one of the most frequently studied concentrations in growth/inactivation kinetic modeling studies conducted in meat products (8, 9, 13, 34, 43).

After the fresh ground meat samples were confirmed for the absence of *L. monocytogenes* ATCC 7644 as well as the presence of any *L. monocytogenes*, the essential oil (EO) applications were conducted. The number of *L. monocytogenes* ATCC 7644 was evaluated in ten ground meat samples (Tab. 1). Preliminary experiments and the growth limits of the bacterium defined the experimental range for levels of each studied factor. In this respect, appropriate concentrations of thyme essential oil were applied by surface spraying, yielding the relevant concentrations: 0.0, 0.08, 0.28, 0.48 and 0.57% (v/w) EO per sample (10 ground meat samples according to the second order design matrix, Tab. 1). After being packed in PVC (polyvinyl chloride film wrapping material), each inoculated sample was stored at the relevant temperature levels (0.0, 2.07, 7.07, 12.07 and 14.14°C), as shown in the second order design matrix (Tab. 1) prior to enumeration of the pathogen cells. All experiments were conducted in triplicate.

Twenty five grams of ground meat samples were homogenized with 225 ml of a sterile maximum recovery solution (Merck, Darmstadt, Germany). Decimal dilutions were prepared in 9 ml sterile maximum recovery diluent solution until 10^7 dilutions. *L. monocytogenes* was enumerated on Oxford Listeria Selective Agar (Merck, Darmstadt, Germany) using the spread-plate method, after incubation at 37°C for 24 h (1). Typical *L. monocytogenes* colonies

formed on Oxford Listeria Selective Agar were counted as colony forming units (cfu) per gram after each incubation period: hours 6, 12, 24, 36, 48, 60, 72, 84, and 96. Bacterial counts were expressed as log₁₀ cfu/g.

The Baranyi model was used to fit the sigmoidal bacterial growth curves at constant temperature conditions (3).

$$y(t) = y_0 + \mu_{\max} F(t) - \frac{1}{m} \ln \left(1 + \frac{\exp[m\mu_{\max} F(t)] - 1}{\exp[m(y_{\max} - y_0)]} \right) \quad (1)$$

where $F(t) = t + \frac{1}{\mu_{\max}} \ln [\exp(-vt) + \exp(-h_0) - \exp(-vt - h_0)]$

and where $y(t) = \ln(x(t))$ (cfu/g) is the logarithm of the cell number *L. monocytogenes* with $x(t)$; y_0 ($\ln(x_0)$); $y_{\max} = \ln(x_{\max})$, x_0 being the initial and $x_{\max}$ the asymptotic cell concentration, respectively; $\mu_{\max}$ the maximum specific growth rate (ln cfu/g/h); m a curvature parameter to characterize the transition from the exponential phase; v a curvature parameter to characterize the transition to the exponential phase, h_0 a dimensionless parameter quantifying the initial physiological state of the cells. From that, λ (h) can be calculated as $h_0/\mu_{\max}$. For the curvature parameters, Baranyi (2) suggested $v = \mu_{\max}$ and $m = 1$.

In response modelling, multiple linear regression analysis was used and the following second-order polynomial equation of function x_i was fitted for each factor assessed at each experimental point:

$$Y - \varepsilon = \beta_0 + \sum_{i=1}^N \beta_i x_i + \sum_{i=1}^N \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ j=i+1}}^N \sum_{j=i+1}^N \beta_{ij} x_i x_j \quad (2)$$

where Y is the estimated response; β_0 is the average value of the response at the center point of the design, β_1 , β_2 , β_{12} , β_{11} and β_{22} are linear, interaction and quadratic terms, respectively.

To develop predictive models for evaluating the effect of EO concentration and storage temperature on *L. monocytogenes* number (log cfu/g) enumerated at different storage times – namely, hours 6, 24, 48, 72, and 96 as well as $\mu_{\max}$ values (that will be described in later sections) – a 2-factor-5-level Central Composite Rotatable Design (CCRD) with two replicates at the center point was used in this study. Each enumeration time represented a different response variable. The two factors, levels and experimental design in terms of coded and uncoded (actual values) can be seen in Table 1. The CCRD is an optimal design which allows calculation, with a minimum number of experiments, with best precision possible of the linear and quadratic effects and interactions each of the two factors chosen. This design is consisted of 2^k factorial points (coded as ± 1 notation), augmented by $2k$ axial points ($\pm \alpha, 0, 0, \dots, 0$), ($0, \pm \alpha, 0, \dots, 0$), ($0, 0, \pm \alpha, \dots, 0$), ..., ($0, 0, 0, \dots, \pm \alpha$) that are located at a specified distance α from the center in each direction on each axis defined by the coded factor levels. n_0 is each center point ($0, 0, \dots, 0$). k is the number of factors. The relationship between coded and actual values of variables was calculated using the following equation:

$$x_i = \frac{z_i - 0.5(z_{i,\max} + z_{i,\min})}{0.5(z_{i,\max} - z_{i,\min})} \quad (3)$$

where z is the actual variable, the subscripts max and min refer to the maximum (12.07°C and 0.48%, respectively) and minimum values (2.07 and 0.08%, respectively) and x is the coded variable. In this study, rotatability and orthogonality were selected since these properties of the design are desirable. The design is rotatable if the variance of the response is constant for all variables at a given distance from the design center (17, 21). The CCD is rotatable if:

$$\alpha = \sqrt[4]{2^k} \quad (4)$$

Orthogonality of design is requisite for the evaluation of linear, quadratic and interaction effects if they are significant, indicating that different variable effects can be estimated independently. The CCRD would be nearly orthogonal if:

$$N_0 = 4N_f^{0.5} - 2k - 2 \quad (5)$$

where N_f is the number of factorial points ($N_f = 2^k$) (35).

The total number of design points in CCRD can be calculated from:

$$N = 2^k (\text{factorial points}) + 2k (\text{axial points}) + n_0 (\text{center points}) \quad (6)$$

where N is the total number of design points, k is the number of factors, n_0 is the number of replicates at the center point. Thus, for this design, the total number of experimental points will be 10 ($k = 2$; $n_0 = 2$). Table 1 shows the CCRD used and the coordinates for $k = 2$ factors.

Because it is not known what the true functional relation is between the responses and the independent variables, the first-order or second-order polynomial expressions approximated the actual response surfaces for a selected experimental region (2.07-12.07°C and 0.08-0.48%). The general model for the case of two independent variables (temperature, °C (x_1) and concentration, % (x_2)) is

$$Y = f(x_1, x_2) + \varepsilon \quad (7)$$

Then, multiple linear regression analysis was used and the following second-order polynomial equation of function x_i was fitted for each factor assessed at each experimental point.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon \quad (8)$$

where Y is the estimated response (different storage times: 6th, 24th, 48th, 72nd, and 96th in which *L. monocytogenes* (log cfu/g) was enumerated as well as $\mu_{\max}$ values (ln cfu/g/h)); β_0 is the average value of the response at the center point of the design, β_1 , β_2 , β_{12} , β_{11} and β_{22} are linear, interaction and quadratic terms, respectively, produced for the prediction models of variables (thyme oil concentration and storage temperature).

Regarding $\mu_{\max}$ values, they were calculated from the Baranyi model [Eq. (1)] and used in RSM modeling [Eq. (2)] so that the performance of RSM based on the Baranyi model could be tested. For this purpose, the $\mu_{\max}$ values were calculated in terms of nine storage (enumeration) times (6th, 12th, 24th, 36th, 48th, 60th, 72nd, 84th, and 96th) using the Baranyi model [Eq. (1)] for each experimental run (Tab. 1). Next, a total of ten $\mu_{\max}$ values were analyzed using CCRD [Eq. (2)] of RSM. In this respect, the effect of the variables; namely, temperature (t) and concentration (c) on $\mu_{\max}$ (y)

values was evaluated using Eq. (9), a combined model constructed from the Eqs. (1) and (2).

$$y(t) = \frac{1}{m} \ln \left\{ 1 + \frac{\exp \left[m \mu_{\max} \left\{ t + \frac{1}{\mu_{\max}} \ln [\exp(-vt) + \exp(-h_0) - \exp(-vt - h_0)] \right\} \right] - 1}{\exp[m(y_{\max} - y_0)]} \right\} + y_0 \quad (9)$$

$$+ \mu_{\max} \left\{ t + \frac{1}{\mu_{\max}} \ln [\exp(-vt) + \exp(-h_0) - \exp(-vt - h_0)] \right\} = \beta_0 + \beta_1 t + \beta_2 c + \beta_{12} tc + \beta_{11} t^2 + \beta_{22} c^2$$

The best fitting models were determined using multiple linear regressions with backward elimination regression (BER), where insignificant factors and interactions were removed from the models and only the variables significant at $P < 0.01$, $P < 0.05$ and $P < 0.1$ levels were selected for the model construction using BER procedure. For this procedure, let the model with all possible covariates be:

$$Y = \beta_0 + \beta_1 X_1 + \dots + \beta_{r-1} X_{r-1} + \varepsilon \quad (10).$$

Next, the following $r-1$ tests are carried out, $H_{0j} : \beta_j = 0$, $j = 1, 2, \dots, r-1$. The lowest partial F-test value F_l corresponding to $H_{0l} : \beta_l = 0$ or t-test value t_l is compared with the preselected significance values F_0 and t_0 . At the second step, one of two possible steps (step 2a and step 2b) can be taken as:

Step 2a:

If $F_l < F_0$ or $t_l < t_0$, then X_l can be deleted and the new original model is

$$Y = \beta_0 + \beta_1 X_1 + \dots + \beta_{l-1} X_{l-1} + \beta_{l+1} X_{l+1} + \dots + \beta_{r-1} X_{r-1} + \varepsilon \quad (11).$$

Go back to step 1.

Step 2b:

If $F_l > F_0$ or $t_l > t_0$, the original model is the model that should be chosen.

Optimization is composed of finding of all values of the process variables involving the experimental responses. The optimum point can be determined by various ways (37, 41). Constructing a model, the layout of the surface contours remains the easiest method to interpret. To visualize the behavior of the phenomenon studied and choose the zone of the study, these curves are analyzed, making a better output possible (35). The established model can be written in the following matrix form:

$$\hat{y} = b_0 + x_k^t b_k + x_k^t \mathbf{B} x_k \quad (12)$$

where $\hat{y}$ is the predicted response, x_k^t is the vector transposed of the punctual coordinates in the work domain considered, $\mathbf{B}$ is the central matrix. The matrix $\mathbf{B}$ is built as follows:

$$\mathbf{B} = \begin{bmatrix} b_{11} & \frac{1}{2} b_{12} \\ \frac{1}{2} b_{12} & b_{22} \end{bmatrix} \quad (13).$$

Calculation of the optimal values of the process parameters was first performed on coded units by using relation 14 and then converted to actual units by Eq. 3.

$$x_s = -\frac{1}{2} \mathbf{B}^{-1} b_k \quad (14).$$

In RSM, optimization is the ultimate aim. Many response surface problems involve the analysis of several responses. Performing simultaneous considerations of multiple responses, an appropriate response surface model is built for each response at the first step, and then a set of operating conditions that, in some sense, optimizes all responses or at least keeps them in desired ranges is estimated (30). In this respect, some of the variables are aimed to be maximized and some to be minimized. However, a competition occurs between these responses in many cases; namely, improving one response may lead another response to deteriorate, further complicating the situation. In order to overcome this problem, several approaches have been developed. In one approach, a constrained optimization procedure is used. In the second one, the contour diagrams of the different response variables is superimposed and in the third approach, the problem of multiple responses is solved through use of a desirability function which combines all the responses into one measurement. Use of desirability functions has three advantages: (1) different scaled responses can be compared with each other, (2) different responses can be simply and quickly transformed to single measurement, and (3) it is possible to simultaneously use qualitative and quantitative responses (12, 19).

The operating conditions, x providing the "most desirable" response values can be found by this method. In this respect, different desirability functions $d_i(Y_i)$ can be used depending on whether a particular response Y_i is to be maximized or minimized (12). Let L_i , U_i and T_i be the lower, upper and target values, respectively, that are desired for response Y_i with L_i , U_i and T_i .

If a response is maximized, then its individual desirability function is with the exponent s that determines how significant it is to hit the target value. For $s = 1$, the desirability function increases linearly towards T_i which indicates a large adequate value for the response; for $s < 1$, the function is convex, and for $s > 1$, the function is concave (15):

$$d_i(\hat{y}_i) = \begin{cases} 0 & \hat{y}_i(x) < L_i \\ \left(\frac{\hat{y}_i(x) - L_i}{T_i - L_i} \right)^s & L_i \leq \hat{y}_i(x) \leq T_i \\ 1 & \hat{y}_i(x) > T_i \end{cases} \quad (15).$$

If a response is to be minimized, then its individual desirability function is with T_i which indicates a small adequate value for the response:

$$d_i(\hat{y}_i) = \begin{cases} 1 & \hat{y}_i(x) < T_i \\ \left(\frac{\hat{y}_i(x) - U_i}{T_i - U_i} \right)^s & T_i \leq \hat{y}_i(x) \leq U_i \\ 0 & \hat{y}_i(x) > U_i \end{cases} \quad (16).$$

Having been computed for each response variable, desirability values were combined into a single desirability index, D . For this purpose, each response was transformed in a dimensionless function, which is called partial desirability function, d_p , which reflects the desirable ranges for each response. The desirable ranges from zero to one (least to most desirable, respectively). The definition of the partial desirability functions allows the global desirability function D to calculate the weighted geometric mean of n individual desirability functions (all transformed responses) [Eq. (17)]. The simultaneous objective function is a geometric mean of all transformed responses (24, 32):

$$D = d_1^{p_1} \times d_2^{p_2} \times d_3^{p_3} \times \dots \times d_n^{p_n} \quad 1/\sum p_i \\ = \left[\prod_{i=1}^n d_i^{p_i} \right]^{1/\sum p_i} \quad (17)$$

where p_i is the weighting of the i_{th} , and normalized in order that $\sum_{i=1}^n p_i = 1$. By the weighting of partial desirability functions, it is possible to enable the optimization process to take the relative importance of each response into consideration. Allowing the examination of the form of the desirability function, it is possible to find the region where the function was close to 1 and determine the compromise optimum conditions. However, the overall function becomes zero in case any response or factor is outside their desirability range (32, 39). In the present study, the desired responses of “number of *L. monocytogenes*” and “ μ_{max} values” were the minimum of the target goal. The same importance was applied to each response during the optimization analysis. The modeling procedure and optimization methodology by RSM (combined the Baranyi model) is diagrammed in Fig. 1.

The computational work including designation of experimental points, randomization, analysis of variance, fitting of the second-order polynomial models and graphical representations (3D surface, studentized residual plots and desirability graphs) as well as optimization was performed using a statistical package, Design-Expert version 7.0 (Stat-Ease Inc., Minneapolis, USA). Regarding non-linear regression analysis, a non-linear regression procedure in Statistica software (Release 5.0, Statsoft Inc., Tulsa, OK, USA) was used to fit each individual set of growth data to the Baranyi model, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e. loss function (observed–predicted). The Quasi-Newton algorithm option of the non-linear regression procedure was used during numerical iteration to search for the calculated parameters of each model. After several iterations in the non-linear procedure, the starting values converged to estimated values of the parameters.

Fig. 1. Steps of modeling and optimization by RSM and a combined model (RSM based on Baranyi model)

Results and discussion

Table 1 shows the coded and uncoded levels of experimental factors. The levels of the factors were selected based on the results of preliminary experiments and limits of factors based on the literature assessments. For temperature, 14.14°C was the upper limit because *L. monocytogenes* is a psychrotropic, although it can optimally develop in the temperature range of 30°C–37°C. In addition, ground meat is stored below 10°C, mainly under refrigerator conditions. The lower limit of temperature was selected as 0°C since it can grow in a temperature range of 1°C–45°C (27, 28). Within the studied concentration range of the thyme essential oil in this study, the lower and upper levels were selected according to the levels reported in the literature and the results of preliminary sensory analysis.

The effects of essential oil (EO) and storage temperature levels on the responses; namely, the *L. monocytogenes* number (log cfu/g) enumerated at differ-

Tab. 2. Anova table for response surface reduced linear and quadratic regression models and effects of the variables on the *L. monocytogenes* number (log cfu/g) enumerated at 6th and 24th hours

Source of Variance	6 th hour					24 th hour				
	SS ^a	DF ^b	MS ^c	F value	p value	SS	DF	MS	F value	p value
Model	0.41	1	0.41	12.20	0.0082	0.79	2	0.39	27.84	0.0005
<i>B</i> (concentration)	0.41	1	0.41	12.20	0.0082	0.67	1	0.67	47.55	0.0002
<i>B</i> ²	BER ^d					0.12	1	0.12	8.13	0.0246
Residual	0.27	8	0.03			0.10	7	0.01		
lack of fit	0.27	7	0.04	257.8	0.0479	0.09	6	0.02	3.35	0.3954
pure error	1.48×10^{-4}	1	1.48×10^{-4}			4.70×10^{-3}	1	4.70×10^{-4}	4	
total corrected	0.67	9				0.89	9			
<i>R</i> ² ^e	0.6040					0.8883				
adj- <i>R</i> ² ^f	0.5545					0.8564				
pred- <i>R</i> ² ^g	0.4646					0.7566				
adequate precision	7.8110					12.946				

Explanations: ^a SS, sum of squares; ^b DF, degree of freedom; ^c MS, mean square; ^d BER, the reduced variable by “backward elimination regression” process; ^e *R*², coefficient of determination; ^f adjusted *R*²; ^g predicted *R*²

Tab. 3. Anova table for response surface reduced quadratic regression models and effects of the variables on the *L. monocytogenes* number (log cfu/g) enumerated at 48th and 72nd hours

Source of Variance	48 th hour					72 nd hour				
	SS ^a	DF ^b	MS ^c	F value	p value	SS	DF	MS	F value	p value
Model	1.05	3	0.35	50.76	0.0001	1.59	3	0.53	37.94	0.0003
<i>A</i> (temperature)	0.06	1	0.06	8.74	0.0254	0.40	1	0.40	28.47	0.0018
<i>B</i> (concentration)	0.88	1	0.88	127.2	0.0001	1.05	1	1.05	75.03	0.0001
<i>B</i> ²	0.11	1	0.11	16.35	0.0068	0.14	1	0.14	10.32	0.0183
Residual	0.04	6	6.93×10^{-3}			0.08	6	0.01		
lack of fit	0.04	5	8.10×10^{-3}	7.74	0.2661	0.08	5	0.02	2114.7	0.0165
pure error	1.05×10^{-3}	1	1.05×10^{-3}			7.94×10^{-6}	1	7.94×10^{-6}		
total corrected	1.10	9				1.68	9			
<i>R</i> ² ^d	0.9621					0.9499				
adj- <i>R</i> ² ^e	0.9431					0.9249				
pred- <i>R</i> ² ^f	0.8651					0.8356				
adequate precision	19.575					16.819				

Explanations: ^a SS, sum of squares; ^b DF, degree of freedom; ^c MS, mean square; ^d *R*², coefficient of determination; ^e adjusted *R*²; ^f predicted *R*²

Tab. 4. Anova table for response surface non-reduced and reduced quadratic regression models and effects of the variables on the *L. monocytogenes* number (log cfu/g) enumerated at 96th and on $\mu_{\max}$ values (ln cfu/g/h)^a

Source of Variance	96 th hour					$\mu_{\max}$ values				
	SS ^b	DF ^c	MS ^d	F value	p value	SS	DF	MS	F value	p value
Model	4.71	5	0.94	153.6	0.0001	6.59×10^{-5}	3	2.20×10^{-5}	76.48	< 0.0001
<i>A</i> (temperature)	2.16	1	2.16	352.1	< 0.0001	4.36×10^{-5}	1	4.36×10^{-5}	152.0	< 0.0001
<i>B</i> (concentration)	1.09	1	1.09	177.0	0.0002	5.08×10^{-6}	1	5.08×10^{-6}	17.69	0.0056
AB	0.05	1	0.05	8.48	0.0436	BER ^e				
<i>A</i> ²	0.88	1	0.88	143.1	0.0003	1.71×10^{-5}	1	1.71×10^{-5}	59.70	0.0002
<i>B</i> ²	0.07	1	0.07	10.97	0.0296	BER				
Residual	0.03	4	6.14×10^{-3}			1.72×10^{-6}	6	2.87×10^{-7}		
lack of fit	0.02	3	7.79×10^{-3}	6.64	0.2762	1.70×10^{-6}	5	3.40×10^{-7}	14.98	0.1936
pure error	1.17×10^{-3}	1	1.17×10^{-3}			2.27×10^{-8}	1	2.27×10^{-8}		
total corrected	4.74	9				6.76×10^{-5}	9			
<i>R</i> ² ^f	0.9948					0.9745				
adj- <i>R</i> ² ^g	0.9883					0.9618				
pred- <i>R</i> ² ^h	0.9639					0.9260				
adequate precision	39.146					24.155				

Explanations: ^a $\mu_{\max}$, the maximum specific growth rate; ^b SS, sum of squares; ^c DF, degree of freedom; ^d MS, mean square; ^e BER, the reduced variable by “backward elimination regression” process; ^f *R*², coefficient of determination; ^g adjusted *R*²; ^h predicted *R*²

ent storage times; 6th, 24th, 48th, 72nd, and 96th are presented in Tables 2-4. Fig. 2 illustrates these effects on 3D-dimensional graphs where the direction of the

effects of the variables on these properties can be seen. The second order regression model equations predicting effects of processing variables are also included

Fig. 2. Effect of temperature and concentration on *L. monocytogenes* number (log cfu/g) enumerated at 6th, 24th, 48th, 72nd, 96th and $\mu_{\max}$ values (the maximum specific growth rate (ln cfu/g/h) evaluated using the combined model, eq. 9) along with the second-order polynomial model equations after “backward elimination regression” procedure

in the Fig. 2. The reason why only the results for the intermediate responses (6th, 24th, 48th, 72nd, and 96th) was analyzed using RSM was that a similar trend was also seen in the other responses (12th, 36th, 60th, and 84th). Results clearly revealed that linear effects of the thyme oil concentration had a significant ($P < 0.01$; 0.05) effect on the *L. monocytogenes* number enumerated at each storage time (Tab. 2-4). The number was remarkably decreased with thyme oil concentration at each storage time (Fig. 2), which indicated that thyme oil could delay the growth of *L. monocytogenes* irrespective of the storage temperature. Such a delay in microbial growth is particularly useful for food safety, which suggested that use of thyme oils could be recommended in short-term storage of products.

RSM was used to determine the optimum treatment and storage conditions of the number of *L. monocytogenes* and the calculated $\mu_{\max}$ values, which is achievable within the range of the tested storage times. Although the desired responses of “*L. monocytogenes* number” and “the $\mu_{\max}$ values” were the minimum of the target goal, the levels of the independent variables that maximize these responses were also presented in this study, as knowing the best growth conditions of

the bacterium with respect to the variables is also very important.

For the optimization process, desirability functions of RSM were used. The resultant optimum operating conditions with the minimization and maximization of the number of *L. monocytogenes* and $\mu_{\max}$ values, the criteria in these optimizations and desirability response surfaces are shown in Fig. 3. The desirability values (D) for the minimization and maximization were calculated to be 0.99 and 0.96 respectively, indicating that any response or factor was inside their desirability range.

By applying the desirability function method, six solutions were obtained for each optimization process (minimization and maximization) covering the criteria; however, the most desirable solutions were presented in this study. Fig. 4 indicates these solutions. For the most desirable ($D = 0.99$) solution for minimization of each response variable, the temperature and concentration levels should be 4.18°C and 0.57%, respectively. At this circumstance, the solution had the lowest number of *L. monocytogenes* (6.12, 5.96, 5.88, 5.81 and 5.40 log cfu/g enumerated at 6th, 24th, 48th, 72nd and 96th, respectively) and the lowest $\mu_{\max}$ value (0.001 ln cfu/g/h) (Fig. 4) to get the highest product

quality in terms of pathogenicity. Although the most desirable solution for maximization of the response variables was not the main goal of this study, the relevant results were also presented in this optimization study since having a knowledge on the conditions under which the number of *L. monocytogenes* would be the highest is also very important. For the most desirable ($D = 0.99$) solution for maximization of each response variable, the temperature and concentration levels should be 14.14°C and 0.02%, respectively. At this point, the solution had the highest number of *L. monocytogenes* (6.73, 6.79, 7.00, 7.29 and 8.09 log cfu/g enumerated at 6th, 24th, 48th, 72nd and 96th, respectively) and the highest $\mu_{\max}$ value (0.011 ln cfu/g/h) (Fig. 4) to get the lowest product quality in terms of the pathogenic load. On the other hand, it was interesting to see that there was a consistent trend between the calculated optimized response values and the storage time; namely, the minimized and maximized response values decreased and increased, respectively, as the storage time (enumeration or incubation time) increased. This indicated the antibacterial effect of thyme oil become more pronounced under the

Fig. 3. Desirability function response surface for the *L. monocytogenes* number (log cfu/g) enumerated at 6th, 24th, 48th, 72nd, 96th and $\mu_{\max}$ values (the maximum specific growth rate, ln cfu/g/h)

Fig. 4. Ramps reports on numerical optimization by desirability function (eq. 17) solutions for obtaining the most desirable numerical optimization by calculating the minimum and maximum response (*L. monocytogenes* number (log cfu/g) enumerated at 6th, 24th, 48th, 72nd, and 96th and $\mu_{\max}$ values (ln cfu/g/h)) values

minimized response conditions with the prolonged storage times.

Fig. 5 indicates bar graphs which show how well each response variable satisfies the criteria. In this figure, the values close to 1 are desired. In this respect, for the minimized responses, excluding the desirability level ($D = 0.916$) of the response (the number of *L. monocytogenes* enumerated at 6th h), all the remaining minimized responses satisfy the criteria ($D = 1$) (Fig. 5a). Taking into account the maximized responses, there was a different case: desirability levels of only two responses (the number of *L. monocytogenes* enumerated at 96th h and $\mu_{\max}$ values) were 1 (Fig. 5b). These results indicated that the desirability levels of the optimized responses generally increased with extending of the storage period, which suggested that it would be better to optimize the conditions effective on growth/survival of *L. monocytogenes* within more prolonged storage time.

Effective bacteriostatic and bactericidal concentration of *Thymus vulgaris* on the growth of the bacterium was reported to be 1% (v/v) (44). Hammer et al. (18)

Fig. 5. Desirability level of each response (*L. monocytogenes* number enumerated at 6th, 24th, 48th, 72nd, and 96th and $\mu_{\max}$ values) after (a) minimization and (b) maximization, as calculated by desirability function (eq. 17)

reported that the concentrations of thyme oil ranging between 0.12 and 0.5% (v/v) inhibited the bacterial growth. In addition, given the nature of CCRD design ($-\alpha$ and $+\alpha$ values in Table 1), the upper limit of thyme essential oil was selected as 0.57% (v/w) because the

exceeded levels (more than 0.6% v/w) in cooked meat were disliked by the panelists in the preliminary sensory analysis. Therefore, the lower and upper limits were selected as 0.0% and 0.57% (v/w), respectively, to observe and determine the optimum conditions for this factor, as well as to navigate the design space. Finally, the survival/growth kinetic was monitored for a period of 6-96 h of incubation (the storage time range for the ground meat samples). This period was selected because approximately after 6 h of incubation the cells of *L. monocytogenes* went into an exponential phase of growth in samples with the thyme oil. After this time, the survival/growth kinetic was monitored for a period up to the extended storage time, namely, 96th h since the *L. monocytogenes* entered into a stationary phase of growth in different storage times and different inhibition rates were observed within a selected period of incubation depending on the different processing conditions in each experimental run (Tab. 1).

The experiments were run in a random order to minimize the effect of uncontrollable variables. Tables 2, 3 and 4 show the ANOVA results used to evaluate the significance of the constructed quadratic models. In addition, model terms were used after elimination of the insignificant ones without damaging model hierarchy and the other statistical parameters related to the adequacy of the models. The lack of fits for the models for *L. monocytogenes* number (log cfu/g) enumerated at 24th, 48th, and 96th and for $\mu_{\max}$ values were insignificant ($P > 0.05$), indicating that the fitted models could describe the variation of the data (7). In Tables 2 and 3, however, the models for 6th and 72nd, the lack of fits were significant ($P > 0.05$), which means that the order of the regression was not secondary; i.e., the model might have not included all appropriate functions of independent variables or the experimental region may be too large for the quadratic model used. On the other hand, it was also reported that a model with significant lack of fit could still be used when a large amount of data was included in the analysis (6, 29). Thus, the high coefficients R^2 have been reported to be considered as evidence of the applicability of the regression model between the ranges of variables included (29).

In this study, residual analysis, R^2 (coefficient of determination), adj- R^2 (adjusted R^2), pred- R^2 (predicted R^2) and adequate precision (adeq-

-precision) values were used to check the adequacy of the models (Tab. 2-4). Fig. 6 shows the plots of studentized residuals versus the run order for the responses of models: 6th, 24th, 48th, 72nd, and 96th and $\mu_{\max}$ values. The plots indicate that the residuals scattered randomly, which means that the generated models were adequate (32). The R^2 and adj- R^2 values were close to each other, which showed that all terms used in the models were necessary in construction of the correct models (7). The predictive capability of the regression model is partly indicated by the pred- R^2 statistic. The pred- R^2 values were close to the adj- R^2 as expected. The R^2 values were ranged from 0.6040 to 0.9948, indicating that majority of the models were adequate and had sufficient predicting ability. Regarding adeq-precision value implying the signal to noise ratio, a ratio greater than 4 is desired. Given this, the ratios (ranging between 7.81 and 39.15 in this study, Tables 2-4) indicate an adequate signal, which indicates that these models can be used to navigate the design space. It should be noted here that the residual analysis, R^2 , adj- R^2 , pred- R^2 and adeq-precision values were improved as the storage time increased.

Fig. 6. Studentized residual plots for *L. monocytogenes* number enumerated at 6th, 24th, 48th, 72nd, 96th and $\mu_{\max}$ values (the maximum specific growth rate)

Fig. 7. Fitting of Baranyi model [eq. (1)] to experimental data (actual data) in factorial, axial and center points (table 1) for the effect of temperature and concentration on *L. monocytogenes* number (log cfu/g) enumerated at 6th, 12th, 24th, 36th, 48th, 60th, 72nd, 84th, and 96th

In this study, the calculated and predicted values were converted into the logarithmic unit and the values were expressed as log cfu/g in Fig. 7. As can be seen, the fitting of the Baranyi model to the experimental data for the effect of thyme oil concentration and storage temperature on *L. monocytogenes* growth in ground meat was performed for three different points; factorial points, axial points and center points. The Baranyi model was adequately fitted to each individual growth curve with R^2 values ranging from 0.84 to 0.95.

In order to determine the optimum operating conditions that yield minimum number of *L. monocytogenes* and $\mu_{\max}$ value, RSM was used. Analysis of variance showed that the process variables – namely, thyme oil concentration and storage temperature – had significant effects on the survival of *L. monocytogenes* present in ground meat. Second order polynomial models were obtained for predicting the number of *L. monocytogenes* and $\mu_{\max}$ values. In addition, the $\mu_{\max}$ values calculated from the Baranyi model [Eq. 1]) could be successfully used in RSM modeling by testing the performance of RSM based on the Baranyi model.

These results are consistent with the information in literature where, in the presence of various essential oils (oregano, *Origanum vulgare*; rosemary, *Rosmarinus officinalis* and thyme, *Thymus vulgaris*), it was reported that food pathogen microorganisms could be inhibited (18, 44). This inhibition effect was attributed to the chemical composition of the tested oil. It was also reported that the phenolic compounds possess high levels of antimicrobial activity. Therefore, the antimicrobial properties of the thyme oil can be related to its high phenolic content, particularly carvacrol, thymol and *p*-cymene (5, 14, 36). Accordingly, Sagdic and Ozturk (42) determined the EO composition of the *Thymus vulgaris* L. and found that carvacrol, linalool, δ -carene, γ -terpinene, *o*-cymene, terpinen-4-ol and thymol were the major components with concentrations of 51.82, 4.22, 1.99, 7.68, 7.55, 2.42 and 2.14%, respectively. Furthermore, it was also reported that the constituents of EO, such as monoterpenes (pinene, limonene and cineole), contribute to the antimicrobial effect, especially against *L. monocytogenes*, rather than the EO itself (31). Regarding the antimicrobial mechanism of these compounds, the main mechanism of action seems to be leakage of intracellular metabolites due to their activity on cell membrane, altering its functions and in some instances, causing swelling and increasing its permeability (10, 12, 25). Penetrating into the cells, these compounds can interact with intracellular sites which cause death of the cell by modification of protein structure (22). In addition, Rasooli et al. (40) morphologically postulated that the cell wall of *L. monocytogenes* lost smoothness and uniformity as the thyme oil concentration increased; and that cell membrane disruption and lack of cytoplasm was evident at an early stage of thyme oil treatment

as a result of the decrease of the cell membrane functionality as a barrier.

As for the effect of storage temperature, the growth of *L. monocytogenes* was not remarkably influenced ($P > 0.05$) by storage temperature in the 6th and 24th of storage time (Tab. 2). However, the effect of temperature became evident ($P < 0.01$) as the storage time prolonged. Accordingly, in the Fig. 2, it can be clearly seen that the number of *L. monocytogenes* decreased as the storage temperature decreased at the 48th, 72nd and 96th of storage time, which can be expected as the bacterium can survive in a temperature range of 1°C-45°C. At first sight, it may appear that studying the effect of temperature may not be so necessary because the effect of temperature is well-known in the literature. However, this is an optimization study and studying effect of any single variable on the pathogen growth may not be very reasonable with respect to inactivation of the pathogen in most cases. Therefore, it was important to find what the most effective combined effect of thyme oil concentration with storage temperature on the growth of the pathogen would be.

The interaction effect was significant ($P < 0.05$) only at the 96th of storage time, increasing the cell number of *L. monocytogenes* (Fig. 2). Finding of the most desirable combination of storage temperature with thyme oil concentration will be discussed later. Regarding quadratic effects, those of the concentration were found to be significant ($P < 0.01$; 0.05) at 24th, 48th, 72nd and 96th and that of the temperature was found to be significant ($P < 0.01$) at 96th of storage time. From the negative signs of the regression coefficients given in Fig. 2, it can be seen that thyme oil concentration caused a further decrease in the cell numbers of *L. monocytogenes* after a certain concentration level at 24th, 48th, 72nd and 96th. Again, from the positive sign of the regression coefficient given in Fig. 2, it is seen that the storage temperature was effective in a further increase in *L. monocytogenes* numbers after a certain temperature level at 96th of storage time.

In this study, the $\mu_{\max}$ values calculated from the Baranyi model [Eq. (1)] were used for RSM modeling [Eq. (2)] so that the performance of RSM based on the Baranyi model could be tested, meaning that the $\mu_{\max}$ values were also as a response variable. As for the effect of temperature and thyme oil concentration on $\mu_{\max}$ values evaluated using the combined model [Eq. (9)], the same trend seen in the 24th, 48th, 72nd and 96th of storage time was also observed in the $\mu_{\max}$ values. Namely, the $\mu_{\max}$ values were significantly ($P < 0.01$) decreased with thyme oil concentration, but increased ($P < 0.01$) with the storage temperature (Tab. 4 and Fig. 2). These results revealed that the combined model [Eq. (9)] could be successfully used to analyze the effect of processing variables on the parameter calculated by the Baranyi model.

In the food industry, the presence of *L. monocytogenes* is not acceptable in food products. Therefore,

finding optimum conditions for the minimum number of *L. monocytogenes* should be of utmost importance. In this study, the most desirable ($D = 0.99$) solution for minimization of each response variable corresponds to temperature of 4.18°C and thyme oil concentration of 0.57% in order to obtain *L. monocytogenes* numbers of 6.12, 5.96, 5.88, 5.81 and 5.41 log cfu/g (enumerated at 6th, 24th, 48th, 72nd and 96th, respectively) and $\mu_{\max}$ value of (0.001 ln cfu/g/h). Given the fact that no study has appeared to be dealing with determination and optimization of the simultaneous effect of essential oil concentration and storage temperature on the growth/survival of *L. monocytogenes* by RSM based on the Baranyi model, the results of this study might be interesting and suggest a promising approach for obtaining the most desirable products in terms of food safety.

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